Green Chemistry and Sustainable Technology

Shiro Kobayashi
Hiroshi Uyama
Jun-ichi Kadokawa *Editors*

Enzymatic Polymerization towards Green Polymer Chemistry

Springer

Green Chemistry and Sustainable Technology

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Preface

Since the middle of the last century, we human beings have enjoyed the industrialized modern society abundant in various products for everyday life, which was realized mainly by the advances of science and technology. We could obtain energy and/or the products very cheaply through utilization of fossil resources. To the end of the last century, however, we have been forced to worry about the global environment; typically, big disasters such as hurricane, typhoon, and heavy rains became to happen more often than before, which is supposed to be owing to the rapid climate change because of the earth warming. This was pointed out to be due partly to the increase of the carbon dioxide emission via energy production from fossil resources like coal and petroleum. COP3 (Kyoto, 1997) was an epoch-making international conference on this problem. From the new century, this movement has been strengthened; COP21 (Paris, 2015) discussed on the problem, and many countries agreed with its importance. To maintain sustainable society, we should increase the use of renewable resources in place of diminishing fossil resources, for mitigating the carbon dioxide emission. Conducting these activities meets with the "carbon-neutral" concept.

In accord with this movement, the concept of "green chemistry" was proposed at the end of the last century in the chemistry field. In producing chemical products, chemists are expected to contribute to reduce the environmental problems. Springer Co. is in the plan of publishing series: "Green Chemistry and Sustainable Technology." Then, we, polymer chemists, should contribute to the polymer chemistry field. For the production of polymer materials, the new method of polymer synthesis "enzymatic polymerization" was created for conducting "green polymer chemistry": the present editors were invited to edit the book *Enzymatic Polymerization towards Green Polymer Chemistry* in the context of the series.

This book has been edited to describe comprehensively the current status of enzymatic polymerization with an explanation of its importance, and to cotribute to realize environmentally desirable earth as well as to maintain sustainable society in the future, hopefully still having global economic growth. Thus, we organized 12 chapters and invited the authors of the respective chapters; all of them have been very active for research in the field worldwide. This book is dedicated to the readers

who are undergraduate/graduate students and chemistry researchers in academia as well as industry fields. We suppose all of them are eager to study and/or to know about enzymatic polymerization, green polymer chemistry, and practical applications for polymer materials productions via green processes.

We believe all the chapters are informative and stimulating to the readers who are pursuing the research not only in the enzymatic polymerization-related area but also in the interdisciplinary areas.

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Chapter 1
Introduction

Shiro Kobayashi, Hiroshi Uyama, and Jun-ichi Kadokawa

Abstract This chapter provides introductory aspects to the readers so that they may understand readily and clearly the significance of the book edition. It is important for polymer chemists to know the present status of "enzymatic polymerization" and "green polymer chemistry." The former involves its historical background and characteristics including enzymatic reaction mechanism. The latter is related with several important "green" aspects, toward which the former is expected to contribute. Brief abstracts of all the chapters are also given for the easier understanding of the whole book.

Keywords Enzymatic polymerization · Green polymer chemistry · Enzymatic reaction mechanism · Reaction selectivity · Sustainable society · Bio-based materials · Polymer recycling

1.1 Introduction

This section deals with the generally important and introductory aspects for this book *Enzymatic Polymerization towards Green Polymer Chemistry*, for all the readers to recognize the significance of the book. The arguments are made based on the fundamental and application viewpoints.

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1.1.1 Historical Background of Enzymatic Polymerization

"Nature is our teacher." In fact, in chemistry field this saying reflects the situation of enzyme catalyst utilized for chemical reactions. Enzymes are natural catalysts, which catalyze all in vivo reactions indispensable for maintaining living systems. In vitro, on the other hand, they are able to catalyze various transformation reactions not only of natural substrates but of unnatural substrates, some of which are currently used for industrial productions like food, pharmacology, medicine, textile areas, etc.

The first enzyme discovered was diastase (amylase) extracted from malt solution by Payen and Persoz in 1833 [1]. Due to the mysterious and profound nature, enzymes and enzymatic functions have been attracted much attention as one of the most actively studied topics in science. In organic chemistry field, esterase catalysis was employed for the ester synthesis for the first time in the 1930s [2]. Then, in vitro enzymatic esterifications or transesterifications to produce esters have been studied since the 1980s in organic chemistry field [3–5].

In polymer chemistry field, "enzymatic polymerization" has been extensively studied and developed as a new method of polymer synthesis for almost these three decades. Enzymatic polymerization is a polymerization reaction using enzyme as catalyst, and it was defined as "chemical polymer synthesis in vitro (in test tubes) via nonbiosynthetic pathways catalyzed by an isolated enzyme" [6, 7]. Since the research advances related to the enzymatic polymerization have been so rapid and paid much attention, they have been reviewed and focused from time to time [6–30].

It is to be mentioned here that the progress of the polymer synthesis and related studies is given as follows with citing innovative works chronologically.

- Concept of macromolecules was established by H. Staudinger (1920s) [31, 32].
- Radical chain mechanism in organic chemistry was proposed (1930) [33].
- Nylon was disclosed via polycondensation (1930s) [34].
- Cationic ring-opening polymerization of tetrahydrofuran (1937) [35].
- Ionic chain mechanism was proposed (1940) [36].
- Discovery of Ziegler-Natta catalysts (1953 & 1955) [37–39].
- Living polymerization method was discovered (1956) [40].
- Solid-phase synthesis method of polypeptides was developed (1963) [41].
- Concept of supramolecular chemistry and supramolecular polymer (1960s) [42].
- Finding of conducting polymers (1977) [43].
- Metathesis catalysts have been developed (since 1950s) [44, 45].

Thus, from the viewpoint of polymerization reactions using catalyst (or initiator), the first period of the historically traditional methods is since the 1930s: radical, polycondensation, ring-opening, and ionic (cationic and anionic) polymerizations (from the 1930s to present); these polymerizations may be called as class 1, and the second period: transition-metal catalyzed polymerizations including Ziegler-Natta catalysts and metathesis catalysts (from the 1950s to present); these polymerizations

as class 2. Then, the enzymatic polymerization may be regarded as class 3, because the in vitro enzymatic catalysis is much different from the catalysis of classes 1 and 2, i.e., the enzymatic catalysis is really of bioorganic and supramolecular chemistry as argued below.

The above *definition of enzymatic polymerization* made at the early stage was strict, and therefore, sometimes became thought to be broader, i.e., enzymatic polymerization includes not only the above defined reactions but also reactions such as using acyltransferase enzyme as catalyst in microbial cells, using enzyme-model catalysts, and using enzyme as catalyst for polymer modifications. This book has been edited according to the broader definition.

1.1.2 Characteristics of Enzymatic Polymerization

Catalyst Enzymes Since so many studies have been accumulated on enzymatic functions, over several thousand kinds of enzymes are known nowadays. According to the Enzyme Commission, all the enzymes are classified into six main groups [46]. Of the six, oxidoreductases, transferases, and hydrolases have been actually employed for various polymerization reactions, since these enzymes are proved highly active catalysts for many purposes and areas in fundamental studies as well as practical applications (Table 1.1) [30]. Epimerases are rarely used for polysaccharide modification as mentioned in Chap. 12. In addition, ligases have been entitled to catalyze a polymerization reaction [47].

Catalysis Mechanism Concerning the reaction mechanism of enzymatic reactions, the following two are important fundamental issues.

First, Fischer proposed "key and lock" theory in 1894, which mentioned the specific relationships between the enzyme and the substrate [48], where the substrate is a "key" and the enzyme plays the role of "lock." This interactive relationship is nowadays known as the molecular recognition; in an in vivo reaction (cycle A, Fig. 1.1), natural substrate is recognized specifically by the enzyme to form the enzyme-substrate complex. With forming the complex, the substrate is activated via transition state by supramolecular interaction to form a new bond to give the product with perfect regio- and/or stereoselectivity control. It is essential, therefore, that the substrate is recognized by the enzyme for proceeding of the reaction. In an in vitro reaction (cycle B, Fig. 1.1), on the other hand, an artificial substrate must be recognized by the enzyme to form the enzyme-artificial substrate complex; then the artificial substrate is activated via transition-state to form a new bond with controlling all the reaction selectivity perfectly, ending up with the product formation.
Second, Pauling suggested in 1946 the specific reason why enzymes cause the catalysis under mild reaction conditions like in living cells [49, 50]. Enzymatic reaction pathway and its energy diagram are shown as Scheme 1.1 and Fig. 1.2, respectively [30]. An enzyme (E) and a substrate (S) form a complex (ES) through a key and lock

Table 1.1 Classification of enzymes, typical example enzymes, macromolecule examples synthesized by enzymatic polymerization as well as modified by enzymatic reaction [30, 47]

Class	Enzymes	Example enzymes	Macromolecules synthesized	Macromolecules modified
1	Oxidoreductases	Peroxidase, laccase, tyrosinase, glucose oxidase	Polyphenols, polyanilines, polythiophenes, vinyl polymers	Polysaccharides, polypeptides, lignins (proteins)
2	Transferases	Phosphorylase glycosyltransferase, acyltransferase	Polysaccharides, cyclic oligosaccharides, polyesters	Polysaccharides, polypeptides (proteins)
3	Hydrolases	Glycosidase (cellulase, amylase, xylanase, chitinase, hyaluronidase), lipase, protease, peptidase	Polysaccharides, polyesters, polycarbonates, polyamides, poly(amino acid)s, polyphosphates, polythioesters	Polysaccharides, polypeptides (proteins)
4	Lyases	Decarboxylase, aldolase, dehydratase		
5	Isomerases	Epimerase, racemase, isomerase		Polysaccharides
6	Ligases	Ligase, synthase, acyl CoA synthase, cyanophycin synthetase	Cyanophycin	

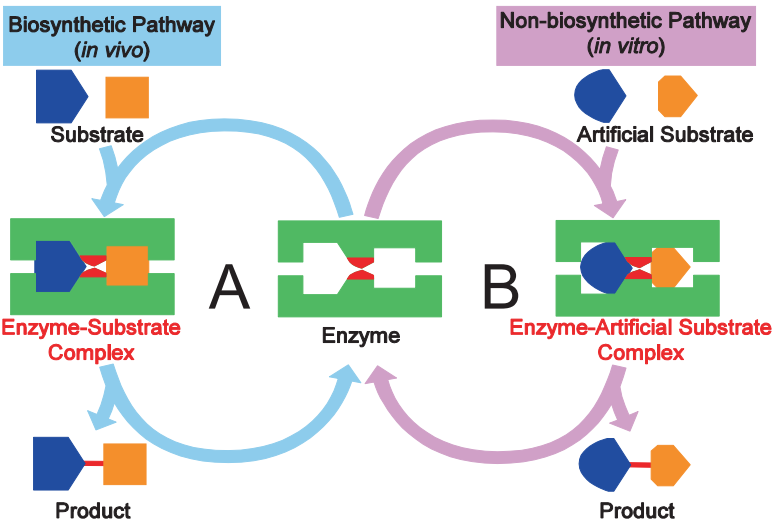

Fig. 1.1 Schematic expression for "key and lock" theory for in vivo enzymatic reactions via biosynthetic pathway (cycle A) and for in vitro enzymatic reactions via nonbiosynthetic pathway (cycle B). (Reproduced with permission from [30]. Copyright 2016 American Chemical Society)

Scheme 1.1 Fundamental reaction pathway with enzymatic catalysis

$$E + S \rightleftharpoons ES \rightleftharpoons [ES]^{\ddagger} \rightleftharpoons EP \rightleftharpoons E + P$$

E: enzyme, S: substrate, P: product

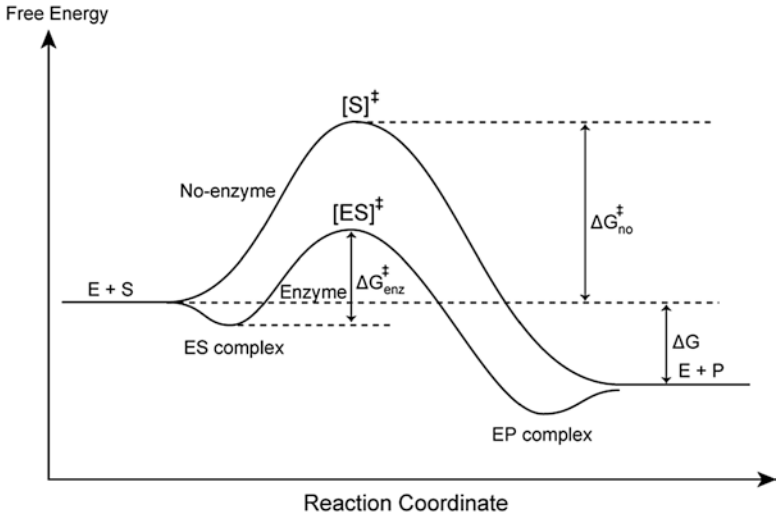

Fig. 1.2 Energy diagram for a chemical reaction: Comparison between an enzyme-catalyzed reaction and a reaction without enzyme. (Reproduced with permission from [18]. Copyright 2009 American Chemical Society)

interaction, which activates the substrate to lead to a transition-state ($[ES]^{\ddagger}$) for the reaction to proceed readily, where the activation energy ($\triangle G_{enz}^{\ddagger}$) is greatly lowered by the stabilization effect of the enzyme, in comparison with that ($\triangle G_{no}^{\ddagger}$) of a reaction without enzyme via a transition-state $[S]^{\ddagger}$, i.e., the enzyme stabilizes the transition-state via complex formation to lower the activation energy.

The enzymatic catalysis normally brings about the rate acceleration of 10^6–10^{12}-fold; however, a specific case reached even 10^{20}-fold [51]. The mechanism of in vivo enzymatic reaction shown in Scheme 1.1 and Fig. 1.1 is generally well accepted [52]. It is also noted that the concept of "catalytic antibody" brought about a new way for reaction selectivity, which is different from that of enzymatic reaction [53]. The difference in mechanistic aspect to cause the selectivity between the enzymatic reaction and the antibody-related reaction is that the former selectively binds the transition state and the latter binds the ground state.

The above key and lock theory does not necessarily mean that the enzyme and substrate relationship requires an absolutely strict combination, but the relationship involves flexibility to some extent. This is the fundamental reason why enzymes are able to catalyze in vitro reactions. So far as the substrate is recognized by the enzyme to form an ES complex, the enzyme-catalyzed reaction is realized in vitro. It is to be paid attention that supramolecular chemistry operates very importantly during the course of reaction, in particular, in the transition-states. These arguments are the cases for all the enzyme-catalyzed synthesis of macromolecules.

As to an important aspect of the enzyme-substrate interactions, monomer of the polymerization must be recognized by the enzyme. It was speculated, therefore, that if the monomer possesses a close structure to that of the transition-state, the monomer is easily recognized by the enzyme, to lead to complex formation and to bring about a transition-state. Then, the polymerization reaction shall proceed smoothly. Based on the speculation, a monomer was newly designed to have the transition-state analogue substrate (TSAS) monomer and was polymerized successfully to produce artificial (synthetic) chitin. The concept of TSAS monomer was proposed for the first time [54] and serves for designing a novel polymerization reaction.

1.1.3 Green Polymer Chemistry

Recently, "green" is a keyword everywhere in the world, due to the importance of the earth environment. A book "green chemistry" was published in 1998; it pointed out the future direction of chemistry research and chemical industry [55]. The book showed 12 philosophical principles to chemists for mitigating the environmental problems yet maintaining the sustainable society. In the polymer chemistry area, "green polymer chemistry" was first noted in 1999 [56]. Among the 12, enzymatic polymerization for green polymer chemistry is concerned with the following issues: reaction conversion must be high (atom economy); starting materials and product polymers are to be of low toxicity or nontoxicity; reaction processes must be designed to lower the toxicity; solvents or other supports are to be minimized in quantity; energy consumption must be minimized to perform the reaction; starting materials are to be renewable rather than depleting resources; using protection and/or deprotection steps for reactions is to be minimized; reactions are hoped to be a catalytic process rather than a mol/mol reaction; product polymers are desirably biodegradable; and chemical disasters are to be prevented. In this context the green character of enzymatic polymerization has often been argued [7, 12, 15, 18–20, 23–30, 47, 56–60].

Owing to the research importance of the area, a new journal *Green Chemistry* was launched in 1999 from The Royal Society of Chemistry. Then, other new journals have been launched like *ACS Sustainable Chemistry & Engineering* (2013) and *Environmental Science & Technology Letters* (2014). Further, the followings are some examples currently published: *Trends in Green Chemistry*, *Green Chemistry Letters and Reviews*, *International Journal of Green Chemistry and Bioprocesses*, *ChemSusChem*, *Environmental Chemistry Letters*, *Current Green Chemistry*, *Green and Sustainable Chemistry*, and so forth.

The appearance of these journals clearly indicates the future direction to which our society should go with taking seriously the environmental and sustainable issues as the most important. Enzymatic polymerization is addressed as a powerful polymer synthesis method for conducting the green polymer chemistry. It involves many advantageous green aspects concerning clean-processes, reaction selectivity, energy savings, natural and renewable resource problems, carbon dioxide emission, etc. These advantageous aspects are described more concretely below [30, 60].

Characteristics of Enzyme Catalyst Enzyme is a nontoxic, renewable natural catalyst, which is free from a metal in most cases. The catalytic activity is very high even in vitro. In some cases, enzyme is robust enough to be used in combination with other chemical catalysts, allowing a new chemo-enzymatic process. An immobilized enzyme can be recovered and repeatedly used. Moreover, enzyme catalyzes a very complicated reaction, when the substrate monomer is adequately designed, as seen particularly in the polysaccharide synthesis; the products like cellulose, hyaluronic acid, chondroitin, etc. are the macromolecules having the most complicated structure ever synthesized in vitro.

The above characteristics are to be noted in particular from the viewpoint that "nature is our teacher." Enzyme-catalyzed reactions often provide us with ideas for biomimetic chemistry, and then, the approach will lead to develop new efficient reaction processes.

It is fortunate that nowadays various enzymes become widely available and the reaction mechanism has increasingly been elucidated due to extensive studies on the area, including X-ray structure analysis, and hence, the enzymatic method will be extended more readily in the future.

Clean Reactions under Mild Conditions with High Selectivity Enzyme-catalyzed reactions normally proceed under mild conditions: at a lower temperature, at around neutral pH, under ordinary pressure, etc. The rate of these reactions is very large in terms of the turnover number. Moreover, the reactions are highly selective in all respects such as enantio-, regio-, chemo- and choro-selectivities [59–63], and hence, they give a clean reaction system with producing no- or minimal by-products. These advantages contribute to energy savings by all means and are normally hard to be achieved by conventional catalytic reactions.

Starting Raw Materials Enzymatic polymerizations are able to use many renewable bio-based materials as starting substrates in place of fossil-based raw materials. They include important platform materials such as cellulose, amylose, lignin, plant-oils, lactic acid, itaconic acid and anhydride, succinic acid and anhydride, sebacic acid, several fatty acids, 1,4-butanediol, sorbitol, glycerol, cardanol, tuliparin, chitin, etc. which shall appear in this book. All of these chemicals are derived from bio-based corn, wheat, sugar cane, cassava, switch grass, some animals, etc. via fermentation and/or chemical/physical treatments. Using the renewable starting materials accords with the concept of "carbon neutral" not to increase the carbon dioxide emission. In addition, environmentally benign reagents can be employed; water, oxygen from air, hydrogen peroxide, carbon dioxide, etc. are applicable.

For reference, during 2011 the sum of 3.5 million tons of bio-based polymers were globally produced, which represents a share of 1.5% of the global polymer production of 235 million tons. The bio-based polymers are expected to be about 12 million tons by 2020, which is compared with the global production of about 400 million tons, the bio-based share being increased to 3%. Thus, the bio-based production capacity will grow faster than the global production [47]. This trend shall contribute to mitigate the global environmental problems with reducing the consumption of fossil resources as well as with contributing to the carbon neutral concept.

Product Polymers Enzymatic polymerization creates new polymers which do not produce via conventional methods due to a very complicated structure. Product polymers from biomasses are nontoxic, and almost all are biodegradable, which are benign to nature. Functionalized polyesters and polysaccharides often provide with value-added products, which are applicable to biomedical and pharmaceutical areas.

Reaction Solvents In vivo enzymatic reactions usually take place in a water solvent system. However, in vitro enzymatic reactions are sometimes robust enough to be carried out not only in an organic solvent but also in a green solvent, like water, supercritical carbon dioxide, and ionic liquids, or in other green solvents.

Polymer Recycling and Degradation First, the case is concerned with the lipase catalysis for polyesters. The ester group is relatively easy for bond-forming and bond-breaking, which is a reversible process. A new method of chemical recycling of polyesters using lipase catalysis was proposed [64–66]. The principle lies in that the ring-opening polymerization system of lactones by lipase catalysis is reversible between linear polymers and cyclic oligomers, which can be controlled by changing the reaction conditions. A continuous flow method combining degradation-repolymerization must be a good way of chemical recycling [67]. Polyester degradation through lipase catalyst is specific to the stereochemistry of polyester backbone as well as branches [68]. Cutinase is also a polyester hydrolase, showing a capability to hydrolyze polyethylene terephthalate (PET, a polyester) to its monomeric units. Cutinase was decorated in strategic positions with sugars to improve a plastic-degrading ability, making it more effective at breaking down PET for the better recycling [69].

Second, enzymatic degradation behaviors of polysaccharides like cellulose by cellulase [70] and chitin by chitinase [71], as well as various nylon polymers and copolymers by nylon hydrodase [72], are well studied by using high-speed atomic force microscopy [70, 71] and gas cluster secondly ion mass spectrometry [72]. These results involve possible application of enzymatic biomass degradation to biofuel developments, fabrication of fibers, and recycling of polyesters.

1.2 Chapter Relations

According to the issues discussed above, we editors made the chapter formation composed of 12 chapters and asked the expert polymer chemists to write the chapter manuscript. The authors of the respective chapter have been very active in the field worldwide and comprehensively wrote/reviewed on the chapter subject including the up-to-date status.

In Chap. 1, the editors described the significance and importance of “enzymatic polymerization” and “green polymer chemistry,” including their historical background. A general scheme of enzymatic polymerization is discussed, and character-

istics of green polymer chemistry are stressed. In addition, a brief description on the contents of 12 chapters is presented. The editors hope that the readers make this book utilized for their further activities.

In Chap. 2, Shoda et al. reviewed the glycoside hydrolase-catalyzed polycondensation of activated glycosyl monomers such as glycosyl fluorides and ring-opening polyaddition of sugar oxazoline monomers, all of which were newly developed reactions. Thus, various kinds of natural polysaccharides were produced for the first time, e.g., cellulose (particularly stressed by the first in vitro synthesis in 1991) and xylan via polycondensation and chitin, hyaluronic acid, and chondroitin via ring-opening polyaddition. Further, these new polymerizations enabled to create various nonnatural polysaccharides. A new concept of "transition state analogue substrate" (TSAS) was also explained.

In Chap. 3, Loos and Kadokawa wrote on the phosphorylase-catalyzed polymerizations to produce polysaccharides. Phosphorylases are rather tolerant with respect to utilizing modified donors and acceptor substrates; they are used for preparation of natural oligo- and polysaccharides, glycoconjugates and their analogues, and for diversification of natural products. Their strict primer-dependence nature allows synthesis of various hybrid materials such as amylose supramolecules of amylose-polymer inclusion complexes.

In Chap. 4, Kimura and Iwata summarized the developments on enzymatic synthesis of polysaccharides by using sucrase (glucansucrase or fructansucrase) as catalyst. Sucrose as monomer is polymerized catalyzed by the former or the latter to produce glucans or fructans, respectively. Product polymers properties, possibility on application of the products as new bio-based materials, and the polymerization mechanism are also discussed.

In Chap. 5, Kobayashi and Uyama described the polyester synthesis via polycondensation reaction with hydrolases as catalyst. Characteristics of lipase catalysis are mentioned from fundamental aspects as well as practical applications. Polycondensation reactions were those of oxyacids or their esters, and of dicarboxylic acids or their esters with alcohols, via dehydration and/or transesterification. Ring-opening addition-condensation polymerization produced unique functional polyesters. Lipase was mainly used as catalyst and also with some protease catalyst.

In Chap. 6, Uyama and Kobayashi dealt with the polyester synthesis via ring-opening polymerizations (ROPs) with catalyst of hydrolases, mainly using lipase. Various cyclic esters, lactones, produced polyesters via lipase-catalyzed ROP, often giving terminal functional polyesters. Lipase catalysis showed unique polymerization behaviors of lactones with different ring-size, which was discussed from ROP mechanistic viewpoint. Lipase catalysis induced enantio-, regio-, and chemo-selective ROPs, which could hardly be achieved by conventional chemical catalysts. ROP of lactones in a variety of media is mentioned for green synthesis of polyesters.

In Chap. 7, Taguchi et al. stated that the natural polyester polyhydroxyalkanoate (PHA) is synthesized via thioester exchange reaction in microbial cells. PHA synthase is the key for producing the various structure-regulated, functional polyesters. In reflecting the importance of bio-based plastics, Kaneka Company built a pilot-scale plant for PHA production in 2011, which is an important news.

In Chap. 8, Numata et al. reviewed the synthesis of polypeptides using proteases enzymes as catalyst. Polypeptides synthesized are of α-peptide linkage structure, and enzymes used are various proteases such as papain, bromelain, α-chymotrypsin, protease K, trypsin, and thermolysin. Starting monomers were mainly natural amino acids and some unnatural amino acids.

In Chap. 9, Uyama reviewed comprehensively the enzymatic oxidative polymerization to aromatic polymers. Phenols, anilines, and thiophenes are mainly used as monomers. As catalyst enzyme, peroxidase containing Fe as active center, laccase, tyrosinase, and bilirubin oxidase (the last three containing Cu as active center) are employed for the polymerization. With using hydrogen peroxide as oxidant, the polymerization of phenols efficiently produce phenolic polymers, most of which are hardly obtained by conventional chemical catalysts. Many product polymers are useful as various functional materials.

In Chap. 10, Higashimura dealt with oxidative polymerization of aromatic monomers catalyzed by enzyme model complexes to produce poly(aromatic)s. The enzyme model complexes used are Fe-containing peroxidase models and Cu-containing monooxygenase and oxidase models, in which H_2O_2 or O_2 is employed as oxidant. The poly(aromatic)s obtained like polyphenols, poly(phenylene oxide)s, polyanilines, and polypyrroles possess excellent physical and chemical properties, as exemplified by "artificial urushi" prepared from cardanol.

In Chap. 11, Zhang and Hollmann presented the synthesis of vinyl polymers via enzymatic oxidative polymerization. The initiating species for the radical polymerization are generated via Fe-containing peroxidase- or Cu-containing laccase-catalyzed radical formations, where H_2O_2 or O_2 are employed as oxidant, with using normally a β-diketone compound together. Parameters to control the polymer properties are introduced and discussed.

In Chap. 12, Cheng covered enzymatic modification of polymers, with focusing literatures for the period 2012–2018 among huge number of studies conducted for research and development and potential industrial applications. The enzymes include mostly hydrolases, oxidoreductases, transferases, and isomerases. The substrates used were polysaccharides, proteins, fats, oils, and lignins, all of which occur abundantly in nature. The types of reaction are polymer hydrolysis and degradation, polymerization, oxidation, glycosylation, crosslinking, and transformation of functional groups. The combination of the biopolymers and enzymes represents opportunities for new product developments and green polymer chemistry.

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Chapter 2
Synthesis of Polysaccharides I: Hydrolase as Catalyst

Shin-ichiro Shoda, Masato Noguchi, Gefei Li, and Shunsaku Kimura

Abstract The glycoside hydrolase-catalyzed polycondensation of activated glycosyl monomers such as glycosyl fluorides and polyaddition of sugar-oxazoline monomers have been reviewed. Various kinds of oligo- and polysaccharides including natural cellulose, xylan, chitin, hyaluronic acid, and specifically modified functional polysaccharides have successfully been prepared by this methodology. Based on the formation of metastable cellulose I by the enzymatic polymerization of β-cellobiosyl fluoride monomer catalyzed by cellulase, a new concept of "choroselective polymerization" for the control in high-order molecular assembly during polymerization was proposed.

The use of sugar oxazolines as a glycosyl monomer with a distorted conformation allowed the polymerization to proceed only in the direction of the product polysaccharides while suppressing hydrolysis. Sugar oxazolines which possess higher potential energy compared with the conventional glycosyl donors enabled us to produce various *N*-acetylglucosamine-containing polysaccharides such as chitin, hyaluronic acid, and chondroitin. A new concept of "transition state analogue substrate" (TSAS) has been introduced to polymerization chemistry.

Keywords Glycoside hydrolase · Functional polysaccharide synthetic cellulose · Choroselective polymerization · Glycosyl fluoride monomer · Artificial chitin · Sugar-oxazoline monomer · Transition state analogue substrate (TSAS)

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2.1 Introduction

Polysaccharides are one of the naturally occurring three major biomacromolecules, together with proteins and nucleic acids, that play important roles, such as structural materials (e.g., cellulose, chitin, and hyaluronic acid) and energy storage (e.g., amylose and glycogen), in living body. Recently, polysaccharide-governed immune response and their potential for clinical use have also been identified [1]. Accordingly, the synthesis of polysaccharides is of keen interest to reveal structure-function relationships and to prepare bioactive and biodegradable materials for research and commercial applications. However, because of the strict requirement for complete regio- and stereoselective glycosidic bond formation between saccharide monomers (Fig. 2.1a and b), synthesis of polysaccharide has long been one of the most challenging yet important topics in macromolecular science [2]. While the automated synthesizers of polynucleotides (DNA, RNA) and polypeptides have already been developed and commercially available, chemical approaches toward polysaccharides which rely upon multiple protection and deprotection steps are inherently limited in scopes [3]. Hence, enzymatic reaction, which requires no protecting groups and proceeds basically in aqueous media, has become an attractive alternative to the conventional synthesis from the viewpoint of green and sustainable chemistry.

In nature, polysaccharides are biosynthesized by the action of glycosyltransferases (GT family, EC 2.4) using sugar nucleotide phosphates as monomers [4]. The employment of these glycosyltransferases for polysaccharide synthesis is, however, currently limited due to their low solubilities, low expression efficiencies, and consequently high cost [5]. In comparison to these glycosyltransferases, another class of enzyme, glycoside hydrolases (GH family, EC 3.2.1), is considerably inexpensive due to the production on an industrial scale and has gained attention for practical synthesis of oligo- and polysaccharides [6,7]. This chapter reviews the glycosidase-catalyzed polymerization toward naturally occurring polysaccharides as well as nonnatural polysaccharide derivatives.

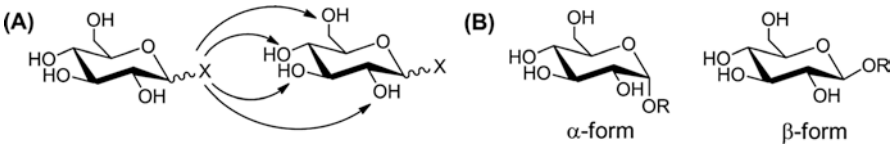


Fig. 2.1 Regio- and stereochemistry of sugar. (**a**) Possible glycosidic bonds in the formation of a disaccharide; (**b**) α- and β-type linkages on the anomeric carbon

2.2 General Introduction of Glycosidases

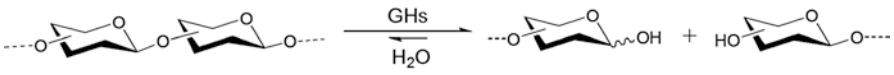
Hydrolases (EC 3) are naturally occurring macromolecular organic catalysts that cleave the bond between part A and part B of the substrate A-B in the presence of water. In general, water (H_2O) is divided to H^+ and OH^- and distributed to the part A and part B, respectively, affording A-OH and B-H (acid-base reaction). According to the principle of microscopic reversibility, hydrolases can catalyze not only hydrolysis but also the reverse reaction (A-OH + B-H → A-B + H_2O) through the same transition state (dehydrative condensation).

Glycosidases (GHs) (also called glycoside hydrolases) are a class of hydrolases that degrade sugar chain by cleaving the glycosidic linkages (Scheme 2.1). Glycosidase-catalyzed synthesis of oligosaccharides by the reverse reaction of hydrolysis using a free sugar as glycosyl donor is a well-known method in the food industry. However, this approach suffers from relatively low yields because the equilibrium is greatly shifted to the direction of thermodynamically stable hydrolyzates.

Therefore, the synthesis of oligo- or polysaccharides by using the reverse reaction of hydrolysis (equilibrium-controlled synthesis) must be done under special reaction conditions to construct a glycosidic bond effectively. The position of the equilibrium can be shifted toward the product by increasing substrate concentration, decreasing the amount of water, and removing the final product from the reaction system, for example, by precipitation or by extraction. However, the yield of polysaccharides would normally be low even if the reaction was carried out under a high substrate concentration, which can be explained by the large negative values of standard Gibbs energy formation of hydrolysis.

The addition of organic solvent or the operation of an enzymatic reaction in organic solvents can enhance the dehydrative condensation toward the products by decreasing the amount of water. However, it is not recommended from the viewpoint of green polymer chemistry to use organic solvents that may increase the environmental stress of the synthetic process. The removal of the products by precipitation or extraction would also require special equipment such as packed columns with activated charcoal for absorption of the resulting products.

On the other hand, glycosidase-catalyzed glycosidic bond formation can be performed under kinetically controlled conditions by using the transglycosylating activity of glycosidases [8] One solution is to introduce a leaving group X into the anomeric center of glycosyl donors. By replacing the reducing hemiacetal end with an appropriate leaving group X, a variety of activated glycosyl substrates can be designed and chemically prepared for glycosidase-catalyzed polymerization (Scheme 2.2). By introducing an electronically negative group X, the partial charge

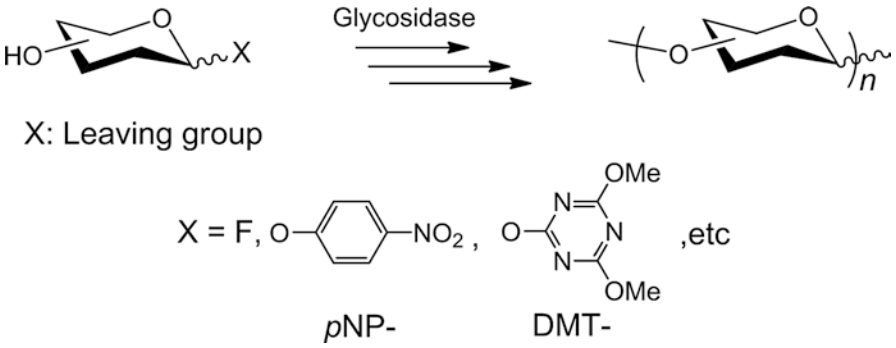


Scheme 2.1 GH-catalyzed hydrolysis of the glycosidic linkage of sugar chain

(δ^+) on the anomeric carbon is increased, and the nucleophilic attack of a hydroxy group of another monosaccharide unit occurs smoothly. In these reactions, the by-product is not H_2O but HX. Polysaccharides of definite structures are produced by repeating a regio- and stereoselective glycosylations between saccharide units.

Glycosyl fluorides, sugars whose anomeric hydroxy group is replaced by a fluorine atom (Scheme 2.2, X = F), are interesting sugar derivatives because fluorine has the smallest covalent radius among all elements [9]. Since glycosyl fluorides were found to be recognized by glycosidase [10], numerous studies on the interaction between glycosyl fluoride and enzymes have been reported [11]. From the viewpoint of organic chemistry, the use of fluorine as a leaving group has the following advantages. First, the size of fluorine atom is small enough to be incorporated in the catalytic site of glycosidases. Second, among glycosyl halides, only glycosyl fluorides are stable in an unprotected form due to the large bond-dissociation energy of the C-F bond, which is necessary for most enzymatic reactions to be carried out in aqueous media.

The higher efficiency of using glycosyl fluorides can be rationalized by considering the number of lone pairs on the fluorine atom. The conventional *O*-glycoside donors such as *p*-nitrophenyl (*p*NP) glycosides have two lone pairs on the anomeric oxygen atom (Fig. 2.2b), whereas the anomeric fluorine atom possesses three lone pairs (Fig. 2.2a). Furthermore, there is a potential risk for the lone pairs on the anomeric oxygen of *p*NP derivatives not to be protonated by an acidic amino acid side chain effectively, because the position of the lone pairs may not be oriented in the proper position when influenced by the location of the bulky *p*NP moiety in the active site of the enzyme. The position of the lone pairs on the fluorine atom has



Scheme 2.2 Glycosidase-catalyzed polymerization by using activated glycosyl donors

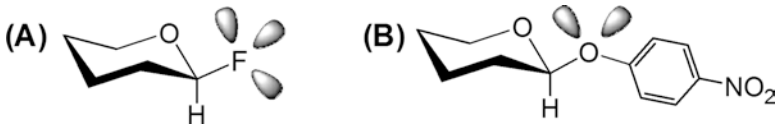


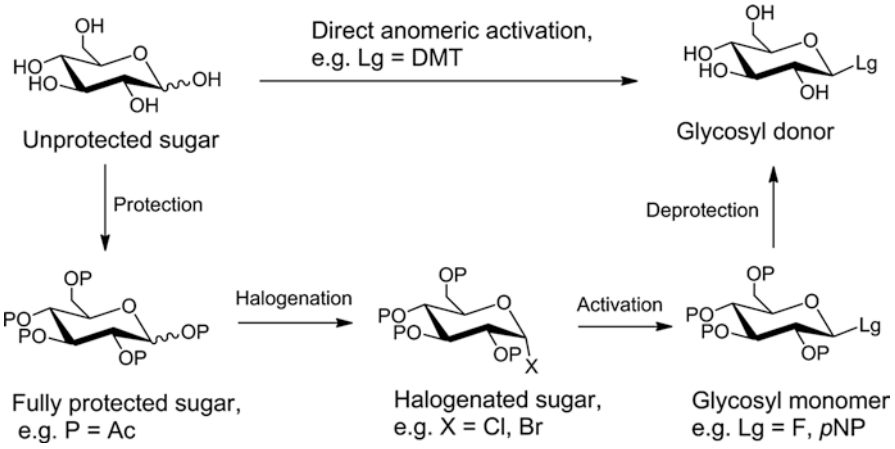
Fig. 2.2 (**a**) Glycosyl fluorides, (**b**) *p*-nitrophenyl (*p*NP-) glycosides

considerable flexibility, which contributes to an efficient protonation from the acidic amino acid side chain in the active site of the enzyme. The selection of glycosyl fluorides as glycosyl donors for glycosidase-catalyzed transglycosylation is quite reasonable, taking their stability and reactivity into consideration.

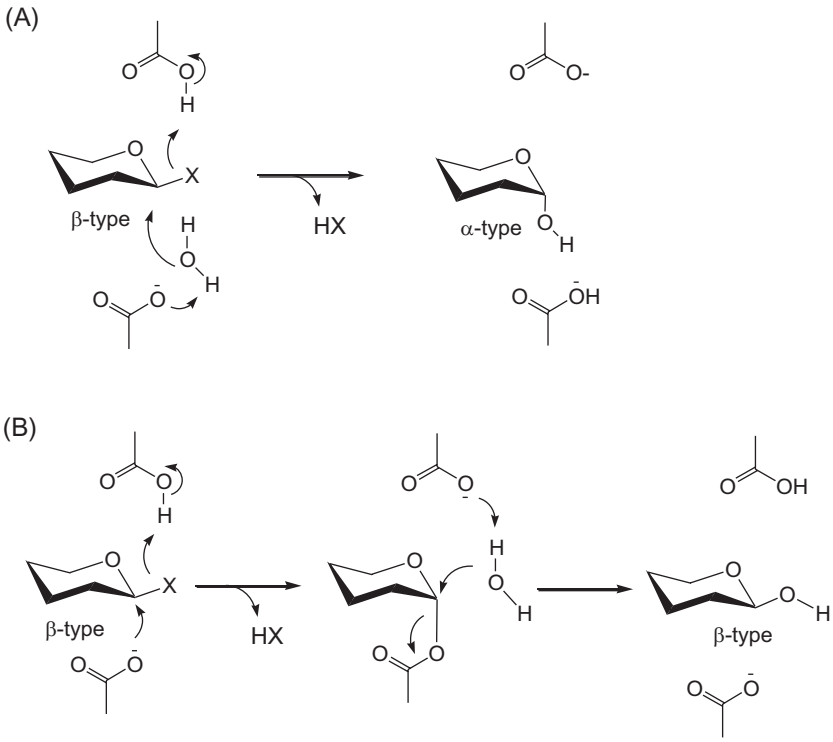
In general, the preparation of *p*-nitrophenyl glycosides or glycosyl fluorides requires a multistep process including the protection of all of the hydroxy groups; the regioselective introduction of a bromine or chlorine into the anomeric center under acidic conditions; the nucleophilic substitution of these halogen atoms by, for example, *p*-nitrophenol or fluoride anion; and the removal of the protecting groups [12] (Scheme 2.3). Recently, the dimethoxytriazinyl (DMT-) glycosides have been developed as a one-step preparable glycosyl donor for glycosidase-catalyzed transglycosylation from the viewpoint of green chemistry where the use of organic solvents and reagents has drastically been reduced (Scheme 2.3) [13].

Glycosidases show perfect stereoselectivity in transglycosylation reactions, giving rise to only one isomer (either of α-isomer or β-isomer) because the formation of the undesired isomer is impossible due to steric hindrance caused by the wall of amino acid residues in the active site of the enzyme. For example, in case of β-glucosidase, the attack of the hydroxy group of a glycosyl acceptor takes place from the β-face of the pyranose ring, leading to stereoselective formation of a β-glycosidic bond.

Glycosidase-catalyzed hydrolysis occurs with either retention or inversion mechanism (Scheme 2.4) [14]. The inverting glycosidases follow a direct displacement reaction in which two carboxylic groups in the active site act simultaneously as an acid and as a general base (Scheme 2.4a). The acid (carboxylic acid) protonates a lone pair on the leaving group X, while the base (carboxylate anion) activates the incoming water leading to a hydrolyzate with inversion of configuration. According to this mechanism, α-type hydrolyzates are obtained starting from β-type



Scheme 2.3 Synthesis of glycosyl monomer for glycosidase-catalyzed polymerization by the direct anomeric activation (above) and via the conventional route (below)



Scheme 2.4 Hydrolysis mechanism of glycosidases. (**a**) Inverting mechanism: the hydrolysis proceeds via a direct displacement of the leaving group X by water. (**b**) Retaining mechanism: a glycosyl-enzyme intermediate is formed that is subsequently attacked by water to complete hydrolysis

glycosides. The hydrolysis catalyzed by retaining enzymes undergoes through a double-displacement process involving a glycosyl-ester intermediate (Scheme 2.4b). Firstly, the nucleophilic residue of enzyme attacks the anomeric center concomitant with protonation of the leaving group X by the acid residue. This results in the cleavage of the C-X bond, forming the glycosyl-enzyme intermediate. The anomeric center of this intermediate is then attacked by water, with assistance from the same acid/base residue. This leads to a product with the same anomeric configuration of the substrate.

Glycosidases are classified into exo-type enzyme and endo-type enzyme. Exo- and endo- refer to the ability of a glycoside hydrolase to cleave a substrate at the end or the middle of a chain [15]. Exo-type glycosidases have a pocket in their catalytic site and are used for enzymatic syntheses of glycosides having a small size (Fig. 2.3a). Endo-type glycosidase, whose shape at the catalytic domain looks like a cleft, shows high catalytic activity for enzymatic syntheses of polysaccharides (Fig. 2.3b).

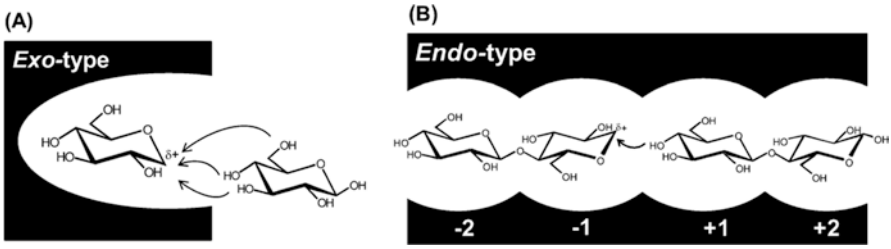


Fig. 2.3 (**a**) Exo-type glycosidases have a pocket in their catalytic site. **(b)** Endo-type glycosidases have a cleft where both a glycosyl donor and a glycosyl acceptor are strictly recognized by the corresponding −1 subsite and + 1 subsite, respectively. (Reprinted with the permission from Ref. [17]. Copyright 2016 American Chemical Society)

The formation of only a single isomer can be realized perfectly regardless of the class of enzyme employed; both exo-type and endo-type glycosidases catalyze a completely stereoselective glycosylation, whereas the regioselectivity of enzyme-catalyzed reactions depends on the class of glycosidase. Exo-type glycosidase normally displays lower regioselectivity toward an acceptor, giving rise to a mixture of regio-isomers (Fig. 2.3a) [16]. In the case of endo-type glycosidases, higher regioselectivity can be achieved. Since both the reducing end of the glycosyl donor and the nonreducing end of the glycosyl acceptor are strictly recognized by the −1 subsite and +1 subsite of endo-type glycosidases, respectively, the hydroxy group that will be incorporated into the resulting glycosidic bond can be located in a suitable position at the +1 subsite of the enzyme to attack the anomeric center located in the −1 subsite (Fig. 2.3b). Consequently, perfect regioselectivity can be guaranteed concerning the resulting glycosidic bond. This is one of the main reasons why endo-type glycosidases have been extensively used as catalysts for synthesis of polysaccharides where perfectly controlled glycosylation reactions must occur repeatedly.

Taking the characteristics of both glycosyl donor and hydrolase into consideration, the combined use of a glycosyl fluoride as monomer and an endo-type glycosidase as catalyst has now become one of the most favorable methods to achieve a polysaccharide synthesis [18]. Finding an appropriate combination of a glycosyl fluoride and an enzyme catalyst is, therefore, key in designing a method for preparation of polysaccharides. For the purpose of finding the best combination of glycosyl fluoride donor and the corresponding glycosidase, a novel enzyme assay for screening glycosidases has been developed by using glycosyl fluorides as enzyme substrates [19]. The method is based on the color change caused by the complex formation of fluoride ion liberated as the result of hydrolysis of glycosyl fluoride and lanthanum-alizarin complexone (La^{3+}-ALC). The assay has a much higher sensitivity compared with the conventional methods using *p*-nitrophenyl glycoside as a screening substrate. According to this enzyme assay screening method, it is possible to find a suitable combination of glycosyl fluoride donor and the corresponding hydrolase by employing the glycosyl donor itself for an enzymatic transglycosylation.

Although the activated sugar units can be polymerized successfully under the repeated glycosidase-catalyzed transglycosylation, in all cases product hydrolysis severely limits conversion. To overcome this problem, enzyme engineering has been implemented to enhance the transglycosylation activity of glycosidase and simultaneously attenuate hydrolysis [20]. Random or site-directed mutations combined with directed evolution have significantly improved the transglycosylation/hydrolysis (T/H) activity ratio of glycosides [21]. In the late 1990s, a new class of glycosidase mutants was introduced in which the active sites of the enzymes are modified such that they lose the ability to hydrolyze their transglycosylation product, thereby driving the reaction in the synthetic direction [22]. These mutant glycosidases, termed "glycosynthases," are rendered hydrolytically incompetent through the replacement of the nucleophilic residue (aspartic or glutamic acid) with an alternative unreactive amino acid (e.g., alanine). These glycosynthases can perform neither hydrolysis nor transglycosylation on the native substrates. However, when used in conjunction with an activated donor having the opposite anomeric configuration to that of the native substrate, glycosidic linkages may be formed (Fig. 2.4). Donors of this configuration mimic the glycosyl enzyme intermediate and take advantage of the vacant cavity created by mutation of the nucleophile. Without risk of hydrolysis, glycosynthases are the ideal tool for the efficient production of oligo- and polysaccharides.

In the following sections, enzymatic polycondensation reactions catalyzed by glycosidases are described according to the nature of catalyst enzymes.

2.3 Cellulase

2.3.1 Enzymatic Polymerization of Cellobiosyl Monomers Catalyzed by Cellulase

Cellulose, which is the most abundant organic compound on earth, has a linear structure of a β(1 → 4)-linked D-glucose repeating units. It is one of the three major structural components of the primary cell walls of green plants, along with hemicellulose and lignin. In nature, cellulose is biosynthesized by the polycondensation of

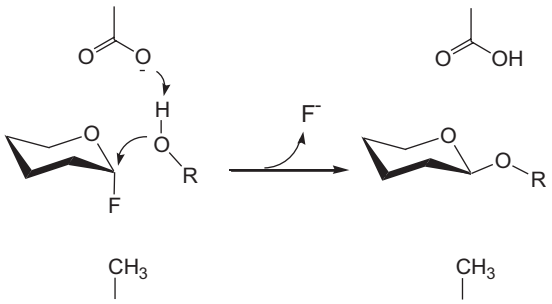
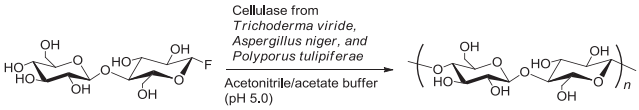
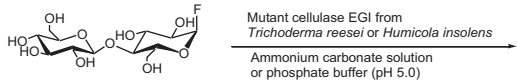
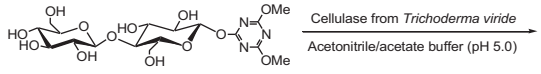
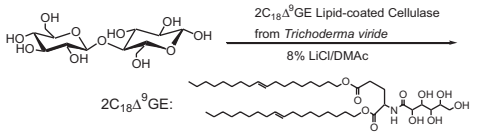


Fig. 2.4 Mechanism of transglycosylation catalyzed by glycosynthases

uridine diphosphate glucose (UDP-glucose) catalyzed by cellulose synthase mainly at the plasma membrane of plants.

In spite of the importance of cellulose, an in vitro chemical synthesis of cellulose had not been achieved in the first challenge of the task in 1941; the in vitro cellulose synthesis has been a central problem in polymer chemistry [23]. In 1991, the first in vitro synthesis of cellulose was achieved via the polymerization of β-cellobiosyl fluoride monomer catalyzed by cellulase from *Trichoderma viride*, an extracellular hydrolase of cellulose (Table 2.1, entry 1) [24–26]. Cellulase catalyzed the in vitro glycosidic bond formation, producing "synthetic cellulose" having a perfectly controlled β-(1 → 4) glycosidic structure. During the polymerization, the glycosidic linkage-forming reaction was repeated, and hence, the cellobiosyl moiety behaved as a glycosyl monomer. Thereafter, hydrolytically inactive mutants of glycosidase (glycosynthase) were developed to improve the transglycosylation yields [22,27]. The mutant enzyme from *Humicola insolens* was capable of transferring disaccharides from α-cellobiosyl fluoride, giving rise to cellulose in good yield (Table 2.1, entry 2). As an alternative of cellobiosyl fluorides, one-step preparable glycosyl monomer having the 4,6-dimethoxy-1,3,5-triazin-2-yl (DMT) leaving group at the anomeric position was developed (Table 2.1, entry 3), where both the glycosyl monomer synthesis and the subsequent polymerization can be achieved in aqueous media without any protection and deprotection steps [28]. Recently, a nonaqueous polycondensation with nonactivated disaccharide monomer under the catalysis of a cellulase-surfactant complex in dimethylacetamide (DMAc)/LiCl was demonstrated to afford cellulose as white powders (Table 2.1, entry 4) [29,30]. The DP value was high (over 100), but the product yield was low (<5%). This is a typical example of classical dehydrative polycondensation, affording water as a by-product.

Table 2.1 Cellulase-catalyzed synthesis of cellulose

Entry	Polymerization scheme	References
1		25, 26
2		27
3		28
4		29, 30

One of the most interesting differences between this enzymatic polymerization and the polymerization via biosynthetic path is that the cellulase-catalyzed polycondensation used a cellobiose derivative as a monomer, rather than a glucose derivative, taking the symmetry of cellulose crystal into consideration. The smallest unit of the cellulose repeating unit structure is "cellobiose" because cellulose has a twofold screw axis due to the two kinds of intramolecular hydrogen bonds between C3-OH with endocyclic oxygen and C6-OH with the C2-OH (Fig. 2.5). It was postulated that cellobiose moiety would be a preferable substrate since it could be recognized by the catalytic site more strongly than a glucose derivative. Furthermore, from the viewpoint of supramolecular interaction between the substrate and amino acids, disaccharide substrates are preferable for the processive movement of chain elongation along the cleft of cellulase. In fact, it is to be noted that disaccharide fluoride monomers were widely used, and the disaccharide structure was confirmed effective [27,31]. A monosaccharide fluoride was not a good substrate for the polysaccharide synthesis [32].

The characteristic feature of synthetic cellulose formation in vitro is that a single glucan chain elongates by interacting with other growing glucan chains. These dynamic events via a nonbiosynthetic path can be realized only by the enzymatic polymerization technique where unprotected glucan chains propagate in aqueous media. These dramatic phenomena prompted us to consider a polymer-polymer interaction during polymer chain elongation in addition to the conventional monomer-polymer interaction, monomer-catalyst interaction, and polymer-catalyst interaction in the field of supramolecular chemistry [33].

Cellulose forms typically two types of allomorphs of high-order molecular structure through self-assembly. One form is the thermodynamically metastable cellulose I, in which cellulose chains are aligned in parallel. Another form is the thermodynamically stable antiparallel cellulose II. Notably, naturally occurring cellulose forms the less stable cellulose I crystalline structure. In vitro, crystalline structure of cellulose synthesized via cellulase-catalyzed polymerization was of the cellulose II structure with crude enzyme [34] and of cellulose I structure with purified enzyme. Metastable cellulose I could be formed due to a kinetically controlled process [35,36]. This was the first example of cellulose I formation via a nonbiosynthetic pathway. Such a control in high-order molecular assembly during polymerization was not reported before; therefore, a new concept of "choroselective polymerization" was proposed [37]. The term "choros" has its origin in a Greek word which means "space."

The self-assembling process of synthetic cellulose during crude cellulase-catalyzed polymerization was investigated in detail in real time and in situ by a

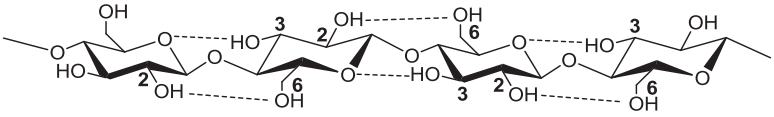


Fig. 2.5 Molecular structure of cellulose and its intramolecular hydrogen bonds

combined small-angle scattering (SAS) methods, together with wide-angle X-ray scattering (WAXS) and field-emission scanning electron microscopy (FE-SEM). The aggregation of the synthetic cellulose was observed and associated with characteristic lengths larger than 200 nm in aqueous media. Further, cellulose molecules created at each active site of enzymes associate themselves around the enzyme associations into cellulose aggregates having surface fractal dimensions D_s, increasing from 2 (smooth surface) to 2.3 (rough surface with fractal structure) as the reaction progressed, extending over a surprisingly wide length scale ranging from ~30 nm to ~30 μm with three orders of magnitude. The construction of this unique self-assembly can be explained by an extremely large number of cellulose molecules repeatedly created at the active site of cellulase [38,39].

Concerning the enzyme catalysts responsible to the polymerization of β-cellobiosyl fluoride, many kinds of cellulases (EC 3.2.1.4) have the possibility of possessing the glucan chain-elongating ability. One of these is endoglucanase II (EGII) from *Trichoderma viride.* EGII is composed of three functional domains: the cellulose-binding domain (CBD), the linker, and the catalytic domain. In the hydrolysis, CBD first binds the crystalline part of cellulose, and then the catalytic domain catalyzes the hydrolysis of cellulose molecules, the linker domain linking these two domains. Using biotechnology, two types of enzymes were prepared from yeast, the one having all domains (EGII) and the other having only catalytic domain lacking CBD and the linker domain ($EGII_{core}$) [40] (Fig. 2.6a and b. Very interestingly, both enzymes showed high polymerization activity for β-cellobiosyl fluoride, giving synthetic cellulose. With progress of time, the produced cellulose gradually disappeared with EGII-induced hydrolysis; however, the cellulose degradation is resisted in the polymerization solution with $EGII_{core}$. These results suggest that the CBD plays an important role for the hydrolysis of the product, but not for the polymerization. The polymerization needs only the function of the catalytic domain.

Another mutant EGII having two sequential catalytic core domains ($EGII_{(core)2}$) was prepared (Fig. 2.6c) [41]. This mutant $EGII_{(core)2}$ catalyzed polymerization of β-cellobiosyl fluoride faster than $EGII_{core}$, affording large spherulites. The resulting large spherulites were composed of platelike crystals radiating from the center of the spherulites. On the other hand, fibrous cellulose was produced from cross-linked

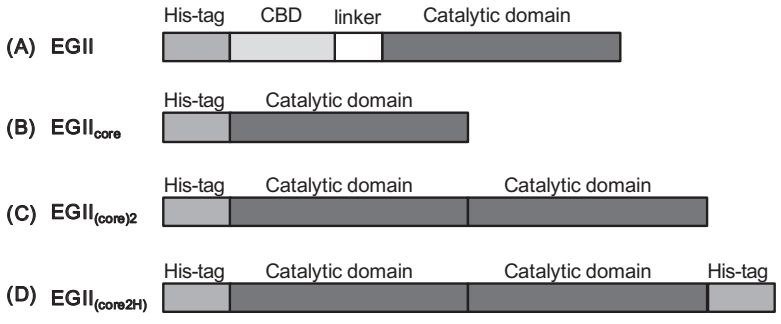


Fig. 2.6 Schematic representation of (**a**) EGII, (**b**) $EGII_{core}$, (**c**) $EGII_{(core)2}$, and (**d**) $EGII_{(core2H)}$

mutant $EGII_{(core2H)}$ (Fig. 2.6d) having two hexameric histidine residues (His-tags) in total on both enzyme chain terminals [42].

The cross-linking molecule used is bisNTA (Fig. 2.7a), which has two nitrilotriacetic acid (NTA) moieties on both terminals of poly(ethylene oxide). The NTA moiety is one of the most utilized ligands which is known to interact with oligohistidine residues (His-tag) through transition metal ions such as Ni [43]. $EGII_{(core2H)}$ mutant enzymes were cross-linked with the help of Ni ions through bisNTA. Using the cross-linked $EGII_{(core2H)}$ as a catalyst for enzymatic polymerization of β-cellobiosyl fluoride, the polymerization proceeded extremely fast, and the fibrous cellulose with high molecular weight was produced. Taken together, the configuration of enzymes in vitro synthesis of cellulose seems to influence the morphology of synthetic cellulose as is the case of in vivo synthesis. To obtain an in-depth understanding of in vitro synthesis of cellulose with regard to geometry of the mutant enzymes, $EGII_{(core)2}$ was immobilized on gold, and its hydrolytic activity [44] and polymerization activity were analyzed [45]. The linker molecules (NAT-SH) (Fig. 2.7b) with thiol at one end and NTA at the other were self-assembled on gold, and $EGII_{(core)2}$ having a His-tag was immobilized on the self-assembled monolayer (SAM) via Ni ion. The hydrolytic activity of the immobilized $EGII_{(core)2}$ was nearly the same on either anchor molecule. The immobilized $EGII_{(core)2}$ apparently retained the inherent hydrolytic activity similar to free $EGII_{(core)2}$. The local high concentration of $EGII_{(core)2}$ on gold probably promoted successive hydrolysis of the transient products, leading to high hydrolytic activity despite immobilization. Two kinds of the mutant enzymes, $EGII_{(core)2}$ and $EGII_{(core2H)}$, were immobilized on the

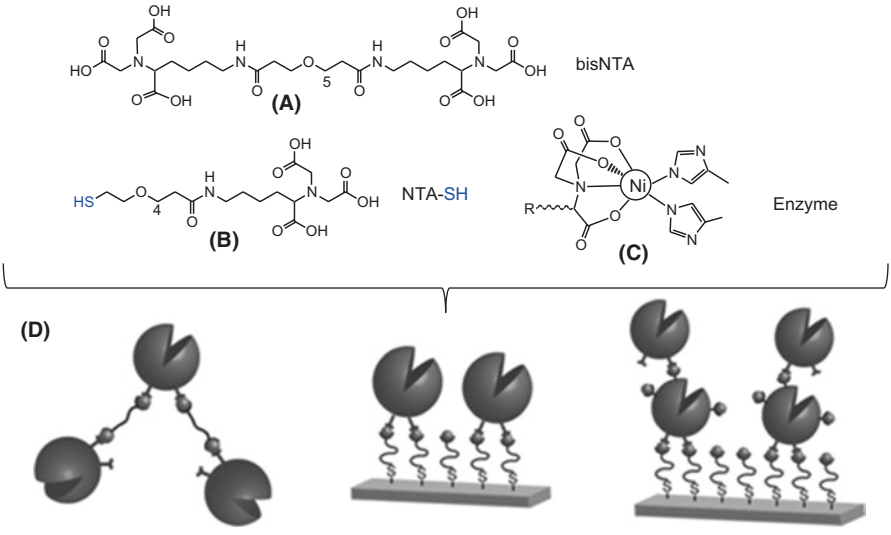


Fig. 2.7 Chemical structure of (**a**) BisNTA; (**b**) NTA-SH; (**c**) complex formed with NTA, Ni, and His-tag; (**d**) schematic illustration of cross-linked enzymes, immobilized enzyme on gold substrate, and cross-linked and immobilized enzymes. (Reprinted with the permission from Ref. [17]. Copyright 2016 American Chemical Society)

NTA-SAM. The crystallinity of the synthesized cellulose by the immobilized $EGII_{(core)2}$ was higher than that by free $EGII_{(core)2}$. The crystallinity of the synthesized cellulose by the immobilized $EGII_{(core2H)}$ was particularly high that can be attributed to the high density with the horizontally immobilized $EGII_{(core2H)}$ on the flat gold substrate. The well-oriented endoglucanase should help in the crystallization of synthesized cellulose. EGII(core2H) was piled up on gold via NTA-Ni-His-tag linkage (Fig. 2.7c), where $EGII_{(core2H)}$ was cross-linked with each other in the vertical direction on gold substrate [46]. The highly crystalline cellulose was synthesized also by this cross-linked enzyme on gold substrate because of high local concentration of $EGII_{(core2H)}$ (Fig. 2.7d).

2.3.2 *Enzymatic Polymerization of Other Saccharide Monomers Catalyzed by Cellulases*

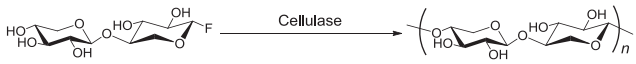
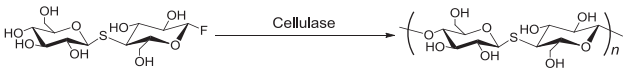
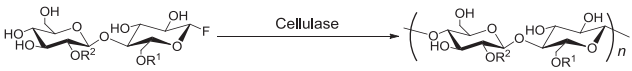
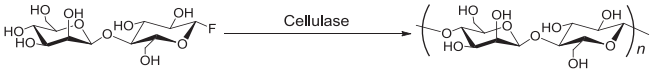
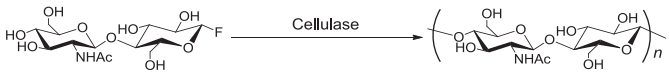
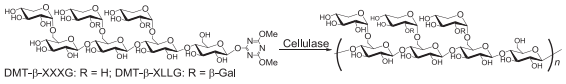
Xylan is one of the most important components of hemicelluloses in plant cell walls. The backbone of naturally occurring xylan is a polycondensation product of xylose through a β-(1 → 4) glycosidic bond. β-Xylobiosyl fluoride monomer was polycondensed by the action of cellulase enzyme. The polymerization proceeded in a mixed solvent of acetonitrile/buffer (pH 5.0) to give a synthetic xylan having β-(1 → 4) glycosidic linkage, with a *M*n of 6.7×10^3 corresponding to the DP value of ~23 (Table 2.2, entry 1) [47]. The resulting xylan has the same structure as natural xylan extracted from esparto grass [48].

As an analogous substrate to β-cellobiosyl fluoride, S-linked β-cellobiosyl fluoride was polymerized by cellulase from *Trichoderma viride* to produce oligosaccharides having an *O*-glycosidic bond and *S*-glycosidic bond alternatingly (hemithiocellodextrins) (Table 2.2, entry 2) [49]. Tetra-, hexa-, octa-, and decasaccharides were isolated from the reaction mixture in 4.5, 7.5, 5.7, and 5.0% yields, respectively.

The development of cellulose-based materials necessitates a precise control of regioselectivity and degree of substitution. However, chemical structures constructed by a polymer reaction are not precisely controlled because such modifications normally give a mixture of randomly substituted polysaccharides. An alternative method for the construction of a modified polysaccharide with well-defined structure is to polymerize a modified saccharide monomer. Based on this strategy, cellulase-catalyzed polymerization with *O*-substituted β-cellobiosyl fluoride monomers was demonstrated.

The 6-*O*-methyl-β-cellobiosyl fluoride (R^1 = Me, R^2 = H) was found to be recognized by cellulase and polymerized, giving rise to the corresponding alternatingly *O*-methylated cellulose derivative (Table 2.2, entry 3) [50,51]. The *M*n of the product was 3.9×10^3, corresponding to n ~7. In comparison with 6-*O*-methyl derivative, 6′-*O*-methyl-β-cellobiosyl fluoride gave oligomers like tetrasaccharide. The cellulase-catalyzed polycondensation of the other methylated β-cellobiosyl fluoride

Table 2.2 Cellulase-catalyzed synthesis of polysaccharide derivatives

Entry	Polymerization scheme	References
1		47
2		49
3	 R^1 = Me, R^2 = H R^1 = H, R^2 = Me;	50, 51, 52
4		52
5		55
6	 DMT-β-XXXG: R = H; DMT-β-XLLG: R = β-Gal	57

derivative, 2′-*O*-methyl-β-cellobiosyl fluoride, also took place in a mixed solvent of acetonitrile/acetate buffer (pH 5.0) to produce an alternating 2′-*O*-methylated cellulose derivative (Table 2.2, entry 3) [52]. These results show that the 6-OMe group and 2′-OMe group of the cellobiose moiety do not have large steric hindrances for the substrate recognition.

A synthetic strategy for hybrid-type oligosaccharides and polysaccharides having an alternating structure composed of two monosaccharide units has been developed by chemoenzymatic procedures [53]. Because of the wide spectrum in substrate recognition of glycoside hydrolases, synthesis of unnatural polysaccharides composed from different two polysaccharide components could be achieved [54]. Such polysaccharides (hybrid polysaccharides) are difficult to synthesize via the biosynthetic path or by the conventional chemical synthesis.

Synthesis of cellulose-mannan hybrid polysaccharide was performed by the enzymatic polymerization of Man-β-(1 → 4)-Glc-β-fluoride (Man: mannose) catalyzed by cellulase from *Trichoderma reesei* (Table 2.2, entry 4) [52]. The MALDI-TOF mass spectrum indicated that the water-insoluble fraction in the products was composed of oligosaccharides up to hexadecasaccharide.

Cellulose-chitin hybrid polysaccharide has also been synthesized based on the same concept [55]. GlcNAc-β-(1 → 4)-Glc-β-fluoride monomer was designed and prepared and polymerized by the action of cellulase (GlcNAc: *N*-acetylglucosamine) (Table 2.2, entry 5). These results clearly show that the −2 subsite and +1 subsite of cellulase can accept even a nongluco-type monosaccharide unit like GlcNAc efficiently.

Although the enzymatic glycosylation of glycosyl fluoride monomers has become a general strong synthetic method for polysaccharide synthesis, the preparation of glycosyl fluorides requires several steps, including protection of the hydroxy groups, activation of the anomeric center by introducing chlorine or bromine, nucleophilic replacement by fluorine as a leaving group, and the removal of the protecting groups (Scheme 2.3). These procedures eventually lower the total yields of the glycosyl donor syntheses. In addition, in case of oligosaccharide donors, the cleavage of the inner glycosidic bonds occurs during the process of glycosyl donor synthesis, affording a mixture of oligosaccharide donors with different molecular weights [56]. These demerits of the conventional glycosyl donor synthesis have hampered the application of enzymatic glycosylation to complex target molecules.

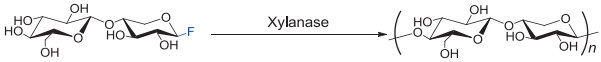
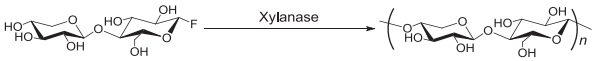
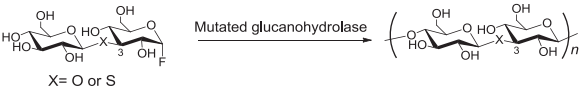
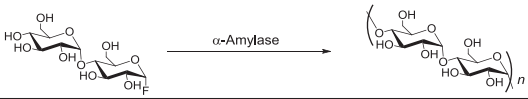
One-step preparable glycosyl donors, 4,6-dimethoxy-1,3,5-triazin-2-yl glycosides (DMT-glycosides), have been developed for enzymatic polymerization. A cellotetraose-backboned heptasaccharide (XXXG) and a nona-saccharide (XLLG) have directly been converted to the corresponding DMT-β-XXXG and DMT-β-XLLG, respectively, by the action of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMT-MM) [57]. Note that the letter X represents a glucopyranose residue that is substituted with a xylopyranose through an α-1,6 glycosidic bond, the letter G represents a nonsubstituted glucopyranose residue, and the letter L represents a glucopyranose residue that is substituted with a galactopyranose β-(1 → 2) xylopyranose through an α-(1 → 6) glycosidic bond. The resulting activated oligosaccharide derivatives were found to polymerize catalyzed by an endo-β-1,4-glucanase as catalyst. The polymerization took place in a complete regio- and stereoselective manner, affording nonnatural polysaccharides having a XXXG-repeating unit and a XLLG-repeating unit, respectively, in the main chain (Table 2.2, entry 6).

2.4 Xylanase, 4-Glucanohydrolase, and Amylase

A cellulose-xylan hybrid polysaccharide has been prepared [58]. There are two possible candidate monomers: Glc-β-(1 → 4)-Xyl-β-fluoride (Table 2.3, entry 1) and Xyl-β-(1 → 4)-Glc-β-fluoride (Table 2.3, entry 2). Both monomers were polymerized by xylanase (EC 3.2.1.32) enzyme catalyst, giving rise to the corresponding cellulose-xylan hybrid-type polysaccharides.

Polysaccharides composed of glucose residues linked through β-(1 → 3)-glycosidic linkages, i.e., (1 → 3)-β-glucans, such as curdlan, laminarin, and schizophyllan, are found in nature. They have increasingly attracted much attention because of their high immune properties and anticancer activities [59]. A 1,3-1,4-D-glucan 4-glucanohydrolase from *Bacillus licheniformis* has been shown to catalyze the polycondensation of β-laminaribiosyl fluoride (Glc-β-(1 → 3) -Glc-β − fluoride) and to lead to alternating 1,3-1,4-β-D-glucotetraose and 1,3-1,4-β-D-glucohexaose in low yields [60]. Thereafter, the mutated glucan endohydrolase E231G was pre-

Table 2.3 Synthesis of polysaccharides catalyzed by xylanase, 4-glucanohydrolase, and amylase

Entry	Polymerization scheme	References
1		58
2		58
3		61
4		62

pared, and the polymerization of α-laminaribiosyl fluoride by this mutated enzyme was performed in a mixed solvent of acetonitrile/acetate buffer (pH 5.0) to produce (1 → 3)-β-glucan with DPs of 28–44 (Table 2.3, entry 3) [61]. The catalysis of this mutated enzyme was extended to the polymerization of 3-thio-laminaribiosyl fluoride, giving rise to the corresponding polysaccharide with DPs of 6–18.

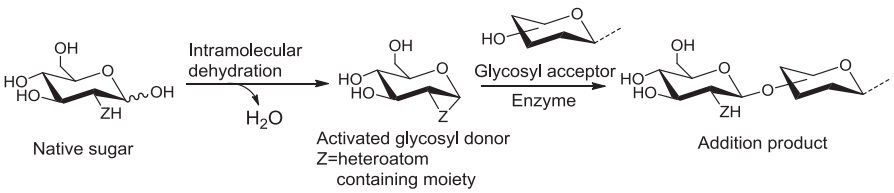
Malto-oligosaccharides are glucose oligomers linked through α-(1 → 4) glycosidic bonds. An activated substrate of α-D-maltosyl fluoride was designed and prepared as a glycosyl monomer based on the double-displacement transfer mechanism of α-amylase (EC 3.2.1.1), and its polymerizability has been investigated. The reaction was carried out in a methanol-phosphate buffer, affording amylose oligomers up to heptasaccharide (Table 2.3, entry 4) [62].

2.5 Glycosidase Catalysis for Ring-Opening Polyadditions

Addition reactions are organic transformations where two or more molecules combine to form larger adducts without liberating any small molecules like H_2O and HX. The glycosidase-catalyzed polyaddition reaction is regarded as a repeating multistep process of an addition reaction of one of the hydroxy groups in a glycosyl monomer to the anomeric center of another monomer [6].

In principle, it is impossible to construct dehydrated skeletons like *O*-glycosidic bonds through an addition reaction because the concept of elimination of H_2O or HX is not included in any addition reactions. Therefore, to design a glycosylation based on an addition reaction, the use of a glycosyl donor having an already dehydrated moiety like an unsaturated bond or a heterocycle is indispensable.

A general scheme of enzymatic addition glycosylation consists of the following two reactions (Scheme 2.5): (1) an intramolecular dehydration of a native sugar (Scheme 2.5, left) to produce activated glycosyl donor containing a heterocyclic moiety Z (Scheme 2.5, center) and (2) addition of glycosyl acceptor catalyzed by an



Scheme 2.5 Addition glycosylation using an intramolecular-dehydrated donor

enzyme, affording the corresponding addition product (Scheme 2.5, right). By using such kind of already dehydrated glycosyl donor, the construction of a dehydrated moiety through an addition reaction becomes possible. Meanwhile, this intramolecularly dehydrated glycosyl donor having higher potential energy can promote the reaction effectively in the direction of product under kinetically controlled conditions. Since the glycosyl donor possesses an intramolecularly dehydrated structure, the reaction proceeds smoothly via a nucleophilic attack of one of the hydroxy groups in the glycosyl acceptor to the anomeric carbon atom of the glycosyl donor, accompanying a ring opening, without liberating any small molecules. This is a typical example of addition reactions where the O^- and the H^+ from the OH in the glycosyl acceptor bind to glycosyl donor's anomeric carbon and the heteroatom unit Z, respectively.

Actually, an addition of water to a reactive intermediate having a heterocycle moiety was proposed for hydrolysis of chitin, a β-(1 → 4)-linked polysaccharide composed of *N*-acetylglucosamines, catalyzed by chitinases (EC 3.2.1.14). Specifically, the glycosidic oxygen is protonated by one of the carboxylic acid residues immediately after the recognition of chitin at the catalytic domain. The carbonyl oxygen of C-2 acetamido group in a chitin unit at the donor site attacks the anomeric carbon from α-side to form an oxazolinium ion transition state stabilized by the other carboxylic acid residue, and the glycosidic linkage is cleaved completely (substrate-assisted catalysis) [63]. A nucleophilic attack by a water molecule from β-side induces the opening of the oxazoline ring, giving rise to the hydrolyzate (Fig. 2.8a).

Sugar oxazolines, which are the intramolecularly dehydrated derivatives of 2-acetamido-2-deoxy sugars (Z = -N=C(CH_3)-O- in Scheme 2.5), are potentially useful glycosyl donors for enzymatic glycosylation by addition reaction because the oxazoline ring can be opened by the attack of a hydroxy group at the anomeric position, regenerating an *N*-acetylglucosaminide moiety. The reasoning for using oxazoline derivatives can be shown in the retro-synthetic analysis of an *N*-acetylglucosaminide unit [63]. First, the sugar oxazoline is recognized and simultaneously protonated by the carboxylic acid residue to form the corresponding oxazolinium ion. Then, the hydroxy group of acceptor attacks the anomeric carbon from β-side to induce the ring opening, giving a new β-type *O*-glycosidic linkage (Fig. 2.8b). The key point for the ring-opening addition is the similarity in the structure of sugar oxazoline and the oxazolium transition state. The pH value for the optimal condition of the hydrolysis reaction was reported to be 8.0 [64]. However, the ring-opening addition took place even under the weak alkaline conditions at

around pH 10–11 while simultaneously suppressing the hydrolysis of the product [65].

Another advantage of using sugar oxazolines is that the transglycosylation proceeds smoothly due to its lower activation energy. Sugar oxazolines are bicyclic molecules having a distorted structure where a six-membered pyranose ring and a five-membered oxazoline ring are fused sharing the anomeric and C-2 positions. Sugar oxazolines possess higher potential energy compared with the conventional glycosyl donors such as *p*-nitrophenyl glycoside. Consequently, the activation energy between the TSAS donor and the product of glycoside becomes much smaller than the case of using the conventional glycosyl donor (Fig. 2.9). Therefore,

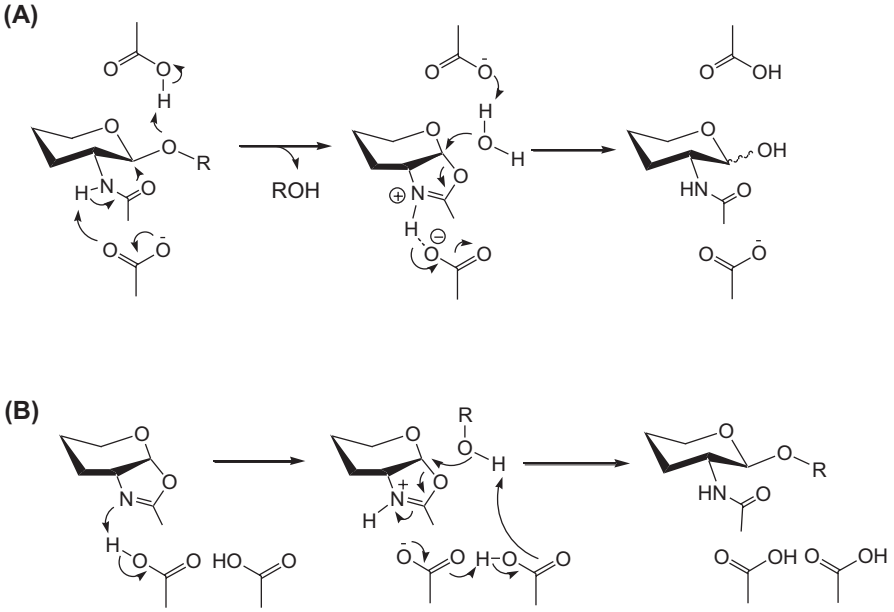


Fig. 2.8 Proposed mechanism for (**a**) hydrolysis by neighboring group participation and (**b**) ring-opening addition of sugar oxazoline in the catalytic site of chitinase. Note that two acidic amino acids synergetically act as proton donor and proton acceptor [2]

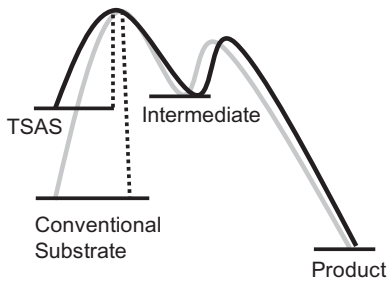


Fig. 2.9 Difference of activation energy of transglycosylations starting from the conventional substrate and the transition state analogue substrate (TSAS)

the glycosylation reactions proceed efficiently even when a chitinase with low activity is employed. It should be noted that the use of sugar oxazolines as a transition state analogue substrate (TSAS) allows the reaction to proceed only in the direction of the glycosylation reaction while suppressing hydrolysis of the product.

Taking the abovementioned requirements for glycosyl donor and enzyme catalysts into consideration, the use of sugar oxazolines as monomers and endo-type glycosidases that can act as a substrate-assisted catalysis has now become the most promising combination to achieve synthesis of *N*-acetylglucosamine-containing polysaccharides. In the following sections, several enzymatic polyaddition reactions catalyzed by *N*-acetylglucosaminidases will be introduced.

2.6 Chitinase

2.6.1 Enzymatic Polymerization of Chitobiose Oxazoline Monomer Catalyzed by Chitinase: Artificial Chitin

Chitin is a β-(1 → 4)-linked *N*-acetyl-D-glucosamine (GlcNAc) polysaccharide that can be biosynthesized by the polymerization of UDP-GlcNAc as substrate monomer with chitin synthase [66]. Chitosan is an *N*-deacetylated product of chitin. Chitin is one of the most abundant and widely found polysaccharides in the animal field. Chitin and chitosan show excellent characteristics of biodegradability, biocompatibility, and in particular low immunogenicity.

In 1995, the first in vitro synthesis of chitin was accomplished via ring-opening polyaddition of a chitobiose oxazoline monomer catalyzed by chitinase and published in 1996 (Fig. 2.10) [65,67–70]. The reaction occurs as a ring-opening polyaddition mode and is promoted under weak alkaline conditions (pH 9.0–11.0). The yield of "artificial chitin" in these works was almost quantitative because the resulting chitin was not hydrolyzed by the chitinase catalyst due to its lower hydrolytic activity under alkaline conditions. The DP value of synthetic chitin was evaluated as 10–20, depending on the reaction conditions [2].

The organization process of crystalline chitin during the chitinase-catalyzed polyaddition of chitobiose oxazoline monomer was monitored by using phase-contrast and polarization microscopy in combination with SEM and TEM (Fig. 2.10) [70]. Under reaction conditions of pH 10.5 and 30 °C, 25 h was required for the complete consumption of the disaccharide monomer. During the first 30 min, a small number of rectangular platelike solids were observed whose width, height, and length of the plates were 25, 10, and 50 nm to 1 μm, respectively. The electron microdiffraction of the resulting plates clearly showed that the products were the thermodynamically stable form of chitin crystal, α-chitin. Therefore, single-crystalline plates of α-chitin were formed, in which polysaccharide chains packed antiparallel and formed intra- and intermolecular hydrogen bonds. The crystal plates grew and stacked on each other as time elapsed (ca. 3 h) and shaped into ribbons,

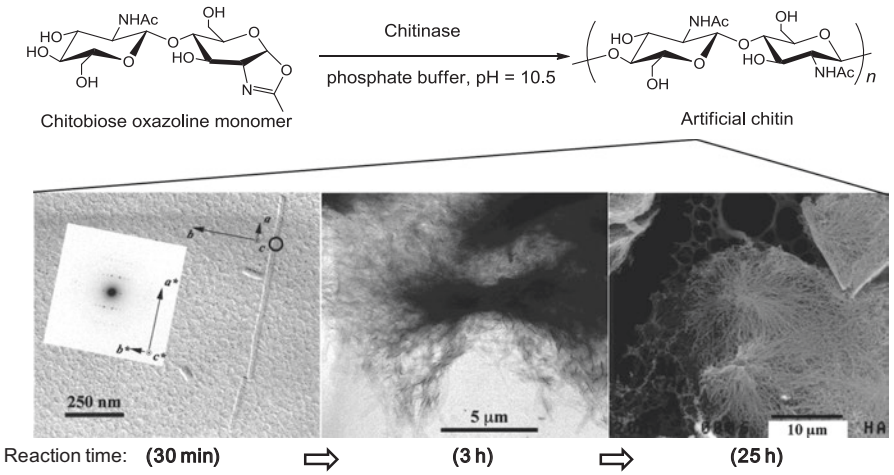


Fig. 2.10 Chitinase-catalyzed ring-opening polyaddition of chitobiose oxazoline and electron microscopy of the resulting artificial chitin. (Reprinted with the permission from ref. [70]. Copyright 2000 American Chemical Society)

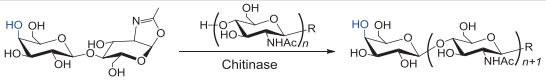
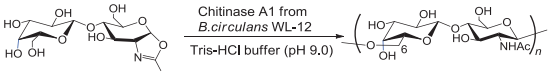
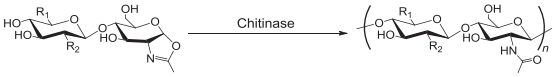
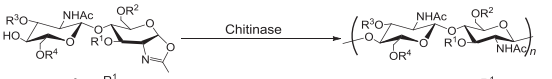
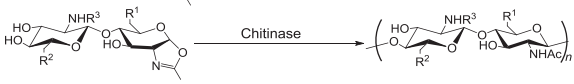
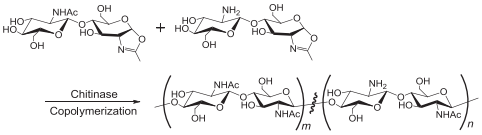
followed by the formation of bundle-like assemblies. After 25 h, the texture of the synthetic chitin spherulites was observed by SEM that revealed the diameters of these spherulites to be 20–50 μm.

A direct method for synthesis of sugar oxazolines from the corresponding *N*-acetyl-2-amino sugars in aqueous media has been developed with chloroformamidinium-type dehydrating reagents (e.g., DMC. 2-chloro-1,3-dimethylimidazolinium chloride; CDMBI, 2-chloro-1,3-dimethyl-1H-benzimidazol-3-ium chloride) [71,72]. This method was applied to one-pot regio- and stereospecific synthesis of chitoheptaose ($(GlcNAc)_7$) by using chitopentaose ($(GlcNAc)_5$) and chitobiose ($(GlcNAc)_2$) as starting materials. The key intermediate, 1,2-oxazoline derivative of $(GlcNAc)_5$, was transglycosylated to a $(GlcNAc)_2$ acceptor catalyzed by a mutant chitinase with lower hydrolyzing activity [73].

2.6.2 Chitinase-Catalyzed Synthesis of Unnatural Oligo- and Polysaccharides

Several artificial disaccharide oxazoline derivatives have been designed as substrates for chitinase-catalyzed synthesis of unnatural oligo- and polysaccharides (Table 2.4). The stepwise elongation of the GlcNAc unit, which was performed via the combined use of chitinase and β-galactosidase catalysis, was demonstrated. Chitinase-catalyzed transglycosylation by using *N*-acetyllactosamine (LacNAc) oxazoline as glycosyl donor and a chitooligosaccharide as glycosyl acceptor allowed the synthesis of unnatural chitooligosaccharides having a galactose unit at the

Table 2.4 Chitinase-catalyzed synthesis of unnatural oligo- and polysaccharides

Entry	Polymerization scheme	References
1		74, 75
2		76
3		55, 77, 78
4		79, 80, 81
5		82, 83, 84
6		85

nonreducing end. Repetition of sequential manipulations using chitinase and β-galactosidase has made it possible to produce chain-length-controlled chitooligosaccharides (Table 2.4, entry 1) [74,75]. Interestingly, in the absence of the glycosyl acceptor, *N*-acetyllactosamine oxazoline was polymerized by chitinase, giving rise to novel oligosaccharides having the β-(1 → 4)-β-(1 → 6)-linked repeating unit in the main chain (Table 2.4, entry 2) [76]. The DP of the resulting oligosaccharides was up to 5 based on the disaccharide. This was the first example of enzymatic glycosylation forming β-(1 → 6)-glycosidic linkage by chitinase catalysis.

Cellulose-chitin hybrid polysaccharide having an alternating Glc and GlcNAc was obtained by the enzymatic polymerization of Glc-β-(1 → 4)-GlcNAc oxazoline monomer catalyzed by a chitinase (Table 2.4, entry 3, $R^1 = CH_2OH$, $R^2 = OH$) [55]. Despite the high crystallinity of both cellulose and chitin homopolymers, the resulting cellulose-chitin hybrid polysaccharide showed no crystalline structure. Similarly, xylan-chitin hybrid polysaccharide having an alternating Xyl and GlcNAc was prepared from Xyl-β-(1 → 4)-GlcNAc oxazoline monomer catalyzed by a chitinase (Table 2.4, entry 3, $R^1 = H$, $R^2 = OH$) [77]. The resulting polysaccharide was water-soluble, and its molecular weight was larger than 1.0×10^4. Chitosan-chitin hybrid-type polysaccharide having a regular alternating sequence of GlcNAc and GlcN was synthesized (Table 2.4, entry 3, $R^1 = CH_2OH$, $R^2 = NH_2$) [78].

Substituted chitin derivatives were also prepared; 3-*O*-methyl-chitobiose oxazoline (Table 2.4, entry 4, R^1 = Me, $R^2 = R^3 = R^4 = H$) or 3′-*O*-methyl-chitobiose oxazoline (Table 2.4, entry 4, R^3 = Me, $R^1 = R^2 = R^4 = H$) gave products of lower degree of polymerization [79]. 6-*O*-Carboxymethylated chitobiose oxazoline

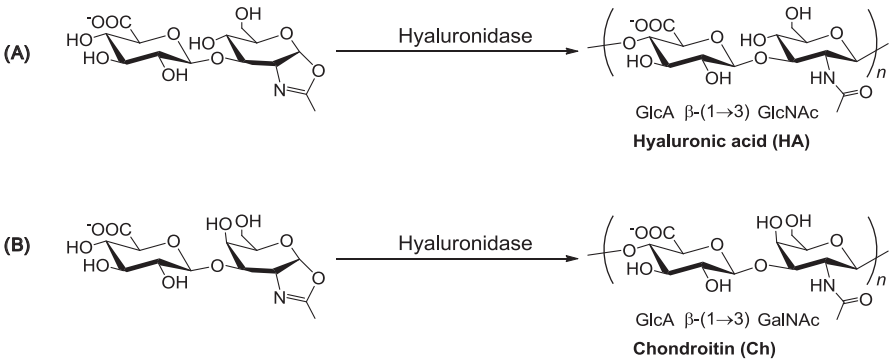
(Table 2.4, entry 4, $R^2 = CH_2COONa$, $R^1 = R^3 = R^4 = H$) and 6′-*O*-carboxymethylated chitobiose oxazoline (Table 2.4, entry 4, $R^4 = CH_2COONa$, $R^1 = R^2 = R^3 = H$) were polymerized by a chitinase, indicating that the introduction of a carboxymethyl group at 6 or 6′ position did not affect the recognition for chitinase [80,81]. Similarly, fluorine-substituted chitobiose oxazoline derivatives at the C-6 and/or C-6′ position could be polymerized by the chitinase catalyst, giving rise to white precipitates of structurally well-defined fluorinated chitins (Table 2.4, entry 5, R^1 and/or R^2 = F, R^3 = Ac) [82,83]. Chitinase-catalyzed polymerization of a TSAS monomer bearing a bulky *N*-sulfonate group at the C-2′ position proceeded homogeneously, due to a good solubility of the resulting polysaccharide (Table 2.4, entry 5, $R^1 = R^2 = OH$, $R^3 = SO_3Na$) [84]. Chitinase from *Serratia marcescens* provided a polysaccharide of *M*n = 4180. With copolymerization of *N*-acetylchitobiose oxazoline monomer with *N,N′*-diacetylchitobiose oxazoline monomer, a tailor-made synthesis of a chitin derivative with controlled deacetylated extent ranging from 0% to 50% was achieved (Table 2.4, entry 6) [85].

2.7 Hyaluronidase

2.7.1 Hyaluronidase-Catalyzed Synthesis of Hyaluronan and Chondroitin

Hyaluronidase (EC 3.2.1.35–36) is an endo-type glycoside hydrolase, which hydrolyzes β-(1 → 4) glycosidic linkage between GlcNAc and GlcA (glucuronic acid) of hyaluronic acid. The hydrolysis mechanism of hyaluronidase is considered similar to that of chitinase due to the presence of GlcNAc at the −1 site of the catalytic center [86,87]. Therefore, based on the concept of a "transition state analogue substrate" (TSAS) monomer proposed first in the synthesis of chitin [65], GlcA-β-(1 → 3)-GlcNAc oxazoline was designed as a monomer for enzymatic polymerization catalyzed by hyaluronidase (Scheme 2.6a). The polymerization afforded the hyaluronic acid (HA) with a high molecular weight *M*n value of 1.74×10^4 in more than 50% yields [88]. HA is present in almost all biological fluids and tissues. In clinical medicine, it is used as a diagnostic marker for many diseases, including cancer, rheumatoid arthritis, and liver pathologies, as well as for supplementation of impaired synovial fluid in arthritic patients by means of intra-articular injections. It is also used in certain ophthalmological and otological surgeries and cosmetic regeneration and reconstruction of soft tissue [89].

Hyaluronidase is known to be an in vivo hydrolysis catalyst of chondroitin (Ch), cleaving its β-(1 → 4) glycosidic linkage. An oxazoline derivative of GlcA-β-(1 → 3)-GalNAc was synthesized, and its polymerization was examined using a hyaluronidase catalyst (Scheme 2.6b) [90]. The corresponding nonsulfated chondroitin of the *M*n value of 5.0×10^3 was obtained.



Scheme 2.6 In vitro synthesis of (**a**) hyaluronic acid (HA) and (**b**) chondroitin (Ch) by using hyaluronidase as enzyme catalyst

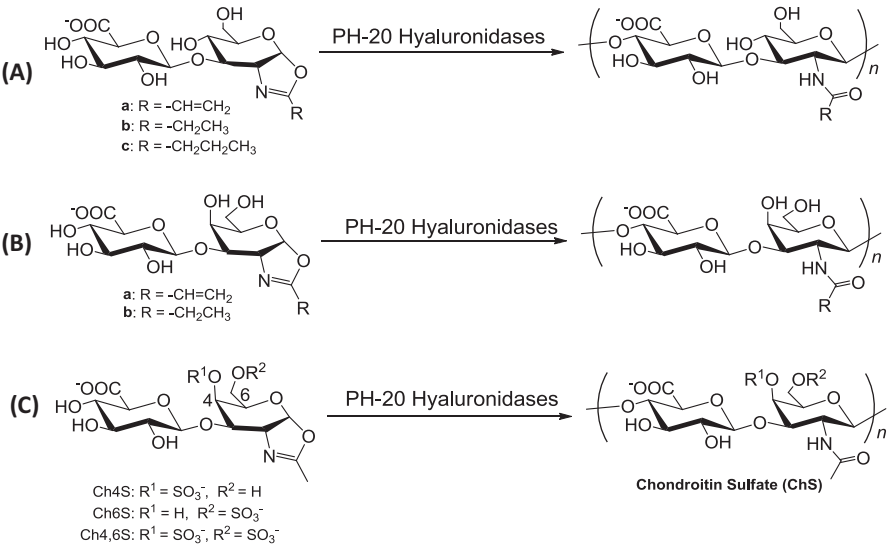
Monomers bearing various substituents at the 2-position (2-vinyl, 2-ethyl, 2-*n*-propyl) were prepared for the production of unnatural HA and Ch bearing the corresponding *N*-acyl groups (Scheme 2.7a and b). Polymerization proceeded successfully with the catalysis of PH-20 HAase, which facilitates penetration for the sperm through the hyaluronan-rich matrix of the oocyte [87].

Chondroitin sulfate is an important structural component of cartilage and provides much of its resistance to compression. Along with glucosamine, chondroitin sulfate has become a widely used dietary supplement for the treatment of osteoarthritis [91]. A structurally well-defined synthetic chondroitin sulfate has been prepared using the hyaluronidase catalyst (Scheme 2.7c) [92]. Among three oxazoline monomers sulfated at C4, C6, and C4 + C6, the monomer sulfated at C4 gave the chondroitin sulfate in good yields. The resulting synthetic chondroitin sulfate has the sulfonate group exclusively at C-4 of the GalNAc unit; the purity of the synthetic ChS-A 100% is to be compared with that of natural ChS-A ~ 80%. The *M*n value ranged from 4.0×10^3 to 1.8×10^4.

2.7.2 Unnatural Hybrid-Type Glycosaminoglycan Synthesis

The use of PH-20 HAase enabled the production of various polysaccharides with well-defined structure through the homopolymerization of sugar-oxazoline monomers. These results imply the possibility of cross reactions, that is, copolymerization of the monomers. First, HA monomers with different substitutes at C2 were copolymerized, affording the corresponding HA_1-co-HA_2 products (Fig. 2.11) [93].

Furthermore, hybrid glycosaminoglycans of hyaluronan-chondroitin (HA-co-Ch) and hyaluronan-chondroitin 4-sulfate (HA-co-Ch4S) can be obtained by enzymatic polymerization using a hyaluronidase catalyst (Fig. 2.11) [94]. *N*-Acetylhyalobiuronate (GlcA-β-(1 → 3)-GlcNAc)-derived oxazoline was copolymerized with *N*-acetylchondrosine (GlcA-β-(1 → 3)-GalNAc)-derived oxazolines by the hyal-



Scheme 2.7 Synthesis of (**a**) *N*-acyl HA derivatives, (**b**) *N*-acyl Ch derivatives, and (**c**) chondroitin sulfate (ChS) by PH-20 HAase-catalyzed polymerization

uronidase catalysis at pH 7.5 and 30 °C, giving rise to the corresponding copolymer with a Mn = 7.4 × 10^3 in 50% yield. Hyaluronidase-catalyzed copolymerization of monomer with *N*-acetylchondrosine oxazoline having a sulfate group at C4 on the GalNAc moiety produced the corresponding copolymer with a Mn = 1.4 × 10^4 in 60% yield. The copolymer compositions can be controlled by varying the comonomer feed ratio.

Hyaluronidase catalyzes multiple enzymatic polymerizations with controlling regio- and stereoselectivity perfectly. This behavior, that is, the single enzyme being effective for multireactions and retaining the enzyme catalytic specificity, is unusual, and hence, hyaluronidase can be considered to be a "supercatalyst" [93].

2.8 Keratanase

Keratanase II obtained from *Bacillus* sp. Ks36 has been used for the enzymatic polymerizations. Even though the details of the enzyme are still unknown, similar ones obtained from *Bacillus circulans* KsT202 have been reported [95]. The enzyme was shown to hydrolyze keratan sulfate between the 4GlcNAc-β-(1 → 3)-Gal structure [96].

Keratan sulfate (KS) is in the class of glycosaminoglycans, which have repeated sulfated disaccharide structures of β-(1 → 3)-linked Gal-β-(1 → 4)-GlcNAc. Besides acting as a constitutive molecule of the extracellular matrices, KS also plays a role as a hydrating and signaling agent in the cornea and cartilage tissues. Inasmuch as

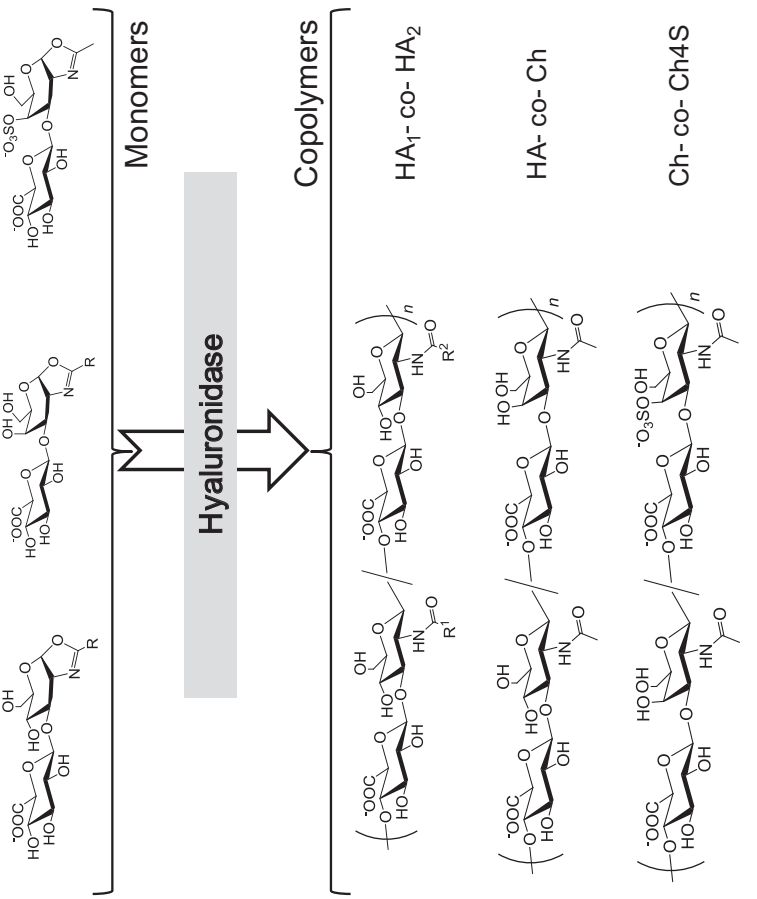


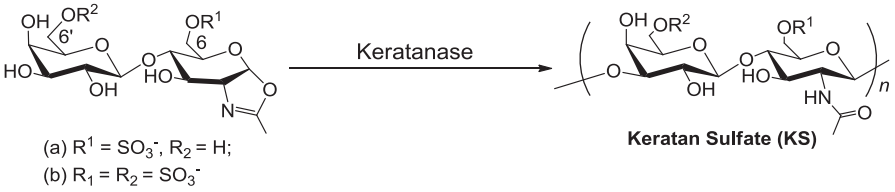
Fig. 2.11 Hyaluronidase-catalyzed copolymerization with the sugar-oxazoline monomers

KS is widely explored in the pharmaceutical industry [97], C-6 sulfated and C-6, 6′ disulfated Gal-β-(1 → 4)-GlcNAc oxazoline monomers have been designed for polymerizations using keratanase II. These two sulfated monomers were polymerized by keratanase II (3.2.1.103), producing the corresponding keratin sulfate oligosaccharides (Scheme 2.8) [98]. Notably, keratanase II catalyzed transglycosylation to form a β-(1 → 3)-glycosidic bond and required the 6-sulfate group in the GlcNAc residue. The information on the catalytic site of keratanase II of KsT202 was further obtained by the examination of the reaction specificity using 6-*O*-sulfonato-Lewis X and its oxazoline derivative as a glycosyl acceptor and donor, respectively. The active site should be constituted as a cleft structure having a steric hindrance around the (+3)(+4) subsites [99].

2.9 Perspective

Enzymatic polymerization that utilizes glycoside hydrolases as catalysts is a promising method not only for synthesis of natural polysaccharides but also for the construction of artificial polysaccharide backbones having definite structures. A variety of monosaccharides (Ⓐ or Ⓑ), disaccharides (Ⓐ-Ⓑ or Ⓐ-Ⓑ), or even larger oligosaccharides are available as refined raw materials from the naturally occurring polysaccharide biomass (Fig. 2.12a). These refined raw materials can be converted to activated glycosyl monomers (Ⓐ-Ⓑ* or Ⓐ-Ⓑ*) via the classical organic reactions with protection and deprotection of the hydroxy groups or the direct anomeric activation methods. The resulting glycosyl monomers are then polymerized by the action of glycoside hydrolases, giving rise to the corresponding polysaccharides having definite structures (Fig. 2.12b). According to this methodology, it has become possible to achieve a process of low environmental impact for production of various natural polysaccharides or functional polysaccharides.

Since the first in vitro synthesis of cellulose was reported by S. Kobayashi et al. in 1991, various kinds of bioactive and structurally complex polysaccharides have successfully been prepared by the enzymatic polymerization of activated glycosyl monomers. Furthermore, recent progress in biotechnology has made it possible to overcome the problems caused by the hydrolysis of the products by wild-type glycosidases; mutated enzymes called glycosynthases were developed with the help of



Scheme 2.8 In vitro synthesis of keratan sulfate (KS) by using keratanase

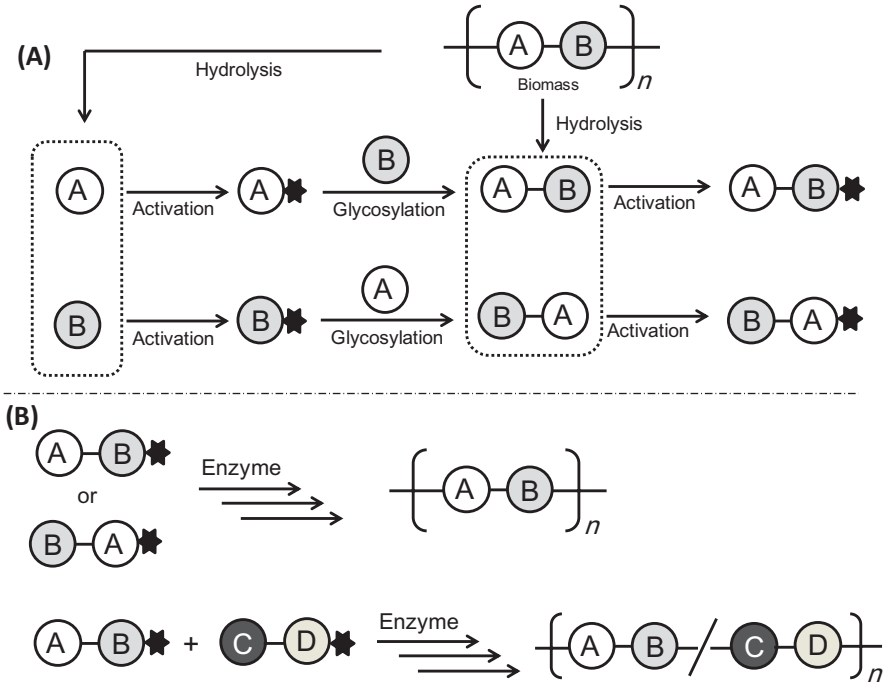


Fig. 2.12 General strategy for the synthesis of glycosyl monomers by the hydrolysis of naturally occurring polysaccharide biomass (**a**) and the enzymatic polymerization toward polysaccharides (**b**)

advanced enzyme engineering such as the site-directed mutation and site-directed evolution technologies.

The enzymatic polymerization catalyzed by glycoside hydrolases are environmentally friendly process because it utilizes a protein, naturally occurring biomacromolecule, whose production requires no strong acid/base or harsh conditions. It is expected that the production of polysaccharides from biomass-derived saccharide monomers catalyzed by glycoside hydrolases will greatly contribute to the future innovation of polymer materials that have been difficult to produce by conventional methodologies, including classical synthetic reactions that have high environmental stress.

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Chapter 3
Synthesis of Polysaccharides II: Phosphorylase as Catalyst

Katja Loos and Jun-ichi Kadokawa

Abstract Oligo- and polysaccharides are important macromolecules in living systems, showing their multifunctional characteristics in the construction of cell walls, energy storage, cell recognition, and their immune response. The chemical synthesis of oligo- and polysaccharides is feasible though it can be laborious since multiple protection, deprotection, and purification steps are required. In contrast to this, phosphorylases are useful synthetic tools for the preparation of natural oligo- and polysaccharides, glycoconjugates, and their analogs. Since phosphorylases are rather tolerant with respect to utilizing modified donors and acceptor substrates, they can be used to prepare oligo- and polysaccharide analogs and for diversification of natural products. Their strict primer-dependence allows synthesis of interesting hybrid materials. Furthermore, enzymatic reaction, such as that using phosphorylase, is one of the most promising environmentally benign technologies with a simple operation under mild conditions, eliminating undesirable side reactions.

Keywords Enzymatic polymerization · Phosphorylase · Polysaccharide · Glycomaterial · Supramolecule

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3.1 Introduction: Overview on Phosphorylase-Catalyzed Enzymatic Polymerization

Polysaccharides are composed of monosaccharide residues linked together through glycosidic bonds, a type of covalent linkage that joins a monosaccharide residue at its anomeric position to another group, typically another saccharide moiety, such that it is principally a result of dehydrative condensation between the components' two hydroxy groups. Polysaccharides in nature are constructed by a wide variety of monosaccharide units linked through the different glycosidic bonds in stereo- and regioarrangements, resulting in quite complicated chemical structures [1, 2]. The structural diversity of polysaccharides leads to providing a whole range of biological functions, in which it is well accepted that a subtle change in their structure has a profound effect on the properties and functions of these molecules.

Polysaccharides are an abundant source of raw materials that are interesting due to the biodegradable, biocompatible, and renewable character. Saccharides are expected to play an increasingly bigger role as raw material in the future and to replace petrol-based materials. Already, polysaccharides find their way in many disparate fields of industry. A short overview is given in Table 3.1.

Conventional chemical synthetic approaches are, in many cases, inadequate to provide substantial quantities of saccharides [3]. The difficulties arise from realizing complete regio- and stereocontrol of the glycosylating process. At present, no such methods are available because, in chemical synthesis, most of the difficulties arise from the laborious regio- and stereochemical control [4–6]. Most synthetic approaches are therefore based on the modification or degradation of naturally occurring polysaccharides resulting in less than perfect products.

In contrast to this, enzymatic approaches have been identified as a powerful tool to synthesize polysaccharides with well-defined structure because enzymatic reaction generally proceeds with highly stereo- and regiocontrolled manners [7–21]. Two approaches have dominated enzyme-catalyzed saccharide synthesis: glycosyl transferase and glycosidase-catalyzed glycosidic bond formation. The first uses the normal biosynthetic machinery of living organisms. In the second, enzymes that normally catalyze transfer of an enzyme-bound glycosyl residue to water are induced to transfer it instead to a different acceptor.

Table 3.1 Polysaccharide-processing industries

Industry	Polysaccharide	Main function
Paper	Cellulose	Structural material
Food	Starch	Thickener, rheological control, texture
Biomedical, pharmaceutical	Dextran	Biocompatibilizer, artificial blood stabilizer, drug carrier
Package	Starch/cellulose derivatives	Reduction of synthetic polymers, increase biodegradability
Coating	Starch	Rheological control
Adhesive	Starch	Adhesive
Textile	Cotton	Structural material

Phosphorylases belong to the class of glycosyltransferases (GTs) which are part of the class of transferases (Enzyme Classification (EC), Class No. 2). These enzymes catalyze reactions in which a group is transferred from one compound to another. Groups that are transferred are Cl, aldehydic or ketonic residues, acyl, glycosyl, alkyl, nitrogenous, phosphorus, and sulfur-containing groups [22]. GTs are important biological catalysts in cellular systems generating complex cell surface glycans involved in adhesion and signaling processes. Recent advances in glycoscience have increased the demands to access significant amount of glycans representing the glycome. GTs catalyze the transfer of a sugar moiety from an activated donor sugar onto saccharide and nonsaccharide acceptors. GTs can be divided into the Leloir and non-Leloir types according to the type of glycosyl donors they use. Non-Leloir GTs typically use glycosyl phosphates as donors, while Leloir GTs utilize sugar nucleotides as donors and transfer the monosaccharide with either retention (retaining enzymes) or inversion (inverting enzymes) of the configuration of the anomeric center. Most of GTs responsible for the biosynthesis of mammalian glycoproteins and glycolipids are Leloir glycosyltransferases. GTs are now playing a key role for in vitro synthesis of oligosaccharides, and the bacterial genome is increasingly utilized for cloning and overexpression of active transferases in glycosylation reactions [23–33].

All of the phosphorylases, which have been found in nature, show strict stereo- and regiospecificities, which thus catalyze the phosphorolysis of a specific glycosidic linkage at the nonreducing end of the saccharide in the presence of inorganic phosphate (Pi) to form a monosaccharide 1-phosphates (Fig. 3.1) [34–36]. Therefore, phosphorylases are classified by the anomeric forms of the resulting monosaccharide 1-phosphates or by the anomeric forms of glycosidic linkages in substrates, which are phosphorolyzed. Alternatively, phosphorylases are classified in terms of retention or inversion at the anomeric position in the reaction. The reversible nature in phosphorylase-catalyzed reactions is conceived because the bond energy of phosphate esters in the product is comparable to that of the glycosidic linkage in the saccharide chain. With respect to reversibility, therefore, phosphorylases catalyze enzymatic glycosylation according to reaction conditions. In the reactions, monosaccharide 1-phosphates act as glycosyl donors, and the monosaccharide residue is transferred from the donor to the nonreducing end of a specific glycosyl acceptor to form glycosidic linkages with strictly controlled stereo- and regioarrangements, with liberating Pi. Some phosphorylases catalyze the reactions for a synthetic way to polysaccharides or oligosaccharides with relatively high DPs via consecutive glycosylations, whereas other phosphorylases only catalyze the reversible phosphorolysis of disaccharide substrates to produce the corresponding monosaccharide 1-phosphates and other monosaccharides. As mentioned mainly in this chapter, only

Fig. 3.1 Reversible phosphorolysis reaction catalyzed by phosphorylase

α-glucan phosphorylase (GP) and cellodextrin phosphorylase (CDP)-catalyzed reactions (EC 2.4.1.1 and 2.4.1.49, respectively) have been used for the practical synthesis of poly- or oligosaccharides with relatively higher DPs, which have also provided polysaccharide- and oligosaccharide-based functional materials [19]. Several works for practical synthesis of oligosaccharides catalyzed by kojibiose phosphorylase (EC 2.4.1.230) have also been reported (vide infra) [37]. Besides them, β-1,3-oligoglucan phosphorylase (EC 2.4.1.30) [38] and β-1,2-oligoglucan phosphorylase [39] also catalyze the phosphorolysis of glucans with the higher DPs than 2. However, there have not been many studies on the synthesis of poly- or oligosaccharides catalyzed by these enzymes.

As abovementioned, GP (systematic name: (1 → 4)-α-D-glucan:phosphate α-D-glucosyltransferase; EC 2.4.1.1) is one of the enzymes which have been used as catalysts for practical synthesis of polysaccharides [40–42]. While this enzyme is responsible for the depolymerization of linear α(1 → 4)-glucosidic chains in vivo, it can also be used to synthesize linear α(1 → 4)-glucosidic chains (amylose) in vitro. The existence of a phosphorylating enzyme in a higher plant was first reported by Iwanoff who observed that an enzyme he found in the germinating vetches, V*icia sativa*, liberates inorganic phosphate from organic phosphorous compounds [43]. Shortly after, the same enzyme was found in other vetches and wheat [44, 45], rice and coleseed [46], barley and malt, etc. Bodńar was the first to report a progressive disappearance of inorganic phosphate (thus the reverse reaction) while incubating suspended flour from ground peas in a phosphate buffer [47]. Cori and Cori demonstrated that animal tissues contain an enzyme which acts upon glycogen as well [48–51]. Cori, Colowick, and Cori suggested that the product of this reaction is α-glucopyranose-1-phosphoric acid (also called Cori-ester), which was confirmed later by Kiessling [52] and Wolfrom and Pletcher [53].

In GPs, glycogen GPs belong to the group of vitamin B_6 enzymes bearing a catalytic mechanism that involves the participation of the phosphate group of pyridoxal-5′-phosphate (PLP). The proposed mechanism is a concerted one with front-side attack as can be seen in Fig. 3.2 [54]. In the forward direction, e.g., phosphorolysis of α(1 → 4)-glycosidic bonds in oligo- or polysaccharides, the reaction is started by protonation of the glycosidic oxygen by orthophosphate, followed by stabilization of the incipient oxocarbonium ion by the phosphate anion and subsequent covalent binding of the phosphate to form α-D-glucose 1-phospate (Glc-1-P). The product, Glc-1-P, dissociates and is replaced by a new incoming phosphate. In the reverse direction, protonation of the phosphate of Glc-1-P destabilizes the glycosidic bond and promotes formation of a glucosyl oxocarbonium ion–phosphate anion pair. In the subsequent step, the phosphate anion becomes essential for promotion of the nucleophilic attack of a terminal glucosyl residue on the carbonium ion. This sequence of reactions brings about α-1,4-glycosidic bond formation and primer elongation. This mechanism accounts for retention of configuration in both directions without requiring sequential double inversion of configuration. It also provides for a plausible explanation of the essential role of pyridoxal-5′-phosphate in glycogen GP catalysis, as the phosphate of the cofactor, pyridoxal-5′-phosphate,

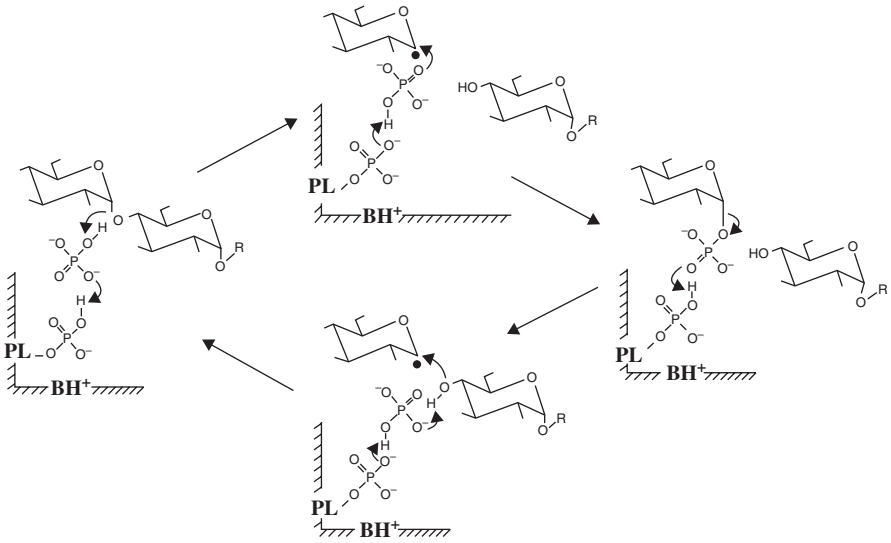

Fig. 3.2 Catalytic mechanism of glycogen phosphorylases. The reaction scheme accounts for the reversibility of phosphorolysis of oligosaccharides (R) in the presence of orthophosphate (upper half) and primer-dependent synthesis in the presence of glucose-1-phosphate (lower half). PL = enzyme-bound pyridoxal; BH^+ = a general base contributed by the enzyme protein. (Reprinted with permission from ref. [54]. Copyright 1990 American Chemical Society)

and the substrate phosphates approach each other within a hydrogen-bond distance allowing proton transfer and making the phosphate of pyridoxal-5′-phosphate into a proton shuttle which recharges the substrate phosphate anion.

The fact that glycogen GP can be used to polymerize amylose was first demonstrated by Schäffner and Specht [55] in 1938 using yeast phosphorylase. Shortly after, the same behavior was also observed for other phosphorylases from yeast by Kiessling [56]; muscles by Cori, Schmidt, and Cori [57]; pea seeds [58]; potatoes by Hanes [58]; and preparations from liver by Ostern and Holmes [59]; Cori, Cori, and Schmidt [60]; and Ostern, Herbert, and Holmes [61]. These results opened up the field of enzymatic polymerizations of amylose using Glc-1-P as monomer and can be considered the first experiments ever to synthesize biological macromolecules in vitro.

One of the remarkable properties of GP is that it is unable to synthesize amylose unless a primer is added (poly- or oligomaltosaccharide); n (Glc-1-P) + primer ⇆ amylose + n (orthophosphate (Pi)). As the GP-catalyzed polymerization is conceived analogously to a living polymerization, accordingly, the molecular weights of the produced amylose can relatively be controlled by the monomer (Glc-1-P)/primer feed ratios. As the complete isolation of amylose from natural starch resources is not facilely achieved, the GP-catalyzed enzymatic polymerization is well accepted as a powerful tool to obtain a pure amylose sample with desired molecular size. Furthermore, a single amylose chain exhibits water solubility but

spontaneously forms an antiparallel double helix to each other owing to its left-handed helical conformation to produce a water-insoluble assembly [62, 63]. By means of this behavior, amylosic materials with hierarchically controlled structure have been fabricated [19, 64–69].

The kinetic behavior of the polymerization of amylose with potato GP with various saccharides as primers was first studied by Hanes [58]. Green and Stumpf [70] failed to detect priming action with maltose but were able to confirm all other results by Hanes. Weibull and Tiselius [71] found that the maltooligosaccharide of lowest molecular weight to exhibit priming activity was maltotriose which was confirmed by Whelan and Bailey [72], who also showed that maltotriose (Glc_3) is the lowest member of the series of oligosaccharides to exhibit priming activity. Whelan and Bailey were also able to clarify the polymerization mechanism of the enzymatic polymerization with GP [72]. Their results showed that the polymerization follows a "multichain" scheme in contrast to a "single-chain" scheme that was also proposed by some authors. In the "multichain" polymerization scheme, the enzyme–substrate complex dissociates after every addition step, whereas in the "single-chain" scheme, each enzyme continuously increases the length of a single primer chain without dissociation.

By studying the polydispersities of amyloses obtained by enzymatic polymerization with potato GP from maltooligosaccharides of various lengths, Pfannemüller and Burchard were able to show that the reaction mechanism of the polymerization with Glc_3 as primer varies from its higher homologues [73]. While the amyloses built by polymerization from maltotetraose (Glc_4) or higher showed a Poisson distribution [74] that can be expected from a polymerization following a "multichain" scheme (random synthesis occurs, and all the primer chains grow at approximately equal rates), a bimodal broad distribution was observed when Glc_3 was used as primer. The authors found that in the case of Glc_3 as a primer, the reaction can be divided into a start reaction and the following propagation, the rate of the first reaction being 400 times slower than the rate of the propagation. Due to this start reaction, not all chains start to grow at the same time which results in a broader distribution. The propagation follows again a "multichain" reaction scheme. Suganuma et al. [75] were able to determine the exact kinetic parameters of the synthetic as well as the phosphorolytic reaction using Glc_3 and higher maltooligosaccharides as primer and were able to confirm the results of Whelan and Bailey and Pfannemüller and Burchard.

Although GP catalysis implies strict stereo- and regiospecificities, i.e., the formation of α(1→4)-glycosidic arrangement, the enzyme exhibits weak specificity for the recognition of monosaccharide structures in the 1-phosphate substrates. Accordingly, GP has also catalyzed glycosylations using analog substrates of Glc-1-P as glycosyl donors to obtain nonnatural oligosaccharides having different monosaccharide residues at the nonreducing end [76–78]. For example, potato GP has been found to recognize α-D-mannose, 2-deoxy-α-D-glucose, α-D-xylose, α-D-glucosamine, and *N*-formyl-α-D-glucosamine 1-phosphates (Man-1-P, dGlc-1-P, Xyl-1-P, GlcN-1-P, and GlcNF-1-P, respectively) as glycosyl donors in glycosylations using Glc_4 as a glycosyl acceptor, to produce nonnatural α-mannosylated,

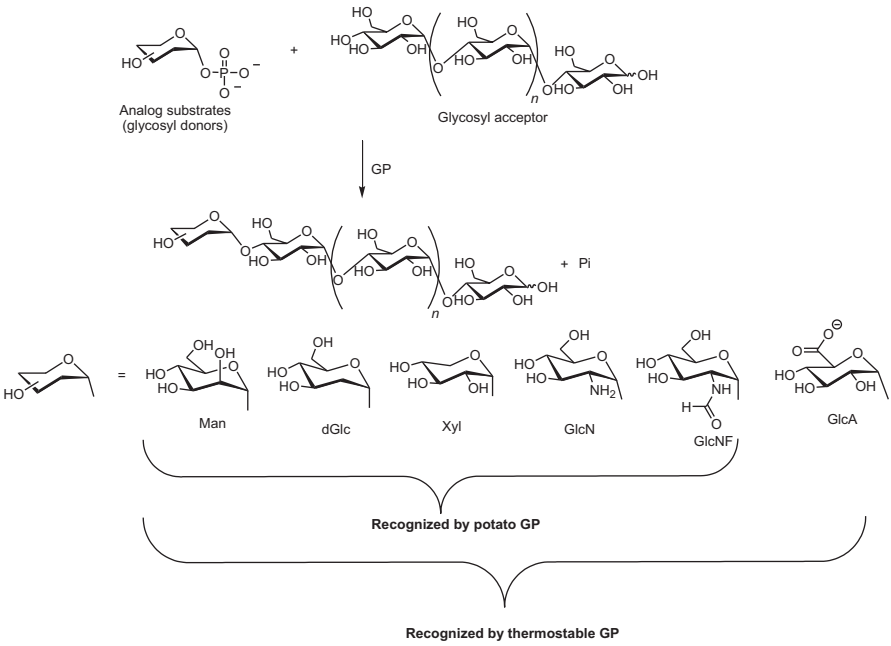

Fig. 3.3 GP-catalyzed enzymatic glycosylations using analog substrates as glycosyl donors to produce nonnatural oligosaccharides

2-deoxy-α-glucosylated, α-xylosylated, α-glucosaminylated, and *N*-formyl-α-glucosaminylated pentasaccharides, respectively (Fig. 3.3) [79–84]. In these reactions, only a monosaccharide residue is transferred from the donor to the acceptor, but consecutive glycosylations, i.e., enzymatic polymerization, are not progressed. The produced α-glucosaminylated Glc_4 by glucosaminylation using GlcN-1-P is a basic oligosaccharide owing to the presence of an amino group at the C-2 position of GlcN unit, while the other products are neutral oligosaccharides. Thermostable GP has shown more tolerance for recognition specificity of the glycosyl donor than that obtained from potato. For example, potato GP does not recognize α-D-glucuronic acid 1-phosphate (GlcA-1-P), while thermostable GP from *Aquifex aeolicus* VF5 [85] recognizes GlcA-1-P and catalyzes glucuronylation of Glc_3 as a glycosyl acceptor to produce an acidic tetrasaccharide having a GlcA residue at the nonreducing end [86].

By means of the enzymatic glucuronylation, dendritic acidic α-glucans have been synthesized [87]. A highly branched cyclic dextrin was employed as a polymeric glycosyl acceptor with plural reactive sites. This material is a water-soluble dextrin, which is produced from amylopectin by cyclization catalyzed by the branching enzyme (EC 2.4.1.18, *Bacillus stearothermophilus*) [88–90]. As the branched dextrin has a number of the nonreducing α(1→4)-glucan ends, this acts as multi-glycosyl acceptors for the GP-catalyzed glucuronylation. The thermostable GP-catalyzed glucuronylation of the branched dextrin ($M_n = 1.25 \times 10^5$, the number

of nonreducing ends = ca. 59) with GlcA-1-P was performed to produce acidic α-glucans. The glucuronylation ratios of the GlcA residues to the nonreducing ends were controlled by the donor/acceptor feed ratios. The subsequent thermostable GP-catalyzed glucosaminylation of the acidic products using GlcN-1-P was examined to obtain dendritic amphoteric α-glucans having both GlcA and GlcN residues at the nonreducing ends [91]. The amphoteric polysaccharides having the different GlcA/GlcN ratios were produced by the successive glucuronylation/glucosaminylation in several glycosyl donor feed ratios of GlcA-1-P/GlcN-1-P. Their inherent isoelectric points were calculated by ζ-potential measurement, which were reasonably changed in accordance with the GlcA/GlcN ratios in the products.

A series of studies on the potato GP-catalyzed glycosylations using the above analog substrates as glycosyl donors has suggested that if a single monosaccharide residue is transferred to the nonreducing end of the maltooligosaccharide acceptor, further glycosylations do not take place because the new structure different from the Glc residue at the nonreducing end is no longer recognized by potato GP. On the other hand, it has been found that when the thermostable GP (from *Aquifex aeolicus* VF5) is alternatively employed as a catalyst in the glycosylations of Glc_3 using excess molar ratios of Man-1-P and GlcN-1-P, consecutive mannosylations/glucosaminylations took place to obtain nonnatural heterooligosaccharides composed of α(1→4)-linked mannose/glucosamine chains at the nonreducing end of Glc_3 [92]. The matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) of the products of the thermostable GP-catalyzed mannosylation/glucosaminylation with a donor to acceptor feed ratio of 10:1 showed several peaks corresponding to the molecular masses of tetra-octa-saccharides having one–five Man or GlcN residues with Glc_3. Further chain elongation for higher DPs, however, was inhibited by Pi produced from the glycosyl donors, which is a naturally occurring substrate for phosphorolysis by GP catalysis as aforementioned.

An attempt was made to remove Pi as a precipitate in the thermostable GP-catalyzed glucosaminylation by conducting the reaction in an ammonium buffer (0.5 M, pH 8.6) containing $MgCl_2$ [93] (Fig. 3.4), based on the fact that Pi forms an insoluble salt with ammonium and magnesium ions [94]. Consequently, the thermostable GP-catalyzed polymerization in the buffer system at 40 °C for 7 days of GlcN-1-P with the Glc_3 primer (30:1) efficiently occurred to produce the α(1→4)-linked glucosamine polymer with a DP of the GlcN units of ~20 (M_n = 3760), that is, an amylose analog aminopolysaccharide, called "amylosamine." The produced polysaccharide is water-soluble and has not formed a controlled higher-order

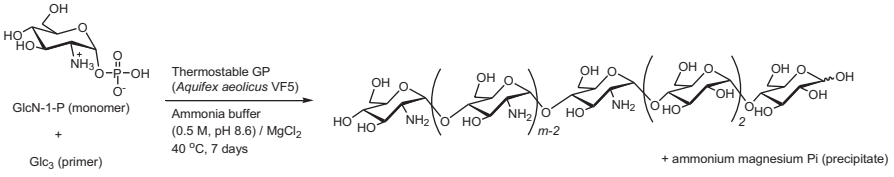

Fig. 3.4 GP-catalyzed enzymatic polymerization of GlcN-1-P in ammonia buffer containing Mg^{2+}

assembly in water as observed for the amylose double helix. It has been found that the cationic amylosamine forms a double helix with anionic amylouronic acid, another amylose analog polysaccharide, by electrostatic interaction [95]. In addition, the thermostable GP-catalyzed enzymatic copolymerizations of Glc-1-P with GlcN-1-P and with Man-1-P have been successfully progressed under the same conditions to obtain nonnatural glucosaminoglucan composed of Glc/GlcN units and mannoglucan composed of Glc/Man units [96, 97]. The thermostable GP-catalyzed enzymatic polymerization of GlcN-1-P using maltooligosaccharide-functionalized amylouronic acid (α(1→4)-linked GlcA polysaccharide with a short α(1→4)-linked Glc chain at the nonreducing end) under the same operation yielded an amylose analog amphoteric block polysaccharide composed of GlcN and GlcA chains [98].

CDP is an enzyme that catalyzes the reversible phosphorolysis of cello-oligosaccharides larger than cellotriose to produce Glc-1-P [99]. Cello-oligosaccharides have been synthesized by the CDP-catalyzed oligomerization using various cellobiose acceptors and Glc-1-P as the glycosyl donor [100, 101]. When cellobiose was used as a glycosyl acceptor, various cello-oligosaccharides ranging from water-soluble products to crystalline assembles were obtained, depending on the concentration of the acceptor. The precision determination of molecular-weight distributions of cello-oligosaccharides synthesized by CDP from *Clostridium stercorarium* or *Clostridium thermocellum* as catalysts was investigated (Fig. 3.5) [102]. The oligocellulose molar mass distribution was analyzed using different methods, such as MALDI-ToF MS. The molar mass distribution of the synthesized oligocellulose was only dependent on the concentration of cellobiose used

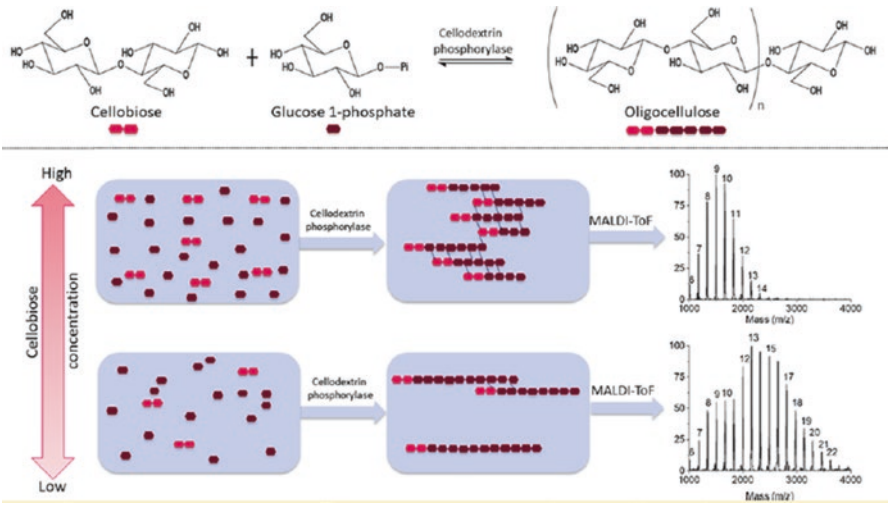

Fig. 3.5 Images and MALDI-ToF MS for molecular-weight distributions of cello-oligosaccharides synthesized by CDP from *Clostridium stercorarium* or *Clostridium thermocellum* (Reprinted with permission from ref [102]. Copyright 2015 American Chemical Society)

in the reaction. Although glucose has been identified as a non-glycosyl acceptor for CDP-catalyzed reaction, a significant amount of insoluble cellulose was precipitated without accumulation of soluble cello-oligosaccharides in this enzymatic reaction system [103]. This result was explained in terms of the large difference in the reactivity of acceptors between glucose and cello-oligosaccharides.

The CDP-catalyzed synthesis of cello-oligosaccharides substituted at their reducing ends has been investigated using various cellobiose derivatives and analogs as the glycosyl acceptors. Cellulose analog oligosaccharides, that is, β(1→3,1→4)-oligosaccharides and thiooligosaccharides, have been synthesized by the CDP-catalyzed oligomerization of Glc-1-P using β(1→3)-linked oligosaccharide acceptors [104, 105]. Moreover, cellobiosylated dimer and trimer and cellobiose-coated polyamidoamine dendrimers have been used as glycosyl acceptors for CDP-catalyzed oligomerization of Glc-1-P to give the corresponding materials composed of cello-oligosaccharide chains at the nonreducing end [106]. Based on the fact that CDP from *Clostridium stercorarium* was found to display a broad donor and acceptor specificity, the enzymatic glycosylations of sophorolipid and glucolipid acceptors with either Glc-1-P or α-D-galactose 1-phosphate as glycosyl donors were achieved to produce novel glycolipids [107]. The transfer of a glucose residue afforded a mixture of products that precipitated from the solution, resulting in near quantitative yields. The transfer of a galactose residue, on the other hand, a single product was generated, which remained in solution at thermodynamic equilibrium.

Kojibiose phosphorylase is an enzyme that catalyzes the phosphorolysis of kojibiose into *β*-D-glucose 1-phosphate and glucose [37]. This enzyme also catalyzes the reverse reaction for chain elongation greater than the disaccharide to synthesize koji-oligosaccharides composed of α(1→2)-linked glucose units. The kojibiose phosphorylase-catalyzed oligomerization has been extensively examined using various glycosyl acceptors to obtain novel oligosaccharides composed of koji-oligosaccharide chains [108–111].

3.2 Enzymatic Preparation of Glycomaterials Using Functional Primers

The GP-catalyzed enzymatic polymerization can be conducted using modified maltooligosaccharide primers, where their reducing ends, which do not take part in the polymerization, are covalently immobilized on various substances, such as low molecular-weight compounds, polymers, and surfaces [19, 64–69].

Pfannemüller et al. showed that it is possible to obtain carbohydrate-containing amphiphiles with various alkyl chains via amide bond formation. For this, maltooligosaccharides were oxidized to the according aldonic acid lactones which could subsequently be coupled to alkylamines [112–119]. Such sugar-based surfactants are important industrial products finding their applications in cosmetics, medical applications, etc. [120–122]. The authors were also able to extend the attached

maltooligosaccharides with enzymatic polymerization with potato GP which resulted in products with very interesting solution properties [123, 124].

Amylosic diblock copolymers have been synthesized by the GP-catalyzed enzymatic polymerization using such polymeric primers having a maltooligosaccharide moiety at the chain end. For example, GP-catalyzed enzymatic polymerization using maltoheptaose (Glc_7)-functionalized polystyrene was performed to produce (amylose-*block*-polystyrene)s [125–127]. Products with various compositions formed micellar aggregates in water and tetrahydrofuran (THF). The analytical results observed the presence of a single-chain, small aggregates, and large micellar species of the block copolymers in THF. Crew-cut micelles, in contrast, were formed in water from the same block copolymers by a single-solvent approach, elevated temperature, and pressure. These crew-cut aggregates were much more uniform than the respective star aggregates in THF. Similarly, amylose-*block*-polyTHF, amylose-*block*-poly(D-lactide), and amylose-*block*-poly(2-vinylpyridine) have been obtained by the GP-catalyzed enzymatic polymerization using the corresponding polymeric primers [128–130].

Amphiphilic amylose-*block*-methoxy-terminated poly(ethylene glycol) (MPEG) has been synthesized by GP-catalyzed enzymatic polymerization using Glc_5-functionalized MPEO primer [131, 132]. A pure amylose is insoluble in chloroform, whereas the product forms reverse micelles, with hydrophobic methyl orange being entrapped in the amylose helix in the micelles in chloroform. Previously various linear block copolymers with PEG – of the AB, ABA and ABC type – with enzymatically polymerized amylose blocks were reported. Ziegast and Pfannemüller converted the hydroxy end groups of PEG into amino groups via tosylation and further reaction with 2-aminoalkylthiolate [133–135]. To the resulting mono- and di-amino-functionalized PEG, maltooligosaccharide lactones were attached and subsequently elongated to amylose via enzymatic polymerization [133]. Pfannemüller et al. performed a very detailed study on the solution properties of the synthesized A-B-A triblock copolymers as they can be considered as model substances for "once broken rod" chains [117]. With static and dynamic light scattering, they found that the flexible joint between the two rigid amylose blocks has no detectable effect on the common static and dynamic properties of the chain. With dielectric measurements it however became obvious that the directional properties of the electric dipoles of the broken rigid chains showed a different behavior to the non-broken rods (pure amylose).

Glycogen is known to be a water-soluble and high molecular-weight natural polysaccharide, composed of linear α(1→4)-glucan chains containing an average of 10 to 14 glucose residues, which are further interlinked by α(1→6)-glycosidic linkages, leading to a highly branched structure and spherical morphology in water [136, 137]. Accordingly, glycogen has the similar chemical structure as the above-mentioned cyclic dextrin, but the molecular weight is much higher. Besides glycogen's role in in vivo phosphorolysis with GP as an energy resource, therefore, it can be also used as polymeric primer for the GP-catalyzed enzymatic polymerizations because of the presence of a number of nonreducing α(1→4)-glucan chain ends. When the GP-catalyzed enzymatic polymerization of Glc-1-P on glycogen

was carried out in aqueous acetate buffer solution, followed by the standing of the reaction mixture under ambient atmosphere for 24 h, the solution fully turned into a hydrogel [138]. Hydrogelation is induced by the formation of double-helix cross-linking points by the elongated amylose chains among glycogen molecules because amyloses are well-known to spontaneously form double helixes in water (Fig. 3.6). The hydrogel was then converted into a cryogel by lyophilization. The scanning electron microscope (SEM) image of the cryogel observed porous morphology. The X-ray diffraction (XRD) profile of the cryogel showed diffraction peaks ascribed to the crystalline structure of amylose double helix. This result indicated that the networks in the cryogels were constructed based on the double helical entanglement of the elongated amylose chains, resulting in the formation of the porous morphologies.

By means of the thermostable GP-catalyzed consecutive glucosaminylation/glucuronylation of glycogen using GlcN-1-P and GlcA-1-P, amphoteric glycogens having both basic GlcN and acidic GlcA residues could also be synthesized (Fig. 3.6) [139]. The functionalities of the GlcA/GlcN residues depended on the feed ratios of glycosyl donors. The GP-catalyzed enzymatic polymerization of Glc-1-P with the non-functionalized, nonreducing ends of the amphoteric glycogens was then examined to produce amphoteric glycogen hydrogels through the formation of double helix cross-link by the elongated amylose chains (Fig. 3.6). The resulting hydrogels exhibited pH-responsive behavior, in which they were solubilized under alkaline conditions and returned to hydrogels upon acidification of the system. Furthermore, the hydrogels exhibited pH-dependent shrinking/swelling behavior.

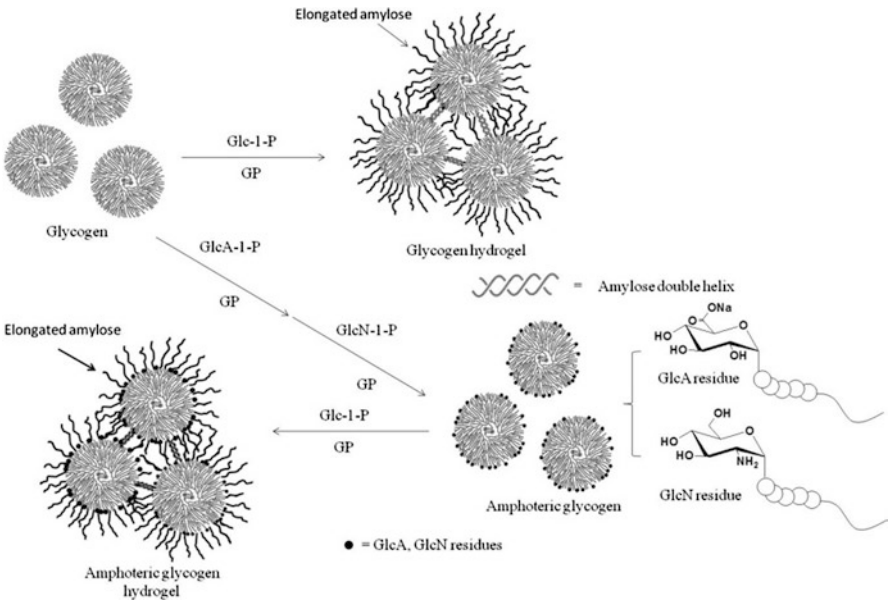

Fig. 3.6 GP-catalyzed enzymatic polymerization using glycogen to produce hydrogel and consecutive enzymatic reactions to produce amphoteric glycogen hydrogel

The GP-catalyzed enzymatic polymerization of Glc-1-P using maltooligosaccharide-grafted polymeric primers, which are prepared by the covalent bond formation at the reducing end on the polymeric substrates, has been performed to produce amylose-grafted polymeric materials (chemoenzymatic approach) [19, 64–69]. For example, a styrene macromonomer having an amylose chain was prepared by the GP-catalyzed enzymatic polymerization using a primer having a polymerizable group at the reducing end. Radical polymerization of the macromonomer produced an amylose-grafted polystyrene [140]. This type of the graft material was also synthesized by radical polymerization of a styrene macromonomer having a maltooligosaccharide, followed by the GP-catalyzed enzymatic polymerization from the nonreducing end of the maltooligosaccharide primer on the product [140, 141]. By the similar approach, amylose-grafted dimethylsiloxane, polyacetylene, and poly(vinyl alcohol) have been synthesized [142–144]. A series of amylose-based star polymers (1, 2, 4, and 8 arms) have been synthesized by GP-catalyzed enzymatic polymerization using Glc_5-functionalized PEG (Fig. 3.7) [145]. The obtained eight-arm primer serves as a gelator when triggered enzymatically.

The chemoenzymatic approach is also appropriately conducted for the amylosic modification of spherical and planner surface, such as Au and Si. The Au and Si surfaces were amino-functionalized with self-assembled monolayers of cystamine and 3-aminopropyldimethylethoxysilane (APDMES), respectively. Glc_7 was covalently attached to the amino-functionalized Au and Si surfaces via reductive amination. Amylose brushes were grown from Glc_7-modified surfaces with GP-catalyzed enzymatic polymerization (Fig. 3.8) [146, 147].

The chemoenzymatic approach has extensively been applied to the synthesis of amylosic conjugates with biopolymeric main-chains. Interestingly, the gelling

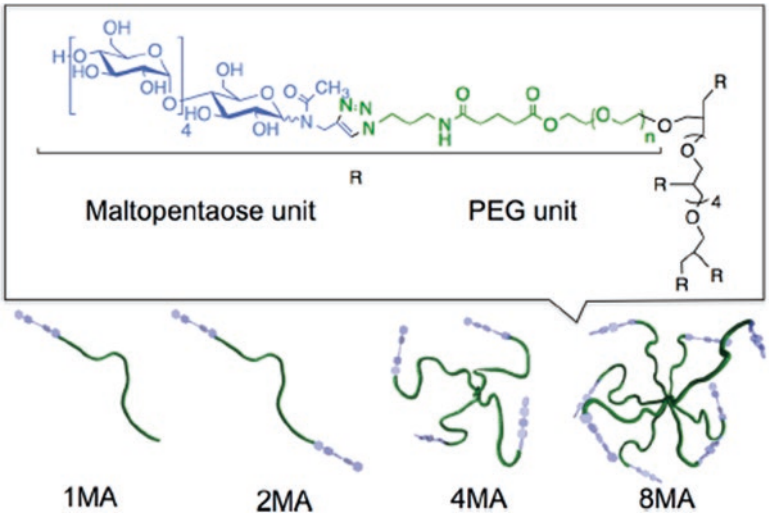

Fig. 3.7 Chemical structures and illustrations of Glc_5-functionalized PEG primers. (Reprinted with permission from ref. [145]. Copyright 2015 American Chemical Society)

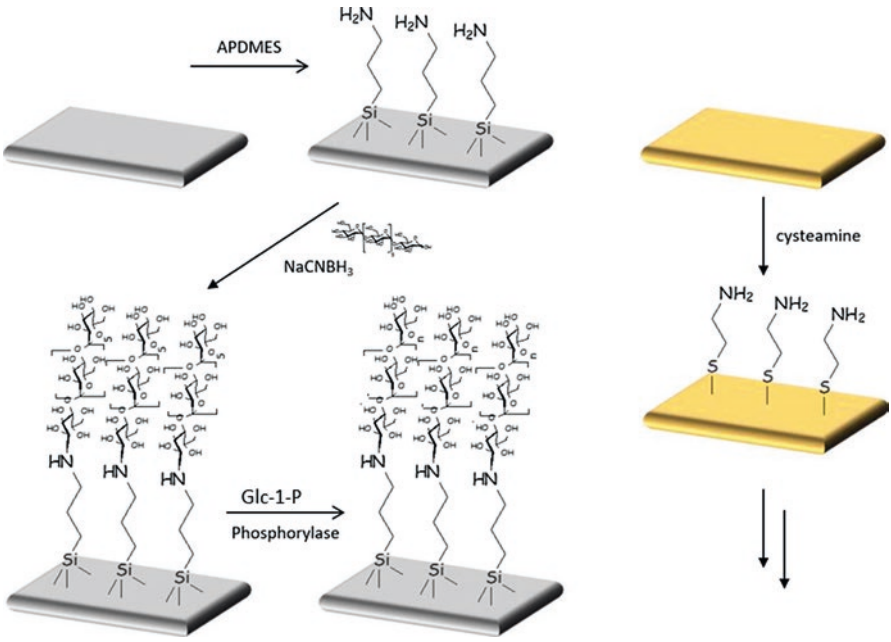

Fig. 3.8 Synthesis of amylose brushes via GP-catalyzed enzymatic polymerization from silica (left) and gold surfaces (right). (Adapted from ref. [146])

system from amylose with suitable biopolymeric components has been reported and is constructed by the formation of amylose double helix as non-covalent cross-linking points through the GP-catalyzed enzymatic polymerization. For example, amylose-grafted heteropolysaccharides composed of abundant polysaccharide main-chains have been synthesized by this approach. The maltooligosaccharides have been introduced onto the main-chain polysaccharides by appropriate chemical reactions, such as reductive amination and condensation. Using the reductive amination, chitosan, a basic polysaccharide with amino groups at the C-2 position in its glucosamine repeating units, was functionalized to obtain a maltooligosaccharide-grafted chitosan, which could be further converted into maltooligosaccharide-grafted chitin by *N*-acetylation. From the maltooligosaccharide primer ends of the chitin/chitosan derivatives, amylose chains were elongated by the GP-catalyzed enzymatic polymerization of Glc-1-P to yield amylose-grafted chitin/chitosan (Fig. 3.9) [148, 149]. A hydrogel of the amylose-grafted chitosan was produced by slowly drying the polymerization mixture at 40–50 °C. An amylose-grafted cellulose was also synthesized using a similar procedure [150]. A partially aminated cellulose derivative at the C-6 position was initially prepared by the successive partial tosylation of its C-6 hydroxy groups, displacement of the tosylates by azido groups, and their subsequent reduction to amine groups. The resulting cellulose derivative was then reacted with a maltooligosaccharide by reductive amination to give a maltooligosaccharide primer-grafted cellulose, which was used for the GP-catalyzed enzymatic polymerization to produce the amylose-grafted cellulose (Fig. 3.9). The produced heteropolysaccha-

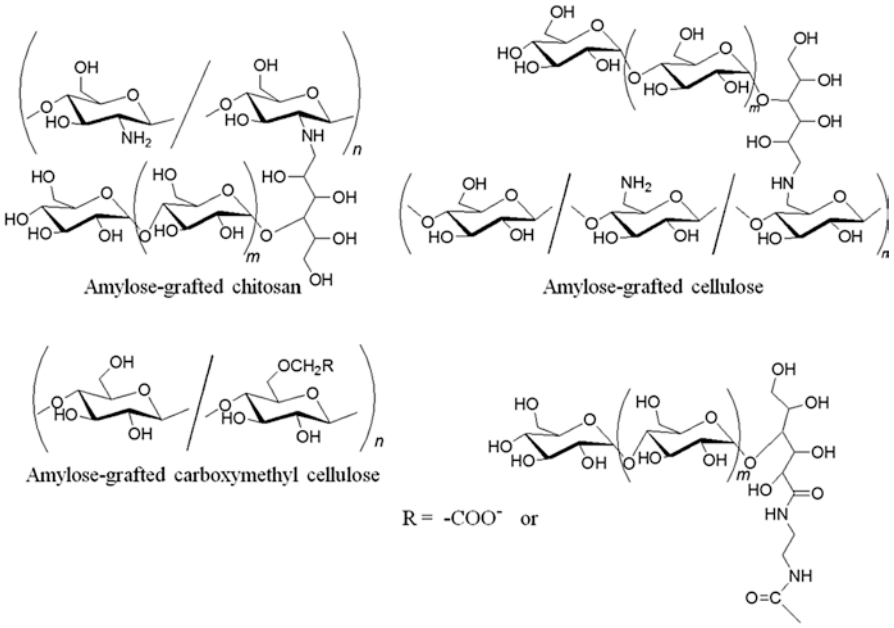

Fig. 3.9 Chemical structures of amylose-grafted chitosan, cellulose, and carboxymethyl cellulose

ride is made up of two representative glucose polymers, cellulose and amylose, which are composed of the same repeating units but linked through opposite stereo-arranged glycosidic bonds, β(1→4)– and α(1→4)–, respectively. The polymerization mixture totally turned into gel form when it was left standing at room temperature for several days. Drying the hydrogel under ambient atmosphere gave a solid material. The addition of water to the solid resulted in hydrogelation again. Such conversion cycle was repeated by the wetting and drying process. Moreover, the reaction mixture of the enzymatic polymerization was spread thinly on a glass plate and subsequently left standing at room temperature, resulting in a film.

Amylose-grafted chitin nanofibers were also fabricated by the chemoenzymatic approach including reductive amination [151]. Nanofibrillated materials from native chitin, so-called chitin nanofibers, have increasingly attracted much attention and are expected to find applications as new functional materials. As native chitin sources are made up of nanofibrous assemblies, several methods for disentanglement of the assemblies have efficiently been developed to fabricate nanofiber dispersions in water. Re-dispersible amidinium chitin nanofibers are also obtained from an amidinated chitin by CO_2 gas bubbling with ultrasonic treatment in water [152]. For the chemoenzymatic approach, maltooligosaccharide primers were first introduced by reductive amination with amino groups present on the amidinium chitin nanofibers partially having amino groups. Elongating of amylose chains on the nanofibers was then carried out by the GP-catalyzed enzymatic polymerization from the maltooligosaccharide graft chains to produce amylose-grafted chitin nanofiber materials. The reaction mixtures turned into hydrogels upon increasing the Glc-1-P/primer

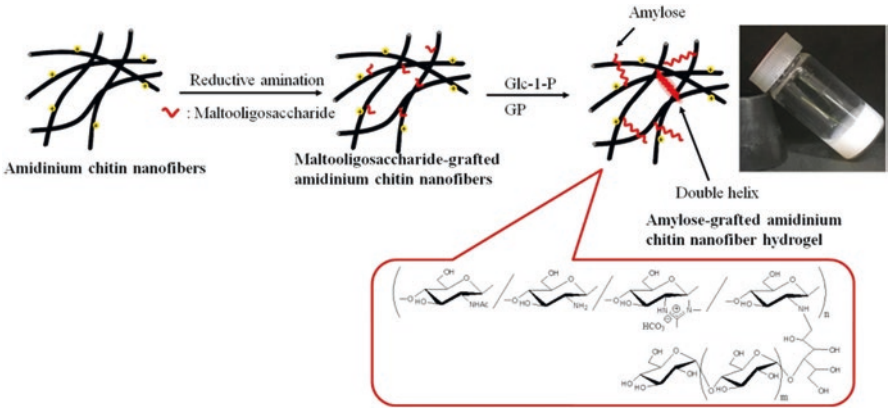

Fig. 3.10 Chemoenzymatic synthesis of amylose-grafted chitin nanofibers

feed ratios (Fig. 3.10). The formation of double helixes from a part of amylose graft chains closely preset among the nanofibers contributed to forming the smaller networks, resulting in hydrogelation. Lyophilization of the hydrogels gave rise to the formation of the controlled microstructures, which were changed from fibrous to porous morphologies in accordance with the molecular weights of the amylose graft chains. Most of the amylose graft chains with the higher molecular weights, which did not take part in double helixes, formed amorphous membranes inside the nanofiber networks by lyophilization, to construct the porous structure.

The condensation reaction using an amine-functionalized maltooligosaccharide at the reducing end was also employed to prepare modified primers from acidic polysaccharides, such as alginate, xanthan gum, and carboxymethyl cellulose (CMC) having carboxylate groups. The GP-catalyzed enzymatic polymerization from the maltooligosaccharide chain ends of the condensation products was then carried out to obtain amylose-grafted alginate, xanthan gum, and CMC (Fig. 3.9) [153–156]. A film of the amylose-grafted CMC was formed by drying the thinly spread alkaline solution (0.040 g polysaccharide in 1.5 mL 0.50 M aqueous NaOH solution). The SEM image of the film showed highly entangled nanofibers. After washing out the residual NaOH from the film by immersion in water, the SEM image showed that the nanofibers were merged at the interface, while the arrangement of fiber was retained.

The chemoenzymatic approach has been successfully conducted to produce amylose-grafted polypeptides. For example, a maltopentaosyl amine was treated with carboxylate groups of poly(L-glutamic acid) using the condensation agent to produce a maltopentaose-grafted poly(L-glutamic acid). The product was employed as a polymeric primer for the GP-catalyzed enzymatic polymerization to obtain amylose-grafted poly(L-glutamic acid) [157]. Another polypeptide, poly(L-lysin) (PLL) having amino groups, was also used for the chemoenzymatic approach. Maltooligosaccharide was introduced into cholesterol-bearing poly(L-lysine) material by reductive amination [158]. The product, ChMaPLL31 (31 maltopentaoses

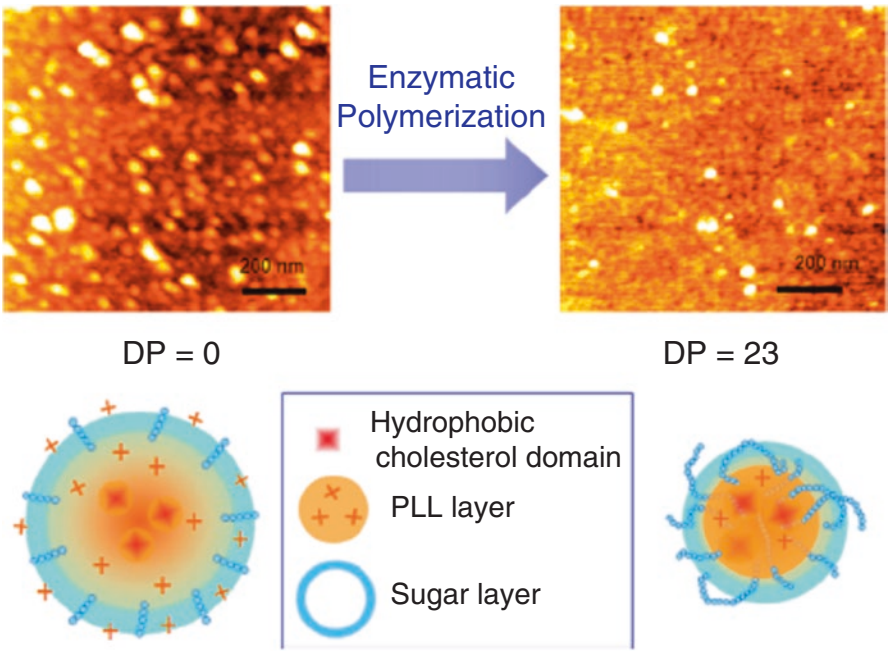

Fig. 3.11 AFM images and schematic illustrations before and after the enzymatic polymerization of ChMaPLL31 nanogels. (Reprinted with permission from ref. [158]. Copyright 2013 American Chemical Society)

per 100 lysine residues), forms positively charged nanogel with an average diameter of 50 nm via self-assembly in water. The nanogel was suited as a primer for the GP-catalyzed enzymatic polymerization. The average diameter after the enzymatic polymerization decreased to 30 nm as observed by AFM measurement (Fig. 3.11). The produced nanogel exhibited a weak negative charge, suggesting the shielding of the cationic charged surfaces by the elongated amylose graft chains.

A natural polypeptide, poly(γ-glutamic acid) (PGA), has been employed for the chemoenzymatic approach [159] because it has carboxylate groups and is a well-known material for multifarious potential applications in foods, pharmaceuticals, healthcare, water treatment, and other fields. Amine-functionalized maltooligosaccharide primers were first introduced on PGA main-chain by condensation using the condensing agent in aqueous NaOH. The GP-catalyzed enzymatic polymerization was then conducted from the primer chain ends of the product to obtain amylose-grafted PGAs (Fig. 3.12). The products formed hydrogels in reaction media depending on Glc-1-P/primer feed ratios. The amylose graft chains formed double helixes, which contributed to constructing network structure as cross-linking points for hydrogelation. The SEM images of the cryogels, which were obtained by lyophilization of the hydrogels, observed regularly controlled porous morphologies. Furthermore, pore sizes increased upon increasing Glc-1-P/primer feed ratios, while the degrees of substitution of primer on the PGA main-chain did not obviously affect pore sizes.

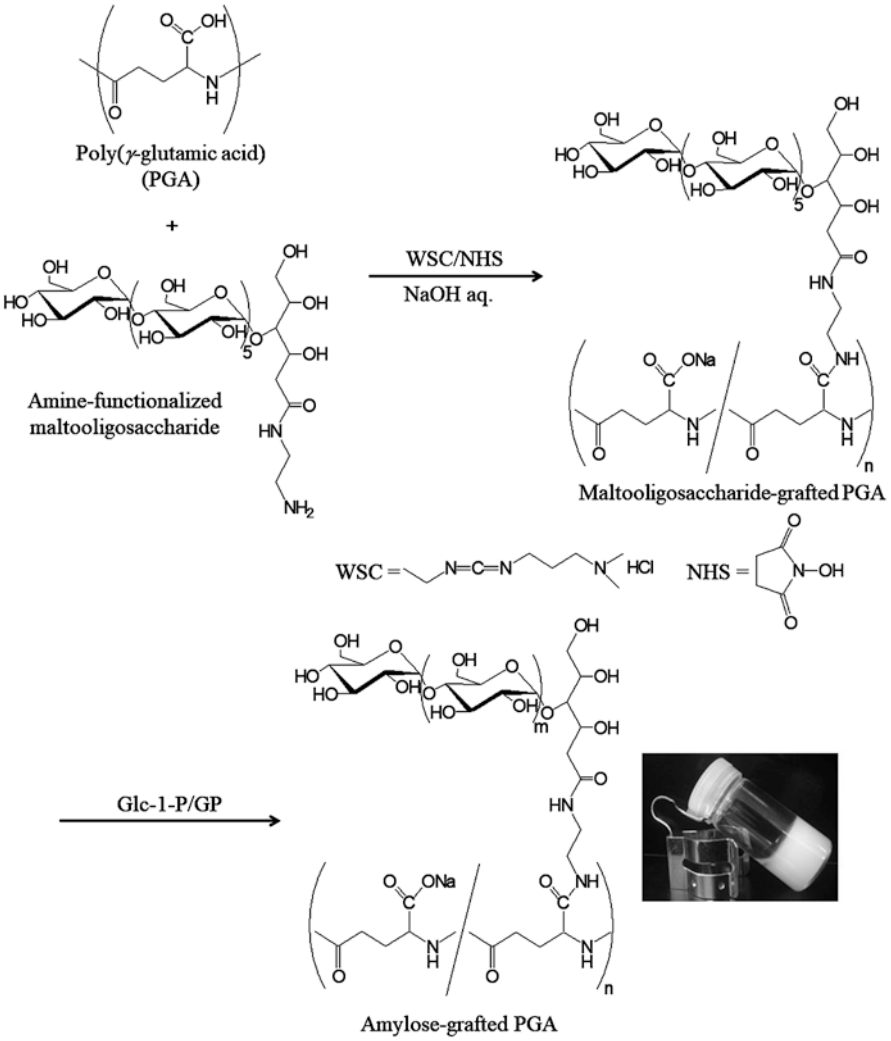

Fig. 3.12 Chemoenzymatic synthesis of amylose-grafted PGA

3.3 Fabrication of Supramolecules and Controlled Assemblies in Phosphorylase-Catalyzed Enzymatic Polymerization Field

Amylose acts as a host molecule owing to its left-handed helical conformation to form inclusion complexes with various guest molecules, typically monomeric and oligomeric low molecular-weight compounds [160, 161]. The driving force for the binding of guest molecules is hydrophobic interactions, as the amylose cavity is hydrophobic, and therefore, in aqueous solvents, hydrophobic guest molecules are

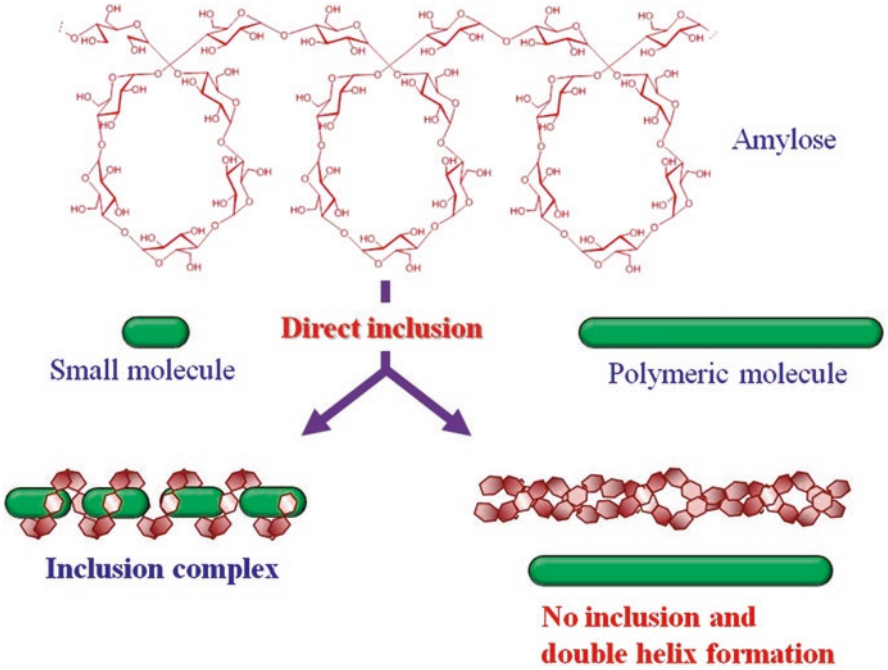

Fig. 3.13 Amylose forms inclusion complex with relatively low molecular-weight (small) hydrophobic molecule but largely does not form it with polymeric molecule

spontaneously included in the amylose cavity (Fig. 3.13). However, a limited number of investigations have been reported on the complexation of amylose with polymeric guest molecules (Fig. 3.13). As the difficulty in the inclusion of polymeric guest molecules into the amylose cavity arises from the necessity for a weak hydrophobic interaction as driving force of the complexation, amylose does not have a sufficient ability to direct the inclusion of long polymeric chains into its cavity. For the direct incorporation of polymeric guests, hydrophilic groups have been introduced at the polymer chain ends, which enhance the complexation ability by amylose in aqueous media [162, 163]. Additional methods for directly forming amylose–polymer inclusion complexes have been achieved by inclusion polymerization and guest-exchange approaches [164–166].

An efficient method for the direct construction of amylose–polymer inclusion complexes has been developed by means of the GP-catalyzed enzymatic polymerization. In this enzymatic polymerization system, which involves dispersion with appropriate hydrophobic guest polymers in an aqueous polymerization solvent, the propagation is progressed with the formation of inclusion complexes between the produced amylose and guest polymers [65, 167–172]. The elongation of the short α(1→4)-glucan (maltooligosaccharide primer) to the longer α(1→4)-glucan (amylose) is considered to provide sufficient field for more facile complexation of polymeric guests compared to the direct complexation between the polymeric amylose

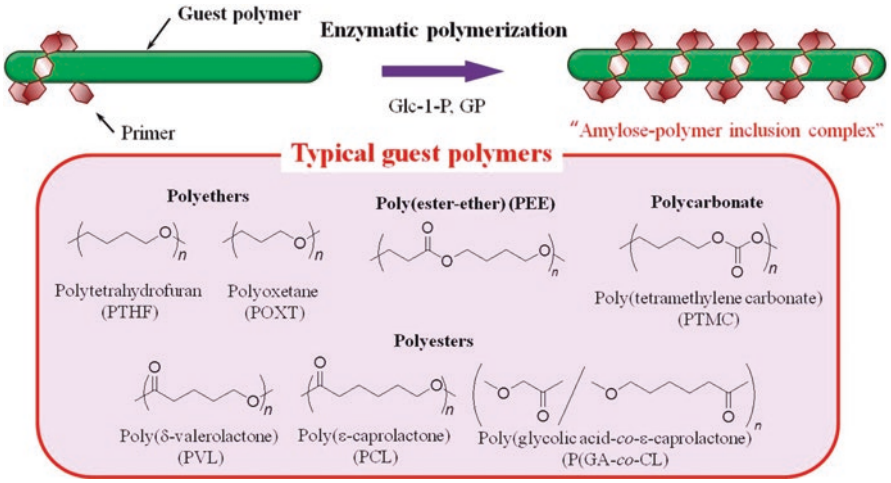

Fig. 3.14 Image for vine-twining polymerization and typical guest polymers

host and guest. The process of this host-guest interaction method for the generation of such inclusion complexes is similar to the way that vines of plants grow and twine around a rod, which is the reason why this polymerization approach has been named "vine-twining polymerization" (Fig. 3.14). Hydrophobic polyethers such as poly(tetrahydrofuran) (PTHF) and poly(oxetane) (POXT) [173, 174]; polyesters such as poly(δ-valerolactone) (PVL), poly(ε-caprolactone) (PCL), and poly(glycolic acid-*co*-ε-caprolactone) [175–177]; polycarbonates such as poly(tetramethylene carbonate) [178]; and poly(ester-ether)s ($-CH_2CH_2C(C{=}O)OCH_2CH_2CH_2CH_2-$) [176] have been found to act as guest polymers and included into amylose using this polymerization technique. To investigate the effect of the structures of polyethers on inclusion complexation in vine-twining polymerization, the GP-catalyzed enzymatic polymerization of G-1-P was conducted using polyethers with different alkyl chain lengths, that is, PTHF (4 methylenes), poly(oxetane) (POXT, 3 methylenes), and PEG (2 methylenes). Consequently, the hydrophobic POXT formed an inclusion complex with amylose, whereas vine-twining polymerization with PEG did not induce inclusion complexation. A hydrophilic poly(ester-ether) ($-CH_2CH_2C(=O)OCH_2CH_2O-$) with a shorter methylene length also did not form an inclusion complex. In addition, complexation via vine-twining polymerization has not been achieved with strongly hydrophobic polymers like a polyether, poly(oxepane) with 6 methylenes, due to their aggregation in aqueous buffer solvents as a result of their longer alkyl chains when compared to PTHF and POXT. It, therefore, is concluded that the moderate hydrophobicity of guest polymers is the important factor in determining whether or not amylose will form inclusion complexes during a vine-twining polymerization.

An attempt of a parallel enzymatic polymerization system was made to achieve the formation of an inclusion complex with a strongly hydrophobic polyester [179]. In this approach, two enzymatic polymerizations, the GP-catalyzed enzymatic

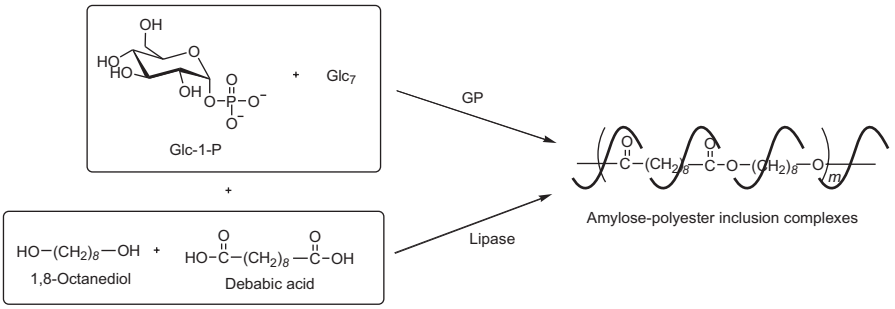

Fig. 3.15 Parallel enzymatic polymerization system to produce amylose-polyester inclusion complex

polymerization of Glc-1-P from the Glc_7 primer to produce amylose and the lipase-catalyzed polycondensation of a diol (1,8-octanediol) and a dicarboxylic acid (sebacic acid) to produce a strongly hydrophobic aliphatic polyester as the guest polymer [180, 181], were simultaneously conducted in an aqueous buffer solvent (Fig. 3.15). The analysis of the product supported the formation of the inclusion complex of amylose with the polyester.

Amylose showed the selective inclusion behavior in certain structures, molecular-weight distributions, and chiralities of the guest polymers in the vine-twining polymerization system. By means of the vine-twining polymerization technique, the selective inclusion complexation toward two resemblant polymers by amylose has been achieved. For example, amylose selectively included one polyether, that is, PTHF from a mixture of PTHF/POXT in the vine-twining polymerization [182]. The selective inclusion by amylose was also found when the vine-twining polymerization was investigated in the presence of a mixture of two resemblant polyesters, PVL/PCL, in which PVL was selectively included by amylose [183].

Amylose also showed selective inclusion behavior toward a specific range of molecular weights of guest polymers in vine-twining polymerization [169]. Synthetic polymers are considered to be mixtures of different molecular-weight analogs, which possess different properties. For example, the molecular weight of PTHF polymers affects its hydrophobicity and water solubility, where low molecular-weight PTHF exhibits water solubility, whereas those with larger molecular weight are hydrophobic and show insolubility in water. When several vine-twining polymerization systems were examined using PTHFs with different average molecular weights, the specific range of molecular weights of all PTHFs was suitably recognized by amylose to form inclusion complexes.

Aside from the chemical structure and molecular weight, amylose showed selectivity toward the chirality in guest polymers in vine-twining polymerization. The selective inclusion of chiral molecules by amylose was achieved using chiral polyesters, poly(lactide)s (PLAs) as guest polymers with three stereoisomers, i.e., poly(L-lactide) (PLLA), poly(D-lactide) (PDLA), and racemic poly(DL-lactide) (PLDLA) [184]. When vine-twining polymerization was conducted using PLLA, an inclusion complex was formed, while from the PDLA and PDLLA, inclusion com-

plexation was not observed. The similar selective inclusion was also observed in vine-twining polymerization using chiral polyalanine (PAlas) stereoisomers as guest polymers [185]. An inclusion complex was formed with poly(D-alanine) (PDAla), whereas inclusion complexes were not obtained with poly(L-alanine) (PLAla) or poly(DL-alanine) (PDLAla). The stereoselective inclusion behavior of amylose based on chirality in vine-twining polymerization is explained by the helical direction of the host and guest polymers. The left-handed helical conformation of PLLA and PDAla is the same direction as that of the host amylose, resulting in their efficient inclusion. In contrast, the opposite and irregular helical conformations of the other stereoisomers are not suitable for binding by the amylose helix.

The vine-twining polymerization approach by the GP-catalyzed enzymatic polymerization has been applied to the preparation of amylosic supramolecular networks based on amylose–polymer inclusion complexes. Such supramolecular network materials, e.g., hydrogels, which are hierarchically composed of inclusion complexes as cross-linking points, were designed as the vine-twining polymerization products by using graft copolymers with hydrophobic graft chains. As shown in Fig. 3.16, the enzymatically produced amylose chains by GP catalysis potentially include the hydrophobic graft chains as guest polymers to form inclusion complexes, which act as cross-linking points to hierarchically construct the supramolecular

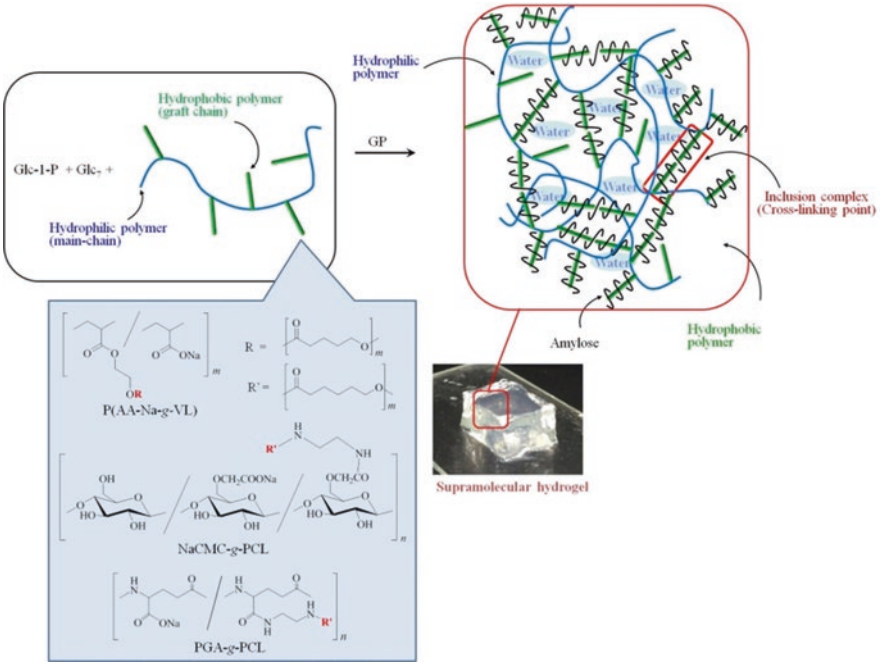

Fig. 3.16 Preparation of amylosic supramolecular networks by vine-twining polymerization using graft copolymers having hydrophilic main-chains and hydrophobic guest graft chains

network structure in aqueous media, giving rise to hydrogels. The hydrophobicity of the graft chains as guest polymers is necessary, while the graft copolymer should generally be water-soluble to efficiently act as a component of hydrogels.

For example, the hierarchical construction of hydrogels was achieved by the GP-catalyzed enzymatic polymerization of Glc-1-P from Glc_7, according to the vine-twining polymerization manner in the presence of a water-soluble graft copolymers composed of hydrophobic PVL or PCL graft chains, that is, poly(acrylic acid sodium salt-*graft*-δ-valerolactone) (P(AA-Na-*g*-VL)) [186], CMC (sodium salt)-*graft*-PCL (NaCMC-*g*-PCL) [187], and poly(γ-glutamic acid-*graft*-ε-caprolactone) (PGA-*g*-PCL) [188]. The enzymatic reaction mixtures completely turned into the hydrogel form, where the hydrophilic main-chains, PAA, NaCMC, and PGA, acted as the main components in the hydrogels. The enzymatically produced amylose included the PVL graft chains between the intermolecular (P(AA-Na-*g*-VL)s to form inclusion complexes as the polymerization progressed, which acted as cross-linking points for hydrogelation. The ability of the resulting hydrogel to reversibly facilitate enzymatic disruption and reproduction was successfully demonstrated by the combined use of β-amylase-catalyzed hydrolysis of the amylose component in the hydrogel, followed by its reformation by the GP-catalyzed enzymatic polymerization. A film was further formed by adding water, followed by drying, to a powdered sample, which was prepared by lyophilization of the hydrogel from NaCMC-*g*-PCL.

The mechanical properties of the hydrogels obtained by the above systems using PAA-Na-*g*-PVL and NaCMC-*g*-PCL, however, were not sufficient for further applications. To improve the mechanical properties of the hydrogels, PGA has been used as the main-chain of a graft copolymer, based on the viewpoint of its better water retention and moisturizing properties. Indeed, vine-twining polymerization using PGA-*g*-PCL resulted in the formation of a hydrogel with self-standing properties, indicating much better mechanical properties compared to the abovementioned hydrogels. It was found, moreover, that the resulting hydrogel showed the macroscopic interfacial healing through the GP-catalyzed enzymatic polymerization. The hydrogel initially formed, as the vine-twining polymerization product, was cut into two pieces, and a sodium acetate buffer solution dissolving Glc-1-P and GP was dropped on the surfaces of the hydrogel pieces. After the surfaces were placed in contact with one another, the materials were left standing for the progress of the GP-catalyzed enzymatic polymerization. Consequently, the two pieces were fused at the contacted area. Such healing behavior of the hydrogels on a macroscopic level was induced by the complexation of the enzymatically produced amyloses with the PCL graft chains at the interface. In addition, a porous cryogel and an ion gel were produced by lyophilization and soaking of the hydrogel in an ionic liquid of 1-butyl-3-methylimidazolium chloride (BMIMCl).

The abovementioned maltooligosaccharide-grafted PGA (Glc_7-grafted PGA) has also been used for vine-twining polymerization to construct amylosic network structure. The GP-catalyzed enzymatic polymerization of Glc-1-P initiated from the primer chain ends of the graft copolymer was carried out in the presence of different feed ratios of a guest polymer, PCL according to vine-twining polymerization man-

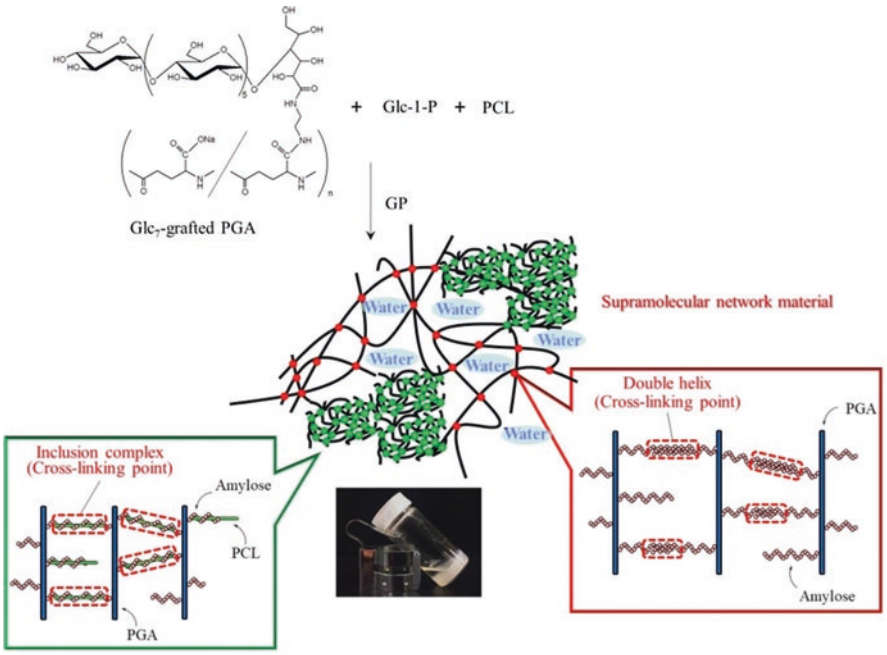

Fig. 3.17 Formation of smaller network structure by inclusion complex and larger network structure by double helix in GP-catalyzed enzymatic polymerization

ner [189]. The predominant formation of double helixes from the amylose graft chains in the products in the absence of PCL (as aforementioned) or in the presence of less amount of PCL gave rise to the hierarchical construction of the lager macroscopic network, leading to hydrogelation of the reaction mixtures. In the presence of larger amount of PCL, on the other hand, the smaller network mostly constructed from inclusion complexes was formed according to the vine-twining polymerization manner, resulting in the production of aggregates with the smaller macroscopic network in the reaction mixtures. Furthermore, it was revealed that in the presence of moderate amount of PCL, inclusion complex was initially formed by the shorter amylose chains with the progress of the GP-catalyzed enzymatic polymerization, and subsequently, double helix was produced by the longer amylose chains (Fig. 3.17).

Supramolecular polymers composed of the continuums of amylose-PTHF and amylose-PLLA inclusion complexes were successfully synthesized by vine-twining polymerizations using Glc_7-*block*-PTHF and Glc_7-*block*-PLLA as primer–guest conjugates (Fig. 3.18a) [190, 191]. In these systems, an enzymatically propagating amylose chain included a PTHF or PLLA segment of another conjugate, and such inclusion complexation among the conjugates consecutively takes place, giving rise to the formation of the linear inclusion supramolecular polymers.

The relative chain orientations of amylose and the two stereoisomers of PLA in inclusion complexes formed in the GP-catalyzed enzymatic polymerization were

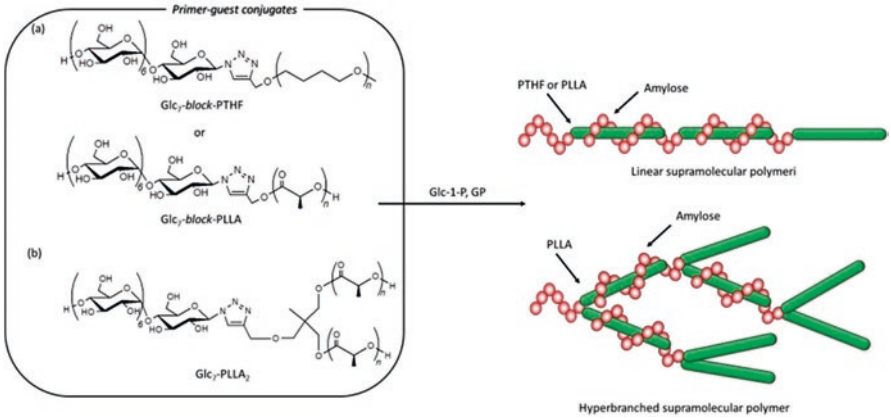

Fig. 3.18 Formation of (a) linear and (b) hyperbranched supramolecular polymers by vine-twining polymerization using primer–guest conjugates

precisely investigated by using four different primer–guest conjugates, which consisted of a Glc_7 segment functionalized at the carboxylate or hydroxy termini of both PLLA and PDLA [129]. The amylose-PLLA supramolecular polymers were formed in the enzymatic polymerization in the presence of both of the two PLLA conjugates, suggesting that the amylose cavity included the guest polymer, regardless of the chain orientation of PLLA. In contrast, the products from two PDLA conjugates were the amylose–PDLA diblock copolymers due to the noninclusion owing to the recognition behavior of amylose for chirality, regardless of the chain orientation of PDLA. The left-handed helixes from both the amylose and PLLA induce inclusion complexation, whereas complexation was not significantly affected by the orientation of the methyl substituents in PLA, which oppositely change according to the relative chain orientation.

Vine-twining polymerization using a branched maltoheptaose-(poly(L-lactide))$_2$ (G_7-$PLLA_2$) conjugate resulted in the production of a hyperbranched supramolecular polymer (Fig. 3.18b) [192]. The hyperbranched product formed an ion gel with BMIMCl, which was further converted into a hydrogel upon exchange of the dispersion media by soaking in water. Lyophilization of the resulting hydrogel produced a porous cryogel.

The CDP-catalyzed enzymatic oligomerization of Glc-1-P has been employed to prepare post-functionalizable two-dimensional crystalline cellulose nanosheets. An azido-functionalized cellulose oligomer was obtained by the enzymatic oligomerization of Glc-1-P using β-glucosyl azide as a primer [193]. The products aligned and formed nanosheets of an average thickness of 5.5 nm with antiparallel chain arrangement (cellulose II allomorph). As the azido groups of the cellulose oligomers at the reducing end were located on the sheet surface, the post-functionalization with 1-ethynylpyrene was achieved by copper(I)-catalyzed Huisgen cycloaddition to produce a pyrene-conjugated nanosheet (Fig. 3.19). The nanosheet has been found to act as an artificial hydrolytic enzyme [194]. The as-prepared nanosheets

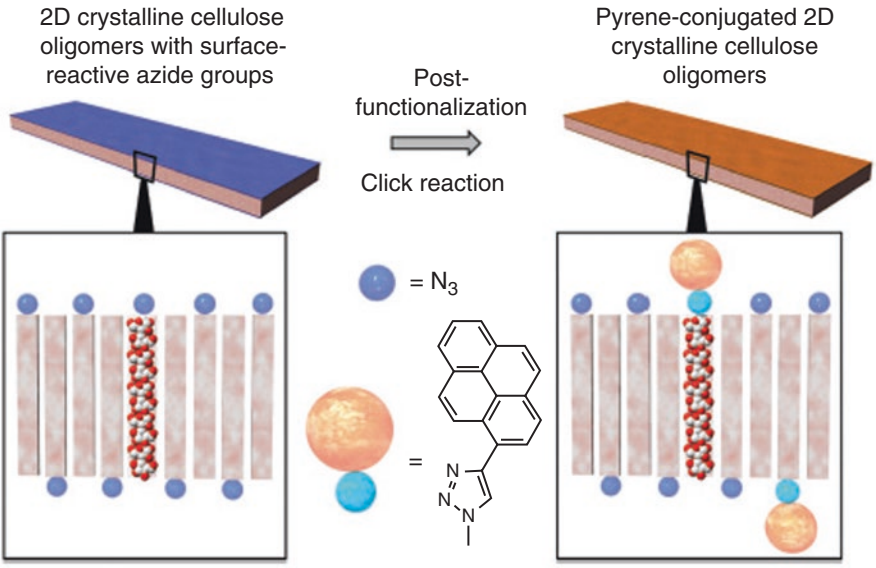

Fig. 3.19 Schematic illustration of the post-functionalization of surface-azidized 2D crystalline cellulose oligomers with 1-ethynyl pyrene through copper(I)-catalyzed Huisgen cycloaddition reactions; cellulose oligomers with azide groups at the reducing end were synthesized by CDP-catalyzed enzymatic oligomerization using Glc-1-P monomers and β-glucosyl azide primers. Reprinted from ref. [193] (Royal Society of Chemistry) under Creative Commons Attribution 3.0 Unported License

exhibited relatively low hydrolytic activities. However, distorted and smaller nanosheets with larger surface areas, which were prepared by sonication-based mechanical treatment, exhibited significantly greater hydrolytic activities. Through the CDP-catalyzed enzymatic oligomerization from the primer 2-(glucosyloxy) ethyl methacrylate (GEMA), a novel type of two-dimensional methacrylate-containing cellulose nanosheets with a thickness of about 6 nm was directly synthesized by a bottom-up method [195]. The obtained nanosheet was covalently incorporated into PEG matrix through thiol−ene Michael addition, fabricating a series of GEMA-cellulose nanosheet-based nanocomposite hydrogels.

The enzymatically synthesized cellulose oligomers by the CDP-catalyzed oligomerization using a cellobiose primer appeared to align perpendicularly to the base plane of the nanoribbons in an antiparallel manner [196]. The analysis of reaction time dependence suggested that the production of nanoribbon network structures was kinetically controlled by the amount of water-insoluble cellulose oligomers produced. A solution state with high macromolecular concentrations, so-called macromolecular crowding, was used to promote the crystallization-driven self-assembly of the enzymatically synthesized cellulose oligomer by CDP catalysis. The enzymatic oligomerization was conducted in the concentrated solutions of water-soluble polymers, such as dextran, PEG, and poly(*N*-vinylpyrrolidone). The

reaction mixtures turned into cellulose oligomer hydrogels composed of well-grown crystalline nanoribbon networks irrespective polymer species [197]. This method was further applied to the one-pot preparation of double network hydrogels composed of the nanoribbons and physically cross-linked gelatin molecules. The effect of solution viscosity on hydrogelation in the enzymatic reaction system was investigated using a highly branched polymer, Ficoll, which created macromolecular crowding conditions with relatively low solution viscosity [198]. The results suggested that a certain level of solution viscosity for the enzymatic synthesis is an essential requirement of hydrogelation. CDP-catalyzed bottom-up synthesis of mechanically and physicochemically stable nanoribbon network hydrogels composed of crystalline cellulose oligomers in which cellulose nanocrystals (CNCs) as model colloidal particles are immobilized spatially [199]. The stiffness of the hydrogels increased with the amount of CNCs incorporated. Protein adsorption property of the cellulose oligomer nanoribbons with primary amino groups was also investigated [200]. The enzymatic synthesis was carried out by the CDP-catalyzed oligomerization using 2-aminoethyl-β-glucose as a primer. The primary amino groups on the nanoribbon surfaces effectively attracted negatively charged proteins but not positively charged ones.

3.4 Amylose Engineering Applications by Phosphorylase-Catalyzed Enzymatic Polymerization

The GP-catalyzed enzymatic polymerization precisely produces amylose and amylosic materials, but the problem in practical application is that Glc-1-P is expensive. To overcome it, an attempt has been made to synthesize Glc-1-P by phosphorolysis of an inexpensive starch catalyzed by *Thermus caldophilus* GK24 phosphorylase [201]. The optimal pH and temperature were 7.0 and 70 °C for the phosphorolytic reaction producing Glc-1-P. Soluble starch (amylopectin, amylose) turned out to be a better substrate giving a higher yield of Glc-1-P than glycogen, potato starch, etc. As a result, Glc-1-P was produced in a good yield (47%) in the reaction containing 5% soluble starch in 0.7 M potassium phosphate at pH 7.0.

Another possible solution to the expensive problem of Glc-1-P is to combine another enzyme that produces Glc-1-P. The combined action by plural enzymes often shows synergistic effect to efficiently produce target products. Sucrose phosphorylase catalyzes phosphorolysis of sucrose in the presence of Pi to produce Glc-1-P and fructose. The Glc-1-P thus produced in situ by this enzyme can be used for the subsequent GP-catalyzed synthesis of amylose (Fig. 3.20). However, the following antagonistic reaction conditions are conceived when these two individual enzymatic reactions should efficiently occur, in which the first reaction should be carried out in high concentration of Pi, while the Pi should be removed as soon as possible from the media of the second reaction. Waldmann et al. have reported in the system for the production of amylose from sucrose, interestingly, Pi produced in the second GP-catalyzed reaction is recycled for the first sucrose phosphorylase-catalyzed

reaction. Therefore, the cooperative action by the two phosphorylases takes place continuously with a constant Pi concentration without any inhibition caused by an accumulation of Pi.

Thermostable sucrose phosphorylase was generated by introducing a random and site-directed mutagenesis on the sucrose phosphorylase gene from *Streptococcus mutans* to increase and used together with the triple-mutant thermostable GP (F39 L/N135S/T706I) originally from potato for the production of amylose from sucrose. These thermostable variants of sucrose phosphorylase and GP were employed to optimize the conditions for the production of amylose from sucrose. The yields of amylose produced the two enzymatic reactions from sucrose were higher than those from Glc-1-P. The molecular weights of the produced amyloses were strictly controlled by the sucrose/primer feed ratios, and their M_w/M_n values were close to 1. The amyloses with molecular weights less than 7.1×10^4 were produced as insoluble particles, whereas those with molecular weights more than 3.05×10^5 were produced in the solution. These results indicated that the properties of amylose differ according to the molecular weights.

For the purpose to produce Glc-1-P in situ, the use of cellobiose phosphorylase combined with GP was also examined (Fig. 3.20). Cellobiose phosphorylase catalyzes phosphorolysis of cellobiose in the presence of Pi to produce Glc-1-P and glucose. When a partially purified cellobiose phosphorylase was incubated with cellobiose and GP in the presence of Pi, various sizes of amylose (from 4.2×10^4 to 7.3×10^5) were produced. However, the yield (38.6%) was not as high as that in the system using sucrose phosphorylase. To improve the yield of amylose, mutarotase and glucose oxidase were added to the initial reaction mixture. The role of these enzymes is to remove glucose derived by the cellobiose-catalyzed reaction, leading to shifting the equilibrium state to phosphorolysis. As the result, the yield of amylose increased to 64.8% by the action of these enzymes.

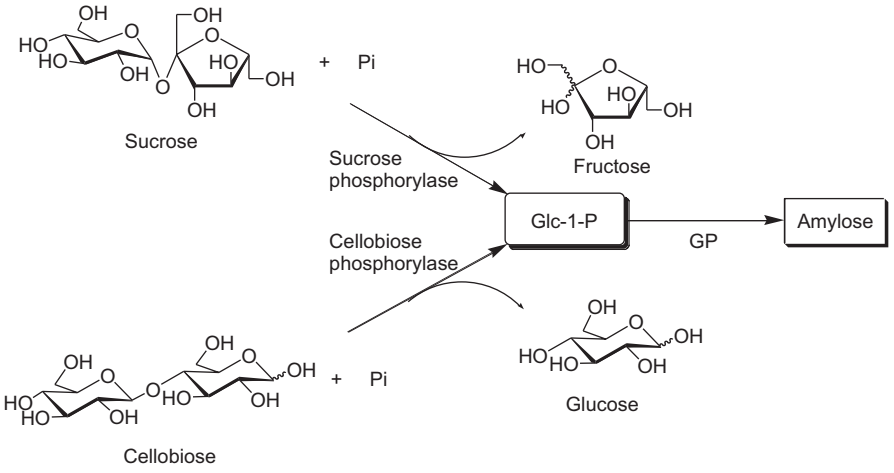

Fig. 3.20 Production of amylose by combined used of sucrose or cellobiose phosphorylase and GP

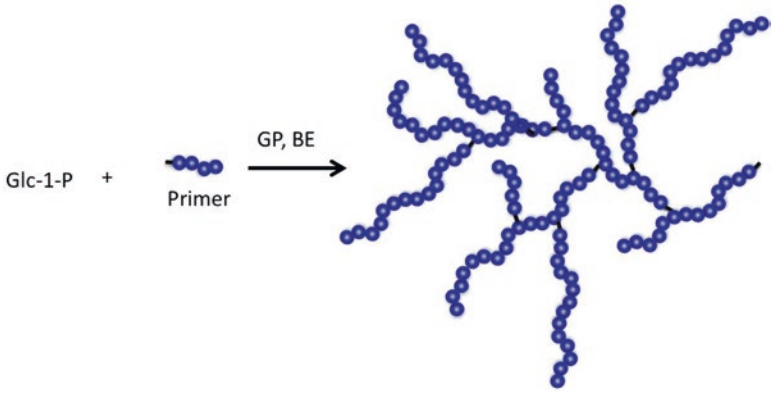

Fig. 3.21 Synthesis of branched glucan by combined use of GP and BE

Branched glucan has been prepared by the combined action of GP and BE on Glc-1-P in the presence of an adequate primer (Fig. 3.21) [202–209]. The molecular weight and branching pattern of the product were controlled by the Glc-1-P/ primer feed ratio and by the relative BE/GP activity ratio, respectively. Thus, various branched glucans were produced by using different BE/GP activity ratios. The produced glucans at high BE/GP activity ratios had more frequently branching points than those produced at low BE/GP ratios. *Deinococcus geothermalis* glycogen-branching enzyme is known to catalyze the redistribution of short α-glucans via inter- and intramolecular chain transfer from α(1→4)-positions to α(1→6)-positions. The combined use of GP and glycogen-branching enzyme, therefore, gave highly branched amylose from Glc-1-P [203].

Amylose-modified silica gels have been prepared by the GP-catalyzed enzymatic polymerization using maltooligosaccharide primers having appropriate functional groups at the reducing end, followed by immobilization on the surface of silica gels, by two different approaches (Fig. 3.22) [210, 211]. In approach I, 1,3-aminopropyltriethoxysilane-modified maltooligosaccharide was used of the GP-catalyzed enzymatic polymerization. The produced amylose derivative was immobilized by reaction with silica gel. In approach II, amylose lactone was first prepared by the GP-catalyzed enzymatic polymerization using maltooligosaccharide having potassium gluconate at reducing end (precursor of lactone), followed by the lactonization. The produced lactone was reacted with 3-aminopropyl-silanized silica gel. The two modified silica gels were reacted with a large excess of 3,5-dimethylphenyl isocyanate to convert hydroxy groups in glucose residues to carbamate derivatives. The products can be used as chiral stationary phases in high-performance liquid chromatography, owing to their intrinsic chirality. The modified silica gels display excellent enantioseparation of ten different racemates. The systems are thus widely used to separate racemic compounds into their enantiomers.

The GP-catalyzed enzymatic polymerization using modified primers has been applied to an enzyme-responsive artificial chaperone system for protein refolding.

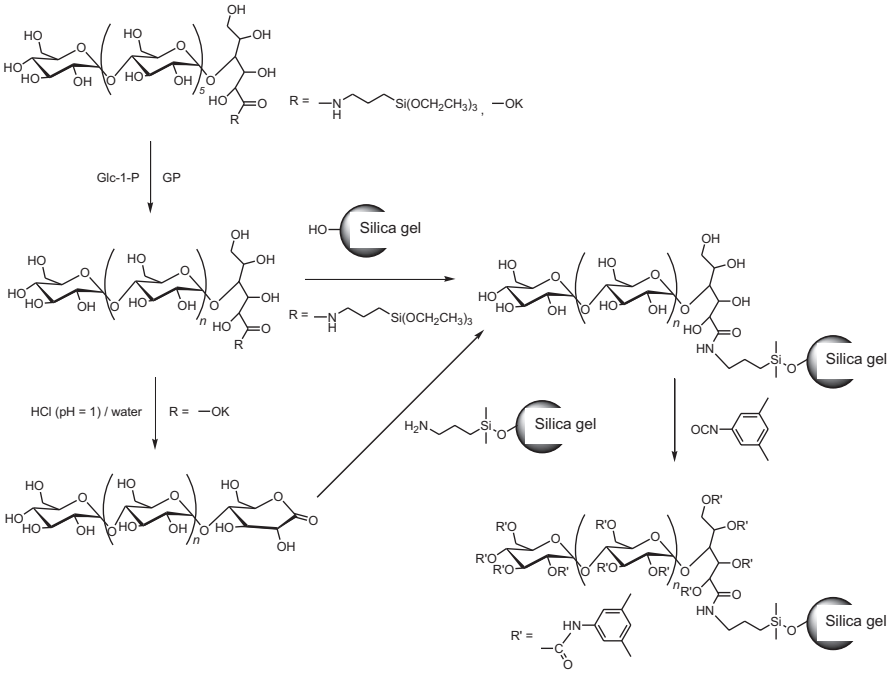

Fig. 3.22 Preparation of amylose-grafted silica gels

Glc_5-*block*-alkyl chain surfactants with different numbers of carbon (e.g., Glc_5C_8, Glc_5C_{12}, and Glc_5C_{16}) have been used as primers for the GP-catalyzed enzymatic polymerization, where the reducing end of Glc_5 is substituted with an alkyl group (C_8, C_{12}, and C_{16}). The primer surfactants formed micelles in water, which dissociated upon the GP-catalyzed enzymatic polymerization [212]. By this property, the micelle-to-vesicle transition of the mixed lipid–primer systems was observed during the enzymatic polymerization. Consequently, Glc_5C_{12} micelles behaved as enzyme-responsive molecular assembly systems. Accordingly, an enzyme-responsive artificial chaperone system by means of the behavior of this surfactant was constructed to enable protein refolding (Fig. 3.23) [213]. The effective refolding of carbonic anhydrase B after either treatment with guanidine hydrochloride or heat denaturation was observed by controlled association between the protein molecules and surfactant primer micelles via the GP-catalyzed enzymatic polymerization.

3.5 Conclusion

Polysaccharides are important biobased materials with applications spanning the whole range of cheap commodity plastics to advanced medical applications. Phosphorylases are excellent biocatalysts for the synthesis of well-defined oligo- and polysaccharides. They also provide environmentally friendly processes, because

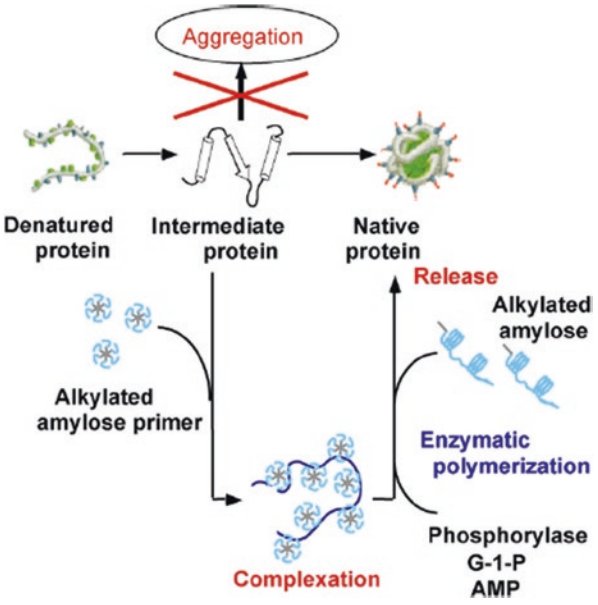

Fig. 3.23 Schematic illustration of artificial chaperone system by GP-catalyzed enzymatic polymerization of Glc_5C_{12} primer. (Reprinted with permission from ref. [213]. Copyright 2009 Elsevier)

the reactions can be operated using unprotected substrates in aqueous media under mild conditions. They are extensively used for the synthesis of oligo- and polysaccharides as standard materials, in hybrid structures, etc.

Advancements in the discovery, production, and characterization of phosphorylases, like high-throughput screening for enzyme evolution and metabolic pathway engineering, have broadened the scope of possibilities and allowed the improved synthesis of oligo- and polysaccharide libraries and hybrid materials thereof. The synthesized hybrid materials show very interesting properties and can be used for various applications.

It can be envisioned that both the discovery of new phosphorylases and the synthesis of new hybrid materials will be the focus of more intensified research in the near future as the possibilities summarized in this review show the great potential of this area of research, based on the viewpoints of several topics, e.g., synthetic method, functional material, biological function, and environmental aspect.

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Chapter 4
Synthesis of Polysaccharides III: Sucrase as Catalyst

Satoshi Kimura and Tadahisa Iwata

Abstract Sucrase-type glycosyltransferases that classified into non-Leloir glycosyltransferases, named glucansucrase and fructansucrase, catalyze in transfer of either a glucose or a fructose from sucrose to produce glucans or fructans. The reactions need only a renewable carbon resource, such as sucrose, and proceed very efficiently, with high yields, with regio- and stereoselectivity, and in one-pot water-based system. This chapter provides an overview of the glucansucrase and fructansucrase enzymes, their reaction, and product specificity. Finally, we discuss the potential applications of α-glucans produced by glucansucrase in new bio-based materials.

Keywords α-Glucan · Fructan · Glucansucrase · Fructansucrase · Glucosyltransferase · Sucrose

4.1 Introduction

Polysaccharides, natural polymers composed of sugar units linked via glycosidic bonds, have been considered as interesting bio-based materials for utilization in many applications such as plastics and biomedical field with currently increasing amount of researches. The advantages are that they are made from renewable resources supporting the trend to reduce the consumption of plastics made from petrochemicals, and with the concept of carbon neutrality, they can be regarded as eco-friendly materials [1]. Plants typically produce polysaccharides such as well-known cellulose and hemicelluloses such as xyloglucan, xylan, and glucomannan. However, hemicelluloses are branched, and they are extracted from wood via alkali or acid process leading to chain degradation that lowers the molecular weight. Besides, microorganisms can synthesize many polysaccharides in the culture

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medium as well such as pullulan from *Aureobasidium pullulans*; curdlan from *Agrobacterium*, *Rhizobium*, and *Cellulomonas*; dextran from *Leuconostoc* and *Streptococcus*; and hyaluronic acid from *Streptococcus* and *Pasteurella* [2]. In any case, however, it is required complicated purification step in order to purify the target polysaccharides, and the limitation of the direct production of polysaccharides by microorganisms is the difficulty in structure and composition control due to the nature of each producer.

In vitro enzymatic polymerization of polysaccharides is convenient and environmentally friendly method for production of polysaccharides. Sucrase-type glycosyltransferases classified into non-Leloir glycosyltransferases have been employed as catalysts for the practical synthesis of polysaccharides by both polymerization and modification. It is to be mentioned that sucrases, e.g., glucansucrase and fructansucrase, belong to glycosidases (EC 3.2.1). These enzymes catalyze in transfer of either a glucose or a fructose moiety of sucrose to produce glucans or fructans of different types with respect to glycosidic linkages and side chains. The reactions proceed very efficiently, with high yields, with regio- and stereoselectivity, and in one-pot water-based system. The background of this convenient synthetic pathway is the high energy of the glycosidic bond of sucrose, which is similar to that of nucleotide-activated sugars. The simplified reaction manners are represented as follows.

Glucansucrase: n Sucrose → Glucan + n Fructose
Fructansucrase: n Sucrose → Fructan + n Glucose

In this chapter, biochemical characterizations of glucansucrases and fructansucrases are summarized, and their recent applications with a focus on in vitro-synthesized α-glucan by glucansucrase are described.

4.2 Glucansucrase

Glucansucrases are extracellular enzymes mainly produced by lactic bacteria *Lactococcus*, *Leuconostoc*, and oral *Streptococcus* [3]. Glucansucrases catalyze the synthesis of high molecular weight D-glucose polymers named glucans from sucrose (Fig. 4.1). Dextran, α(1 → 6)-glucan is the first reported and most common glucan synthesized by a kind of glucansucrase, was one of the first biopolymer to be produced on an industrial scale in 1948 [4]. The glucansucrase responsible for the synthesis of dextran was first reported in *Leuconostoc* [5] and was named as dextransucrase (EC 2.4.1.5). Amylosucrase (EC 2.4.1.4) is the most extensively studied glucansucrase [6–8]. Amylosucrase was found in the genus *Neisseria* and named because of its enzymatic conversion of sucrose to a glycogen- or amylopectin-like polymer [9]. Until now, amylosucrase gene from *Neisseria polysaccharea* was cloned and heterologously expressed in *Escherichia coli* [10], followed by reports of its three-dimensional structure [11, 12].

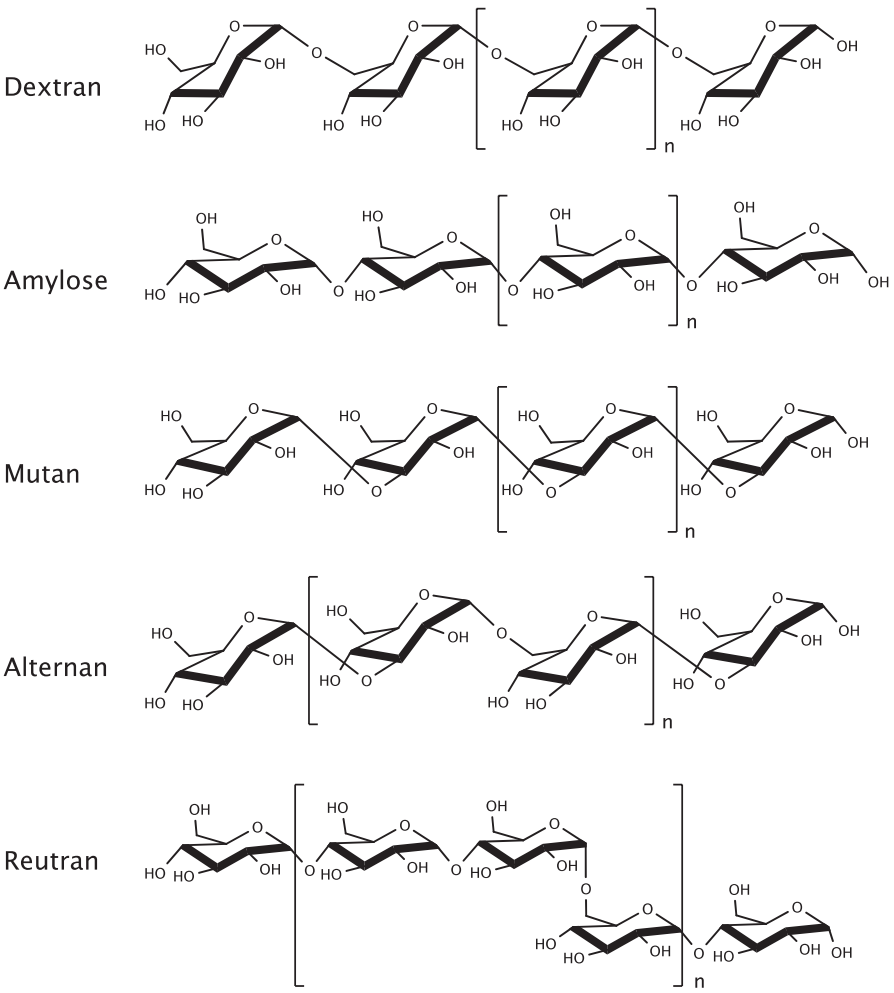

Fig. 4.1 The basic structures of the α-glucans synthesized by glucansucrases. The α-glucans are classified according the dominant linkage type in the main chain

Glucansucrases of oral streptococci, like *Streptococcus mutans*, play a key role in the cariogenesis process, as the synthesized glucans enhance the attachment and colonization of cariogenic bacteria [13, 14]. Therefore, in order to develop vaccines against dental caries, studies for the isolation of the gene encoding for these glucansucrases of oral streptococci were initiated more than 40 years ago. The first genes encoded glucansucrases (named *gtf*) were cloned from *Streptococcus downei*, using γ phage as cloning vector and screening on sucrose-containing medium [15]. The term GTF (glucosyltransferases) has been the one preferred by researchers with oral bacteria; this term does carry to confusion with the nucleotide sugar-dependent intercellular glycosyltransferases [16]. In the sucrase-type enzymes, energy

necessary to catalyze all the reactions comes only from the cleavage of the glycosidic bond of sucrose. The mediation of nucleotide-activated sugars or cofactors is not necessary. The term glucansucrase has been favorably used in recent years, the specificity of the enzyme being indicated by a name derived from the main glucan produced, e.g., α(1 → 6)-glucan (dextransucrase) and α(1 → 4)-glucan (amylosucrase).

A long-standing question about glucansucrase relates to the mechanisms of chain elongation and the determinants of type of glycosidic linkage introduced to the growing glucan chain. As nucleotide sequences of glucansucrases became available, multiple alignments of the deduced amino acid sequences revealed conserved unique features which may explain which amino acid residues or domains are responsible for these properties. Of the oral streptococci, *S. mutans* produces three distinct glucansucrases, while in *S. sobrinus*, the closely related *S. downei* and *S. salivarius* each produce four glucansucrase genes (Table 4.1) [17]. In contrast, *S. gordonii*, *S. sanguinis*, and *S. oralis* make only a single glucansucrase.

4.2.1 Catalytic Mechanism of Glucansucrase

The sequences of about 500 different glucansucrase genes including ORF fragment are listed on the CAZY database of Carbohydrate Active Enzymes (http://www.cazy.org/) [18–20]. And more than 60 glucansucrases have been biochemically characterized. In the CAZY classification system, all glucansucrases except amylosucrases are classified as family GH70. Amylosucrase is the only enzyme of family GH13 displaying polymerase activity and is clearly unique in the family GH13 that mainly contains starch-degrading enzymes. These enzymes from families GH13 and GH70 are also known as the part of the α-amylase superfamily and are classified in clan GHH [18–20]. In the α-amylase enzymes, the central catalytic core of these enzymes is predicted to have alternating α-helixes and β-sheets, in an arrangement the same as found in the (β/α)8 barrel found in amylase. Although these enzymes catalyze transglycosylation or hydrolysis reactions on differently linked α-glucan polymers, they use the same set of key amino acid residues to catalyze their reaction [21–24]. During the 2000s, the role of these residues and the mechanism of the reaction have been extensively studied in GH13 enzymes such as

Table 4.1 Glucansucrases in *Streptococcus salivarius* (ATCC 25975)

Enzyme	Mw (10^{-3} x M_r)	Glucan (water solubility)	
GtfJ	168	100% α(1→3)-	(insoluble)
GtfK	176	100% α(1→6)-	(soluble)
GtfL	157	50% α(1→6)- / 50% α(1→3)-	(insoluble)
GtfM	171	95% α(1→6)-	(soluble)

Neisseria polysaccharea amylosucrase [25], *Aspergillus oryzae* α-amylase [26], and *Bacillus circulans* cyclodextrin glucanotransferase [27]. A decade later, final evidence for the catalytic mechanism of GH70 glucansucrase came from the three-dimensional structure and its complexes with sucrose or maltose [23].

General reaction mechanism for glucansucrase is shown in Fig. 4.2. Based on the sequence homology within the α-amylase superfamily, the corresponding catalytic residues in glucansucrases had been identified, and the mechanism was proposed to be similar [24]. The most important catalytic residues are a nucleophile (aspartate), a general acid/base (glutamate), and a transition-state stabilizer (aspartate). This mechanism is based on a detailed structural analysis of *B. circulans* 251 CGTase in complex with intact substrate and on a covalent intermediate of the same enzyme [27]. In this mechanism, first the glycosidic linkage of the sucrose is cleaved, resulting in a covalent α-glucosyl-enzyme intermediate; in the second half-reaction, the glucosyl moiety is transferred to an acceptor with retention of the α-anomeric configuration. After formation of the covalent intermediate, fructose is released, and the glucosyl moiety is transferred to an accepting sugar (transglycosylation) or to a water molecule (hydrolysis). The excellent review articles have been also published to provide detailed information of structure and function of glucansucrase [28–30].

4.2.2 Synthesis of α-Glucans by Glucansucrases

Different kinds of α-glucans with different sizes and structures, depending on the glucansucrase-producing bacterium, are synthesized. Based on their main glycosidic linkage type, these α-glucans are divided into five categories: dextran, amylose, mutan, alternan, and reuteran (Fig. 4.1). This structural variability results in a wide range of physicochemical properties, which may be suitable for different applications.

Dextran Dextran is a water-soluble α-glucan mainly composed of α(1 → 6) linkage connected by varying amounts and arrangements of α(1 → 2), α(1 → 3), and

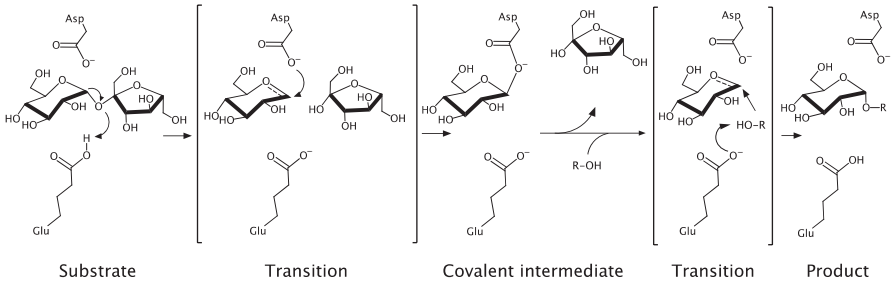

Fig. 4.2 General reaction mechanism of glucansucrase catalyst to produce glucan from sucrose with liberation of fructose

Table 4.2 Examples of dextran synthesized by glucansucrases from sucrose

Enzyme	Linkage compositon (%)				Ref.
	α(1→2)	α(1→3)	α(1→4)	α(1→6)	
Leuconstoc mesenteroides NRRL B-512F DSRS		5		95	[40]
Leuconstoc citreum B-1299 DSRE	5	10	3	81	[72]
Leuconstoc citreum B-1299 BSR-A	37			63	[73]
Leuconstoc citreum BSR-B		50		50	[74]
Weissella cibaria DSRWC				100	[39]
Lactobacillus reuteri 180 Gtf180		31		69	[47]
Streotococcus mutanss GS5 GtfD		30		70	[37]
Streotococcus salivarius GtfJ				100	[12]

α(1 → 4) linkages (Fig. 4.1, Table 4.2). The glucansucrase responsible for the synthesis of dextran is designated as dextransucrase and was first reported in *Leuconostoc* [5]. Subsequently, dextransucrases from various species of the genera *Lactobacillus*, *Streptococcus*, and *Weissella* were also identified and characterized [31–33]. The dextran is widely used in separation technology, biotechnology, and several applications in medicine [34, 35]. Dextran for clinical and technical applications is marketed in most developed countries all over the world. Of industrial relevance is the dextran produced by *Leuconostoc mesenteroides* NRR B- 512F DSR-S; the dextransucrase of this strain converts sucrose into a high molar mass (up to 1 MDa) polymer with 95% α(1 → 6) linkages in the main chains and 5% α(1 → 3)-branching linkages [36–38]. This native or partially degraded dextran and its derivatives have found many industrial applications in medicine (e.g., blood plasma expander, anticoagulant, and antithrombotic agents), pharmacy (e.g., lubricant and carrier), food (e.g., thickening, stabilizing, and gelling agent), and biotechnology (e.g., chromatography matrix) [38–40].

Another intensively studied dextransucrase is the *Lactobacillus reuteri* 180 Gtf180 GS producing an α-glucan with a high molecular weight of 30 MDa containing 69% of α(1 → 6) linkages plus single α(1 → 3) linkages in linear (21%) and branched (13%) orientations [41]. Notably, different α-glucans with unique highly branched structures have been reported in *Leuconostoc* strains. The *Leuconostoc citreum* NRRL B-1299 was found to synthesize a dextran polymer with mostly α(1 → 6) linkages but also containing about 30% of α(1 → 2) linkages, as well as a limited amount of α(1 → 3)-branching linkages [42–44]. This strain encodes six different glucansucrases, namely, DSR-A, DSR-B, DSR-E, DSR-M, DSR-P, and BRS-A [43].

Amylose Amylosucrase can catalyze three types of enzymatic reactions when sucrose is the only substrate: polymerization, isomerization, and hydrolysis [45].

The polymerization reaction, a unique characteristic of amylosucrase, synthesizes α-glucan with only α(1 → 4) linkages and has no need for any primer. Recombinant amylosucrase from *Neisseria polysaccharea* synthesizes amylose with DP of 35–58 in vitro. By changing only the initial sucrose concentration, it was possible to obtain amyloses with different morphology and structure [46]. Simultaneously, amylosucrase produces a certain number of sucrose isomers, turanose and trehalulose, through isomerization reactions, and catalyzes a hydrolysis reaction releasing glucose and fructose from sucrose [45]. In addition, in the presence of sucrose and extra glycosyl acceptors, amylosucrase has transglycosylation capacity to attach glucose molecules from sucrose to glycosyl acceptors, such as glycogen [47, 48], starch [49], and flavanone [50]. These unique reactions make it a vital transglucosylation tool in producing novel polysaccharides and carbohydrate-based bioactive compounds.

Using the self-assembly process of α(1 → 4)-glucans produced by amylosucrase, several amylose microbeads and their applications were reported. Amylose-single-walled carbon nanotube microbeads are the first exploration of enzymatic synthesis of microparticles by amylosucrase [51]. Amylose magnetic microbeads were also prepared by the similar enzymatic approach in the presence of iron oxide nanoparticles [52]. The produced microbeads had a well-defined spherical shape and exhibited excellent magnetic responses and dispersibility in aqueous solutions.

Mutan Mutan polymers are water-insoluble α-glucan mainly composed of α(1 → 3) linkage in the linear backbone and minor amounts of α(1 → 6) linkages. Mutansucrase-synthesizing mutan polymers are mainly found in *Streptococcus* strains. The ability of *Streptococcus mutans* and *Streptococcus sobrinus* to convert sucrose into water-insoluble glucans was found to be important in the etiology of dental caries by facilitating the colonization of tooth surfaces [53]. Consequently, glucansucrases are regarded as potential targets for anticaries drugs [54]. Mutansucrases have also been found in *Lactobacillus* and *Leuconostoc* strains [55, 56]. Mutan is regarded as a potentially low-cost polymer, which may be used to develop new bio-based materials [57, 58]. In particular, chemical modification of mutans to ester derivatives has shown to improve the thermoplasticity of this polysaccharide (see below for further details) [57, 58]. Moreover, chemically sulfated mutan showed fibrinolytic, anti-inflammatory, and antimicrobial properties [59–62]. The use of mutan for a variety of applications as fibers, films, and resins has been patented. The in vitro-synthesized mutan by recombinant mutansucrase (GtfJ from *S. salivarius* ATCC 25975) showed only α(1 → 3) linkage without any branching structure, which is difficult to obtain from native sources.

A transmission electron micrograph of in vitro-synthesized mutan is shown in Fig. 4.3 (Kimura and Iwata unpublished data). The synthetic mutan are wavy fibril-like crystals, which aggregated into plates. By electron diffraction analysis, it was indicated that the glucan chain was not extended parallel to the fibril axis as with other fibrillar polysaccharides, such as cellulose and chitin, but folded in the fibrils

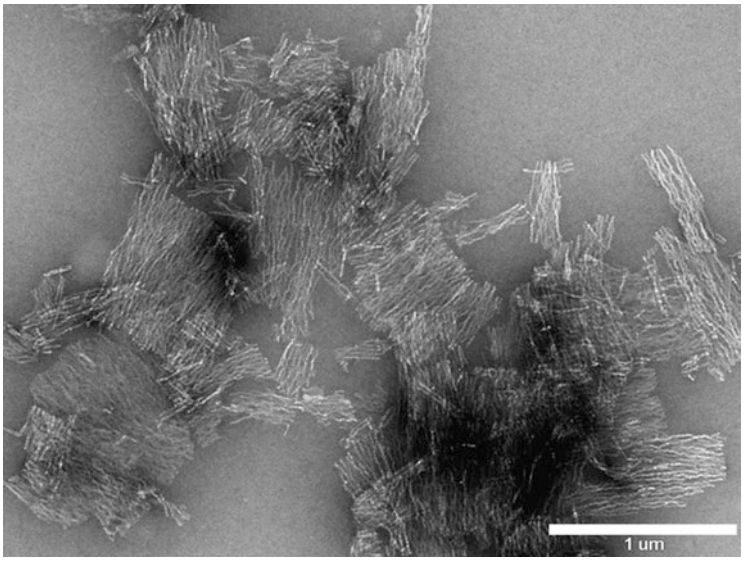

Fig. 4.3 Transmission electron micrograph of in vitro-synthesized mutan. (Kimura and Iwata unpublished data)

[63]. Several structural studies of mutan-like glucans have been reported by X-ray diffraction analysis [64–67]. In either case, however, regenerated mutan derived from the precipitate of an alkaline extraction from fungi cell walls was used, and the detailed structures of mutan have not been elucidated. The in vitro-synthesized mutan was a pure and completely linear α(1 → 3)-glucan without branches; for the first time, it was confirmed the morphology of native mutan [63].

Alternan Alternan, with alternating α(1 → 6) and α(1 → 3) linkages, was found in *L. mesenteroides* NRRL B-1355 [68, 69]. Due to its backbone structure of alternating α(1 → 6) and α(1 → 3) linkages, alternan is resistant to enzymatic digestion by most known mammalian and microbial hydrolytic enzymes [70]. These properties make alternan valuable as low-calorie food additive [71]. So far the only enzymes reported to hydrolyze alternan are isomaltodextranases and alternanase [70–73].

Reuteran Reuteran is a water-soluble branched α-glucan mainly composed of α(1 → 4)-glucan segments interconnected by single α(1 → 6) linkage. Only two reuteransucrase have been characterized, both of them present in *Lactobacillus reuteri* strains and producing reuteran polymers differing in the amount of α(1 → 4) and α(1 → 6) linkages [74, 75]. Reuteran has been described as a potentially health-promoting food ingredient.

4.3 Fructansucrase

Fructansucrases transfer the fructose units of sucrose onto polysaccharides or appropriate acceptors with release of glucose. Fructans, thus produced, are either levan composed of β(2 → 6)-linked fructose residues by levansucrase or inulin composed of β(2 → 1)-linked fructose residues by inulosucrase. Figure 4.4 has shown the typical structure of levan and inulin. When sucrose is used as the acceptor in the initial priming reaction, synthesized fructans contain a nonreducing glucose unit at the end of the chain. In bacteria, fructansucrases are extracellular enzymes; levansucrases are widely distributed in both gram-positive and gram-negative bacteria, while inulosucrases are exclusively present in lactic acid bacteria. Most of the research of fructansucrases has been performed on levansucrases, in particular on enzymes from *Bacillus* spp. [76–79] and *Zymomonas* spp. [80–83].

Fructansucrases cleave the glycosidic bond of sucrose and use the released energy to couple a fructose unit (i) to a growing fructan chain (transfructosylation), (ii) to sucrose, (iii) to water (hydrolysis), or (iv) to another acceptor (such as raffinose) [84, 85]. Because sucrose is used as the acceptor in the initial priming reaction, bacterial fructans contain a nonreducing glucose unit at the end of the chain [86] (Fig. 4.4). In the initial reaction of fructansucrases, the fructose of a sucrose molecule is coupled by the enzyme to another nonreducing fructose with a free

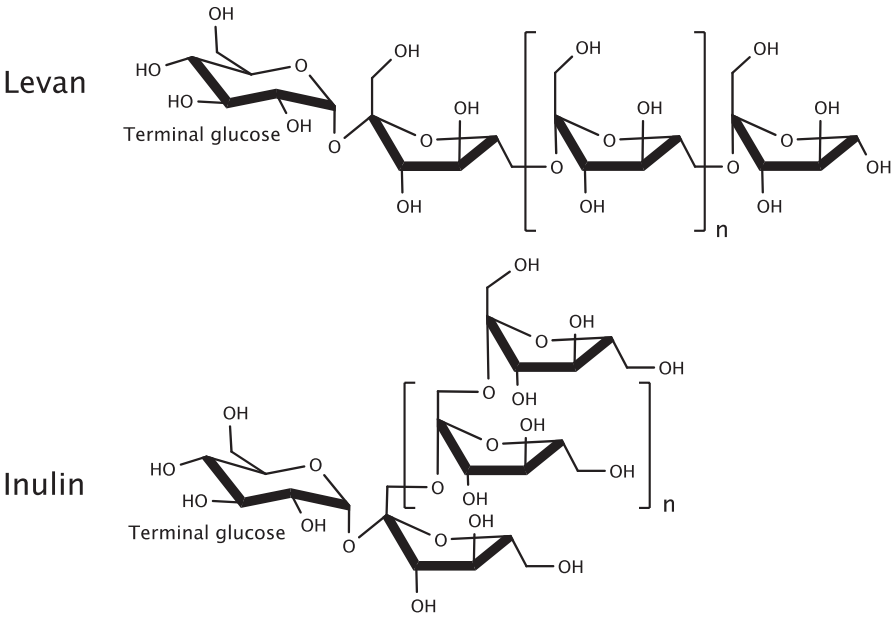

Fig. 4.4 Structure of levan and inulin synthesized by fructansucrase from sucrose. When sucrose is used as the acceptor in the initial priming reaction, synthesized fructans contain a nonreducing glucose unit at the end of the chain (terminal glucose)

primary alcohol at position C2, acting as an acceptor substrate, e.g., sucrose, raffinose, or a fructan molecule [87, 88]. This is also referred to as the priming reaction. In subsequent steps, the enzyme elongates the primer. A clear difference between fructansucrase and glucansucrase enzymes is the fact that glucansucrase enzymes cannot use sucrose as an acceptor but rather the cleaved glucose residue. The molecular masses of the fructans produced show a large variation, from 2×10^4 to 50×10^6 Da [28]. There are some reports that the molecular mass of the fructan produced is dependent on incubation conditions, the temperature, salinity, and sucrose concentration [89–91]. Sucrose analogues with a similar glycosidic linkage to sucrose have been used for the synthesis of new poly- and oligosaccharides by fructansucrase. For example, a wide range of fructansucrases recognize most of the sucrose analogues, such as those which were composed of galactose, mannose, xylose, fucose, and rhamnose in place of glucose, giving rise to novel poly- and oligosaccharides [92–97].

4.4 In Vitro-Synthesized α-Glucan as New Bio-Based Materials

The usage of naturally obtained mutan in the material application had not attracted any attention until now as the mutan has a branched structure; they lack a uniform composition, and purification costs are relatively high. The GtfJ enzyme, a kind of glucansucrase from *Streptococcus salivarius*, can effectively catalyze the one-pot water-based enzymatic polymerization of linear α(1 → 3)-glucan without branches. The synthesis process is environmentally friendly with a reaction in water medium, without organic solvent, and also convenient: only mixing sucrose solution with enzyme and storing at designated temperature. In addition, the molecular weight of the mutan can be adjusted by reaction conditions. Thus, in vitro-synthesized mutan can be considered as a potentially low-cost polymer for future prospect. A disadvantage of mutan, like all other polysaccharides, is the insolubility against most organic solvents and its thermally unprocessability. Up until now, many researchers have been attempting to improve this drawback by various techniques. One interesting method is the introduction of acyl groups to the hydroxyl groups of sugar units, or esterification, which raised the thermoplastic properties of cellulose known as so-called cellulose acetate and also improved the thermal properties and solubility of other polysaccharides in reported researches recently.

The series of mutan ester derivatives with Mw of about 200 kDa having different acid chain length were synthesized, and their thermal and mechanical properties of mutan ester films were investigated [58]. Hopefully, this material could be a substitution product to come on board replacing petroleum-derived plastics so as to avoid the uncertainty and sensitivity from oil's booms and busts, mitigate the environmental issues, and create the sustainability for future generations. Glass transition and melting behaviors of mutan ester series from C2 (acetate) to C8 (octanoate) are

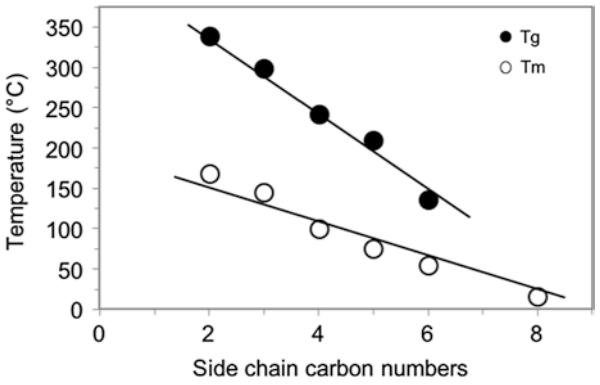

Fig. 4.5 Glass transition (T_g) and melting temperature (T_m) of mutan esters series from C2 (acetate) to C8 (octanoate). Redrawn on the basis of the reference [58]

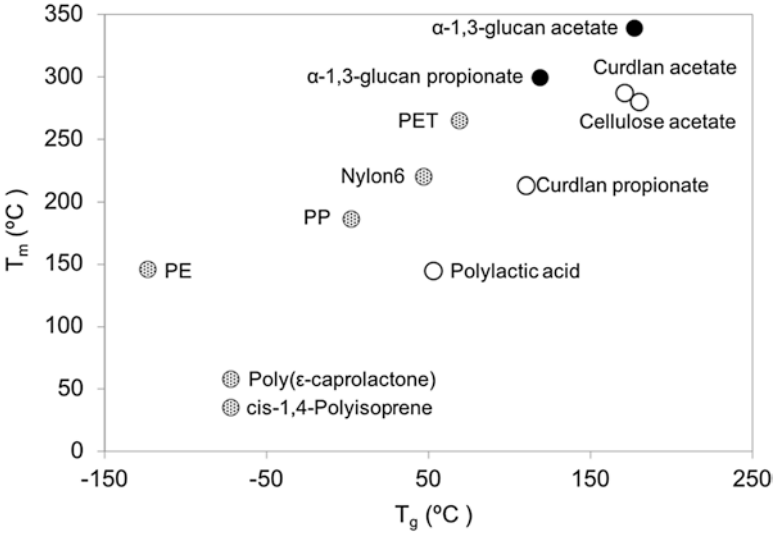

Fig. 4.6 Comparison of glass transition (T_g) and melting temperature (T_m) between those of mutan acetate and propionate, esters of other polysaccharides, and commercially available polymers. PE, PP, and PET are polyethylene, polypropylene, and polyethylene terephthalate, respectively. Redrawn on the basis of the reference [57]

shown in Fig. 4.5. The thermal and mechanical properties of mutan esters and its degree of crystallinity can be controlled by changing the length of its ester chain. Figure 4.6 shows the superior melting and glass transition temperature of mutan acetate and propionate over commercially available petroleum-based thermoplastics and currently interesting bio-based polymers; thus these materials are regarded as promising candidates for future thermoplastic application [57]. It was showed that unbranched mutan can be conveniently produced by green method using sucrose as a low-cost material. Furthermore, fully substituted mutan esters which

their thermal and mechanical properties can be adjusted are of interest for developing new thermoplastic materials.

4.5 Conclusions

Sucrase-type (non-Leloir-type) enzymes can catalyze to produce both polysaccharides and oligosaccharides using sucrose as a renewable cheap substrate via one-pot water-based reaction. The sucrase-type enzymes can be a very efficient tool to provide the synthesis of tailor-made glucan, fructan polysaccharides, and oligosaccharides for a wide range of applications. Several enzymatic processes with these enzymes already have been established for polysaccharide and oligosaccharide synthesis, some of which are applied industrially for a variety of applications. Further breakthroughs in this field are expected in the future, with enzyme engineering approaches increasingly allowing new construction of mutant enzymes and the discovery of new types of sucrase enzymes.

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Chapter 5
Synthesis of Polyesters I: Hydrolase as Catalyst for Polycondensation (Condensation Polymerization)

Shiro Kobayashi and Hiroshi Uyama

Abstract Synthesis of polyesters via enzymatic polymerization is described comprehensively in up-to-dated review manner, in which the polymerization is of polycondensation type using hydrolases mainly lipase as catalyst. First, characteristics of lipase catalysis are discussed: catalyst nature for green polymer chemistry including the catalysis mechanism, immobilization of lipases, role of surfactants for lipase catalysis, and so forth. Then, the lipase-catalyzed polycondensation synthesis of polyesters is argued according to the types of polymerization reactions: via dehydration of α- and ω-oxyacids and of dicarboxylic acids, via transesterification using carboxylic acid esters, and via ring-opening addition-condensation polymerization using cyclic anhydrides or cyclic esters as a monomer component. Other polymers like polyamides, polyamines, polycarbonates, and sulfur-containing polymers were synthesized by lipase catalyst. These reaction results indicate that lipase catalysts induce various polycondensation reactions to produce a variety of new polyesters. Further, protease which catalyzes primarily the peptide bond cleavage and bond formation catalyzed also the polyester production via polycondensation.

Keywords Lipase catalyst · Polyester synthesis · Polycondensation · Dehydration polymerization · Transesterification polymerization · Ring-opening addition-condensation polymerization · Protease catalyst

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5.1 Introduction

Polycondensation is an abbreviated expression of condensation polymerization. Polycondensation and ring-opening polymerization are two major reaction modes for the polyester synthesis. Both of them are catalyzed by enzymes. Synthesis of polyesters via polycondensation is described in this Chap. 5 and that via ring-opening polymerization is in the next Chap. 6. Polycondensation to produce polyesters is catalyzed by hydrolase enzymes such as lipase and protease.

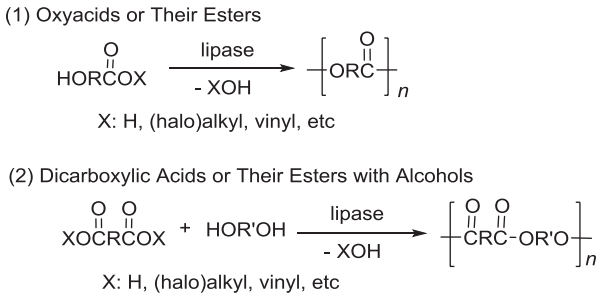
Such polycondensation reactions are very extensively studied and extremely important in the polymer synthesis field, in particular by lipase catalyst, and hence have been reviewed or focused many times for more than these two decades; related publications are typically shown [1–29].

Thus, polyester synthesis using hydrolases (mainly EC 3.1.1.3) as catalyst is given in Chaps. 5 and 6, and that using acyltransferases (EC 2.3.1) as catalyst in Chap. 7.

5.2 Lipase-Catalyzed Polycondensation

Synthesis of polyesters is most often achieved by using lipase as catalyst. Depending upon the substrate structure, two reaction modes of polycondensation are typically given as Scheme 5.1. During the reaction small molecules (XOH) are eliminated. Condensation reactions are basically reversible processes, and hence, it is necessary to remove or reduce a by-product water or an alcohol from the reaction mixture for producing the product polymer effectively.

It is notable that enzyme-catalyzed polyester synthesis was first achieved via polycondensation reaction in the middle of the 1980s by using lipase as catalyst [30, 31]. The catalysis of lipase in vitro in the polycondensation to produce polyester is via the ester bond formation, whereas the hydrolysis of ester compounds in vivo is a reverse reaction, i.e., via the ester bond cleavage.



Scheme 5.1 Two reaction modes of polyester synthesis via polycondensation

5.2.1 *Lipase as Catalyst*

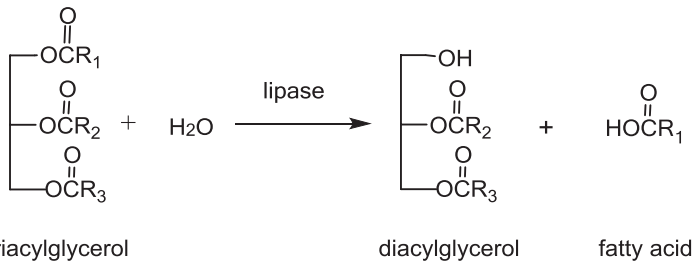
Catalyst Functions of Lipase Lipase (triacylglycerol lipase, EC 3.1.1.3) belongs to a hydrolase enzyme, which catalyzes the hydrolysis of fatty acid esters (ester bond cleavage) normally in an aqueous environment in living systems (in vivo). Scheme 5.2 represents a typical fundamental reaction in vivo [32]. It is stressed here again that the enzymatic reaction is reversible in principle, and hence, the polyester synthesis (ester bond formation) in vitro via polycondensation is a reverse reaction of the hydrolysis in vivo.

Generally, lipases play indispensable role to industry; hydrolyzing carboxylic ester bonds that are used in many of applications for food, detergent, pharmaceutical, and materials sciences. Here from fundamental scientific viewpoint, lipase is a typical enzyme catalyst for polycondensation synthesis of polyester, similarly to ring-opening polymerization of polyester synthesis as seen in the next chapter.

It is to be paid attention that in vitro lipase catalysis was observed in many years ago, in the 1930s [14, 33], and the catalysis became used later with much attention in organic chemistry field [34–36]. Lipase is by far the most well-known enzyme among others to catalyze the polyester synthesis, and hence, in this section some important aspects of lipase are described.

So far, various lipases of the different origin have been employed. For examples, industrial lipases derived from *Candida cylindracea* (abbreviated as lipase CC), *Candida rugosa* (lipase CR), *Burkholderia cepacia* (lipase BC), *Pseudomonas fluorescens* (lipase PF), and porcine pancreas (PPL) were used [37–39]. In addition, *Aspergillus niger* (lipase A), *Penicillium roqueforti* (lipase PR), *Pseudomonas aeruginosa* (lipase PA), *Pseudomonas cepacia* (lipase PC), *Rhizopus delemer* (lipase RD), *Candida antarctica* (lipase CA), and *Rhizomucor miehei* (lipase RM) were active in polyester synthesis [40, 41]. *Candida antarctica* lipase B (CALB) immobilized on acrylic resin is commercially called as Novozym 435.

Due to developments of X-ray crystallographic analysis technique as well as of isolation and crystallization techniques of enzymes, three-dimensional structures of enzymes were elucidated. Such structure determination of CALB was reported [42]; its 3D structure was given [43] and also referred [16]. CALB is constituted of 317 amino acid residues having a formula weight of 33,273. The active center has a



Scheme 5.2 In vivo catalysis function of lipase

catalytic triad, serine (Ser105)-histidine (His224)-aspartic acid (Asp187), containing a large hydrophobic pocket above the Ser-His-Asp triad and a medium-sized pocket below it. In the catalysis pathway, the acyl moiety of the substrate is considered to lie in the large subsite, while the leaving group/nucleophile moiety to lie in the medium pocket. The catalytic triad, Ser-His-Asp, is common to serine hydrolases like lipases and esterases.

Well-accepted catalytic mechanism involving the triad is illustrated in Fig. 5.1, where an ester RC(=O)-OR' (substrate) is hydrolyzed when a nucleophile Nu-H is water (HO-H) and trans-esterified when Nu-H is an alcohol (R"O-H). The catalyst site is $-CH_2OH$ of Ser residue [16]. During the reaction, the imidazole group of His

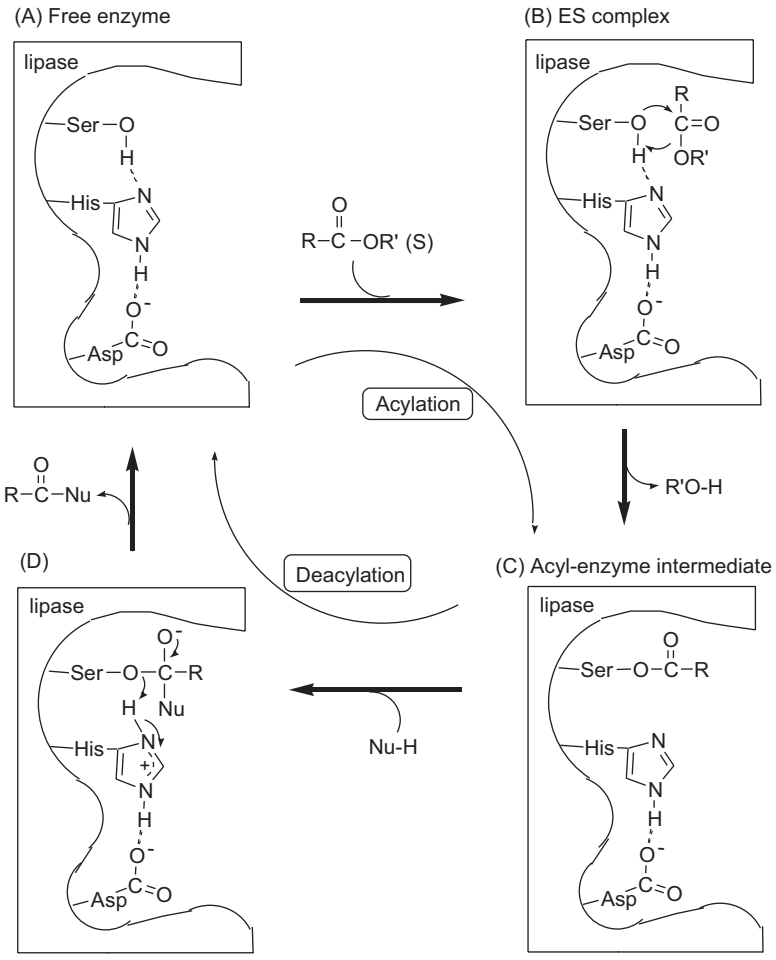


Fig. 5.1 Illustration of the mechanism of lipase-catalyzed hydrolysis (Nu-H = H_2O) and transesterification (Nu-H = R"O-H). (Reproduced with permission from [16]. Copyright 2010 The Japan Academy)

residue acts as a general base catalyst to pull the proton from $–CH_2OH$ and increases the nucleophilicity of the oxygen to attack the carbonyl carbon of the substrate in the stage of ES complex. At the same time, the carboxylate group of Asp residue helps the imidazole group to pull the proton, and the acyl-enzyme intermediate is formed with liberating R'O-H (acylating step). Then, in the deacylating step, also the imidazole and carboxylate groups act like a general acid/base mode to facilitate the production of RC(=O)-Nu.

Furthermore, lipase involves several catalysis aspects from "green chemistry" viewpoint:

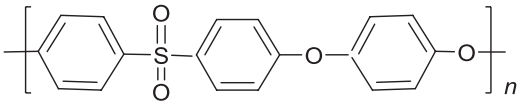
- High reaction-selective catalysis for acylation from alcohols or amines to give esters or amides.
- High enantioselective hydrolysis and resolution of esters or amides.
- Reactions under mild reaction conditions.
- Reactions in aqueous solution, organic media, or other green solvents like supercritical CO_2 and ionic liquids.
- High selectivity in enantiomer formation from a racemic mixture, etc.
- Also, lipase itself is derived from renewable resources.
- Lipase is often used to catalyze a polymerization reaction using starting materials from renewable resources like biomasses, giving rise to useful polymers.

Lipase involves possibilities to be used as catalyst in vitro for industrial production of polyesters, polyamides, poly(ester-amide)s, etc.; it is recovered after the reaction and reused for many cycles. So, lipase is strongly hoped to be highly active as catalyst, be physically, thermally, and hydrolytically stable, and be stored for a longer time and handled conveniently. For realizing these hopes, lipase is modified or treated in various methods; one of typical methods is immobilization.

Immobilization Lipase has been immobilized via various ways. In general, immobilization is achieved via chemical covalent binding of enzyme to the support or via physical or chemical adsorption of enzyme on the surface of the support. Lipase CR was employed for both approaches [44]. First, polyethylene glycol (PEG)-diacyl chloride (ClCO-PEG-COCl) was attached to -OH group of cellulose surface via ester bond formation and then allowed to react with the enzyme to form amide covalent bond via the reaction between –COCl and Enz-NH_2 group (sample 1). Second, poly(acrylic acid) (PAA) was grafted onto the cellulose surface through –OH group and then allowed the enzyme adsorption via the interaction between $–CO_2H$ and Enz-NH_2 groups (sample 2). Both surface polymer chains are compatible with aqueous and organic media. The stability of the enzymes of samples 1 and 2 toward several organic solvent was improved compared with the free enzyme. The sample 1 enzyme showed much better thermal stability than sample 2. Sample 1 showed higher catalytic activity at elevated temperatures than sample 2 and free lipase form. Both sample 1 and 2 lipases were repeatedly used for four cycles.

It is to be noted that the support substance is very important and can be one of the following many materials, for example, polyacrylic resins, ion-exchange resins, cellulose, alumina, celite, porous glass particles, silica, alumina, poly(ethylene glycol)

Scheme 5.3 Structure of PES



(PEG), hollow fiber membrane, poly(vinyl alcohol) cryogels, etc. [45, 46]. A poly(ether-sulfone) (PES, Scheme 5.3) was employed as a supporting material for CALB immobilization [47]. The PES membrane (around 2 mm in thick) was prepared from 20% of the optimized mixtures of PES, PSf (polysulfone), and PEG dissolved in 80% *N*-methylpyrrolidone as solvent. CALB was immobilized on the PES membrane, and the immobilized CALB showed twice higher than the native enzyme in *p*-nitrophenol palmitate hydrolyzing catalyst, indicating an excellent support material of PES for CALB immobilization.

Enzymes are generally limited to hydrophilic reaction media, because they usually are not soluble and active in hydrophobic media. However, chemical modification of enzymes using an amphiphilic synthetic polymer like poly(ethylene glycol) (PEG) made various enzymes soluble and active in highly hydrophobic organic solvents. The activated polymers can be attached to enzymes in aqueous buffer solutions, and once enzymes are modified, they become soluble and active in various organic solvents such as benzene, toluene, and chlorinated hydrocarbons and exhibit high enzymatic activities in these organic solvents. Modified hydrolytic enzymes catalyzed the reverse reaction of hydrolysis in organic solvents; the modified lipase catalyzed various ester synthesis reactions. Such lipase also catalyzed ester exchange between an ester and an alcohol, between an ester and a carboxylic acid, and between two esters in organic solvents. When the two substrates for ester exchange were liquid, the reaction could take place without organic solvents. The modified lipase catalyzed an ester exchange reaction between trilaurin and triolein when dissolved in these substrates. The modified enzyme was extremely thermostable in its substrates [48–52].

Moreover, the lipase from *Burkholderia cepacia* adsorbed on macroporous resin NKA was investigated by combined strategies of bioimprinting and interfacial activation to enhance its catalytic performance. The activity of the derived lipase was 20–47% enhancement over the free lipase powder. The derived lipase exhibited a thermal stability over a wide range of temperature (from 30 to 70 °C) and a strong tolerance to organic solvents such as methanol, ethanol, and acetone [53]. PMMA nanoparticles obtained by miniemulsion polymerization proved to be a good support for the CALB enzyme immobilization, particularly in terms of hydrolysis catalyst activity [54].

Encapsulation is another effective way of immobilization [55]: Lipase PC was spontaneously complexed by simply mixing with nanogels of cholesterol-bearing pullulan (CHP). The enzyme increased catalytic activity after complexation, in several times more by *k*cat values, and the thermal stability of the lipase also increased upon complexation. It is stated as a new type of nano-encapsulation of enzyme inside a hydrogel matrix [55]. In relation to encapsulation, a vesicle (self-assembled capsule) can hold enzymes or drug components inside and act as a “nanoreactor” or “nanofactory” [56, 57]. Self-assembled nanofactories formulate with polymeric

vesicles with an intrinsically permeable membrane. The vesicles, glyco-polymer liposomes (CAPsomes), are composed of carbohydrate-*b*-poly(propylene glycol) and show molecular-weight-depended permeability. This property enables CAPsomes to act as biocatalyst nanoreactors, protecting encapsulated enzymes from degradation while acting on low-molecular-weight substrates. In tumor-bearing mice, combined treatment with enzyme-loaded CAPsomes and doxorubicin prodrug inhibits tumor growth in these mice without any observable toxicity. The results demonstrated in vivo therapeutic efficacy of CAPsomes as nanofactories for enzyme prodrug cancer therapy [56].

Lipases were immobilized onto a photo-cross-linked polymer network, showing the activity recovery 76% and 41% for entrapment and adsorption methods, respectively. Both immobilized enzymes were very stable, retaining more than 60% of their activity. In the ring-opening polymerization of ϵ-caprolactone, polymerization rates were clearly affected as monomer conversions of 58% and 49%, and the highest molecular weights (M_n) obtained were 7890 and 5600 for entrapment and adsorption methods, respectively [58]. Nanofibrous membranes containing reactive carboxyl groups were fabricated from poly(acrylonitrile-*co*-maleic acid) by the electrospinning process. The morphology and fiber diameter were 100 to 600 nm. Lipase CR was covalently immobilized onto the membrane surface. The properties of the immobilized lipases on the nanofibrous and hollow fiber membranes were as follows. Compared with the hollow fiber membrane, the enzyme loading and the activity retention of the immobilized lipase on the nanofibrous membrane increased from 2.36 to 21.2 mg/g and from 34 to 38%, respectively [59].

CALB was complexed with iron oxide nanoparticles followed by interfacial assembly at the surface of an oil-in-water emulsion to lead to a hierarchical assembly immobilization, which brought about increasing the catalytic efficiency compared with the native enzyme and Novozym 435 and about enhanced thermal and pH stability. Very specific aspect is that the iron-containing lipase can be magnetically recovered across five times [60]. In addition, immobilization employing magnetic microparticles is reported, where lipase RM was adsorbed onto hydrophobic surfaces of magnetic microparticles, and then, the surface amino acids of lipase can be tailored on the previously modified hydrophilic surface of the particles to suit biomolecule conjugation, as seen in Fig. 5.2 [61].

Lipase CR was immobilized via physical adsorption onto an ethylene−vinyl alcohol polymer (EVAL) functionalized with acyl chlorides. To alter the hydrophobicity, three long aliphatic fatty acid chlorides (C_8, C_{12}, C_{18}) were employed, EVAL-OH group was esterified, and the obtained EVAL functionalization degrees were ranged from 5% to 65%. The enzyme−polymer affinity increased with both the length of the alkyl chain and the matrix hydrophobicity. The esterified polymers showed a tendency to give segregated hydrophilic and hydrophobic domains. Desorption experiments showed that lipase CR may be adsorbed in a closed form on the polymer hydrophilic domains and in an open, active structure on the hydrophobic ones, an image of which is shown in Fig. 5.3. The best results were found for the EVAL-C_{18} 13% matrix that showed hyperactivation with both the soluble and insoluble substrate. This supported biocatalyst retained its activity for repetitive cycles [62].

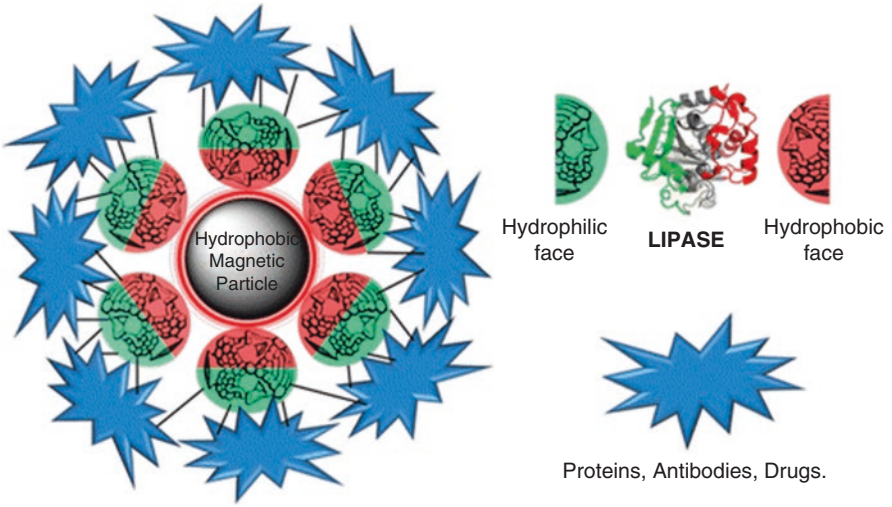


Fig. 5.2 Image of the immobilization of lipase onto hydrophobic magnetic particles. (Reproduced with permission from [61]. Copyright 2013 American Chemical Society)

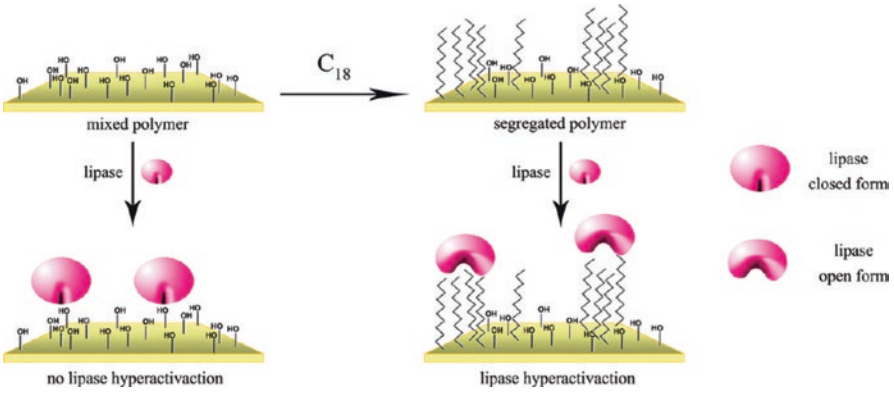


Fig. 5.3 Image of hydrophobicity influence on lipase catalyst activity. (Reproduced with permission from [62]. Copyright 2012 American Chemical Society)

A recent paper reported a lipase immobilization for catalysis using graphene oxide (GO) supports, which opens the lipase lid and maintains it in an open conformation in order to expose its active site [63]. The study belongs to immobilization via physical adsorption of enzyme on the surface of the support, and the lipase from *Alcaligenes* sp. (QLM) was used, which is an extracellular enzyme. The morphologies of bare GO sheets and GO sheets containing surface-bound QLM (GO+QLM) were visualized using AFM. The height profile corresponding to bare GO sheets (Fig. 5.4a) features a stable plateau near 1 nm (Fig. 5.4d). Upon putative lipase immobilization (Fig. 5.4c), a rougher surface topography emerges alongside a height profile plateau at 2–3 nm (Fig. 5.4e), confirming that protein has been successfully

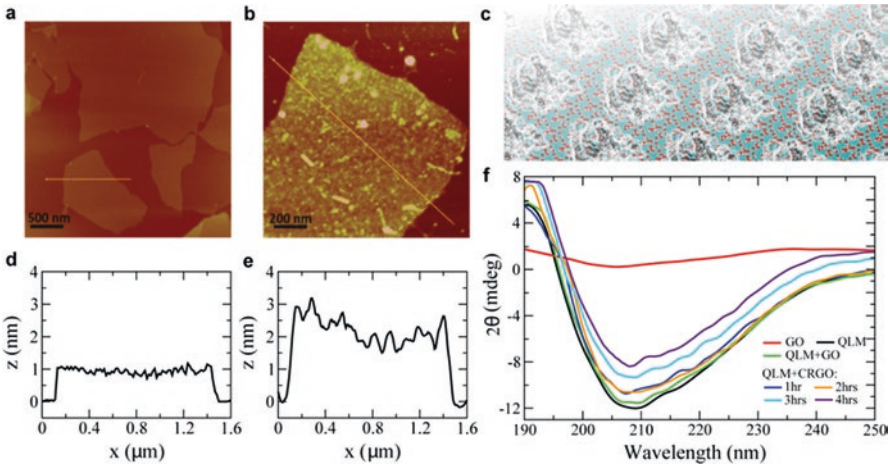


Fig. 5.4 Binding of lipase to graphenes. AFM images of (**a**) bare GO and (**b**) lipase-bound GO. (**c**) Illustration of lipase adsorption onto GO obtained from molecular modeling. (**d**, **e**) Height profiles of representative cross sections taken from (a) and (b), respectively. The GO surface became rough upon lipase binding, and the corresponding height profile exhibits a doubling or tripling of sample thickness. (**f**) Far-UV CD spectra for lipase bound to different hydrophobic surfaces. A general decrease in ellipticity at 208 nm was observed, implying a decrease in α-helical content upon adsorption. (Reproduced with permission from [63]. Copyright 2016 American Chemical Society)

deposited. These dramatic changes in the height profile probably correspond to the formation of a sandwich-like structure between two GO+QLM monolayers. Each GO+QLM sheet should be dotted with protein adsorbates on both sides, enabling the cohesion of multilayered complexes bridged by lipase molecules. Figure 5.4 e shows a molecular model for how QLM's binding to single GO sheets proceeds. Increasing hydrophobic surface of graphene increased lipase activity due to opening of the helical lid present on lipase, which was examined by the hydrolysis experiment of canola oil and *p*-nitrophenyl palmitate. The molecular mechanism of lid opening revealed in molecular dynamics simulations showed the role of hydrophobic interactions at the interface. The open and active form of lipase can be achieved and tuned with an optimized activity through chemical reduction of GO. This research looks a very good step toward designing nanomaterials as a platform for enhancing enzyme immobilization/activity. These tailor-made graphene-based nanosupports for effective immobilization may be a big challenge to control biophysicochemical interactions at the nano−bio interface. From the polymerization viewpoint, it is of interest to examine the catalytic activity of the present lipase for a polymerization reaction.

Protein Engineering Enzyme is modified by protein engineering, in order to alter the catalytic activity toward an increased number of substrate to yield a new polymeric product or to change the nature of active site of the catalyst. A reference example in polymer synthesis area is a study of using a mutated enzyme of endoglucanase II, in which cellulose-binding domain was delated [64]. The mutated enzyme catalyzed the enzymatic polymerization of β-cellobiosyl fluoride to produce highly

crystalline artificial cellulose with lacking the hydrolytic activity, in contrast to the artificial cellulose obtained by the un-mutated enzyme [65].

Surfactant (Detergent) As seen above, the catalytic activity of lipase is sensitive to hydrophobic/hydrophilic nature of the enzyme surface. Accordingly, in the presence of various surfactants, enzyme was subjected to immobilization procedure [66]. Lipase CR and CALA as well as CALB (lipase isoforms A and B from lipase CA) were adsorbed on monoaminoethyl-*N*-aminoethyl (MANAE)-agarose beads as support in the presence of detergent Triton-X 100 to have individual lipase molecules. Then, one fraction was washed to eliminate the detergent, and both preparations were treated with glutaraldehyde for covalent cross-linking. The presence of detergent during the cross-linking of the lipases to the support permitted an increase in the activity (in some instances, even by a tenfold factor). The activity increase was speculated as that the lipase active center of the serine residue is more exposed to the medium by the detergent (with opening the lid), which makes the substrate easier to access to the active center. Hydrophobic area of detergent tends to help for lid opening, causing the higher activity [60]. Other surfactants are, for example, Span 85 (sorbitan trioleate) and propylene glycol stearate [67].

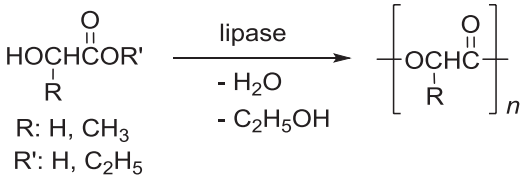
The influences of detergent using 17 kinds including nonionic, anionic, cationic, and zwitterionic detergents were investigated on the enzymatic activity and thermal stability of *Thermomyces lanuginosus* lipase (lipase Tl) [67]. For all detergents, low concentrations enhanced the activity of lipase Tl toward *p*-nitrophenyl butyrate hydrolysis by more than an order of magnitude. It was concluded that lipase-detergent interactions occur at many independent levels and are governed by a combination of general and structurally specific interactions.

5.2.2 *Polycondensation of Oxyacids or Their Esters*

A general reaction mode of oxyacids is shown as reaction (1) (X = H) in Scheme 5.1. In this section, we describe their reactions as two parts with dividing them into α-oxyacids and ω-oxyacids.

α-Oxyacids or Their Esters The smallest molecule of α-oxyacid, glycolic acid (R = R' = H, Scheme 5.4), was polymerized via lipase-catalyzed polycondensation with dehydration in an organic solvent to produce oligo(glycolic acid). Lipase from *Aspergillus niger* gave the best result for the polycondensation of glycolic acid

Scheme 5.4 Polycondensation of α-oxyacids or their esters



among the unmodified enzymes used. PEG-modified lipase gave relatively high yields. By using ethyl glycolate (R = H, R' = C_2H_5) as monomer, PEG-modified lipase catalyzed the polycondensation in 1,4-dioxane effectively to give the pentamer of poly(glycolic acid) with transesterification [51].

In the lipase-catalyzed polycondensation of lactic acid (R = CH_3, R' = H, Scheme 5.4), porcine pancreatic lipase (PPL) showed better conversions of lactic acid monomer and higher molecular weight poly(lactic acid) (PLA) than those with Lipozyme IM20. The highest molecular weight achieved was 1423 at 80% conversion by PPL. Blends of enzymatically prepared PLA with polystyrene yielded very good films in terms of tensile strength, elongation, and optical properties [68].

From the viewpoint of green polymer chemistry, PLA is derived from lactic acid, lactate, or its cyclic dimer of lactide as monomers, which are very promising alternate starting biobased and biodegradable materials in place of petro-based materials for practical polymer production (Fig. 5.5). In the production of PLA, impurities in lactic acid or lactide much affect the various properties of resulting PLA; these effects are discussed from a variety of analytical and characterization methods [69]. It might be possible, however, that if PLA is produced via enzymatic process, impurity problems may be much mitigated, because enzymatic catalysis is very high in substrate selectivity, i.e., impurities would less bother the synthesis reaction. It is to be added here that lactic acid was used extensively as an important starting biobased material for the production of various functional polymers [70, 71].

Novel enzymatic oligomerizations of alkyl lactates (RLa) were disclosed, employing lipase [72] and protease [73], respectively, as catalyst. These studies revealed new information on the mechanism for enantioselection aspects. Polycondensation of alkyl D-lactates (RDLa, α-oxyacid ester) involving transesterification catalyzed by lipase (Novozym 435) at 50 °C produced oligo(D-lactic acid)s (oligoDLA) up to 82% yields with n = 2–7 (Scheme 5.5). Primary alkyl lactates of

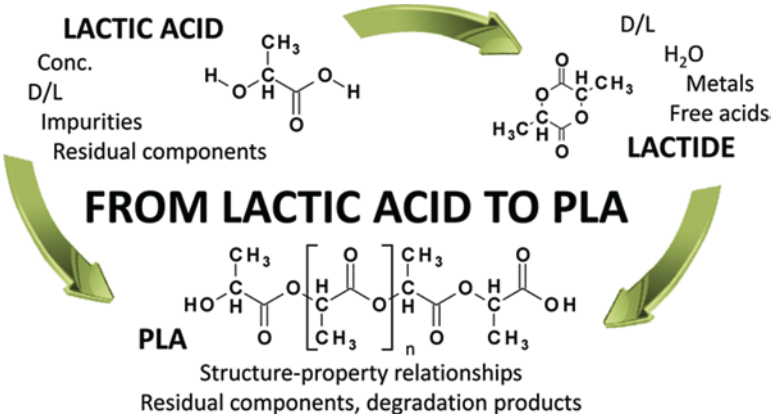
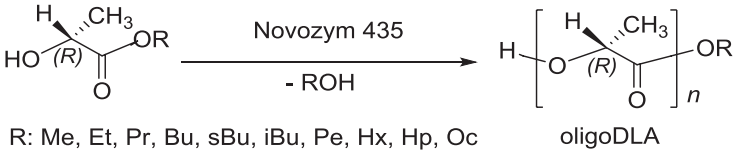
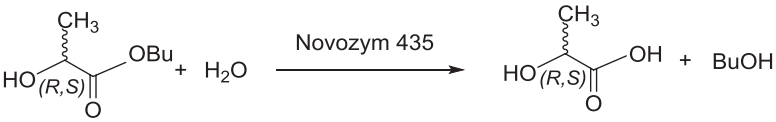


Fig. 5.5 PLA synthesis processes. (Reproduced with permission from [69]. Copyright 2011 American Chemical Society)



Scheme 5.5 Oligomerization of alkyl D-lactates



Scheme 5.6 Hydrolysis of BuLa

R = Et-, Pr-, and Bu- showed a higher reactivity than longer alkyl lactates like R = Pe-, Hx-, Hp-, and Oc-, and a secondary alkyl lactate of sBuDLa showed a reduced reactivity. These results suggested that there is an appropriate hydrophobic nature as well as stereochemistry of acyl group in monomer substrate for the enzyme to enable the enzyme catalysis. L-Lactates did not show oligomerization reactivity, i.e., enantioselection for D-isomers is very severe [72].

Michaelis-Menten Eq. (5.1) and for simplicity a pseudo-first-order rate Eq. (5.2) were applied for the reaction analysis:

$$\mathrm{E} + \mathrm{S} \overset{k'}{\rightleftarrows} \mathrm{ES} \overset{k_{\mathrm{cat}}}{\rightarrow} \mathrm{P} + \mathrm{E} \tag{5.1}$$

$$-\frac{\mathrm{d}[\mathrm{S}]}{\mathrm{dt}} = k'[\mathrm{E}][\mathrm{S}] = k[\mathrm{S}] \quad \left(k'[\mathrm{E}] = k\right) \tag{5.2}$$

where E, S, and P denote enzyme, substrate, and product, respectively. Plots of integrated form of Eq. (5.2) gave k values ($\times 10^4$ s^{-1}): MeDLa (3.7), EtDLa (4.4), PrDLa (3.7), and BuDLa (3.4).

The inhibition function of EtLLa toward the oligomerization of EtDLa was found "competitive." As a model reaction, the following hydrolysis experiments of BuDLa and BuLLa were conducted in THF (Scheme 5.6) [72]. Interestingly, in contrast to the oligomerization, Novozym 435 induced the hydrolysis of both BuDLa and BuLLa substrates. The rough values ($\times 10^4$ L mol^{-1} s^{-1}) are $k = 2.1$ for BuDLa and $k = 0.92$ for BuLLa at 50 °C; the D-isomer proceeded about 2.3 times faster than the L-isomer.

The above observations suggest the mechanistic aspects of lipase (Novozym 435) catalysis as follows: Enantioselection is operated by deacylation step as shown in Fig. 5.6 [72], where only the dimer formation is shown for simplicity. First, the monomer (substrate) is to be activated by the enzyme with forming *(R)*-acyl-enzyme intermediate in step (a) (enzyme-activated monomer: **EM**) ("acylation of lipase"). Onto the activated carbonyl carbon of **EM**, OH group of the D-lactate nucleophili-

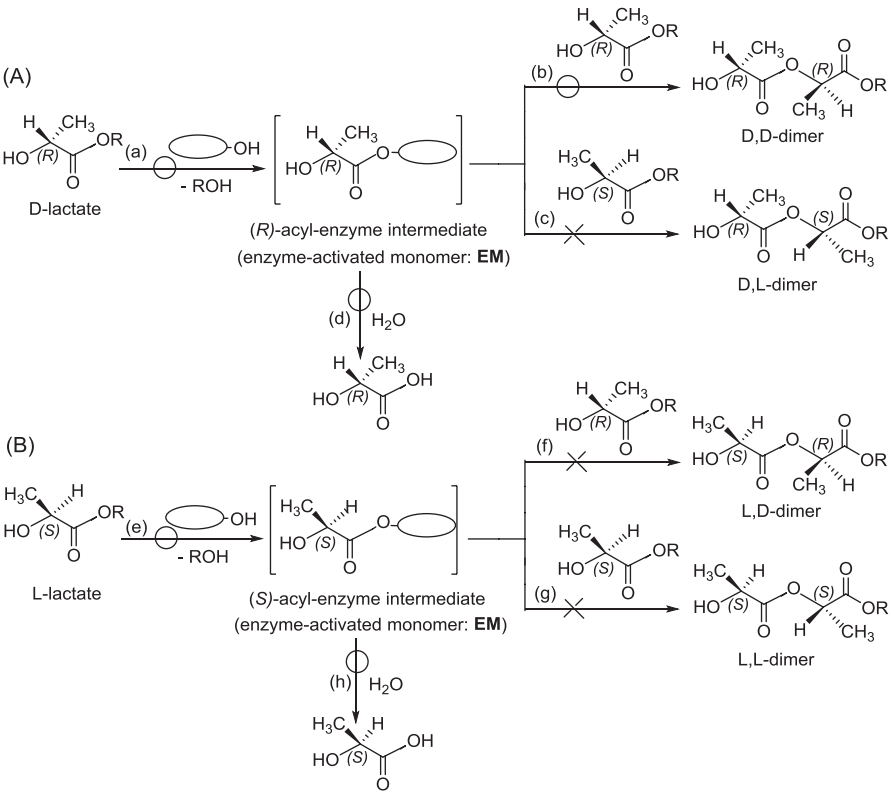


Fig. 5.6 Lipase-catalyzed oligomerization pathways of D-lactates (**A**) and L-lactates (**B**): acyl-enzyme intermediate formation steps (a and e), subsequent dimer formation steps (b, c, f, and g), and hydrolysis steps (d and h). ○ denotes that the step takes place, whereas × denotes that the step does not take place. (In steps b, c, d, f, g, and h, the leaving group of lipase is omitted). (Reproduced with permission from [72]. Copyright 2010 American Chemical Society)

cally attacks to form an ester bond with liberating lipase enzyme, giving rise to D,D-dimer in step (b) ("deacylation of lipase"). If, in place of the D-lactate monomer, OH group of D,D-dimer attacks **EM**, D,D,D-trimer will be formed, and the repetition of this type of reaction ends up with the formation of higher D-oligomers. Since the L-lactate was not consumed, the reaction of **EM** with OH group of L-lactate does not occur; reaction of step (c) does not take place. On the other hand, hydrolysis of D-lactate also needs activation to form **EM**. Then, **EM** reacts with water to give D-lactic acid shown in step (d).

On the other hand, alkyl L-lactate monomers (B) were not consumed in the oligomerization. Whereas, in the hydrolysis, alkyl L-lactates were hydrolyzed to give L-lactic acid in step (h). This is a clear indication that step (e) actually took place to produce *(S)*-acyl-enzyme intermediate **EM**. However, neither OH group of D-lactate nor OH group of L-lactate was allowed to attack **EM** to give L,D-dimer via step (f) or L,L-dimer via step (g).

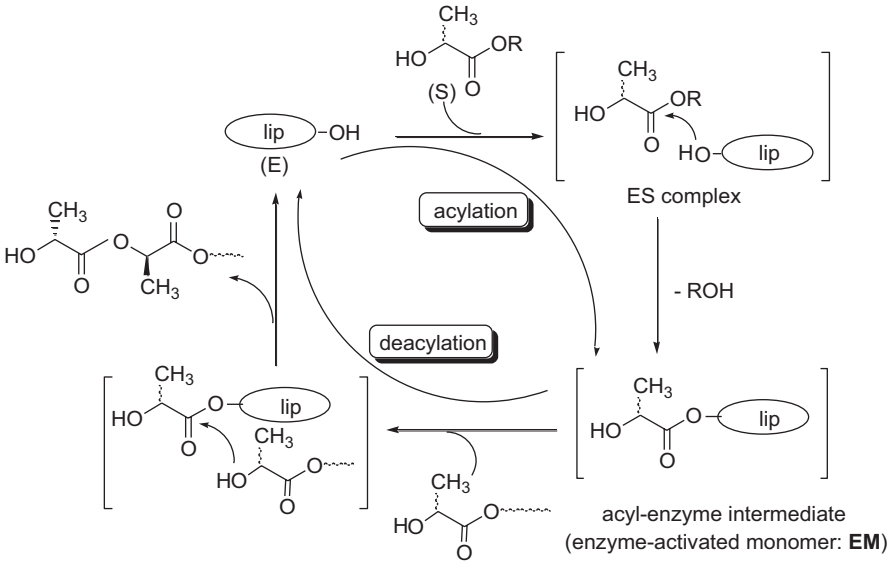


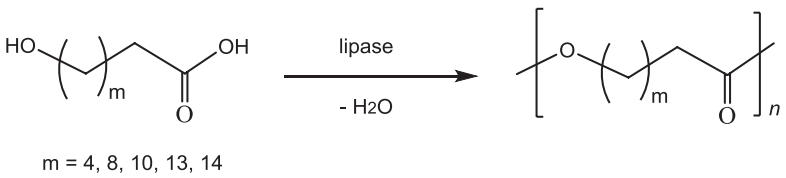
Fig. 5.7 General illustrative mechanism of lipase-catalyzed oligomerization of alkyl lactates. (Reproduced with permission from [72]. Copyright 2010 American Chemical Society)

Hydrolysis steps (d) and (h) (both deacylations) are nonselective due to no chirality in water molecule, whereas esterification steps (b), (c), (f), and (g) (all deacylations) are enantioselective. The above results demonstrate that "the enantioselection is governed by the deacylation step"; among four steps, only step (b) is allowed to give D,D-dimer. However, the **EM** formation, both steps (a) and (e), is possible from all alkyl D- and L-lactate monomers. This "enantioselection mechanism" is a clear-cut new finding.

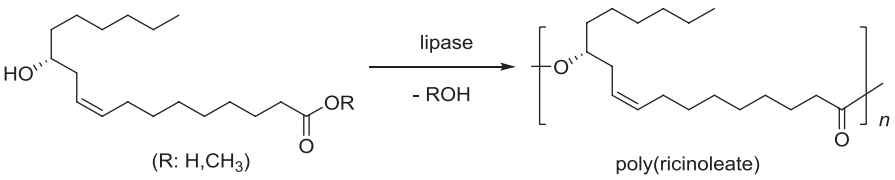
More generally, Fig. 5.7 shows a reaction mechanism of lipase (Novozym 435)-catalyzed oligomerization of alkyl lactates (RLa). The acylation step of RLa takes place regardless of D- or L-isomer, and therefore, their hydrolysis is catalyzed by the lipase. In the oligomerization, however, the reaction of *(R)*-acyl-enzyme intermediate (**EM**) is possible only with OH group of D-lactate or D-oligoLA and not with L-lactate or L-oligoLA. *(S)*-Acyl-enzyme intermediate, on the other hand, does not react with OH group of D-, L-lactates or D-, L-oligoLA. Namely, the deacylation step governs the enantioselection in the oligomerization.

These behaviors of lipase catalysis are to be compared with those of protease catalysis, where the enantioselection of the latter is much loose than the former [73]. This situation is mentioned in the Sect. 5.3.

The above D-selective reaction of alkyl lactates by lipase catalysis was applied for optical resolution of D, L-isomers [74]. Typically, a mixture containing 90.4% of *n*-butyl L-lactate (BuLLa) and 9.6% of D-lactates (BuDLa) was incubated with an immobilized lipase, and then, the purity of BuLLa was increased to 98.6%, indicating that the lipase catalysis provides with a good method for enantio-purification.



Scheme 5.7 Dehydration polycondensation of ω-oxyacids



Scheme 5.8 Dehydration (R = H) or transesterification (R = CH_3) polycondensation of ricinoleic acid or its ester

ω-Oxyacids or Their Esters Polycondensation of an ω-oxyacid (m ≥ 1 in Scheme 5.7) was reported first in 1985, describing a poly(ethylene glycol)-modified (PEG) lipase-catalyzed dehydration polycondensation of 10-hydroxydecanoic acid (m = 8), a hydrophobic monomer, in a benzene solution [31]. The resulted polyester structure is considered linear; degree of polymerization (DP) was not high. In this polymerization, modification of the lipase with PEG (mol. wt. 5000) was very important and made the reaction proceed in a hydrophobic medium.

It is to be noted that the dehydration polycondensation occurred in water solvent catalyzed by lipases PA, CC, PC, and PF; in water the lipase-catalyzed dehydration reaction of ω-oxyacids (m = 9, 13 in Scheme 5.7) at 35–75 °C for 24 h gave the corresponding polyesters with M_n ~1000 in 5–69% yields. Normally, this type of dehydration reaction is in equilibrium with the starting side and the product side, and hence, the water solvent makes the reaction proceeding disfavor because of the water product, due to the "law of mass action." Yet, lipase catalysis enabled the reaction forward; this concept *dehydration in water* is considered as a new finding in organic chemistry [75, 76].

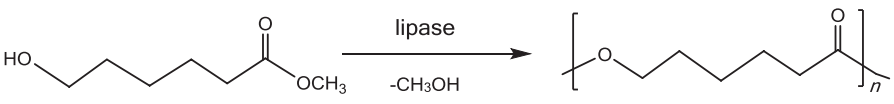
Dehydration polycondensation of an oxyacid, ricinoleic acid (a main component from castor oil), was catalyzed by lipase at 35 °C in an organic solvent to give a polyester with M_n ~1000 in good yields (R = H, Scheme 5.8) [77]. Methyl ricinoleate was polymerized via transesterification with immobilized lipase PC catalyst in bulk in the presence of molecular sieves at 80 °C for 7 days. Polyricinoleate was of high mol. wt. $M_w \sim 10^5$, a viscous liquid at room temperature with a glass transition temperature (T_g) of −74.8 °C, and was biodegraded by activated sludge. Polyricinoleate was readily cured using a dicumyl peroxide at 170 °C for 30 min to produce a chloroform-insoluble cross-linked polyricinoleate. Its M_w value was very high ~10^5 (R = CH_3, Scheme 5.8) [78].

Regioselective oligocondensation of cholic acid (an oxyacid) by lipase CA catalyst was achieved. A mixture of cholic acid, 11-methacryloylaminoundecanoic acid, and the lipase produced a radically polymerizable oligo(cholic acid ester) baring methacryloyl end group. This new monomer was radically polymerized to give a corresponding comb polymer having several cholic acid ester units as side chains [79].

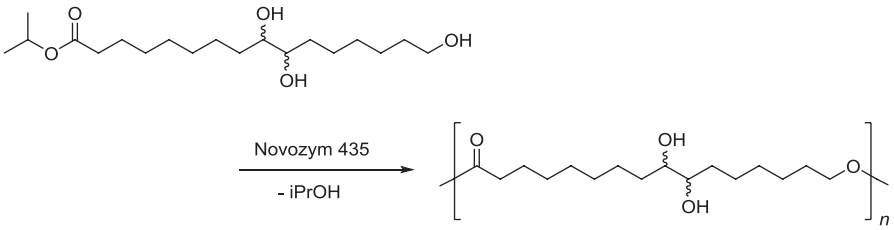
Hydrophobic oxyacids were efficiently condensation-polymerized by lipase CA as catalyst. In the polymerization of 16-hydroxyhexadecanoic acid (m = 14, Scheme 5.7), 12-hydroxydodecanoic acid (m = 10), or 10-hydroxydecanoic acid (m = 8) under vacuum at a higher temperature (90 °C) in bulk for 24 h, the DP value of the product polymer was beyond 100, whereas the polyester with lower molecular weight (DP ~ 63) was formed from 6-hydroxyhexanoic acid (m = 4) under the similar reaction conditions [80]. Immobilized *Humicola insolens* (HiC)- and Novozym 435 (N435)-catalyzed homopolymerizations of ω-hydroxyalkanoic acids (ωHA) were investigated. ωHAs examined were hexanoic acid (m = 4), 10-hydroxydecanoic acid (m = 8), 12-hydroxydodecanoic acid (m = 10), and 16-hydroxyhexadecanoic acid (m = 14) (Scheme 5.7) [81]. HiC's activity for ωHA substrates with 12 and 16 carbons was high C16 > C12, but not C10-ωHA and C6-ωHA were polymerized. In contrast, N435's activity for ωHA substrates was C16 = C12 > C10, but not C6-ωHA was polymerized. HiC-AO (Amberzyme oxirane resin)- and N435-catalyzed C16-ωHA homopolymerization at 70 °C for 8 h gave polyesters with M_n values ~ 4.0×10^4 and ~ 2.6×10^4, respectively, which are quite high. These results show that the catalytic activity of these lipases is very sensitive to the length of the substrate monomer; hydrophobic nature of the monomer is operative.

The lipase CC-catalyzed polycondensation of 11-hydroxyundecanoic acid (m = 9, Scheme 5.7) in hexane produced a polyester, the molecular weight of which was up to 3.5×10^4. A time-conversion course study of the polymerization process revealed that oligomers were formed relatively rapidly and that these oligomers then condensed to generate higher molecular weight polyesters [82]. Novozym 435 was also an efficient catalyst for the dehydration polycondensation of an oxyacid, *cis*-9,10-epoxy-18-hydroxyoctadecanoic acid, carried out in toluene in the presence of molecular sieves at 75 °C for 68 h to give the polyester with the highest M_w 2.0×10^4 (M_w/M_n = 2.2). It is noticeable that the epoxy functional group was not affected by the enzyme catalysis [83].

The transesterification polycondensation of methyl 6-hydroxyhexanoate, an ω-oxyacid ester, with lipase catalyst in hexane at 70 °C for more than 50 days gave the polyester with DP value up to 100 (Scheme 5.9, see also reaction (1) in Scheme 5.1). PPL-catalyzed polymerization of methyl 5-hydroxypentanoate produced the polyester with DP value of 29 [39].



Scheme 5.9 Transesterification polycondensation of methyl 6-hydroxyhexanoate

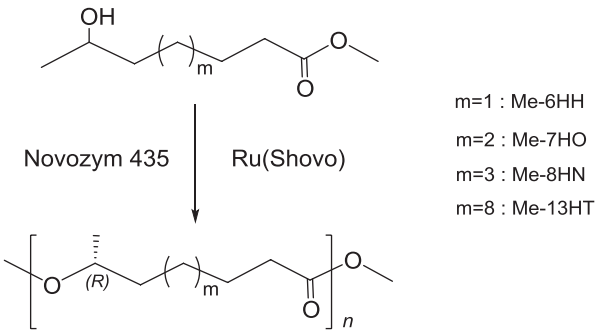


Scheme 5.10 Regioselective transesterification polycondensation of isopropyl aleuriteate

Various hydroxyesters, ethyl esters of 3- and 4-hydroxybutyric acids, 5- and 6-hydroxyhexanoic acids, 5-hydroxylauric acid, and 15-hydroxypentadecanoic acid, were polymerized by lipase PS catalyst via transesterification to give the corresponding polyesters with molecular weight of several thousands [84]. Novozym 435 induced a regioselective polycondensation of isopropyl aleuriteate, where the only primary alcohol was reacted at 90 °C in a toluene/2,4-dimethyl-3-pentanol mixed solvent, giving rise to the polymer of M_n 5600 in 43% yields (Scheme 5.10) [85].

With using a chemo-enzymatic method, new polyesters having rotaxanes in the side chain were prepared. Terminal-functionalized polymers like macromonomers and telechelics are important and often used as prepolymers for synthesis of functional polymers. The lipase CA-catalyzed polycondensation of 12-hydroxydodecanoic acid in the presence of 11-methacryloylaminoundecanoic acid produced a methacrylamide-group-containing polyester macromonomer, in which a β-cyclodextrin is contained in the side chain [86]. 12-Hydroxydodecanoic acid and methyl 12-hydroxystearate (both from seed oil) were copolymerized in polycondensation manner catalyzed by Novozym 435 in toluene in the presence of molecular sieves at 90 °C. During the reaction water as well as methanol liberated. After several days, the copolymer was obtained in good yields, having M_w ~1.0×10^5 showing elasticity and biodegradability [87].

An optically active oligoester was obtained by enantioselective polycondensation of racemic 10-hydroxyundecanoic acid, a secondary alcohol, by lipase CR catalyst. The resulting oligomer (mol. weight ~1000) was enriched in the (*S*) enantiomer with 60% enantio-excess (ee), and the residual monomer was recovered with 33% ee favoring *(R)* enantiomer. It was speculated that the enantioselection is caused at the deacylating step, the attack of substrate OH group onto the acyl carbon of acylated enzyme (see also Fig. 5.1 for reference) [88]. Lipase PPL catalyzed transesterification polycondensation of racemic ε-substituted-ε-hydroxy ester monomers ($HOCHR(CH_2)_4COOR'$: R = Me, Et, Ph; R' = Me, CH_2CCl_3) to produce optically active oligomers (degree of polymerization DP < 6); enantioselection occurred. With increasing bulkiness of the substituent, in the order Me < Et < Ph, the enzymatic reaction becomes slower, yet the enantioselectivity becomes higher. Copolycondensation between the monomer 1, R = H, R‘ = Me, and three monomers 2, R = H, R‘ = Me; 3, R = H, R‘ = Et; and 4, R = H, R‘ = Ph, gave copolyesters with DP values 8.7, 7.0, and 5.4, respectively. Optically active copolymers were obtained, being higher in molecular weight than the analogous homopolymers [89].



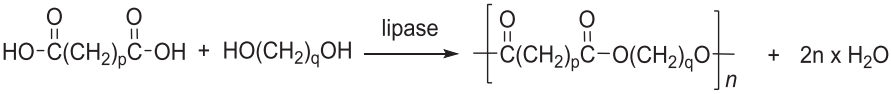
Scheme 5.11 Procedure of ITC method

Iterative tandem catalysis (ITC), a method of synthesizing chiral polyesters via the transesterification polycondensation of racemic monomers, was investigated. The reaction uses racemic oxyacid ester monomers (AB type monomers) having a secondary hydroxy group and a methyl ester moiety (Scheme 5.11). The concurrent actions of an enantioselective acylation catalyst (Novozym 435) and a racemization catalyst (Ru(Shvo)) brought about the high conversion of the racemic monomers to enantio-enriched D-polymers. Monomers used were typically methyl 6-hydroxyheptanoate (Me-6HH), methyl 7-hydroxyoctanoate (Me-7HO), methyl 8-hydroxynonanoate (Me-8HN), and methyl 13-hydroxytetradecanoate (Me-13HT). Using isopropyl esters under the selected reaction conditions, the polymerization produced chiral polymers with monomer conversion (up to 99%), high enantiomeric excess (ee, up to 92%), and molecular weight (up to 16.3×10^3) [90], (see also 5.2.3 for DKR method).

A chemo-enzymatic method was applied to prepare an oleic acid (a vegetable oil)-based polyester: First, oleic acid (C-18 mono-ene acid) was epoxidized by Novozym 435 catalyst with H_2O_2 oxidant, and then the intermolecular ring-opening addition between the epoxide group and CO_2H group thermally took place to produce the poly(oleic acid)-based polyester. It was further cross-linked by a diisocyanate compound to give a biodegradable tough material of polyester [91].

5.2.3 Polycondensation of Dicarboxylic Acids or Their Esters with Alcohols

Via Dehydration This type of lipase-catalyzed polycondensation of reaction (2) (X = H, Scheme 5.1) was reported first in 1984 on the dehydration reaction between a dicarboxylic acid and a diol to produce polyester oligomers (Scheme 5.12). Lipase from *Aspergillus niger* catalyzed the synthesis of oligoesters from various dicarboxylic acids and diols. For example, the product oligoesters synthesized from 1,13-tridecanedioic acid (p = 11) and 1,3-propanediol (q = 3) contained pentamer and heptamer as dominant components, both end groups being hydroxy [30].



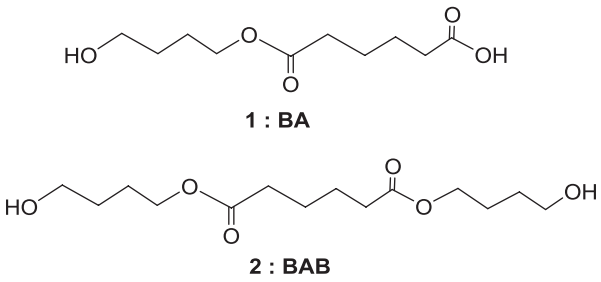
Scheme 5.12 Dehydration polycondensation between a dicarboxylic acid and a glycol

Dehydration polycondensation of adipic acid and 1,4-butanediol in diisopropyl ether gave a polyester with a degree of polymerization (DP) of 20 (p = q = 4) [92]. Higher molecular weight polyesters were enzymatically obtained by polycondensation of sebacic acid (p = 8) and 1,4-butanediol (q = 4) under vacuum. In the lipase MM-catalyzed polymerization in hydrophobic solvents of high boiling point such as diphenyl ether, the molecular weight of polyesters from various combinations of diacids and glycols reached the value higher than 4×10^4 [93–95].

Lipase CA efficiently catalyzed the polycondensation of dicarboxylic acids and glycols without solvent under mild reaction conditions at 60 °C. Methylene chain length of the monomers greatly affected the polymer yield and molecular weight. The polymer with molecular weight higher than 1×10^4 was obtained by the reaction under vacuum [96]. In the polymerization between dicarboxylic acids and glycols, effects of various combination of the monomers (p = 2, 4, 6, 8, and 12 and q = 2, 3, 4, 5, 6, 8, 10, and 12 in Scheme 5.12) as well as several lipase catalysts were examined. The best results were observed in terms of yields and M_n values for the reaction of sebacic acid (p = 8) and 1,5-pentanediol with lipase CA catalyst at 60 °C for 48 h, giving rise to the product polyester in 86% yields with M_n 14,000. In order for the reaction to proceed, it was necessary for the monomers to have appropriate hydrophobicity (e.g., p, q > 4). These syntheses afforded a variety of biodegradable aliphatic polyesters via nontoxic enzyme catalysis under mild reaction conditions, and hence, it was addressed to provide a good example of "Green Polymer Chemistry" [97].

Catalytic activity of lipases, immobilized *Humicola insolens* (HiC) and Novozym 435 (N435), was studied on the dehydration polycondensation of α,ω-*n*-alkane diols and α,ω-*n*-alkane diacids with varying chain length (Scheme 5.12). HiC on Amberzyme oxirane (AO) resin (i.e., HiC-AO) activity for the polymerization of sebacic acid (p = 8) with the diols (q = 3, 4, 5, 6, and 8) was C8 > C6, where C3, C4, and C5 diols were not polymerized. N435's relative activity for diol substrates was C8 > C6 > C5 > C4 > C3. HiC-AO activity for the polymerizations of 1,8-octanediol with the diacids with 6-, 8-, 9-, 10-, and 13-carbon chain lengths was C13 > C10, where HiC showed little activity for C6, C8, and C9 diacid polymerizations. N435 displayed similar activity for all these diacid chain lengths. Thus, N435 has a broader substrate promiscuity than HiC-AO. HiC-AO- and N435-catalyzed copolymerization of 1,8-octanediol/C13-diacid at 8 h gave polymers with M_n of 11.0×10^3 and 9.6×10^3, respectively [81].

Reaction pathway of Novozym 435-catalyzed dehydration polycondensation between adipic acid (A, p = 4) and 1,4-butanediol (B, q = 4) (see Scheme 5.13) was studied. The polyester chain formation involves a step-growth mechanism utilizing a key synthon, the simplest adduct of 1,4-butanediol and adipic acid, 6-carboxy-11-



Scheme 5.13 Structures of adducts **1** and **2**

hydroxy-7-oxaundecanoic acid (**1**), termed as *BA*. In the solvent-free reaction, enzyme-catalyzed esterification of *BA* with 1,4-butanediol forms *BAB* (**2**), and stepwise addition of *AB* gives $B(AB)_2$, $B(AB)_3$, etc. These reactions to give the oligomers with both B-terminals at the early stage are due to the hard solubility of the acid monomer. The polymerization pathway involves a key-adduct **1**, which becomes acylated by the enzyme catalysis. Propagation of the polymer chain proceeds via reaction with a hydroxy-terminated species, releasing the enzyme site, i.e., propagation via stepwise fashion [92, 98].

Effects of substrates and solvent on the ester-chain formation, polydispersity, and end-group structure were examined. Diphenyl ether shows a high boiling point with hydrophobic nature, and hence, it is a preferred solvent for the polycondensation of adipic acid (p = 4) and 1,8-octanediol (q = 8), giving M_n of $2{,}85 \times 10^4$ (48 h, 70 °C). Monomers having longer alkylene chain length of diacids (sebacic and adipic acids) and diols (1,8-octanediol and 1,6-hexanediol) showed a higher reactivity than the reactions of shorter chain derivatives, indicating that lipase catalysis is generally more effective for a hydrophobic substrate [99].

As to the reaction solvent, the enzymatic reaction takes place even in ionic liquids [100]; in an ionic liquid, lipase was active and transesterification polycondensation between diethyl adipate or diethyl sebacate and 1,4-butanediol occurred to give the product polyester [101].

"Dehydration reaction in water solvent" was achieved for the first time in organic chemistry field, which was also mentioned above [75, 76]. Dehydration reaction is generally achieved in nonaqueous media, because the product water of the dehydration is in equilibrium with starting materials and the solvent water disfavors the dehydration to proceed in an aqueous medium due to the "law of mass action." Nevertheless, lipase catalysis enabled a dehydration polycondensation between several α,ω-dicarboxylic acids and glycols in water at 45 °C, to afford a polyester in good yields. Lipase CA and other lipases were active for these dehydration polycondensations (Scheme 5.12). In the polymerization of a dicarboxylic acid and a glycol, the polymerization behavior was greatly depending on the methylene chain length of the monomers. For example, from sebacic acid and 1,8-octanediol (p = q = 8, in H_2O solvent), the polyester with $M_n = 1700$ in 43% yields at 45 °C for 24 h by lipase CA catalyst was obtained, whereas no polymer formation was observed from sebacic acid and 1,6-hexanediol, suggesting that the combination of the monomers

with appropriate hydrophobicity is important for the polymer production by dehydration. This finding of *dehydration in water* is a new aspect and attracted organic chemists with much interests (see also the above 5.2.2) [75, 76].

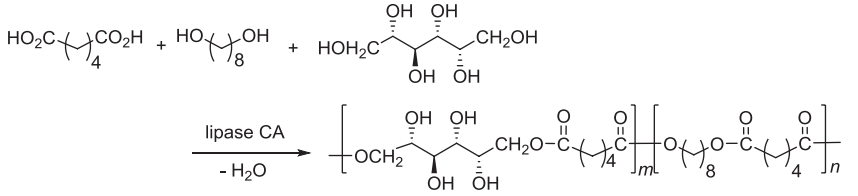
CALB was covalently immobilized onto epoxy-activated macroporous poly(methyl methacrylate) Amberzyme beads with a poly(glycidyl methacrylate) outer region. In bulk Amberzyme-CALB-catalyzed polycondensation between glycerol (0.1 equiv), 1,8-octanediol (0.4 equiv), and adipic acid (0.5 equiv) at 90 °C for 24 h gave higher molecular weight polyester of $M_w \sim 4.0 \times 10^4$ [102].

For obtaining information on commercialization of the environmentally friendly synthetic process, the scale-up experiments of the Novozym 435-catalyzed polyester synthesis from adipic acid and glycerol (a triol) were performed on a 500 g scale. The reaction was carried out in a heated, solvent-free system, and the influence of various reaction conditions (i.e., temperature 60–90 °C, reduced pressure, enzyme concentration, reactants ratio, stirrer type, stirring rate, and reaction time) on the substrate conversion and molecular weight of the product was investigated. Conversions were higher than 90%, and molecular weights were in the desired M_n range of 2000–3000. High hydroxyl functionality of the product polymer is expected to cause new function. And, a scale-up to 200 kg polyester yields was also examined [103, 104].

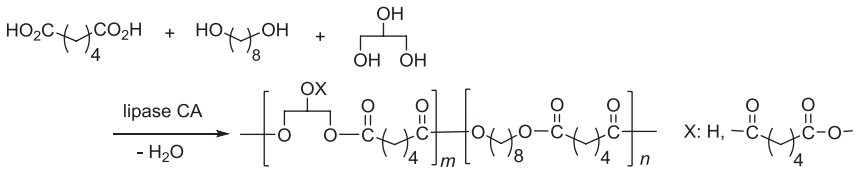
Dehydration polycondensation of adipic acid and 1,8-octanediol in the presence of L-malic acid (L-MA) in organic media was achieved with Novozym 435 catalyst. The molecular weight reached to a maximum of 17,400 at 80 °C in isooctane at an L-MA feed ratio in the diacids of 40 mol %. The M_w increased from 3200 to 16,600 when the reaction time was extended from 6 to 48 h at 70 °C. The hydrophilicity, thermal stability, and crystallizability of the copolymer were also investigated [105]. As green polymerization cycle, the preparation of partially renewable aliphatic polyesters based on 1,8-octanediol and biobased long-chain diacids, namely, 1,12-dodecanedioic and 1,14-tetradecanedioic acid, was performed. It involved CALB-catalyzed prepolymerization combined with low-temperature post-polymerization, in the melt or solid state [106].

The direct dehydration polycondensation between adipic acid and sorbitol, a polyol sugar component, with Novozym 435 catalyst in bulk was performed at 90 °C for 48 h. The product poly(sorbityl adipate) was water-soluble. The M_n and M_w values were ~1.1×10^4 and ~1.7×10^4, respectively. In the polymer, sorbitol was esterified at primary alcohol group of 1- and 6-positions with high regioselectivity (~85%). In place of sorbitol, glycerol was employed; however, the M_n and M_w values were lower, 2500 and 3700, respectively. To obtain a water-insoluble sorbitol copolyester, adipic acid, 1,8-octanediol, and sorbitol (molar ratio 50:35:15) were terpolymerized at 90 °C for 42 h (reaction (A), Scheme 5.14). The methanol-insoluble part (80%) had M_w of 1.17×10^5. Another terpolymerization of adipic acid, 1,8-octanediol, and glycerol (molar ratio 50:40:10) was performed in bulk at 70 °C, to give a polyester (reaction (B), Scheme 5.14). The product polymer showed the monomer ratio in the 50:41:9, respectively, and the values for M_w and M_w/M_n of $7{,}56 \times 10^4$ and 3.1, respectively. The product contained 90% methanol-insoluble parts, showing few cross-links. The selectivity at glycerol primary alcohol sites was only 66%; therefore, the product was highly branched; 27% of glycerol units were

(A) Terpolymer from adipic acid, 1,8-octanediol, and sorbitol



(B) Terpolymer from adipic acid, 1,8-octanediol, and glycerol



Scheme 5.14 Terpolymerizations of adipic acid, 1,8-octanediol and (**A**) sorbitol, and (**B**) glycerol

for branched sites. Physical properties of the resulting polyesters containing sorbitol or glycerol were characterized in detail [107, 108].

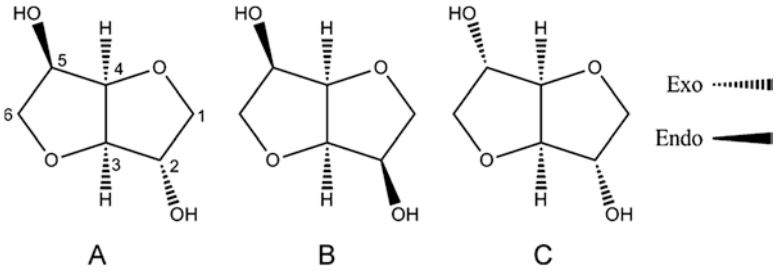
Lipase CA catalyzed the bulk dehydration polycondensation of the monomers, adipic acid (A_2), 1,8-octanediol (B_2), and glycerol ($B'B_2$) and gave hyperbranched polyesters at 70 °C for 42 h. With monomer feed molar ratio A_2: B_2: $B'B_2$ = 1.0:0.8:0.2, linear copolyesters were formed during the first 18 h, and extending the reaction time to 42 h gave hyperbranched copolymers with dendritic glycerol units. The regioselectivity for esterification at the primary glycerol positions ranged from 77 to 82%. Variation of glycerol in the monomer feed gave copolymers with degree of branching from 9 to 58% [109]. A similar dehydration polycondensation to produce terpolymers was conducted by using monomers, adipic acid (A_2), 1,8-octanediol (B_2), and trimethyrolpropane (B_3) with lipase CA catalyst in bulk at 70 °C for 42 h. As an example, with a feed ratio (A_2:B_2:B_3 = 1:0.5:0.5), a hyperbranched copolyester with 53% B_3/A_2 units was obtained in 80% yields having M_w 14,100 (M_w/M_n = 5.3) [110]. CALB (N435)-catalyzed one-pot copolymerization of linoleic acid (LA), glycerol (G), and 1,18-cis-9-octanedecenedioic acid (oleic diacid, OD) yielded cross-linkable unsaturated polyesters. The reaction efficiently formed poly(OD-*co*-G-*co*-LA). For comonomer feed ratio OD:G:LA = 1:1:0.67, M_n reached ~ 9500 in 8 h, trisubstituted G-units increased to 64%, and monomer was well consumed. By varying the feed ratio of LA, polymeric triglyceride-type structures were formed with controlling chain length and trisubstituted G-unit content [111]. "Sweet polyesters" were prepared from natural sugar polyols. The polyols used were C4-carbon, erythritol, C5-carbon, xylitol and ribitol, and C6-carbon, mannitol, glucitol, and galactitol. The terpolymerization was performed with Novozym 435 catalyst in bulk for the combination of a polyol, adipic acid, and 1,8-octanediol under vacuum at 90 °C. The M_w value of the product polyol-polyester ranged from 1.1 × 10^4 (D-galactitol) to 7.3 × 10^4 (D-mannitol), having a branching structure. Primary alcohol groups are more reactive than the secondary ones [112].

Cutinase (EC 3.1.1.74) catalyzed a dehydration polycondensation. A glycol like 1,4-butanediol, 1,6-hexanediol, 1,8-octanediol, and 1,4-cyclohexanedimethanol (1,4-CHDM) and a diacid like adipic acid, succinic acid, suberic acid, and sebacic acid were combined for the polycondensation at 70 °C for 48 h under vacuum. In all reactions the monomers were consumed quantitatively. With fixing the adipic acid component, polyesters from 1,4-butanediol, 1,6-hexanediol, and 1,8- octanediol showed M_n values of 2700, 7000, and 12000, respectively. With fixing the 1,4-CHDM component, polyesters from succinic acid, adipic acid, suberic acid, and sebacic acid possessed M_n values of 900, 4000, 5000, and 19000, respectively. There was a tendency for both glycols and diacids that the higher the hydrophobicity, the higher the molecular weight of the product polyester [113]. ω-Carboxy fatty acid monomers, 1,18-*cis*-9-octadecenedioic, 1,22-*cis*-9-docosenedioic, and 1,18-*cis*-9,10-epoxy-octadecanedioic acids, were synthesized from oleic, erucic, and epoxy stearic acids by whole-cell biotransformations catalyzed by *C. tropicalis* ATCC20962. The polycondensation of the ω-carboxy fatty acid monomers and diol monomers to polyesters was carried out with using Novozym 435 catalyst, giving rise to corresponding polyesters with unsaturated and epoxidized repeat units and M_w values ranging from 25000 to 57000 [114].

CALB catalyzed the synthesis of aliphatic polyesters (PEs) in the following way: from diols (1,4-butanediol and 1,8-octanediol) and diacids or their derivatives (diethyl succinate, sebacic acid, 1,12-dodecanedioic acid, and 1,14-tetradecanedioic acid), in order to produce poly(butylene succinate) (PE 4.4), poly(octylene sebacate) (PE 8.10), poly(octylene dodecanate) (PE 8.12), and poly(octylene tetradecanate) (PE 8.14). The two-stage procedures were suggested, both sustainable and in accordance with the principles of "green" polymerization. The first comprised an enzymatic prepolymerization under vacuum in diphenyl ether solvent, whereas a low-temperature post-polymerization step [solid state polymerization (SSP)] followed in order to upgrade the PEs quality. In the first synthesized prepolymers, the M_n attained was from 3700 to 8000 with yields reaching even 97%. Next, SSP of PE 4.4 and PE 8.12 took place under vacuum or flowing nitrogen and lasted 10–48 h, at temperatures close to the prepolymer melting point (4 °C ~ 14 °C). The solid state finishing led to increase in the molecular weight, and it also contributed to improvement of the physical characteristics and the thermal properties [115].

With the CALB-catalyzed synthesis, linear ester oligomers and cyclic ester oligomers from succinic acid in combination with a di-anhydro hexitol (DAH) in toluene were produced. The conversion was highest for isomannide and decreases in the order isomannide (B) > isosorbide (A), isoidide (C) (Scheme 5.15). The maximum conversions under optimized conditions were in ~ 90% yields for linear oligomers [116].

Some polyester thermosets based on photocurable prepolymers composed of itaconic acid (IA) and various polyols were developed by using CALB catalyst at 90 °C or by thermal dehydration reaction at 145 °C. The reaction components were IA, a dicarboxylic acid like succinic acid and adipic acid, and a polyol like 1,4-cyclohexanedimethanol, a PEG-diol, and sorbitol. Dimethyl itaconate was an ideal monomer for enzymatic polymerization, as demonstrated by the synthesis of



Scheme 5.15 Structures of (**A**) isosorbide, (**B**) isomannide, and (**C**) isoidide

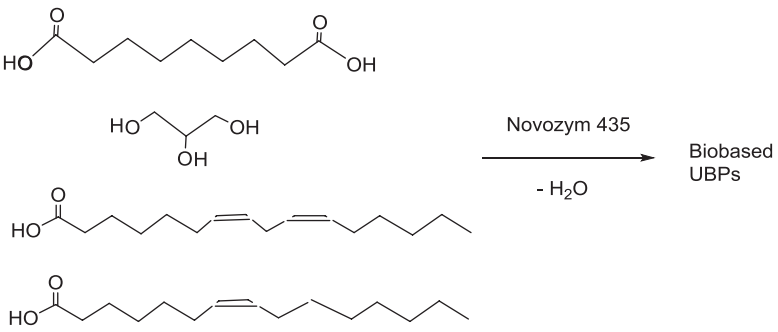
linear poly(1,4-cyclohexanedimethanol itaconate), poly(PEG itaconate), and poly(3-methyl-1,5-pentanediol itaconate-*co*-3-methyl-1,5-pentanediol adipate). Photolysis of the polyesters gave cured polyesters, whose physical properties were examined for proving the usefulness as future biomaterials [117].

By using oleic diacid having a reactive double bond and glycerol, the synthesis and structure of poly(oleic diacid-*co*-glycerol) were compared in the cases of catalysts, Novozym 435 (N435) and dibutyl tin oxide (DBTO). With employing N435 catalyst and an oleic diacid-to-glycerol molar ratio of 1.0:1.0, the M_n values were 6000 at 6 h and 9100 at 24 h with low branching degree (% of glycerol 13%–16%). With N435 catalyst, resulting polyesters were not cross-linked. In contrast, with DBTO catalyst, an oleic diacid-to-glycerol molar ratio of 1.0:1.0 polyester M_n of 1700 was obtained at 6 h, and, thereafter, a gel was formed due to cross-linking. Thus, N435's catalyst ability to deter cross-linking due to steric hindrance enabled to give soluble and hyperbranched copolyesters [118].

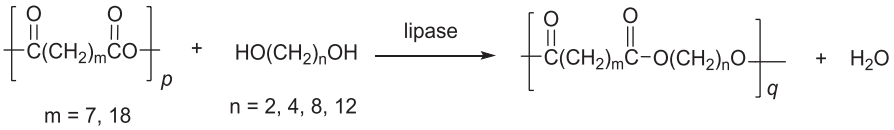
Direct production of highly branched polyesters was achieved via a one-pot, enzyme-catalyzed dehydration bulk polymerization. Biobased feed components in the form of glycerol, pentaerythritol, azelaic acid, and tall oil fatty acid (TOFA) were polymerized using CALB as catalyst, and the potential for the enzymatic synthesis of alkyds was investigated. Biobased unsaturated branched polyesters (UBPs) were synthesized in a one-pot process from TOFA, glycerol, and azelaic acid as a renewable alternative to the phthalic acid or anhydride normally applied for alkyd synthesis. The UBPs were prepared at 90 °C directly from the mono- and difunctional carboxylic acids (Scheme 5.16) [119]. Pentaerythritol was also used as the alcohol component for the similar reactions to give various UBPs with more highly branched structure. The post-polymerization cross-linking of these UBPs showed their potential as binders in alkyds.

Biobased aromatic-aliphatic oligoesters were synthesized via CALB-catalyzed dehydration polycondensation reaction of aromatic dicarboxylic acids with linear diols with varying chain length. The acids used were terephthalic acid, isophthalic acid, and phthalic acid, and the glycols were $HO(CH_2)_mOH$ (m = 2, 4, 6, 8, 10, and 12). The dehydration polycondensations of the acid with the glycol were very unreactive; conversions were very low [120].

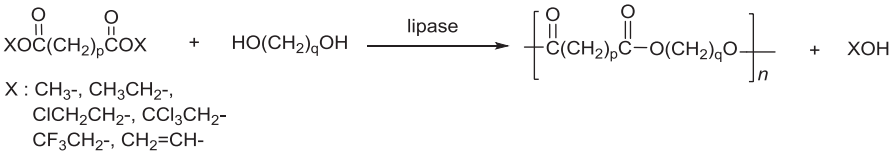
As an additional type, the reaction of a linear polyanhydride, such as poly(azelaic anhydride), and a glycol, such as 1,8-octanediol, was induced with lipase CA catalyst at 30–60 °C in bulk or in toluene involving the dehydration-insertion to give a



Scheme 5.16 CALB-catalyzed preparation of UBPs by one-pot bulk polymerization between azelaic acid, glycerol, and TOFA



Scheme 5.17 Polycondensation involving dehydration as well as transesterification



Scheme 5.18 Transesterification polycondensation reaction

polyester with molecular weight of several thousands (Scheme 5.17). It is to be noted that this type of reaction is of dehydration polycondensation, yet this is a kind of transesterification polycondensation type as well [121].

Via Transesterification Polycondensation normally needs activation of carboxylic acid group for better reaction results. The activation is achieved ordinarily by esterification of the acid group as shown in Scheme 5.18. In the early studies, alkyl or haloalkyl esters and later vinyl esters have been developed.

A lipase-catalyzed high enantioselective polymerization was reported in 1989; the reaction of bis(2,2,2-trichloroethyl) *trans*-3,4-epoxyadipate with 1,4-butanediol in anhydrous diethyl ether using porcine pancreas lipase (PPL) catalyst gave a highly optically active polyester. The feed molar ratio of the racemic diester to the diol was adjusted to 2:1, resulting in producing the (−)-polymer to show enantiomeric purity >96%. The molecular weight was estimated as 5300. From the unchanged (+)-monomer, enantiomeric purity was higher than 95% [122].

1,4-Butanediol (BD) and diethyl sebacate (DES) were copolymerized with bicyclic acetalized D-glucose derivatives (Glux) by polycondensation both in the melt at high temperature and in solution at mild temperature mediated by CALB (Fig. 5.8).

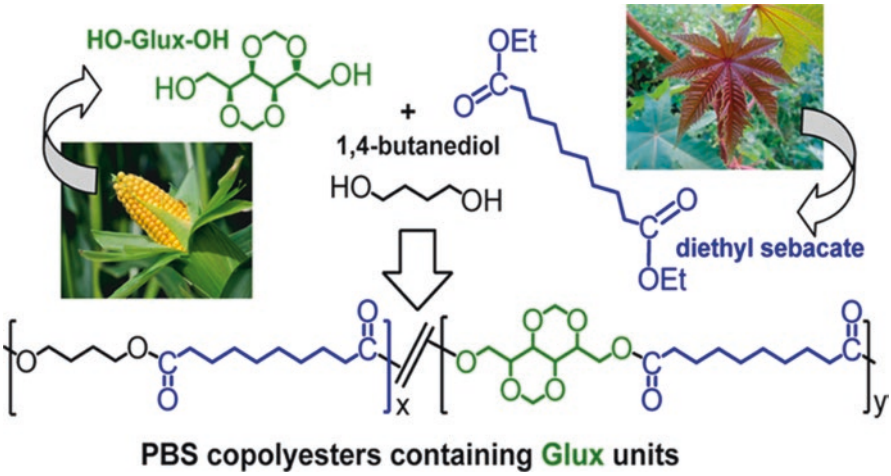


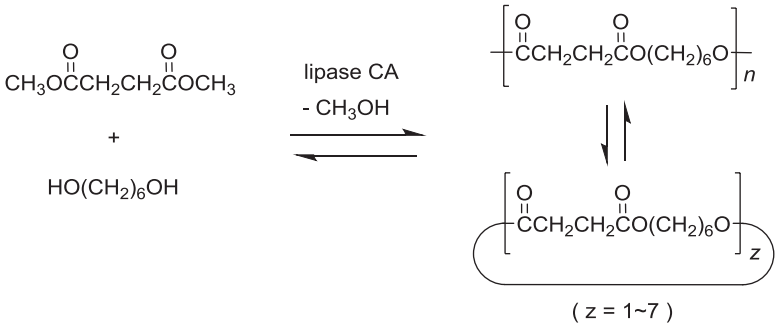
Fig. 5.8 Illustration of the synthesis processes. (Reproduced with permission from [123]. Copyright 2015 American Chemical Society)

Two series of random copolyesters (PB_xGlux_ySeb and $PBSeb_xGlux_y$) were prepared differing in which D-glucose derivative (Glux diol or Glux diester) was used as comonomer. Both methods were found to be effective for polymerization although significant higher molecular weights were achieved by melt polycondensation. The thermal properties of the copolyesters were largely dependent on composition and also on the functionality of the replacing Glux unit [123].

Cutinase belongs also to a hydrolase enzyme like lipase. Cutinase 1 from *Thermobifida cellulosilytica* (The_cut1) was found as an efficient biocatalyst in polycondensation reactions. Under thin film conditions, the covalently immobilized enzyme catalyzes the synthesis of oligoesters of dimethyl adipate with different polyols leading to higher M_w (~1900) and M_n (~1000), if compared to lipase B from *Candida antarctica* (CALB) or cutinase from *Humicola insolens* (HiC). Computational analysis disclosed the structural features that make The_cut1 readily accessible to substrates and optimally suited for covalent immobilization. As lipases and other cutinase enzymes, The_cut1 presents hydrophobic superficial regions around the active site. These observations and molecular dynamics simulations may allow to study a systematic comparison of functional differences between cutinases and lipases [124].

Microwave energy (MWe) effects on the lipase-catalyzed transesterification polymerization were studied. The reaction examined is the polymer synthesis from dimethyl succinate (DMS) and 1,4-butanediol (BD) to give the corresponding polyester in bulk or in solution. In terms of monomer conversion, MWe effects were not well observed: Results showed that MWe enabled the biocatalyzed synthesis of polyesters and prepolymers in a similar way to that reported using conventional heating with an oil bath [125].

Polycondensation with CALB catalyst for the synthesis of polyester prodrugs of ketoprofen was studied, giving rise to the linear polyesters with pendant ketoprofen



Scheme 5.19 Product polymers of ring-chain equilibrium

groups based on ketoprofen glycerol ester, poly(ethylene glycol), and divinyl sebacate. The polyester had M_w reaching to 7000 and could be a promising prodrug with extended pharmacological effects by delayed release of ketoprofen [126, 127].

An important phenomenon of the ring-chain equilibrium of the product was observed in the polycondensation between dimethyl succinate and 1,6-hexanediol catalyzed by lipase CA in toluene at 60 °C, reaching the ring-chain (cyclic-linear structure) equilibrium of the product polymer (Scheme 5.19) [128]. Adsorption of methanol by molecular sieves or eliminating methanol by nitrogen bubbling shifted to the thermodynamic equilibrium. Polyesters with the molecular weight about several thousands were produced from α,*ω*-alkylene dicarboxylic acid dialkyl esters, and regardless of the monomer structure, cyclic oligomers were formed. In the polymerization of dimethyl terephthalate and diethylene glycol catalyzed by lipase CA in toluene for producing a terephthalate polymer, the distribution of the macrocyclic species obeyed the Jacobson-Stockmayer theory, in terms of ring-chain equilibrium [129].

In the lipase-catalyzed polycondensation of dimethyl terephthalate and diethylene glycol, a cyclic dimer was selectively formed. The cyclic dimer formed (%) in the products was examined on reaction temperature, reaction time, and extent of reaction (*p*): at 50 °C, 4 h, 0.30 *p*, 2%; 8 h, 0.62 *p*, 24%; and 24 h, 0.99 *p*, 64%, and at 80 °C, 4 h, 0.95 *p*, 88%; 8 h, 0.98 *p*, 80%; and 24 h, 0.99 *p*, 99%. So, at 80 °C the cyclic dimer was almost quantitatively formed. It was considered that among several factors the selective cyclic dimer formation is ascribed to a driving force of a *π*-*π* stacking of the aromatic rings together with a relative flexibility of the diol segment and also of the nature of the enzyme catalytic site [130].

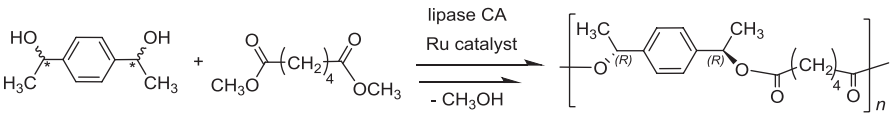
For the preparation of copolyesters, from dimethyl succinate and 1,4-butanediol by CALB-catalyzed transesterification polycondensation, cyclic(butylene succinate) oligomers $c(BS)_n$ were first produced. They are a mixture of dimer, trimer, and tetramers as the main components, whereas higher size cycles, $c(BS)_n$, as well as the monomer were present in much smaller amount. Then, the ring-opening copolymerization of $c(BS)_n$ and ε-caprolactone (CL) with CALB catalyst gave the copolyester, *co*P(BS_xCL_y), with different unit composition [131]. By using chemo-enzymatic method, α,ω-dicarboxylic acid dimethyl esters having unsaturated C_{18}, C_{20}, C_{26} alkylene chains were epoxidized via chemo-enzymatical oxidation with hydrogen

peroxide/methyl acetate with lipase CA catalyst. Polycondensations of these dimethyl esters with a diol by the lipase catalysis gave the linear unsaturated and epoxidized polyesters with molecular weight of 1950–3300 and melting point of 47–75 °C from a 1,3-propanediol substrate and with molecular weight of 7900–11,600 and melting point of 55–74 °C from a 1,4-butanediol substrate [132]. Novozym 435-catalyzed synthesis of poly(butylene succinate) (PBS) via polycondensation was achieved using a monophasic reaction mixture of dimethyl succinate and 1,4-butanediol in bulk and in solution. Diphenyl ether was a preferred solvent to give a higher molecular weight PBS; at 60, 70, 80, and 90 °C after 24 h, M_n values of PBS were 2000, 4000, 8000, and 7000, respectively. The reaction at 95 °C after 21 h gave PBS with M_n value of 38,000 [133].

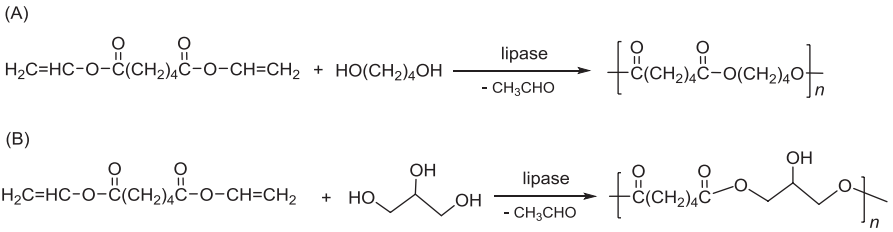
In an ionic liquid, a green solvent, such as 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF_4]), a similar polycondensation between diethyl adipate or diethyl sebacate and 1,4-butanediol gave the polyester having M_n up to 1500 in good yields. Since the ionic liquid is nonvolatile, ethanol was removed under vacuum during the reaction. Lipase CA-catalyzed polycondensation of dimethyl adipate or dimethyl sebacate with 1,4-butanediol was performed in an ionic liquid such as [bmim][BF_4], [bmim][PF_6], and [bmim][$(CF_3SO_2)_2N$] at 70 °C for 24 h to give a higher molecular weight polyester, M_n reaching several thousands. Using ionic liquids as solvent involves the wide range of tunability of solvent hydrophilicity and monomer solubility [101, 134].

In a supercritical fluoroform solvent, polycondensation of bis(2,2,2-trichloroethyl) adipate with 1,4-butanediol using PPL catalyst took place, giving rise to the polymer with molecular weight of several thousands [135].

As to the structure of the alkoxy component (XO- group in Scheme 5.18), transesterifications by lipase catalyst are affected and often very slow because of the reversible nature of the reaction. To shift the equilibrium toward the product polymer more effectively, activation of esters was conducted by using a halogenated alcohol (XOH) like 2-chloroethanol, 2,2,2-trifluoroethanol, and 2,2,2-trichloroethanol. Compared with methanol or ethanol, they increased the electrophilicity of the acyl carbonyl and avoided significant alcoholysis of the products by decreasing the nucleophilicity of the leaving alkoxy group. Lipase PF-catalyzed reaction of bis(2-chloroethyl) succinate and 1,4-butanediol carried out at 37 °C gave the polyester with the highest M_n of 1570 [136]. Polycondensation of bis(2,2,2-trichloroethyl) glutarate and 1,4-butanediol proceeded with PPL catalyst at room temperature in diethyl ether to produce the polyesters with molecular weight of 8200 [137]. In the PPL-catalyzed polymerization of bis(2,2,2-trifluoroethyl) glutarate with 1,4-butanediol in 1,2-dimethoxybenzene, a periodical vacuum method for removing 2,2,2-trifluoroethanol from the reaction mixture increased the molecular weight to ~ 40,000 [138]. Also, the vacuum technique was effective to shift the reaction equilibrium, thus increasing the molecular weight. The lipase-catalyzed polycondensation between bis(2,2,2-trifluoroethyl) sebacate and aliphatic diols was performed at 37 °C. The elimination of the product 2,2,2-trifluoroethanol was critical for obtaining the higher molecular weight polyesters; the polyester from 1,4-butanediol reached the highest M_w of 46,400 [94].



Scheme 5.20 DKR method to prepare the optically active polyester from the racemic monomer



Scheme 5.21 Lipase-catalyzed synthesis of (**A**) poly(butylene adipate) from divinyl adipate and 1,4-butanediol and (**B**) poly(glycerol adipate) (PGA) from divinyl adipate and glycerol

Dynamic kinetic resolution (DKR) method was newly developed to synthesize an optically active polyester from a racemic monomer via polycondensation [139]. A mixture of racemic secondary diol, α,α'-dimethyl-1,4-benzenedimethanol, was enzymatically copolymerized with dimethyl adipate (Scheme 5.20). Because of the enantioselectivity of lipase CA, only the hydroxy groups at the (*R*) center preferentially reacted to form the ester bond with liberation of methanol. The reactivity ratio was estimated as $(R)/(S) = \sim 1 \times 10^6$. In situ racemization from the (*S*) to the (*R*) configuration by Ru catalysis allowed the polymerization to high conversion, that is, the enzymatic polymerization and the metal-catalyzed racemization occurred concurrently. The DKR polymerization was carried out for 4 days; during the reaction, molecular weight increased to 3000–4000, and the optical rotation of the reaction mixture increased from – 0.6° to 128°. In the final stage, all product polymers will be end-capped with (*R*) stereocenters (chain stoppers) (see also [90] and Scheme 5.11).

As mentioned above, polycondensation is, in principle, a reversible reaction. However, an irreversible polyester-formation process was disclosed; for this process, a divinyl ester was employed for the first time in 1994 as the activated acid forms in the enzyme-catalyzed polyester synthesis. A vinyl ester proceeds much faster than an alkyl ester or a haloalkyl ester to form the desired product in higher yields, where the product of vinyl alcohol tautomerizes to acetaldehyde. Thus, the lipase PF-catalyzed polycondensation of divinyl adipate and 1,4-butanediol was performed at 45 °C in diisopropyl ether for 48 h to afford a polyester with M_n of 6.7×10^3 with liberating acetaldehyde (reaction (A), Scheme 5.21). While, the use of adipic acid or diethyl adipate did not produce the polymeric materials under the similar reaction conditions. As a diol, ethylene glycol, 1,6-hexanediol, and 1,10-decanediol were also reacted to give the corresponding polyester with molecular weight of several thousands [140]. The similar polycondensation of divinyl adipate and 1,4-butanediol with lipase PC catalyst produced the polyester with M_n of 2.1×10^4 [141]. With varying the molar ratio of the divinyl ester and the glycol, a

macromonomer having the glycol, the dicarboxylic acids, or the acid-alcohol end structure are expected to be produced.

Novozym 435-catalyzed transesterification polymerization from divinyl adipate and unprotected glycerol produced poly(glycerol adipate) (PGA) with molar mass (12×10^3), having a pendant hydroxyl group which imparts a hydrophilic character to this water-insoluble polymer (reaction (B), Scheme 5.21). Using the lipase as catalyst suppressed cross-linking at the pendant glyceryl hydroxyl through steric hindrance at the active site, thus producing polymers with low degrees of branching (5–30%) because of the regioselective reaction, and removes the need for any pre- or post-polymerization protection/deprotection reactions. Due to the ability of the synthetic route to produce a controlled structure, the generated PGA may emerge as a useful biodegradable polymer platform for various applications [142] (see also Scheme 5.15). It was argued that during the lipase-catalyzed polymerization of divinyl esters and glycols, there was a competition between the enzymatic transesterification and hydrolysis of the vinyl end group, and hence, the polymer growth might be disturbed with this side reaction [143].

A batch-stirred reactor was developed to minimize temperature and mass-transfer effects. Using the reactor, the polycondensation became very fast; within 1 h at 60 °C, poly(1,4-butylene adipate) with the molecular weight of 2.3×10^4 was obtained [144]. The CALB-catalyzed polycondensation produced aliphatic polyesters having pendant azide groups. The grafting reaction to the N_3-functional polyester was carried out quantitatively at room temperature using copper-catalyzed azide-alkyne cycloaddition (CuAAC, "click" reaction) with monoalkyne-functional poly(ethylene oxide) (alkyne-PEO, M_n = 750). Both enzymatic polycondensation and "click" reaction were performed in sequential one-pot reaction. The graft copolymer was surface-active and self-assembled in water [145].

A combinatorial approach was applied for biocatalytic production of polyesters. A library of polyesters was synthesized in 96 deep-well plates from a combination of divinyl esters and glycols with lipases of different origin. In the screening experiments, lipase CA was the most active biocatalyst for the polyester production. As an acyl acceptor, 2,2,2-trifluoroethyl esters and vinyl esters were examined, and it was stated that the former produced the polymer of higher molecular weight [146].

Supercritical carbon dioxide ($scCO_2$) was shown to be a good solvent for the lipase-catalyzed polycondensation of divinyl adipate and 1,4-butanediol. Quantitative consumption of both monomers was achieved to give the polyester with M_n of 3.9×10^3 [147] (see also [135] for supercritical fluids).

Aromatic diacid divinyl esters produced aromatic polyesters via lipase CA-catalyzed polycodensation. Divinyl esters of isophthalic acid, terephthalic acid, and *p*-phenylene diacetic acid were polymerized with various glycols to give aromatic polyesters with the highest M_n of 7200 [148]. Enzymatic polycondensation of divinyl esters with aromatic diols also afforded the aromatic polyesters [149]. Biobased aromatic-aliphatic oligoesters were synthesized via CALB-catalyzed transesterification polycondensations of aromatic dicarboxylic acid methyl esters with linear diols with varying chain length. The acids used were terephthalic acid, isophthalic acid, and phthalic acid, and the glycols were $HO(CH_2)_mOH$ (m = 2, 4, 6,

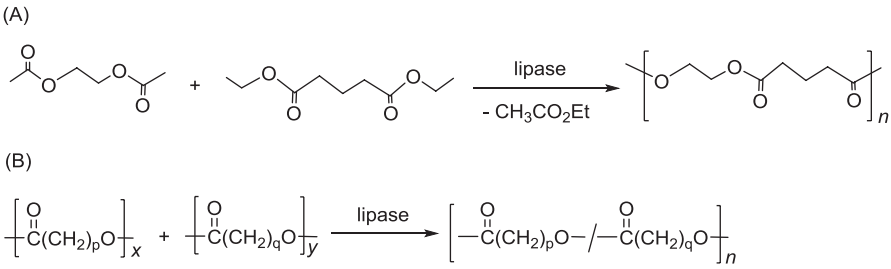
8, 10, and 12). In contrast to the dehydration polycondensation, the transesterification gave better results; the highest conversions of dimethyl isophthalate with 1,4-butanediol and 1,10-decanediol (m = 4 and 10) were 88 and 87%, respectively, M_w being ~ 1500 [120].

Novozym 435-catalyzed polycondensation of ketone-containing diesters and diacids combined with di(ethylene glycol) gave biodegradable and amorphous polyketoesters with M_n values of up to 10.1×10^3. By including ketone groups in the repeat unit, facile post-polymerization modifications were possible by reaction with oxyamine-tethered ligands through the formation of an oxime linkage. Upon reaction with molecules containing oxyamines, these polymers can easily have a diverse set of side chains appended without co-reagents or catalysts. The chemoselective oxime-forming coupling strategy is compatible with physiological conditions and can be done in the presence of a wide range of functional groups and biomolecules such as proteins and nucleic acids [150].

Silicone-containing polyesters were produced with lipase catalyst to give the polymers between 74 and 95% yields via polytransesterification. A lipase from *C. rugosa* was only successful in performing esterifications using carboxy-modified silicones that possessed alkyl chains greater than three methylene units between the carbonyl and the dimethylsiloxy groups [151].

Renewable green monomers were polymerized by using CALB as the biocatalyst. Commercially available succinate, itaconate, and 1,4-butanediol were enzymatically copolymerized in solution via a two-stage method. The chemical structures of the obtained products, poly(butylene succinate) (PBS) and poly(butylene succinate-*co*-itaconate) (PBSI) having reactive vinylene group, were confirmed. Values of M_n reached to ~6500 in 90% yields [152]. Diesters and diols were converted into aliphatic polyesters and polycarbonates by lipase *Candida* sp. 99–125 catalysis, with β-cyclodextrin acting as supporting architecture without using organic solvents. The polytransesterification was a much greener process, being solvent-free and metal residues-free. The lipase showed a high catalytic activity for bulk polymerization of diesters and diols with various numbers of methylene groups in their chains. β-Cyclodextrin encircled the linear polymer chain and maintained the chain in a proper configuration to avoid its coagulation. Lipase initiated the polymerization and β-cyclodextrin threaded onto the polymer chain to control the structure for producing high molecular weight polyesters up to 62,100 obtained at 70 °C. The corresponding polyesters showed an excellent thermal stability till 350 °C [153].

The enzymatic synthesis of poly(ethylene glutarate) (PEG) was achieved from diethyl glutarate and ethylene glycol diacetate without solvent (reaction (A), Scheme 5.22). The reactions were catalyzed by CALB at 40 °C, for 18 h in water bath with mechanical stirring or 1 h in ultrasonic bath followed by 6 h in vacuum. The application of ultrasound intensified the polyesterification reaction with reducing the reaction time from 24 h to 7 h. The highest degree of polymerization (DP) was 31 with a monomer conversion of 97%. The ultrasound treatment demonstrated to be an effective green approach to intensify the polyesterification enhancement and brought about a high DP [154]. Ultrasound-assisted CALB-catalyzed synthesis of poly(ethylene glutarate) (PEG) (reaction (A)), poly(ethylene malonate) (PEM), and



Scheme 5.22 Transesterification synthesis of (**A**) PEG and (**B**) a copolyester from a mixture of two homo-polyesters

poly(ethylene phthalate) (PEP) was conducted in solvent-free conditions. The synthesis of these polyesters was based on the ester-ester exchange reaction between ethylene glycol diacetate and diethyl glutarate, dibenzyl malonate, as well as di-*n*-octyl phthalate to produce PEG, PEM, and PEP, respectively. The effect of ultrasound indicated to improve the synthesis of all polyesters. Ultrasound, as a green solvent-free technology, showed high potentiality to strengthen the polyester synthesis [155].

It is interesting to note that the transesterification frequently occurs not only between monomers (reaction (A)) but also between polymers during the polymerization; in fact, from a mixture of two homo-polyesters, a copolyester was obtained by lipase catalysis (reaction (B), Scheme 5.22). For example, a mixture of poly(ε-caprolactone) and poly(1,4-butylene sebacate) ($M_w = 1.1 \times 10^4$) was subjected to react at 90 °C in toluene for 48 h by lipase CA catalyst to give a copolyester with $M_w = 3.0 \times 10^4$ [156].

In the enzymatic transesterification synthesis, various reaction parameters were examined for preparing fully biobased poly(3-hydroxybutyrate-*co*-butylene succinate) (poly(HB-*co*-BS)) copolyesters. In CALB-catalyzed reactions, copolyesters were produced in solution, via a one-step or a two-step process from 1,4-butanediol, diethyl succinate, and the synthesized telechelic hydroxylated poly(3-hydroxybutyrate) oligomers (PHB-diol) (Fig. 5.9). The influences of the ester/hydroxyl functionality ratio, catalyst amount, PHB-diol oligomer chain length, hydroxybutyrate (HB) and butylene succinate (BS) contents, and the nature of the solvent were investigated. The two-step process allowed the synthesis of copolyesters of M_n (up to 18,000), compared with the one-step process ($M_n \sim 8000$). The highest M_n was obtained with diphenyl ether as solvent, compared with dibenzyl ether or anisole. During the two-step process, the transesterification rate between the HB and BS segments was influenced by the factors of the catalyst amount, molar mass of the PHB-diol oligomer, the solvent, and the HB/BS ratio. Tendencies toward block or random macromolecular architectures were also observed. Immobilized CALB-catalyzed copolyesters were thermally stable up to 200 °C. The crystalline structure of the poly(HB-*co*-BS) copolyesters depended on the HB/BS ratio and the average sequence length of the segments. The crystalline content, T_m and T_c decreased with increasing HB content and the randomness of the copolymer structure [157].

CALB-catalyzed transesterification synthesis of (co)polyesters, poly(1,4-butylene succinate-*ran*-2,3-butylene succinate) (PBB'S), was examined via copoly-

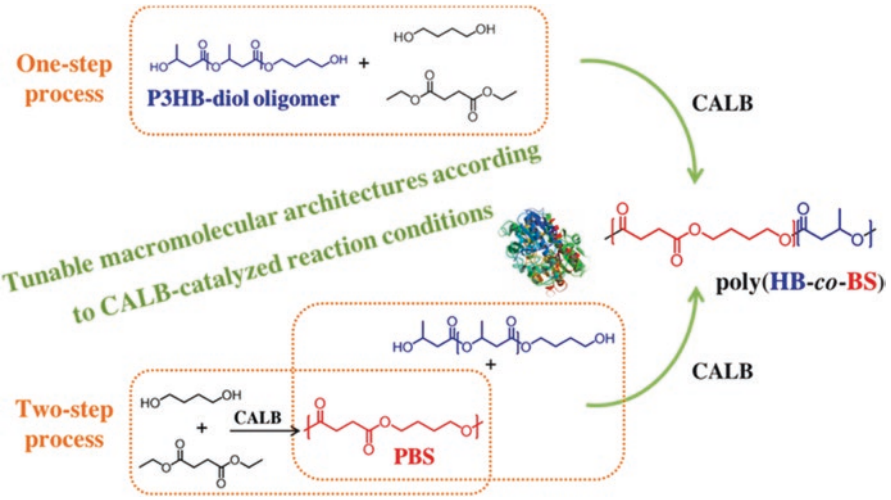
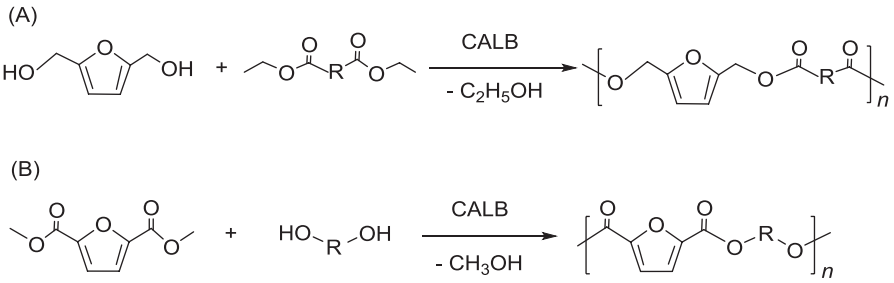


Fig. 5.9 Enzymatic synthesis of poly(HB-*co*-BS) with immobilized CALB (N435) catalyst via one-step process or two-step process. (Reproduced with permission from [157]. Copyright 2016 American Chemical Society)

merization of different 1,4-butanediol (1,4-BDO)/2,3-butanediol (2,3-BDO) molar ratios, with diethyl succinate (DES). The units of (DES-1,4-BDO) and (DES-2,3-BDO) were incorporated randomly in PBB'S. M_w decreased with increasing 2,3-BDO content, from 26 to 6 × 10^3. Increase in 2,3-BDO content raised the T_g and reduced the degree of crystallinity, leading to amorphous materials. The copolyesters exhibited a good thermal stability, higher than 250 °C, depending on the 1,4-BDO/2,3-BDO ratio [158]. Enzymatic (co)polymerizations of biobased short aliphatic diols and diethyl carboxylates were investigated in solution using CALB as biocatalyst. CALB showed a better affinity for 1,4-butanediol (1,4-BDO) compared to 1,3-propanediol (1,3-PDO), whereas no preference was observed between diethyl succinate (DES) and diethyl adipate (DEA). In addition, two series of random copolyesters, poly(butylene succinate-*ran*-butylene adipate) (PBSA) and poly(propylene succinate-*ran*-butylene succinate) (PPBS), were synthesized at different compositions. Their compositions were similar to the feed ones. Nevertheless, this activity difference decreased the M_w of PPBS from 24 to 11 × 10^3 for 1,3-PDO content varying from 0 to 100 mol %. Moreover, 1,3-PDO induced an increase of T_g and a decrease of the crystallization rate. On the other side, the reduction of the diethyl carboxylate chain length from diethyl adipate to diethyl succinate did not induce a clear tendency with M_n varying from 22 to 12 × 10^3 with the succinate content. Both copolyesters exhibited an isodimorphic co-crystallization behavior [159].

The CALB-catalyzed polymerization of various diacid ethyl esters was conducted with 2,5- bis(hydroxymethyl)furan, which is a highly valuable biobased rigid diol resembling aromatic monomers (reaction (A), Scheme 5.23). Novel biobased furan polyesters with M_n around 2000 were obtained. All product polyesters were semicrystalline. The degree of crystallinity ranged from 34 to 65%. The T_m was around 43–86 °C. These polyesters were thermally stable up to 250 °C, show-



Scheme 5.23 Synthesis of biobased furan polyesters via transesterification: (**A**) From 2,5-bis(hydroxymethyl)furan and (**B**) from dimethyl 2,5-furandicarboxylate

ing two decomposition steps. The T_{max} at the major degradation step was around 276–332 °C. The T_g of 2,5-bis(hydroxymethyl)furan-based polyesters was around −38–4 °C, being significantly higher than that of the aliphatic counterparts [160].

Biobased dimethyl 2,5-furandicarboxylate was polymerized with various (potentially) renewable aliphatic alcohols by CALB catalyst at 80 °C in diphenyl ether (reaction (B), Scheme 5.23) [161]. As alcohols, diols, HO-$(CH_2)_m$-OH (m = 3, 4, 6, 8, 10), HO-$(CH_2)_2O(CH_2)_2$-OH, 2,3-butanediol, and isosorbide, triol (glycerol), and hexaol (sorbitol) were used. 1,3-Propanediol, isosorbide, glycerol, and sorbitol produced ester oligomers ($M_n < 500$), whereas diols (m = 6, 8, 10) gave the corresponding polyesters with higher molecular weight in good yields, M_n reaching to 2.37×10^4 in 94% yields in the case of 1,10-decanediol. The structure-property relationships of a series of relevant furanic-aliphatic polyesters were studied by comparing the crystalline/thermal properties.

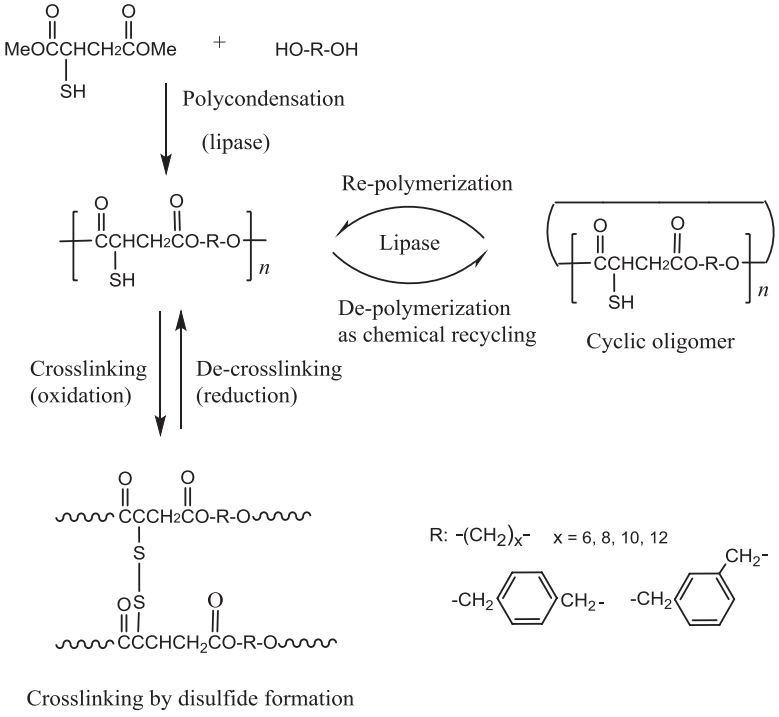
2,5-Furandicarboxylic acid (2,5-FDCA) can be prepared straight from renewable resources like furfural and hydroxymethylfurfural. Since the polyesters derived from FDCA monomers are a new class of aliphatic-aromatic bio-polyesters and involve the potentials for wide applications, the progress and fundamental aspects for their synthesis and thermal properties have been reviewed (see reaction (B), Scheme 5.23) [162].

Novozym 435 catalyzed aliphatic polyester synthesis from isosorbide (see Scheme 5.15). Using diethyl ester derivatives of diacid comonomers gave copolyesters of isosorbide with highest isolated yield and molecular weights. The length of the diacid aliphatic chain was less restrictive but with a clear preference for longer aliphatic chains. The M_w values were higher than 40,000, which was unexpected, because isosorbide has two chemically distinct secondary hydroxyl groups. This is the first example in which isosorbide polyesters were regioselectively synthesized by enzyme catalysis, to lead to large possibilities for the important biomaterials [163].

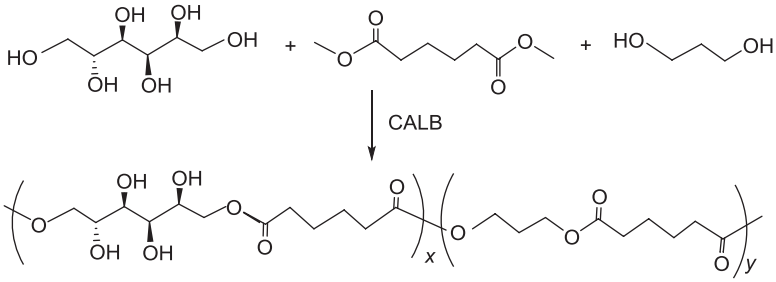
A mercapto group was applied for a cross-linkable group. Lipase CA-catalyzed polycondensation of 1,6-hexanediol and dimethyl 2-mercaptosuccinate at 70 °C in bulk gave an aliphatic polyester having free pendant mercapto groups with M_w 14,000 in good yields. The mercapto group content could be controlled by copolymerization with other monomers. The polyester was readily cross-linked by the air oxidation via the disulfide linkage formation in dimethyl sulfoxide [164]. Reversibly

cross-linkable aliphatic and aromatic polyesters with free pendant mercapto groups were prepared by lipase-catalyzed polycondensation of various diols and dimethyl mercaptosuccinate (Scheme 5.24). The CALB-catalyzed reaction in an organic solvent at 70 °C produced the polyesters with M_w 17,000~27,000 in high yields without any reaction at the mercapto group. The polyesters were easily cross-linked to form gels or films by air oxidation of the pendant free mercapto groups via forming a disulfide bond. The cross-linked polyesters were readily de-cross-linked by reduction with tributylphosphine to regenerate polyesters with free mercapto groups almost quantitatively. For chemical recycling, the polyesters with free mercapto groups were depolymerized into cyclic oligomers, which were repolymerized to form polyesters with the similar M_w and polymer structure as the initial polymers [165].

Renewable polyesters derived from a sugar alcohol (i.e., sorbitol) were synthesized by solvent-free transesterification polycondensation, in order to prepare linear polyesters with pendant hydroxyl groups along the polymer backbone (Scheme 5.25). The performance of the sustainable biocatalyst SPRIN liposorb CALB (trade name for the immobilized form of CALB) and the organo-base catalyst 1,5,7-triazabicyclo[4,4,0]dec-5-ene (TBD) were compared with two metal-based catalysts: dibutyl tin oxide (DBTO) and scandium trifluoromethanesulfonate [scandium triflate, $Sc(OTf)_3$]. For the four catalytic systems, the efficiency and selectivity



Scheme 5.24 Synthesis, cross-linking, and chemical recycling of polyester with pendant mercapto groups

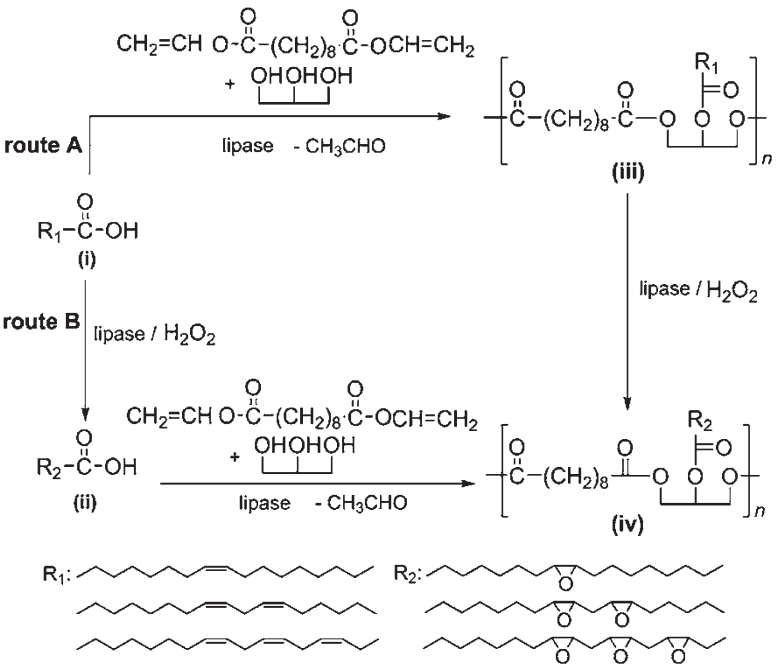


Scheme 5.25 Synthesis of poly(1,3-propylene-*co*-sorbitol adipate)s

for the incorporation of sorbitol were studied. Especially, the biocatalyst SPRIN succeeded in incorporating sorbitol in a selective way without side reactions, leading to close to linear polyesters (sorbitol 3.4 mol% incorporated, M_n 12.2 × 10^3). By using a renewable hydroxyl-reactive curing agent based on L-lysine, transparent and glossy poly(ester urethane) networks were synthesized, offering a tangible example of biobased coatings [166]. In a similar way, the synthesis of aliphatic polyesters from biobased, commercially available sorbitol, 1,10-decanediol (DD), and dimethyl adipate (DMA) was carried out with solvent-free at ~100–105 °C by using the SPRIN as catalyst. The polycondensation reactions gave polyesters with close to linear structure, typically with the feed ratio, DMA: DD: sorb. = 1:0.9:0.2; sorbitol was incorporated in 3.2 mol% in the copolymer having M_n 8.2 × 10^3 [167].

As shown in Scheme 5.21, divinyl esters are good monomers for the lipase-catalyzed regioselective polycondensation using polyols. Triols such as glycerol were regioselectively polymerized at a primary hydroxy group with divinyl adipate by lipase catalyst to produce a linear polyester linked through mainly primary hydroxy groups having also a secondary hydroxy group (5–10%) in the main chain. The polymer contained pendant hydroxy groups, with no evidence of network structure, having M_w value from ~3000 to 14,000 [168]. The reaction of divinyl sebacate and glycerol with lipase CA catalyst produced water-soluble polyesters at 60 °C. From products, the chloroform-soluble part with M_w of 19,000 was isolated in 63% yields, which indicated the regioselectivity of primary OH/secondary OH ratio of 74/26. At a lower temperature of 45 °C, however, the regioselectivity was perfectly controlled to give a linear polymer consisting exclusively of 1,3-glyceride unit [169]. The lipase CA catalysis gave a sugar-containing polyester regioselectively from divinyl sebacate and sorbitol, in which sorbitol was exclusively reacted at the primary OH group of 1- and 6-positions at 60 °C. Mannitol and *meso*-erythritol analogously behaved [170].

Reactive polyesters were prepared by the lipase-catalyzed polycondensation of divinyl sebacate with glycerol in the presence of an unsaturated higher fatty acid **(i)** such as linolenic acid obtained from renewable plant oils (route A, Scheme 5.26). Product polyester **(iii)** is biodegradable and contains an unsaturated fatty acid moiety in the side chain. Curing of **(iii)** induced by oxidation with cobalt naphthenate catalyst or thermal treatment gave a cross-linked transparent film. Biodegradability



Scheme 5.26 Two routes of epoxide-containing polyester synthesis using a vinyl ester with lipase catalyst

of the film was tested by biochemical oxygen demand (BOD) measurement [171, 172]. In addition, epoxide-containing polyesters were enzymatically synthesized via two routes, A and B. In route A, **(iii)** was enzymatically epoxidized to give **(iv)**, and in route B the lipase-catalyzed epoxidation of the fatty acid **(i)** was first applied to give **(ii)**, and lipase-catalyzed condensation polymerization of **(ii)** was performed to produce reactive polyester **(iv)**. Curing of **(iv)** proceeded thermally, yielding transparent polymeric films with high gloss surface. Pencil scratch hardness of film **(iv)** from **(ii)** was higher than that from **(iii)**. Both films showed good biodegradability [171–173]. Similarly, an unsaturated vegetable oil of oleic acid was epoxidized with lipase/H_2O_2, and the intermolecular ring-opening addition reaction between carboxylic acid group and epoxy group gave poly(oleic acid), a thermally stable materials, up to 300 °C [91].

Lipase-catalyzed transesterification allowed the synthesis of biobased epoxy resins. The epoxy functional polyester resins with various architectures (linear, tri-branched, and tetra-branched) were synthesized through polycondensation of fatty acids derived from epoxidized soybean oil and linseed oil with three different hydroxyl cores. The transesterification between a glycol, triol, or tetraol and a mono-, di-, or tri-epoxy fatty acid methyl ester produced macromonomers containing 2–12 epoxides, which are linked through an ester bond (Fig. 5.10). The cationic polymerization of these macromonomers gave new polymer thermosets with T_g values ranging from −25 to over 100 °C [174].

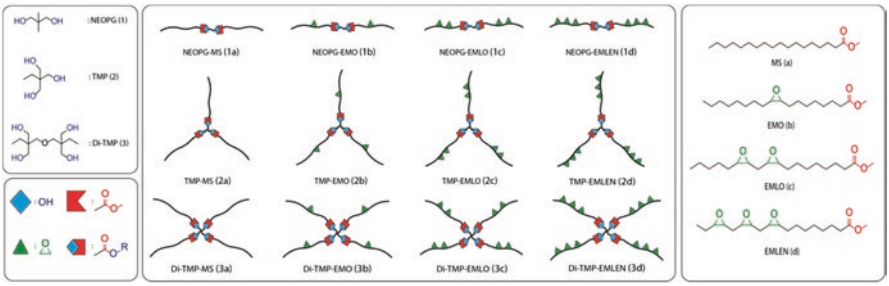
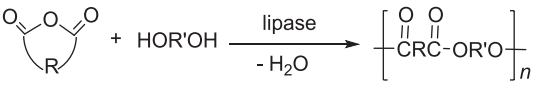


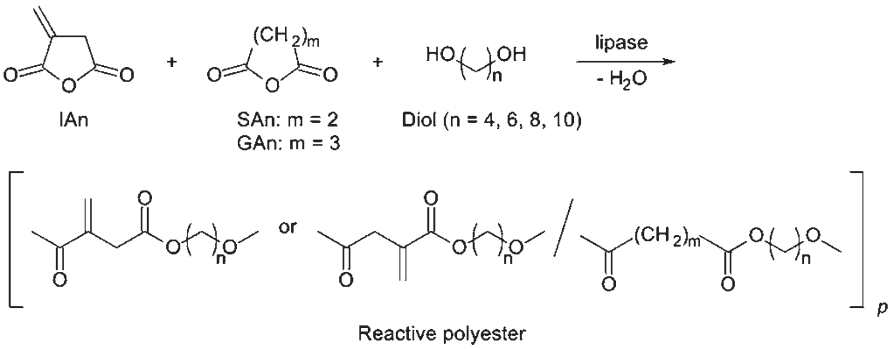
Fig. 5.10 Schematic representation of the reactants used in the synthesis (upper left side and right side) and products formed by the combination of those (center). (Reproduced with permission from [174]. Copyright 2016 American Chemical Society)

The CALB-catalyzed copolymerization of ethyl glycolate (EGA) with diethyl sebacate (DES) and 1,4-butanediol (BD) resulted in the formation of poly(butylene-*co*-sebacate-*co*-glycolate) (PBSG) copolyesters with M_w up to 28,000 and typical polydispersity between 1.2 and 1.8. This type of aliphatic copolyesters consisting of diester, diol, and glycolate repeat units were enzymatically synthesized for the first time. The synthesized copolymers contained 10–40 mol% glycolate (GA) units depending on the monomer feed ratio. PBSG copolyesters were hydrolytically degradable, and doxorubicin-encapsulated PBSG nanoparticles exhibited controlled release delivery of the drug [175]. CALB catalyzed the copolymerization of isosorbide (IS) or isomannide (IM) (see Scheme 5.15) with diethyl adipate and fractions of different unsaturated diester monomers (diethyl itaconate, diethyl fumarate, diethyl glutaconate, and diethyl hydromuconate) to produce unsaturated polyesters in one step. Unsaturated polyesters from IS and IM showed M_w values 4000 ~ 16,000 when fumarate or glutaconate was used in 5 mol% for the adipate [176].

To obtain information on the factors that affect stability and transport efficiency, multifunctional amphiphilic dendronized and hyperbranched polymers were synthesized using the "enzymatic polymerization" and "click chemistry." For the base polymer, a polycondensation synthesis from polyethylene glycol diethyl ester derivative (M_n ~1000) and azide glycerol was conducted with Novozym 435 catalyst to give the azide group-containing polyester. Onto the polyester, a polyglycerol dendron as hydrophilic part or a long alkyl chain (C_{18}) as hydrophobic part was introduced via click chemistry to result in the polyester (M_n 2600 ~ 10,500). The comparative cytotoxicity profile of the resulting polymers was studied, and their transport potential was also explored with Nile red as a model dye. Dendronized polymers proved to be superior nanocarriers [177]. PEG- and azidotriglycerol-based dendronized polymers were prepared using Novozym 435 as a biocatalyst, which is for the further applications of cyanine 3 dye, an amphiphilic fluorescent dye, e.g., for its encapsulation. The resulting polymers having azido and hydroxyl functionalities available in the backbone were further grafted with alkyl chains and polyglycerol dendrons using esterification and "click" chemistry approach. These polymers form stable micelles at micromolar concentrations. The polymers were nontoxic up to a concentration of 500 μg/ml [178].

Scheme 5.27 Typical reaction mode of ROACP





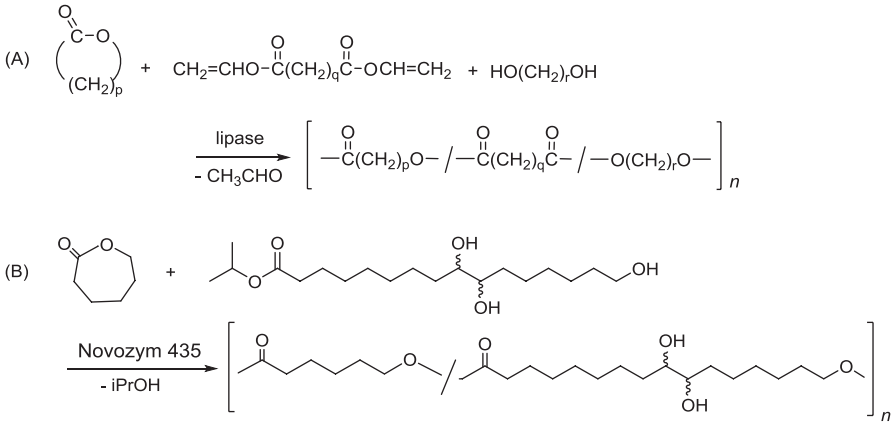
Scheme 5.28 Synthesis of reactive polyesters from IAn via ROACP

5.2.4 *Ring-Opening Addition-Condensation Polymerization (ROACP)*

This type of polymerization, ring-opening addition-condensation polymerization (ROACP), involves two modes of polymerization: ring-opening and addition-condensation.

Carboxylic Anhydrides and Diols for ROACP One of the typical reaction modes of ROACP is given in Scheme 5.27. It was disclosed in 1993 that reaction of a cyclic acid anhydride, R = $-(CH_2)_l-$, and a glycol, R' = $-(CH_2)_m-$, took place via ROACP by lipase PF catalyst in an organic solvent (benzene, toluene, *i*-Pr_2O, or Bu_2O) at room temperature, giving rise to a polyester (l = 2, m = 8) having M_n 2300 (M_w/M_n = 1.4) in 42% yields. The reaction involves the ring-opening addition as well as the dehydration condensation. Glycols of m = 8, 10, and 12 produced the polyesters, whereas an acid anhydride (l = 3) did not give a polyester with any glycols [179]. Various cyclic anhydrides, succinic, glutaric, and diglycolic anhydrides, were polymerized by lipase CA catalyst with α,ω-alkylene glycols in toluene at 60 °C to give the polyesters with M_n reaching 1.0×10^4. This ROACP involving dehydration proceeded also in water and in $scCO_2$ [180]. Significance of the reaction "dehydration in water" was already noted [76].

Such polymerization was extended to itaconic anhydride (IAn) as a new carboxylic anhydride monomer for lipase-catalyzed ROACP to produce reactive polyesters (Scheme 5.28) [181]. ROACP reaction of three components, IAn, succinic anhydride (SAn), or glutaric anhydride (GAn), and a diol at 25 °C in toluene produced reactive polyesters in good to high yields. As diols, 1,4-butane, 1,6-hexane, 1,8-octane, and 1,10-decane diols were used. From the SAn reactions, polyesters with molecular weight (M_n) values of 650–3510 with 1.3–2.6 IAn units per molecule were obtained. From the GAn reactions, these values were 560–3690 and 1.2–



Scheme 5.29 (**A**) ROACP terpolymerization using a lactone monomer and (**B**) ROACP copolymerization using ε-caprolactone

3.1, respectively. Cross-linking reaction at the vinylidene group(s) of a product polyester showed reactive nature, giving a cross-linked hard solid polyester. These polyesters derived from renewable starting materials involve possible applications as macromonomer, telechelics, or cross-linking reagent, and the vinylidene group(s) can be used for practical applications via further modification reactions. Regioselectivity at the carbonyl group of IAn was about the same (~50%) for both α- and β-positions of IAn by lipase catalysis, whereas about 90% for β-selectivity with Sn(II) catalyst and without catalyst. ROACP between two components, IAn and a diol, did not give polyesters, because the reactivity of IAn is smaller [181].

Cyclic Esters for ROACP Terpolymerization of a lactone (in place of an acid anhydride), a divinyl ester, and a glycol produced an ester terpolymer with M_n higher than 1×10^4, which belongs to a ROACP (reaction (A), Scheme 5.29). Lipases showed high catalytic activity for the terpolymerization involving both ring-opening and condensation polymerization simultaneously in one pot to produce an ester terpolymer, without involving homopolymer formation [182]. A similar terpolymerization was performed using three kinds of monomers, *ω*-pentadecalactone, diethyl succinate, and 1,4-butanediol, by CALB catalyst desirably at 95 °C via a two-stage vacuum technique. The polymerization was examined under various reaction conditions, and the product terpolyester reached M_w 77,000 with M_w/M_n between 1.7 and 4.0 [183]; it is to be noted that these similar terpolymerizations are sometimes called as copolymerizations.

Novozym 435-catalyzed copolymerization of ε-CL with isopropyl aleuriteate gave a random copolyester, having M_n up to 10,600 in ~70% yields via ROACP type (reaction (B), Scheme 5.29) [85].

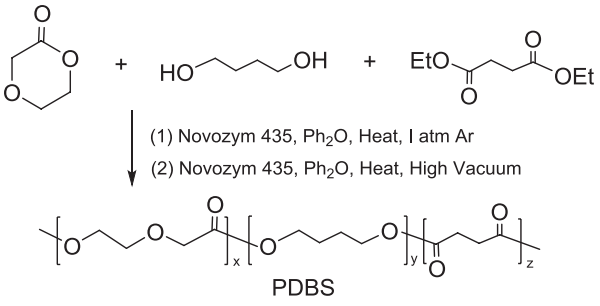
Copolymerization of L-lactide (LLA) with a diester (diethyl adipate or dodecanedioate) and a diol (1,6-hexanediol or 1,4-butanediol) was studied with using CALB catalyst. Aliphatic lactate-bearing copolyesters were synthesized; the resul-

tant copolymers had M_w up to 38,000, containing L-lactate units up to 53 mol%, C_6–C_{12} diester units, and C_4–C_6 alkylene units in the polymer chains. The LLA-diester-diol copolymers were obtained in good yields (70–85%), and all purified copolymers were optically active. LLA-diethyl adipate-1,6-hexanediol copolymers were hydrolytically degradable. Depending on their structure and composition, the copolymers exhibit a wide range of physical properties [184].

CALB catalyzed the synthesis of copolymerization of ω-pentadecalactone (PDL, 16-membered), 1,4-butanediol, and dialkyl succinate to produce poly(PDL-*co*-butylene-*co*-succinate) copolymers with various compositions. The monomer unit ratio in the copolymers could be controlled by adjusting monomer feed ratio, having nearly random distribution of PDL, butylene, and succinate repeat units, linked by ester groups. Thermal stability of the copolymers is composition-dependent, increasing with increasing PDL unit content. All copolymers are highly crystalline irrespective of their composition. M_w values of the copolymers are 1.6 ~ 10.6 × 10^3 [185]. CALB also catalyzed the ROACP synthesis of semicrystalline diepoxy functional macromonomers using glycidol, pentadecalactone, and adipic acid. Macromonomers having epoxy functions at both ends with molecular weight from 1400 to 2700 were obtained with conversion higher than 95%. The macromonomers were used for photopolymerization and copolymerized cationically with a cycloaliphatic diepoxide to afford a durable crystalline film [186].

In addition, Scheme 5.30 shows a typical ROACP to produce a polyester, poly(*p*-dioxanone-*co*-butylene-*co*-succinate) copolyesters (PDBS), where ethanol is evolved during the reaction. The reaction was carried out in diphenyl ether solvent via two steps at 70 °C for 24 h, followed by treatment under 150 Pa pressure or 48 h with Novozym 435 catalyst (5 wt% for total monomer). Typically, a 1:1:1 feed ratio of the three monomers gave a polyester containing *p*-dioxanone (PDO) unit in 30 mol% with M_n 25,500 (PDI 1.30) in quantitative yields. T_g and T_m values of the polyester were argued in terms of PDO content. The biodegradation of the polyester was very rapid, suggesting its potential to biomedical application [187].

Enzymatic cooperation owing to two hydrolases in the two-step tandem polymerization of bulky ibuprofen-containing hydroxyacid methyl ester (HAEP) and ε-CL catalyzed by a lipase (CALB) and a protease from *Bacillus subtilis* (BSP) was



Scheme 5.30 Synthesis route to PDBS copolyester via ROACP

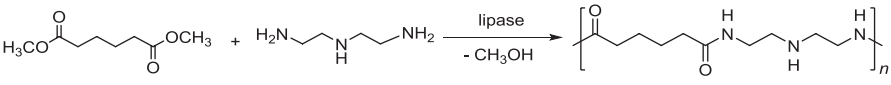
achieved. The tandem process improved the molecular weight of the resultant ibuprofen-containing polyester from 3130 (CALB) and 720 (BSP) to 9200 (CALB/BSP). CALB mainly catalyzed ROP of ε-CL under the initiation of HAEP to form homopolymer of ε-CL with relatively low molecular weight, while BSP catalyzed the subsequent polycondensation of the ROP product to yield copolymers with increased molecular weight. Based on the enzymatic cooperative tandem polymerization, valuable chiral (*R*)- or (*S*)-ibuprofen-containing polyesters were tailor-made in high yields and ee values (see also Scheme 5.11 for tandem polymerization). The in vitro drug release behavior and degradation of the prepared chiral polymeric prodrug were also examined [188].

5.2.5 Polycondensation to Polymers Other Than Polyesters

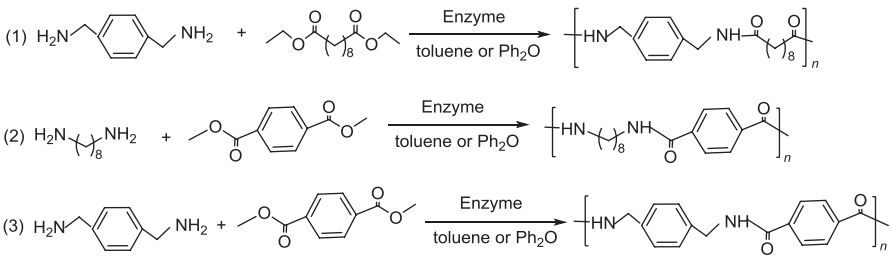
Lipase is active as catalyst for the synthesis of not only polyesters but also other polymers such as polyamides, polyamines, sulfur-containing polymers, polycarbonates, and so forth.

Synthesis of Polyamides Lipases are also effective as catalyst for polyamide synthesis [189]. For example, the reaction of dimethyl adipate and diethylene triamine produced the poly(aminoamide) with less branching at 50–110 °C with M_n ~3800 (Scheme 5.31). A chemical method to prepare similar polymers needs a higher reaction temperature ~180 °C to give the polymer with more branching having M_n ~3000. Other carboxylic acids such as malonic acid and fumaric acid were used for similar polymers via dehydration. As amines, triethylene tetramine, tetraethylene pentamine, and triethylene glycol diamine were also employed.

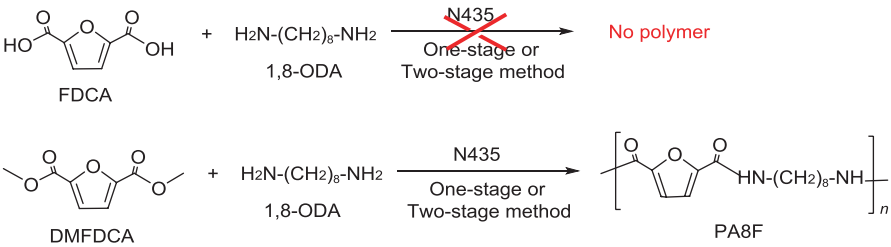
Lipase-catalyzed polycondensation to lead to aliphatic-aromatic oligoamides was studied (Scheme 5.32) [190]. The reaction of *p*-xylylenediamine and diethyl sebacate resulted in oligo(*p*-xylylene sebacamide) with high melting temperatures (223–230 °C) (reaction 1), and the enzymatic polycondensation of dimethyl terephthalate and 1,8-diaminooctane led to oligo(octamethylene terephthalamide) with two melting temperatures at 186 and 218 °C (reaction 2). Also, oligoamides were formed from the enzymatic reaction of dimethyl terephthalate and *p*-xylylenediamine (reaction 3). All reactions were catalyzed by an enzyme like CALB, cutinase, or CLEA cutinase. Reactions catalyzed by CALB showed higher conversion than reactions catalyzed by these cutinases. The highest DP, n = 15, was achieved in a one-step and two-step synthesis of oligo(*p*-xylylene sebacamide) catalyzed by CLEA cutinase [190].



Scheme 5.31 Lipase-catalyzed polyamide synthesis



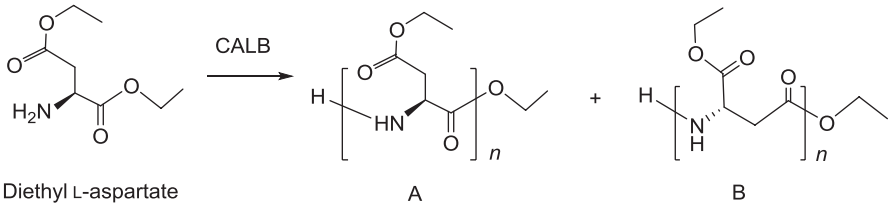
Scheme 5.32 Outline of the lipase-catalyzed synthesis of aliphatic-aromatic polyamides



Scheme 5.33 N435-catalyzed synthesis of sustainable PA8F from FDCA or DMFDCA and 1,8-ODA using the one-stage method or the two-stage method

Furan-2,5-dicarboxylic acid (FDCA)-based furanic-aliphatic polyamides are promising sustainable alternatives to polyphthalamides (semiaromatic polyamides) and applied as high performance materials with great commercial interest. So, poly(octamethylene furanamide) (PA8F), an analog to poly(octamethylene terephthalamide) (PA8T), was produced via Novozym 435 (N435)-catalyzed polymerization, using a one-stage method in toluene and a temperature-varied two-stage method in diphenyl ether, respectively (Scheme 5.33). The enzymatic polymerization of DMFDCA resulted in PA8F with M_w up to 54,000. The one-stage enzymatic polymerization in toluene indicated that the molecular weights of PA8F increased significantly with the concentration of N435, with an optimal reaction temperature of 90 °C. The temperature-varied, two-stage enzymatic polymerization in diphenyl ether yields PA8F with higher molecular weights, as compared to the one-stage procedure, at higher reaction temperatures (up to 140 °C). Compared to PA8T, the obtained PA8F possesses a similar T_g and similar crystal structures [191].

2,5-Furandicarboxylic acid (FDCA)-based semiaromatic polyamides were produced via N435-catalyzed polycondensation of biobased dimethyl 2,5-furandicarboxylate (DMFDCA) and aliphatic diamines differing in chain length (H_2N-$(CH_2)_x$-NH_2; x = 4, 6, 8, 10, 12), using a one-stage method at 90 °C in toluene (see also Scheme 5.33). The obtained polyamides PAXF reached M_W ranging from 15,800 to 48,300; and N435 shows the highest selectivity toward 1,8-octanediame (x = 8), the yield being ~70%. This enzymatic method gave the polyamides of higher molecular weight in higher yields, compared with a conventional method. The biobased polyamides PAXF are attractive as engineering thermoplastics and high performance materials [192].



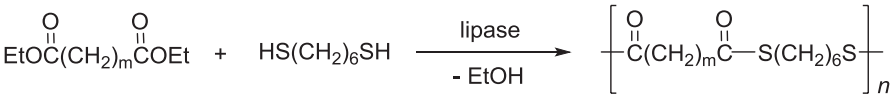
Scheme 5.34 CALB-catalyzed polymerization of diethyl aspartate. (**A**) α-linkage, (**B**) β-linkage

CALB catalyzed the polycondensation of β-alanine esters to give poly-β-alanine; -(C=O$CH_2CH_2NH)_n$-. In the reaction, the effect of different solvents, reaction temperature, and substrate/enzyme concentration on polymer yield and DP was determined. Also the effect of methyl group substituent at α- and β-position of β-alanine was studied. Cross-linked enzyme aggregates of CALB converted β-alanine esters into polymers in high yield reaching to 90% and with high DP up to 53 units [193].

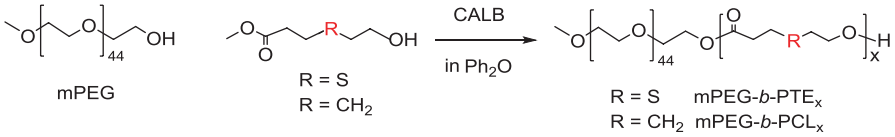
Lipase (CALB)-catalyzed polymerization of diethyl D- or L-aspartate was carried out without using solvent. The reaction gave chiral D- and L-polyaspartates in ~80% yields with an average degree of polymerization (DP_{avg}) up to 60. The polymer structure was of 96% β-linkages, α-linkages being of very small amount, indicating good regioselectivity. The complexation of enantiopure D- and L-polyaspartates was not a stereocomplex but a homocomplex. Other properties were also examined [194]. On the other hand, the bulk polymerization of L-aspartic acid diethyl ester catalyzed by CALB at 80 °C for 24 h produced primarily (~95%) α-linked poly(L-aspartate) in 70% yields with DP_{avg} = 50 and regioselectivity (α/β) = 94:6 (Scheme 5.34). These results suggested that polymerization proceeds in a controlled manner by a chain-growth mechanism up to 90% conversion. Thereafter, competition between chain-growth and step mechanisms occurred [195].

Synthesis of Polyamines CALB is an efficient catalyst for copolymerization of diesters with amino-substituted diols to form poly(amine-*co*-esters) in one step via the ester linkage formation [189].

Synthesis of Sulfur-Containing Polymers Transesterification polycondensation of 11-mercaptoundecanoic acid (11-MU) with lipase CA catalyst in bulk gave an poly(thioester). The reaction at 110 °C for 48 h produced poly(11-MU) with M_w of 34,000 in high yields. The T_m value of 104.5 °C was about 20 °C higher than that of the corresponding polyoxyester. Poly(11-MU) was readily transformed by lipase into the cyclic oligomers mainly of the dimer, which were readily repolymerized by lipase via ring-opening polymerization as a sustainable chemical recycling [196]. A poly(thioester) was prepared by lipase CA catalyst via the direct transesterification of 1,6-hexanedithiol and a diester with eliminating ethanol in bulk (Scheme 5.35, m = 1–8). The product poly(thioester) possessed M_w ~1.0×10^4. Both the melting point and crystallization temperature were higher than those of the corresponding poly(oxyester)s [197].



Scheme 5.35 Transesterification polymerization of a diester and a dithiol to poly(thioester)s



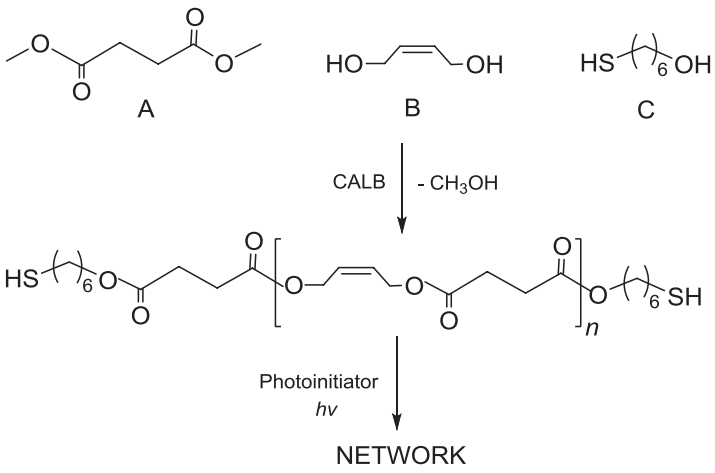
Scheme 5.36 Lipase-catalyzed synthesis of mPEG-*b*-PTE and mPEG-*b*-PCL block copolymers

The lipase CA-catalyzed ring-opening addition-condensation polymerization (ROACP) of ε-caprolactone (ε-CL) with 11-mercaptoundecanoic acid or 3-mercaptopropionic acid under reduced pressure produced the copolymer of an ester-thioester structure with molecular weight higher than 2×10^4. The transesterification between poly(ε-CL) and 11-mercaptoundecanoic acid or 3-mercaptopropionic acid was also observed [198].

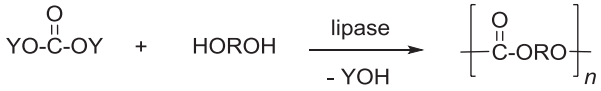
CALB catalyzed the one-step polycondensation of methyl 3-((2-hydroxyethyl) thio)propanoate (MHETP) with monomethoxy poly(ethylene glycol) (mPEG, M_n = 1980) to produce novel oxidation-responsive amphiphilic diblock copolymers with β-thioether ester groups. The amphiphilic poly(ethylene glycol)-*b*-poly(β-thioether ester) (mPEG-*b*-PTE, M_n = 2700–4560) diblock copolymers (Scheme 5.36) self-assembled to form nanosized micelles in aqueous solution with low critical micelle concentration (CMC). In vitro oxidation studies indicated that the mPEG-*b*-PTE micelles could be destroyed by the oxidation stimuli due to the oxidized thioether groups of hydrophobic PTE segment, due to –S- to –S(=O)- by H_2O_2 oxidant, and further hydrolysis of the ester bonds. The oxidation insensitive poly(ethylene glycol)-*b*-poly(3-caprolactone) (mPEG-*b*-PCL) diblock copolymer was also prepared for comparison (Scheme 5.36). These amphiphilic block copolymers mPEG-*b*-PTE have potentials as a new type of oxidation-responsive polymeric materials [199].

α,ω-Thiol telechelic oligoesters having internal alkenes were prepared using selective lipase catalysis and were subsequently cross-linked by thiol-ene chemistry, yielding alkene functional networks. The oligoesters were produced by CALB-catalyzed polymerization of dimethyl succinate (A), *cis*-2-butene-1,4-diol (B), and 6-mercapto-1-hexanol (C) at 60 °C (Scheme 5.37). The oligomers obtained by the feed ratio, e.g., 3:2:2 and 5:4:2, have the structure with –SH group at both ends, with molecular weight 600 ~1100. They contain the reactive thiol-ene system and form films. UV irradiation to the films caused curing by cross-linking to give polymer networks, showing good thermal and mechanical properties [200].

Synthesis of Polycarbonates The lipase-catalyzed polycondensation of a carbonic acid diester and a glycol gave polycarbonates; the simplest reaction mode is given as Scheme 5.38. CALB-catalyzed transesterification between diethyl carbonate



Scheme 5.37 Chemo-enzymatic route to alkene functional networks



Scheme 5.38 Reaction mode of polycarbonate synthesis

(Y = C_2H_5) and 1,6-hexanediol (R = $-(CH_2)_6$-) to produce poly(hexamethylene carbonate) was investigated under various reaction conditions. It proceeded via two stages: the first to yield oligomers and the second to give a higher molecular weight polymer, reaching to $M_w > 25{,}000$, $M_w/M_n \leq 2.2$ [201].

Polycarbonate synthesis reactions produced aromatic polycarbonates of DP greater than 20 [149] and aliphatic polycarbonates of molecular weight higher than 4×10^4 [202, 203]. The CALB-catalyzed transesterification among three components, diethyl carbonate, a diester, and a diol, produced aliphatic poly(carbonate-*co*-ester)s with about 1:1 molar ratio of the ester-to-carbonate repeat units. Molecular weight M_w value reached 59,000 at a reaction temperature 90 °C. A carbonate-ester transesterification reaction between poly(butylene carbonate) and poly(butylene succinate) was also catalyzed by CALB at 95 °C to give a block type copolymer [204].

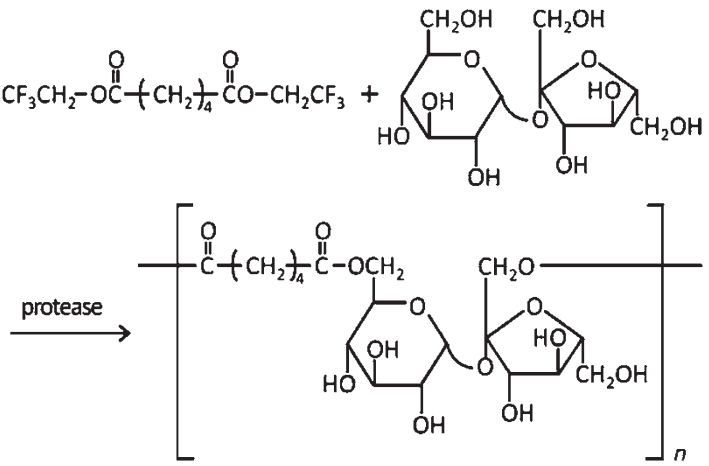
CALB was used to promote synthesis of aliphatic poly(carbonate-*co*-ester) copolymers from dialkyl carbonate, diol, and lactone monomers via ROACP mode reaction. The copolymerization was carried out in two stages: the first-stage oligomerization under low vacuum, followed by the second-stage polymerization under high vacuum. Therefore, copolymerization of ω-pentadecalactone (PDL), diethyl carbonate (DEC), and 1,4-butanediol (BD) yielded PDL-DEC-BD copolymers poly(PDL-*co*-butylene-*co*-carbonate) with a M_w of whole product up to 33,000 in ~ 92% yields. The copolymer compositions were controlled by adjusting the monomer feed ratio [205]. CALB-catalyzed condensations gave high-purity, metal-free, polycarbonate polyols. Terpolymerizations of diethyl carbonate (D) with

1,8-octanediol (O) and tris-(hydroxymethyl)ethane (T) were performed in bulk at temperature 80 °C using a reduced pressure. With D/O/T monomer feed ratio 3:0.9:0.1, the highest values of dendritic T-unit content (83%) and M_w (23900) were attained. At short reaction times (e.g., 4 h), highly functional linear terpolymers were formed. Increase in reaction time from 4 to 8, 12, 24, and 30 h resulted in increased dendritic unit content (0 ~ 48%), relative M_w (2100 to 39,000), and M_w/M_n (1.5–5.6); this synthesis of polyol polycarbonates enabled control of critical polymer structure [206].

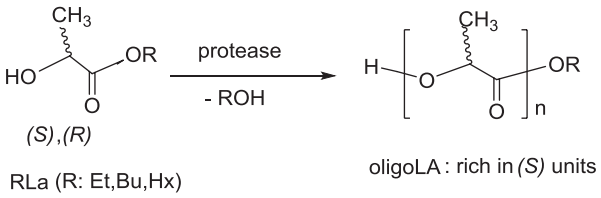
5.3 Protease-Catalyzed Polycondensation for Polyester Synthesis

Proteases (EC 3.4) are hydrolysis enzymes, primarily catalyzing the hydrolysis of proteins for L-amino acid residues [207]. Similarly to the lipase catalysis, however, proteases catalyze the ester bond formation, leading to polyester production [6, 8, 15, 25].

Some proteases exhibit esterase activity to produce polyesters. Polycondensation of bis(2,2,2-trifluoroethyl) adipate with sucrose catalyzed by an alkaline protease from *Bacillus* sp. resulted in an alternating linear polyester soluble in water and polar organic solvents with M_n 1600 (M_w / M_n= 1.31). Sucrose reacted regioselectively at 6- and 1′- positions and behaved like a diol; cross-linking did not take place (Scheme 5.39) [208]. Protease is effective for aromatic polyester synthesis via transesterification in THF; a terephthalic acid diester and 1,4-butanediol produced oligoesters, e.g., molecular weight from 400 to 1000 from various aromatic diesters and diols [209].



Scheme 5.39 Synthesis of a sucrose-containing linear polyester with protease catalyst



Scheme 5.40 Enantioselective oligomerization of lactates

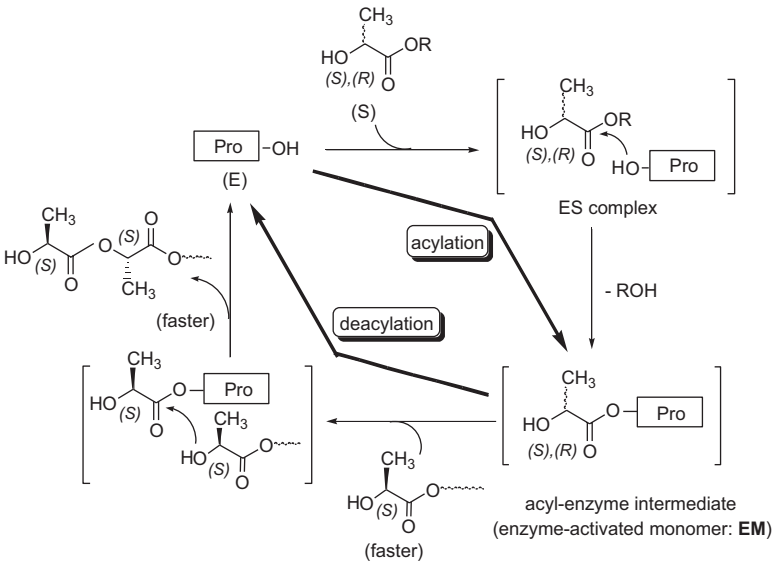


Fig. 5.11 An illustrative mechanism of protease-catalyzed L-enantioselective oligomerization of alkyl L,D-lactates. (Reproduced with permission from [73]. Copyright 2011 American Chemical Society)

Protease catalyzed the oligomerization of alkyl D- and L-lactates (RDLa and RLLa) (Scheme 5.40) [73]. Four proteases examined showed catalyst activity to give preferentially oligo(L-lactic acid)s (oligoLLa)s; dimer ~ pentamer), with moderate to high yields. The enantioselection was L−/D-selective (56/28 to 25/4 in conversion % ratio). L-Enantioselection of the protease is opposite to that of the lipase (Scheme 5.5) [72]; it is less strict compared with that of lipase, whose D-enantioselection is perfect.

In the hydrolysis reaction of ethyl D- and L-lactates, (EtDLa)s and (EtLLa)s, catalyzed by protease, EtLLa was consumed little faster than EtDLa. The mechanism of the protease-catalyzed oligomerization (Fig. 5.11) is explained similarly to that of lipase as seen in Fig. 5.7, since protease contains a similar triad, Ser-His-Asp, Ser being as an active center. These observations imply that the acylation step proceeds almost in the same rate both in the hydrolysis and oligomerization, and thus, in the oligomerization the deacylation step governs the preferential L-enantioselection.

The opposite enantioselection between protease and lipase was argued in the PLA depolymerizing hydrolysis [207, 210]. These two enzymes belong to serine hydrolases, possessing a catalytic triad of serine, histidine, and aspartic acid; the catalytic-active site of the two classes, however, is of topological mirror image structure [207, 211–213]. This catalyst site situation is considered responsible for the opposite selection, where protease is PLLA-preferential and lipase is PDLA-specific [210]. The enantioselection of Novozym 435 was perfect, whereas that of proteases was less selective. This selectivity difference is probably because in living system the substrate of lipase is an ester having an ester linkage like RLa, whereas the substrate of protease is a protein having an amide linkage.

It is to be noted that the protease-catalyzed polycondensation synthesis of polyamides produced in almost all cases α-polypeptides among various polyamides, which is described in detail in Chap. 8.

5.4 Conclusion

Lipase is one of the most important and extensively used enzymes: in particular, it is an indispensable enzyme for polyester synthesis in fundamental as well as application purposes. Naming of lipase is based on the origin and the catalyst functions of lipase are mentioned first. Its modification via immobilization processes is, then, described in detail, and also the general catalysis mechanism is fully discussed for explaining the importance toward *green polymer chemistry.*

Lipase-catalyzed synthesis of polyesters via polycondensation (condensation polymerization) has been described in a comprehensive review manner with up-to-dated research results. α- and ω-Oxyacids or their esters gave various polyesters with lipase catalyst via dehydration polycondensation (DHP) or transesterification polycondensation (TEP). Using dicarboxylic acids or their esters as monomer reacted with alcohols produced a variety of functionalized polyesters via a DHP or TEP process; actually polycondensations via the combination of diacids and glycols are the most extensively investigated, and hence, a wide variety of functional polyesters have been produced. Some specific aspects of lipase-catalyzed polymerizations were argued, including "dynamic kinetic resolution" ("iterative tandem catalysis"), "DHP in water solvent," "enantioselection is operated at deacylation step," and so forth. Ring-opening addition-condensation polymerization (ROACP) was achieved with using a cyclic ester, an acid anhydride, etc. as a monomer to give another class of polyesters. Other polymers than polyesters were obtained also by lipase catalysis. Protease, another hydrolase enzyme, catalyzed the polycondensation to give polyesters besides polyamides via transesterification.

The lipase catalysis provides so many value-added functional polyesters via an environmentally permissible process, which is desirable toward "green polymer chemistry." Lipase catalyst allows to use renewable resource-based (biobased) starting materials in place of diminishing fossil-based raw materials. Enzymatic polyester synthesis is highly selective in almost all reactions (chemo-, regio-, stereo-, enantio-, etc.), occurring under mild conditions, and proceeding in a clean way.

Products polyesters prepared from renewable starting materials are biodegradable and can be recycled. These situations involve big possibility for new practical applications in industry. Thus, further developments of this field shall definitely contribute to maintain the sustainable society in the future.

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Chapter 6
Synthesis of Polyesters II: Hydrolase as Catalyst for Ring-Opening Polymerization

Hiroshi Uyama and Shiro Kobayashi

Abstract This chapter reviews enzymatic lipase-catalyzed ring-opening polymerizations (ROPs) to polyesters. A variety of cyclic esters are subjected to lipase- catalyzed ROP. Lipase catalysis shows unique polymerization behaviors of lactones with different ring sizes. ROP mechanism of lactones by lipase catalyst is mentioned, which applies to preparation of terminal functional polyesters. Lipase catalysis induces enantio-, regio-, and chemoselective ROPs, which can hardly be achieved by conventional chemical catalysts. ROP of cyclic esters in a variety of media is mentioned for green synthesis of polyesters. ROP of lactones is combined with living radical polymerizations, yielding designed block copolymers. ROP of other cyclic monomers, mainly cyclic carbonate, is also mentioned in this chapter.

Keywords Cyclic monomer · Enzymatic polymerization · Lactone · Lipase · Polyester · Ring-opening polymerization

6.1 Introduction

Many studies concerning syntheses of aliphatic polyesters by fermentation and chemical processes have been made in viewpoint of biodegradable materials. Another approach to synthesis of biodegradable polyesters has been developed by polymerization using lipase as catalyst [1–7]. It is generally accepted that an enzymatic reaction is virtually reversible, and hence, the equilibrium can be controlled by appropriately selecting the reaction conditions. Based on this concept, many of hydrolases, which are enzymes catalyzing a bond-cleavage reaction by hydrolysis,

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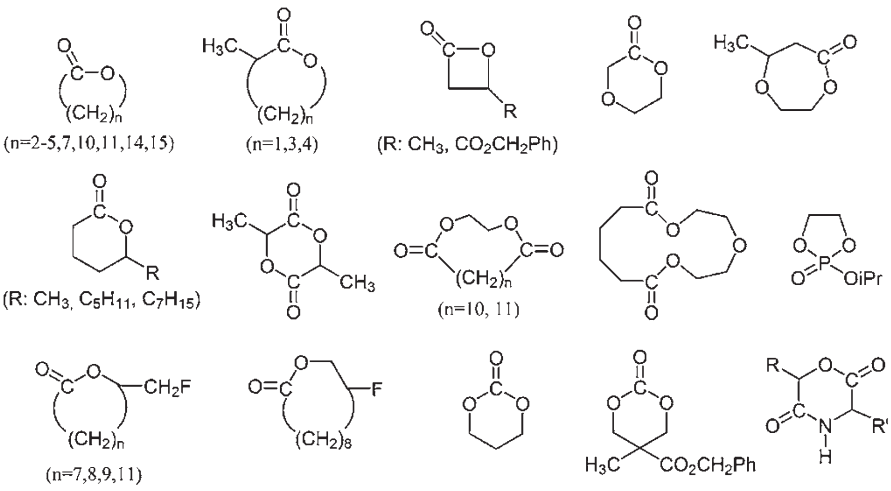

Fig. 6.1 Typical cyclic monomers for enzymatic ROP

have been employed as catalyst for the reverse reaction of hydrolysis, leading to polymer production by a bond-forming reaction.

Lipase is an enzyme which catalyzes the hydrolysis of fatty acid esters normally in an aqueous environment in living systems. However, lipases are sometimes stable in organic solvents and can be used as catalyst for esterifications and transesterifications. By utilizing such catalytic specificities of lipase, functional aliphatic polyesters have been synthesized by various polymerization modes, i.e., as seen for the polycondensation mode in the previous chapter.

Ring-opening polymerizations (ROPs) are one of the typical routes to polymers in chemical industries. Enzyme catalysis has been used for ROP of various cyclic monomers, mainly cyclic esters (lactones) to polyester syntheses. Typical examples of cyclic monomers are shown in Fig. 6.1.

6.2 Enzymatic ROP of Cyclic Esters

Various cyclic esters (lactones) with different ring sizes have been subjected to lipase-catalyzed ROP. Lipase catalyzed the ROP of 4- to 17-membered non- substituted lactones (Fig. 6.2). In 1993, it was first demonstrated that medium-size lactones, δ-valerolactone (δ-VL, six-membered) and ε-caprolactone (ε-CL, seven- membered), were polymerized by *Burkholderia cepacia* lipase (lipase BC), *Candida cylindracea* lipase (lipase CC), and *Pseudomonas fluorescens* PF (lipase PF) and porcine pancreas lipase (PPL) [8, 9]. Later, various cyclic esters with different ring size and structure were polymerized via the lipase catalysis. The polymerization was conducted in bulk, an organic solvent, or a binary solvent system.

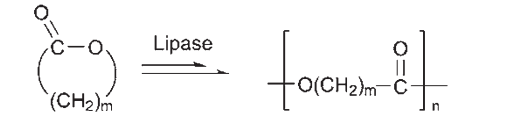

Fig. 6.2 Lipase-catalyzed ROP of non-substituted lactones

β-Propiolactone (β-PL, four-membered) was polymerized by *Pseudomonas* family lipases as catalyst in bulk, yielding a mixture of linear and cyclic oligomers with molecular weight of several hundreds, whereas poly(β-PL) of high molecular weight (molecular weight > 5×10^4) was obtained by using lipase CR as catalyst [10–14].

Substituted four-membered lactones were polymerized via the lipase catalysis. The lipase-catalyzed polymerization of β-butyrolactone (β-BL) produced poly(β-hydroxybutyrate) (PHB) [13], which is a polyester having similar structure produced in vivo by bacteria for an energy-storage substance. The molecular weight of PHB reached 7300 in the polymerization using PPL as catalyst at 100 °C [15]. *Candida rugosa* lipase (lipase CR) also showed high catalytic activity toward the polymerization at a high temperature. The resulting products contained a significant amount of cyclic oligo(β-hydroxybutyrate)s, which were formed by the lipase-catalyzed reaction of linear PHB [16].

Poly(malic acid) is a biodegradable and bioadsorbable water-soluble polyester having a carboxylic acid in the side chain. The chemoenzymatic synthesis of poly(malic acid) was achieved by the lipase-catalyzed polymerization of benzyl β-malolactonate, followed by the debenzylation. The molecular weight of poly(benzyl β-malolactonate) increased by the copolymerization with a small amount of β-PL using lipase CR catalyst. Propyl malolactone (β-propyloxycarbonyl-β-PL) was also polymerized with lipase CR catalyst in toluene and bulk to produce the polymer with molecular weight of 5×10^3 quantitatively. The enzymatic polymerization took place much faster than the thermal polymerization [17].

Five-membered unsubstituted lactone, γ-butyrolactone (γ-BL), is not polymerized by conventional chemical catalysts. However, oligomer formation from γ-BL was observed by using PPL or *Pseudomonas* sp. lipase as catalyst [18, 13]. Enzymatic polymerization of six-membered lactones, δ-VL, was reported. δ-VL was polymerized by various lipases of different origin [19, 8]. The molecular weight of the enzymatically obtained polymer was relatively low (less than 2000).

ε-CL (seven-membered lactone) is industrially manufactured, and its oligomer having a hydroxy group at both ends is widely used as soft segment of polyurethanes. High molecular weight poly(ε-CL) is a commercially available biodegradable plastic. So far, various commercially available lipases have catalyzed the ε-CL polymerization. In the case of crude industrial lipases (PPL, lipases CR, BC, and PF), a large amount of catalyst (often more than 40 weight % for ε-CL) was required

for the efficient production of the polymer [20, 21]. A novel thermophilic lipase from *Fervidobacterium nodosum* for ROP of ε-CL was developed [22]. This enzyme could effectively catalyzed ROP at high temperature and showed the highest activity at 90 °C. *Yarrowia lipolytica* lipase (lipase YL) immobilized on the selected supports catalyzed ROP of ε-CL to prepare poly(ε-CL) polyol for polyurethane [23].

Candida antarctica lipase (lipase CA) showed high catalytic activity toward the ε-CL polymerization; a very small amount of lipase CA (less than 1 weight % for ε-CL) was enough to induce the polymerization [24]. Under the appropriate conditions, poly(ε-CL) with the molecular weight close to 10^5 was obtained [25]. During the polymerization of ε-CL, the degradation simultaneously took place. The polymerization in bulk produced the linear polymer, whereas the main product obtained in organic solvents was of cyclic structure, suggesting that the intramolecular condensation took place during the polymerization. Lipase CA mutants were developed to improve the catalytic activity. Nearly threefold increase in catalytic efficiency for the ε-CL ROP and 30% increased poly(ε-CL) molar mass were achieved [26]. Lipase CA induced the polymerization of α-methyl substituted six- and seven-membered lactones at 45 °C for 24 h to give the corresponding polyesters with molecular weight of 1.1×10^4 and 8.4×10^3, respectively [27].

Lipase catalysis induced ROP of a nine-membered lactone (8-octanolide, OL). The polymerization at 75 °C for 10 days produced the polymer with molecular weight of 1.6×10^4 [28, 29]. Lipases BC and CA showed the high catalytic activity for the polymerization. The lipase catalysis was effective for the ROP of macrolides such as 11-undecanolide (12-membered, UDL) [30], 12-dodecanolide (13- membered, DDL) [31], 15-pentadecanolide (16-membered, PDL) [32, 29, 33], and 16-hexadecanolide (17-membered, HDL) [34]. Various lipases catalyzed the polymerization of these macrolides. For the polymerization of DDL, the activity order of the catalyst was lipase BC > lipase PF > lipase CR > PPL. The lipase CA-catalyzed polymerization of PDL proceeded fast in toluene to produce a high molecular weight polymer with the molecular weight higher than 8×10^4. Enzymatic ROP of macrolides (UDL, DDL, and PDL) proceeded even in an aqueous medium.

Macrocyclic nonadecalactone and tricosalactone were subjected to lipase CA-catalyzed ROP to give the corresponding polyesters with high molecular weight [35]. The melting point of the products was higher than 100 °C, and their mechanical properties displayed the resemblance to polyethylene. A 24-membered lactone derived from natural sophorolipid was polymerized via lipase-catalyzed polymerization to give a glycolipid-based polyester. The reaction proceeded in two modes depending on the reacted position of a hydroxyl group with the formation of monoacylated products to oligomers and polymers [36]. A 26-membered sophorolipid lactone having a double bond was enzymatically prepared and subjected to ring-opening metathesis polymerization with a Ru catalyst, giving rise to poly(sophorolipid) with molecular weight $> 1 \times 10^5$ [37]. For the applications of glycolipid biomaterials, properties of the solid-state product were examined in detail [38].

Macrocyclic esters having 24- to 84-ring atoms were polymerized by polymer-supported lipase CA catalyst. Typically, the polymerization was carried out in tolu-

ene at 70 °C for 20 h with 7.5% w/w of the lipase catalyst, affording the polyesters with molecular weight up to 6.3×10^4 in very high yields. These monomers are strainless, and hence, the polymerization was discussed in terms of a type of entropically driven ROP [39]. The polymerization of a bile acid (cholic acid) macrocyclic ester monomer was performed in toluene at 80 °C for 24–72 h by lipase CA catalyst to produce polymers with molecular weight of $1–3 \times 10^4$ in moderate yields. The reaction is considered to proceed via transesterification mode. In order to obtain polymers efficiently, the high concentration of the starting monomer was chosen to favor the polymerization in the ring-chain equilibrium in the ROP [39]. Lipase CA-catalyzed cyclic butylene 2,5-furandicarboxylate and butylene succinate oligomer mixtures produced the corresponding polyester with molecular weight higher than 10^4 [40].

Lipases CA, BC, and PF catalyzed the polymerization of cyclic diester-type lactones, ethylene dodecanoate, and ethylene tridecanoate to give the corresponding polyesters [41]. The enzyme origin affected the polymerization behaviors; in using lipase BC catalyst, these bislactones polymerized faster than ε-CL and DDL, whereas the reactivity of these cyclic diesters was in the middle of ε-CL and DDL in using lipase CA.

Poly(ε-CL) with molecular weight larger than 7.0×10^4 was enzymatically obtained from the corresponding oligomer at 70 °C in toluene [42, 25]. A cyclic dimer (14-membered) of ε-CL was polymerized by lipase CA to produce poly(ε-CL) with molecular weight of 8.9×10^4 at high temperature quantitatively, and cyclic oligomers of larger ring size were also polymerized via lipase catalysis [25, 43, 44].

A cyclic oligomer prepared from 1,4-butanediol and dimethyl succinate was polymerized by a lipase catalyst to give poly(butylene succinate) (PBS) with molecular weight of 1.3×10^5. Interestingly, the molecular weight of the polymer from the cyclic oligomer was higher than that obtained by the direct polycondensation [45]. High molecular weight poly(ethylene succinate) (PES) was efficiently obtained by lipase CA-catalyzed ROP of cyclic oligo(ethylene succinate). This cyclic monomer was copolymerized with lactide and ε-CL to produce the corresponding ester copolymers [46].

Lactide (LA), a six-membered cyclic dimer of lactic acid, is a very important starting monomer for industrial production of poly(lactic acid) (PLA), one of the most famous green plastics and bioabsorbable materials. Lactide was not enzymatically polymerized under mild reaction conditions; however, poly(lactic acid) with the molecular weight higher than 1×10^5 was formed using *Pseudomonas* species as catalyst at higher temperatures (80–130 °C), although the product yield was low. DL-LA (DLLA) gave the higher molecular weight in comparison with LL-LA (LLA) and DD-LA (DLA) monomers [47]. Noticeably, lipase CA was reported not to catalyze the polymerization of LLA, whereas DLA was enantioselectively polymerized at 70 °C for 3 days to produce polyDLA with molecular weight of 3.3×10^3 [48]. Mutants of lipase CA for the DLA ROP were created and their activity was 90-fold higher than that of the wild type [49]. Free lipase CA and immobilized one on chitin and chitosan were used for ROP of LLA to produce polyLLA with high molecular weight [50].

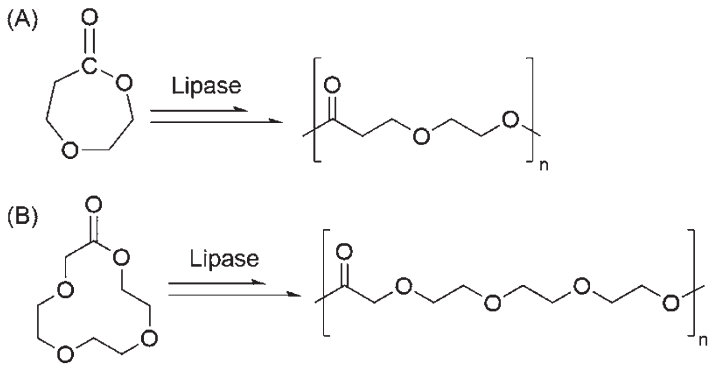

Fig. 6.3 Enzymatic synthesis of ether-containing polyesters by ROP

Lipase CA efficiently catalyzed the ROP of 1,5-dioxepan-2-one (DXO) to produce the corresponding poly(ester-*alt*-ether) (Fig. 6.3a) [51, 52]. A linear relationship between the monomer conversion and the molecular weight of the polymer was observed. The monomer consumption followed a first-order rate law, suggesting no termination and chain-transfer reaction. The enzymatic polymerizability of DXO was much larger than that of ε-CL.

In synthesis of polyesters via ROP, metal catalysts are often used. For medical applications of polyesters, however, there has been concern about harmful effects of the metallic residues. Enzymatic synthesis of a metal-free polyester was demonstrated by the polymerization of 1,4-dioxan-2-one (DO) using lipase CA [19]. Under appropriate reaction conditions, the high molecular weight polymer (molecular weight = 4. 1 × 10^4) was obtained. A 12-membered lactone, 2-oxo-12-crown-4-ether (OC), showed high reactivity for lipase CA-catalyzed polymerization to produce a water-soluble polyester with molecular weight of 3.5 × 10^3 (Fig. 6.3b) [53].

It is well known that catalytic site of lipase is a serine-residue and lipase- catalyzed reactions proceed via an acyl-enzyme intermediate. The enzymatic polymerization of lactones is explained by considering the following reactions as the principal reaction course (Fig. 6.4). The key step is the reaction of lactone with lipase involving the ring-opening of the lactone to give an acyl-enzyme intermediate ("enzyme-activated monomer," EM). The initiation is a nucleophilic attack of water, which is contained partly in the enzyme, onto the acyl carbon of the intermediate to produce ω-hydroxycarboxylic acid (n = 1), the shortest propagating species. In the propagation stage, the intermediate is nucleophilically attacked by the terminal hydroxyl group of a propagating polymer to produce a one-unit-more elongated polymer chain. The kinetics of the polymerization showed that the rate-determining step of the overall polymerization is the formation of the enzyme-activated monomer. Thus, the polymerization probably proceeds via an "activated-monomer mechanism" [54, 55, 1].

Generally, ring-chain (cyclic-linear structure) equilibrium exists in ROP of various cyclic monomers, and such an equilibrium was observed also for enzymatic ROP [56] as well as enzymatic polycondensation [57]. In some cases of lipase- cata-

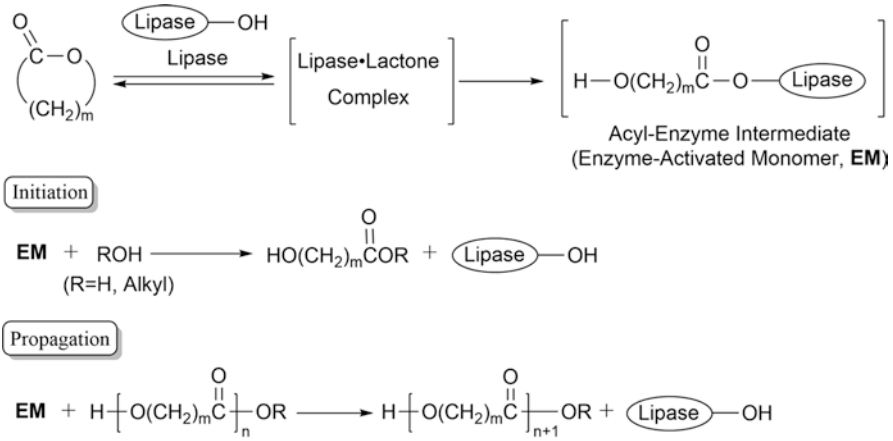

Fig. 6.4 Postulated mechanism of lipase-catalyzed ROP of lactones

lyzed ROP of lactones such as ε-CL [9] and β-PL [11], cyclic oligomers were formed in addition to major products of linear polyesters. Only a small amount of the cyclic dimer was formed in the lipase CA-catalyzed polymerization of ε-CL in bulk, whereas the polymerization in acetonitrile, THF, or 1,4-dioxane afforded the cyclic products. The formation of macrocycles up to the 23 monomer units was produced in 1,4-dioxane [56]. These unique behaviors are explained by the backbiting reaction of a polymer acyl-enzyme intermediate.

Reactivity of cyclic compounds generally depends on their ring size; small and intermediate ring-size compounds possess higher ring-opening reactivity than macrocyclic lactones (macrolides) due to their larger ring strain. Table 6.1 summarizes dipole moment values and reactivities of lactones with different ring sizes. The dipole moment (an indication of ring strain) of the macrolides is lower than that of δ-VL and ε-CL and close to that of an acyclic fatty acid ester (butyl caproate). The rate constant of the macrolides in anionic polymerization is much smaller than that of δ-VL and ε-CL. These data clearly show that the macrolides have much lower ring strain and, hence, show less anionic reactivity (from alkaline hydrolysis) and polymerizability (from propagation) than the medium-size lactones.

On the other hand, the macrolides showed unusual reactivity by enzyme catalyst. Lipase PF-catalyzed polymerization of the macrolides proceeded much faster than that of ε-CL. The lipase-catalyzed polymerizability of lactones was quantitatively evaluated by Michaelis-Menten kinetics. For all monomers, linearity was observed in the Hanes-Woolf plot, indicating that the polymerization followed Michaelis-Menten kinetics. The $V_{max(lactone)}$ and $V_{max(lactone)}/K_{m(lactone)}$ values increased with the ring size of lactone, whereas the $K_{m(lactone)}$ values scarcely changed. These data imply that the enzymatic polymerizability increased as a function of the ring size, and the large enzymatic polymerizability is governed mainly by the reaction rate (V_{max}), but not to the binding abilities, i.e., the reaction process of the lipase-lactone complex to the acyl-enzyme intermediate is the key step of the polymerization. For the

Table 6.1 Dipole moments and reactivities of unsubstituted lactones

		Rate constant		Michaelis-Menten kinetics[c]		
Lactone (ring size)	Dipole moment	Alkaline hydrolysis[a]	Propagation[b]	$K_{m(lactone)}$	$V_{max(lactone)}$	$V_{max(lactone)}/K_{m(lactone)}$
	Cm	$\times 10^4$, $L \cdot mol^{-1} \cdot s^{-1}$	10^3, s^{-1}	$mol \cdot L^{-1}$	$\times 10^2$, $mol \cdot L^{-1} \cdot h^{-1}$	$\times 10^2$, h^{-1}
δ-VL(6)	4.22	55,000	–	–	–	–
ε-CL(7)	4.45	2550	120	0.61	0.66	1.1
UDL(12)	1.86	3.3	2.2	0.58	0.78	1.4
DDL(13)	1.86	6.0	15	1.1	2.3	2.1
PDL(16)	1.86	6.5	–	0.80	6.5	8.1
HDL(17)	–	–	–	0.63	7.2	11
Butyl caproate	1.75	8.4	–	–	–	–

[a]Alkaline: NaOH. Measured in 1,4-dioxane/water (60/40 vol%) at 0 °C

[b]Measured using NaOMe initiator (0.06 mol amount) in THF at 0 °C

[c]Kinetics of the polymerization was carried out using lipase PF (200 mg) as catalyst in the presence of 1-octanol (0.03 $mol \cdot L^{-1}$) in diisopropyl ether (10 mL) at 60 °C

Zn-catalyzed anionic polymerizability of lactones, the reverse reactivity with large difference was reported; much higher reactivities of δ-VL and ε-CL (more than 2000 times) for the microlides were found [58]. These behaviors are similar to those of alkaline hydrolysis.

Immobilized lipase CA (Novozym 435) is the most used enzyme for enzymatic synthesis of polyesters. The structure of lipase CA was determined by X-ray crystallographic analysis [59]. Lipase CA also exhibited higher catalytic activity than ε-CL for ROP of macrolides. Kinetic parameters are as follows: initial rates ($x10^5$, $L \cdot mol^{-1} \cdot h^{-1} \cdot mg^{-1}$) were 2300 for ε-CL, 48 for OL, 1600 for DDL, and 4700 for PDL. For these four monomers, the corresponding relative values for lipase PF were 1.3, 1.8, 2.5, and 8.5, and those for lipase BC were 0.42, 2.2, 4.8, and 11 [20, 28, 29].

Recently, the low-cost immobilized enzyme from lipase CA immobilized on a hydrophobic support was developed and used as catalyst for ROP of PDL to give the high molecular weight polyPDL [60]. An amorphous silica material was used as matrix to immobilize lipase CA by cross-linking method for ROP of ε-CL. The activity for ROP increased 17% by the immobilization process.

Catalysis of lipase CA for ROP of nine lactones was investigated according to Michaelis-Menten kinetics in detail [61]. The values of K_m were in a narrow range between 0.09 and 0.73 $mol \cdot L^{-1}$, suggesting close affinities of lipase CA for all lactones. On the other hand, the values of V_{max} varied between 0.07 and 6.10 $mol \cdot L^{-1} \cdot h^{-1}$ and did not show systematic trend. These behaviors are partially similar to those with lipase PF [62–64], suggesting that the key step in the lipase-catalyzed ROP of lactones is the step from the lipase-lactone complex to form EM in Fig. 6.4. The most reactive monomer (eight-membered) showed almost 200 times more reactive than the least reactive monomer (11-membered). The trend of the relative rates for the monomer ring sizes was complicated; there was no monotonous change depending on the ring size. These results suggest that various physical properties, dipole moment, ring strain, transoid and cisoid structure of lactones [65], conformational strain, and transannular interactions should be taken into account for explanation of ROP behaviors with enzyme and chemical catalysts [61]. Combination of docking and molecular dynamics studies provided information about the unique reactivity difference of lactones in lipase-catalyzed ROP. Cisoid or transoid conformation of the ester bond would govern the reactivity by the lipase catalysis [66]. The turnover number (TON, mol of ε-CL converted to polymer per mol of enzyme per min) was examined for immobilized lipase CA and *Humicola insolens* cutinase (HIC). The value for lipase CA was close to that for HIC [67].

Lipase CA was immobilized on a cross-linked macroporous copolymer from glycidyl methacrylate and ethylene glycol dimethacrylate. Effects of pore size, specific surface area, specific volume, and particle size on the polymerization of ε-CL were systematically investigated. Eighty percent of the enzyme was covalently immobilized on the macroporous resin. The decrease of the diameter, the increase of pore size, and specific surface area led to the improvement of the catalytic activity [68]. Reuse of commercially available lipase CA for ROP of ε-CL was examined. Up to ten cycles, high molecular weight poly(ε-CL) with similar polyindex was obtained [69]. Chemically immobilized lipase on photo-cross-linked chitin was developed for the polymerization of ε-CL [70]. Lipase CA was also immobilized on

montmorillonite and sepiolite nanoclays. An organo-modified clay catalyzed ROP of ε-CL to produce poly(ε-CL), and clay nanohybrids and the poly(ε-CL) chains were effectively grafted on the clay [71]. Lipase CA was immobilized onto surface-modified rice husk ashes by covalent binding and used for ROP of ε-CL. The immobilized enzyme showed catalytic activity even at 30 °C [72, 73]. Poly(glycidol) was used as matrix resin for immobilization of lipase CA, and the immobilized lipase CA catalyzed ROP of ε-CL in toluene [73].

As shown in Fig. 6.4, alcohols can initiate lipase-catalyzed ROP of lactones. Polyglycidols having primary OH groups were employed as multifunctional initiator for polymerization of ε-CL by enzymatic and chemical catalysts. Lipase CA and Zn(II) 2-ethylhexanoate were used as enzymatic and chemical catalysts, respectively. The formed polymer architectures were of core-shell structure, and the structural difference was discussed for these processes [74]. Enzymatic ROP was applied to synthesize hyperbranched aliphatic copolyesters by lipase CA-catalyzed copolymerization of ε-CL with 2,2-bis(hydroxymethyl)butyric acid. In preparing the AB_2 polyesters, the degree of branching and the density of functional end group were controlled [75].

The microwave irradiation was applied for lipase CA-catalyzed polymerization of ε-CL; the polymerization rate was decelerated in boiling nonpolar solvents such as toluene and benzene by the irradiation, whereas the rate was moderately accelerated in a boiling polar solvent, diethyl ether [76]. The effects of the detailed reaction parameters such as reaction temperature, time, and microwave intensity were examined [77]. The polymerization reaction under optimal conditions (90 °C, 240 min, and 50 W) afforded poly(ε-CL) with molecular weight of 2.1×10^4.

A three-step enzymatic reaction sequence for the synthesis of poly(ε-CL) was designed running in a fed-batch operation [78]. Cyclohexanol was used as the starting substrate, and the first two steps consisted of an oxidation of cyclohexanol to cyclohexanone catalyzed by alcohol dehydrogenase and an enzymatic Baeyer-Villiger oxidation of cyclohexanone to ε-CL. In an aqueous solution, ε-CL was hydrolyzed by lipase CA, and the extraction and subsequent polymerization produced poly(ε-CL) of high molecular weight.

6.3 Enzymatic Copolymerization of Cyclic Esters

Lipase catalyzed ring-opening copolymerization of cyclic monomers. In 1993, the first example of the enzymatic ring-opening copolymerization of lactones was demonstrated; δ-valerolactone (δ-VL) and ε-CL were copolymerized by lipase PF catalyst [79]. The resulting copolymer was of random structure having both units. Random ester copolymers were also enzymatically synthesized from other combinations: ε-CL–OL, ε-CL–PDL, and OL–DDL. The formation of the random copolymers in spite of the different enzymatic polymerizability of these lactones suggests that the intermolecular transesterifications of the polyesters frequently took place during the copolymerization. The lipase-catalyzed copolymerization of ε-CL with

PDL and DLA was also examined [32]. By utilizing this specific lipase catalysis, random ester copolymers were synthesized by the lipase-catalyzed polymerization of macrolides in the presence of poly(ε-CL) [80]. Lipase CA-catalyzed ring- opening copolymerization of DO with PDL in toluene or diphenyl ether at 70 °C gave a copolyester of poly(DO-*co*-PDL) with molecular weight higher than 3×10^4 [81]. In the copolymerization of OC with PDL, OC polymerized five times faster than PDL. Interestingly, the product copolymer was of the random structure.

In the ring-opening copolymerization of ε-CL with DLLA by using lipase CA as catalyst, DLLA was consumed more rapidly than ε-CL in the initial reaction stage, which was incorporated into the LA dimer-wise. As the copolymerization proceeded, the relative amount of ε-CL in the copolymer increased. The nonrandom copolymer structure disappeared with time, probably due to the lipase-catalyzed transesterification reaction. The macrocyclic compound was formed during the copolymerization [82].

The copolymerization of the cyclic dimeric monomers, DLLA, LLA, and glycolic acid (GA) by using lipase BC catalyst was studied. The copolymerization of LA and GA (8:2 molar ratio) was conducted in bulk at 100 or 130 °C. Under these reaction conditions, the copolymer was formed without lipase (control experiment); the molecular weight of the copolymer was much smaller than that obtained in the presence of the lipase catalyst. In case of the copolymerization at 130 °C for 2 days, a cyclic random copolymer with molecular weight of 2.1×10^4 was formed, whereas the formation of the linear polymer with molecular weight of 1.2×10^4 was found at 100 °C for 7 days, suggesting the complicated reactions [83]. When multifunctional alcohols like ethylene glycol, pentaerythritol, inositol, and polyglyceline were used as initiator in the lipase PS-polymerization of LLA, DLA, and DLLA at 140 °C, various branched polymers were obtained [84].

Lipase-catalyzed copolymerization of divinyl esters, glycols, and lactones produced ester copolymers with molecular weight higher than 1×10^4 (Fig. 6.5) [85]. Lipases BC and CA showed high catalytic activity for this copolymerization. ^{13}C NMR analysis showed that the resulting product was not a mixture of homopolymers, but a copolymer derived from the monomers, indicating that two different modes of polymerization, polycondensation and ROP, simultaneously take place through enzyme catalysis in one pot to produce ester copolymers (see also 5.2.4

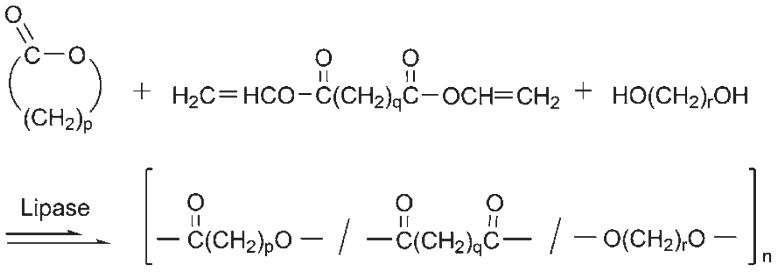

Fig. 6.5 Lipase-catalyzed copolymerization of lactones, divinyl esters, and glycols

Ring-Opening Addition-Condensation Polymerization (ROACP) in the previous chapter). Furthermore, this result strongly suggests the frequent occurrence of transesterification between the resulting polyesters during the polymerization.

6.4 Enantioselective and Chemoselective ROP

Lipase catalysis is often used for enantioselective production of chiral compounds. Lipase induced enantioselective ROP of racemic lactones. In the lipase-catalyzed polymerization of racemic β-butyrolactone (β-BL), the enantioselectivity was low; an enantioselective polymerization of β-BL occurred by using thermophilic lipase to give the (*R*)-enriched polymer with 20–37% ee [14]. The enantioselectivity was greatly improved by the copolymerization with 7- or 13-membered non-substituted lactone using lipase CA catalyst; the ee value reached ca. 70% in the copolymerization of β-BL with DDL (Fig. 6.6) [86]. It is to be noted that in the case of lipase CA catalyst, the (*S*)-isomer was preferentially reacted to give the (*S*)-enriched optically active copolymer. The lipase CA-catalyzed copolymerization of δ-caprolactone (six-membered) with DDL enantioselectively proceeded, yielding the (*R*)-enriched optically active polyester with ee of 76%.

Optically active polyesters were synthesized by lipase CA-catalyzed ROP of racemic 4-methyl, ethyl-, or propyl-ε-caprolactone (Fig. 6.7). The S-isomer was enantioselectively polymerized to produce the polyester with >95% ee [87]. Quantitative reactivity of 4-substituted-ε-caprolactone using lipase CA as catalyst was analyzed [88]. The polymerization rate decreased by a factor of 2 upon the introduction of a methyl substituent at the 4-position. Furthermore, 4-ethyl-ε- caprolactone polymerized 5 times slower than the 4-methyl-ε-caprolactone. This reac-

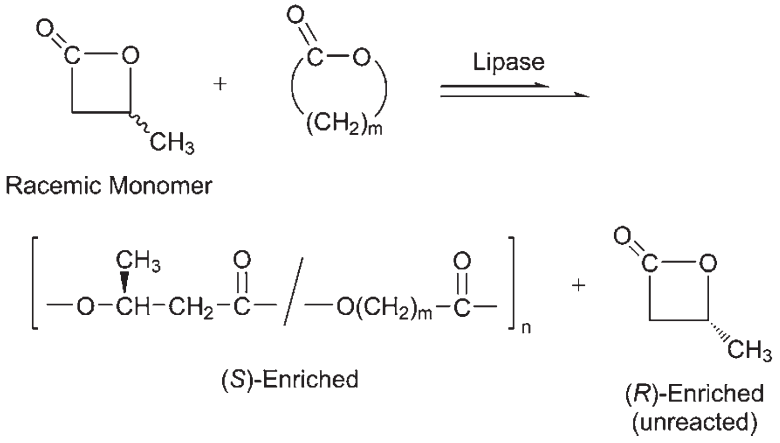

Fig. 6.6 Lipase-catalyzed enantioselective copolymerization of β-BL with non-substituted lactones

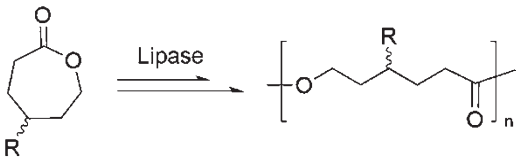

Fig. 6.7 Enantioselective ROP of 4-substituted-ε-CL

tivity difference is strongly related to the enantioselectivity. Interestingly, lipase CA displayed the S-selectivity for 4-methyl or ethyl-ε-caprolactone; the enantioselectivity was changed to the (*R*)-enantiomer in the case of 4-propyl-ε-caprolactone. Lipase BC induced the enantioselective polymerization of 3-methyl-4-oxa-6- hexanolide (MOHEL). The initial reaction rate of the S-isomer was seven times larger than that of the R-isomer, indicating that the enantioselective polymerization of MOHEL took place through lipase catalysis [62].

Iterative tandem catalysis (ITC) was applied for lipase-catalyzed ROP of lactones. ITC means a polymerization in which the chain growth is effectuated by a combination of two different catalytic processes that are both compatible and complementary. By a combination of lipase CA-catalyzed ROP of 6-MeCL and Ru-catalyzed racemization of a propagating secondary alcohol, optically pure *(R)* oligoesters were obtained [89, 90]. This process is on the basis of dynamic kinetic resolution (DKR), and the polymerization was performed in one pot. First, lipase CA enantioselectively catalyzed the ring-opening of an *(S)* monomer with the benzyl alcohol initiator. For the lipase-catalyzed enantioselective propagation, the terminal *(S)* alcohol is less favored to react with the monomer via ring-opening, and hence the Ru-catalyzed racemization of the terminal took place. Then, the terminal *(R)* alcohol was selectively reacted with the monomer by lipase catalysis to facilitate one monomer-unit elongated. This reaction cycle repeated to produce the *(R)* oligoester from the racemic monomer. This new approach can be regarded as a flexible and convenient tool for obtaining chiral macromolecules from racemic or prochiral monomers.

A combination of simultaneous dynamic kinetic resolution (DKR) of a secondary alcohol and ROP of ε-CL using lipase CA and Ru catalysts was reported [91]. Racemic 1-phenylethanol (PhE) was used as model alcohol and incorporated into the terminal of poly(εCL) under DKR conditions. The incorporation of 75% PhE was achieved, and the ee value of the product ((*R*)-PhE-poly(ε-CL)) was over 99%. This methodology is applicable for simple one-step production of polymers containing a optically pure group at the terminal, which has large application potentials for release of chiral species in medical fields [91].

Reactive polyesters were enzymatically synthesized. Lipase catalysis chemoselectively induced the ROP of a lactone having *exo*-methylene group to produce a polyester having the reactive *exo*-methylene group in the main chain (Fig. 6.8) [92, 93]. This is in contrast to the anionic polymerization; the vinyl polymerization of this monomer took place by a conventional anionic initiator or catalyst.

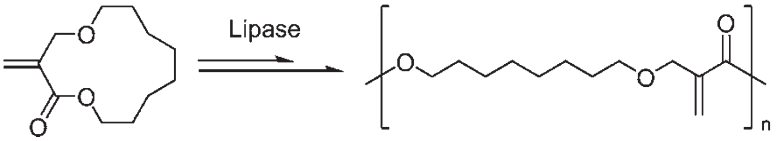

Fig. 6.8 Lipase-catalyzed chemoselective ROP of a lactone having *exo*-methylene group

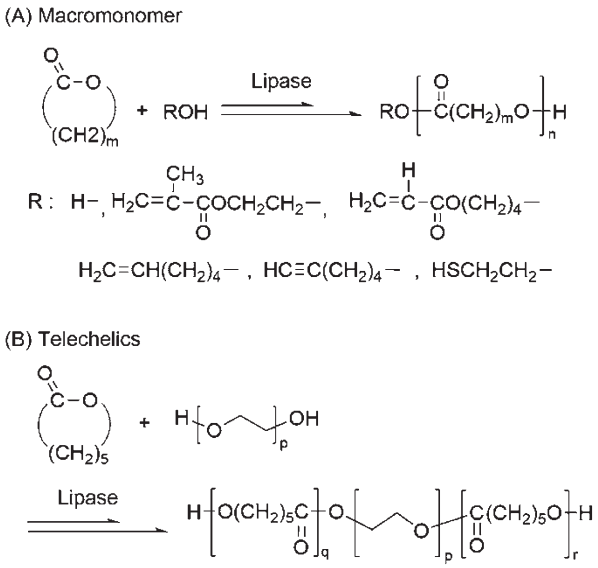

Fig. 6.9 Lipase-catalyzed synthesis of polyester macromonomers and telechelics via ROP

6.5 Enzymatic Terminal Functionalization via ROP

Since terminal-functionalized polymers such as macromonomers and telechelics are very important as prepolymer for construction of functional materials. Single-step functionalization of polymer terminal was achieved via lipase catalysis. Alcohols could initiate the ROP of lactones by lipase catalyst. The lipase CA-catalyzed polymerization of DDL in the presence of 2-hydroxyethyl methacrylate gave the methacryl-type polyester macromonomer, in which 2-hydroxyethyl methacrylate acted as initiator to introduce the methacryloyl group quantitatively at the polymer terminal ("initiator method") (Fig. 6.9a) [94]. This methodology was expanded to synthesis of ω-alkenyl- and alkynyl-type macromonomers by using 5-hexen-1-ol and 5-hexyn-1-ol as initiator.

Lipase CA-catalyzed ROP of ε-CL or 1,5-dioxepane-2-one (DXO) in the presence of HEMA produced the macromonomer. By changing the feed ratio of the monomer and alcohol, the macromonomer with various molecular weights was obtained. The amphiphilic nature of the macromonomer was controlled by the

copolymerization to give an amphiphilic poly(ε-CL-*co*-DXO) macromonomer [52]. 4-Penten-2-ol could also initiate lipase CA-catalyzed ROP of ε-CL or DXO, to give a macromonomer having a double bond. Propargyl alcohol and 4- dibenzocyclooctynol efficiently initiated ROP of ε-CL by lipase CA [95]. The resulting alkyne group of the polymer was sequentially functionalized in a post-polymerization using metal- free and copper-catalyzed click chemistry.

Poly(ethylene glycol) and poly(ε-CL)-diol were used as initiator for ROP of ε-CL or DXO to give triblock polyesters with a hydroxyl group at both ends (Fig. 6.9b) [96]. The methacryl-type polyester macromonomer was produced via lipase-catalyzed ROP of PDL using HEMA as initiator, which was subjected to radical polymerization, leading to polymers with brush structure [97]. Lipase from *Candida* sp. 99–125 catalyzed ROP of ε-CL in the presence of 6-mercapto-1- hexanol to produce thiol-terminated poly(ε-CL). Interestingly, this enzyme remarkably showed chemoselectivity between hydroxyl and thiol groups [98]. A porous material was obtained from a mixture of polyPDL and HEMA-initiated polyPDL by thermally induced phase separation method, which has large potential as scaffolds for bone tissue engineering [99].

Polyester-sugar or polyester-polysaccharide conjugates were regioselectively synthesized via enzyme catalysis (Fig. 6.10). Lipase CA-catalyzed polymerization of ε-CL in the presence of alkyl glucopyranosides produced polyesters bearing a sugar at the polymer terminal [100, 101]. In the initiation step, the primary hydroxyl group of the glucopyranoside was regioselectively acylated. Enzymatic selective monosubstitution of a hydroxyl-functional dendrimer was demonstrated. Lipase CA-catalyzed polymerization of ε-CL in the presence of the first-generation dendrimer gave the poly(ε-CL)-monosubstituted dendrimer [102]. Ibuprofen, an important chiral 2-arylpropionic acid class of nonsteroidal anti-inflammatory drug, initiated lipase CA-catalyzed ROP of ε-CL, followed by polycondensation using alkaline protease from *Bacillus subtilis* to produce biodegradable polyesters with the drug pendants [103].

Lipase-catalyzed ROP of ε-CL was examined to coat the hydrophilic cellulose-fiber surface with the hydrophobic poly(ε-CL) polyester chains by utilizing the

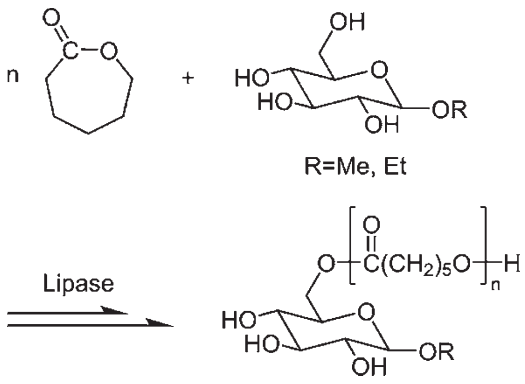

Fig. 6.10 Regioselective initiation of lipase-catalyzed ROP of ε-CL on alkyl glucopyranosides

cellulose-binding module-lipase CA conjugate as catalyst. The hydrophobicity of the surface did not arise from the covalently attached poly(ε-CL) to the surface hydroxyl groups, but rather from the surface-deposited polymers, which could be easily extracted [104].

The initiation process in the lipase-catalyzed ROP under various reaction conditions in bulk or in toluene strongly relates to synthesize end-functionalized polymers by introducing the functionality into polymers via ROP (initiator method) [105]. In the lipase-catalyzed ROP of lactones, an initiator nucleophile species can be water, an alcohol, an amine, or a thiol (see also Fig. 6.9a). Initiation reaction behaviors in lipase CA-catalyzed ROP of ε-CL using water, benzyl alcohol, and other Br-containing primary alcohols as initiators were investigated. Among these nucleophiles, water was the most reactive; thus the removal of water in the reaction system was important to achieve the high functionality to synthesized end- functionalized polymers.

In case of the lipase CA-catalyzed ROP of ε-CL or PDL in the presence of HEMA in bulk at high temperature such as 80 °C, the complicated reaction behavior was found [106]. Not only the HEMA-initiated ROP but also transesterification among the product polymers, monomer, and HEMA took place extensively after long reaction time. By utilizing the transesterification, di-methacrylated polyesters were obtained in one-pot reaction. For the selective preparation of the desired macromonomer, the lipase-catalyzed ROP should be performed under mild conditions and terminated at a lowed monomer conversion. The effect of the initiator structure on the transesterification was examined. HEMA and 2-hydroxyethyl acrylate (HEA) were used as initiator [107]. In both cases, the transesterification occurred extensively at 80 °C in bulk, and the reaction rate was 15 times faster on the HEA- initiated system. Thiol end-functionalization was achieved by using lipase CA catalyst for ROP of ε-CL with 2-mercaptoethanol initiator [108].

An alcohol attached on the gold surface was used as initiator for lipase-catalyzed ROP of ε-CL and 1,4-dioxan-2-one (DO). The resulting system can be applied for biocompatible/biodegradable coating materials in biomedical areas such as passive or active coatings of stents. This method would be beneficial in the applications where the minimization of harmful species is critical [109]. A similar surface- initiated polymerization was reported for the in situ solid-phase synthesis of biocompatible poly(3-hydroxybutyrate) [110]. A macroinitiator of a linear or a four-arm star-shaped polyglycidol was used for synthesis of densely grafted poly(glycidol-*graft*- ε-CL) and loosely grafted poly[(glycidol-*graft*-ε-CL)-*co*-glycidol] copolymers via lipase-catalyzed ROP of ε-CL or Sn-catalyzed chemical process using ε-CL monomer [111]. In comparison with linear poly(ε-CL), the latter showed much enhanced degradability, probably due to high concentration of hydroxyl groups at the polyglycidol backbone.

A methacryl-type polyester macromonomer was synthesized by lipase PF-catalyzed polymerization of DDL using vinyl methacrylate as terminator ("terminator method"), in which the vinyl ester terminator was present from the beginning of the reaction (Fig. 6.11a) [112]. The polymerization in the presence of vinyl 10-undecanoate produced the ω-alkenyl-type macromonomer.

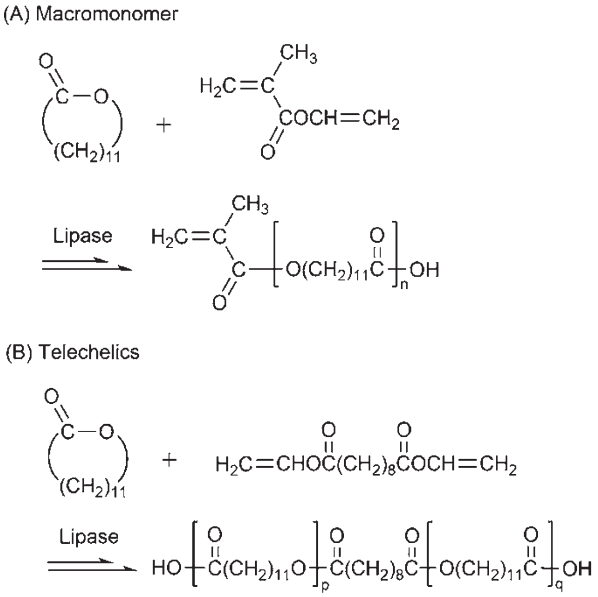

Fig. 6.11 Lipase-catalyzed synthesis of polyester macromonomer and telechelics by terminator method

In using divinyl sebacate as terminator, the telechelic polyester having a carboxylic acid group at both ends was obtained (Fig. 6.11b) [113]. Telechelic polyesters containing a thiol group were prepared by an enzymatic one-pot system. The lipase CA-catalyzed ROP of PDL in the presence of 6-mercaptohexanol initiator and subsequent termination by γ-thiobutyrolactone produced a telechelic polyester with thiol groups at both ends [114]. The obtained telechelic polymer was used for the preparation of semicrystalline polymer networks [115]. When vinyl acrylate was used as terminator, poly(PDL) with thiol group at one end acrylate group at another end was formed.

A combination of initiator and terminator method provided amphiphilic macromonomers containing poly(ε-CL); ROP of ε-CL was initiated via lipase catalysis from hydroxyl group-containing initiators like a hexahydroxyl dendrimer and ethyl glucopyranoside, followed by introduction of a (meth)acryloyl polymerizable group by termination with vinyl (meth)acrylate [116–118].

6.6 Solvent for Enzymatic ROP

As described above, lipase-catalyzed ROP of lactones proceeded in bulk as well as organic solvents, typically toluene, heptane, 1,4-dioxane, diisopropyl ether, and dibutyl ether. In addition, water, supercritical carbon dioxide ($scCO_2$),

and ionic liquids, which are regarded as typical "green solvents," were also employed as solvent.

In the polymerization of ε-CL catalyzed by lipase CA in organic solvents, the polymer was obtained efficiently in solvents having *log* P (a parameter of hydrophobicity) values from 1.9 to 4.5, whereas solvents with *log* P from −1.1 to 0.5 showed the low propagation rate [119]. Among the solvents examined, toluene was the best solvent to produce high molecular weight poly(ε-CL) efficiently. Variation in the ratio of toluene to ε-CL in the reaction at 70 °C showed that the monomer conversion and polymer molecular weight were the largest for ratio about 2:1. Furthermore, lipase CA could be reused for the polymerization. In the range of five cycles, the catalytic activity hardly changed. The kinetics of the ε-CL bulk polymerization by lipase CA showed linear relationships between the monomer conversion and the molecular weight of the polymer; however, the total number of the polymer chains was not constant during the polymerization. The monomer consumption apparently followed a first-order rate law.

A closed variable volume reactor was used in the lipase CA-catalyzed ROP of PDL [120], which enabled usage of dichloromethane and chloroform at the reaction temperature higher than the boiling point. The high molecular weight polyPDL was obtained in high yields even for short reaction time.

Lipase catalysis induced ROP of ε-CL, OL, UDL, DDL, and PDL in water. Macrolides (UDL, DDL, and PDL) were polymerized by lipase in water to produce the corresponding polyesters with relatively low molecular weight in high yields. In terms of the yield and molecular weight of the product, lipase BC showed the highest activity [121, 62]. On the other hand, ε-CL and OL were not subjected to lipase-catalyzed ROP in water, due to no formation of an emulsion-like system. A mechanistic study on lipase-catalyzed ROP of PDL in aqueous biphasic medium was reported [122]. A model was proposed assuming Michaelis-Menten interfacial kinetics followed by chain extension via lipase-catalyzed polycondensation. Polyester particles were formed by the lipase CA-catalyzed ROP of ε-CL, UDL, and PDL in water [123].

Lipase-catalyzed ROP of lactones in a miniemulsion containing a surfactant was reported [124]. Viscous mixing of PDL, a nonionic surfactant having a poly(ethylene glycol) chain (Lutensol AT50), water, and hexadecane afforded the miniemulsion, in which PDL was polymerized by addition of lipase PS at 45 or 60 °C. The PDL monomer was consumed quantitatively, and the polyPDL nanoparticles with diameter less than 100 nm were formed. PolyPDL showed a bimodal molecular weight distribution. In the polymerization in the presence of an unsaturated alcohol or acid such as linoleic acid, it was introduced at the polymer terminal via extensive esterifications to give a functionalized polyPDL. HDL was also polymerized in the miniemulsion system by lipase PS [125]. ROP of PDL in high internal phase emulsion (HIPE) using *Thermomyces lanuginosus* lipase was examined [126]. The enzyme present in the dispersed aqueous phase of HIPE effectively catalyzed ROP to produce polyPDL.

Lipase CA-catalyzed ROP of lactones was examined in a stable polymersome with bilayer structure, which was formed by a polystyrene-polyisocyanopeptide

block copolymer [127]. Four lactones, ε-CL, OL, DDL, and a cyclic diester, 1,4,7,10-tetraoxacyclotetradecane-11,14-one, were used for varying the nature of the monomers. The lipase catalyst was located both in the water pool and in the bilayer and catalyzed the ROP of the lactones. OL and DDL were polymerized in the polymersome water pool similarly as in a free water system, but the resulting product had low molecular weight, probably due to sterically less accessible of the monomer to the catalyst.

$scCO_2$, one of the most typical green solvents, could be used as solvent for lipase-catalyzed ROP of lactones. The polymerization of ε-CL and the copolymerization of ε-CL and DDL using lipase CA as catalyst produced the polymer with molecular weight higher than 1×10^4 [128]. A further work reported the synthesis of poly(ε-CL) having higher molecular weight reaching 7.4×10^4, and the repeated use of enzyme and $scCO_2$ were achieved [129]. The kinetic study showed that the polymerization was approximately first order with respect to monomer up to 80% conversion. Effects of water content in $scCO_2$ on the reaction rate and the molecular weight of the product were examined. The molecular weight reached 5×10^4 in case of the dry reaction system, but the molecular weight could not be controlled owing to the large degree of transesterification, forming both linear polymers (intermolecular transesterification) and cyclic compounds (intramolecular transesterification), the behavior of which was similarly observed in conventional solvents [130]. Lipase CA-catalyzed ROP of PDL using $scCO_2$ as solvent and dichloromethane or chloroform as cosolvents produced the high molecular weight polymer [131].

Lipase catalysis also induced the degradation of poly(ε-CL) in $scCO_2$ to form the oligomer of ε-CL [132, 133]. The oligomer with molecular weight less than 500 was polymerized by lipase CA to yield poly(ε-CL) with molecular weight higher than 8×10^4 [133]. $scCO_2$ was used as solvent for chemoenzymatic synthesis of a block copolymer of ε-CL and MMA, which was obtained by the combination of lipase-catalyzed ROP of ε-CL and atom transfer radical polymerization (ATRP) of MMA [134–136]. Hyperbranched polyesters were prepared by lipase CA-catalyzed ROP of δ-VL or ε-CL in the presence of 2,2-bis(hydroxymethyl)butyric acid in 1,1,1,2-tetrafluoroethane (R-134a), a kind of green solvent, or $scCO_2$ [137].

Room-temperature ionic liquids have received much attention as green designer solvents. Ionic liquids are nonvolatile, thermally stable, and highly polar liquids, which allow to dissolve many organic, inorganic, metallo-organic compounds, and also polymeric materials [138]. Ionic liquid solvents such as 1-butyl-3- methylimidazolium salts ([bmim][X^-]) have been used for lipase-catalyzed synthesis of polyesters. ε-CL was polymerized in [bmim][BF_4] [139], [bmim][PF_6], or [bmim][$(CF_3SO_2)_2N$] by lipase CA catalyst to produce poly(ε-CL) with molecular weight of several thousands in good yields [140]. For lactide, high temperature was required in lipase CA-catalyzed ROP; PLA with molecular weight of 3.6×10^4 was formed in the anhydrous ionic liquid and the molecular weight decreased by the addition of water [141]. Hyperbranched polyLLA was synthesized by lipase CA-catalyzed ROP in the presence of bis(hydroxymethyl)butyric acid with use of [bmim][PF_6] as solvent. The degree of branching could be controlled by the reaction conditions [142].

ROP of ε-CL also proceeded in various ionic liquids such as 1-ethyl-3- methylimidazolium tetrafluoroborate, 1-butyl-3-methylimidazolium tetrafluoroborate, 1-butylpyridinium tetrafluoroborate, 1-butylpyridinium trifluoroacetate, and 1-ethyl-3-methylimidazolium nitrate by using lipases YL and CR and PPL [143]. Dicationic type of ionic liquids also promoted the lipase-catalyzed ROP of ε-CL [144]. For the polymerization of ε-CL in an ionic liquid, the small amount was coated on an immobilized lipase CA [145]. By selection of an appropriate ionic liquid, the polymer yield and molecular weight increased. It was reported that use of an ionic liquid as solvent and ultrasonic irradiation technique were combined in the lipase CA-catalyzed ROP of ε-CL. Comparison of ionic liquids and organic solvents for lipase CA-catalyzed ROP of ε-CL and lactide was examined. At dilute concentration, 1-butyl-3-methylimidazolium hexafluorophosphate and *N,N*-dimethylacetamide (DMA) produced the polyesters with relatively high molecular weight in moderate yields [146]. The enzymatic copolymerization of ε-CL and DLLA in 1-butyl-3-methylimidazolium tetrafluoroborate and 1-butyl-3- methylimidazolium hexafluorophosphate using lipase CA catalyst was examined [147]. The reaction conditions greatly affected the microstructure, and ε-CL was the most active comonomer which enhanced the polymerizability of DLLA to increase the molecular weight of the copolymer.

Compared to conventional nonsonicated operation, the sonication enhanced the polymerization rate by more than twofold and the turnover number of the lipase catalyst by more than threefold [148]. A continuous flow system for lipase- catalyzed ROP of ε-CL was developed by using a microreactor, in which an immobilized lipase CA was filled in the channel of the reactor. The large improvement of the polymerization rate was found in comparison with the conventional batch system. Furthermore, the molecular weight of the product was high [149]. For lipase CA-catalyzed ROP of ε-CL in the presence of benzyl alcohol, the functionalization ratio of the polymer terminal was much higher than that in the batch system [150].

6.7 Chemoenzymatic Synthesis of Functional Polymers

Chemoenzymatic routes for production of functional polymers have been developed. Multiarm heteroblock star-type copolymers of PLA and poly(ε-CL) were prepared via a chemoenzymatic route. ROP of ε-CL was regioselectively initiated from hydroxyl group at 6 position of ethyl glucopyranoside by PPL catalyst, and the terminal hydroxyl group was acetylated with by vinyl acetate by the same lipase catalyst. Second, Sn-catalyzed ROP of lactide was initiated from the remaining hydroxyl group of ethyl glucopyranoside to produce the block copolymer consisting one poly(ε-CL) arm and three PLA arms [151].

A different type of polymerization methods including enzymatic polymerization is useful for production of functional polymers [152]. A combination of enzymatic ROP of lactones and atom transfer radical polymerization (ATRP) was reported

[153]. Such a combination of two different consecutively proceeding reactions is referred to as *cascade polymerization* [154] and allows a versatile synthesis of block copolymers consisting of a polyester chain and a vinyl polymer chain by using a designed initiator [154, 152, 155–157]. Lipase CA-catalyzed ROP of ε-CL involving the initiation of the hydroxyl group of the designed initiator produced Br-terminated poly(ε-CL), followed by the Cu-catalyzed ATRP of styrene to produce poly(ε-CL-*block*-St) [152]. One-pot chemoenzymatic cascade synthesis of block copolymers combining lipase-catalyzed ROP of ε-CL and ATRP of alkyl methacrylate monomer was examined. ATRP system showed inhibitory effect on the enzymatic activity. Methyl methacrylate was interfered with enzymatic ROP by transesterification, whereas *t*-butyl methacrylate was inert [158].

This principle was extended to synthesis of optically active block copolymers. An enantioselective ROP from a combination of a bifunctional initiator having hydroxyl and bromide groups, an racemic monomer of 4-methyl-ε-caprolactone, and lipase CA produced an optically active polyester with the bromide terminal, which was subjected to ATRP of methyl methacrylate (MMA), yielding the block copolymer containing a chiral polyester chain [154]. By optimization of the reaction conditions, side reactions such as the homopolymer formation were minimized to less than 5% with taking an optimized enzyme drying procedure [155].

Branched polymers with or without polyMMA chain from a poly(ε-CL) macromonomer were produced via a chemoenzymatic pathway. 2-Hydroxyethyl α-bromoisobutyrate was used as a bifunctional initiator to synthesize a bromide-containing poly(ε-CL) macromonomer, to which a polymerizable end group was introduced by in situ enzymatic acrylation with vinyl acrylate. Subsequent ATRP of the acrylate-type macromonomer afforded the branched polymer [156].

Another living radical polymerization, a nitroxide-mediated radical process, was similarly used for one-pot chemoenzymatic synthesis of a block copolymer. Metal-free poly(ε-CL-*block*-St) was obtained in two consecutive polymerization steps via corresponding route B and in a one-pot cascade approach without intermediate transformation or work-up step via corresponding route A (Fig. 6.12). By combination of enantioselective ROP via lipase catalysis, a chiral block copolymer from 4-alkyl ε-CL and styrene with high enantiomeric excess was formed [157]. Reversible addition-fragmentation chain-transfer (RAFT) polymerization is also

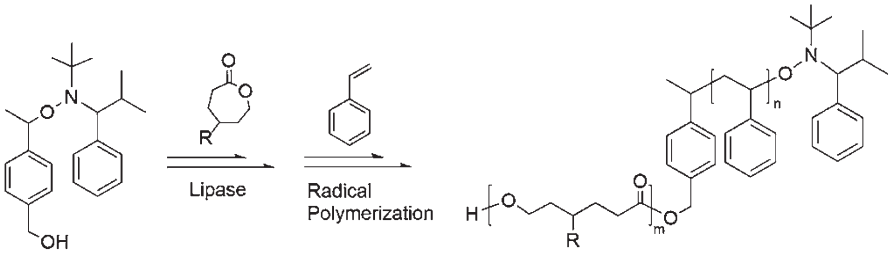

Fig. 6.12 Metal-free block copolymer synthesis by combination of lipase catalysis and nitroxide-mediated radical process

popular among living radical polymerization methods. Diblock copolymers of polyPDL-block-polyacrylate were prepared by combination of lipase CA-catalyzed ROP of PDL and RAFT of acrylate [159].

$scCO_2$ was used as solvent for a spontaneous one-step chemoenzymatic synthesis of block copolymers. A combination of $scCO_2$, lipase CA, ε-CL, MMA, CuCl, and 2,2′-bipyridine afforded poly(ε-CL-*block*-MMA) in a moderate yield. Each homopolymer was produced only in small amount (less than 10%). An amphiphilic block copolymer was prepared from a fluorooctyl methacrylate (FOMA) in $scCO_2$ by a sequential monomer addition technique [135]. A poly(ε-CL) macroinitiator was obtained via enzymatic ROP using the bifunctional initiator. Subsequently, FOMA was polymerized from the macroinitiator to produce poly(ε-CL-*block*-FOMA).

2,2,2-Trichloroethanol was used as initiator for a combination of enzymatic ROP and ATRP. Trichloromethyl group-terminated poly(ε-CL) was first prepared by lipase CA-catalyzed ROP of ε-CL, and subsequently styrene (St) was polymerized with this macroinitiator via carbon-chlorine bond cleavage via ATRP to produce poly(ε-CL-*block*-St) [160]. Instead of St, glycidyl methacrylate (GMA) was used to prepare an amphiphilic block copolymer. The resulting block copolymer formed polymeric micelles with hydrodynamic diameter around 100–200 nm.[161]. The trichloromethyl group-terminated polyDDL was similarly prepared by lipase catalyst, which was converted to a block copolymer of polyDDL and PSt via ATRP [162].

Heterografted molecular bottle brushes (HMBB) were prepared via a chemoenzymatic process combining enzymatic ROP and ATRP [163]. Polyglycidol was used as multifunctional initiator for lipase CA-catalyzed ROP of ε-CL, followed by the selective enzymatic acetylation with vinyl acetate. The remaining hydroxyl group at the backbone was reacted with 2-bromo-2-methylpropionyl bromide to introduce the bromide group in the polymer. Subsequently MMA or *n*-butyl methacrylate was polymerized via ATRP with CuBr/2,2′-bipyridyl catalyst to produce HMBB with the designed polymer blocks.

Liquid crystalline polymers were prepared via a chemoenzymatic route. Preparation of a monomer, 6-(4-methoxybiphenyl-4′-oxy)hexyl vinyl hexanedioate, was prepared with lipase CA catalyst [164]. For synthesis of multifunctional poly(meth)acrylates, functional monomers of (meth)acrylates were prepared by lipase CA-catalyzed transesterification of MMA and methyl acrylate with various functional alcohols. These monomers were radically polymerized by AIBN initiator to give poly(meth)acrylates via cascade reactions [165].

A microbial telechelic polyester of low molecular weight, hydroxylated poly[*(R)*-3-hydroxybutyrate] (PHB-diol), was used as initiator for lipase CA-catalyzed polymerization of ε-CL. The initiation took place regioselectively at the primary hydroxyl group of PHB-diol in toluene or 1,4-dioxane at 70 °C, affording the diblock polyester consisting of the PHB block with molecular weight of 2.4×10^3 and the poly(ε-CL) block with molecular weight of 1.5×10^3. Glass transition temperature and melting point of the block copolymers were tuned by varying the content of the two blocks and their molecular weight [166].

6.8 Other Cyclic Monomers

A new monomer of the *O*-carboxylic anhydride derived from lactic acid (lacOCA) was found polymerized by lipase catalyst via ROP fashion with liberation of carbon dioxide [167]. Lipase PS and lipase CA were used for ROP of lacOCA at 80 °C for several hours to give poly(lactic acid) (PLA) having relatively high molecular weight in good yields. Both lipases showed similar catalytic activity for ROP of lacOCA, and lacOCA is much more reactive with lipase catalysis than the lactide monomer. The lipase showed slightly higher catalyst activity for L-lacOCA than for D-lacOCA; enantioselection was scarcely observed.

In addition to polyesters, lipase catalysis produced other polymers such as polycarbonates, polyphosphates, polythioesters, etc. which were synthesized by ROP (Fig. 6.1). Lipase catalyzed the ring-opening polymerization of cyclic carbonates. Trimethylene carbonate (six-membered, TMC) was polymerized by lipases BC, CA, PF, and PPL to produce the corresponding polycarbonate without involving elimination of carbon dioxide (Fig. 6.13) [168, 169]. The detailed tuning of the reaction conditions enabled the increase of the molecular weight by adding a solvent and molecular sieves [170]. High molecular weight poly(TMC) (molecular weight $> 1 \times 10^5$) was obtained by using a small amount of PPL catalyst (0.1 or 0.25 weight % for TMC) at 100 °C [171].

Cyclic dimers of carbonate were polymerized by lipase CA to produce polycarbonates with high molecular weight, which were also obtained by lipase-catalyzed polycondensation of 1,4-butanediol or 1,6-hexanediol with diphenyl carbonate under the dilute conditions [172]. Another cyclic carbonate dimer, 6,14-dimethyl-1,3,9,11-tetraoxa-6,14-diaza-cyclohexadecane-2,10-dione, was also subjected to lipase CA-catalyzed ROP to produce the polycarbonate with molecular weight up to 1.2×10^4 [173]. Lipase CA-catalyzed ROP of a 26-membered macrocyclic carbonate, cyclobis(decamethylene carbonate), was reported [174]. Unlike in the case of lipase-catalyzed ROP of lactones with different ring sizes, the reactivity of cyclobis(decamethylene carbonate) was much lower than that of six-membered cyclic carbonate.

An enantiomerically pure seven-membered cyclic carbonate having a ketal group, which was derived from L-tartaric acid, was polymerized by lipase catalyst. Lipase CA showed efficient catalytic activity. Deprotection of the ketal group resulted in a hydroxyl group in the polycarbonate chain [175]. ROP of cyclic carbonate oligomers and their ring-opening copolymerization with lactones were catalyzed by lipase CA, producing corresponding polycarbonates [176]. Ring-opening copolymerization of a substituted TMC with 1,4-dioxan-2-one or with TMC by lipase catalyst was also reported to give a poly(carbonate-*co*-ester) or a substituted poly(carbonate) [177, 178]. A degradable carbonate copolymer with micelle forma-

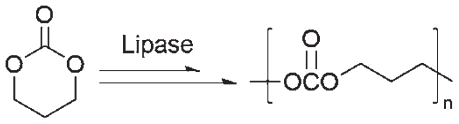

Fig. 6.13 Lipase-catalyzed ring-opening polymerization of trimethylene carbonate

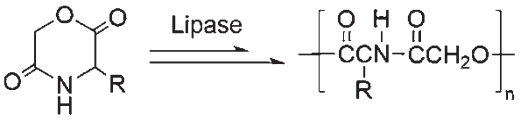

Fig. 6.14 Enzymatic synthesis of poly(ester-*alt*-amide) from six-membered cyclic depsipeptide

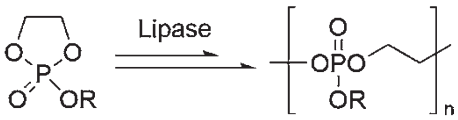

Fig. 6.15 Enzymatic ring-opening polymerization of five-membered cyclic phosphate to polyphosphate

tion ability for pH-dependent controlled drug release was synthesized via a chemoenzymatic route [179]. Poly(PEG-*co*-cyclic acetal) (PECA), an α,ω-glycol synthesized chemically, was used as initiator for lipase-catalyzed ROP of TMC to give a ABA-type block copolymer.

Poly(ester-*alt*-amide) was prepared by the lipase-catalyzed ROP of a six- membered cyclic depsipeptide, 3*(S)*-isopropylmorpholine-2,5-dione (IPMD), in bulk (Fig. 6.14). Lipase PS and PPL showed high catalytic activity to produce the polymer with molecular weight up to 3×10^4, but lipase CA was almost inactive for ROP of IPMD. PPL catalyzed ring-opening copolymerization of IPMD with D,L- lactide to produce a copolymer with molecular weight of 1×10^4 [180–182]. A monomer of MD derivative, 6-*(S)*-methylmorpholin-2,5-dione, was also polymerized by lipase catalyst [183]. ROP of a large cyclic ester-urethane oligomer was achieved by lipase CA to yield a poly(ester-urethane) with molecular weight up to 1×10^5 [44].

A five-membered cyclic phosphate was subjected to lipase-catalyzed ROP, yielding polyphosphate (Fig. 6.15) [184, 185]. A polymer with thioester linkage was synthesized by lipase CA-catalyzed ring-opening addition-condensation polymerization between ε-CL and a mercaptoalkanoic acid under vacuum produced poly(ε-CL) containing thioester groups in the backbone [186, 187]. An 18-membered monomer of 1,6-hexanedithiol and sebacate was prepared and polymerized via ROP catalyzed by lipase CA in bulk in the presence of molecular sieves to give poly(thioester) with molecular weight of 1.2×10^5 [188]. This cyclic monomer was enzymatically copolymerized with the thioester monomer, giving rise to poly(thioester-*co*-ester).

6.9 Conclusion

In this chapter, enzymatic ROP of cyclic esters to polyesters is mainly reviewed. Enzymes are natural renewable and environmentally benign catalysts. In this regard, the enzymatic polymerization is a valuable and promising method for conducting "green polymer chemistry." Additionally, ROP of cyclic esters can afford

biodegradable polymers which are alternative to conventional plastics for solution of their global environmental issues. Aliphatic biodegradable polyesters are also obtained by chemical catalyst; however, enzyme catalysis provides metal-free these materials under milder conditions. Additionally, lipase catalysis specifically produces functional polymers by enantio-, regio-, and chemoselective polymerizations. Such green nature in enzymatic ROP is significant in the viewpoints of clean reaction processes without or with small by-products to afford biodegradable plastics under mild conditions, some of which can hardly be obtained by conventional chemical catalysts.

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Chapter 7
Synthesis of Polyesters III: Acyltransferase as Catalyst

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Abstract The natural polyester polyhydroxyalkanoate (PHA) is synthesized as an energy storage via thioester exchange reaction in microbial cells. The thermal and mechanical properties of PHA can be varied by modifying the monomeric composition, molecular weight, and chemical modification. To date, many efforts have been made to understand the polymerization mechanism and industrialization of PHA. PHA synthase (Acyltransferase; EC2.3) is the key player for making stereochemically regulated polyesters. PHA synthase should be one of the most important targets for the synthetic biology of PHA. In 2017, a major breakthrough occurred in the PHA research field, whereby the tertiary structures of two PHA synthases from the class I enzyme have been solved. Based on the crystal structures of the PHA synthases, the detailed reaction mechanism of PHA synthase is discussed in this chapter. Common and unique structural elements are extracted through structure-function relationships between both enzymes. Additionally, function-based studies of PHA synthases are introduced as another milestone. The discovery of a lactate-polymerizing enzyme (LPE) evolved from a PHA synthase is a typical case. The effectiveness of the evolutionary engineering of PHA synthases is demonstrated through case studies including the creation of new polyesters as well as tailor-made PHA production.

Keywords Beneficial mutation · Domain structure · Evolutionary engineering · Lactate-polymerizing enzyme · Open-closed form · Polyhydroxyalkanoate · Polyhydroxyalkanoate synthase · Polymerization mechanism · Substrate specificity · Tertiary structure

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7.1 Preface

Recently, bio-based materials have been synthesized through biorefinery technologies from naturally occurring polysaccharides or plant oils. Polyhydroxyalkanoates (PHAs) are bacterial storage polyesters that can be used as bio-based and biodegradable plastics and have attracted considerable research interests as an alternative to petroleum-derived plastics. Biodegradable and/or compostable plastic has been in increasing demand, particularly in the USA and Europe. In fact, KANEKA Company in Japan built a pilot-scale plant for PHA (Biodegradable Polymer PHBH™) production in 2011. A representative polymer of PHA is a homopolymer of optically active (*R*)-3-hydroxybutyrate, poly(3-hydroxybutyrate) [P(3HB)], which was discovered by Dr. Maurice Lemoigne from the Institute of Pasteur, France, in the 1920s [1]. P(3HB) can be extracted from fermented bacterial cells, and the resulting product resembles some commodity plastics such as petroleum-derived polypropylene. Over 160 different monomeric constituents have been identified so far in the PHA family, giving rise to polymers with diverse properties [2, 3].

Generally, many biopolymers with ordered monomer sequences are synthesized via template-dependent polymerization. For instance, proteins are representative sequence-ordered polymers that are synthesized by incorporating amino acid monomers into assigned positions using mRNA as a template. On the other hand, PHAs are polymerized synthesized via polymerization in a non-template-dependent manner by catalysis of PHA synthase (acyltransferase), a key enzyme relevant for polymer synthesis [4]. In the process of PHA synthesis, the transfer of an acetyl group from one molecule to another is a fundamental biochemical process. PHA synthase belongs to the acetyltransferase superfamily, which catalyzes the transfer of the acetyl group from acetyl coenzyme A (as the "donor") to a counterpart (as the "acceptor"). From the perspective of structural biology, the acetyltransferase superfamily is a case study on how a common acetyltransferase domain evolved to serve a wide variety of functions. The term acyl-XYZ is a general expression of acyl groups. Here we use the concrete terms fitting to individual cases like 'acetyl'-CoA corresponding to the alkyl-chain-lengths.

In principle, monomers for PHA, 3-hydroxyalkanoic acids (3HAs), are randomly copolymerized with different monomeric constituents tightly depending on the substrate specificity of PHA synthase as well as monomer flux generated in bacterial cells. PHA synthase-catalyzed polymerization that can proceed in the water system should be the ultimate green chemistry over chemical polymerization, which frequently requires organic solvents and high-energy environments. Basically, the chemical polymerization proceeds via a release of water molecules upon ester-bond formation. Therefore, elimination of water molecules is necessary to obtain long-chain polymers. In this light, coenzyme A (CoA) of the PHA precursor is the preferable form for efficient operation of the thioester exchange reaction in a biological system to elongate the polymer chain. Thus, the polymer products tend to achieve ultrahigh molecular weights and have extremely high chirality.

In the green polymerization system, the creation of designer PHA synthases is an attractive project in order to create new polymers incorporating new monomers as

well as tailor-made biosynthesis of the PHAs with designable properties. To this end, how can we engineer the PHA synthase? Since 1999, evolutionary engineering of the enzyme has been extensively applied to the alteration of functional properties without any tertiary structure information on PHA synthase [5, 6]. For the last few decades, beneficial mutations closely related to the activity and substrate specificity of three kinds of PHA synthases have been addressed through an evolutionary engineering approach [5, 6]. This evolutionary lineage of enzymes can be considered the most important key point for accomplishing the synthetic biology of polymeric material manufacturing. It should be of great interest to achieve the incorporation of new and unnatural monomers into the polymeric backbone. The word "unnatural" here means that the constituent itself is not natural among PHA members but is a naturally occurring substance. A typical example is lactic acid (LA). Given that a lactate-polymerizing enzyme (LPE) has been artificially evolved from one of the PHA synthases, LA-based polymers could be synthesized *in vivo* by using LPE [7–9]. This major breakthrough has opened the door to expand the diversity of monomers that can be incorporated as unnatural new building blocks into the polymeric backbones [10, 11].

Great efforts have long been made to perform a mechanistic study on PHA synthase- catalyzed polymerization via solving the crystal structure. Most currently, we have made another breakthrough, namely, the successful solution of tertiary structures of two enzymes that belong to class I PHA synthase [12]. One PHA synthase is derived from *Cupriavidus necator* (formally known as *Ralstonia eutropha*) [13–15], and another one is derived from *Chromobacterium* sp. USM2 [16]. The former is the most-studied PHA synthase, and the latter is a PHA synthase with the highest enzymatic activity reported to date. Such a monumental achievement would provide the PHA research community with the structure-function relationship of natural and evolved PHA synthases. In this chapter, first, the molecular basis and mechanistic study of PHA synthase will be described based on the solved crystal structures. As a second topic, achievements in the evolutionary engineering of PHA synthases will be discussed.

7.2 Mechanistic Studies of Polymerization for PHA Synthesis Based on Biochemical Findings

In the past, various biochemical studies were performed using the most-studied class I synthase from *Cupriavidus necator* ($PhaC_{Cn}$) and class III synthase from *Chromatium vinosum* ($PhaCE_{Cv}$). Since all PHA synthases contain a PhaC box sequence ([GS]-X-C-X-[GA]-G) [17] and show a high sequence similarity to prokaryotic lipases, the catalytic mechanism of PhaC was proposed using lipase as a template [18]. Like PhaCs, lipases also belong to the superfamily of α-/β-hydrolase, which possesses a catalytic triad of Ser, Asp, and His.

Two types of catalytic mechanisms were proposed for PhaC: (i) the non-processive ping-pong model and (ii) the processive model [4]. In both proposals, the

active site in PhaC is comprised of catalytic Cys, His, and Asp, forming a catalytic triad. The conserved Cys works as a nucleophile attacking the thiol group of the thioester of substrate acyl-CoA [19–22]. The catalytic His acts as a general base catalyst to the catalytic Cys, could assist in the deprotonation of the side-chain thiol group, and accelerates the nucleophilic reaction [18, 21]. Last, the negatively charged Asp was proposed to activate the 3-hydroxyl group of the substrate acyl-CoA, enhancing the nucleophilic reaction [18, 21]. In simpler terms, Cys is the nucleophile, His is the initiator, and Asp is the elongator.

The non-processive ping-pong model requires an active site that comprises two catalytic Cys residues from two PhaC monomers to come close together for chain transfer elongation of PHA, whereas in the processive model, only a single set of active sites from a single PhaC monomer is required. Both models share the same initiation step, where the Cys is activated through deprotonation, assisted by His, allowing the activated nucleophilic Cys to attack the thioester carbon, and subsequently releases CoA from the HB-CoA substrate as a by-product. This process forms a covalent intermediate of HB-Cys. In the ping-pong model, the second substrate HB-CoA enters the active site formed by the PhaC dimer and is covalently bound to the free Cys through the same initiation reaction. When both Cys residues are occupied, the Asp might act as a general base catalyst to attack the hydroxyl group of HB-Cys, which subsequently activates the HB bound to the first Cys to attack the thioester bond of the second HB-Cys, forming a $(HB)_2$-Cys covalent intermediate. The free Cys will then attack another newly entered substrate similar to the initiation step. Again, the Asp from this active site will activate the substrate and initiate a chain transfer reaction, imitating a ping-pong game (Fig. 7.1a). In the processive model, the elongation step starts with the activation of HB-CoA by Asp, resulting in an attack on the thioester bond of HB-Cys. Then, a $(HB)_2$-CoA non-covalent intermediate is formed. The unbound Cys is once again free to attack the thioester of $(HB)_2$-CoA, forming the covalent intermediate of $(HB)_2$-Cys, and releases CoA (Fig. 7.1b).

7.3 Tertiary Structures of PHA Synthases

The molecular structure of an enzyme always relates to its functions and provides valuable insights into understanding its working mechanism. The structural information is often useful for designing beneficial mutations and modifying substrate-binding pockets, with the goal of engineering a robust high-performance enzyme for industrial applications.

PHA synthase (PhaC) is the key enzyme involved in the polymerization reaction of PHA, a family of bacterial thermoplastic polyesters with properties like commodity plastics. PHA is being developed as a bio-based and biodegradable alternative for petrochemical plastics. Lack of structural information of PHA synthases has greatly hindered the progress in understanding its catalytic mechanism, which is important for the design and synthesis of superior bioplastics comparable to

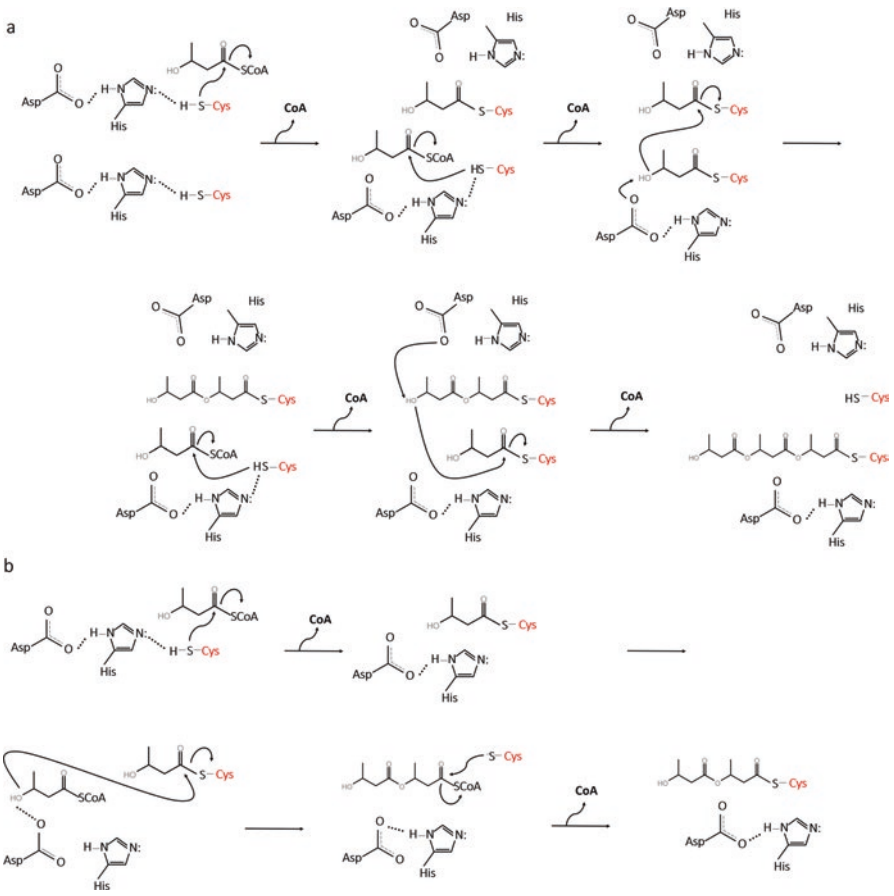

Fig. 7.1 Proposed catalytic mechanism of PHA synthase (**a**) Non-processive ping-pong model. (**b**) Processive model

synthetic plastics. The most desired PhaC crystal structure is that of *Cupriavidus necator* H16 (formerly known as *Alcaligenes eutrophus* and *Ralstonia eutropha*) which is the model bacterium used in PHA research. Many studies on PHA biosynthesis have been done using this model bacterium. The PhaC of *C. necator* ($PhaC_{Cn}$) is grouped into the class I PHA synthase. PhaCs in this group are composed of an approximately 60 kDa single subunit polypeptide that forms a homodimer and polymerizes preferentially short-chain length (SCL) PHA monomers such as 3-hydroxybutyryl-CoA. In addition to class I, there are three other classes of PhaCs [12]. The crystal structure of the catalytic domain of *C. necator* PhaC ($PhaC_{Cn}$-CAT) at a resolution of 1.80 Å was recently published by two independent groups (PDB 5T6O [13] and PDB 5HZ2 [14]). At approximately the same time, the catalytic domain structure of another interesting class I PhaC from *Chromobacterium* sp. USM2 ($PhaC_{Cs}$) was also published. This latter structure of $PhaC_{Cs}$-CAT was determined at a much higher resolution of 1.48 Å (PDB 5XAV) [16]. Interestingly,

all three crystals were grown after the partial degradation of the protein during crystallization. The full-length PhaCs could not be crystallized, probably because of the mobility of their N-terminal domain. In this chapter, we will discuss on the crystal structures of the catalytic domain of the PhaCs from *Chromobacterium* sp. USM2 and *C. necator* H16. This new structural information will then be compared with the proposed catalytic mechanisms of PHA synthases that are widely used to date [18, 21]. In addition, this chapter will also discuss the effects of mutations on the properties of PhaCs, especially with regard to the synthesis of new polyesters.

7.3.1 Crystal Structures of PhaC from Cupriavidus necator

The structures of the catalytic domain of the PhaC from *C. necator* ($PhaC_{Cn}$-CAT) were determined by two independent groups from the Massachusetts Institute of Technology (MIT) and Kyungpook National University (KNU). Both groups published almost the same crystal structures at 1.80 Å resolution [13, 14]. In the $PhaC_{Cn}$-CAT structure reported by Wittenborn et al. [13], the catalytic Cys319 had been mutated to Ala in order to improve protein stability. Their structure (PDB 5T6O) consisted of residues 201–368 and 378–589, whereas residues 369–377 were disordered (Fig. 7.2a). This disordered region was part of the protein structure that was flexible and, thus, not visible in the electron density map of the crystal. The other group was successful in determining the crystal structure of $PhaC_{Cn}$-CAT (PDB 5HZ2) using the wild-type $PhaC_{Cn}$ [14]. The KNU group reported that the full- length $PhaC_{Cn}$ protein succumbed to proteolysis, which occurred at Arg192. Therefore, by substituting the Arg192 with Ala, they claimed that the full-length $PhaC_{Cn}$ crystal was successfully obtained, though it was diffracted poorly despite multiple trials [15]. The structure of $PhaC_{Cn}$-CAT from KNU consisted of residues 202–589 and displayed a D-loop, which appeared to be disordered in the previously reported structure by the group from MIT (Fig. 7.2b). The length between the catalytic Cys in a dimer of $PhaC_{Cn}$-CAT measures approximately 33 Å, while the total length of the dimeric $PhaC_{Cn}$-CAT is approximately 99 Å (~10 nm), with both the width and height at approximately 50 Å (~5 nm) each (Fig. 7.2a, b).

Overall, both the crystal structures of $PhaC_{Cn}$-CATs show the same conformation, adopting an α-/β-hydrolase fold core subdomain (residues 214–346, 472–589) and a CAP subdomain (residues 347–471) (Fig. 7.4c). $PhaC_{Cn}$-CAT forms dimers mediated by the CAP subdomains. The residues involved in the dimerization are Asp401, Val403, Val407, Val408, and Leu412 from one protomer as well as Ile357, Val360, and Glu364 from another protomer. Mutational analyses of these residues showed a lower dimer ratio and decreased activity compared to that of the wild-type $PhaC_{Cn}$ [14]. The catalytic triad residues (Cys319, Asp480, and His508) of $PhaC_{Cn}$ were positioned close to each other and formed hydrogen bonds in the active site [13, 14]. Unfortunately, the architecture of the catalytic triad was incomplete, as the Cys319 was mutated to Ala in 5T6O and the imidazole ring was flipped from the others in 5HZ2 (Fig. 7.3c). The flipped imidazole ring is not favorable, as it will

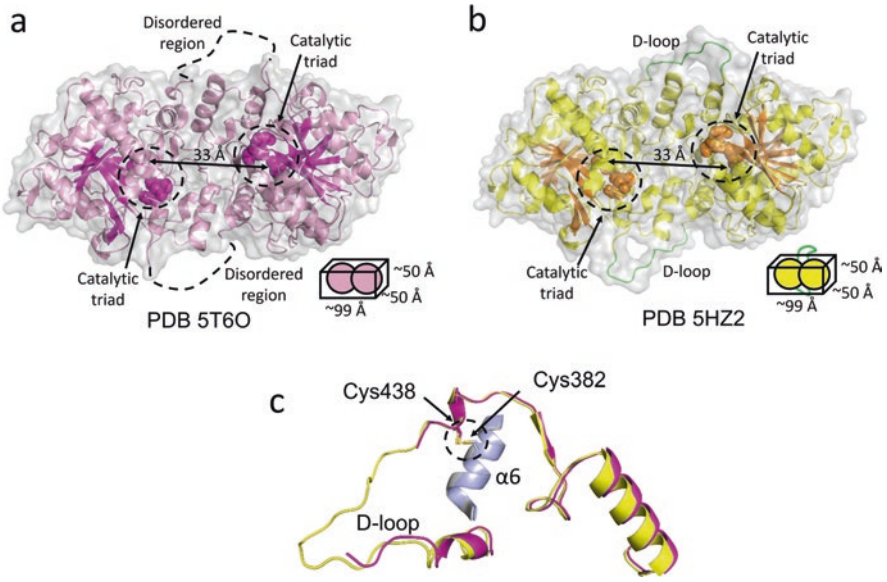

Fig. 7.2 Crystal structures of dimeric $PhaC_{Cn}$-CAT
(**a**) $PhaC_{Cn}$-CAT was deposited in PDB as 5T6O; the catalytic Cys was mutated to Ala to improve protein stability. The disordered region is shown as a dotted line. (**b**) $PhaC_{Cn}$-CAT was deposited in PDB as 5HZ2, with a D-loop (green), which was derived as the disordered region in (**a**). (**c**) A disulfide bond was observed between a pair of non-conserved Cys residues in both structures of $PhaC_{Cn}$-CAT, which might stabilize a small opening for substrate entry. The distance of the catalytic Cys319 in the dimer is approximately 33 Å. $PhaC_{Cn}$-CAT (PDB 5T6O) is color-coded in pink and magenta; $PhaC_{Cn}$-CAT (PDB 5HZ2) is color-coded in orange and yellow

affect the interaction between Asp with the Nδ atom of His [13, 16]. Additionally, another ring nitrogen atom, Nε of the His is positioned at a close distance (3.8 Å) to the Cys (S_γ) and, at the same time, bound to a water molecule (2.8 Å) in the structure of $PhaC_{Cs}$-CAT [16]. These observations would not be possible if the imidazole ring of the His was flipped around and changed the positions of the imidazole ring nitrogen atoms.

A detailed analysis of both crystal structures of $PhaC_{Cn}$-CAT revealed the presence of a non-conserved Cys-Cys disulfide bond between Cys382 and Cys438 (Fig. 7.2c) [16]. Physiologically, the occurrence of such a disulfide bond is not possible because the bacterial cytoplasm is maintained at a reduced state in the redox potential. It is highly possible that the observed disulfide bond formation in the crystal structures of $PhaC_{Cn}$-CAT is an artifact that occurred during crystallization. As it takes time for the crystals to grow, the proteins become oxidized and result in the formation of the disulfide bond. This disulfide bond is not observed in the structure of $PhaC_{Cs}$-CAT as $PhaC_{Cs}$ does not possess such Cys residues. The artificial disulfide bond probably is the key factor in stabilizing the partially open form of $PhaC_{Cn}$-CAT through the unfolding of αA and ηA helices of $PhaC_{Cs}$-CAT into the

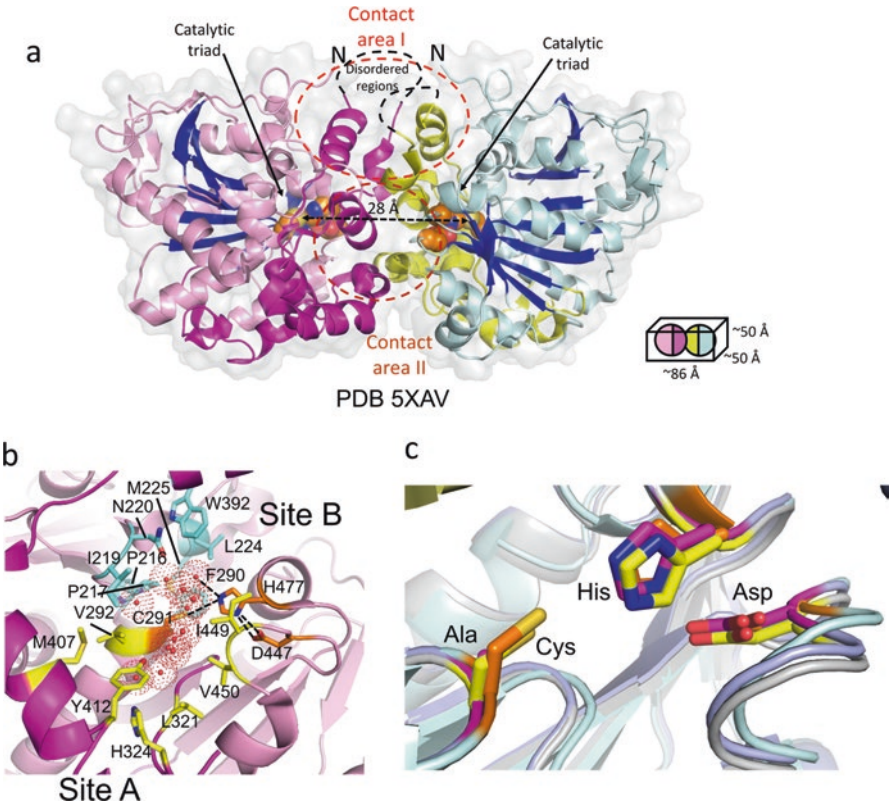

Fig. 7.3 Crystal structures of dimeric PhaC$_{Cs}$-CAT
(**a**) The dimeric structure of PhaC$_{Cs}$-CAT comprises protomer A (core, pink; CAP, magenta) and protomer B (core, cyan; CAP, yellow). The interactions of the dimer are mediated mainly by the CAP subdomain. The N-terminal ends of both protomers are located at the same direction. (**b**) Water molecules (red spheres with surface dots) are observed around the nucleophilic Cys291. Part of the CAP subdomain has been removed to show the water cavity. Water molecules are divided into Site A (yellow residues) and Site B (cyan residues) by Cys291. Hydrophobic amino acids are mostly found in the cavity, except for His324, Tyr412 (Site A), and Asn220 (Site B). Hydrogen bonds involving catalytic triad residues are shown as dotted lines. (**c**) The overlay of the catalytic triad of all three PhaC-CATs. The Cys319 was mutated to Ala in PDB 5T6O (magenta sticks); the imidazole ring was flipped from others in PDB 5HZ2 (yellow sticks); the catalytic triad in PDB 5XAV shows a more accurate and complete architecture (orange sticks)

D-loop of the PhaC$_{Cn}$-CAT. Through the opening, a possible substrate access channel filled with water was deduced from the structure [13]. Additionally, a putative product egress route was proposed; although the amino acid residues in the proposed route are conserved, a certain extent of conformational changes is required for the polymerization reaction to occur [13]. Another important feature of PhaC, its substrate specificity, was also explained using the crystal structure. A possible acyl moiety-binding pocket composed of mainly hydrophobic residues, i.e., Pro245,

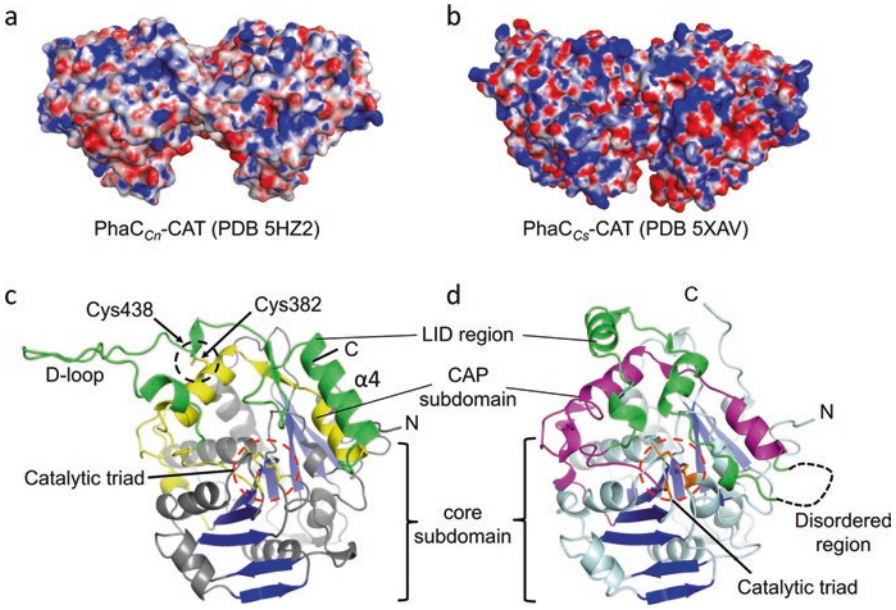

Fig. 7.4 Comparison of PhaC$_{Cn}$-CAT and PhaC$_{Cs}$-CAT
(**a**) The electrostatic surface potential representation of PhaC$_{Cn}$-CAT (PDB 5HZ2) and (**b**) PhaC$_{Cs}$-CAT (PDB 5XAV). Blue represents a basic/positively charged surface; red represents an acidic/negatively charged surface; white represents a neutral/hydrophobic surface. (**c**) The monomer structures of PhaC$_{Cn}$-CAT, the core subdomain (gray and blue), the CAP subdomain (yellow), and the LID region (green) are shown. (**d**) The monomer structures of PhaC$_{Cs}$-CAT, the core subdomain (cyan and blue), the CAP subdomain (magenta), and the LID region (green) are shown. A major difference is observed at the LID region (both green) and the non-conserved disulfide bond in (**c**)

Ile252, Leu253, Phe318, Thr393, and Trp425, was proposed to stabilize the 3HB moiety of the substrate [14].

7.3.2 Crystal Structure of PhaC from Chromobacterium sp. USM2

In addition to the structure of PhaC$_{Cn}$-CAT from the most-studied class I PhaC$_{Cn}$, the structure of the most active PhaC from *Chromobacterium* sp. USM2 (PhaC$_{Cs}$-CAT) was determined, by a structural group from Nara Institute of Science and Technology (NAIST), at a resolution of 1.48 Å [16]. The catalytic domain of the PhaC$_{Cs}$-CAT is characterized by an amino acid sequence that belongs to the superfamily of α-/β-hydrolase. This domain exists as a globular structure. Structural analysis revealed that the PhaC$_{Cs}$-CAT (residues 175–567) is folded into α-/β-core subdomain (residues 186–318 and 439–562) and CAP subdomain (residues 319–438) (Fig. 7.3a, 7.4b). The α-/β-core subdomain adopts the regular α-/β-hydrolase fold with the

central β-sheet displaying a left-handed superhelical twist and the first β-strand crossing the eighth β-strand (β9) at ~90° and the final strand (β11) at ~180°. The CAP subdomain projects topologically from the α-/β-core subdomain and completely covers the active site. As expected, the active site consists of the catalytic triad residues (Cys291, His477, and Asp447), which have been confirmed before by primary sequence analysis of many PHA synthases and mutation studies [23, 24].

A common characteristic of the members of α-/β-hydrolase superfamily is the possession of the important catalytic triad residues, which have been identified as Cys291, His477, and Asp447 in $PhaC_{Cs}$. Even though these residues are not close to each other in the primary amino acid sequence, they are brought closer together to form the catalytic pocket through hydrogen bonds (Fig. 7.3b, c). In the observed structure of $PhaC_{Cs}$-CAT, the catalytic pocket was completely covered by the CAP subdomain, which essentially blocks the substrates from entering. Therefore, this structure was called a closed conformation, unlike the partially open conformation that was observed for the *C. necator* $PhaC_{Cn}$-CAT. The 1.48 Å high-resolution structure of $PhaC_{Cs}$-CAT also allowed the visualization of water molecules both inside and outside of the catalytic pocket. The presence of the trapped free water molecules in the catalytic pocket is an indication that the LID region (residues 327–386), which is part of the CAP subdomain (residues 319–438), is a dynamic and flexible structure allowing water to get inside the catalytic pocket (Fig. 7.3b, 7.4d). Water molecules may play an important role in the polymerization process at the active site [25]. Water molecules may also play a role in the stabilization of the growing PHA chains [26], and the presence of water in PHA granules is well documented [27]. The water molecules were observed at both sides of the catalytic Cys and divided into two groups accordingly, namely, Site A and Site B. These sites are two possible cavities for the allocation of the acyl moiety of (*R*)-hydroxyalkanoyl-CoA substrate during the nucleophilic reaction. For instance, the acyl moiety, such as the 3HB moiety of the 3HB-CoA substrate, fits into these sites, and the thioester bond of *R*-hydroxyalkanoyl-CoA can be positioned close enough to the catalytic Cys, allowing the nucleophilic reaction to occur. This positioning of the acyl moiety also allows the formation of an acyl-S-intermediate and subsequent CoA release from the active site at the end of the nucleophilic reaction. Site A is formed by polar Tyr412, His324, and other hydrophobic residues, i.e., Val292, Leu321, Met407, and Ile449. On the other hand, Site B comprises nonpolar residues, Pro216, Pro217, Ile219, Leu224, and Met225, Phe292, Trp392, and only one polar residue, Asn220 [16]. The water molecules can also be used as a clue to search for the possible entry pathway for the water-soluble (*R*)-hydroxyalkanoyl-CoA substrates; however, this is not the case for $PhaC_{Cs}$-CAT, as the catalytic site is completely covered.

In general, PHA synthases are believed to be more active in the dimeric form and exist in a dynamic equilibrium between monomer and dimer in solution [28–31]. $PhaC_{Cs}$-CAT forms a face-to-face dimer with a pseudo-dyad axis with the N-terminal end of both protomers facing each other, suggesting that the N-terminal domain might enhance the oligomerization of PhaC through direct contact [16]. However, the exact function of the N-terminal domain remains unknown as it is absent in the current structure. The dimeric conformation of $PhaC_{Cs}$-CAT is most likely mediated

by the CAP subdomain through intermolecular interactions such as hydrophobic, salt bridging, and aromatic stacking interactions. Two intimate contact areas can be identified from the structure, i.e., contact areas I and II. Contact area I forms a hydrophobic cluster with nonpolar residues, Leu369, Trp371, Pro386, Phe387, and Leu390, in both molecules A and B. Contact area II forms salt bridges (Arg365 and Glu329) inside the interface composed of Phe332, Phe333, His448, and Leu451. Notably, there is no connection between the two catalytic Cys in the current dimer structure because they are separated by quite a distance (the S_γ atom of Cys291 residues are at a distance of 28.1 Å) for the PHA chain transfer reaction to occur [16].

7.3.3 *Structural Differences Between PhaC$_{Cn}$-CAT and PhaC$_{Cs}$-CAT*

Since both PhaC$_{Cs}$ and PhaC$_{Cn}$ share an amino acid sequence identity of 46%, it would be interesting to compare the structure of PhaC$_{Cs}$-CAT with PhaC$_{Cn}$-CAT. In the structural comparison, the catalytic triad and α-/β-core subdomains of all three structures showed good alignment with each other [16]. However, some differences were noted. The first major difference is in the conformational changes of part of the CAP subdomain, known as the LID region (residues Pro327–Pro386 in PhaC$_{Cs}$-CAT; residues Thr335–Pro419 in PhaC$_{Cn}$-CAT) (Fig. 7.4c, d). The conformational changes are marked by two events, i.e., the unfolding of αA and ηA helices in PhaC$_{Cs}$-CAT into a flexible D-loop in PhaC$_{Cn}$-CAT and the folding of αB' and ηB' in PhaC$_{Cs}$-CAT into a long α4 helix in PhaC$_{Cn}$-CAT (Fig. 7.4c). These conformational changes result in a small opening for the substrate entry into the catalytic pocket, making PhaC$_{Cn}$-CAT a partially open structure [16].

The second difference between the crystal structures of PhaC$_{Cs}$-CAT and PhaC$_{Cn}$-CAT is their dimeric arrangement. In the structural comparison, by aligning one protomer (molecule A from both PhaC-CATs) from each dimer, another protomer (molecule B) of PhaC$_{Cn}$-CAT was swung ~40 Å and rotated ~120 Å from PhaC$_{Cs}$-CAT [16]. Additionally, the N-terminal ends of both protomers in PhaC$_{Cs}$-CAT are located at the same direction with a distance of 19.2 Å, whereas the N-termini of PhaC$_{Cn}$-CAT are distanced further at 55.1 Å, although still facing the same direction [16]. This phenomenon suggests that the N-terminal domain is involved in enhancing the dimerization of PhaCs through direct interaction, which was supported by the size exclusion analysis of full-length PhaC$_{Cs}$ and PhaC$_{Cs}$-CAT [16]. In contrast, the small angle X-ray scattering data from full-length PhaC$_{Cn}$ suggests that the N-terminal domains of PhaC$_{Cn}$ (PhaC$_{Cn}$-ND) are localized at opposite sites from each other without direct contact [15].

Since the PHA granule is hydrophobic, it might be useful to identify the hydrophobic patches on the surface of PhaC-CATs to understand the localization of PhaCs on PHA granules. However, both PhaC-CATs showed evenly distributed charged surfaces, and no obvious hydrophobic patches were observed (Fig. 7.4a, b). The only difference between the two is that PhaC$_{Cn}$-CAT is more hydrophobic com-

pared to that of $PhaC_{Cs}$-CAT, and this might be the reason for the better protein solubility and expression of $PhaC_{Cs}$. It is also worth mentioning that this information is based solely on the structure's lack of an N-terminal domain. To obtain the full picture, the unique structures of the N-terminal domain of PhaCs are essential to fill the knowledge gaps.

7.3.4 Proposed Catalytic Mechanisms Based on Crystal Structures

The previously proposed non-processive ping-pong mechanism requires that the catalytic triad residues from both protomers in a dimeric PhaC come close to the interface for substrate transfer reactions (Fig. 7.1a). However, both the current crystal structures of $PhaC_{Cn}$ and $PhaC_{Cs}$ turn out to be different for such a reaction to occur. Therefore, the currently available structures of the catalytic domain of the 2 PhaCs favors the processive catalytic mechanism (Fig. 7.1b). However, if it is shown in the future that the full-length PhaC adopts a completely different conformation due to its flexible N-terminal domain, the reaction mechanism needs to be revisited.

All three crystal structures, however, do provide an accurate picture of the active site with the three conserved amino acid residues forming the catalytic triad (Cys-His-Asp). As expected, these three amino acids interact with each other through hydrogen bonds in the published crystal structures (PDB 5T6O, 5HZ2 & 5XAV) [13, 14, 16]. Among these three structures, 5XAV shows the most accurate picture of the catalytic triad because, in one structure (5T6O), the important catalytic Cys has been mutated to Ala to stabilize the protein. In the other structure (5HZ2), the imidazole ring of His is represented in a flipped form, which deviates from that shown in the other two structures (Fig. 7.3c). In the catalytic pocket, the position of the side-chain imidazole ring of the catalytic His is close enough to extract a hydrogen/proton from the side-chain thiol group of the catalytic center, Cys. In other words, the deprotonation of Cys allows nucleophilic attacks on the thioester carbon of the acyl-CoA substrate, which results in the formation of an acyl-Cys intermediate. The catalytic Asp was proposed to act as a general base, which is responsible for accelerating the deprotonation of the 3-hydroxyl group of the incoming acyl-CoA substrates in the PHA elongation step [18, 24].

The processive model involving a single set of catalytic triads. (**a**) In this model, the main difference is the role of His in the elongation, which was previously proposed to be the role of Asp. (**b**) In this model, the hydroxyl group of the subsequent 3HB-CoA substrate is involved in the elongation. (**c**) In this alternative model, the hydroxyl group of a second 3HB-CoA attacks the thioester bond of 3HB-Cys to produce $(3HB)_2$-CoA. Then, this non-covalent intermediate leaves the active site

The new crystal structures from $PhaC_{Cn}$ and $PhaC_{Cs}$ have led to the proposition of three modified catalytic mechanisms [13, 14, 16]. While the initiation step involv-

ing acylation of Cys through His in the catalytic triad remains largely the same, the proposed elongation steps are different (Fig. 7.5). In the model based on the structure of the partially open form $PhaC_{Cn}$-CAT (PDB 5T6O), the major difference is the involvement of His in the activation of the hydroxyl group of incoming 3HB-CoA for product elongation (Fig. 5a) [13]. In the model based on the closed form of the $PhaC_{Cs}$-CAT structure (PDB 5XAV), the hydroxyl group from the second 3HB-CoA attacks the 3HB-Cys covalent intermediate forming the non-covalent intermediate $(3HB)_2$-CoA. Then, the free thiol Cys attacks the thioester carbon of $(3HB)_2$-CoA and forms a covalent intermediate of $(3HB)_2$-Cys (Fig. 7.5b) [16]. The role of Asp in the activation of the incoming substrate is still elusive as the exact geometry of the Asp-substrate interaction is not revealed in the free-form structure. These two proposed mechanisms agree with previous reports of a covalent intermediate observed using saturated trimer CoA (sT-CoA), a substrate analogue of $(3HB)_3$-CoA, in which the terminal hydroxyl group has been replaced by a hydrogen, making it a non-hydrolyzable analogue [19, 20, 32]. In the model based on the other structure of $PhaC_{Cn}$-CAT (PDB 5HZ2), the hydroxyl group of the subsequent substrates enters and attacks the 3HB-Cys covalent intermediate and forms a non- covalent intermediate $(3HB)_2$-CoA, which then leaves the catalytic pocket. The cycle repeats with a new incoming 3HB-CoA, which forms a 3HB-Cys covalent intermediate in the catalytic pocket, followed by the reentry of previously released $(3HB)_2$-

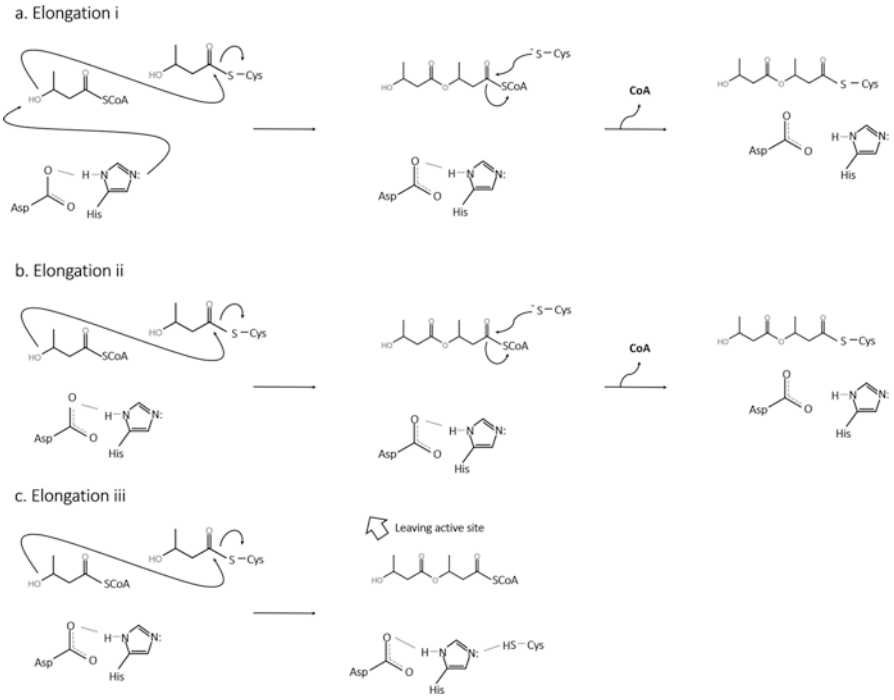

Fig. 7.5 Proposed catalytic mechanism of PHA synthase

CoA into the active site to produce $(3HB)_3$-CoA and so on (Fig. 7.5c) [14]. However, this proposal contradicts the presence of sT-CoA, which has been shown to be stably bound to the catalytic Cys. In this case, the elongation process will stop once $(3HB)_3$-CoA (like the non-hydrolyzable sT-CoA) interacts with the catalytic Cys; thus, the elongation process would require another active site through an intersubunit reaction. Although several modified catalytic mechanisms have been proposed based on the newly published structures of PhaCs, the validity of the proposals is still debatable. There are still many questions that remain unanswered, for instance, the exact role of Asp in the catalytic triad and the effect of the highly variable N-terminal domain, which is absent in all the structures.

It took almost three decades since the cloning of the first PhaC gene by three independent groups in the late 1980s until the successful crystallization of partial PhaC by another three independent groups. This achievement marks a significant milestone in the research and development of PHA even though the crystal structures of the two PhaCs are incomplete and different. The crystal structures of the catalytic domain of the class I PhaC from *Chromobacterium* sp. USM2 (PDB 5XAV) and *C. necator* (PDB 5T6O; PDB 5HZ2) share a high similarity in the α-/β-core subdomain, which contains the conserved catalytic triad (Cys-His-Asp). However, a big difference was observed in the CAP subdomain structures of the two PhaCs; in $PhaC_{Cs}$-CAT, the CAP subdomain was in a closed form, while in $PhaC_{Cn}$-CAT, it was in a partially open form, probably because of the artificial disulfide bridge in the latter. However, the open and closed forms do provide some insight into the possible conformational changes that may occur during the PhaC-substrate interactions. The crystal structure of a complete PhaC is much needed to get a better understanding of the polymerization mechanism and the subsequent PHA granule biogenesis.

7.4 Functional Alteration of PHA Synthase for Tailor-Made Polyester Synthesis

PHA synthase is a key enzyme for the polyester synthesis in terms of quality and quantity. So far, many researchers have focused on properties of the enzyme, such as activity and substrate specificity, to regulate the synthesized PHA characters (monomer composition and molecular weight) as well as its productivity (content in bacterial cells) (Fig. 7.6). In this chapter, the history of functional alteration of PHA synthases is summarized.

Polymer content depends on the enzyme activity. Monomer composition is governed by the enzyme substrate specificity. Molecular weight is affected by the protein expression.

As shown in Table 7.1, PHA synthases can be classified into three groups based on substrate specificity. PHA synthase derived from *Ralstonia eutropha* (= *Cupriavidus necator*) can polymerize short-chain-length-3-hydroxyacyl-coenzyme As (SCL-3HA-CoAs) such as 3-hydroxybutyryl-coenzyme A (3HB-CoA) and

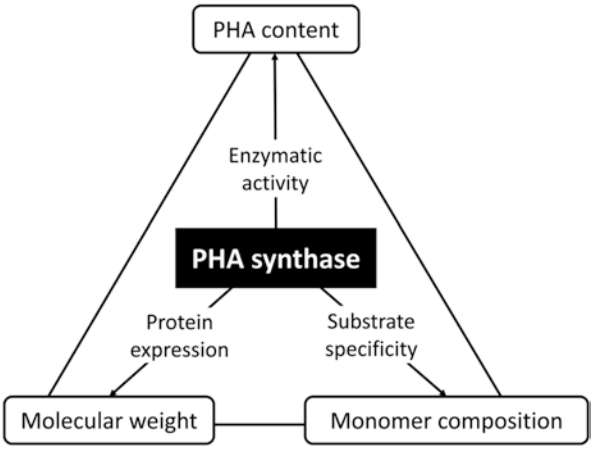

Fig. 7.6 Properties of PHA related to PHA synthase

Table 7.1 Classes of PHA synthases and varieties of PHAs

Substrate specificity[a]	Class[b]	Subunit(s)[c]	Microorganism[d]	Polymers produced[e]
SCL-3HA-CoA (C3-C5)	I	PhaC	*Ralstonia eutropha*	P(3HB), P(3HB-*co*-3 HV)
			Chromobacterium sp. USM2	
	III	PhaC, PhaE	*Allochromatium vinosum*	
	IV	PhaC, PhaR	*Bacillus megaterium*	
			Bacillus cereus YB-4	
MCL-3HA-CoA (C6-C14)	II	PhaC	*Pseudomonas oleovorans*	P(3HA)
			Pseudomonas putida	
SCL-MCL-3HA-CoA (C3-C14)	I	PhaC	*Aeromonas caviae*	P(3HB-*co*-3HA)
	II	PhaC	*Pseudomonas* sp. 61–3	

[a]Substrate preferred by the PHA synthase. *SCL-3HA-CoA (C3-C5)* short-chain-length-3-hydroxyacyl-coenzyme A (3–5 carbons in length), *MCL-3HA-CoA (C6-C14)* medium-chain-length-3-hydroxyacyl-coenzyme A (6–14 carbons in length), *SCL-MCL-3HA-CoA (C3-C14)* short-medium-chain-length-3-hydroxyacyl-coenzyme A (3–14 carbons in length)
[b]Class of PHA synthase
[c]Name of the PHA synthase subunit
[d]Native microorganisms where the PHA synthases and polymers were found
[e]Polymers produced. *P(3HB)* poly(3-hydroxybutyrate), *P(3HB-co-3HV)* poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), *P(3HA)* poly(3-hydroxyalkanoate), *P(3HB-co-3HA)* poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoate)

3-hydroxyvaleryl-coenzyme A (3HV-CoA). On the other hand, PHA synthases derived from most *Pseudomonads* polymerize medium-chain-length-3-hydroxyacyl-coenzyme As (MCL-3HA-CoAs) with relatively long side chains. PHA synthases of *Aeromonas caviae* and *Pseudomonas* sp. 61–3 have wide substrate specificities and can polymerize both SCL- and MCL-3HA-CoAs [12].

Evolutionary engineering is a powerful method to change the properties of PHA synthase without any information on tertiary structure. So far, beneficial mutants of PHA synthases were gained by the combination of error-prone PCR-based mutagenesis and screening methods based on the Nile red stain [33]. At the initial stage starting from 1999, beneficial sites and amino acids related to the enzymatic performances (activity, substrate specificity, thermostability, and so on) were explored at the point mutation level by error-prone PCR-based mutagenesis. In the next stage, site-specific saturation mutagenesis at the hot positions was conducted in a parallel way to optimize the improvement of enzymatic performances.

This function-based enzyme engineering strategy was extensively applied to class I and class II PHA synthases. Table 7.2 and Fig. 7.7 show the summaries of beneficial mutations that provide the target enzymes with better functional performances. As stated in the following section, many beneficial mutations causing the improved enzyme performances were gained for the individual PHA synthases through the evolutional program. In Sections 7.3.1 and 7.3.2, typical functional alterations of PHA synthases are introduced.

7.4.1 Improvement in Activity, Thermostability, and Expression

The first trial of *in vitro* evolution was conducted for the class I PHA synthase derived from *R. eutropha* ($PhaC_{Re}$ which is the same as $PhaC_{Cn}$ described in 7.1–7.2) by error-prone PCR-based mutagenesis. $PhaC_{Re}$ is the most widely studied PHA synthase and was selected as the first target for the model study. *Escherichia coli*-carrying $phaC_{Re}$, $phaA_{Re}$, and $phaB_{Re}$ genes (*phaA* and *phaB* are monomer-supplying enzyme genes) can produce P(3-hydroxybutyrate) [P(3HB)], which is the most typical PHA (Fig. 7.9, Pathway I). Mutants of $PhaC_{Re}$s generated by error-prone PCR were expressed in *E. coli* cells together with $PhaA_{Re}$ and $PhaB_{Re}$, and then the recombinant strains produced P(3HB)s at various accumulation levels. There was a good correlation between P(3HB) accumulation level and $PhaC_{Re}$ activity for the selected seven mutants. This means that high-throughput screening (*in vivo*) is possible for exploring the beneficial mutants without a time-consuming enzymatic assay (*in vitro*). However, it was too difficult to gain the positive mutant enzyme starting from the highly active wild-type PHA synthase. Notably, the suppression mutagenesis approach was effective to obtain beneficial mutations causing enhanced activity of $PhaC_{Re}$. In fact, two positive mutants were obtained by using one mutant harboring the Ser80Pro (S80P) mutation as a starter for the second mutation. The first mutant, Phe420Ser (F420S), exhibited a 2.4-fold increase in specific activity toward 3HB-CoA compared to the wild type [35]. This activity improvement contributed to the increased polymer productivity *in vivo*. This positive mutation presumably occurred due to the productive dimerization appropriate for enzyme activation of $PhaC_{Re}$, considering the previous report on the mutation related to the

Table 7.2 Studies on engineered PHA synthases and polyesters production by using them

Year	Enzyme source[a]	Method[b]	Mutation	Polyester[c]	Changed enzyme property	References
2001	*Ralstonia eutropha*	Random mutagenesis	–	P(3HB)	Activity	[34]
2002	*Ralstonia eutropha*	Intragenic suppression mutagenesis	F420S, S80P	P(3HB)	Activity, thermostability	[35]
	Aeromonas caviae	Random mutagenesis	N149S, D171G	P(3HB)	Activity, substrate specificity	[36]
	Ralstonia eutropha and *Pseudomonas aeruginosa*	Gene shuffling	–	P(3HB) P(3HB-*co*-3HA)	Activity, substrate specificity	[37]
	Aeromonas caviae	Random mutagenesis (*in vivo*)	V214G, F518I,	P(3HB-*co*-3HHx)	Activity, substrate specificity	[23]
			F362I + F518I			
			D459V + A513C			
2003	*Pseudomonas* sp. 61-3	Random mutagenesis, site-specific saturation mutagenesis, recombination	S325C/T Q481M/K/R S325C/T + Q481M/K/R	P(3HB)	Activity, substrate specificity	[38]
	Pseudomonas resinovorans	Site-specific chimeragenesis	–	P(3HA)	Substrate specificity	[39]
2004	*Pseudomonas* sp. 61-3	Random mutagenesis, site-specific saturation mutagenesis, recombination	S325C/T Q481M/K/R S325C/T + Q481M/K/R	P(3HB-*co*-3HA)	Activity, substrate specificity	[40]
	Pseudomonas putida GPo1	Localized semi-random mutagenesis	–	P(3HA)	Activity, substrate specificity	[41]
	Pseudomonas oleovorans	PCR-mediated random chimeragenesis	–	P(3HA)	Activity, substrate specificity	[42]
	Ralstonia eutropha	Site-specific saturation mutagenesis	A510X	P(3HB) P(3HB-*co*-3HA)	Activity, substrate specificity	[43]

(continued)

Table 7.2 (continued)

Year	Enzyme source[a]	Method[b]	Mutation	Polyester[c]	Changed enzyme property	References
2005	*Ralstonia eutropha*	Intragenic suppression mutagenesis, site-specific saturation mutagenesis	G4X	P(3HB)	Protein expression	[44]
	Ralstonia eutropha	Recombination	G4D + F420S	P(3HB)	Activity	[45]
	Pseudomonas sp. 61-3	Site-specific saturation mutagenesis, recombination	E130X	P(3HB) P(3HB-*co*-3HHx)	Activity	[46]
2006	*Pseudomonas* sp. 61-3	Site-specific saturation mutagenesis	S477X	P(3HB) P(3HB-*co*-3HA)	Activity, substrate specificity	[47]
2007	*Aeromonas caviae*	Site-specific saturation mutagenesis	A505X	P(3HB) P(3HB-*co*-3HHx)	Activity, substrate specificity	[48]
	Aeromonas caviae	Site-specific saturation mutagenesis	N149S, D171G N149S + D171G	P(3HB-*co*-3HHx)	Activity, substrate specificity	[49]
2008	*Pseudomonas* sp. 61-3	–	S325 T + Q481K	P(3HB-*co*-LA)	Activity, substrate specificity	[7]
2009	*Pseudomonas* sp. 61-3	Site-specific saturation mutagenesis	S477X + Q481X	P(3HB-*co*-3HA)	Activity, substrate specificity	[50]
	Ralstonia eutropha and *Aeromonas caviae*	Chimeragenesis	–	P(3HB-*co*-3HHx)	Activity, substrate specificity	[51]
	Aeromonas caviae	–	N149S + D171G	P(3HB-*co*-3H4MV)	Activity, substrate specificity	[52]
2010	*Pseudomonas* sp. 61-3	Site-specific saturation mutagenesis	F392X	P(3HB-*co*-LA)	Activity, substrate specificity	[53]
	Pseudomonas sp. 61-3	Site-specific saturation mutagenesis	Q508X	P(3HB-*co*-LA)	Activity, substrate specificity	[54]
	Ralstonia eutropha and *Pseudomonas putida*	Chimeragenesis	–	P(3HB-*co*-3HHx-*co*-3HO)	Activity, substrate specificity	[55]

2011	*Pseudomonas stutzeri* 1317 (PhaC2)	Site-specific mutagenesis	S326 T + Q482K	P(3HB-*co*-3HA)	Activity, substrate specificity	[56]
	Pseudomonas sp. 61-3	–	S325 T + Q481K	P(GL-*co*-3HA)	Activity, substrate specificity	[57]
	Ralstonia eutropha	–	Wild type	P(2HB-*co*-3HB)	–	[58]
2012	*Ralstonia eutropha*	Chimeragenesis	–	P(3HB)	Activity, thermostability	[59]
	Aeromonas caviae	Random mutagenesis, site-specific saturation mutagenesis	D171X	P(3HB-*co*-3HHx)	Activity, substrate specificity	[60]
	Pseudomonas sp. SG4502	–	S324 T + Q480K	P(3HB-*co*-LA)	Activity, thermostability	[61]
	Pseudomonas sp. 61-3	–	S325 T + Q481K	P(2HB) P(2HB-*co*-LA)	Activity, substrate specificity	[62]
2013	*Ralstonia eutropha*	Site-specific saturation mutagenesis	A510X	P(3HB-*co*-LA)	Activity, substrate specificity	[63]
	Chromobacterium sp. USM2	Site-specific saturation mutagenesis	A479X	P(3HB-*co*-3HHx)	Activity, substrate specificity	[64]
	Ralstonia eutropha	–	S80P + F420S	P(3HB-*co*-3HP-*co*-5 HV)	Activity, substrate specificity	[65]
2014	*Pseudomonas putida* GPo1	Random mutagenesis, site-specific saturation mutagenesis	Q481M, S482G, L484 V, A547V	P(3HB-*co*-3HA)	Activity, substrate specificity	[66]
2015	*Aeromonas caviae*	–	N149S + D171G	P(3HB-*co*-3H2MB)	Activity, substrate specificity	[67]

(continued)

Table 7.2 (continued)

Year	Enzyme source[a]	Method[b]	Mutation	Polyester[c]	Changed enzyme property	References
2017	*Pseudomonas* sp. 61-3		S325 T + Q481K	P(3HB-*co*-3H3PhP), P(3HB-*co*-3H4PhB)	Activity, substrate specificity	[68]
	Pseudomonas sp. 61-3	–	S325 T + Q481K	P(3HB-*co*-2HP-*co*-2H3MB-*co*-2H3MV-*co*-2H4MV-*co*-2H3PhP)	Activity, substrate specificity	[69]
	Pseudomonas sp. 61-3	–	S325 T + S477X + Q481X	P(2HB)	Activity	[70]
	Aeromonas caviae + *Ralstonia eutropha*	Chimeragenesis	–	P(2HB-*b*-3HB)	Activity, substrate specificity	[71]

[a]Bacterial strain from which the PHA synthase was derived

[b]Random mutagenesis: the exploration of beneficial positions and amino acid substitutions by means of error-prone PCR
Site-specific saturation mutagenesis: the construction of a set of all amino acid substituted variants occurring at beneficial positions
Localized mutagenesis: creation of mutations at localized regions related to meaningful properties
Chimeragenesis: construction of chimeric combinations between heterogeneous enzymes at the interpositions that share common structural contexts such as secondary structures
Gene shuffling: efficient generation of superior mutants by recombining beneficial mutations

[c]3HHx, 3-hydroxyhexianoate; *LA (2HP)* lactate (2-hydroxypropionate), *3H4MV* 3-hydroxy-4-methylvalerate, *3HO* 3-hydroxyoctanoate, *3HP* 3-hydroxypropionate, *5HV* 5-hydroxyvalerate, *3H2MB* 3-hydroxy-2-methylbutyrate, *3H3PhP* 3-hydroxy-3-phenylpropionate, *3H4PhP* 3-hydroxy-4-phenylpropionate, *2H3MB* 2-hydroxy-3-methylbutyrate, *2H3MV* 2-hydroxy-3-methylvalerate, *2H4MV* 2-hydroxy-4-methylvalerate, and *2H3PhP* 2-hydroxy-3-phenylpropionate

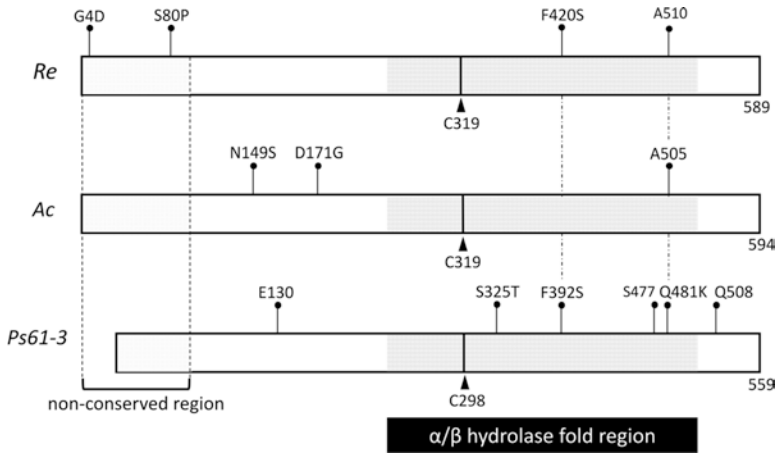

Fig. 7.7 Beneficial mutations of PHA synthases
PHA synthase consisted of α-/β-hydrolase fold region which plays a crucial catalytic role for enzyme activity exhibition. The N-terminal region is assumed to coordinate enzyme catalysis and substrate recognition in the polymerization [12]. C319 and C296 function as catalytic center sites of PHA synthases. Beneficial mutations related to activity, expression, thermostability, and substrate specificity are mapped on the whole regions of individual PHA synthases. A compatibility in activity improvement between F420S and F392S was confirmed at the corresponding positions (vertically dotted line). In a similar way, substitutions of amino acids at the Q481, A510, and A505 positions affect the enzyme substrate specificity

lag-phase reduction in the $PhaC_{Re}$ reaction [29, 45]. To verify the adaptive possibility of a beneficial mutation across classes of PHA synthase, F420S of class I $PhaC_{Re}$ was applied to the class II PHA synthase of *Pseudomonas* sp. 61–3 ($PhaC_{Ps}$). Phe392Ser (F392S) of $PhaC_{Ps}$, which corresponds to F420S of $PhaC_{Re}$ (Fig. 7.7), resulted in an increased polymer content and an increased co-monomer fraction compared to wild-type $PhaC_{Ps}$ [53], indicating that a beneficial mutation can work properly in different classes of PHA synthase. Another positive mutant, $PhaC_{Re}$(G4D), was obtained with an increased expression level [44]. This improved expression was presumed to contribute to the increased polymer production. In addition, the molecular weight of P(3HB) polymerized by $PhaC_{Re}$(G4D) tended to be higher, suggesting that G4D enhanced the polymer chain elongation. The higher expression of $PhaC_{Re}$(G4D) was observed for not only various *E. coli* strains (JM109, DH5, and HB101) but also gram-positive *Corynebacterium glutamicum*, indicating that G4D is a host-independent-type mutation. Further substitution of the G4 residue with other amino acids by saturation mutagenesis yielded various other G4X mutants, which exhibited a higher P(3HB) content and higher molecular weights [44].

Thermostability of PHA synthase should be an important factor in order to realize sustainable production of the target polyesters during the cultivation. The variant of $PhaC_{Re}$ carrying the S80P mutation used for suppression mutagenesis exhibited higher thermostability, although the reason is unclear [35]. In the other study on the

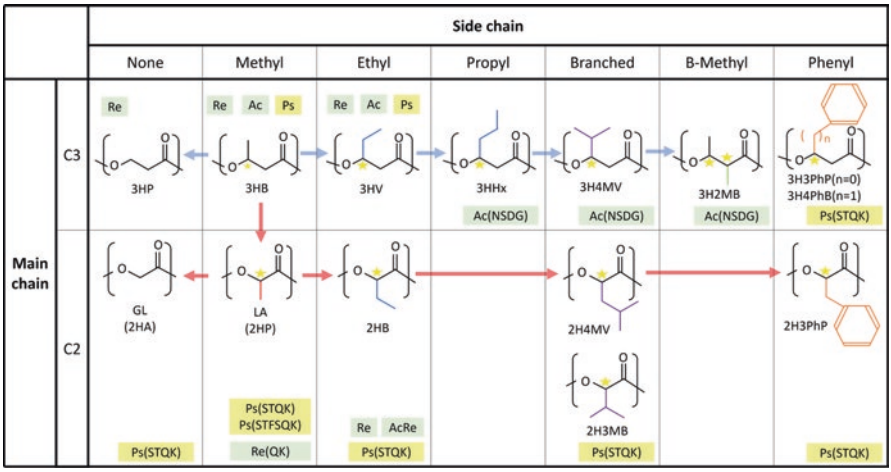

Fig. 7.8 Expansion of monomers by using native and engineered PHA synthases
Chemical structures of monomers are lined up based on the main chain and side chain. 3HB is the most common monomer in microbial polyesters (PHAs). Monomer structures are diversified from 3HB, as shown in the blue line. Transition from 3HB to LA could be achieved by using the engineered PHA synthase, $PhaC_{Ps}$(STQK)/lactate-polymerizing enzyme (LPE). Red arrows indicate the expansion of recognizable monomers by PHA synthases starting from LA

improvement of thermostability, Shue et al. attempted to carry out a chimeragenesis approach [59]. Thermophilic PhaC from *Cupriavidus* sp. stain S-6 ($PhaC_{Csp}$) and mesophilic $PhaC_{Re}$ was combined, and one chimeric enzyme $PhaC_{Re\beta}$, which was $PhaC_{Re}$ bearing 30 mutations derived from $PhaC_{Csp}$, showed P(3HB) accumulation at 45 °C with high productivity. $PhaC_{Re\beta}$ exhibited increased thermostability, 127-fold compared to that of the wild type, indicating that the thermostability of $PhaC_{Csp}$ was successfully inherited by $PhaC_{Re}$. In addition, Tajima et al. identified a natural thermostable PHA synthase from the thermotolerant bacterium *Pseudomonas* sp. SG4502 [61]. These studies indicate that improved thermostability can be achieved in both engineered and native PHA synthases.

7.4.2 Substrate Specificity Alteration (at the Side Chain)

Substrate specificity alteration was successfully achieved mainly for two PHA synthases of *A. caviae*, class I $PhaC_{Ac}$ and class II $PhaC_{Ps}$. These PHA synthases serve as key catalysts for synthesizing 3HB-based copolyesters with MCL-3HA monomers. By applying an *in vitro* evolutionary technique to a limited region of the $phaC_{Ac}$ gene, two beneficial mutations, N149S and D171G, were isolated [36]. These mutations exhibited an increase in the enzymatic activity toward 3HB-CoA compared to the wild-type enzyme *in vitro* assay and enhanced the accumulation of P(3HB-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] (6.5-fold and threefold)

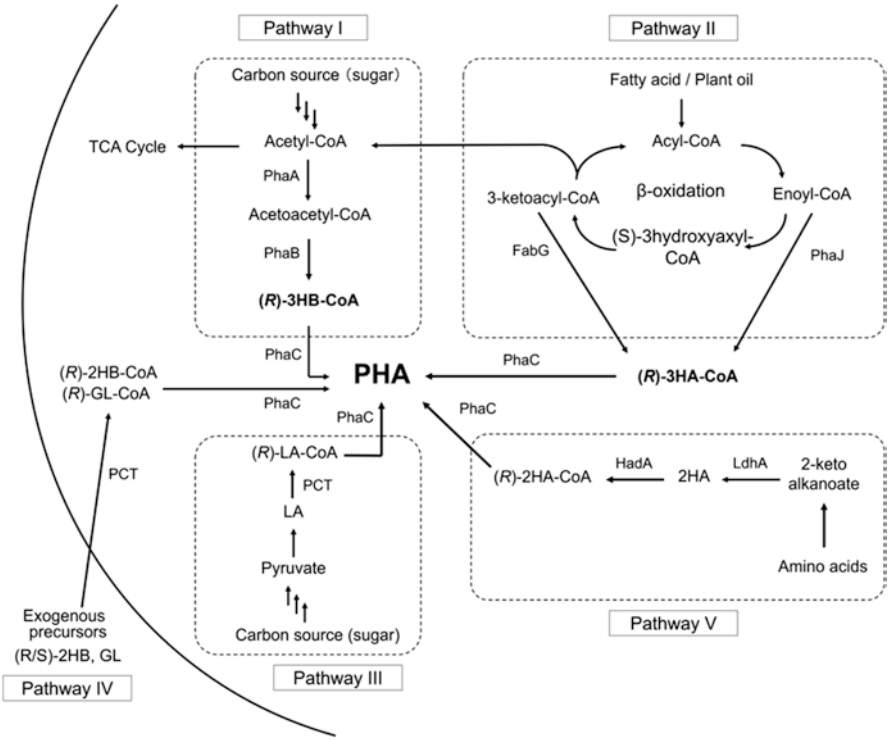

Fig. 7.9 PHA biosynthesis pathways
The target PHAs are synthesized by the five metabolic pathways illustrated. Pathway I is an acetyl-CoA condensation pathway to generate (*R*)-3HB-CoA. Pathway II is composed of the β-oxidation pathway to produce (*R*)-3HA-CoAs. Pathway III is the new pathway for the synthesis of (*R*)-LA-CoA. Pathway IV supplies (*R*)-2HB-CoA and (*R*)-GL-CoA by the addition of the corresponding precursors. Pathway V is an amino acid derivative pathway to generate (*R*)-2HA-CoAs including an aromatic group

in vivo (Fig. 7.9, Pathways I and II). At the same time, the change in substrate specificity was demonstrated by data showing that the fraction of 3HHx (MCL-3HA monomer) was increased up to 18 mol% (N149S) and 16 mol% (D171G) from 10 mol% (wild type). Basically, class I $PhaC_{Ac}$ preferentially polymerizes SCL- 3HA monomers over MCL-3HA monomers, indicating that the two mutants obtained a higher substrate specificity toward the MCL-3HA unit. The combination of Asn149Ser (N149S) and Asp171Gly (D171G) further increased the 3HHx fraction, probably due to the synergetic effect [50]. The $PhaC_{Ac}$(NSDG) mutant has been used as an industrial enzyme for P(3HB-*co*-3HHx) production at KANEKA Company. $PhaC_{Ac}$(NSDG) contributed the increase in the comonomer fraction of 3-hydroxy-4-methylvalerate harboring a branched side chain [53] and 3-hydroxy-2-methylbutyrate (3H2MB) harboring a beta-methylated side chain [68], indicating that the NSDG mutant has reactivity toward MCL-3HA monomers with various side-chain structures.

On the other hand, class II $PhaC_{Ps}$ has a contrasted nature to $PhaC_{Ac}$, which means that $PhaC_{Ps}$ preferentially polymerizes MCL-3HA monomers over SCL-3HA monomers. To reinforce the reactivity toward the 3HB monomer, *in vitro* evolutionary engineering based on error-prone PCR was employed. As a result, four beneficial mutations at position Glu130 (E130), Ser325 (S325), Ser477 (S477), and Gln481 (Q481), which are presumably located close to the active center Cys296, were identified [38, 40, 46, 47]. An *in vitro* activity assay revealed that E130 and S325 contribute to the enzyme activity, and the remaining Q477 and Q481 contribute to the substrate specificity. Via site-specific saturated mutagenesis and a combination of superior mutations, the activity toward 3HB-CoA was increased up to 720-fold. The 3HB fraction in P(3HA-*co*-3HB) was increased from 14 mol% (wild-type enzyme) to a maximum of 70 mol% (engineered enzyme) (Fig. 7.9, Pathways I and II), indicating that the molecular evolution of PHA synthase ($PhaC_{Ps}$) changed the enzyme substrate specificity.

7.5 Creation of New Polyesters by Engineered PHA Synthases

7.5.1 Alteration of Main-Chain Substrate Specificity (2-OH, LA Incorporation)

As described before, the substrate specificity of PHA synthase is closely related to the monomeric composition of the copolymers. In fact, many achievements were obtained for PHA synthases by enzyme evolutionary engineering (Table 7.1). Accordingly, tailor-made synthesis of copolymers with desired properties can be achieved by using engineered PHA synthases. These beneficial mutants mainly exhibit the alteration of side-chain-based substrate specificity represented by 3-hydroxyacyl (3-OH) monomer substrates. In contrast, the alteration of main-chain substrate specificity from 3-OH to 2-OH was one of the big challenges in the research field of PHA to expand biopolymer diversity. Lactate (LA) was selected as a first target of the 2-OH unit, and two pioneering works found a trace activity toward lactyl-CoA (LA-CoA) in PHA synthase from *Allochromatium vinosum* (class III) [72, 32]. However, at that time, unfortunately, the polymer-containing LA unit was not generated.

In 2008, the discovery of LPE paved the way for synthesis of the LA-based polymer [7]. First, Taguchi et al. attempted to explore natural and/or engineered PHA synthase(s) with a lactate-polymerizing activity based on the *in vitro* polymerization system using chemically synthesized LA-CoA as a monomer substrate. In the water-organic solvent two-phase *in vitro* system, LA-polymerizing activity was assayed based on the generation of a polymer-like precipitant. Among PHA synthases, only one engineered PHA synthase derived from *Pseudomonas* sp. 61–3, $PhaC_{Ps}$(STQK), termed LPE (lactate-polymerizing enzyme), clearly exhibited poly-

mer- like precipitation (wild-type $PhaC_{Ps}$ had a trace level) under the condition of the coexistence of LA-CoA and 3HB-CoA. Analysis of the precipitate revealed that LA was incorporated into the polymer chain together with the 3HB unit. The proof of concept for establishing that LPE was also functional in living cells was carried out. To transfer the LA-based polymer synthesis system into bacterial cells, an artificial metabolic pathway was constructed (Fig. 7.9). The key point was the utilization of propionyl-CoA transferase (PCT) for supplying the LA-CoA monomer, and PCT was expressed in *E. coli* together with LPE, $PhaA_{Re}$ and $PhaB_{Re}$. As a result, incorporation of the LA unit into the polymer chain was first found in *E. coli*. However, its molar fraction was too low, namely, 6 mol%. To enhance the LA fraction, a second-generation LPE was generated by adding the beneficial mutation (F392S) found in $PhaC_{Re}$ in the prototype LPE. This evolved LPE(STFSQK) exhibited further enhancements in the LA fraction in the copolymers as well as polymer production [53]. This LA enhancement was synergistically reinforced by combination of other strategies such as anaerobic cultivation for LA increase and metabolic pathway redirection (*ΔpflAΔdld*) [73]. These approaches allowed us to tune the LA fraction in the range of 0–99 mol%. Interestingly, an extremely high LA fraction (99.3 mol%) was observed in *Corynebacterium glutamicum* without 3HB-CoA-supplying enzyme genes, suggesting that the different monomer fluxes depended on the host cell [74]. These achievements imply the establishment of a microbial platform for the one-step production of polylactide (PLA) and its copolyesters by using LPE and evolved LPE.

It is of interest to promote the evolution of $PhaC_{Re}$ to LPE by introducing the mutation(s) that converted $PhaC_{Ps}$ into LPE. Amino acid substitution at position Ala510 (A510) of $PhaC_{Re}$, which corresponds to Gln481 (Q481) of LPE [$PhaC_{Ps}$(STQK)], was carried out (Fig. 7.7). Among 19 $PhaC_{Re}$(A510X) mutants, 15 mutants synthesized P(LA-*co*-3HB), indicating that the 510 residue plays a critical role in LA polymerization. In addition, it was revealed that the LA-polymerizing ability could be transferred between different classes (from class II to class I) [63].

7.5.2 Diversification of Monomers for Creating New Polyesters

2HB and GL After a major breakthrough in the enzyme reactivity, namely, the transition from 3-OH to 2-OH in the main chain of monomers, it is of interest to address the range of 2-OH monomers other than LA accessible by LPEs (STQK and STQKFS). First, 2-hydroxybutyrate (2HB) and glycolate (GL) should be targeted, as shown in Fig. 7.8. Homopolyesters and copolyesters containing 2HB and GL are bio-based polyesters like PLA. In particular, GL-based polyesters are often applied for biomedical materials. By blending the chiral homopolymers of the L-form and D-form, homopolymers of 2HB can form a stereocomplex in homogeneous combination and heterostereocomplex in heterogeneous combination with PLA.

2HB-based polymers were successfully synthesized by $PhaC_{Re}$ (wild type), LPE(STQK), LPE(STSRQK), and the chimeric enzyme of $PhaC_{Ac}$ and $PhaC_{Re}$, termed AcRe12 [58, 62, 70, 71] (Fig. 7.8). In 2011, it was confirmed that $PhaC_{Re}$ polymerized P(2HB-*co*-3HB) *in vitro* from a mixture of (*R*)-2HB-CoA and (*R*)-3HB-CoA. However, at this time, the 2HB incorporation was very weak, and the polymerizing reaction was inhibited by the presence of (*S*)-2HB-CoA [58]. To explore a more efficient enzyme for the 2HB-based polymer production, the utilization of LPE was attempted. LPEs exhibit superior reactivity toward 2HB, and the P(2HB) homopolymer could be synthesized *in vivo* from endogenous racemic 2HB by expressing PCT and LPEs in *E. coli* [62, 70] (Fig. 7.9, Pathway IV). Recently, it was reported that chimeric enzyme AcRe12 can polymerize the block copolymer P(2HB-*b*-3HB) *in vivo*. This was the first report that chimeric PHA synthase possesses significant activity toward 2-OH monomers [71].

GL-based polymers were also successfully synthesized by LPE [57]. In addition to the 2HB-based polymer, the accumulation of P(GL-*co*-3HAs) with 17 mol% of GL fraction was confirmed *in vivo* using recombinant *E. coli* from exogenous glycolate and dodecanoate (Fig. 7.9, Pathway IV). Thus, LPE can recognize 2-OH monomers with no side chain and an ethyl side chain as well as methyl side chain (LA, 2-hydroxypropionate) (Fig. 7.8).

2H4MV, 2H3PhP, and 2H3MB A side-chain variety of 2-OH monomers was further explored. Amino acid derivatives, which have a 2HA backbone with various side-chain structures corresponding with each amino acid, were the next candidates. Mizuno et al. first investigated the LPE acceptance of the 2-hydroxy-4-metylvalerate (2H4MV) unit derived from leucine supplied via 2H4MV dehydrogenase (LdhA) and 2H4MV-CoA transferase (HadA) reactions (Fig. 7.9, Pathway V). Further investigation determined that not only the 2H4MV unit but also 2-hydroxy-3-phenylpropionate (2H3PhP), 2-hydroxy-3-metylvalerate (2H3MB), and 2-hydroxy-3-metylvalerate (2H3MV) derived from phenylalanine, valine, and isoleucine were incorporated into the polymer as P(3HB-*co*-2HAs), suggesting that LPE(STQK) can recognize 2HA with bulky side chains [69].

7.6 Properties of Polyesters Produced by Native and Engineered PHA Synthases

The final goal of PHA research work is commercialization of the polymeric materials. The typical example is PHBH, P(3HB-*co*-3HHx), manufactured by KANEKA Company in Japan. In the fermentation process for production of the PHBH, NSDG mutant of $PhaC_{Ac}$ has been actually utilized in the microbial platform. Additionally, PLA and its copolyesters can be biosynthesized by means of LPEs evolved from PHA synthases. In such a sense, engineering of the PHA synthase provides us the effective achievements related to the application of PHAs. Table 7.3 summarizes the up version and properties of PHA-related polyesters. Expansion of the variety of monomeric constituents has been extensively updated for creating new polyesters based on PHA synthase alteration.

Table 7.3 Properties of conventional PHAs, new type of PHAs, and chemosynthesized polymers

	T_m [a]	T_g [b]	Young's modulus	Tensile strength	Elongation to break	References
	[°C]	[°C]	[MPa]	[MPa]	[%]	
P(3HB)	177	4	3500	43	5	[75]
Ultrahigh-molecular-weight PHB	185	4	–	400	35	[75]
P(3HB-*co*-20 mol% 3HV)	145	-1	800	20	50	[75]
P(3HB-*co*-10 mol% 3HHx)	127	-1	–	21	400	[75]
P(3HB-*co*-6 mol% 3HA)[c]	133	-8	200	17	680	[75]
P(3HB-*co*-15 mol% 3H4MV)	150	3	–	20	250	[53]
P(3HB-*co*-9 mol% 3H3PhP)	135	15	–	–	–	[76]
P(3HB-*co*-23 mol% 3H2MB)	140	-1	–	–	–	[67]
P(3HB-*co*-29 mol% LA)	158	25	154	7	156	[53]
P(2HB)	99	30	75	9	170	[62]
P(86 mol% 2HB-*co*-LA)	99	31	466	19	115	[62]
P(3HB-*co*-22 mol% 2HA)[d]	154	7	–	–	–	[69]
P(3HD)	70	−46	118	8	226	[77]
PLA (D-form)	153	60	1020	52	2	[53]
Polypropylene	176	−10	1700	38	400	[75]
Low-density polyethylene (LDPE)	130	−30	200	10	620	[75]
Poly(ε-caprolactone)	58 to 65	−65 to −60	210 to 440	21 to 42	300 to 1000	[77]

[a]Melting temperature, [b]glass transition temperature, [c]3HA units (3-hydroxyoctanoate (< 1 mol%), 3-hydroxydecanoate (3 mol%), 3-hydroxydodecanoate (3 mol%), 3-hydroxy-*cis*-5-dodecanoate (< 1 mol%)), [d]2HA units (2HP (LA) (1 mol%), 2H3MB (1 mol%), 2H4MV (14 mol%), 2H3PhP (6 mol%))

7.7 Future Perspectives

The structure-function relation study of PHA synthase is crucial to better understand the reaction mechanism of polymerization for PHA synthesis and to improve PHA from the viewpoints of academic and industrial progress. In this sense, solution of the tertiary structures of two class I PHA synthases provides us with various

insights into the aforementioned subject. In both crystal structures, one can find the common structural properties in the catalytic domain such as the conserved catalytic triad (Cys-His-Asp) in the α-/β-core subdomain. On the other hand, a big difference is observed in the CAP subdomain structures of the two PHA synthases. In particular, the presence of the open-closed mode found in $PhaC_{Cs}$ is characteristic in creating the dynamic conformational change upon the $PhaC_{Cs}$-substrate interaction. Such structure-based interpretation is available for discussing the various enzyme functions. With the available structural information of PhaCs, another catalytic mechanism was proposed based on a modelled structure of class I PhaC from *Aquitalea* sp. USM4 [81]. Crystal structures of enzyme-substrate complexes together with the complete whole structures of PHA synthases should be required for further understanding the polymerization mechanism of PHA synthase and rational enzyme engineering.

Prior to the presentation of crystal structures of PHA synthases, evolutionary engineering has been applied to three kinds of PHA synthase focusing on the activity improvement, substrate specificity alteration, thermostability improvement, and expression improvement. Even with no information on enzyme tertiary structure, these achievements strongly evidence that evolutionary engineering is a powerful toolbox for improving the enzymatic properties related to PHA biosynthesis. For more than 20 years, a large library of class II-categorized PHA synthase mutants that result in alterations of monomeric composition, sequence regulation such as the development of a block copolymer [71] and the use of various molecular weights, and enhanced polymer production has been increasing. A typical case is the discovery of LPE, which can synthesize LA-based polymers. This case may be fortunate and now could be the time to take a great leap forward, as commented in the article [78]. Most recently, mechanistic model on polymerization of LA-based polymer has been demonstrated based on biochemical and kinetic studies [79]. The pioneering study has led to further expansion of the range of structural diversity of PHA members other than LA-based polymers. Along this line, one can expect to synthesize chiral copolymers with various monomer compositions, owing to the extremely high enantioselectivity and broad substrate specificity of the class II PHA synthase. In this chapter, it is a good starting point to have a discussion on the relationship between 3D-structure and mutation effects based on both milestones. For example, several key amino acid residues closely related to the enzymatic performances can be commonly addressed from four representative class I and class II PHA synthases [80].

For what is PHA synthase studied? The answer is simple. Needless to say, PHA synthase should be an essential machinery for PHA biosynthesis in practical applications. It seems to be clear that the evolutionary engineering of “biocatalysts” will be a key approach, analogous to the fact that the field of chemical polymeric materials has moved forward via the development of advanced “chemical catalysts” like Ziegler-Natta catalysts. With the current PHA synthase structure now unveiled, we are moving closer to tailor-made PHA to meet various needs.

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Chapter 8
Synthesis of Polypeptides

Kousuke Tsuchiya, Yu Miyagi, Takaaki Miyamoto, Prashant G. Gudeangadi, and Keiji Numata

Abstract Proteases (EC 3.4), enzymes originally used to cleave the amide bonds of proteins by hydrolysis, have been utilized for the enzymatic synthesis of peptide compounds. This enzymatic synthesis of polypeptides is a biomass-based, environmentally benign, atom-economical, and stereo-/regioselective method that can replace petroleum-derived chemical polypeptide syntheses. Enzymatic polymerization of amino acid derivatives using proteases proceeds via the reverse reaction of hydrolysis, which is aminolysis, in an equilibrium. Thermodynamic and kinetic controls in the aminolysis reaction rationally optimize enzymatic polymerization efficiency. Polymerization is regulated by the substrate specificity of proteases, namely, a combination of amino acid monomers and proteases. A great number of polypeptides, including homopolymers, random/block copolymers, and specific polymer architectures, such as star-shaped polymers, are synthesized by a protease-catalyzed polymerization technique. In this chapter, versatile designs and syntheses of polypeptide materials using various types of proteases are entirely reviewed in detail.

Keywords Polypeptide · Protease · Amino acid · Aminolysis · Protein

8.1 Introduction

Polypeptides are physiologically essential components as the main proteins in living systems. Proteins are synthesized via the translation of mRNA sequential information by ribosomes in the central dogma. The diversity of their physiological functions and properties is associated with their primary, secondary, and higher-order structures. In addition to proteins possessing well-defined amino acid

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sequences, artificial polypeptide materials, including homopolymers, random or block copolymers, and special polymeric architectures, such as star-shaped polymers, have attracted intensive attention as bio-based polymeric materials. The material properties of these polypeptides can be tuned by amino acid monomer units and their sequences, promising useful applications in various fields.

There are three approaches to synthesizing artificial polypeptides: chemical, biological, and biochemical synthetic methods. The chemical method includes solid phase peptide synthesis (SPPS), which was developed by Merrifield,[1] and ring opening polymerization of amino acid *N*-carboxyanhydrides (NCA).[2] In the SPPS method, polypeptides are synthesized on insoluble solid supports to propagate the peptide chain in a stepwise manner, enabling precise control of the amino acid sequence of polypeptides. On the other hand, the polymerization of amino acid NCA derivatives can provide polypeptides of higher molecular weight; however, this method is only available for the synthesis of homopolymers and random/block copolymers. These chemical synthetic methods employ the polymerization of amino acid derivatives, which are chemically synthesized in advance with organic solvents. In spite of their beneficial advantages, a recent trend to avoid petroleum-derived chemicals for a future sustainable society pushes us to pursue an environmentally benign alternative method of polypeptide synthesis.

The biological method includes the biosynthesis of proteins in vivo, which is generally processed by combination of biomacromolecules, such as enzymes. A key enzyme involved in biosynthesis is ligase. Free amino acids are activated by aminoacyl-tRNA synthetases to generate amino-acid-ligated tRNAs, and then, they iteratively react with the C-terminal of a propagating peptide according to the order that the mRNA regulates by its codon sequence. Since gene engineering technology was established, many natural and engineered proteins have been biosynthesized in living cells, particularly in genetically modified microbes. Biosynthesis using a sophisticated protein expression system in nature is a powerful way to strictly control amino acid sequences based on the codon sequences preserved in mRNA. Desired polypeptide sequences can be obtained by designing proper DNA constructs to transform host genes. Not only natural proteins but also artificial polypeptides can be designed for biosynthesis. Tirrell et al. pioneered the biosynthesis of desired polypeptides using living organisms and demonstrated that artificial polypeptides were successfully designed and expressed by in vivo biosynthesis using microbes transformed with plasmids encoding target sequences. The synthetic polypeptides consisting of periodic sequences, such as $GluAsp(Glu_{17}Asp)_4GluGlu$ or $[(AlaGly)_3ProGluGly]_4$, were designed and encoded in plasmid DNA to transform *Escherichia coli* [3, 4]. This biosynthetic technique enables the precise, sophisticated design of functional polypeptides; however, practical use of this biosynthesis system suffers from some disadvantages. Expression of the target proteins is occasionally suppressed to a low level by various factors, mainly because of the cytotoxicity of the expressed proteins. A tedious purification process to isolate target polypeptides from cell lysates also hampers cost-effective production.

The biochemical method uses enzymes as catalysts in vitro to synthesize peptides by enzyme-mediated polymerizations. Enzymatic polymerization is another promising candidate for an alternative green synthetic method for polypeptide synthesis. In contrast to biosynthesis with ligase, the enzymes used for the enzymatic polymerization of amino acid monomers are proteases, which originally cleave the peptide bonds in polypeptides. This protease-catalyzed hydrolysis of polypeptides is a reversible reaction; hence, the reverse reaction can propagate peptide chains under optimized reaction conditions as described in the next section. Protease-catalyzed polymerization has several advantages over conventional chemical synthetic methods. Amino acid monomers, generally in a form of ester, are polymerized in the presence of a protease in an aqueous buffer solution, resulting in the formation of polypeptides with only small alcoholic by-products. Therefore, this enzymatic polymerization is considered as an eco-friendly synthetic method with high atom economy compared to conventional condensation techniques. In addition, the substrate specificity of proteases rationally recognizes an appropriate chiral isomer (generally the L-isomer) and reacting groups attached to an α-carbon. This feature allows enzymatic polymerization to proceed in a stereo- and regioselective manner. Some amino acids, therefore, can be polymerized without protection of the reactive side groups. In addition, the enzymatic reaction is readily scalable because the synthetic protocol simply mixes amino acid monomers and proteases in moderate conditions.

Enzymatic amide formation has been continually developed to synthesize small peptide compounds since the 1950s [5]. To date, many comprehensive studies on enzymatic peptide synthesis have not only elucidated the mechanisms of the involved reactions but also synthesized novel functional polypeptide materials. Because protease-catalyzed aminolysis generates amide bonds, an enzymatic polymerization technique using proteases can be applied to not only polypeptides but also other synthetic polyamides. Indeed, some reports have employed unnatural amino acids for enzymatic synthesis in copolymerization with natural amino acids as described below (Sect. 8.5.6 *Unnatural Amino Acids*). In this chapter, the target building blocks are mainly focused on polypeptides [6–8], and not only fundamental aspects, including polymerization mechanisms and characteristics of proteases, but also structural variations of polypeptides synthesized by enzymatic polymerization are thoroughly reviewed.

8.2 Mechanisms of Enzymatic Reactions

Proteases catalyze not only the hydrolysis of peptide bonds but also their aminolysis, which results in the formation of a peptide bond between two amino acids. There are two strategies to synthesize peptides by proteases [9–16], that is, thermodynamically and kinetically controlled syntheses.

Fig. 8.1 Equilibrium in thermodynamically controlled peptide synthesis

8.2.1 Thermodynamic Control

Thermodynamically controlled synthesis of peptides can be applied to all proteases. This synthetic method focuses on an equilibrium between the hydrolysis and aminolysis reactions. As shown in Fig. 8.1, protease-catalyzed peptide bond formation can be divided into two equilibrium reactions. One reaction is the equilibrium between the ionization and deionization of carboxylic acid/amine, and the other is the equilibrium between hydrolysis and aminolysis. Thus, these two steps must be considered to obtain peptides from amino acids with free carboxylic acid and amine groups. In the first step, non-charged substrates must be formed by the deionization of carboxylic acid and amine groups. Subsequently, the aminolysis reaction occurs via the formation of an acyl intermediate within proteases. Therefore, proteases are involved in the second step to accelerate the hydrolysis/aminolysis reaction. When neutral amino acids are used as the starting substrates, the first equilibrium tends to drive to the left side. This effect can be explained by the equilibrium constant and the ΔG of the reaction [17]. In cases using two unprotected amino acids, the equilibrium constant of the first step is $10^{-7.5}$, and the ΔG is approximately 10 kcal·mol^{-1}, which means that the equilibrium favors not the nonionic but rather the ionic reactants. The equilibrium constant of the second step is $10^{3.7}$, and its ΔG is about -5 kcal mol^{-1}. Therefore, the equilibrium constant of the whole reaction is $10^{-3.8}$, and its ΔG is approximately 5 kcal·mol^{-1}. This value indicates that the equilibrium reaction proceeds toward an endothermic reverse reaction. Great disincentives to forming peptide bonds in this step are a slow reaction speed and a low yield because of the low equilibrium constant and positive ΔG. When the aminolysis reaction in homopolymerization proceeds, the equilibrium constant of the reaction becomes lower, and the ΔG of the reaction becomes higher. Hence, it is difficult to obtain the polypeptide by iterative aminolysis reactions. Thermodynamically controlled synthesis is a method to control the thermodynamic equilibrium constants of these two elementary reactions. There are two strategies to increase the equilibrium constant of aminolysis. One strategy is the control of the first reaction, and the other is the control of the second reaction.

In the first step, deionization of an ionized substrate occurs by proton transfer from ammonium to carboxylate groups at a pH above its isoelectric point. However, amino acids exist as zwitterions at pH 5–9, a pH at which most proteases show high catalytic activity. In this pH range, the neutral form of amino acids is a minor substrate because the equilibrium constant of first step is much lower than 1. In contrast, the equilibrium constant of the reaction between N-protected and C-protected amino acids is $10^{-4.0}$. This value is still lower than 1, but it is much higher than that

of protection-free amino acids. Therefore, the combination of N- and C-protected amino acids is favorable for driving the equilibrium toward neutral forms. This method is limited to the synthesis of oligopeptides via a coupling between two reactants and is not favorable for the synthesis of polypeptides, which generally result from multistep reactions. There are also other factors that can control the first equilibrium reaction, such as pH and ionic strength. For example, the addition of water-miscible organic solvents induces a reduction in ionic strength and thereby a decrease in the frequency of the proton transfer from ammonium to carboxylate groups. By using the N- or C-protected amino acids as substituents and organic/water-miscible solution as solvents, the equilibrium constant can increase, and the equilibrium constant can move to the right side, namely, peptide synthesis. Homandberg et al. examined the equilibrium constant of the reaction between benzyloxycarbonyl-protected tryptophan (Z-Trp) and glycinamide (Gly-NH_2) in water, 60 v/v% triethylene glycol solution, or 85 v/v% 1,4-butanediol solution [18]. The equilibrium constant in 85 v/v% 1,4-butanediol solution showed the largest value. The reaction rate and yields can be controlled by the content of organic solvent and water.

Precipitation of peptide products is useful to effectively drive the second equilibrium toward the right (Fig. 8.1). When products precipitate from the solution, the concentration of products dissolved in the solution becomes lower, and the equilibrium shifts toward the right side. In this way, peptide formation proceeds by removing products from a system. In other words, by using a biphasic condition, products can be removed from water media. The reaction occurs around the surface between water and organic layers. A nonpolar organic solvent, such as ethyl acetate, chloroform, and toluene, is used as the organic layer. Cassells and Halling reported protease-catalyzed peptide synthesis in water/organic two-phase systems [19]. The reaction rate of Z-Phe-Phe-OMe synthesis with a silica-supported thermolysin in a reaction mixture composed predominantly of ethyl acetate was as fast as that with thermolysin in the corresponding high-water emulsion system. In this case, the reaction proceeds as follows: substrates dissolved in the organic layer diffused to water media in which proteases exist and reacted to afford products. Substrates are continuously supplied from the organic layer to water media, whereas products are removed from water to the organic layer. This removal of the peptide product drives the equilibrium toward aminolysis and prevents hydrolysis of the product. However, the reaction rate is very slow because of the slow diffusion of the substrate, and the reaction suffers from denaturing of proteases at the surface.

Hydrolysis of peptides is induced by nucleophilic attack of water molecules (H_2O). Reducing the amount of H_2O or the activity of H_2O is a useful method to prevent hydrolysis. An organic solvent suppresses the activity of H_2O. However, proteases do not maintain their structures and functions in organic solvent. Therefore, water-miscible organic solvents (such as ethanol, dimethylsulfoxide, *N*,*N*-dimethylformamide, dioxane, acetone, and acetonitrile) are used with 2–5% water as the equilibrium condition [18, 20, 21].

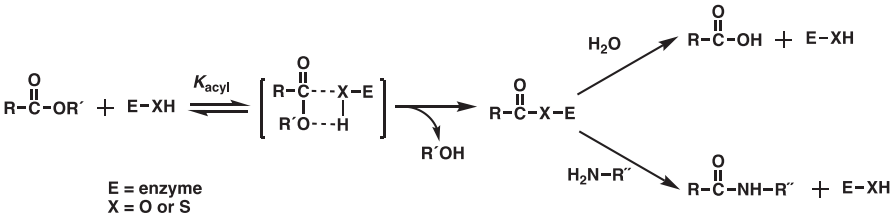

Fig. 8.2 Mechanism of kinetically controlled peptide synthesis

8.2.2 Kinetic Control

The synthetic scheme of kinetically controlled peptide synthesis is shown in Fig. 8.2 [22]. An ester group is generally introduced at a carboxyl end and used as a mildly activated acyl donor. The ester donor rapidly reacts at the catalytic center in proteases and an acyl-protease intermediate forms. Subsequently, the acyl-protease intermediate is nucleophilically attacked by nucleophiles, i.e., H_2O or an amino group of another amino acid substrate. Thus, hydrolysis/aminolysis occurs by deacylation with H_2O and free amino groups. Hydrolysis and aminolysis are not reversible reactions but rather cooperative reactions in kinetically controlled peptide synthesis. Therefore, the degree of the nucleophilic attack affects the rate of hydrolysis/aminolysis, which is why this effect is called "kinetic control." At the same time, thermodynamic hydrolysis proceeds. Nucleophilic hydrolysis and aminolysis are much faster than the equilibrium reaction. In the initial stage of the reaction, the concentration of peptide product rapidly increases. In contrast, in the latter half of the reaction, the concentration of peptide product reaches a plateau. Qin et al. reported the polymerization of L-Glu diethyl ester catalyzed by papain [23]. They examined the relationship between consumption of the starting material and reaction times. The L-Glu diethyl ester was consumed immediately after the reaction started. The yield reached 80% by 20 min and did not significantly increase afterward. This result indicates that the aminolysis reaction rapidly occurs under kinetically controlled peptide synthesis.

Serine or cysteine proteases can be used for kinetically controlled synthesis to create peptide bonds. The hydroxyl group of serine and the thiol group of cysteine play an important role in forming the acyl-protease intermediate. Figure 8.3 shows molecular behaviors around the active site of serine proteases. When carboxylic ester comes close to the catalytic center, a tetrahedral intermediate forms with a C–O covalent bond between the ester and the catalytic serine residue. Next, the acyl-protease intermediate is generated by releasing an alcohol corresponding to the ester groups of the starting substrates. Subsequently, the tetrahedral intermediate is formed again by nucleophilic attack of nucleophiles. Finally, an amide bond is formed through the equilibrium between the tetrahedral intermediate and a product.

There are many factors that enhance the rate of aminolysis. The concentration of amine nucleophilic groups, pH, and temperature affect the yield of peptide products. The rate of nucleophilic attack by amines to form acyl-protease intermediates is increased by raising the concentration of amine substrates. The amine nucleophilic

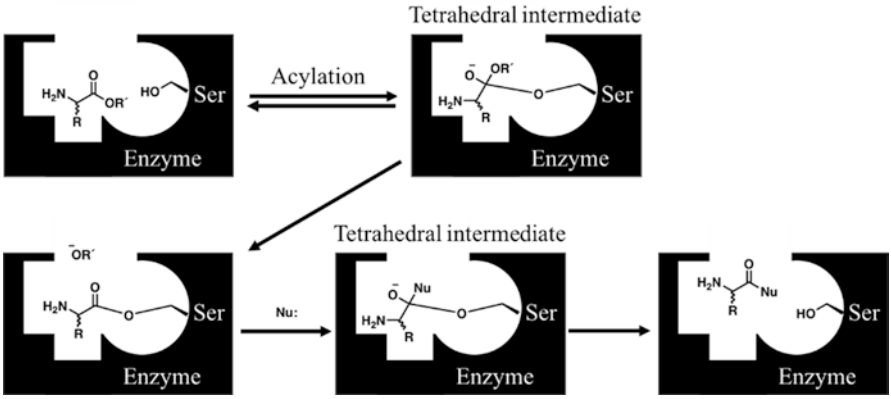

Fig. 8.3 The behavior of acyl donors and nucleophiles at the active site of serine proteases

group exists in its reactive neutral form at high pH. A suitable pH is in a range of 5–9 for the enzymatic reaction in the thermodynamic control method, whereas the kinetic control method needs a higher pH condition [24]. In addition, higher temperature induces a lower reaction rate of aminolysis. Based on these characteristics of aminolysis by proteases, frozen aqueous synthesis has been developed by many research groups [25–27]. In frozen aqueous conditions, namely, −20 to 5 °C, the rate of hydrolysis is reduced, and the yield of peptide formation is increased.

In contrast to the thermodynamic control method, the use of biphasic solution is inappropriate for kinetically controlled synthesis [28–31]. Proteases exist in an aqueous phase, whereas amino acid ester substrates exist in both phases. Therefore, the concentration of acyl-protease intermediates is low and hardly controlled in this system. On the other hand, the activity of H_2O toward the acyl-protease is reduced in a miscible water/organic solvent system, which suppresses the competing hydrolysis reaction. Kisee et al. [32] and Viswanathan et al. [33] reported a miscible water/organic (methanol, ethanol, tetrahydrofuran, and so on) solvent system. The yield of resulting peptides was dependent on the content of water and organic solvent. Thus, the water/organic solvent system is an interesting reaction system. However, it is noted that organic solvent has the potential to cause protease denaturation and dysfunction.

8.2.3 Specificity of Proteases

The substrate specificity of proteases affects the rate of aminolysis/hydrolysis. An acyl ester donor is recognized as a substrate at the active sites of proteases, and acyl-proteases are formed. Therefore, the affinity of acyl esters to proteases is the most important in forming acyl-protease intermediates. Furthermore, the affinity of amine nucleophilic groups to the catalytic site depends on the species of amino acids. Fastrez and Fersht reported a comparison of the deacylation rate of Acyl-Phe-chymotrypsin.

[34] The rate of aminolysis with L-glycine amide (Gly-NH_2) was 11 times faster than that of hydrolysis with H_2O, whereas the rate of aminolysis with L-alanine amide (Ala-NH_2) was 44 times faster than that of hydrolysis with H_2O. This result means that the affinity of Ala-NH_2 to chymotrypsin is higher than that of Gly-NH_2 to chymotrypsin. The substrate specificity of proteases can be evaluated by the partition constant *p*. The partition constant *p* is calculated from the reaction rates of hydrolysis and aminolysis. Schellenberger reported on the partition constant of nucleophilic amino acids using maleyl-(3-carboxyacryloyl)-Phe-chymotrypsin as an acyl-protease [35]. The order of nucleophilicity toward the protease is Arg-NH_2 > Leu-NH_2 > Val-NH_2 > Ala-NH_2 > Gly-NH_2 > H_2O. Since the affinity of amino acids to proteases affected the reaction rate of aminolysis, the yields and degree of polymerization (DP) of the polypeptides depend on the selection of proteases and amino acids.

Proteases have some subsites that align on both sides of the catalytic site, inducing substrate specificity [36, 37]. These subsites determine the cleavage point of hydrolysis and the reaction rate of aminolysis. Schechter and Berger reported the subsite of papain [36]. The size of the binding site of papain was estimated using the oligo(L-Ala) with or without D-Ala; the existence of D-Ala reduces the rate of hydrolysis. Based on this experimental result, the size of the papain subsite was estimated at over 25 Å. Furthermore, they proposed that there are four subsites (S1–S4) located on one side and three subsites (S1′–S3′) located on the other (Fig. 8.4), and each site is able to accommodate one amino acid residue of a substrate (P1–P4 and P1′–P3′). Peptide bonds far from the D-Ala residue were labile to papain-catalyzed hydrolysis, whereas peptide bonds next to the D-Ala residue (P1 and P1′ position) hardly reacted. This experimental result indicates that the chirality of amino acid substrates is most importantly recognized in S_1 and S'_1. On the other hand, D-residues are acceptable to some extent in occupying the subsites away from

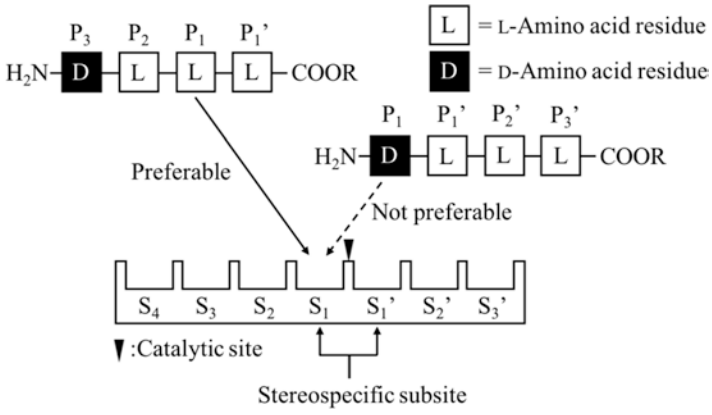

Fig. 8.4 Schematic illustration of the catalytic site (*black triangle*) and subsites (denoted as Sn and Sn′) in the papain active site. Each subsite can accommodate an amino acid residue in polypeptide substrates (denoted as Pn and Pn′), and subsites S1 and S1′ strictly regulate the stereospecificity of oligoalanine substrates. Peptide bonds next to the L-alanine residue are preferred for hydrolytic cleavage compared to the bonds next to the D-alanine residue [36]

the catalytic center. Furthermore, they concluded that active site S_2 tends to bind the hydrophobic amino acids, such as Phe, Leu, and Val. In the case of enzymatic polymerization, this specificity affects the efficiency of the reaction. Detailed insights into the specificity of subsites in each protease are summarized in Sect. 8.4.

8.3 General Synthetic Procedure for Enzymatic Polymerization

The homopolymerization and copolymerization of α-amino acids by kinetically controlled enzymatic polymerization have been reported by many research groups. The general procedure of protease-catalyzed polypeptide synthesis is as follows:

- 1. C-terminal-activated amino acid is dissolved in a buffer solution.
- 2. A solution of protease in buffer solution is added.
- 3. The mixture is stirred at a certain temperature for several hours.
- 4. After the reaction, the protease is removed, and the polypeptide is isolated.

Amino acid esters or oligopeptide esters [38], such as methyl, ethyl, and benzyl ester derivatives, have been used as a monomer for enzymatic homo- [39–43] and copolymerization [44, 45]. To maintain pH conditions, aqueous buffer solutions are used as a reaction media. A suitable range of pH for protease function is 5–9. For example, phosphate, carbonate, and citrate buffers have been used as a reaction solvent. Generally, 25–60 °C is suitable for enzymatic polymerizations because of proteases' thermal stability. The final concentration of amino acid substrate needs to be high enough to overcome the hydrolysis reaction competing with aminolysis.

After the reaction, there are several methods to isolate the resultant polypeptides from the polymerization solution. If the polypeptides are insoluble in the polymerization solution, we can obtain the peptides as a precipitate. In this case, the precipitate is washed with water to remove the remaining proteases and unreacted monomers. On the other hand, when the polypeptides are soluble in the polymerization solution, the polypeptides, proteases, unreacted monomers, and by-products are dissolved in the reaction solution. Thus, an additional isolation process is required to separate the polypeptides from other components. If the molecular weight of the polypeptides, which is generally less than 5000 in enzymatic polymerization, is much smaller than that of the protease, the protease can be removed from the mixture by centrifuge ultrafiltration with a suitable molecular weight cut-off (MWCO) membrane filter. Qin et al. isolated polyLys and N_ε-protected polyLys with different solubilities in a buffer solution with the following procedures [23]. In the case of water-soluble polyLys, the mixture was centrifuged using a commercially available centrifugal filter with a 3000 MWCO membrane, and then the filtrate was dialyzed to isolate the polyLys product. In contrast, in the case of water-insoluble N_ε-protected polyLys, the precipitate was easily collected by simple centrifugation of the reaction mixture, and purification was done by washing with a pH = 5 diluted HCl solution three times.

8.4 Enzymes Available for Enzymatic Polypeptide Synthesis

The enzymes used in enzymatic polypeptide synthesis include cysteine proteases (papain and bromelain), serine proteases (α-chymotrypsin, proteinase K, and trypsin), and metalloproteases (thermolysin). In addition to these proteases, enzymes with distinct properties (carboxypeptidase Y, D-aminopeptidase, alkaline D-peptidase, and lipase) are also available. The choice of the enzyme is one of the key factors in successful polypeptide synthesis because each enzyme has its own substrate specificity and optimal condition for catalytic activity.

8.4.1 Papain

Papain is found in papaya fruit (*Carica papaya*) and is one of the most investigated plant cysteine proteases in terms of structure-function relationships. It consists of a single polypeptide chain with 212 amino acid residues (23,429 Da). The three-dimensional structure exhibits two domains with a V-shaped cleft at which substrates can bind (Fig. 8.5). On the top of the V-shaped cleft, Cys25 and His159 form the catalytic diad. Although Cys25 and His159 are essential for catalysis, additional residues, including Gln19 [46], Asn175 [47], and Trp177 [48], have been suggested

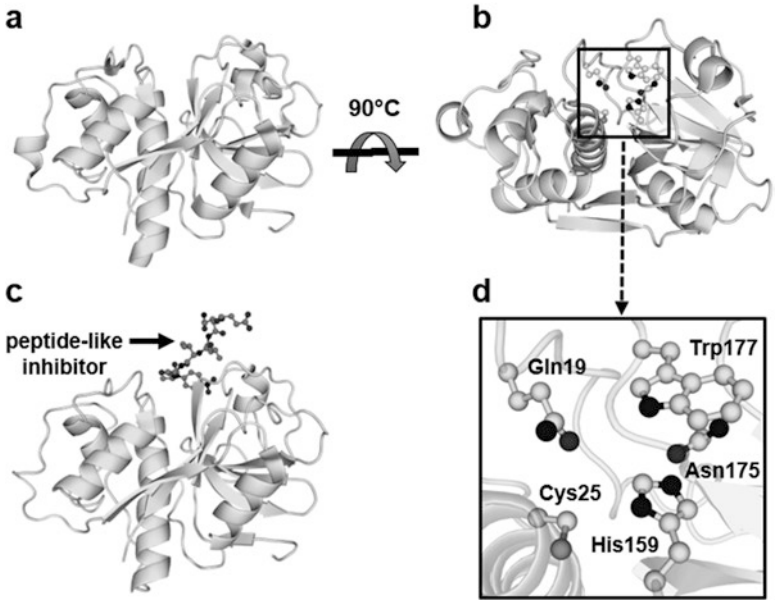

Fig. 8.5 (**a**) Crystal structure of papain (PDB: 1PPN), (**b**) a 90 °C-rotated view of (**a**), (**c**) crystal structure of papain with a peptide-like substrate (inhibitor, succinyl-QVVAA-*p*-nitroanilide) (PDB: 1PIP), (**d**) an enlarged view of the boxed area in (**b**)

to play an important role in catalytic activity. Schechter and Berger proposed that the papain active site contains seven subsites as described above (Fig. 8.4) [36]. Two decades later, Turk and co-workers redefined five subsites (S1–S3 and S1′–S2′) [49]. The subsites are located on both sides of the catalytic site, three on the N-terminal side (S1–S3) and two on the C-terminal side (S1′–S2′). Substrate specificity is mainly determined by the S2 subsite, which prefers hydrophobic amino acid residues, such as Val, Phe, Tyr, and Leu [50]. Although the S1 subsite exhibits some preference for Arg and Lys [51], the other sites lack the selectivity for amino acid residues of the substrate. In addition, the subsites (except for S1 and S1′) have been shown to accept D-amino acid residues [36]. Taken together, papain favors substrates possessing hydrophobic residues at the P2 position, but it exhibits broad substrate specificity.

In the field of enzymatic polypeptide synthesis, papain has been widely used owing to its availability at low cost and broad substrate specificity. To date, papain has successfully catalyzed the polymerization of various α-amino acid derivatives. Papain-catalyzed polymerization has often been performed at 40 °C in alkaline buffer solution to obtain a high yield and DP of peptide products. Geng and co-workers examined the papain-catalyzed polymerization of a side chain-protected L-Glu ethyl ester (γ-ethyl L-Glu-OEt) at various pH to elucidate the relationship between reaction pH and product yield [40]. This study indicated that the suitable pH range was between 5.5 and 8.0, giving an over 60% product yield and an average DP (DP_{avg}) of 8.7 ± 0.3 (determined by ^{1}HNMR). Since papain maintains its catalytic activity in the presence of organic solvents, water-miscible cosolvents can be applied to papain-catalyzed polymerization of poorly water-soluble amino acid substrates. The polymerization of L-Phe methyl ester (L-Phe-OMe) was achieved by papain in 0.25 M sodium phosphate buffer containing 10% dimethylsulfoxide, 20% methanol, and 18% acetonitrile, resulting in 38%, 50%, and 50% of the product yield, respectively [33].

The efficiency of papain-catalyzed polymerization (i.e., yield and DP of peptide products) can be influenced not only by reaction conditions (temperature, pH, and concentrations of the enzyme and substrate) but also by substrate properties. Schwab and co-workers investigated papain-catalyzed homopolymerizations using four hydrophobic amino acid methyl esters (L-Leu-OMe, L-Tyr-OMe, L-Phe-OMe, and L-Trp-OMe) as the substrate [43]. Based on the resulting yield and DP of each homopolymer, the reactivity of the substrates was classified as L-Tyr-OMe > L-Leu-OMe > L-Phe-OMe > L-Trp-OMe. Another comparative study using different L-Ala and Gly esters (methyl, ethyl, benzyl, and *tert*-butyl esters) revealed that the yield of poly(L-Ala) and polyGly increased in the order of *tert*-butyl (no polymer was obtained) < methyl < ethyl < benzyl esters [52]. The highest yield obtained from the benzyl esters was likely due to the higher affinity of papain toward aromatic substrates. Proper choice of the ester group can enhance substrate affinity toward papain, leading to efficient polymerization. In addition to the ester group, the side chain-protecting group can affect polymerization efficiency. Qin and co-workers reported the polymerization of side chain-protected L-Lys derivatives (N_ε-Z-L-Lys-OMe and N_ε-Boc-L-Lys-OMe) catalyzed by papain [23]. The resulting poly(N_ε-Z-L-

Lys) and poly(N_ε-Boc-L-Lys) exhibited narrower molecular weight distributions and higher DP_{avg} in comparison to the products obtained from the polymerization of unprotected L-Lys-OMe. The benefit of side chain protection of L-Lys is improved binding affinity for the S2 subsite, which prefers bulky hydrophobic side chains. Another benefit is the precipitation of products, which shifts the equilibrium toward product formation and decreases the propensity toward unfavorable reactions, such as transamidation and hydrolysis. Moreover, the precipitated products are easily isolated from the reaction media by centrifugation.

Reports on papain-catalyzed copolymerization of different amino acid esters have been increasing. Uyama and co-workers first demonstrated that papain successfully catalyzed the copolymerization of γ-ethyl-L-Glu-OEt with various amino acid esters, including L-Met-OMe, L-Ala-OEt, L-Leu-OEt, L-Phe-OEt, L-Tyr-OEt, L-Phe-OEt, and β-ethyl L-Asp-OEt [44]. Papain was also used to synthesize several random copolymers, such as poly(L-Glu-*co*-L-Cys) [53], poly(L-Tyr-*co*-L-Lys) [54], poly(L-Lys-*co*-L-Ala) [54], and poly(L-Ala-*co*-Gly) [52]. Another report showed papain-catalyzed binary and ternary copolymerization using four hydrophobic amino acid methyl esters (L-Leu-OMe, L-Tyr-OMe, L-Phe-OMe, and L-Trp-OMe) [43]. It was also reported that papain enabled the conversion of a dipeptide (L-Ala-Gly-OEt) monomer into a polypeptide with an alternating sequence [38]. From the examples introduced above, one can conclude that papain is the most versatile enzyme in enzymatic peptide synthesis and broad substrate specificity is its most important property. This broad specificity can contribute to product variety with respect to amino acid sequences.

8.4.2 Bromelain

Specific enzymes derived from the pineapple plant are known as bromelains. Among these enzymes, stem bromelain, which is present in plant stem extracts, is generally used for enzymatic peptide synthesis. Stem bromelain is referred to as bromelain in this chapter. Bromelain is a cysteine protease of the papain family. It is a glycosylated, monomeric protein of 24.5 kDa that contains seven Cys residues and most likely three disulfide bonds. The oligosaccharide component has been shown to provide enzymatic stability and tolerance to denaturants, including guanidine hydrochloride [55], urea [56], and sodium dodecyl sulfate (SDS) [57]. The enzyme's secondary structure is maintained between pH 7–10, but the enzyme is irreversibly denatured above pH 10 [58]. Although bromelain exhibits broad substrate specificity similar to papain, bromelain has been reported to prefer polar amino acid residues at the P1 and P1′ positions of substrates [59]. Additionally, it shows high hydrolysis efficiency toward substrates containing Arg and His at P2 and Pro at P3 [51].

Qin and co-workers used four proteases (papain, bromelain, α-chymotrypsin, and trypsin) for poly(L-Lys) synthesis and compared their activity [60]. Among them, bromelain showed the highest value of both DP_{avg} (4.1) and monomer conversion

(84 ± 2%) by maintaining the reaction pH between 7.6 and 7.8. This study also indicated that bromelain was sensitive to moderate pH although it catalyzed polymerizations over a wide range of temperatures (10–40 °C). Monomer conversion varied from 23 ± 1% to 76 ± 2% and 46 ± 5% for pH values of 6, 7, and 8, respectively. DP_{avg} also varied between 2.8 and 4.1 from pH 6 to 9. Interestingly, bromelain was shown to be more efficient than papain for poly(L-Lys) synthesis, even though both enzymes are known to exhibit high catalytic activity against substrates containing Lys at the Pl position. The different efficiency between these enzymes is explained by their different preferences for the Lys residue at the P2 position: bromelain shows a higher preference for Lys than papain does. This example suggests that bromelain is an efficient enzyme to catalyze the homopolymerization of polar amino acids, although further comparative studies using various amino acid substrates and proteases are needed.

Bromelain-catalyzed polymerization of hydrophobic L-Phe-OEt was shown to proceed in 20% MeOH-containing phosphate buffer and provided maximum product yields (40–45%) at pH 7–8 and 40 °C [33]. In another study, bromelain displayed relatively high catalytic ability for copolymerizations of L-Leu-OEt with γ-ethyl L-Glu-OEt compared to α-chymotrypsin and protease SG [45]. Moreover, the catalytic ability of bromelain was comparable to that of papain, which favors hydrophobic amino acid residues, such as Leu. These studies indicate that bromelain can efficiently catalyze the polymerization of hydrophobic amino acid substrates as well as polar ones.

8.4.3 α-Chymotrypsin

Chymotrypsin, a serine protease, is produced in an inactive form (chymotrypsinogen) by the acinar cells of the pancreas and then converted into its active form of α- or γ-type chymotrypsin. α-Chymotrypsin contains three separate polypeptide chains linked by five disulfide bridges. His57, Asp102, and Ser195, located at the entrance of a substrate-binding pocket (Fig. 8.6), form the catalytic triad that is essential for hydrolysis/aminolysis reactions. In addition to these three residues,

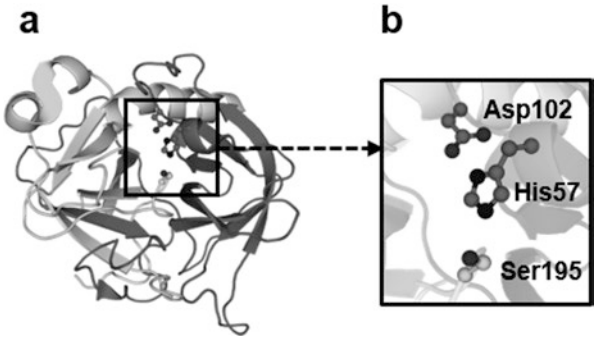

Fig. 8.6 (**a**) Crystal structure of α-chymotrypsin (PDB: 1YPH), (**b**) an enlarged view of the boxed area in (**a**)

Ser214 contributes to catalytic activity by forming hydrogen bonds with Asp102 and stabilizing the charge of the buried Asp102 [61]. Gly193 and Ser195 are also considered to be key residues for catalysis activity because they serve as the oxyanion hole that stabilizes a tetrahedral intermediate [62]. The substrate specificity of α-chymotrypsin originates from the P1–S1 interaction. S1 is a hydrophobic pocket with high specificity for aromatic (Phe, Trp, and Tyr) and bulky nonpolar (Met) side chains of the substrate [62].

In addition to cysteine proteases (papain and bromelain), serine proteases, such as α-chymotrypsin, can be applied to kinetically controlled peptide synthesis. In the 1950s, Brenner and co-workers found that α-chymotrypsin polymerizes the isopropyl esters of L-Met, L-Thr, L-Phe, and L-Tyr [63, 64]. Recently, α-chymotrypsin was used for the homopolymerization of L-Cys-OEt [27]. Since disulfide bond formation and ester hydrolysis simultaneously occurred for L-Cys-OEt under an unfrozen condition, polymerization was performed under a frozen condition at −20 °C. α-Chymotrypsin-catalyzed polymerization successfully provided poly(L-Cys) with DP ranging from 6 to 11, although papain did not catalyze the polymerization of L-Cys-OEt at −20 °C. In another report, α-chymotrypsin was reported to catalyze the rapid conversion of L-Lys-L-Leu-OEt to poly(L-Lys-*alt*-L-Leu) at pH 8.5, whereas L-Lys-L-Leu-OEt polymerization by papain resulted in random sequence oligopeptides [65]. This finding indicated that sequence-controlled polymerization was achieved by α-chymotrypsin but not by papain. α-Chymotrypsin can be an alternative catalyst for enzymatic peptide synthesis, particularly when substrates contain a thiol group that inhibits the catalytic activity of cysteine proteases.

8.4.4 Proteinase K

Proteinase K is a serine protease and the main proteolytic enzyme produced by the fungus *Tritirachium album* Limber [66]. The designation "K" was chosen to indicate that it can even hydrolyze native keratin [67]. Proteinase K is composed of a single polypeptide chain with 278 amino acids (MW 28,930). It has a catalytic triad (Asp39, His69, and Ser224) and requires calcium ions for its full activity [68]. One characteristic of proteinase K is its broad substrate specificity, although its S1 subsite prefers aromatic and hydrophobic amino acids [69]. Another feature is that proteinase K retains its activity at relatively high temperatures and in the presence of denaturants, including urea, 0.5% (w/v) SDS, and 1% (w/v) Triton X (surfactant based on 4-octylphenol ethoxylates) [70]. These easy-to-use features make proteinase K one of the most widely used proteases in molecular biology.

Ageitos et al. first investigated proteinase K-catalyzed synthesis of poly(L-Phe) [71]. The highest DP_{avg} of peptide products (12 ± 0.5) was obtained from the polymerization performed with 0.6 M L-Phe-OEt and 1.0 mg/mL proteinase K in sodium phosphate buffer (pH 8.0) at 40 °C for 3 hours. Furthermore, a star-shaped polypeptide was successfully synthesized by the proteinase K-catalyzed polymerization of L-Phe-OEt with a trifunctional terminal modifier, tris(2-aminoethyl)amine (TREN)

[71]. Other star-shaped polypeptides were also constructed by proteinase K [72]. The star-shaped polypeptides consist of the three-armed TREN center and cationic polypeptide branches of poly(L-Lys), poly(L-Arg), or poly(L-Lys-*co*-L-Arg). Proteinase K was shown to catalyze copolymerization of L-Cys-OEt with L-His-OEt or γ-ethyl L-Glu-OEt. The resultant poly(L-Cys-*co*-L-His) and poly(L-Cys-*co*-γ-ethyl-L-Glu) exhibited DP_{avg} values of 7.3 ± 1.1 and 9.5 ± 0.6, respectively [73]. So far, reports on proteinase K-catalyzed polymerization are limited compared to those on papain. This limitation might be due to the relatively higher cost of proteinase K compared to papain.

8.4.5 Trypsin

Trypsin is a serine protease that preferentially cleaves peptide bonds after Arg or Lys residues of substrates. It has a catalytic triad consisting of His57, Asp102, and Ser195. Trypsin strongly prefers substrates that possess an Arg or Lys residue at the P1 position [74], whereas it exhibits decreased catalytic activity for substrates with an Arg, Ile, Leu, Lys, or Phe residue at P2 and Pro at P3 [50]. The residue at P4 does not affect activity [75]. There are a few reports on enzymatic polypeptide synthesis catalyzed by trypsin. Poly(L-Arg) was synthesized by trypsin-catalyzed polymerization [76]. More than 40% of the substrate was converted into L-Arg-L-Arg within 1 h at 25 °C in 0.2 M carbonate buffer (pH 10). Trypsin was also shown to catalyze the polymerization of L-Lys-OEt [77]. The resultant poly(L-Lys) ranged from a dimer to an octamer. The highest monomer conversion was 70.7%, which was afforded by an optimal reaction condition (1 mM trypsin, 200 μM L-Lys-OEt, pH 10, 25 °C, 2 h). Interestingly, an addition of NaCl into the reaction mixture enhanced monomer conversion by 30% without affecting the polymerization degree, although the reasons for this enhancement were unclear. Trypsin is useful for the polymerization of polar substrates, including Arg and Lys, since it shows relatively high specificity against such residues.

8.4.6 Thermolysin

Thermolysin is a thermostable zinc metalloproteinase isolated from *Bacillus thermoproteolyticus* Rokko [78]. In addition to the Zn^{2+} ion, this enzyme binds four Ca^{2+} ions for its thermal stability [79]. The overall structure is divided into an N-terminal beta-sheet-rich domain and a C-terminal alpha-helix-rich domain. The two domains are spanned by a central alpha-helix located at a cleft running across the middle of the molecule. The central helix contains a zinc-binding HEXXH motif that is essential for hydrolysis activity. In the HEXXH motif, the two histidine residues coordinate with a Zn^{2+} ion, and the glutamate residue plays an important role in the hydrolysis reaction [80].

Although it is very rare to exploit thermolysin as a catalyst for amino acid polymerization, this enzyme has been applied to the industrial-scale synthesis of Z-L-Asp-L-Phe-OMe, the precursor of an artificial sweetener, aspartame [81]. Owing to this successful example, thermolysin has been extensively studied in terms of its catalytic ability for condensation reactions affording oligopeptides [82–85]. Wayne and Fruton investigated the substrate specificity of thermolysin in the condensation reaction [86]. Substrates with Phe or Leu at the P1′ position were efficiently condensed into oligopeptides by thermolysin. Additionally, the presence of aromatic residues (Phe and Trp) at P1 and hydrophobic residues (Ala, Met, and Phe) at P2 enhanced the condensation efficiency. Since thermolysin-catalyzed condensation proceeds through a thermodynamically controlled mechanism, the reaction conditions must be optimized to shift the equilibrium toward peptide bond formation. Kitaguchi and Klibanov have proposed an improved cosolvent system consisting of 90% organic solvent (*tert*-amyl alcohol), 9% water mimic (formamide or ethylene glycol), and 1% water that provides a high yield of peptide product [87]. Another approach is non-covalent immobilization of thermolysin by absorption on a porous support material (Celite) in a hydrophobic organic solvent, such as toluene [88]. The Celite-immobilized enzyme maintained its catalytic activity even in hydrophobic organic solvents owing to an aqueous phase around the Celite surface, thereby enabling highly efficient peptide condensation. Ulijn and co-workers have employed thermolysin for SPPS in which an amine substrate was linked to a solid support [89]. The immobilized amine substrate contributes to an equilibrium shift toward condensation, and its hydrophobicity is critical for efficiency [90]. One advantage of this strategy is that the reactions can achieve high conversion efficiency in bulk aqueous solution without needing an organic cosolvent and activated carboxylic acid. Although thermolysin has often been used for condensation reactions in previous studies, polymerization catalyzed by this enzyme is also interesting.

8.4.7 Others

In addition to the proteases described above, several enzymes with distinct properties are of interest as a catalyst for enzymatic polypeptide synthesis. For example, exopeptidases cleaving only the C-terminal amide bond are distinguishable from endo-type proteases, such as papain, which digest amide bonds within the polypeptide backbone. Exopeptidases, including carboxypeptidase Y (CPDY) [91], are expected to prevent unexpected hydrolysis of polypeptide backbones during polymerization, leading to a narrow molecular weight distribution of products. Indeed, CPDY-catalyzed polymerization successfully provided poly(L-Leu) with a relatively narrow molecular weight distribution [92]. Another example is D-stereospecific peptidase, which recognizes D-amino acid residues of substrates. Owing to its specificity for D-amino acids, D-stereospecific peptidase can be applied to the synthesis of peptides consisting of D-amino acids. Komeda and Asano demonstrated poly(D-Phe) synthesis catalyzed by alkaline D-peptidase from *Bacillus cereus* [93].

Synthesis of poly(D-Ala) was also achieved by immobilized D-aminopeptidase from *Ochrobactrum anthropi* in organic solvents [94]. Polymerization of amino acid esters can also be catalyzed by hydrolases, such as lipase. Lipase-catalyzed polymerization of L-Asp diethyl ester afforded high molecular weight polypeptides with a high yield, although peptide bond formation proceeded less regioselectively compared to protease-catalyzed polymerization [95, 96]. These distinct enzymes can be utilized as a special tool to provide novel peptides that are difficult to synthesize by commonly used proteases.

8.5 Amino Acid Esters for Enzymatic Polymerization

To date, there have been a great number of reports on the synthesis of polypeptides via enzymatic polymerization of various amino acid derivatives, especially amino acid esters. The amino acids used in enzymatic polymerization can be roughly classified into six different types in terms of their side groups, namely, hydrophobic, aromatic, acidic, basic, neutral, and unnatural amino acids (Fig. 8.7). These amino acids differ in their chemical and physical properties, and hence, enzymatic polymerization conditions differ for each amino acid.

8.5.1 Hydrophobic Amino Acids

Hydrophobic amino acids are appropriate substrates for enzymatic polymerization using proteases with a relatively high affinity for hydrophobic amino acids. One great advantage to using hydrophobic amino acids for enzymatic polymerization is that the resulting polypeptides precipitate from water due to their hydrophobicity. This precipitation is useful for easy isolation of the polypeptides obtained by simple

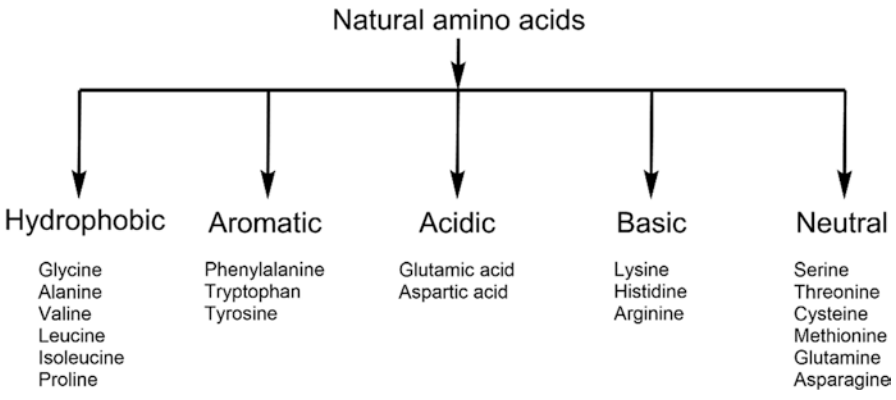

Fig. 8.7 Classification of natural amino acids based on their side group characteristics

centrifugation or filtration. The feature also restricts further chain elongation and hydrolysis of the obtained polypeptides after precipitation. The molecular weight of the obtained polypeptides is up to 3000 Da. However, fine-tuning of the reaction parameters, such as temperature and pH, can improve further polypeptide elongation.

There have been many reports on the enzymatic polymerization of hydrophobic amino acid esters, such as Ala and Leu. As a pioneering work of enzymatic polymerization, enzymatic synthesis of poly(L-Leu) was reported by Dannenberg and Smith using proteinase I from the bovine lung [97]. They found that the yield of polypeptide products remarkably relied on the pH of the reaction medium, which was monitored by tracking amine consumption using a ninhydrin assay. Poly(L-Leu) was also synthesized by the polymerization of L-Leu methyl ester using papain, and the average DP of poly(L-Leu) was 8–9 [98]. They revealed that the reaction proceeded with a pronounced induction period at a low substrate concentration before the precipitation of poly(L-Leu). The induction period can be shortened by the addition of a corresponding dimer or trimer, which implies that dimerization rate is very slow and that chain growth is rapid from the trimer onward. This finding means that chain elongation proceeds stepwise.

Ala derivatives are another hydrophobic amino acid monomer used in enzymatic polymerization. Poly(L-Ala) tends to adopt water-insoluble β-sheet structures, which facilitate rapid precipitation during enzymatic polymerization. Poly(L-Ala) can be readily synthesized by papain-catalyzed polymerization of L-Ala ethyl ester [42]. The reaction parameters influence the resulting poly(L-Ala) chain length to a certain extent. This influence was investigated by the papain-catalyzed polymerization of L-Ala ethyl ester in two different pH buffer solutions (i.e., 1 M sodium phosphate buffer at pH 7.0 and 1 M sodium carbonate buffer at pH 11.0). A higher yield (67.1%) was obtained at pH 7.0 than at pH 11.0 (35.7%). Interestingly, maximal DP increased at pH 11.0 (DP_{max} = 16) compared to that obtained at pH 7.0 (DP_{max} = 11) as determined by MALDI-TOF mass spectrometry. The resultant poly(L-Ala) formed characteristic microfibrils with the predominantly β-sheet structure, which was confirmed by atomic force microscopy and infrared (IR) spectroscopy.

As a highly promising structural material, silk fibers from animal species, such as silkworms and spiders, have attracted a wide range of attention from the scientific community due to their extraordinary strength and toughness. Silk proteins have a relatively longer repetitive domain between their conserved N- and C-terminal domains. In spider dragline silk proteins, an oligo(L-Ala) sequence in the repetitive domain forms β-sheet structures, whereas a glycine-rich domain forms random coil and helical structures. The higher-order structures constructed by these domains in silk fibers realize the excellent mechanical property of silk fibers in nature. Polypeptides mimicking spider silk proteins were synthesized utilizing enzymatic polymerization as shown in Fig. 8.8 [99]. The β-sheet-forming poly(L-Ala) motif was prepared by papain-catalyzed polymerization. The DP of poly(L-Ala) was controlled by adding poor organic solvents, such as methanol, in aqueous media to match the oligo(L-Ala) sequence length in spider silk proteins. On the other hand, a

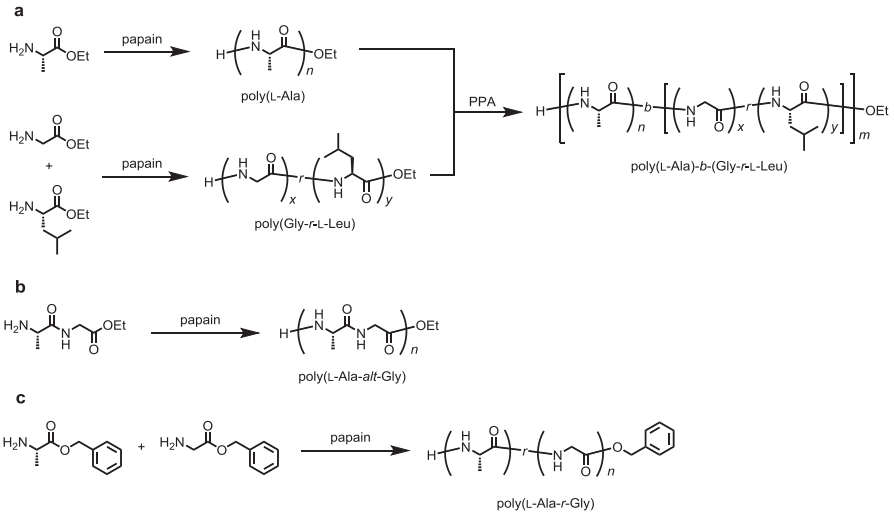

Fig. 8.8 (**a**) Synthesis of multiblock polypeptides inspired by spider dragline silk proteins via enzymatic polymerization followed by polycondensation, (**b**)enzymatic polymerization of L-AlaGly dipeptide ethyl ester affording alternating AlaGly sequence, (**c**) random copolymerization of L-Ala and Gly benzyl esters catalyzed by papain

more flexible glycine-rich motif was prepared by random copolymerization of Gly and L-Leu esters using papain. This poly(Gly-*r*-L-Leu) acts as a "soft" motif expressed between the "hard" polyAla motifs in spider silk proteins. The post-polycondensation of these hard and soft polypeptide motifs was accomplished using polyphosphoric acid (PPA) as a condensing agent at an elevated temperature. The resulting multiblock polypeptide, poly(L-Ala)-*b*-poly(Gly-*r*-L-Leu), adopted characteristic secondary structures and crystallinity based on the polyAla and Gly-rich sequences similar to natural spider silks. This chemically ligated tandem sequence is a biomimetic polypeptide of spider dragline silks, proving that enzymatic polymerization is greatly applicable to the synthesis of polypeptide motifs in structural proteins.

Another β-sheet-forming peptide motif is GAGAGX, which is seen in *Bombyx mori* silkworm silk proteins. Two strategies were used to synthesize polypeptides similar to the GAGAGX motif (Fig. 8.8). Qin et al. used a dipeptide ester L-alanylglycine ethyl ester (L-AlaGly–OEt) to obtain a polypeptide with a strictly alternating sequence poly(L-Ala-*alt*-Gly) via enzymatic polymerization [38]. Stoichiometrically equivalent quantities of alternating L-Ala and Gly units were obtained, which was confirmed by MALDI-TOF mass spectrometry. To obtain alternating sequences of polypeptides, dipeptide esters can be used as monomers in enzymatic polymerization. On the other hand, Ageitos and co-workers used the strategy of copolymerization of L-Ala and Gly esters for the synthesis of polypeptides containing motifs similar to GAGAG [52]. This technique cannot control the exact sequence but can fine-tune the Gly/Ala content of the resulting polypeptides.

Papain shows less affinity for Gly than Ala, resulting in a significant mismatch between Gly and Ala reactivities. To overcome this mismatch, L-Ala and Gly benzyl esters with similar reactivities were used for papain-catalyzed copolymerization. By changing the feed ratio of L-Ala/Gly benzyl esters, the compositions of L-Ala/Gly in the copolypeptides could be controlled. The resulting polypeptides differed in secondary structures and thermal stabilities depending on their L-Ala content.

8.5.2 Aromatic Amino Acids

Aromatic amino acids also exhibit a high affinity for proteases because of their hydrophobic aromatic side group. These aromatic amino acid esters are good substrates for enzymatic polymerization, but rapid precipitation usually causes a low DP for the resulting polypeptides. Enzymatic polymerization of poly(L-Phe) was reported by Dannenberg and Smith using proteinase I from the bovine lung [97]. The DP_{avg} of the obtained poly(L-Phe) was 6. Gross and co-workers reported enzymatic synthesis of poly(L-Phe) using bromelain in various reaction conditions [33]. Ageitos et al. reported enzymatic polymerization of L-Phe esters using proteinase K [71]. In aqueous solutions, the obtained linear poly(L-Phe) showed a unique self-assembly property. In addition to linear poly(L-Phe), three-armed, star-shaped oligoPhe was synthesized by polymerization of L-Phe ethyl ester with tris(2-aminoethyl) amine. This star-shaped poly(L-Phe) self-assembled to form fibrous networks.

Tyrosine is an aromatic amino acid with a relatively polar side group. The phenol moiety of Tyr is polar and reactive; therefore, polypeptides containing Tyr as a monomer unit can be easily functionalized by further chemical modification. Adhesive polypeptides from the copolymerization of L-Tyr and L-Lys ethyl esters were developed using enzymatic polymerization, which was inspired by a natural adhesive protein, foot protein 5 (Mefp-5), produced by a blue mussel (*Mytilus edulis*) [54]. This polymerization was achieved by two-step synthesis (Fig. 8.9). First, copolypeptides

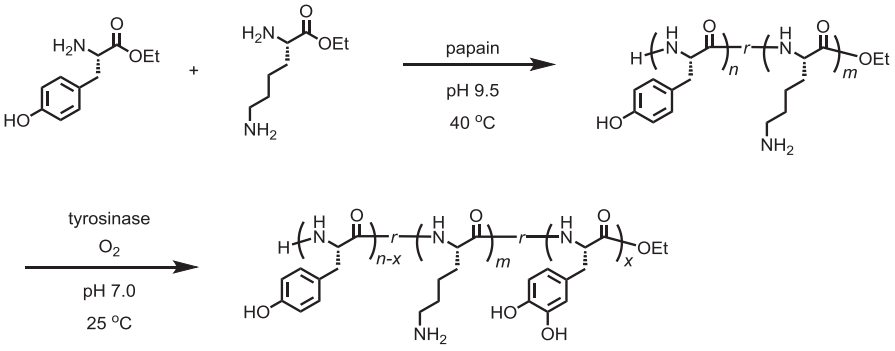

Fig. 8.9 Two-step enzymatic synthesis of adhesive polypeptides consisting of L-Tyr, L-Lys, and L-DOPA residues

of L-Tyr and L-Lys units were synthesized by papain-catalyzed polymerization, and then, enzymatic conversion of L-Tyr to L-3,4-dihydroxyphenylalanine (DOPA) was carried out using tyrosinase. The poly(L-Tyr-*r*-L-Lys-*r*-L-DOPA) with a Tyr/Lys/DOPA ratio of 50/25/25 at pH 12 showed excellent adhesiveness (adhesion strength, 0.95 MPa; Young's modulus, 6 MPa), and this adhesiveness was higher than that of a commercially available superglue as determined by an adhesive lap joint shear strength test using mica sheets. Lampel et al. demonstrated that another functional polypeptide material, tyrosine-containing tripeptides prepared by enzymatic synthesis, acted as a polymeric pigment with tunable coloration ability [100].

8.5.3 Acidic Amino Acids

Glutamic acid and aspartic acid are acidic and hydrophilic amino acids since they contain a carboxylic acid residue in their side chain. There are no reports of successful enzymatic polymerization of Glu and Asp monomers with a free carboxylic acid side group. This lack of successful polymerization is probably because the carboxylic acids interact with the catalytic center of proteases by undesired proton transfer. However, the conversion of carboxylic acid to its corresponding ester causes a polarity change, causing the amino acid to become hydrophobic, which allows Glu to be a good substrate. Indeed, L-Glu diethyl ester is frequently used for enzymatic polymerization using various proteases. The polymerization of L-Glu diethyl ester to give poly(γ-ethyl L-glutamate) [poly(L-Glu-OEt)] using proteases, including papain, bromelain, and α-chymotrypsin, was reported by Uyama et al. (Fig. 8.10) [44]. Poly(L-Glu-OEt) was generated as white precipitate, and the average DP was estimated at approximately 9 by ^{1}H NMR spectroscopy. The polymerization of

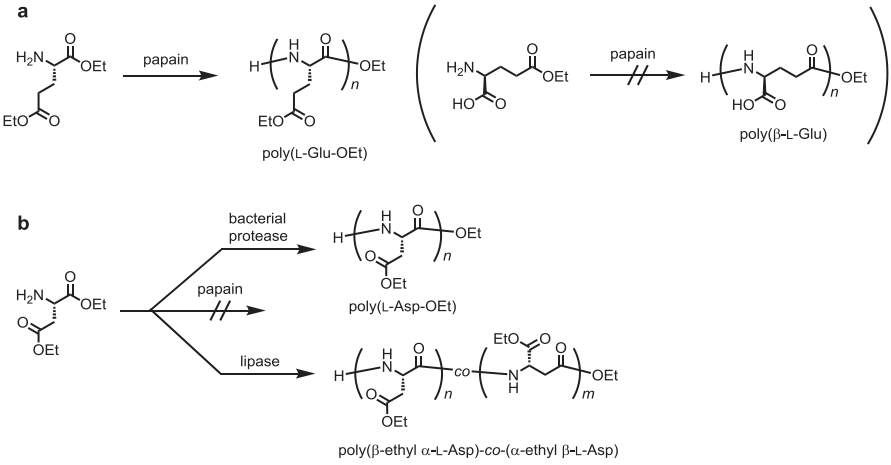

Fig. 8.10 (**a**) Papain-catalyzed polymerization of L-Glu diethyl ester with remarkable regioselectivity, (**b**) enzymatic polymerization of L-Asp diethyl ester using various enzymes

γ-methyl L-glutamate, which possesses an ethyl ester group only at the side chain, did not proceed under similar reaction conditions using papain. This fact revealed that protease-catalyzed polymerization regioselectively proceeds to generate an α-peptide linkage in the resulting polymer backbone. Copolymerization of L-Glu diethyl ester with L-Met methyl ester, L-Ala ethyl ester, L-Leu ethyl ester, L-Phe ethyl ester, L-Tyr ethyl ester, and L-Asp diethyl ester proceeded using papain, providing various random copolypeptides.

The other acidic amino acid, aspartic acid, is an analog of glutamic acid with only one less methylene group. However, the reactivities of Glu and Asp in enzymatic polymerization are quite different. Geng et al. reported that papain-catalyzed polymerization of L-Glu diethyl ester in 0.9 M phosphate buffer for 10 min resulted in poly(L-Glu-OEt) at 80% yield [40]. In contrast, L-Asp diethyl ester was not polymerized in the presence of papain, probably because of the poor affinity of papain for Asp substrates. Matsumura et al. reported that polymerization of L-Asp diethyl ester by alkalophilic proteinase from *Streptomyces* sp. at a temperature between 4 and 50 °C yielded poly[(β-ethyl α-L-aspartate)-*co*-(α-ethyl β-L-aspartate)] [101]. Soeda et al. later polymerized L-Asp diethyl ester by a bacterial protease from *Bacillus subtilis* (BS) using an organic solvent at a temperature ranging from 30 to 50 °C (Fig. 8.10). The resulting α-linked poly(β-ethyl α-L-aspartate) [poly(L-Asp-OEt)] with a weight average molecular weight (M_w) up to 3700 was obtained at 85% yield [102]. Lipase, a subclass of esterases that hydrolyzes lipids, can also catalyze the polymerization of Asp ester derivatives. Zhang et al. reported that lipase (*Candida antarctica lipase B*) catalyzed the polymerization of diethyl D- or L-Asp diethyl ester, providing poly(α-ethyl β-D-aspartate) or poly(α-ethyl β-L-aspartate) in a solvent-free condition, respectively. The DP_{avg} was found to be 60 with up to 96% β-linkages, indicating that lipase provided dominantly amide bonds with β-linkages (Fig. 8.10) [95]. However, recently, Gross and co-workers reported the polymerization of L-Asp diethyl ester with improved regioselectivity using an immobilized lipase at 80 °C for 24 h [96]. The reaction successfully yielded approximately 95% α-linked poly(L-Asp-OEt) with a 70% yield, and the DP_{avg} was approximately 50. They concluded that polymerization proceeds in a controlled manner by a chain-growth mechanism with up to 90% conversion, and then, a step-growth mechanism competes with the chain-growth mechanism.

8.5.4 Basic Amino Acids

Amino acids possessing a basic side group, such as L-Lys, L-Arg, and L-His, fall in this category. No protection of the amine group in the side chain is required for the enzymatic polymerization of these amino acids because of sufficient regioselectivity in protease-catalyzed polymerization. The resulting cationic polypeptides have been employed as cell-penetrating peptides and peptide carriers for gene delivery systems [103, 104]. Enzymatic synthesis of poly(L-Lys) from L-Lys ethyl ester in an aqueous medium using four proteases (papain, bromelain, α-chymotrypsin, and

trypsin) was studied to determine their activity in Lys polymerization at pH values ranging from 6 to 11 [60]. Bromelain was found to be the most effective protease since it gave the highest yield and DP_{avg} of poly(L-Lys). Qin et al. further explored the polymerization of N_ε-protected L-Lys esters (Fig. 8.11). This protection of the ε-amino group improved the yield of the resulting N_ε-protected poly(L-Lys) by enhancing the hydrophobicity of the polymer, which facilitated a water-insoluble precipitate [23]. The protecting groups of the ε-amino group included Boc and Z groups. Poly(N_ε-Boc-L-Lys) and poly(N_ε-Z-L-Lys) resulted from enzymatic polymerization of the corresponding Lys monomers. These poly(L-Lys) with hydrophobic moieties were easily collected by centrifuging the precipitates.

Diblock and random polypeptides of L-Lys and L-Ala were synthesized by one-pot enzymatic polymerization using papain [105]. Characterization by ^{1}H NMR spectroscopy and MALDI-TOF mass spectrometry revealed that the sequential addition of an L-Lys ethyl ester followed by an L-Ala ethyl ester resulted in the formation of diblock polypeptides. Observation by optical microscope clearly revealed that the crystal morphology of the copolymers was dependent on the monomer distribution in the copolymers and the pH of the solution. Diblock copolypeptides formed cubic or hexagonal crystals with a hollow structure at pH 3.0. This type of unique crystal with low cytotoxicity can be used as a biomedical material, e.g., as a carrier for drug delivery systems. Utilizing dipeptide esters as a monomer is another method to synthesize the copolypeptide, as reported by Gross and co-workers [38, 65]. An alternating copolypeptide of L-Lys and L-Leu units was enzymatically synthesized using α-chymotrypsin at pH 8.5 [65]. The resulting poly(L-Lys-*alt*-L-Leu) in the aqueous reaction solution underwent a sol-gel transition in which β-sheet structures assembled into a nanofibril network. This polypeptide hydrogel system can be a promising stimulus-responsive material that has potential in biomedical applications.

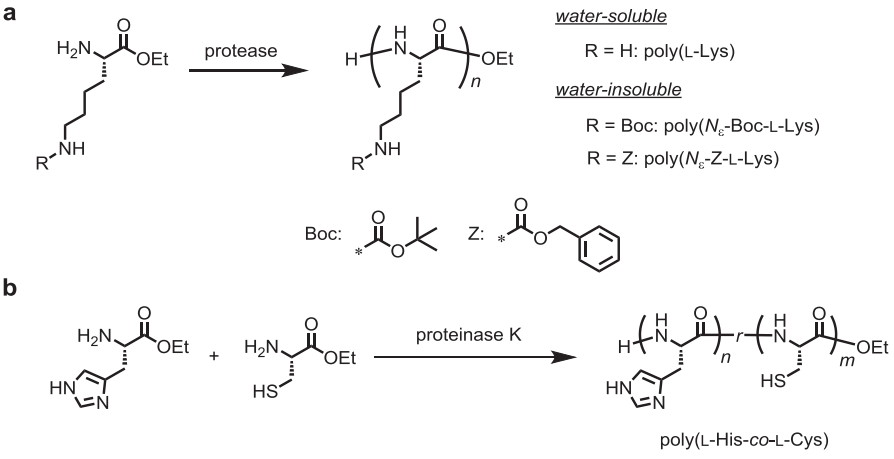

Fig. 8.11 (**a**) Enzymatic polymerization of L-Lys ethyl ester derivatives, (**b**) proteinase K-catalyzed copolymerization of L-His and L-Cys

There have been a few reports on the enzymatic polymerization of Arg and His. The homo- and copolymerization of L-Arg ethyl ester was conducted using proteinase K in a condition similar to the polymerization of Lys esters [72]. Ma et al. reported the copolymerization of L-Cys and L-His ethyl esters with changing ratios of His/Cys using proteinase K (Fig. 8.11) [73]. The resulting copolypeptides with various His/Cys ratios were successfully obtained in 40–50% yields. Poly(L-His-*co*-L-Cys), inspired by a catalytic diad of cysteine proteases, exhibited protease-like activity to cleave an amide bond using fluorescein isothiocyanate-labeled casein as a substrate. This feature was utilized to peel off living cells from a culture plate to obtain a monolayer cell sheet.

8.5.5 Neutral Polar Amino Acids

Neutral amino acids include various side groups. This category focuses on amino acids with polar side groups rather than those with hydrophobic side groups, which were already described in the section on hydrophobic amino acids. Amino acids containing a sulfur in their side groups, namely, cysteine and methionine, play an important role in both natural proteins and synthetic polypeptides because of their specific functionality and availability as reactive residues for chemical modification. Cysteine has a reactive thiol group in the side chain that can be easily oxidized to form a disulfide bond. This feature hampers polypeptide formation during the polymerization process by undesired cross-linking of the polypeptide via disulfide bonds. Therefore, the thiol group of Cys must be protected prior to polymerization in conventional synthetic methods. Narai-Kanayama et al. reported that the poly(L-Cys) was synthesized by enzymatic polymerization of L-Cys ethyl ester using α-chymotrypsin in a frozen aqueous medium [27]. The thiol groups of the obtained polymer were intact during polymerization and purification, indicating that the poly-Cys was successfully synthesized without a tedious protection/deprotection process. The DP of the obtained polyCys ranges from 6 to 11 as confirmed by MALDI-TOF mass spectrometry. Ma et al. synthesized poly(L-Cys) by enzymatic polymerization using proteinase K, and the DP of the polyCys was as high as 17 [106].

Methionine is another sulfur-containing amino acid with a sulfide moiety in the side group. The sulfide group can be utilized for modification of the polypeptide by chemical reactions, such as oxidation and alkylation by electrophiles. One early example was the enzymatic synthesis of poly(L-Met) and its derivatization (Fig. 8.12) [107, 108]. Poly(L-Met) was synthesized from L-Met methyl ester by papain-catalyzed polymerization in a citrate buffer at pH 5.5. However, characterization of the product was limited due to the insolubility of the obtained poly(L-Met) in common solvents. The chemical conversion of sulfide groups in L-Met residues to sulfoxide or sulfone by treatment with acids increased the water solubility of the resulting polypeptide, poly(L-methionine sulfoxide). From ^{1}H NMR spectroscopy, the DP_{avg} was found to be up to 8.

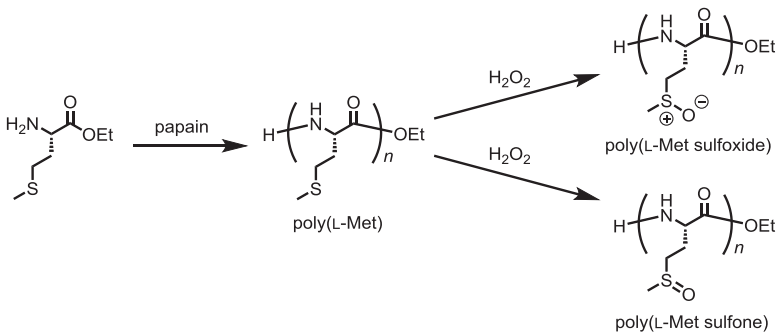

Fig. 8.12 Papain-catalyzed synthesis of poly(L-Met) and its oxidation to sulfoxide and sulfone derivatives

Serine and threonine, which possess a hydroxy group in their side chain, were used in enzymatic polymerization without protecting the hydroxy group, as demonstrated in early reports [63, 64]. To date, there are no reports on the enzymatic polymerization of glutamine and asparagine derivatives, although some short peptides containing these amino acids were synthesized by protease-catalyzed coupling reactions [109, 110].

8.5.6 Unnatural Amino Acids

Incorporation of unnatural motifs into the polypeptide backbone is an innovative method to fabricate various polypeptide materials with novel functionalities. The polymerization of unnatural amino acid esters using proteases was demonstrated in order to synthesize polypeptides containing unnatural structures, although proteases show poor substrate specificity for these substrates. Yazawa et al. demonstrated that ester derivatives of ω-aminoalkanoic acids, monomer units of nylon, were not polymerized alone using papain but were successfully introduced into polypeptides via papain-catalyzed copolymerization with natural amino acids, such as L-Leu and L-Glu esters, in a phosphate buffer [111, 112]. In general, polypeptides exhibit neither melting behavior nor glass transition because of intermolecular multiple hydrogen bonds, resulting in a lack of thermoplasticity. The insertion of nylon units into the polypeptide is assumed to drastically reduce intermolecular interactions via hydrogen bonds and induce melting behavior in the polypeptides. Papain-catalyzed copolymerization of L-Glu diethyl ester with various nylon monomers, including ethyl 3-aminopropionate, ethyl 4-aminobutyrate, and methyl 6-aminohexanoate, afforded polypeptides containing nylon units [111]. Only papain can polymerize copolymerization among many proteases due to its relatively broad substrate specificity. In the resulting polypeptides, one nylon unit was incorporated at the C-terminal of poly(L-Glu-OEt)s as evidenced by ^{1}H NMR spectroscopy and MALDI-TOF

mass spectrometry. On the other hand, in the case of the copolymerization of L-Leu and nylon esters using papain, approximately 15 mol% of the nylon unit was introduced into the poly(L-Leu) backbone [112]. The obtained copolymers showed a broad melting behavior at approximately 200 °C below the decomposition temperature. This result indicates that the introduction of artificial units, such as nylon, into the polypeptide backbone imparted thermoplasticity (Figs. 8.13 and 8.14).

Another example of an artificial amino acid is 2-aminoisobutyric acid (Aib), an α,α-disubstituted amino acid that has a tendency to form helical structures [113, 114]. Enzymatic polymerization of Aib ethyl esters resulted in no polymer formation since Aib is hardly recognized by the catalytic sites of proteases, even in copolymerization with natural amino acid esters. To overcome the incompatibility, the Aib unit was sandwiched in between L-Ala residues to form a tripeptide ester, which resulted in effective recognition by papain (Fig. 8.13b). Enzymatic polymerization of the Aib-containing tripeptide ester, L-Ala-Aib-L-Ala ethyl ester,

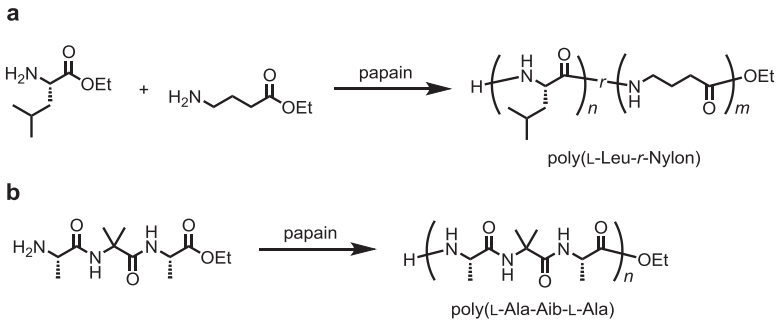

Fig. 8.13 Introduction of unnatural amino acid units in polypeptide backbones via enzymatic polymerization using (**a**) 4-aminobutyric acid (nylon) and (**b**) 2-aminoisobutyric acid (Aib)

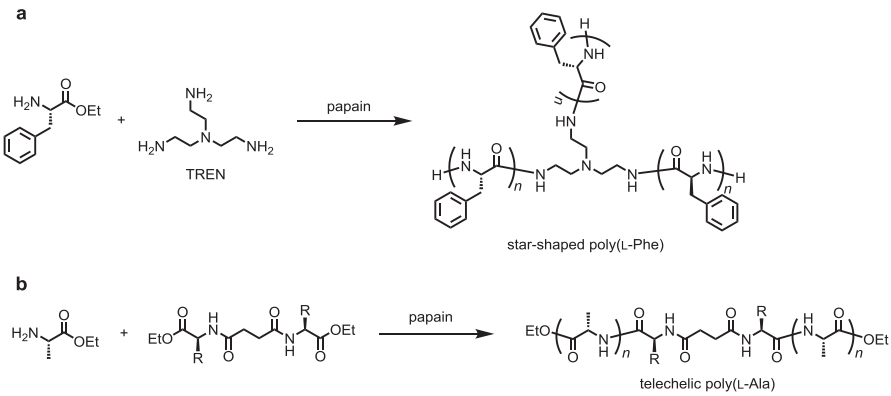

Fig. 8.14 Enzymatic synthesis of special polypeptide architectures; (**a**) three-armed, star-shaped poly(L-Phe), (**b**) telechelic poly(L-Ala)

proceeded smoothly in the presence of papain [115]. IR and circular dichroism (CD) analyses revealed that the obtained poly(L-Ala-Aib-L-Ala) adopted a helical conformation in both solid and solution states. The tripeptide strategy is an effective method to incorporate unnatural amino acids, which are poorly recognized by proteases, into polypeptides. Because the introduction of artificial synthetic amino acids into polypeptide backbones can tune their physical properties, it is useful to produce novel polypeptide materials with a distinctive property compared to natural polypeptides (proteins).

Using multifunctional monomers can afford polymers with a variety of special architectures. These include special structures, such as star-shaped polymers, hyperbranched polymers, polymer brushes, and cross-linked gels, that cannot be synthesized by biological methods. Two types of special polypeptides have been reported using enzymatic polymerization: three-armed, star-shaped polypeptides and telechelic polypeptides. The three-armed, star-shaped polypeptide was formed by polymerization of L-Phe ethyl ester in the presence of TREN catalyzed by proteinase K in an aqueous buffer [71]. The triamine TREN acts as a trifunctional terminal modifier at the C-terminus of the polypeptide. ^{1}H NMR proved that the resulting polyPhe was a star-shaped polypeptide with a TREN core. Another three-armed star-shaped polypeptide consisting of L-Lys, L-Arg, or a copolymer of the two was prepared by proteinase K-catalyzed polymerization in the presence of TREN [72]. Cationic star-shaped polypeptides are frequently used as peptide/plasmid DNA complexes for gene delivery. The high efficiency of gene transfection into human embryonic kidney 293 cells was confirmed using these cationic star-shaped polypeptides.

Telechelic polypeptide architecture, where two polypeptide chains propagate from two initiation points in N- to C-terminal direction, was synthesized by enzymatic polymerization [116]. In this study, a novel telechelic bifunctional compound with an amino acid ester at both terminals was prepared, and then, papain-catalyzed enzymatic polymerization of Gly or L-Ala ethyl ester in the presence of this bifunctional compound was performed. The formation of telechelic poly(L-Ala) and polyGly was confirmed by ^{1}H NMR spectroscopy and MALDI-TOF mass spectrometry. From atomic force microscopy observations, the crystals of telechelic poly(L-Ala) showed long nanofibrils with a high aspect ratio in contrast to the authentic linear poly(L-Ala), which assembled into granule-like crystals. Due to its self-assembling feature into a specific structure, telechelic poly(L-Ala) was utilized as a reinforcing agent for structural materials, such as regenerated silk fibroin films [117, 118].

8.6 Perspective

Enzymatic polymerization of amino acid derivatives using proteases is a powerful way to synthesize various types of polypeptides with a green synthetic protocol. The broad structural versatility of enzymatically synthesized polypeptides in recent studies is beneficial for realizing polypeptide materials in our forthcoming sustainable society. Further improvement of this technique is inevitable for practical

applications of polypeptide materials. It should be noted that the molecular weight of the obtained polypeptides remains in an oligomeric range except for some examples. On the other hand, enzymatic polymerization of some amino acids, such as proline and glutamine, has not yet been achieved, which hampers the design of specific sequences of polypeptides. Developing more efficient polymerization systems will allow us to control the synthesis of well-defined polypeptides with high molecular weight and fine-tune amino acid sequences.

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Chapter 9
Synthesis of Poly(aromatic)s I: Oxidoreductase as Catalyst

Hiroshi Uyama

Abstract This chapter reviews enzymatic oxidative polymerization to aromatic polymers. Phenols, anilines, and thiophenes are subjected to the enzymatic oxidative polymerization. Peroxidases with the use of hydrogen peroxide as oxidant efficiently induce the oxidative coupling of phenols to phenolic polymers, most of which are hardly obtained by conventional chemical catalysts. The enzymatic oxidative polymerizations have merits of using nontoxic catalysts and mild reaction conditions, and the specific enzyme catalysis provides regio- and chemoselective polymerizations to produce functional materials. Laccase and peroxidase are useful for production of cross-linked polymers such as artificial urushi and biopolymer hydrogel.

Keywords Aniline · Enzymatic polymerization · Laccase · Oxidative polymerization · Peroxidase · Phenol

9.1 Introduction

Phenol-formaldehyde resins using prepolymers such as novolaks and resols are widely used in industrial fields. These resins show excellent toughness and thermal-resistant properties, but the general concern over the toxicity of formaldehyde has resulted in limitations on their preparation and use. Therefore, an alternative process for the synthesis of phenolic polymers avoiding the use of formaldehyde is strongly desired.

In living cells, various oxidoreductases play an important role in maintaining the metabolism of living systems. All enzymes of this class catalyze oxidoreduction reactions. The substrate that is oxidized is regarded as hydrogen donor. This chapter deals with enzymatic oxidative polymerizations of aromatic compounds, mainly

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phenolic derivatives and their applications. The oxidative polymerization of phenol derivatives via enzyme catalysis is regarded as preparation of a new class of phenolic polymers without the use of toxic formaldehyde under mild conditions.

9.2 Enzymatic Oxidative Polymerization of Phenols

So far, several oxidoreductases, peroxidase (EC 1.11.1.7), laccase (EC 1.10.3.2), bilirubin oxidase (EC 1.3.3.5), etc., have been reported to catalyze an in vitro oxidative polymerization of aromatic compounds, and among them, peroxidase is most often used.

9.2.1 Peroxidase

Peroxidase is an enzyme whose catalysis is an oxidation of a donor to an oxidized donor by the action of hydrogen peroxide, liberating two water molecules. Horseradish peroxidase (HRP) is a single-chain β-type hemoprotein containing Fe as active center that catalyzes the decomposition of hydrogen peroxide at the expense of aromatic proton donors. Catalytic cycle of HRP for a phenol substrate is shown in Fig. 9.1 [1]. HRP and soybean peroxidase (SBP) are commercially available in industrial fields.

HRP is active as catalyst for oxidative polymerization of phenolic compounds. The reaction proceeds via oxidative coupling between radical species [2–5]. Peroxidase-catalyzed oxidative coupling of phenols proceeds fast in aqueous solutions, giving rise to the formation of oligomeric compounds. However, the resulting oligomers have not well been characterized, since most of them show low solubility toward common organic solvents and water.

In the case of phenol, the simplest and most important phenolic compound in industrial fields, conventional polymerization catalysts afford an insoluble product with noncontrolled structure since phenol is a multifunctional monomer for oxidative polymerization [6]. Phenol was subjected to the oxidative polymerization using HRP or SBP as catalyst in a mixture of 1,4-dioxane and buffer, yielding a polymer consisting of phenylene and oxyphenylene units (Fig. 9.2). The polymer showed low solubility; it was partly soluble in DMF and DMSO and insoluble in other common organic solvents. On the other hand, aqueous methanol afforded the DMF-soluble polymer with molecular weight of 2.1×10^3–6.0×10^3 in good yields [4, 7–11]. The solubility of the resulting polymer strongly depended on the buffer pH and content of the mixed solvent. The resulting phenolic polymer showed relatively high thermal stability, and no clear glass transition temperature (T_g) was observed below 300 °C.

The control of the polymer structure was achieved by solvent engineering. The ratio of phenylene and oxyphenylene units was strongly dependent on the solvent

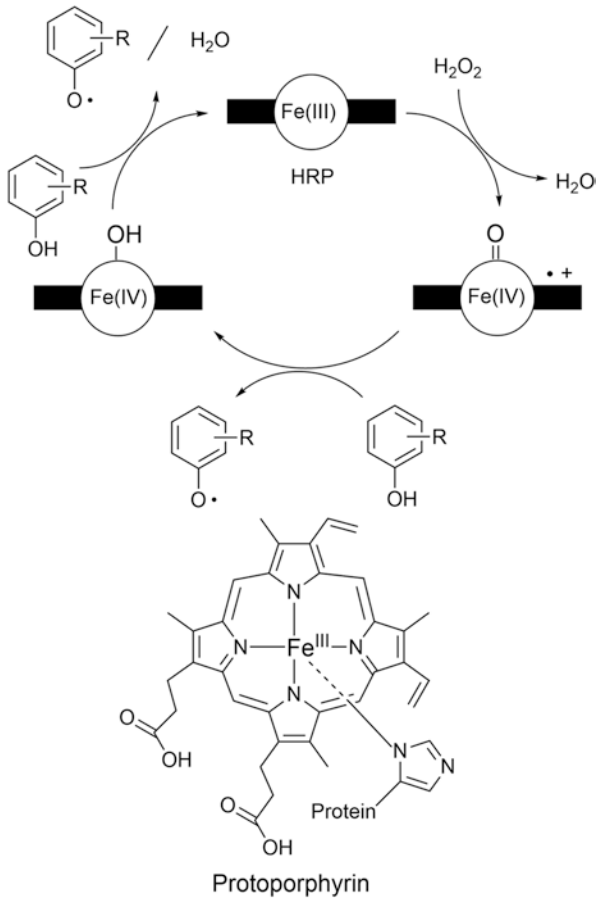


Fig. 9.1 Catalytic cycle of HRP

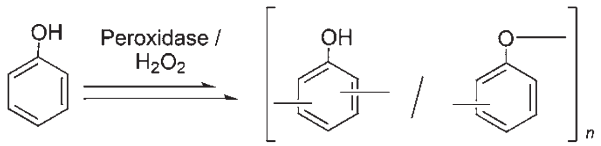


Fig. 9.2 Peroxidase-catalyzed oxidative polymerization of phenol

composition. In the HRP-catalyzed polymerization of phenol in a mixture of methanol and buffer, the oxyphenylene unit increased by increasing the methanol content, while the buffer pH scarcely influenced the polymer structure. The polymer solubility increased with increasing the oxyphenylene unit content [10]. Molecular weight control of the polymer was achieved by the copolymerization with 2,4-dimethylphenol to give a soluble oligomer with a molecular weight of 5×10^2 [12].

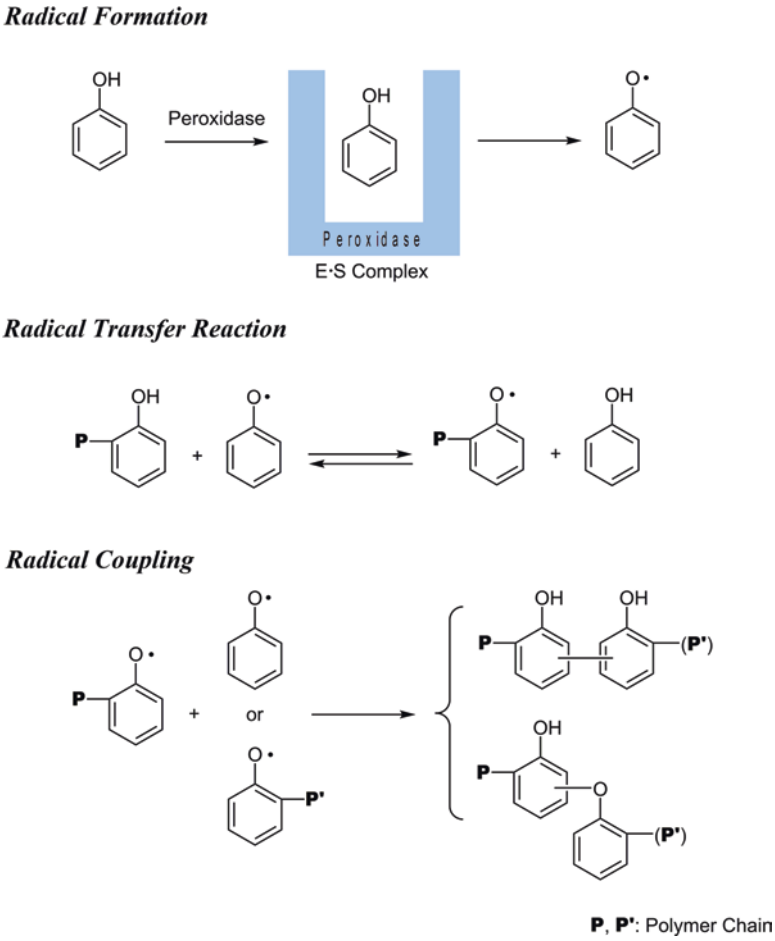


Fig. 9.3 Proposed mechanism of peroxidase-catalyzed oxidative polymerization of phenol

The proposed polymerization mechanism is shown in Fig. 9.3. A phenoxy free radical is first formed, then two molecules of the radical species dimerize via coupling. Since peroxidase often does not recognize larger molecules, a radical transfer reaction between a monomeric phenoxy radical and a phenolic polymer takes place to give the polymeric radical species. In the propagation step, such propagating radicals are subjected to oxidative coupling, producing polymers of higher molecular weight.

The enzymatic polymerization of phenols in aqueous solutions often resulted in the low yield of the insoluble polymer. The peroxidase-catalyzed polymerization of phenol took place in the presence of 2,6-di-*O*-methyl-α-cyclodextrin (DM-α-CD) in a buffer. Only a catalytic amount of DM-α-CD was necessary to induce the polymerization efficiently [13]. Poly(ethylene glycol) (PEG) was found to act as template for an oxidative polymerization of phenol in water. The presence of the PEG template in an aqueous medium greatly improved the regioselectivity of the polymerization,

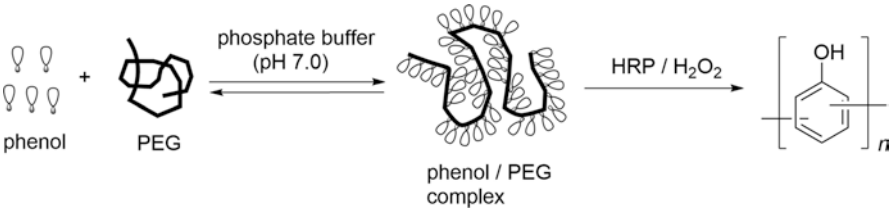


Fig. 9.4 Regioselective enzymatic polymerization of phenol using PEG as template

yielding a phenol polymer with the phenylene unit content higher than 90% (Fig. 9.4) [14, 15]. During the reaction, the polymer was produced in high yields as precipitates in complexing with PEG. The molecular weight of PEG strongly affected the polymer yield. The unit molar ratio of the phenolic polymer and PEG was ca. 1:1. The FT-IR and DSC analyses exhibited the formation of the miscible complex of the phenolic polymer and PEG by hydrogen-bonding interaction. PEG monododecyl ether, a commercially available nonionic surfactant, was also a good template for the polymerization of phenol in water. By using PEG-poly(propylene glycol) (PPG)-PEG triblock copolymer (Pluronic) with the high PEG content as template, the phenolic polymer with ultrahigh molecular weight (molecular weight > 10^6) was formed [16]. The regioselectivity was also high (the phenylene unit content of 86%). This polymerization method did not involve the use of organic solvents; hence it is regarded as being environmentally benign system.

Dispersion polymerization is a good tool to prepare the particles with controlled structure. Morphology of the enzymatically synthesized phenolic polymers was tuned under the selected reaction conditions. Monodisperse polymer particles in the submicron range were produced by the HRP-catalyzed dispersion polymerization of phenol in a mixture of 1,4-dioxane and phosphate buffer (3:2 v/v) using poly(vinyl methyl ether) as stabilizer [17, 18]. The particle size could be controlled by the stabilizer concentration and solvent composition. Thermal treatment of these particles afforded uniform carbon particles. The polymer particles were also prepared by HRP-catalyzed polymerization of phenol in the presence of PEG in an aqueous 1,4-dioxane, and palladium was loaded on the particle surface. This palladium complex showed high catalytic activity for Heck reactions of acrylic acid with aryl iodides [19]. When sodium dodecyl sulfate was used for the enzymatic polymerization of phenol in a phosphate buffer, the resulting polymer was partly soluble in common organic solvent [20]. The modification of the product by epichlorohydrin and the subsequent functionalization by triethylenetetramine and immobilization of palladium afforded the catalyst for Heck reaction.

A bienzymatic system was developed as catalyst for the oxidative polymerization of phenol [21]. The HRP-catalyzed polymerization of phenol in the presence of glucose oxidase (GOD) and glucose gave the polymer in a moderate yield, in which hydrogen peroxide was formed in situ by the oxidative reaction of glucose catalyzed by GOD (Fig. 9.5). In this system, no successive addition of hydrogen peroxide was involved.

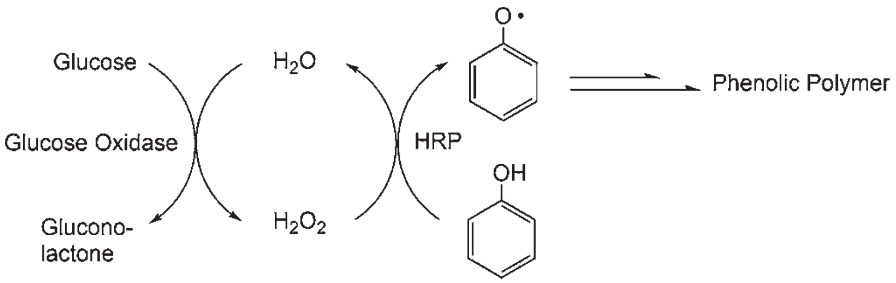


Fig. 9.5 Oxidative polymerization of phenol using glucose oxidase and peroxidase in the presence of glucose

HRP-catalyzed polymerization of phenol was conducted in the presence of TEMPO-oxidized nanocellulose to produce the nanocomposites of the phenol polymer and cellulose [22]. The phenol polymer in the nanocomposites existed as spherical or near-spherical clusters in the size of ca. 0.1 μm. FT-IR analysis showed the physical and chemical interaction between both components. Carbon nanotubes (CNTs) were used as template for the HRP-catalyzed polymerization of phenol in water to functionalize the CNT surface. The polymerization was conducted in the presence of the *p*-hydroquinone (HQ)-linked CNTs, in which the phenolic polymer was grafted through the HQ moiety on the CNT surface. The phenol monomer was regioselectively polymerized to possess mainly the thermally stable oxyphenylene unit [23]. A phenolic polymer-graft-PEG was synthesized by the enzymatic polymerization of phenol, followed by anionic polymerization of ethylene oxide [24]. The obtained graft copolymer was soluble in water, ethanol, DMF, THF, and methylene dichloride.

The polymerization behaviors and properties of the phenolic polymers depended on the monomer structure, solvent composition, and enzyme origin. In the HRP-catalyzed polymerization of *p*-*n*-alkylphenols in aqueous 1,4-dioxane, the polymer yield increased as the chain length of the alkyl group increased from 1 to 5 [25, 26]. HRP catalyzed the oxidative polymerization of all cresol isomers, whereas among *o*-, *m*-, and *p*-isopropylphenol isomers, only *p*-isopropylphenol polymerized by HRP catalysis [27]. Poly(*p*-*n*-alkylphenol)s prepared in aqueous 1,4-dioxane showed low solubility toward organic solvents, whereas a soluble oligomer with molecular weight lower than 1×10^3 was formed from *p*-ethylphenol using aqueous DMF [28]. Poly(4-*t*-butylphenol) (poly(4-TBP)) enzymatically synthesized in aqueous 1,4-dioxane showed T_g and melting point (T_m) at 182 °C and 244 °C, respectively. The product structure from *p*-cresol and *p*-propylphenol was studied in details by using NMR. A coupling mechanism of *p*-cresol was discussed from the structure of the dimers produced at the initial stage of polymerization [29, 30]. The structure and biodegradable properties of the polymers from various *p*-substituted phenols were examined [31–33]. The polymer particles of submicron size from *m*-cresol, *p*-cresol, and *p*-phenylphenol were obtained in the dispersion polymerization system as described above [17, 18].

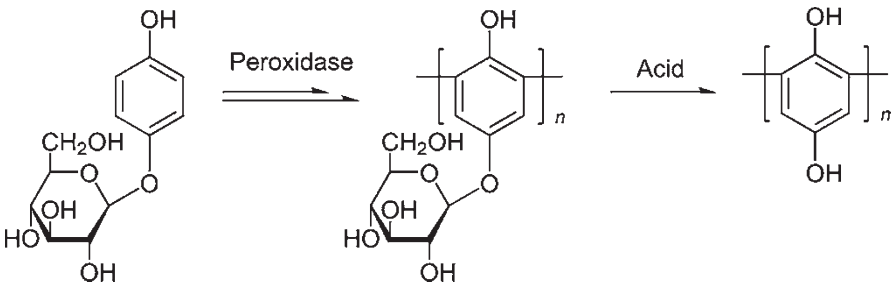


Fig. 9.6 Chemoenzymatic synthesis of poly(hydroquinone)

A natural phenol glucoside, 4-hydroxyphenyl β-*D*-glucopyranoside (arbutin), was subjected to regioselective oxidative polymerization using a peroxidase catalyst in a buffer solution, yielding the water-soluble polymer consisting of 2,6-phenylene units, in turn converted to poly(hydroquinone) by acidic deglycosylation (Fig. 9.6) [34]. Another route for the chemoenzymatic synthesis of poly(hydroquinone) was the SBP-catalyzed polymerization of 4-hydroxyphenyl benzoate, followed by alkaline hydrolysis [35]. Monodisperse nanoparticles of an oligomer from ferulic acid, bi-based phenolic compound, were formed by HRP-catalyzed polymerization in water [36].

Chemoenzymatic synthesis of a new class of poly(amino acid), poly(tyrosine) containing no peptide bonds, was achieved by the peroxidase-catalyzed oxidative polymerization of tyrosine ethyl esters, followed by alkaline hydrolysis. The resulting poly(amino acid) is different from the peptide-type poly(tyrosine) and soluble only in water. The oxidative homopolymerization of *N*-acetyltyrosine and the copolymerization of *N*-acetyltyrosine with 4-hydroxyphenyl *β*-D-glucopyranoside (arbutin) catalyzed by HRP were reported [37]. Oxidative polymerization of tyrosol by HRP afforded an insoluble product consisting of a mixture of linear oligomers (up to 11-mer). When this oligomer was loaded on porous poly(L-lactic acid) scaffold, a significant increase in the alkaline phosphatase activity compared to poly(L-lactic acid) alone, while tyrosol was completely inactive.

HRP catalysis induced a chemoselective polymerization of a phenol derivative having a methacryloyl group. Only the phenol moiety was polymerized to give a polymer having the methacryloyl group in the side chain. The resulting polymer was readily cured thermally and photochemically (Fig. 9.7) [38]. Phenolic monomers containing vinyl groups like 4′-hydroxy-*N*-methacryloyl anilide, *N*-methacryloyl-11-aminoundecanoyl-4-hydroxy anilide, and 4-hydroxyphenyl-*N*-maleimide were also chemoselectively polymerized at the phenol moiety by HRP catalyst. The polymerization proceeded in a buffer in the presence of cyclodextrin. The resulting phenolic polymers having the vinyl group were copolymerized with methyl methacrylate or styrene [39, 40], and the as-obtained polymers were subsequently cross-linked by a radical initiator [41].

A phenol with an acetylenic substituent in the meta position was also chemoselectively polymerized by HRP to give a polymer bearing acetylenic groups (Fig. 9.8)

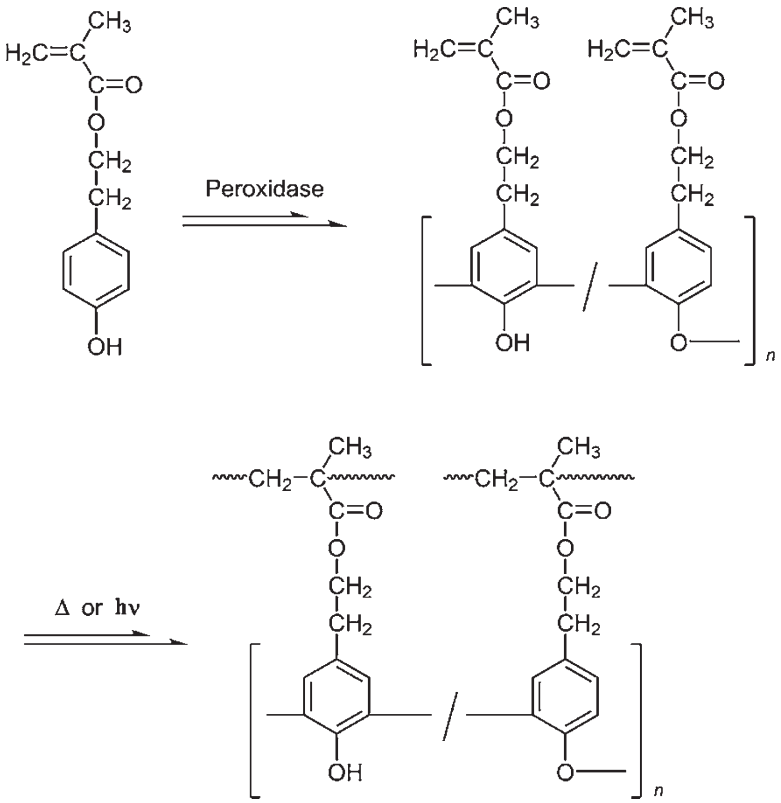


Fig. 9.7 Chemoselective enzymatic polymerization of methacrylate-containing phenol

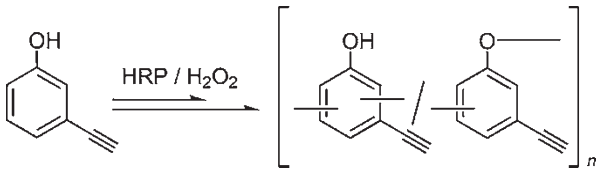


Fig. 9.8 Chemoselective enzymatic polymerization of acetylene-containing phenol

[42]. For comparison, the reaction of the monomer using a copper/amine catalyst, a conventional catalyst for an oxidative coupling, was performed, producing a diacetylene derivative exclusively. The resulting polymer was converted to a carbon polymer in much higher yields than enzymatically synthesized poly(*m*-cresol), suggesting a large potential as precursor of functional carbon materials.

A phenolic polymer soluble in acetone, DMF, DMSO, and methanol was formed from bisphenol-A via peroxidase catalysis [43]. The polymer was produced in higher yields using SBP as a catalyst and showed T_g at 154 °C. Peroxidase also induced the polymerization of an industrial product, bisphenol-F, consisting of

2,2′-, 2,4′-, and 4,4′-dihydroxydiphenylmethanes. Under the selected reaction conditions, the quantitative formation of a soluble phenolic polymer was achieved. Among the isomers, 2,4′- and 4,4′-dihydroxydiphenylmethanes were polymerized to give the corresponding polymers in high yields, whereas no polymerization of the 2,2′-isomer occurred. In the case of 4,4′-dihydroxyphenyl monomers, the bridge structure enormously affected the polymerization behaviors and the thermal properties of the resulting polymers.

Coprinus cinereus peroxidase (CiP), a fungal peroxidase, was used as catalyst for the polymerization of bisphenol-A in aqueous 2-propanol solution [44]. The yield, molecular weight, and structure of the polymer depended on the solvent composition. The polymer was mixed with a diazonaphthoquinone derivative to form a film and applied to a photoresist on the silicon wafer by UV irradiation. Sharply contrasted patterns were obtained from the polymer with molecular weight of 3×10^3.

α-Hydroxy-ω-hydroxyoligo(1,4-oxyphenylene)s were formed in the HRP-catalyzed oxidative polymerization of 4,4′-oxybisphenol in an aqueous methanol [45]. During the reaction, hydroquinone was formed. Figure 9.9 shows the postulated mechanism of the trimer formation; the redistribution and/or rearrangement of the quinone-ketal intermediate takes place, involving the elimination of hydroquinone.

Ortho-imine substituted phenol, (*E*)-2-((p-tolylimino)methyl)phenol, was polymerized using by HRP in aqueous organic solvents and hydrogen peroxide [46]. Different parameters such as solvent system, pH, and reaction temperature on the polymerization were investigated. Ethanol/pH 6.0 buffer (50:50 vol%) at 25 °C for 24 h under air was found to be the optimum polymerization condition to produce the polymer containing phenylene and oxyphenylene repeat units with molecular weight of 6.1×10^3 in 65%. 3,3′-((1*E*,1′*E*)-(1,3-Phenylenebis(azanylylidene)) bis(methanylylidene))diphenol, imine-substituted bisphenol derivative, was also polymerized by HRP in an equivolume mixture of an organic solvent (acetone, methanol, ethanol, dichloromethane, 1,4-dioxane, and tetrahydrofuran) and phosphate buffer (pH = 5.0, 6.0, 7.0, 8.0, and 9.0) [47]. The optimum reaction condition in terms of the highest yield (81%) and molecular weight of 1.1×10^4 was achieved in an equivolume mixture of tetrahydrofuran/pH 7.0 phosphate buffer medium.

(*E*)-2-((2-Phenylhydrazono)methyl)phenol was polymerized by HRP to yield the polymer with hydrazone functionality on the side chain [48]. The optimum polymerization condition with the highest yield (84%) and molecular weight of 8.0×10^3 was achieved by using an equivolume mixture of methanol and pH 6.0 buffer under air. The product was thermally robust and exhibited 5% mass loss at 375 °C.

Oxidative polymerization of phenols was conducted not only in the monophasic solvents but in interfacial solvents such as micelles, reverse micelles, and Langmuir trough systems [49]. *p*-Phenylphenol was polymerized in an aqueous surfactant solution, yielding the polymer with relatively narrow molecular weight [50]. For the HRP-catalyzed polymerization of phenol, sodium dodecylbenzenesulfonate was used to produce the polymer in high yields over a wide pH range from 4 to 10 [51]. The polymer showed good solubility toward organic solvents such as DMF, DMSO,

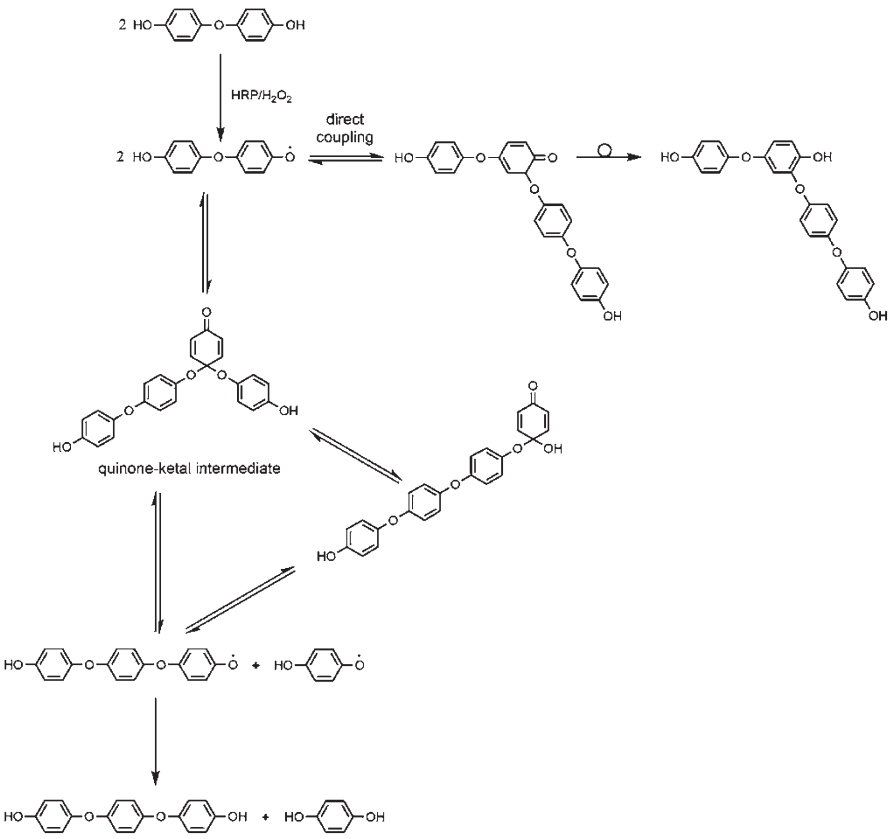


Fig. 9.9 Postulated mechanism of (1,4-oxyphenylene) trimer formation by enzymatic polymerization of 4,4′-oxybisphenol

acetone, and THF [52]. Guaiacol was polymerized by HRP in an aqueous micelle system [53]. The thermal antioxidant behavior of the obtained phenolic polymer for polypropylene (PP) was evaluated. The oxidation induction time of virgin PP was significantly improved, suggesting that enzymatically synthesized poly(guaicol) exhibited higher antioxidative performance than the corresponding monomer. Moreover, it showed good resistance against aging of PP.

Ionic liquids are effective as cosolvent for the enzymatic oxidative polymerization of phenols. In a mixture of a phosphate buffer and 1-butyl-3-methylimidazolium tetrafluoroborate or 1-butyl-3-methylpyridinium tetrafluoroborate, *p*-cresol, 1-naphthol, 2-naphthol, and *p*-phenylphenol were polymerized by SBP to give the polymers in good yields [54, 55]. For *p*-phenylphenol, the polymerization proceeded regioselectively to exclusively produce 2,2′-bi-(4-phenylphenol).

HRP-catalyzed oxidative polymerization of 4-hexyloxyphenol was conducted in an isooctane solvent. HRP was modified by ion-pairing with an anionic surfactant (aerosol AT, AOT), and *t*-butyl hydroperoxide was used as oxidant instead of hydro-

gen peroxide. The product polymer had the C-C coupled structure. The turnover number was about 5×10^3 [56]. *p*-Methoxyphenol was polymerized by HRP catalyst in an aqueous micelle system in the presence of sodium dodecyl sulfate. The resulting polymer showed good antioxidant properties [57]. HRP-catalyzed polymerization of 4-hydroxybenzene-diazosulfonate monomer produced the water-soluble polymer [58]. 4-Aminophenol was polymerized by HRP in water, and the resulting polymer showed good adsorption capacity for silver ions, which were reduced to form silver nanoparticles with face-centered cubic structure [59].

Urushi can be regarded as the only example of practical natural paints utilizing in vitro enzymatic catalysis for hardening. Main important components of urushi are known to be "urushiols," whose structure is a catechol derivative with unsaturated hydrocarbon chains consisting of a mixture of monoenes, dienes, and trienes at 3- or 4-position of catechol. Film-forming of urushiols proceeds under air at room temperature without organic solvents; hence, urushi seems very desirable for coating materials from environmental standpoints [60]. However, modeling study of urushi has been limited mainly due to the difficulty in chemical preparation of the urushiol. An urushiol analogue, an unsaturated higher alkyl group-containing phenolic monomer, was subjected to an oxidative polymerization by HRP to produce a soluble prepolymer, which was and further cured thermally or by cobalt catalyst to yield the cross-linked film with high gloss surface [61]. HRP-catalyzed copolymerization of 4-TBP and 4-ferrocenylphenol in an aqueous 1,4-dioxane produced the corresponding copolymer, which was further modified for preparation of inclusion complexes with β-cyclodextrin [62].

Schiff bases containing a phenol group, 4-(benzylidene-amino)-phenol and 4-[(anthracen-9-ylmethylene)-amino]-phenol, were polymerized by HRP in a mixture of 1,4-dioxane and phosphate buffer [63]. Large redshift was observed for the polymer from the latter, suggesting the increase of the conjugation length. HRP also catalyzed the oxidative polymerization of 4-[(4-phenylazo-phenyimino)-methyl] phenol, which possesses Schiff base and azo groups, yielding the polymer with the large potential for electronic and optic active materials [64]. A phenolic polymer from 4-(2-aminoethyl)phenol (tyramine) was prepared in capsules [65]. HRP-loaded capsules were first prepared via layer-by-layer assembly of polyelectrolites, poly(sodium 4-styrenesulfonate), and poly(allylamine hydrochloride). The selective permeability of the capsule wall allowed the monomer to penetrate, and the enzymatic oxidation polymerization of tyramine took place, while the product polymer and HRP remained in the capsule interior. This biocatalytic method provided a new way to synthesize functional materials in the microcapsules and to modify permeation properties of the microcapsule wall. Enzymatically synthesized poly(tyramine) selectively adsorbed platinum and palladium in HCl solution [66]. HRP also catalyzed the polymerization of 4-hydroxyphenylacetic acid on the polyelectrolyte capsule wall, on which HRP was assembled by layer-by-layer technique as both a catalyst and a coupling template [67]. The phenolic polymer formed the layers of 70–200 nm thickness and modified the permeability properties of the capsule wall.

The efficient phenolic polymer production was achieved by the peroxidase-catalyzed polymerization of *m*-alkyl substituted phenols in aqueous methanol. The mixed ratio of methanol and buffer greatly affected the yields and the molecular

weight of the polymer. The enzyme source greatly affected the polymerization pattern of *m*-substituted monomers. Using SBP catalyst, the polymer yield increased as a function of the bulkiness of the substituent, whereas the opposite tendency was observed when HRP was the catalyst. These enzymatically synthesized phenolic polymers were applied to positive-type photoresists for printed wire boards, because of their high solubility toward alkaline solution and high thermal stability [68, 69]. Various *m*-substituted phenols were oxidatively polymerized in an aqueous buffer in the presence of an equimolar amount of cyclodextrins to give the product polymers in high yields [70]. CiP-catalyzed copolymerization of *m*-cresol and 2,2′-methylenebis[6-(2-hydroxy-5-methylbenzyl)-*p*-cresol was examined in an aqueous acetone [71]. The photoresist was prepared by mixing the copolymer with a diazonaphthoquinone derivative. The copolymer with high hydroxyl value showed remarkably improved dissolution characteristics.

Cardanol, a main component obtained by thermal treatment of cashew nut shell liquid (CNSL), is a phenol derivative having mainly the meta substituent (R) of a C_{15} unsaturated hydrocarbon chain with one to three double bonds as the major. Since CNSL is nearly one-third of the total nut weight, much amount of CNSL is obtained as by-products from mechanical processes for the edible use of the cashew kernel. Only a small part of cardanol obtained in the production of cashew kernel is used in industrial fields, though it has various potential industrial utilizations such as resins, friction-lining materials, and surface coatings. Therefore, development of new applications for cardanol is very attractive [72–74]. A new cross-linkable polymer was synthesized by the SBP-catalyzed polymerization of cardanol (Fig. 9.10). The curing of the polymer took place in the presence of cobalt naphthenate catalyst at room temperature or thermal treatment (150 °C for 30 min) to form yellowish transparent films (“artificial urushi” in a broad sense, see also Fig. 9.14), which properties were similar to those of a traditional Japanese lacquer.

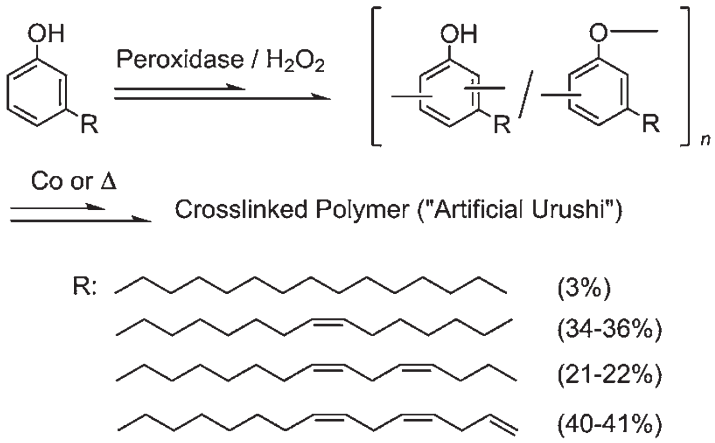


Fig. 9.10 Enzymatic oxidative polymerization of cardanol to artificial urushi

When HRP was used as catalyst for the polymerization of cardanol, the reaction took place in the presence of a redox mediator (phenothiazine derivative) to give the polymer [75]. CiP was also effective as catalyst for the polymerization of cardanol [76]. An equivolume mixture of *t*-butanol and phosphate buffer afforded the highest yield [77]. The curing of the polymer by using methyl ethyl ketone peroxide as initiator and cobalt naphthenate as accelerator was also examined [78]. Anacardic acid is another component of CNSL. SBP polymerized anacardic acid in an aqueous 2-propanol in the presence of phenothiazine-10-propionic acid (mediator) [79]. The polymer was cross-linked on a solid surface to form the coating film, which showed good antibiofouling effect for Gram-positive and Gram-negative bacteria.

Cardol is one of major components derived from CNSL having a C15 unsaturated hydrocarbon chain with 1–3 double bonds at a meta position of resorcinol. Its oxidative polymerization was carried out for the first time using *Coprinus cinereus* peroxidase. Under appropriate conditions, poly(cardol) with molecular weight of 1.4×10^4 was successfully obtained in 66% yield. Compared to poly(cardanol), poly(cardol) was rapidly cured at room temperature within 4 h to give cross-linked dry coatings with dark brown color. The curing rate of poly(cardol) was higher than that of poly(cardanol) irrespective of curing methods. TG analysis showed that poly(cardol) was more thermostable than poly(cardanol) obtained by curing at room temperature [77].

HRP catalyzed the oxidative polymerization of 2-hydroxycarbazole in a mixture of 1,4-dioxane and phosphate buffer [80]. The optical and electrochemical band gaps of the polymer were dramatically lower than those of 2-hydroxy-carbazole. The polymer showed good conductivity by doping with iodine vapor. *o*-Methoxyphenols (apocynin, vanillin, and 4-methylguaiacol) were polymerized by SBP catalyst in an aqueous buffer [81]. A variety of oligophenols (dimer to pentamer) as well as some of their oxidation products including quinones and demethylated quinones were formed. The oxidation of guaiacol is often used as tool of enzyme assay. The structure of the product obtained by HRP-catalyzed polymerization of guaiacol was examined; trimers in addition to the hitherto known dimeric products were isolated and characterized by NMR [82]. Micelle-templated polymeric nanowires were produced by HRP-catalyzed polymerization of guaiacol in a solution containing an ionic surfactant [83]. The TEM image of the product showed the formation of the nanowire.

HRP-catalyzed oxidative polymerization of *o*-, *m*-, and *p*-bromophenols were examined in water [84]. During the polymerization, the precipitate including HRP was formed, which was due to the strong interaction between HRP and the product polymer. For the meta isomer, the lowest precipitation yield was observed. The addition of PEG suppressed the precipitation, leading to the enhancement of the oxidation, whereas the highest inactivation of HRP took place in the copolymerization. In the SBP-catalyzed polymerization of phenol, the enzyme activity in the precipitate remained [85]. By the addition of a surfactant (Triton X-100), the adsorption of SBP was reversible.

Poly(2,6-dimethyl-1,4-oxyphenylene) (poly(phenylene oxide), PPO) is a material widely used as high-performance engineering plastics, thanks to its excellent chemical and physical properties, e.g., a high T_g (ca. 210 °C) and mechanically tough property [6]. PPO was first prepared from 2,6-dimethylphenol monomer using a copper/amine catalyst system. 2,6-Dimethylphenol was polymerized via HRP catalysis to give a polymer exclusively consisting of 1,4-oxyphenylene unit [86], while small amount of Mannich base and 3,5,3′5’-tetramethyl-4,4′-diphenoquinone units are always contained in the chemically prepared PPO. HRP was immobilized on silica nanorods and used as catalyst for polymerization of 2,6-dimethylphenol [87]. Substantially the enhanced enzymatic activity and reusability were found in comparison with those with the free enzyme.

HRP and SBP induced a new type of oxidative polymerization of the 4-hydroxybenzoic acid derivatives, 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid), and 3,5-dimethyl-4-hydroxybenzoic acid [88]. The polymerization involved elimination of carbon dioxide and hydrogen from the monomer to give PPO derivatives with high molecular weight up to 1.5×10^4 (Fig. 9.11). Demethylation of the polymer from syringic acid by boron tribromide catalyst gave poly(2,6-dihydroxy-1,4-oxyphenylene), which was thermally stable below 300 °C under nitrogen [89].

Coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol, CoA) is a phenolic lignin monomer (monolignol) contained in plant cell walls, whose dehydrogenated polymers (DHP, coniferyl alcohol polymers) are regarded as synthetic lignin, a model of cell walls. CoA was polymerized by HRP in a pectin solution in order to mimic the lignification that is the final step of biosynthesis of plant cell walls. The polymerization behaviors and product structures with cluster formation were examined in detail by various physical methods [90]. CoA was also polymerized by HRP in the presence of α-cyclodextrin (α-CD) in a phosphate buffer. The presence of α-CD led to DHP with 8-O-4′-richer linkages, compared with that prepared without any additives. This is probably because the inclusion complex formation between CoA and α-CD suppresses the formation of other linkages like 8-5′ and 8-8′ ones due to steric hindrance of the complex [91]. The peroxidase-catalyzed copolymerization of coniferyl alcohol and sinapyl alcohol in different ratios was conducted to examine the monolignol coupling mechanism [92]. CiP produced the copolymer in higher yield than HRP or SBP. Modification of sulfomethylated alkali lignin catalyzed by HRP was examined [93]. By this enzymatic treatment, the carboxyl group

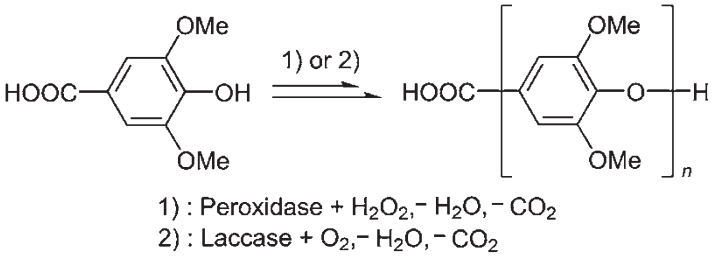


Fig. 9.11 Enzymatic oxidative polymerization of syringic acid to PPO

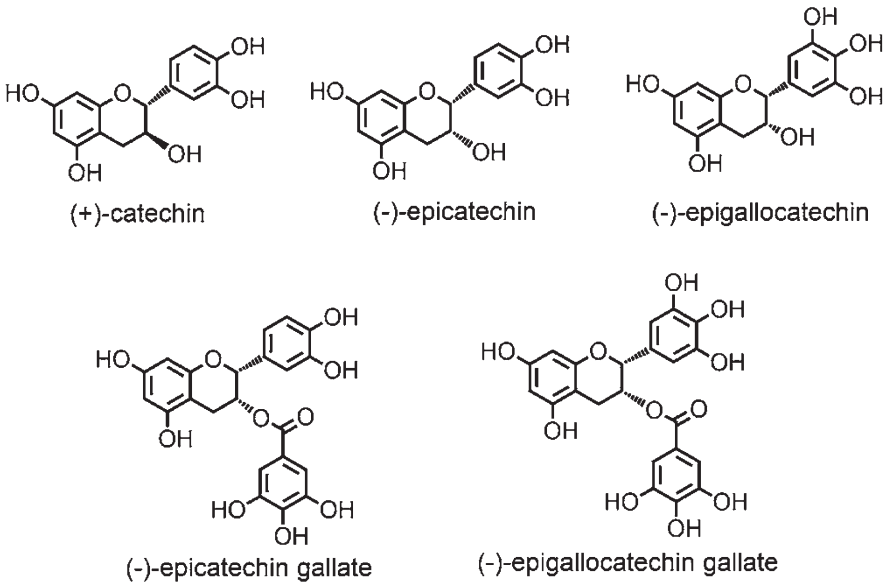
content increased, while the contents of the phenolic group and methoxy group decreased.

Peroxidase-catalyzed oxidative polymerization of 2-naphthol in a reverse micellar system gave the polymer in single and interconnected microspheres [94]. The polymer showed the fluorescence characteristics of the 2-naphthol chromophore. The polymerization of 8-hydroxyquinoline-5-sulfonate was studied by in situ NMR spectroscopy, and the polymerization mechanism was discussed in detail [95]. 1,5-Dihydronaphtahalene (1,5-DHN) was polymerized by HRP to produce poly(1,5-DHN) consisting of the 1,5-dihydroxynaphthalene unit, 1(or 5)-hydroxy-5(or 1)-oxynaphthalene unit, and 1,5-dioxynaphthalene unit [96]. HRP-catalyzed polymerization of 2,6-DHN proceeded regioselectively at the aromatic ring to give the polymer exclusively with the 2,6-dihydroxynaphthalene unit. Reaction of poly(1,5-DHN) with Al(Et)(2-methyl-8-quinolinolato)$_2$ afforded the Al-O bond formation, yielding a photoluminescent-aluminum complex.

Peroxidase efficiently induces an oxidative coupling of polyphenolic compounds with more than two hydroxyl groups on the aromatic ring(s). During the reaction, an unstable *o*-quinone intermediate is formed, which is converted to poly(catechol) [97]. Nanoscale polymer patterning was reported to be fabricated by the enzymatic oxidative polymerization of caffeic acid on 4-aminothiolphenol-functionalized gold surface with dip-pen nanolithography technique [98].

Bioactive polyphenols are present in a variety of plants and used as important components of human and animal diets. Flavonoids are a broad class of low molecular weight secondary plant polyphenolics, which are benzo-γ-pyrone derivatives consisting of phenolic and pyran rings. Their biological and pharmacological effects including antioxidant, antimutagenic, anticarcinogenic, antiviral, and anti-inflammatory properties have been demonstrated in numerous human, animal, and in vitro studies [99, 100]. Enzyme-catalyzed oxidative polymerization of flavonoid compounds and evaluation of the bio-related properties of the products have been studied [5, 101].

Major components of polyphenols in green tea are flavanols, commonly known as catechins; the major catechins in green tea are (+)-catechin, (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG) (Fig. 9.12). Numerous biological activities have been reported for green tea and its contents, among them, the preventive effects against cancer are most notable. Peroxidase catalyzed the oxidative polymerization of catechin to produce the oligomer with degree of polymerization less than five [102]. Kinetic study on the oxidation of catechin by peroxidase from strawberries showed highly efficient catalytic activity of the enzyme at low concentration of hydrogen peroxide [103, 104]. The reaction products obtained by HRP catalyst were analyzed by reversed-phase and size-exclusion chromatographies [103, 104]. The products obtained by using HRP and polyphenol oxidase as catalyst were comparably studied. Both enzymes produced the similar products [105]. The formation of two unusual dimers was detected in the product produced by HRP; one was the dicarboxylic acid compound with C-C linkage between C-6′ of B-ring and C-8′′ of



C-ring and C-8″ of D ring.

Fig. 9.12 Structure of main catechin derivatives

D-ring, which was formed by *ortho* cleavage of the E-ring, and another was the dimer of C-C linkage between C-2 of C-ring and C-8″ of D ring.

Similar to most of phenol and monosubstituted phenols, enzymatic polymerizations of natural polyphenols in a mixed solvent of polar solvent and buffer have been investigated. The HRP-catalyzed polymerization of catechin in an equivolume mixture of 1,4-dioxane and buffer (pH 7) produced the polymer with molecular weight of 3.0×10^3 [106]. Using methanol as cosolvent improved the polymer yield and molecular weight. A water-soluble oligomer was formed by HRP-catalyzed polymerization of catechin using a polyelectrolyte like a sulfonated polystyrene as template and a surfactant like sodium dodecylbenzenesulfonate [107].

Superoxide anion scavenging activity of the enzymatically synthesized poly(catechin) was evaluated. Poly(catechin), synthesized by HRP catalyst, greatly scavenged superoxide anion in a concentration-dependent manner and almost completely scavenged at 200 μM of a catechin unit concentration [108]. It is known that catechin showed prooxidant property in concentrations lower than 300 μM. These results demonstrated that the enzymatically synthesized poly(catechin) possessed much higher potential for superoxide anion scavenging, compared with intact catechin. Furthermore, the enzymatically synthesized poly(catechin) also showed much greater inhibition activity against human low-density lipoprotein (LDL) oxidation in a concentration dependent manner, comparing to the catechin monomer. Chelating ability of polymers enzymatically synthesized from phenol, catechol, and

pyrogallol was examined [109]. The binding capacity of the polymer from phenol for copper ion was larger than that from catechol or pyrogallol. Gold ion was selectively reduced by the phenol group in poly(pyrogallol) in acid media to form gold particles.

Enzymatic oxidative coupling was applied for enzyme-triggered supramolecular polymerization and assembly by the elaborate design of a supramonomer [110, 111]. Upon selective host-guest complexation followed by specific HRP-catalyzed coupling of phenol-containing supramonomer, the preparation of the supramolecular polymers was rapidly and effectively achieved. Moreover, the enzymatically prepared supramolecular polymers exhibited excellent concentration-dependent hierarchical self-assembly behavior.

9.2.2 Laccase

Laccase is an enzyme containing copper as active center. Laccase catalyzed an oxidative polymerization of various phenols such as phenol, *m*-cresol, bisphenol-A (BPA), and 4-TBP with oxygen as an oxidant [112]. The reaction was carried out in an aqueous alcoholic solvent (pH 5, acetate buffer) at room temperature with using laccases from *Pycnoporus coccineus* (PCL) as catalyst. The product polymers were obtained in high yields, and the number-average molecular weights were 2.3×10^3, 1.5×10^4, 2.1×10^4, and 1.9×10^3, for phenol, *m*-cresol, BPA, and 4-TBP, respectively. The polymer structure consisted of both phenylene and oxyphenylene units. The unit ratios of polymers for these four substrates were 48:52, 46:54, 45:55, and 52:48, respectively. For 4-TBP, 2-propanol content in the solvent (from 30% to 60%) controlled the unit ratio from 65:35 to 46:54.

In order to find the optimal operational conditions for maximum initial reaction rate in the laccase-catalyzed polymerization of BPA, a multistep response surface methodology (RSM) was applied [113]. The enzymatic polymerization rate of BPA was studied through RSM, based on the measurements of the initial dissolved oxygen consumption rate in a closed batch system. The optimal conditions were evaluated to be 748 mg/L, 32.2 °C, and 15.9% for monomer concentration, temperature, and solvent content, respectively. These results were in good agreement with the observed responses.

o-, *m*-, and *p*-Methoxyphenols as well as 2,6-dimethoxyphenol were oxidatively polymerized by a fungal laccase catalyst using oxygen in air as oxidant [114]. From *o*-methoxyphenol, a polymer with molecular weight of $7–11 \times 10^3$ was obtained. Polymerization rate was correlated with the energy of the highest occupied molecular orbitals (E_{HOMO}) of the phenol monomers. Laccase-catalyzed oxidative polymerization of 1-naphthol took place in aqueous acetone to produce the polymer having the molecular weight of several thousands [115].

Linear-dendritic (amphiphilic block copolymer of poly(ethylene glycol) and poly(benzyl ether) dendron)/laccase complexes catalyzed the polymerization of DL-tyrosine in a water solvent, which is regarded as the enzyme-catalyzed green

synthesis of an unnatural poly(amino acid) under environmentally friendly conditions [116]. The polymer possessed a mixed structure of phenylene and oxyphenylene units. Depending on the reaction conditions, the molecular weight reached 8.2×10^4, and the polymer yield was in the range of 45% and 69%. It was also found that the linear-dendritic laccase complexes induced the further chain growth upon addition of the fresh monomer to the preformed poly(tyrosine).

Reaction behaviors in the laccase catalyzed polymerization of coniferyl alcohol depended on the origin of the enzyme. PCL and *Coriolus versicolor* laccase showed a high catalytic activity to produce the dehydrogenative insoluble polymer, whereas laccase from *Rhus vernicifera* showed a much reduced activity [117]. In the case of laccase from *Trametes versicolor* (TvL), the molecular weight increase was observed by the treatment of soluble lignin [118].

The parameter effect in the laccase-catalyzed polymerization of sodium lignosulfonates (SLS) was compared with that of the chemical treatment by Mn(III). It was found that the enzymatic process was more efficient than a Mn(III) catalyst. SLSs of different initial molecular weight (1.8×10^4, 4.3×10^3, and 2.5×10^3) were employed. SLS with the highest molecular weight produced the polymer with increased molecular weight (1.1×10^5) [119].

A new oxidative polymerization of 4-fluoroguaiacol (4-fluoro-2-methoxyphenol) was achieved using TvL as catalyst in acetone or methanol and buffer (pH 5) mixture media with oxygen as oxidant to give poly(2-methoxy-1,4-phenylene oxide) [120]. The polymer having low molecular weight up to seven repeat units was derived by defluorination during phenyl-oxy propagation. The polymer was a novel photoluminescent material displaying fluorescence with emissions in blue, green, and red upon applied UV light frequencies. Laccase also catalyzed the oxidative polymerization of syringic acid to poly(2,6-dimethoxy-1,4-phenylene oxide) involving carbon dioxide elimination (Fig. 9.10). Oxidative polymerization of 1,8-dihydroxynaphthalene was examined by using HRP and laccase as catalyst. MS analysis showed the formation of C-C coupling polymer. HRP produced higher molecular weight product, whereas laccase yielded only the oligomer. The obtained data are useful for an optimized synthetic protocol for a potential structural model of *Ascomyces* allomelanins.

The laccase-catalyzed polymerization of a lignin-based macromonomer, lignocatechol, was carried out in an ethanol/phosphate buffer solvent system to give cross-linked polymers in good yields. The copolymerization was also performed with urushiol to afford the corresponding copolymers in high yields. The polymerization was estimated to proceed through quinone radical intermediate at catechol ring to give the cross-linked polymers [121]. The oxidative coupling of aromatics with short nonpolar chains took place by laccase onto flax fibers and nanofibrillated cellulose with different lignin contents. Laccase facilitated the surface coupling to produce materials with different levels of hydrophobicity, increasing the resistance to water absorption. The highest hydrophobization levels of flax fibers was achieved by coupling dodecyl 3,4,5-trihydroxybenzoate, which yielded water contact angle of 80–96 degrees and water absorption times of 73 min [122].

Laccase also acted as catalyst for oxidative polymerization of polyphenolic compounds. Soluble poly(catechin) with molecular weight of around 3×10^3 was obtained in a mixture of acetone and acetate buffer (pH 5). The polymer synthesized in 20% acetone showed low solubility toward DMF, whereas the polymer obtained in the acetone content less than 5% was completely soluble in DMF. In the UV-vis spectrum of poly(catechin) in methanol, a broad peak centered at 370 nm was observed. In alkaline solution, this peak redshifted, and the peak intensity became larger than that in methanol. In the ESR spectrum of poly(catechin), a singlet peak at $g = 1.982$ was detected, whereas the catechin monomer possessed no ESR peak [123]. The polymerization of catechin greatly enhanced the antioxidant property. Poly(catechin) exhibited a much enhanced superoxide anion scavenging activity compared with the catechin monomer. Similarly, poly(catechin) showed greatly amplified xanthine oxidase inhibitory activity [123].

Furthermore, flavonoid compounds including rutin [124] and quercetin [125] were enzymatically polymerized primarily for the enhancement of biological activities such as antioxidant properties. The laccase-catalyzed polymerization of a water-soluble rutin derivative in an equivolume mixture of methanol and acetate buffer produced the polymer with molecular weight of 1×10^4 in a high yield. Superoxide scavenging nature of poly(rutin) was much improved as compared with rutin itself. A similar result was observed for LDL oxidation [124]. From poly(rutin) obtained by the laccase catalyst, rutin polymer fraction (RPF) was separated, which had 6–8 monomer units. Bioactivity of RPF showed more effectively suppressed adipogenesis in 3 T3-L1 adipocytes compared to monomeric rutin, indicating that enzyme-catalyzed polymerization improved bioactivity of flavonoids [126].

Oxidative polymerization of flavonoid compounds, quercetin and kaempferol, was examined by using laccase as catalyst. The polymer aggregate of quercetin with relatively low molecular weight was formed, which exhibited the higher antioxidant activity than the monomer quercetin. The product aggregate from kaempferol reached larger sizes and higher antioxidant activity in the beginning of the polymerization [127].

Among many polymers from substituted catechol or phenol compounds, "urushi" is a very unique natural macromolecule. Urushi is the cured and cross-linked material of urushiol harvested from a special urushi tree (*Rhus vernicifera*). It is the only example in nature that is polymerized with catalyst of natural enzyme of laccase and cured via oxidation in air. It is a traditional Japanese lacquer (more widely, oriental lacquer) known from ancient time, going back to more than 9000 years ago in Japan. This is understandable from the following view that Japan means the country name, while "japan" possesses meanings such as "urushi lacquer," "urushi wares," or "to coat urushi." In Japan, there are many national treasures coated with urushi, which is brilliant and tough and looks soft. Accordingly, chemical studies on structure of urushiol and its hardening mechanism to urushi have been performed by excellent chemists since more than a century ago [128–135]. Urushiol is found not only in Japan but in China and Korea, while laccol (from Vietnam, Taiwan) and thitsiol (from Thailand and Myanmar) are the main components in other oriental

lacquer trees. It is noted, by the way, that Yoshida discovered laccase enzyme in 1883 [128]. Natural urushi is formed by laccase-catalyzed polymerization of urushiol in air, oxygen gas as oxidant, followed by curing via slow air oxidation of the polymerized urushiol [136–139]. Urushiol is of catechol structure having an unsaturated C_{15} hydrocarbon chain at 3-position (Fig. 9.13) [136, 137]. Urushi sap is obtained from the urushi tree, which is known to cause allergy for human skin, like a so-called "poison ivy" tree often found in the USA or European countries.

Since natural urushi is very expensive and difficult to prepare in vitro, its chemistry was mimicked for approaching to "artificial urushi" (or "man-made urushi") [139] with expectation of cheaper cost for practical applications. Natural urushiol analogues having a catechol structure were newly prepared for this purpose (Fig. 9.14) [138]. 4-Hydroxymethylcatechol was esterified by an unsaturated alkenyl carboxylic acid by lipase catalyst to give 4-substituted catechol, which was called as an "urushiol analogue" (UA). UA was cured by laccase-catalyzed cross-linking reaction in air, giving rise to "artificial urushi" [138, 140]. This modeling is the first example of the single-step synthesis of urushi-like cured film from a

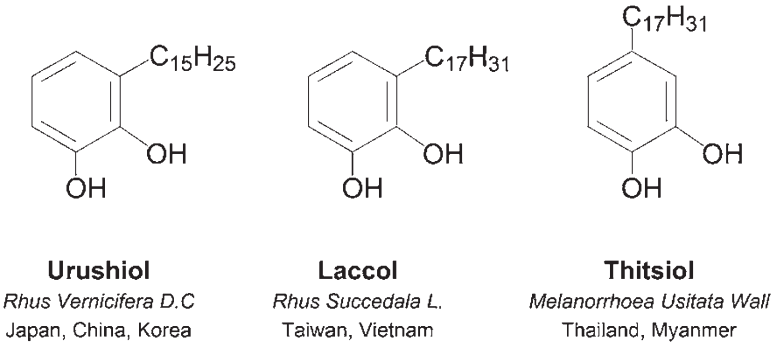


Fig. 9.13 Structure of urushiol and its analogues

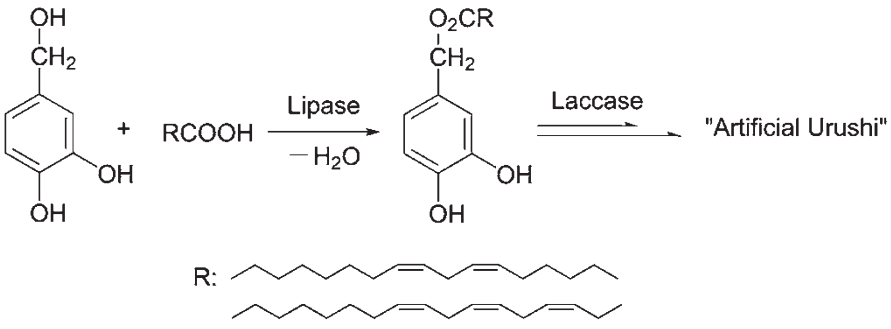


Fig. 9.14 Enzymatic curing of urushiol analogue to artificial urushi

monomeric phenol derivative. The enzymatic cross-linking of UA proceeded when R is a diene or triene. The reaction was carried out in the presence of acetone powder (AP, an acetone insoluble part of the natural urushi sap containing mainly polysaccharide and glycoprotein) at 30 °C under 80% relative humidity for 24 h. AP is believed to act as an emulsifier of oily urushiol and aqueous laccase solution. The curing of the triene-type UA was measured by a dynamic microhardness tester. The curing was slow at the beginning, and after 2 weeks, the hardness suddenly increased. Later the value reached about 150 Nmm^{-2}. The pencil scratch hardness was H, which is hard enough for practical usages. The hardness and gloss values of the cured film were compatible with those to natural urushi coatings. UA from the diene, on the other hand, showed very low hardness.

Laccase catalysis was examined for three natural phenolic lipids obtained from oriental lacquer trees (Fig. 9.13) [141]. Urushiol and laccol, both having an unsaturated hydrocarbon chain, C_{15} and C_{17}, respectively, at 3-position of catechol, were effectively cured in the presence of laccase enzymes to produce the cross-linked polymeric films with high-gloss surface and hardness properties. On the other hand, thitsiol having an unsaturated hydrocarbon chain (C_{17}) at 4-position of catechol was slowly cured with laccase, and the hardness attained was low.

Copolymerization of urushiol with a lignin-based macromonomer (lignocatechol) was examined by laccase catalyst. Both urushiol and lignocatechol were soluble in the mixed solvent of ethanol and phosphate buffer (70:30 vol%). The copolymerization was completed within 12 h to give the polymer with high thermal stability because of the cross-linked structure in good yields [121].

9.2.3 Other Enzymes

Tyrosinase (polyphenol oxidase, EC 1.10.3.1) and bilirubin oxidase also have copper species as active center. Tyrosinase and bilirubin oxidase can catalyze oxidative polymerizations of phenolic compounds by using oxygen gas (air) as an oxidant. There is a recent review paper available on tyrosinase enzyme from microbial origin with emphasis on their biochemical properties and discussing their current and potential applications for pharmaceutical, food bioprocessing, and environmental technology [142].

Tyrosinase from *Ustilago maydis* was used as catalyst for the oxidative polymerization of flavonoid compounds, quercetin and kaempferol. The as-obtained polymers showed strong scavenging effect on reactive oxygen species and inhibition of lipoperoxidation [127]. Bilirubin oxidase catalyzed the polymerization of 1,5-dihydroxynaphthalene in aqueous organic solvents to produce the insoluble polymer [143]. The UV spectrum of the film exhibited a wide band from 300 to 470 nm, showing a long π-conjugation structure. After treatment of the polymer with $HClO_4$, the polymer had an electroconductivity of 10^{-3} S/cm.

9.3 Enzymatic Oxidative Polymerization of Anilines and Thiophenes

In addition to phenols, other aromatic compounds such as aniline, thiophene, and pyrrole compounds are available for oxidative polymerization. Polyaniline (PANI) is extensively studied because of its wide potential for many technological applications such as organic lightweight batteries, microelectronics, optical displays, electromagnetic shieldings, and anticorrosion coatings. PANI can be prepared by either chemical or electrochemical oxidation polymerization of aniline monomer. Enzymatic polymerization provides an alternative method of a "green process," which is usually carried out at room temperature in aqueous organic solvents around neutral pH.

PANI is a typical example for the enzymatic polymerization using oxidoreductase enzymes [144]. Bilirubin oxidase [145] and HRP together with using H_2O_2 oxidant [146] were used as catalyst for an oxidative polymerization of aniline. In the latter case, sulfonated polystyrene (SPS) acted as polyanionic template, and the resulting polymer was complexed to the SPS [146]. Lignosulfonate was also used as a natural polyelectrolyte [147]. The reduction/oxidation reversibility of the PANI/SPS complex was demonstrated. The conductivity of the PANI/SPS complex was measured to be 0.005 S/cm. The value increased to 0.15 S/cm with HCl doping. The enzymatic approach is claimed to offer unsurpassed ease of synthesis, processability, stability (electrical and chemical), and environmental compatibility [148]. Instead of SPS, vesicles were employed as soft material template [149]. Micellar laccase [150] and GOD [151] were used to catalyze the polymerization of aniline. Ionic liquid was used to immobilize, and the polymerization took place at the ionic liquid/aqueous interface, where the aniline monomer, H_2O_2 oxidant, and dodecylbenzene sulfonic acid were present in the aqueous phase [152].

The conductivity of PANI is related with the backbone structure of PANI [153]. The branched structure lowered the conductivity [154], on the other hand, a linear structure with the help of a template like SPS led to the conducting materials [155]. HRP-catalyzed oxidative polymerization of aniline was carried out in the presence of a template, poly(acrylic acid) (PAA), and of a chiral compound, 10-camphorsulfonic acid (CSA) to generate a helical conformation [156]. Chiral PANI was also prepared by micellar peroxidase-catalyzed synthesis in the presence of dodecylbenzenesulfonic acid [157].

Poly(aniline-*co*-3-aminobenzeneboronic acid) was prepared via HRP-catalyzed oxidative copolymerization in the presence of SPS at pH 4.5, which was used as a boronic acid-based sensor for saccharide molecules [158]. PANI colloid particles were prepared by enzymatic polymerization of aniline, which shows potential applications for smart devices such as thermochromic windows, temperature-responsive electrorheological fluids, actuators, and colloids for separation technique [159].

Laccase isolated from the fungi, *Trametes hirsuta*, catalyzed the oxidative polymerization of aniline in micellar solutions of the anionic surfactant sodium dodecylbenzenesulfonate (SDBS), in which the atmospheric oxygen served as an oxidizing agent. The polymerization gave the stable dispersion of the electroactive

PANI/SDBS complexes. The laccase-catalyzed polymerization was kinetically controllable, and the mechanism was distinctly different from that of chemical polymerization. The antistatic properties of the prepared PANI/SDBS complex were studied. It was noted that the laccase-catalyzed synthesis of conducting PANI has advantages as compared with both peroxidase-catalyzed and chemical methods due to the use of air oxygen as oxidant in the aniline polymerization [160].

The enzyme-based imprinting approach using metal ions as the target analyte was investigated. HRP-catalyzed polymerization of various aromatic compounds [161] was performed in the presence of metal ions Cu(II), Ni(II), and Fe(III) as imprinting templates [162]. A new class of polyaromatics was synthesized by peroxidase-catalyzed oxidative copolymerization of phenol with *o*-phenylenediamine [8]. Poly(*p*-methoxyphenol-phenylamine) was synthesized by HRP-catalyzed copolymerization of *p*-methoxyphenol and phenylamine [163]. The product copolymer exhibited excellent antioxidation for various base oils ability at elevated temperatures.

Laccase-mediated system based on potassium octocyanomolybdate (4+) (redox mediator) was first used for acceleration of the enzymatic aniline polymerization [164]. The enzymatic reaction yielded oxidized octocyanomolybdate (5+) which can oxidize the aniline monomer to the aniline radical cation. In this system, the formation of conducting PANI with the concomitant regeneration of the redox mediator was observed. The presence of the mediator accelerated the polymerization of the monomer. The conducting PANI synthesized by the laccase-mediator method had the conductivity approximately five times of the polymer prepared by the laccase-catalyzed method.

PANI/multi-walled carbon nanotubes (PANI/MWCNT) composite was prepared by using fungal laccase, potassium octocyanomolybdate (4+), and atmospheric oxygen as catalyst, redox mediator, and terminal oxidant, respectively [165]. The enzymatic catalysis allowed conducting process of the oxidative aniline polymerization under environmentally friendly and rather mild conditions (aqueous slightly acidic solution and room temperature) without toxic by-products formation such as benzidine. The obtained PANI/MWCNT composite with PANI content of 49 wt% had high specific capacitance of 440 F/g measured by cyclic voltammetry technique with potential scan rate of 5 mV/s.

The in situ enzymatic polymerization of aniline onto MWCNT and carboxylated MWCNT (COOH-MWCNT) was examined [166]. The polymerization was catalyzed by HRP at room temperature in aqueous medium of pH 4. The as-obtained nanocomposites showed higher conductivity than pure PANI, which may be due to the strong interaction between the PANI chains and MWCNT.

The enzymatic polymerization of aniline to PANI with the use of TvL as catalyst was investigated in an aqueous medium containing unilamellar vesicles with an average diameter of about 80 nm [167]. The reaction yielded mainly overoxidized products, which had a lower amount of unpaired electrons in comparison with the products obtained with HRP isoenzyme C/H_2O_2 (the emeraldine salt form of PANI). The color changed from green (emeraldine salt) to blue (emeraldine base) upon exposure to ammonia gas, demonstrating the expected ammonia sensing properties.

PANI/Ag nanocomposites were successfully synthesized via in situ HRP-catalyzed polymerization of aniline based on 2-aminothiophenol (2-ATP)-capped Ag nanoparticles [168]. Smaller particles sizes were obtained with the enzymatic method than that with chemical oxidation.

The fabrication of cellulose/PANI composites was examined. PANI-based aqueous suspensions containing a variety of PANI contents ranging between 5 and 80 wt% were prepared through in situ polymerization of aniline in a nanocellulose suspension, which may find potential applications in flexible electrodes, antistatic coatings, and electrical conductors [169].

DNA-templated polymerization of 3-aminophenylboronic acid was examined by using HRP as catalyst [170]. Fluorescence property was induced within the as-obtained DNA-polymer conjugate using the excellent intercalating propensity of berberine toward base pairs of DNA. This fluorescent conjugate was used as cell imaging probe owing to the presence of boronic acid groups having cell adhesive property.

The chemical and enzymatic deposition of PANI films by in situ polymerization was studied [171]. The film formation and polymerization processes were simultaneously monitored by the evolution of the open circuit potential and quartz crystal microbalance measurements. The mechanism of the film formation on substrates by the enzymatic polymerization was proposed as follows. Before the hydrogen peroxide addition, some enzyme and aniline cations were adsorbed on the surface. After adding the hydrogen peroxide, the polymerization started in the solution, and it is highly likely that the PANI film grew from aggregation of particles by electrostatic adsorption and weak van der Waals forces.

Fully sulfonated PANI was synthesized by the peroxidase-mediated polymerization of aniline-2-sulfonic acid [172]. The sulfonated PANI obtained from chloroperoxidase, HRP, and chemical catalyst showed no differences in their UV-vis and FTIR spectra. The catalytic activity (k(cat)) was higher with chloroperoxidase than with HRP, but due to a higher affinity constant (K_M), the catalytic efficiency (k(cat)/K_M) of chloroperoxidase was lower than for HRP.

A novel H_2O_2 biosensor based on HRP-induced deposition of PANI on the designed graphene-carbon nanotube-nafion/gold-platinum alloy nanoparticles modified by glassy carbon electrode was constructed [173]. The enzymatically induced deposition of PANI provides a general platform for design of novel electrochemical biosensors. The enzymatic method realized highly efficient catalyzed deposition of PANI on the designed electrode, which improved the sensitivity and detection limit for H_2O_2 determination. A novel glucose biosensor was developed based on a 4-aminothiophenol/Au nanoparticle/GOD-HRP/6-mercapto-1-hexanol-11-mercaptoundecanoic acid/Au electrode [174]. The oxidative polymerization of aniline was induced by HRP and H_2O_2, in which the reduction of O_2 accompanied was by the oxidation of glucose into gluconic acid via GOD. The as-obtained biosensor exhibited wide linear range, high sensitivity, and good selectivity.

GOD from *Penicillium vitale* was immobilized on the carbon rod electrode by cross-linking with glutaraldehyde to produce a GOD-electrode [175]. The immobilized GOD was used for polymerization of aniline by taking a high concentration of

hydrogen peroxide produced during the catalytic action of the immobilized GOD and locally lowered pH due to the formation of gluconic acid. The GOD layer, which was self-encapsulated within the formed PANI matrix (GOD/PANI-electrode), showed an increase in the upper detection limit, optimal pH region for operation, and stability of GOD-based electrode modified by PANI in comparison with an unmodified GOD-electrode.

HRP was used as catalyst for the polymerization process leading to a water-soluble poly(3,4-ethylenedioxythiophene) (PEDOT) [176]. The temperature and pH of the essential polymerization conditions were 4 °C and 2, respectively. A similar HRP-catalyzed polymerization of EDOT gave a two-phase reaction system. The enzyme and EDOT formed droplets in the solution to trigger the polymerization in the interface, giving rise to the water-soluble conducting PEDOT. Toward the end of the polymerization, the polymer aqueous phase and the HRP/EDOT phase were separated, and the latter one acted as biocatalyst, which can be recycled and reused [177].

HRP-catalyzed oxidative polymerization of (3-thienyl)-ethoxy-4-butylsulfonate in an aqueous buffer produced the water-soluble polythiophene, which was effective for making hybrid solar cells [178]. The photoluminescence quenching behavior of the as-obtained polymer/titanium dioxide composite implied that the excitons dissociated and separated successfully at the interface.

Enzymatically induced formation of polythiophene layer over GOD-modified electrode was constructed [179]. Apparent K_m value of GOD-/polythiophene-modified electrode increased by prolongation of the polymerization duration. The enzymatic polymerization of thiophene could be applied by tuning of K_m and other kinetic parameters for GOD-based glucose biosensors.

PEDOT/poly(styrenesulfonate) (PEDOT/PSS) was synthesized by denatured catalase (no enzymatic activity) or iron-containing protein, transferrin [180]. Ion-containing proteins may be widely used as oxidants for the synthesis of conducting polymers.

An ultrasensitive assay for electrical biosensing of a DNA was developed by using target-guided formation of PANI base on HRP-catalyzed method [181]. PANI deposition was performed using the hybridized DNA strands as template. The hybridized DNA resulted in a negatively charged surface originated from the phosphate group, which provides a local environment of high acidity facilitating a predominantly head-to-tail coupling during the polymerization.

Laccase was used as a catalyst for an aniline dimer, PADPA (*p*-aminodiphenylamine) for the oxidative polymerization in aqueous SDBS micellar solutions [182]. The rate of the aniline dimer oxidation was markedly higher than the rate of the aniline oxidation under the similar conditions, suggesting that the aniline dimer formation may be the rate-limiting stage of the enzyme-catalyzed aniline polymerization. MALDI-TOF analysis showed the formation of the aniline oligomers with the polymerization degree of 4–22. TEM measurement showed that the PANI nanoparticles had a granular shape. PADPA was polymerized to poly(PADPA) at 25 °C with TvL/O_2 in the presence of vesicles formed using sodium bis(2-ethylhexyl) sulfosuccinate (AOT) as template [183]. The polymerization of PADPA pro-

ceeded much faster, and considerably fewer enzymes were required to complete the monomer conversion. Turbidity measurement indicated that PADPA was strongly bound to the vesicle surface before the polymerization. Such binding was confirmed by molecular dynamics simulations, supporting the assumption that the reactions which lead to poly(PADPA) are localized on the vesicle surface. Poly(PADPA) obtained resembled the emeraldine salt form of PANI in its polaron state with the high content of unpaired electrons. However, there were also notable spectroscopic differences between PANI and the enzymatically prepared poly(PADPA). Structure of poly(PADPA) might be similar to chemically synthesized poly(PADPA) obtained with ammonium peroxydisulfate as the oxidant in a equivolume mixture of ethanol and 0.2 M sulfuric acid [184].

Laccase-mediated system based on potassium octocyanomolybdate (4+) was used for acceleration of the enzymatic polymerization of aniline to give conducting PANI. The enzymatic reaction yielded oxidized octocyanomolybdate (5+) which could oxidize the aniline monomer to the aniline radical cation. Comparison of catalyst and mediator of laccase in the oxidative polymerization of aniline showed that the presence of the mediator accelerated the polymerization. The mediator system produced PANI in higher yield. The conductivity of PANI synthesized by the laccase-mediator method showed approximately five times as great as that by the laccase-catalyzed method. PANI/SDBS complexes synthesized by the both methods possessed a granular structure but differed in the particle size [164]. The aniline polymerization to PANI with TvL/O_2 system was investigated in an aqueous medium containing unilamellar vesicles with average diameter of 80 nm formed from AOT. Compared with the use of HRP isoenzyme C/H_2O_2, notable differences were found in the kinetics of the reaction, as well as in the characteristics of the PANI obtained. Under comparable optimal conditions, the reaction with TvL was much slower than with HRP [167].

The polymerization of pyrrole with the TvL and O_2 system in aqueous solution at pH 3.5 was found to be regulated by anionic vesicles formed from AOT to afford polypyrrole (PPy) [185–187]. The polymeric product obtained in the presence of the vesicular template had high absorption at $\lambda \approx 450$ and 1000 nm, which is indicative for PPy in its conductive bipolaron state. Absence of unpaired electrons in the bipolaronic PPy product was supported by EPR measurement. Furthermore, the FTIR analysis of the isolated PPy was comparable with that of chemically or electrochemically synthesized PPy.

GOD was used as catalyst for oxidative polymerization of aniline in aqueous solution to produce PANI nanoparticles [188]. The concentration of aniline, the enzyme, and the polymerization time significantly affected the PANI nanoparticle formation. The nanoparticles were obtained even at pH 6, and the formed PANI nanoparticles were pure from surfactants and interfering species.

9.4 Enzymatic Oxidative Coupling of Phenolic Compounds on Polymers

A polymer conjugate was prepared by laccase-catalyzed oxidation of catechin in the presence of poly(allylamine), leading to poly(allylamine)-catechin conjugate. The conjugate showed a good antioxidant property against LDL peroxidation induced by a free radical [189]. The conjugation of catechin on poly(ε-lysine) proceeded in the presence of laccase (Fig. 9.15), and the product poly(ε-lysine)-catechin conjugate showed greatly improved inhibition effects against disease-related enzymes, collagenase, hyaluronidase, and xanthine oxidase. While, the catechin monomer showed very low inhibition activity. The amplified activities might offer high potential as a therapeutic agent for prevention of various enzyme-related diseases [190].

Phenol-containing precursor, poly(amino acid)s, poly(α-glutamine), poly(α/β-asparagine), and poly(γ-glutamine) derivatives were subjected to oxidative coupling by HRP catalyst to produce a new class of soluble poly(amino acid)s without formation of insoluble gels. The process is suggested to be useful for development of new functional polymeric materials from renewable resources [191].

A tyramine-containing hyaluronan derivative was intermolecularly coupled by HRP to yield a cross-linked hydrogel (Fig. 9.16) [192]. The sequential injection of this hyaluronan derivative and peroxidase formed biodegradable hydrogels in vivo, offering high potential as a promising biomaterial for drug delivery and tissue engineering.

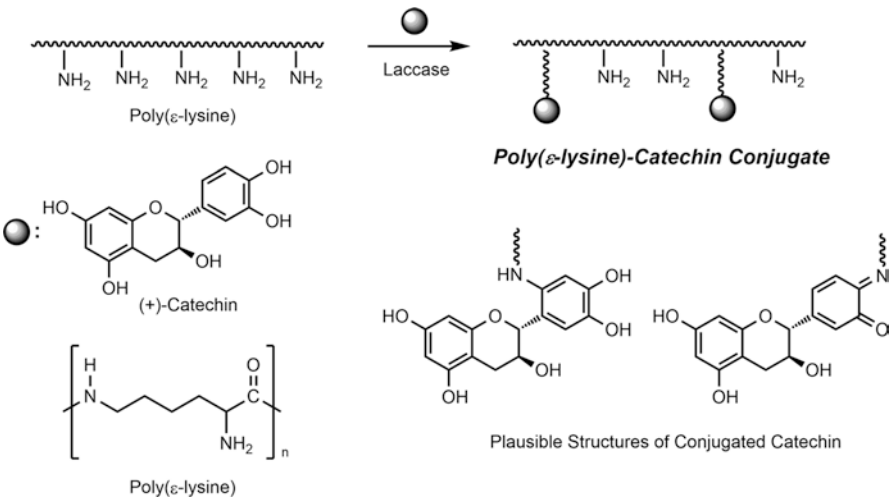


Fig. 9.15 Enzymatic conjugation of catechin on poly(ε-lysine)

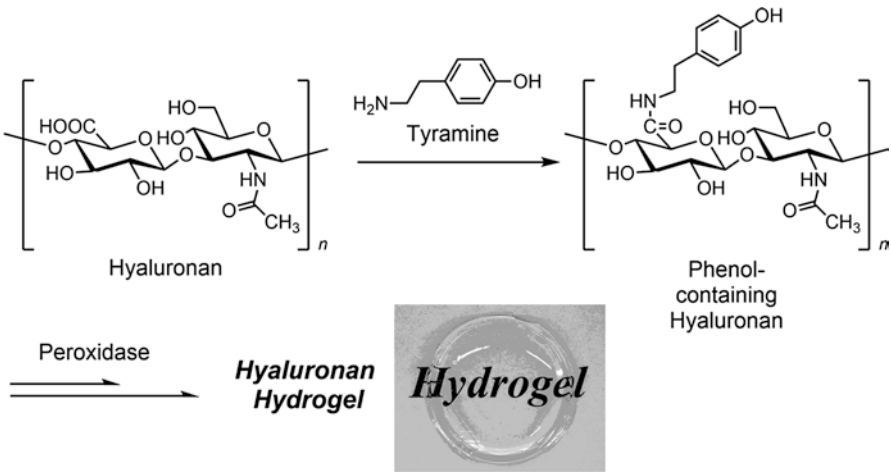


Fig. 9.16 Enzymatic cross-linking of phenol-containing hyaluronan to hyaluronan hydrogel

HRP catalyzed the cross-linking of fibroin and tyramine-substituted hyaluronic acid to form biocompatible hydrogels with tunable mechanical properties similar to that of native tissues [193]. The as-obtained hydrogels were assessed using unconfined compression and IR spectroscopy to show the physical properties over time in relation to polymer concentration. The hydrogels provide a biologically relevant system with controllable temporal stiffening and elasticity, thus offering enhanced tunable scaffolds in tissue engineering.

A microfluidic platform used for on-chip encapsulation of cells in stable microgels was developed by HRP-catalyzed coupling of tyramine-substituted dextran in water [194]. The obtained dextran-based microgels remained stable and sustained cell metabolic activity, providing a novel biomaterial class for the on-chip production of cell-laden microgels.

Laccase was used to catalyze the curing of synthetic polymers having phenol moiety in the side chain [195]. The phenol-containing polymer was prepared by the radical copolymerization of a phenol-containing methacrylate monomer and another methacrylate monomer. The copolymer film on a glass slide became insoluble in any solvents after curing with laccase/O_2 catalyst system. The cross-linking was confirmed by UV-visible spectrum; a strong absorption at 278 nm of the precursor copolymer shifted to 296 nm, indicating the C-C bond formation at the phenolic moieties.

Laccase was used for preparation of lignin-core hyperbranched copolymers [196]. Laccase-assisted copolymerization of kraft lignin with methylhydroquinone and a trithiol is produced. Tyrosinase was employed for modification of macromolecules. Mushroom tyrosinase is an oxidation enzyme capable of converting low molecular weight phenols and accessible tyrosyl residues of proteins like gelatin into *o*-quinones. These *o*-quinones are reactive and undergo nonenzymatic reactions

with a variety of nucleophiles. The primary amino groups of chitosan reacted with the quinone, giving rise to chitosan-natural phenol or chitosan-catechin conjugates via a Michael-type addition and/or a Schiff base formation [197–199].

Tyrosinase effectively catalyzed several reactions for the conjugate formation between chitosan and various proteins: cytochrome C [200], organophosphorus hydrolase [199], histidine-tagged chloramphenicol acetyltransferase [200], gelatin [201, 202], green fluorescent protein [203], silk fibroin [204–206], and silk sericin [207]. These tyrosinase-catalyzed reactions enabled proteins to be covalently tethered to a three-dimensional chitosan gel network. This covalent coupling allowed the easy fabrication of biocatalytically active hydrogel-based membranes, films, and coatings for a wide range of applications. Combining the biodegradable and biocompatible properties of chitosan, these novel hybrid biomaterials are good candidates as a promising material for gene and drug delivery as well as for regenerative medicine and tissue engineering. Tyrosinase also catalyzed the oxidative coupling of soluble lignin fragments to afford the insoluble polymers [208].

Designed peptides of Kcoil and Ecoil are known to hetero-dimerize in a highly specific and stable fashion to adopt a coiled-coil structure. Kcoil-functionalized chitosan was prepared using the tyrosinase-catalyzed oxidation of a tyrosine-containing Kcoil peptide. From the surface plasmon resonance investigation of coil-tagged epidermal growth factor (EGF)/Kcoil chitosan interactions, it was found that the conjugate could capture Ecoil-tagged EGF via coiled-coil-mediated interaction. In this case, the coiled-coil interaction was relatively low, and some improvements were essential for the general usage. This approach seems to provide with multiple added values to chitosan [209].

9.5 Conclusion

In this chapter, enzymatic syntheses of aromatic polymers such as phenolic polymers, polyanilines, and polythiophenes are overviewed. For the enzymatic oxidative polymerizations, the following advantages should be claimed in comparison with chemical processes: (i) nontoxic catalyst and mild reaction conditions and (ii) regio- and chemoselective polymerizations to produce useful polymers with precisely controlled structures, which may satisfy the increasing demands in the production of high-performance polymers in materials science. Last but not least, the enzymatic oxidative polymerization provides a great opportunity for using non-petrochemical renewable resources as substrates. This aspect allows the enzymatic polymerization to contribute to global sustainability and also to be a measure against depletion of scarce resources. The enzymatic polymerization has a large potential as an environmentally friendly synthetic process, providing a good example to achieve the goal of “green polymer chemistry.”

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Chapter 10
Synthesis of Poly(aromatic)s II: Enzyme-Model Complexes as Catalyst

Hideyuki Higashimura

Abstract This chapter deals with oxidative polymerization of aromatic monomers catalyzed by enzyme-model complexes to produce poly(aromatic)s. The enzyme-model complexes include Fe/porphyrin complexes and Fe/*N*,*N*′-bis(salicylidene) ethylenediamine complexes as Fe-containing peroxidase-models, Cu complexes having three nitrogen coordination atoms as Cu-containing monooxygenase models, and multinuclear Cu complexes as Cu-containing oxidase models. By using the enzyme-model complex catalysts, the aromatic monomers such as phenols, anilines, and pyrroles can be polymerized with H_2O_2 or O_2 as oxidants at ordinary temperatures in environmentally benign manners. The obtained poly(aromatic)s like polyphenols, poly(phenylene oxide)s, polyanilines, and polypyrroles possess excellent characteristics in mechanical strength, heat-resistance, and electric property. Enzyme-model catalysts have the following advantages in comparison with enzyme catalysts: (1) lower cost that is important in practical use, (2) applicability in various reaction conditions and monomers, and (3) possibility to express unique functions that have not seen even in enzymes. Hence, oxidative polymerization of aromatic monomers by enzyme-model complex catalysts would be expected as one of the new synthetic methods for advanced materials in green polymer chemistry.

Keywords Oxidative polymerization · Enzyme model · Peroxidase model · Tyrosinase model · Phenol · Aniline · Poly(aromatic) · Polyphenol · Poly(phenylene oxide) · Polyaniline

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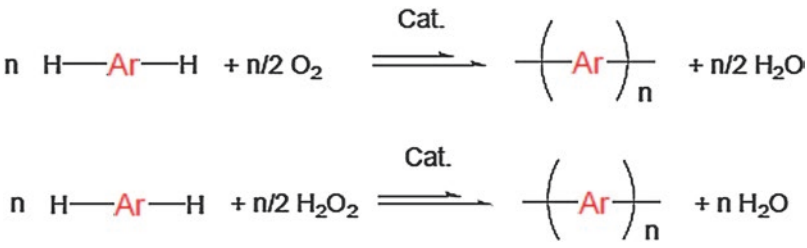
10.1 Introduction

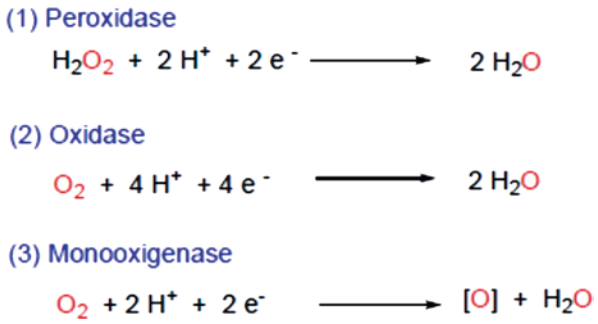
Poly(aromatic)s possess excellent characteristics in terms of mechanical strength, heat resistance, and electric property and thereby have been indispensable for advanced materials in the frontier fields of electronics, information communication, automobile, space, medicine, etc. For their synthesis, catalytic oxidative polymerization of aromatic monomers with O_2 or H_2O_2 as oxidants (Scheme 10.1) is one of the most environmentally benign methods, because the reaction temperatures are moderate and the by-products are water only [1]. The reaction mechanism of oxidative polymerization is generally considered as the coupling of radical species generated by one-electron oxidation from aromatic monomers.

Enzyme-catalyzed oxidative polymerization of aromatic monomers such as phenols, anilines, pyrroles, and so on has be recently attracted from the following reasons: (1) mild reaction conditions regarding temperatures, pressures, and pHs; (2) high coupling selectivity in substrate and chemoselectivity; and (3) nontoxic natural catalysts for ecological requirements (see Chap. 9) [2]. The enzymes applicable to oxidative polymerization catalysts are oxidoreductases that work in oxidative metabolism, including peroxidases, oxidases, and monooxygenases (Scheme 10.2), while dioxygenases ($O_2 \rightarrow 2$ [O]) generally cleavage aromatic rings. These oxidoreductases contain Fe or Cu complexes as the active centers (Fig. 10.1) and play important roles in the reaction with H_2O_2 or O_2 [3, 4].

Peroxidases perform twice one-electron oxidation with H_2O_2 to give two water molecules. Then, two molecules of substrates are oxidized to give two radical molecules ($2\ SH \rightarrow 2\ S\bullet + 2H^+ + 2e^-$), followed by radical coupling with each other ($2\ S\bullet \rightarrow S\text{-}S$). Representatively, horseradish peroxidase (HRP) and soybean peroxidase (SBP) are often employed for in vitro experiments, both of which have heme, an Fe/porphyrin complex (Fig. 10.1a), at the active sites. Although the Fe/porphyrin complex is contained in oxidases (e.g., cytochrome c oxidase) and monooxygenases (e.g., cytochrome P-450), the Fe-containing oxidases and monooxygenases have been little employed so far as the oxidative polymerization catalysts. Oxidases carry out one-electron oxidation four times with O_2, and as the typical polymerization catalysts, laccase and bilirubin oxidase containing tetranuclear Cu complexes (Fig. 10.1c) can oxidize four molecules of substrates,



Scheme 10.1 Catalytic oxidative polymerization of aromatic monomers with O_2 or H_2O_2 as oxidants



Scheme 10.2 Reactons with H_2O_2 or O_2 catalyzed by peroxidase, oxidase, and monooxigenase

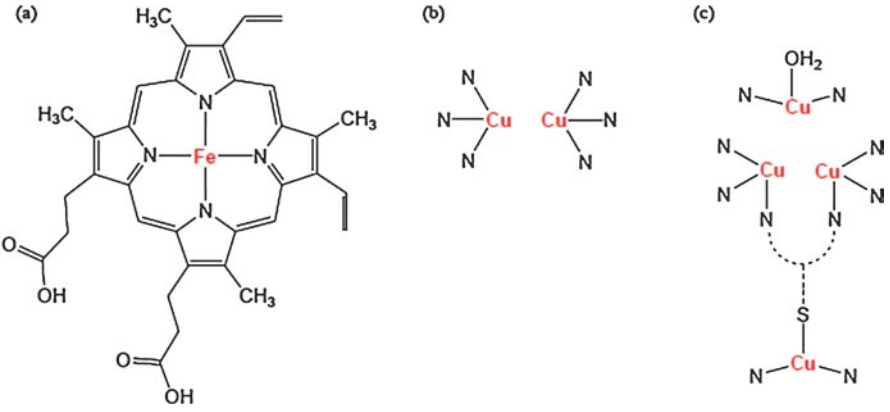
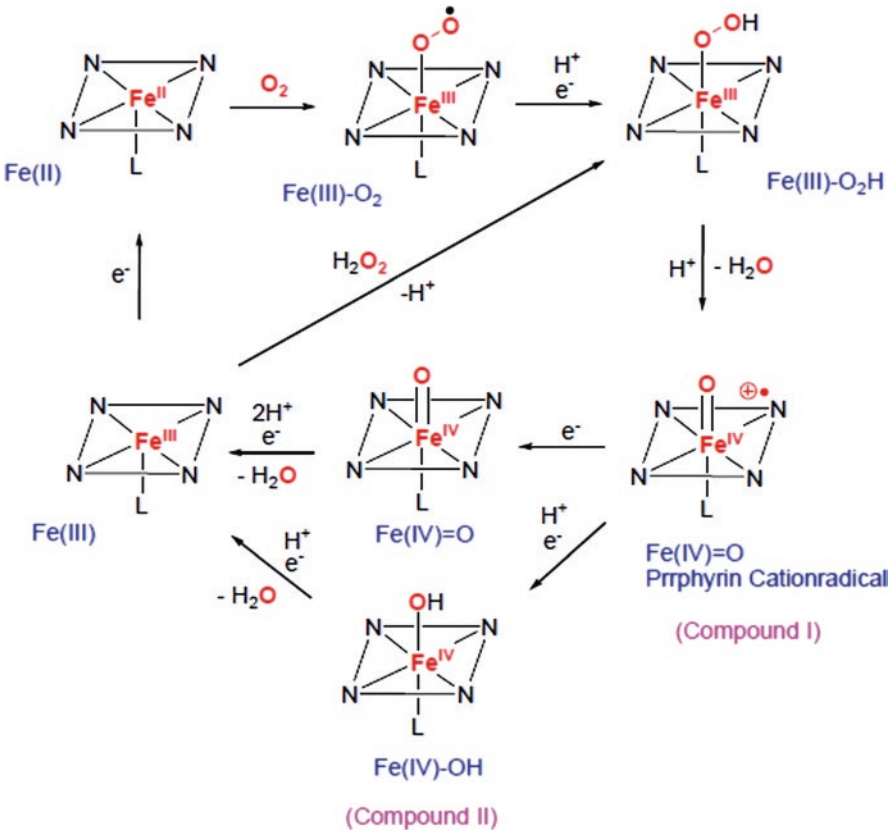


Fig. 10.1 Typical metal complexes as active sites of metalloenzymes: (**a**) heme (a Fe/porphyrin complex), (**b**) a dinuclear Cu complex, and (**c**) a tetranuclear Cu complex

leading to oxidative coupling. Monooxygenase generates one atom of activated oxygen from O_2 with two electrons and two protons; for example, tyrosinase with a dicopper active center (Fig. 10.1b) performs monooxygenation of the phenol moiety in tyrosine, resulting in formation of melanin.

The reaction mechanisms of oxidoreductases have been deeply understood through studying the structures and reactivities of their model complexes. The Fe/porphyrin complex is the active site of various Fe-containing oxidoreductases, but despite the diverse functions, they share common mechanistic bases (Scheme 10.3) [3]. First, Fe(III) complex is a resting state and reduced by one-electron to give Fe(II) complex, which reacts with O_2 to Fe(III)-O_2 complex, followed by one-electron reduction to Fe(III)-O_2H complex. Next, the O-O bond cleavage gives Fe(IV)=O/porphyrin cation radical (Compound I), which is the active oxygen intermediate of monooxygenases. Finally, the one-electron reduction affords Fe(IV)=O or Fe(IV)-OH complex (Compound II), and the further one-electron reduction regenerates Fe(III) complex; totally four-electron reduction of O_2 is conducted in



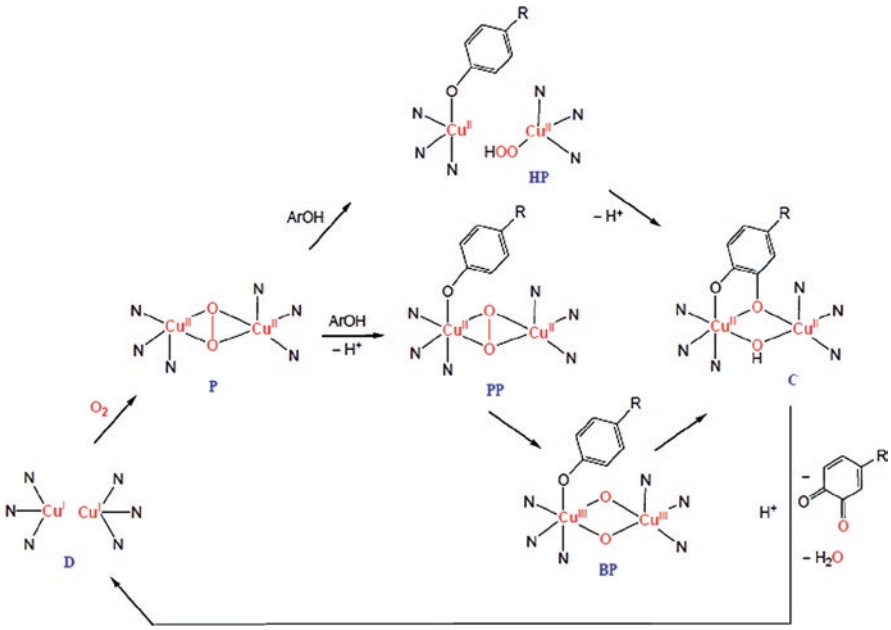
Scheme 10.3 Reaction mechanisms of Fe/porphyrin complexes in Fe-containing oxidoreductases

oxidases. In the reaction with O_2, oxidases and monooxygenases need strong reductants for the O_2-activation step from Fe(III) complex to Compound I.

However, for peroxidase, Fe(III) complex can directly react with H_2O_2 by a shunt path to afford Compound I and the following Compound II. Hence, peroxidases are the most employed as oxidative polymerization catalysts in Fe-containing oxidoreductases, owing to unnecessariness of strong reductants.

Cu-containing oxidases and monooxygenases have various structures of the active sites such as mononuclear, dinuclear, and tetranuclear Cu complexes, although peroxidases with Cu active centers do not exist. The reaction mechanisms of tyrosinase, a monooxygenase having a dinuclear Cu complex, and laccase, an oxidase possessing a tetranuclear Cu complex, are addressed here [4].

In the monooxygenation by tyrosinase (Scheme 10.4), the formation of μ-η^2:η^2-peroxo-dinuclear Cu(II) complex (**P**) as the active oxygen species is characteristic. Dinuclear Cu(I) complex (**D**) reacts with O_2 to give **P**, followed mainly by two



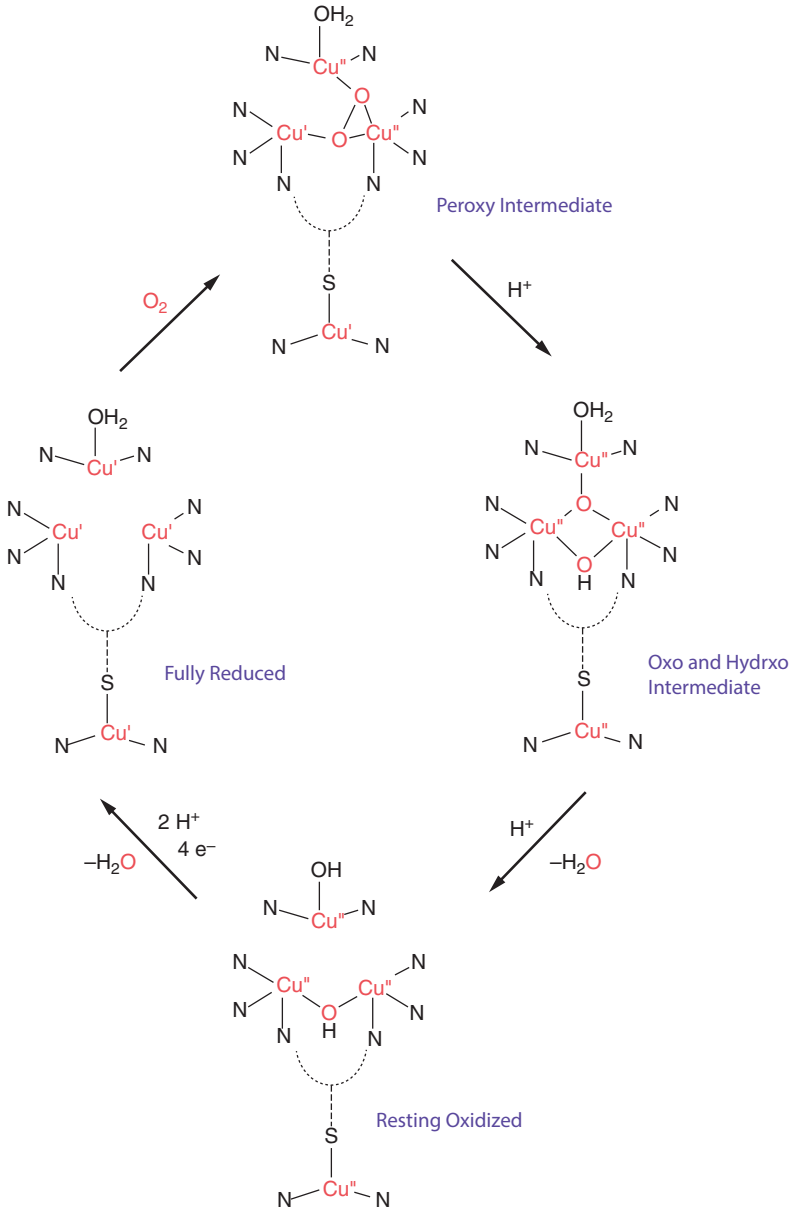
Scheme 10.4 Proposed reaction mechanisms of a dinuclear Cu complex in tyrosinase

proposed mechanisms. One [5] is that, because **P** is not electrophilic but nucleophilic, the reaction of **P** with a phenol affords phenoxo-Cu(II) complex and hydroperoxo-Cu(II) complex (**HP**), in which oxygenation at *o*-position of the phenol occurs. For the other [6], a phenolate coordinates with **P** (**PP**), followed by isomerization from **PP** to phenolate-bond bis(μ-oxo) dicopper complex (**BP**), leading to *ortho*-oxygenation. In both mechanisms, catecolato complex (**C**) is formed and regenerates **D** with formation of *o*-quinone, which is polymerized to produce melanin.

For the oxidation by laccase (Scheme 10.5) [4], tetranuclear Cu(I) complex (fully reduced) reacts with O_2 to give peroxo-Cu(II)$_2$Cu(I) and Cu(I) moieties (peroxo-intermediate), in which O-O bond cleavage affords μ_3-oxo-μ_2-hydroxo-Cu(II)$_3$ and Cu(II) moieties (oxo- and hydroxo-intermediate), followed by dehydration leading to μ-hydroxo-Cu(II)$_2$ and two Cu(II) moieties (resting oxidized). The tetranuclear Cu(II) complex can conduct one-electron oxidation with four molecules of substrates, regenerating the fully reduced complex. Thus, such tetranuclear Cu complexes seem suitable for four-electron oxidation with O_2.

This chapter deals with oxidative polymerization of aromatic monomers catalyzed enzyme-model metal complexes, shown in the following:

- Fe-Containing Peroxidase-Model Complex Catalysts
  - Fe/porphyrin complex catalysts
  - Fe/*N*,*N*′-bis(salicylidene)ethylenediamine complex catalysts



Scheme 10.5 Proposed reaction mechanism of a tetranuclear Cu complex in laccase

- Cu-Containing Monooxygenase- and Oxidase-Model Complex Catalysts
  - Cu complex catalysts having three nitrogen coordination atoms
  - Multinuclear Cu complex catalysts

10.2 Fe-Containing Peroxidase-Model Complex Catalysts

Peroxidases such as HRP and SBP possess the Fe/porphyrin complex at the active cites, in which four heteroatoms coordinate with one Fe ion in a square planar structure. As the Fe-containing peroxidase-model complexes, not only Fe/porphyrin complexes but also Fe/*N,N′*-bis(salicylidene)ethylenediamine (Fe(salen)) complexes are described here.

10.2.1 Fe/Porphyrin Complex Catalysts

Free heme with Fe(II) center (Fig. 10.1a) is unstable and rapidly oxidized to hematin having Fe(III)OH center (R = H in Fig. 10.2a), so hematin is employed as less expensive catalysts in oxidative polymerization than peroxidase catalysts. Akkara and coworkers reported in 2000 that oxidative polymerization of 4-ethylphenol catalyzed by hematin with H_2O_2 as the oxidant in a mixture of *N,N*-dimethylformamide (DMF) and pH 11 buffer solution produced a polymer as the insoluble part in a 72% yield (Scheme 10.6a) [7]. The polymer has a 5-ethyl-2-hydroxy-1,3-phenylene unit

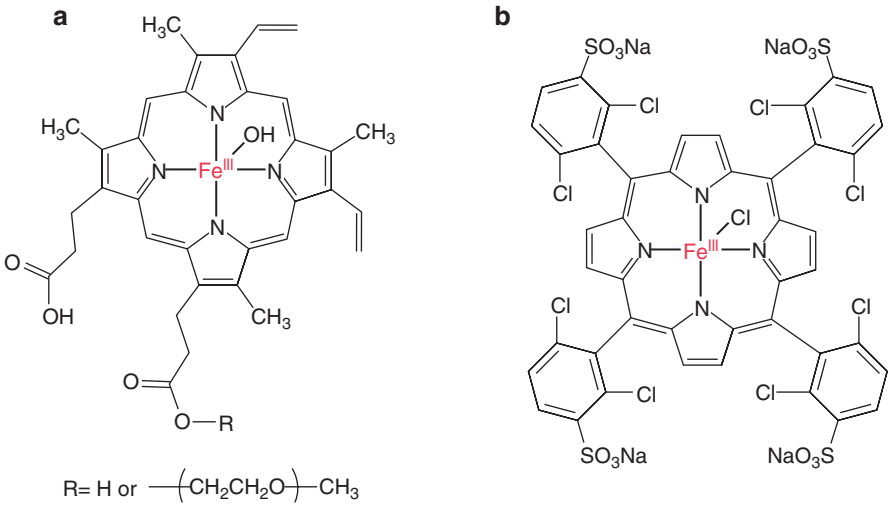
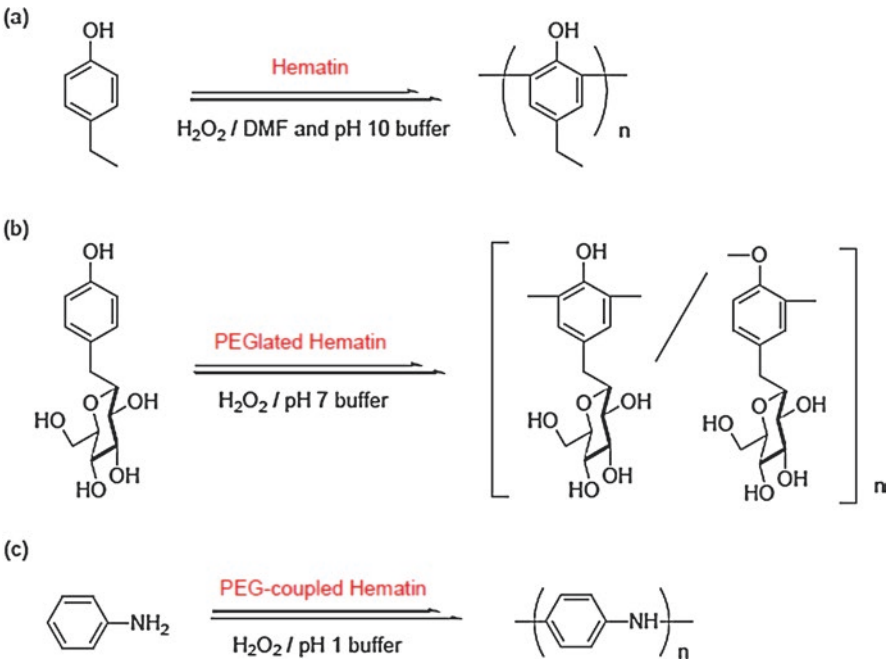


Fig. 10.2 Fe/porphyrin complexes: (**a**) hematins and (**b**) a water-soluble complex



Scheme 10.6 Oxidative polymerization catalyzed by hematin or modified hematins

(C–C coupling structures) form ^{1}H-nuclear magnetic resonance (^{1}H-NMR) and Fourier-transform infrared (FT-IR) spectroscopies. Although no other structures were not described, 4-ethyl-1,2-oxyphenylene units (C–O coupling structures) may be contained in the polymer. The number-averaged molecular weight (M_n) and weight-averaged molecular weight (M_w) by gel permeation chromatography (GPC) are 1150 and 1680, respectively. High pH value of the buffer resulted in high yield of the polymer, and below 5 at the pH, no polymers were obtained, because hematin shows low solubility in acidic conditions.

To improve the low solubility of hematin, the modification with polyethylene glycol (PEG) has been proposed for oxidative polymerization catalysts. A PEGylated hematin was synthesized from hematin and PEG-monomethyl ether (R = $-(CH_2CH_2O)_nCH_3$ in Fig. 10.2a) [8], and by the catalyst, polymerization of arbutin was performed with H_2O_2 in pH 7.0 buffer solution (Scheme 10.6b). The polymer was obtained in an 80% yield and had hydroxyphenylene or oxyphenylene units with M_n of ~5100. The catalysis by hematin itself gave only a small amount of oligomers with M_n of ~500 due to its low solubility. The HRP-catalyzed polymerization afforded polymers with M_n of ~2000 [9], because HRP allows only monomeric and low-molecular-weight oligomers to access the compounds I and II at the active sites (Scheme 10.3).

Polyaniline, one of the typical conducting polymers, was synthesized by HRP-catalyzed polymerization of aniline with H_2O_2 in the presence of a template such as poly(styrenesulfonate) (SPS) under pH 4.3 buffer conditions [10]. The use of SPS promotes C-N coupling at *p*-position of aniline even under such weakly acidic conditions by preferentially aligning aniline monomers onto SPS, although strongly acidic environments are believed to be necessary for linear polyaniline with high conductivity. On the other hand, another PEG-coupled hematin catalyst [11] can be used under strongly acidic conditions, and without any templates, the catalytic oxidative polymerization of aniline in pH 1.0 buffer produced polyaniline (Scheme 10.6c) [12]. The resulting polymer had conductivity of ~10^{-1} S/cm and possessed the structure resembling most with the ones prepared by using equivalent oxidants.

As a water-soluble iron/porphyrin complex, Fe/*meso*-tetra(2,6-dichloro-3-sulfonatophenyl)porphyrin complex (Fig. 10.2b) [13] was employed as the catalyst for oxidative coupling of humic phenolic substrates like caffeic acid and *p*-coumaric acid. The dimers from C–O and C–C couplings were detected by gas chromatography-mass spectrometry (GC-MS), but no oligomers lager than tetramers were formed.

The insolubility of hematin at low pH conditions is due to extensive aggregation, and to prevent the phenomenon, using micellar environments with sodium dodecylbenzenesulfonate (DBSA) was demonstrated (Fig. 10.3) [14]. A micellar solution was prepared by addition of DBSA in pH 3.5 buffer solution, forming hydrophobic core and hydrophilic shell structures. Hematin was loaded into the micelles, and in the nanoreactors, pyrrole was subjected to oxidative polymerization with H_2O_2. The polypyrrole synthesized at 4 °C showed conductivity of 10^{-1} S/cm, which is roughly four orders of magnitude higher than the one at 30 °C.

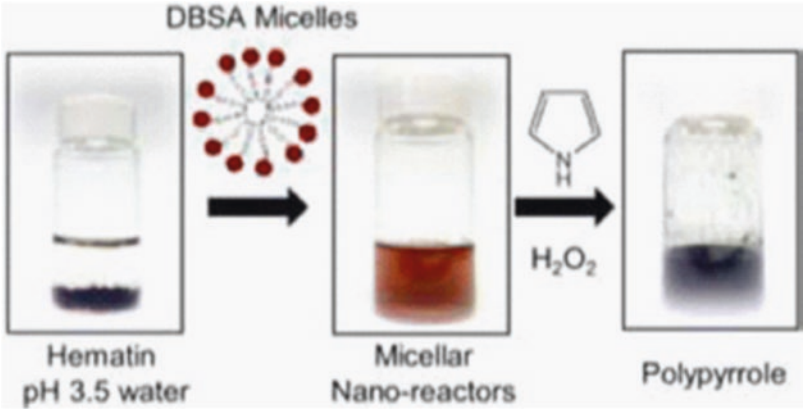


Fig. 10.3 Using micellar nanoreactors of DBSA for hematin-catalyzed synthesis of polypyrrole. (Reprinted with permission from Ref. [14]. Copyright © 2012 American Chemical Society)

10.2.2 *Fe/N,N'-Bis(salicylidene)ethylenediamine Complex Catalysts*

A Fe/porphyrin complex is a direct model of peroxidases but a little difficult to synthesize, while Fe(salen) complex is easily prepared from cheap materials of salicylaldehyde, ethylenediamine, and Fe salt. Fe(salen) complex is available in a reduced Fe(II) form (Fig. 10.4a) and an oxidized Fe(III) dinuclear form (Fig. 10.4b), and its solubility is higher in organic solvents but lower in water than peroxidases.

The first study for Fe(salen)-catalyzed oxidative polymerization of phenols is reported in the Japanese patent applied by the author and a coworker in 1994 [15]. For one of the examples, phenol was polymerized by the use of Fe(salen) catalyst with H_2O_2 as the oxidant in ethyleneglycol monomethylether/NaOH aq to produce an insoluble polymer in a 28% yield (Scheme 10.7a). The polymer obtained by Fe(salen) catalysis is speculated to have hydroxyphenylene (C–C coupling) and oxyphenylene (C–O coupling) units, resembling with the ones by HRP catalysis [16]. It seems to involve almost no significant difference for the coupling selectivity in oxidative polymerization of phenols between peroxidase catalysts and their model catalysts.

A soluble polyphenol from bisphenol-A was obtained by Fe(salen)-catalyzed oxidative polymerization, consisting of C–C and C–O coupling structures [17]. The polymer by Fe(salen) catalysis in pyridine/DMF possessed M_n of 33,000, while the ones by HRP catalysis in methanol/pH 7 buffer had M_n of 3800 [18]. Peroxidase-model catalysts can produce higher-molecular-weight polymers owing to easier accessibility to substrates than peroxidase catalysts. By Fe(salen)-catalyzed oxidative coupling of the polyphenols prepared by HRP, ultrahigh-molecular-weight polyphenols were synthesized: the M_n values increased from 3800 to 350,000 for poly(bisphenol-A) and from 2500 to 420,000 for poly(*m*-cresol) (Scheme 10.7b).

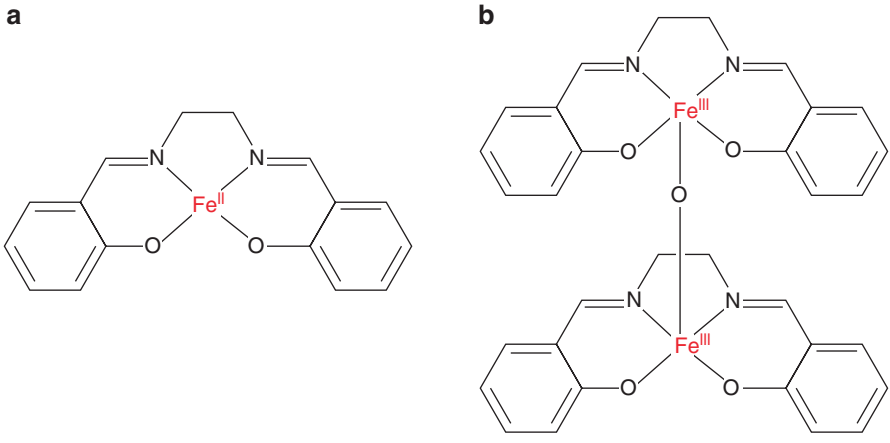
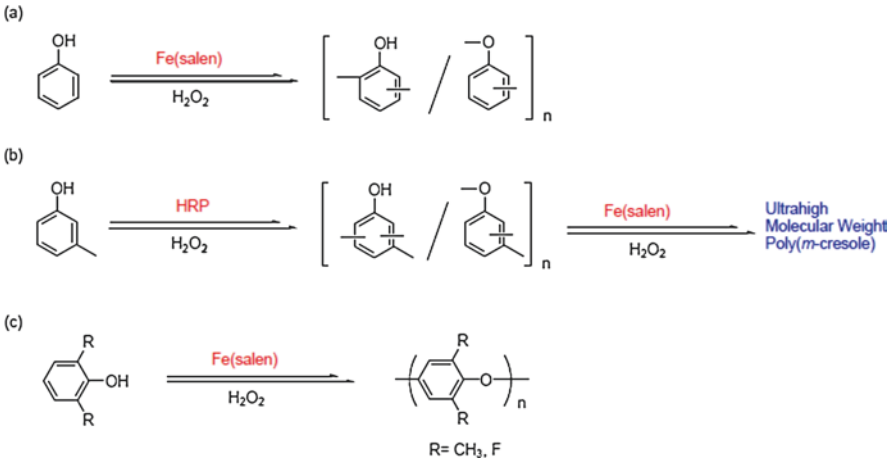


Fig. 10.4 Fe(salen) complex: (**a**) a reduced form and (**b**) an oxidized dinuclear form



Scheme 10.7 Fe(salen)-catalyzed oxidative polymerization of phenols

Poly(2,6-dimethyl-1,4-phenylene oxide) (P-2,6-Me_2P), the alloy of which with polystyrene (PSt) is widely used as one of the engineering plastics, was synthesized by Fe(salen)-catalyzed oxidative polymerization. The polymerization of 2,6-dimethylphenol (2,6-Me_2P) was carried out by the use of Fe(salen) catalyst with H_2O_2 in 1,4-dioxane/pyridine to afford P-2,6-Me_2P with M_n of 12,000 in an 89% yield (R = CH_3 in Scheme 10.7c) [19]. 2,6-Difluorophenol (2,6-F_2P) was also polymerized by Fe(salen) catalyst with H_2O_2 in 1,4-dioxane to produce poly(2,6-difluoro-1,4-phenylene oxide) (P-2,6-F_2P) in a 79% yield (R = F in Scheme 10.7c) [20]. The resulting polymer was partially soluble in tetrahydrofuran (THF) and showed crystallinity with a melting point at ~250 °C for the first time.

Phenol-containing poly(amino acid)s were subjected to further polymerization via Fe(salen)-catalyzed oxidative coupling with H_2O_2 (Fig. 10.5) [21]. Poly(L-tyrosine) was not applicable to the oxidative coupling, because of the short spacer length between the polymer backbone and the phenol group. Therefore, a new α-polypeptide having a phenol group in the side chain was prepared from reaction of poly(γ-ethyl-α-L-glutaminate) with tyramine and employed as the monomer in the Fe(salen)-catalyzed polymerization leading to a high-molecular-weight poly(amino acid) with M_w of 2.7×10^6.

"Urushi" is a unique natural material, formed from polymerization and curing process of "urushiol," which is a mixture of catechol derivatives having saturated or unsaturated hydrocarbon groups (Fig. 10.6a), harvested from a special urushi tree (*Rhus vernicifera*) [2]. Natural urushi is manufactured by the laccase-catalyzed polymerization of urushiol in air to give a prepolymer, followed by curing via slow air oxidation of the prepolymer. In Japan, there are many national treasures coated with urushi, showing hardness enough to be brilliantly polished and high durability. However, the expense of urushi has limited its use, and thus, "artificial urushi" made from monohydroxyarene derivatives with saturated or unsaturated hydrocarbon

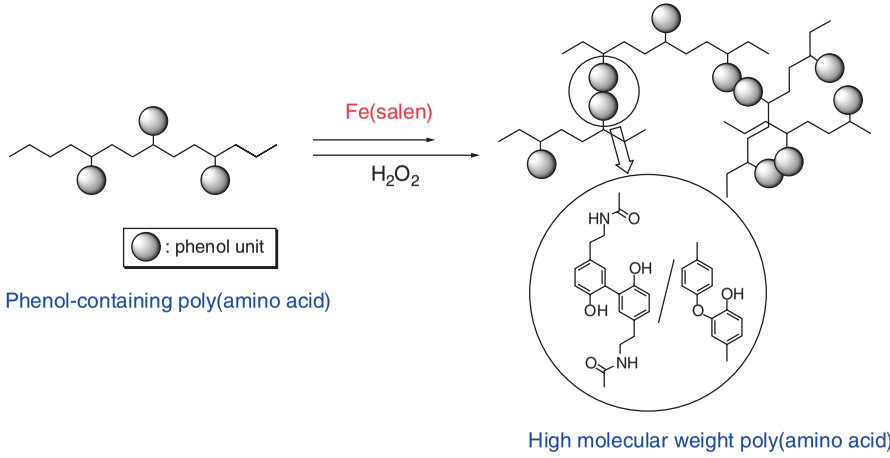


Fig. 10.5 Oxidative coupling of phenol-containing poly(amino acid)s by Fe(salen) catalyst. (Reprinted with permission from Ref. [21]. Copyright © 2004 American Chemical Society)

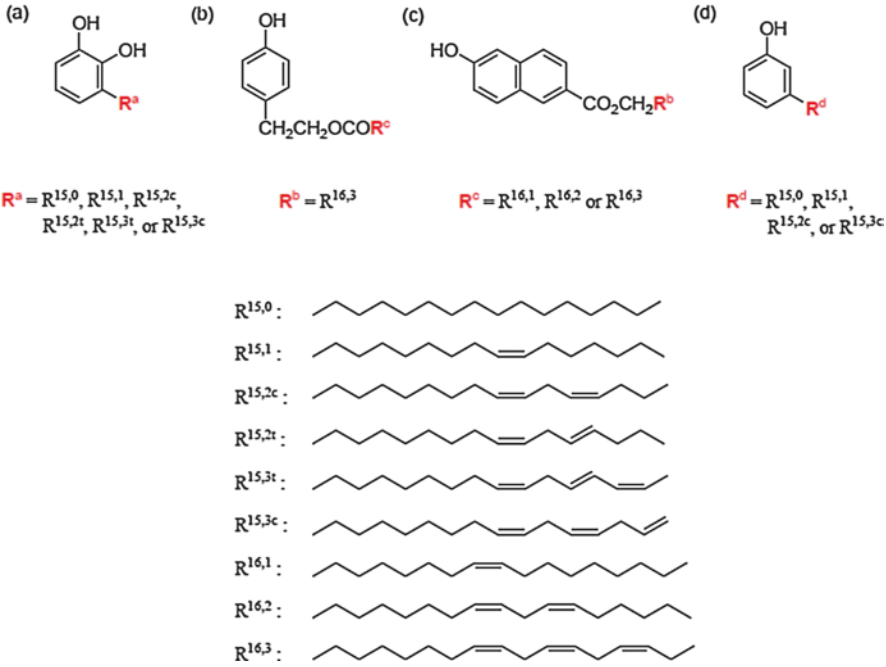


Fig. 10.6 Structures of (**a**) natural urushiol and (**b**)–(**d**) urushiol analogues

groups as urushiol analogues (Fig. 10.6b–d) has been demonstrated by Kobayashi and coworkers. The monohydroxyarenes are oxidized neither by laccase with O_2 owing to the high oxidation potentials, nor enough by HRP with H_2O_2 due to the bulkiness of substituted groups. However, the substrates can be polymerized by Fe(salen) catalyst with H_2O_2 to afford prepolymers, followed by curing via cobalt catalysis or thermal treatment to produce artificial urushi.

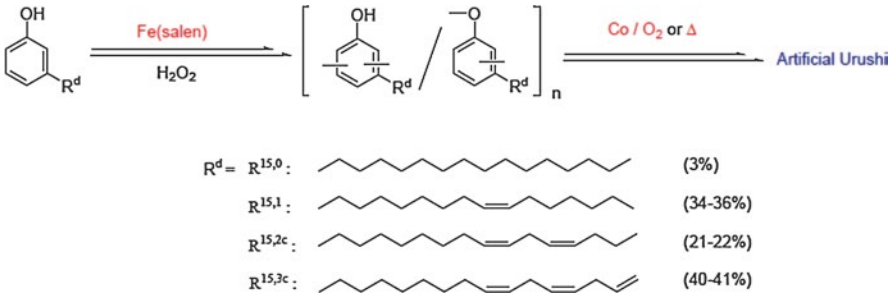
The phenol derivative as the urushiol analogue (Fig. 10.6b) was synthesized lipase-catalyzed esterification of 4-hydroxyphenethyl alcohol with fatty acids, in which the primary hydroxy group was selectively acylated [22]. Fe(salen)-catalyzed oxidative polymerization of the analogue was performed to afford the oily soluble prepolymer in a high yield. The prepolymer was cross-linked to produce artificial urushi, having a high-gloss surface and hardness of 140 N/mm^2. The urushiol analogue with the naphthol structure (Fig. 10.6c) was also prepared by acylation of hydroxynaphthoic acids with unsaturated alcohols resulting from reduction of natural oils [23]. The analogue was polymerized by Fe(salen) catalyst with H_2O_2 to afford the soluble polynaphthol, followed by cross-linking to produce a highly glossy film with hardness of nearly 200 N/mm^2.

Cardanol (Fig. 10.6d), the main component obtained by thermal treatment of cashew nut shell liquid (CNSL), is a phenol derivative mainly having *m*-substituents of C_{15} unsaturated hydrocarbon chains with mostly 1–3 double bonds [24]. It is widely used practically for resins, laminations, surface coating, paints, etc., owing

to the availability and low cost and thus has become a good candidate of the phenolic monomer for artificial urushi. The oxidative polymerization of cardanol was carried out by the use of Fe(salen) catalyst with H_2O_2 in organic solvents like 1,4-dioxane, THF, and toluene at 30 °C, affording the soluble prepolymers with molecular weights of several thousands in 32–72% yields (Scheme 10.8). The unsaturated moieties did not react during the polymerization, and the reaction took place on the phenol moieties. After the polymerization, the curing of the prepolymers occurred in the presence of cobalt naphthenate catalyst at room temperature or thermal treatment (150 °C for 30 min) to form yellowish transparent films, artificial urushi, whose properties are similar to those of a traditional Japanese lacquer. The resulting cross-linked film exhibited good elastic properties comparable with natural urushi. FT-IR monitoring of the curing showed that the cross-linking mechanism is similar to that of the oil autoxidation.

Various metal-containing catalysts were examined in comparison with Fe(salen) catalyst for the oxidative polymerization of CNSL containing cardanol mainly [25]. The other metal M(salen) (M = Mn, Co, Ni, Cu, Zn, and V=O) complexes had the catalytic activity, but Fe(salen) complex showed the highest activity. Co-, Cu-, Fe-, and V-acetyl acetone complexes and Mn-, Cu-, and Fe-phthalocyanine complexes were also lower than Fe(salen) complex in the catalyst activity. The curing behaviors of the prepolymer synthesized from CNSL by Fe(salen) catalysis, a commercially available CNSL-formaldehyde resin, and a natural urushi sample were investigated. The poly(CNSL) produced the cured film with good viscoelastic properties, comparable to the other two films, in which the glass transition temperature (T_g) of the cured film was ca. 90 °C (Fig. 10.7).

Natural urushi sap is polymerized in a water-in-urushiol (w/o) emulsion system, and accordingly, this system is mimicked for the polymerization of cardanol [26]. The combination of a weak amine base and a weak carboxylic acid functions as efficient emulsifier for a water-in-cardanol (w/o) emulsion (Fig. 10.8a), which is subjected to Fe(salen)-catalyzed oxidative polymerization to afford a prepolymer emulsion. The emulsion is stable for storage, but after the curing by thermal annealing or by cobalt naphthenate-catalyzed cross-linking in air, artificial urushi was produced (Fig. 10.8b). The rheological properties of the cardanol monomer and the prepolymer emulsions resulted in excellent film formation on the substrate, and



Scheme 10.8 Artificial urushi obtained from cardanol

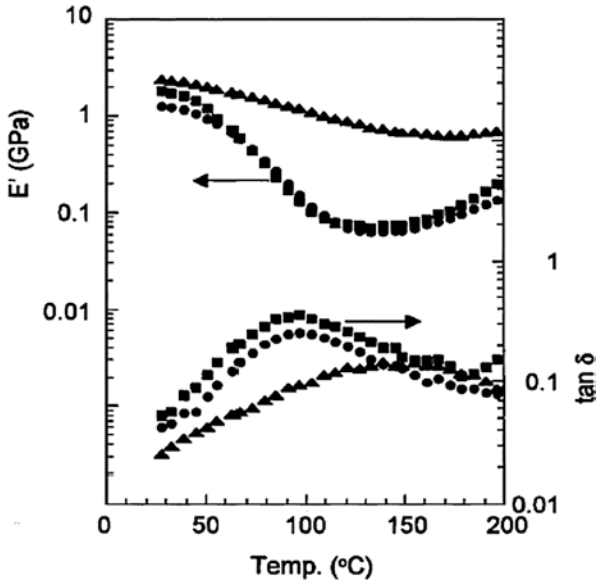


Fig. 10.7 Dynamic viscoelasticity of the cured films from poly(CNSL) (circle), CNSL-formaldehyde resin (square), and natural urushi (triangle). (Reprinted with permission from Ref. [25]. Copyright © 2004 Elsevier Science Ltd)

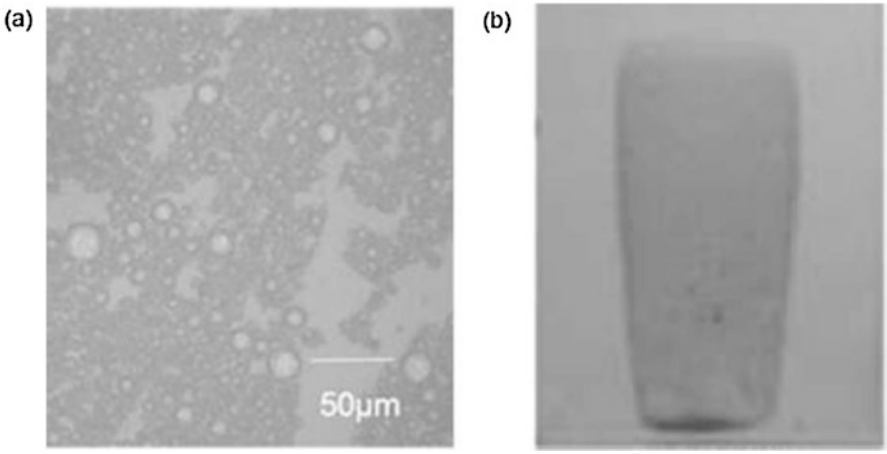


Fig. 10.8 (**a**) The mixtures of water/cardanol (50/50) and (**b**) the subsequent film cured by cobalt naphthenate in air at room temperature for 3 days. (Reprinted with permission from Ref. [26]. Copyright © 2016 Springer Nature)

the physical properties of the cured films showed sufficient hardness for use as a coating layer.

10.3 Cu-Containing Monooxygenase- and Oxidase-Model Complex Catalysts

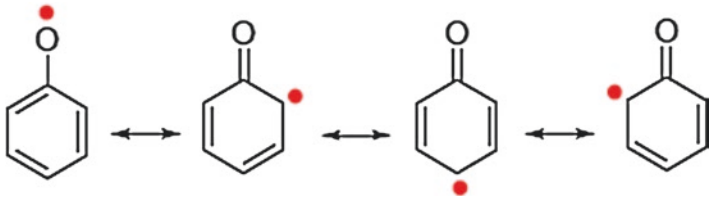
As the model complexes of Cu-containing monooxygenases and oxidases for oxidative polymerization catalysts, two types of Cu complexes are focused on: Cu complexes with three nitrogen-coordinated ligands and multinuclear Cu complexes. The former Cu complexes are derived from the active centers of monooxygenases such as tyrosinase, consisting of one Cu atom and three nitrogen coordination atoms of imidazole groups in histidines. The latter Cu complexes are inspired from oxidases like laccases, which have tetranuclear Cu complexes seeming appropriate to four-electron reduction of O_2.

10.3.1 Cu Complex Catalysts Having Three Nitrogen Coordination Atoms

10.3.1.1 Catalyst Design for Regioselective Oxidative Polymerization of 2- and/or 6-Unsubstituted Phenols

Copper catalysts for oxidative polymerization of phenols have been developed, not for the model catalysts of Cu-containing enzymes but for the practical catalysts of manufacturing engineering plastics. In 1959, Hay and coworkers discovered a Cu/pyridine catalyst in oxidative polymerization of 2,6-Me_2P under O_2 to produce P-2,6-Me_2P [27]. P-2,6-Me_2P is the first example for oxidative polymerization to synthesize a linear phenolic polymer with high molecular weight. After the discovery, various catalysts such as Cu/diamine catalysts were developed, and P-2,6-Me_2P was found completely miscible with PSt [28]. Now, P-2,6-Me_2P/PSt alloy is widely used as engineering plastics with sales approaching 1 billion dollar per year.

Conventional Cu catalysts, typically Cu/diamine catalysts, are producing P-2,6-Me_2P industrially, but are not able to give linear polymers from the phenols having at least one *ortho*-position unsubstituted. Because the phenoxy radical intermediates have such resonance structures as shown in Scheme 10.9, not only *para*-positions but also open *ortho*-positions are reactive for phenoxy radical coupling. Therefore, two substituents at 2,6-positions of phenols were necessary for synthesizing linear polymers through oxidative polymerization by the conventional Cu catalysts. Iron catalysts such as peroxidases and their model complexes changed coupling selectivity by solvent effect to some extent; however the catalysts showed little ability for controlling the regioselectivity in coupling of the phenols unsubstituted at least one *o*-position [29].



Scheme 10.9 Resonance structures of phenoxy radical

Then, the reason why these Cu and Fe catalysts are not able to control the coupling selectivity of 2- and/or 6-unsubstituted phenols is speculated as follows: Cu(I)/diamine complexes reacted with O_2 to give bis(μ-oxo) dicopper(III) complexes [30]. In the reaction of HRP with H_2O_2, Fe(IV)=O intermediates were formed (Scheme 10.3) [31]. These active oxygen complexes were used to the reaction with phenols to afford "free" phenoxy radicals. These data suggest that the regioselective coupling cannot be achieved by the catalysts generating "radical or electrophilic" active oxygen complexes.

Based on this view (Fig. 10.9), a "basic" μ-η^2:η^2-peroxo-dicopper(II) complex **1** as the active oxygen species of tyrosinase was focused on [32]. Thus, the authors and coworkers proposed a working hypothesis as follows: complex **1** abstracts protons (not hydrogen atoms) from phenols to give phenoxo-copper(II) complex **2**, equivalent to phenoxy radical–copper(I) complex **3**. Intermediates **2** and/or **3** are not "free" radicals but "controlled" radicals. If a catalyst generates (and regenerates) only a basic active oxygen intermediate and it reacts with phenols to give the "controlled" radicals without formation of the "free" radicals, regioselectivity of the subsequent coupling will be entirely regulated. Although the attempt to avoid coupling at the open *o*-position of 2-methylphenol with Cu/2-alkylpyridine catalysts in terms of "controlled" radicals has been reported [33], satisfactory effects were not observed, probably involving generation of "free" radicals. The above concept is characterized by the exclusive formation of "controlled" phenoxy radicals, and hence, the new concept was termed as "radical-controlled" oxidative polymerization [34, 35].

The Cu complexes having three nitrogen coordination atoms were selected as the tyrosinase model: (hydrotris(3,5-diphenyl-1-pyrazolyl)borate) copper (Cu(Tpzb)) complex and (1,4,7-R_3-1,4,7-triazacyclononane) copper ($Cu(L^R)$: R = isopropyl (iPr), cyclohexyl (cHex), and *n*-butyl (nBu)) complexes (Fig. 10.10).

10.3.1.2 Oxidative Polymerization of 4-Phenoxyphenol by Tyrosinase-Model Catalysts

First, "radical-controlled" oxidative polymerization catalyzed by tyrosinase-model complexes was applied to 4-phenoxyphenol (PPL) as the monomer (Scheme 10.10). This was the first synthesis of crystalline poly(1,4-phenylene oxide) (PPO) having a

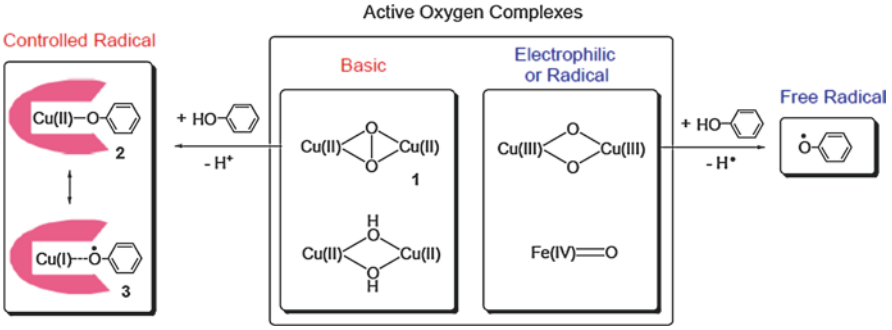


Fig. 10.9 A working hypothesis for controlling phenoxy radical coupling

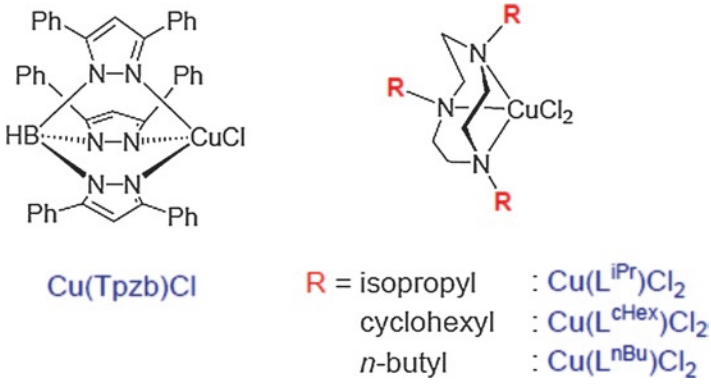
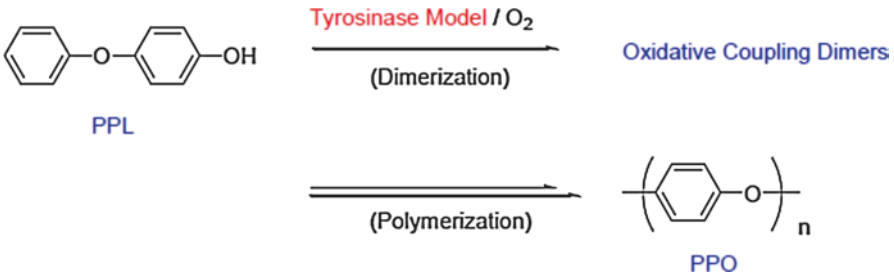


Fig. 10.10 Cu complexes having three nitrogen coordination atoms as tyrosinase models



Scheme 10.10 Oxidative polymerization of PPL by tyrosinase-model catalyst

melting point by the catalytic oxidative polymerization method, although the synthesis by polycondensation with eliminating a halogenated salt at 200 °C was reported [36].

The ratio of oxidative coupling dimers formed at the initial stage of polymerization of PPL was investigated to evaluate the coupling selectivity (Table 10.1). The polymerization catalyzed by Cu(Tpzb)Cl, Cu(L^{iPr}) Cl_2, Cu(L^{cHex}) Cl_2, or Cu(L^{nBu}) Cl_2 was

Table 10.1 Dimer ratio at the initial stage of oxidative polymerization of PPL

Entry	Catalyst	Oxidant	Solvent	Time	Conv.[e]	Yield[f]	Dimer ratio (%)			
				(h)	(%)	(%)	p-4	o-4	oo-22	oo-13
1	Cu(Tpzb)Cl[a]	O_2	Toluene	0.25	13	9	91	9	0	0
2	Cu(Tpzb)Cl[a]	O_2	THF	1.7	11	7	91	9	0	0
3	Cu(L^{iPr})Cl_2[a]	O_2	Toluene	0.2	9	8	93	7	0	0
4	Cu(L^{iPr})Cl_2[a]	O_2	THF	7.5	12	9	89	7	1	3
5	Cu(L^{cHex})Cl_2[a]	O_2	Toluene	0.2	7	7	95	5	0	0
6	Cu(L^{nBu})Cl_2[a]	O_2	Toluene	0.2	12	12	90	9	0	1
7	CuCl/teed[a]	O_2	Toluene	0.02	17	12	79	6	2	13
8	–[d]	AIBN	Toluene	120	27	15	82	4	2	12
9	HRP[b]	H_2O_2	Dioxane/buffer(8/2)	0.25	11	7	37	17	8	38
10	Fe(salen)[b]	H_2O_2	Dioxane/buffer(8/2)	0.25	12	7	41	15	7	37
11	–[d]	AIBN	Dioxane/buffer(8/2)	96	8	6	54	12	4	30
12	Tyrosinase[c]	Air	Acetone/buffer(5/5)	1	14	<0.1	–	–	–	–
13	–[d]	AIBN	Acetone/buffer(5/5)	96	13	6	22	9	10	59

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[a]Polymerization catalyzed by Cu complex and 2,6-diphenylpyridine in solvent under dioxygen (1 atm) at 40 °C
[b]Polymerization by HRP or Fe(salen) in dioxane/pH = 7 buffer with H_2O_2 at 25 °C
[c]Reaction by tyrosinase in acetone/pH = 7 buffer under air (1 atm) at 25 °C
[d]Oxidized by AIBN under nitrogen
[e]Conversion of PPL
[f]Total yield of dimers

carried out under O_2 in toluene or THF at 40 °C (entries 1–6). Oxidative polymerization catalyzed by CuCl/N,N,N′,N′-tetraethylethylenediamine (teed) complex, which was the sole catalyst reported for oxidative coupling of PPL [37], HRP, Fe(salen), or tyrosinase, was also performed (entries 7, 9, 10, 12). As a model system of free phenoxy radical coupling, oxidation of PPL by an equimolar amount of 2,2′-azobisisobutyronitrile (AIBN) was examined in the same solvent (entries 8, 11, 13).

In the case of CuCl/teed catalyst (entry 7), four dimers were detected (Fig. 10.11): p-4 and o-4 are obtained from the C–O coupling, and products oo-22 and oo-13 are from the C–C coupling. Almost none of phenol and 4-(4-phenoxyphenoxy)phenol, a phenol trimer, were observed. Considerable amounts of the two C–C coupling dimers of oo-22 and oo-13 were detected, and consequently, p-4 selectivity was low (79%). These dimer ratios were very similar to those via free radical coupling by AIBN oxidation (entry 8), in which the formation of the C–C coupling dimers is characteristic.

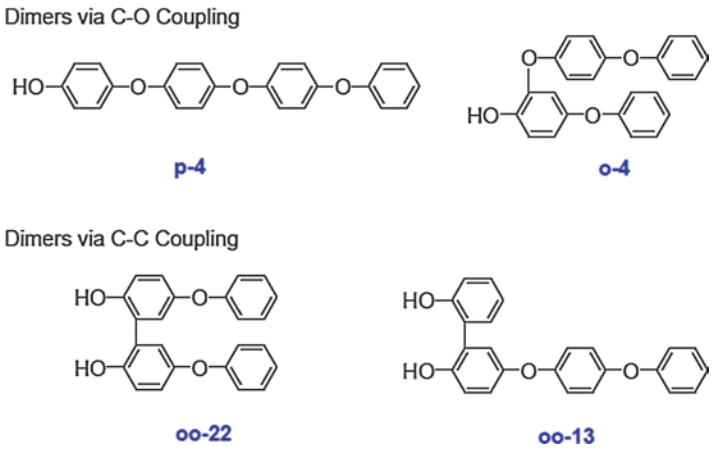

Fig. 10.11 Dimers from C–O and C–C Coupling of PPL

However, for the Cu(Tpzb) in toluene and in THF, and for the Cu(L^{iPr}), Cu(L^{cHex}), and Cu(L^{nBu}) in toluene (entries 1–3, 5, and 6), none or very little of the C–C coupling dimers were generated, and high regioselectivity of p-4 was achieved (max. 95%). In entries of 3, 5, and 6, the order of p-4 selectivity was Cu(L^{nBu}) (90%), Cu(L^{iPr}) (93%), and Cu(L^{cHex}) (95%) in good agreement with that of steric hindrance of the substituents. These data show that the regioselectivity of phenoxy radical coupling can be controlled by these catalysts. On the other hand, the dimerization catalyzed by the Cu(L^{iPr}) in THF (entry 4) gave the C–C coupling dimers to some extent.

In the case of the HRP and Fe(salen) catalysts (entries 9 and 10), the dimer compositions were close to that by AIBN in the same solvent (entry 11), suggesting that HRP and Fe(salen) induced free radical pathways in the oxidative coupling. The oxidation catalyzed by tyrosinase gave almost none of the dimers and insoluble dark-brown materials (entry 12), whereas the AIBN oxidation produced the dimers (entry 13).

The polymers were isolated as methanol-insoluble parts (Table 10.2). In the cases with little or no C–C dimer formation (entries 1–3, 5, 6), white powdery polymers were obtained, and the M_n and M_w were 600–1500 and 700–4700, respectively. The FT-IR spectra of the polymers were very similar to that of PPO synthesized by the dehalogenated polycondensation [36]. From the differential scanning calorimetry (DSC) analysis, the polymers showed melting temperatures (T_m) at 171–194 °C. In the cases with considerable amounts of the C–C dimers (entries 4, 7–10), brownish polymeric materials were formed and showed no clear melting points in the DSC traces.

Based on the above data, the reaction mechanisms for the Cu catalysts are postulated as follows (Scheme 10.11). First, the starting Cu(II) chloride complex **6** reacts with PPL to give phenoxo-copper(II) complex **2**, equivalent to phenoxy radical–copper(I) complex **3**. The complex **2** from 4-fluorophenol was determined by X-ray crystallography, and the complex **2** from 2,6-dimethylphenol gave the oxidative

Table 10.2 Polymers obtained from oxidative polymerization of PPL[a]

Entry	Catalyst	Oxidant	Solvent	Time	Conv.[b]	Yield[c]	M_n	M_w	T_m[d]
				(h)	(%)	(%)			(°C)
1	Cu(Tpzb)Cl	O_2	Toluene	14	55	4	700	1100	186
2	Cu(Tpzb)Cl	O_2	THF	70	91	54	1500	2900	194
3	$Cu(L^{iPr})Cl_2$	O_2	Toluene	19	98	89	1200	4700	171
4	$Cu(L^{iPr})Cl_2$	O_2	THF	81	86	52	3800	13,700	Nd[f]
5	$Cu(L^{cHex})Cl_2$	O_2	Toluene	210	76	28	600	700	186
6	$Cu(L^{nBu})Cl_2$	O_2	Toluene	23	87	40	800	1100	184
7	CuCl/teed	O_2	Toluene	24	100	77	5400	29,100	Nd[f]
8	–	AIBN	Toluene	380	69	8	1600	3100	Nd[f]
9	HRP	H_2O_2	Dioxane/buffer(8/2)	24	99	43	–[e]	–[e]	Nd[f]
10	Fe(salen)	H_2O_2	Dioxane/buffer(8/2)	24	100	97	–[e]	–[e]	Nd[f]

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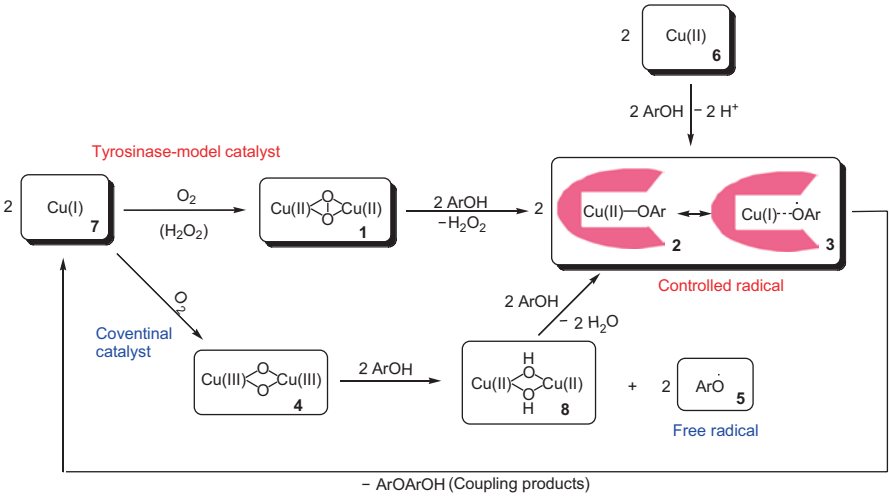
[a]The reaction conditions are shown in Table 10.1
[b]Conversion of PPL
[c]Yield of polymers
[d]Temperature of the largest endothermic peak in the second scan of DSC
[e]Insoluble
[f]Not detected



Scheme 10.11 Proposed reaction mechanisms for Cu catalysis

coupling product, indicating that **2** possesses a radical resonance structure **3** [38]. Regioselective coupling takes place between two molecules of **2** and/or **3** to produce Cu(I) complexes **7** as well as the phenylene oxide with *p*-linkage selectively, because the steric hindrance of the catalysts blocks the *o*-coupling.

In the case of the Cu(Tpzb) complex, formation of μ-η^2:η^2-peroxo-dicopper(II) complex **1** from **7** under O_2 [32] was confirmed in both toluene and THF. For the

Cu(L^{iPr}) complex, it was reported that **7** afforded complex **1** in toluene [39], and **1** reacted with HBF_4 to yield H_2O_2, working as the base [40]. A similar complex **1** reacted with 4-fluorophenol to give complex **2** [5], and the complex **7** was proved to react not only with O_2 but also with H_2O_2 to form the complex **1** [35]. These data strongly indicate that μ-η^2:η^2-peroxo-dicopper(II) complex **1** is formed and abstracts protons from phenols to regenerate **2** and H_2O_2. Hence, this catalytic system would allow only the regioselective coupling process from **2** and/or **3** and completely exclude free radical coupling, which is thus recognized as "radical-controlled" oxidative polymerization. The Cu(L^{cHex}) and Cu(L^{nBu}) catalysts possess the similar reaction mechanism as the Cu(L^{iPr}) one.

For the Cu(L^{iPr}) catalysis, the reaction intermediate was analyzed by electron spin resonance (ESR) measurement [41]. After the reactions of Cu(L^{iPr})Cl_2 with PPL and phenol were performed in toluene under O_2, the reaction mixture was quickly cooled and measured by ESR (Fig. 10.12). In the both samples, similar signals to each other were ascribed to mononuclear Cu(II) complexes, which would be phenoxo-copper(II) complexes **2** by comparison with that for 4-fluorophenol [5]. From the spin intensities, the detected species are regarded as the major reaction intermediates. Hence, the rate-determining step in the Cu(L^{iPr}) catalysis would be the coupling process from the controlled radical species **2** and/or **3**. The phenoxo complex **2** would contribute to the stability, and once two molecules of the species come close to each other, phenoxy radical complex **3** would lead to the oxidative coupling.

With the Cu(L^{iPr}) complex in THF [39] and CuCl/teed complex, **7** gave bis(μ-oxo) dicopper(III) complex **4** under O_2, and then, **4** abstracted hydrogen atoms from phenols to give bis(μ-hydroxo) copper(II) complex **8** and the radical coupling product [30]. These data indicate that bis(μ-oxo) dicopper(III) complex **4** is formed,

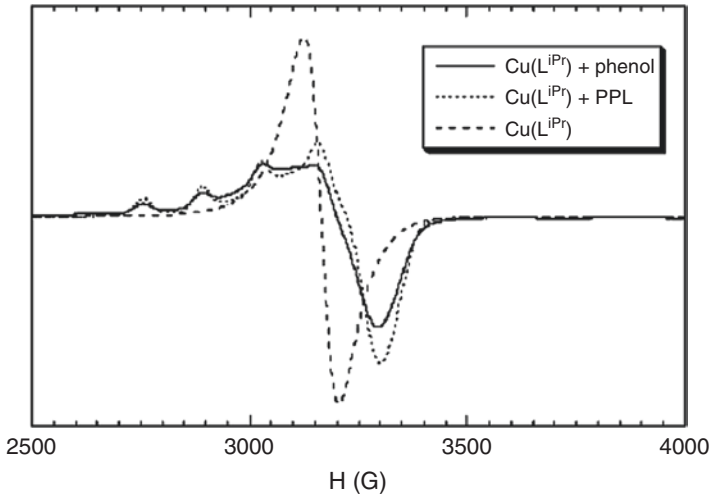


Fig. 10.12 ESR spectra of Cu(L^{iPr})-catalyzed reaction mixture for phenol and PPL (solid line and dotted line, respectively) and Cu(L^{iPr})Cl_2 solution (broken line). (Reprinted with permission from Ref. [41]. Copyright ©2005 Wiley periodicals, Inc)

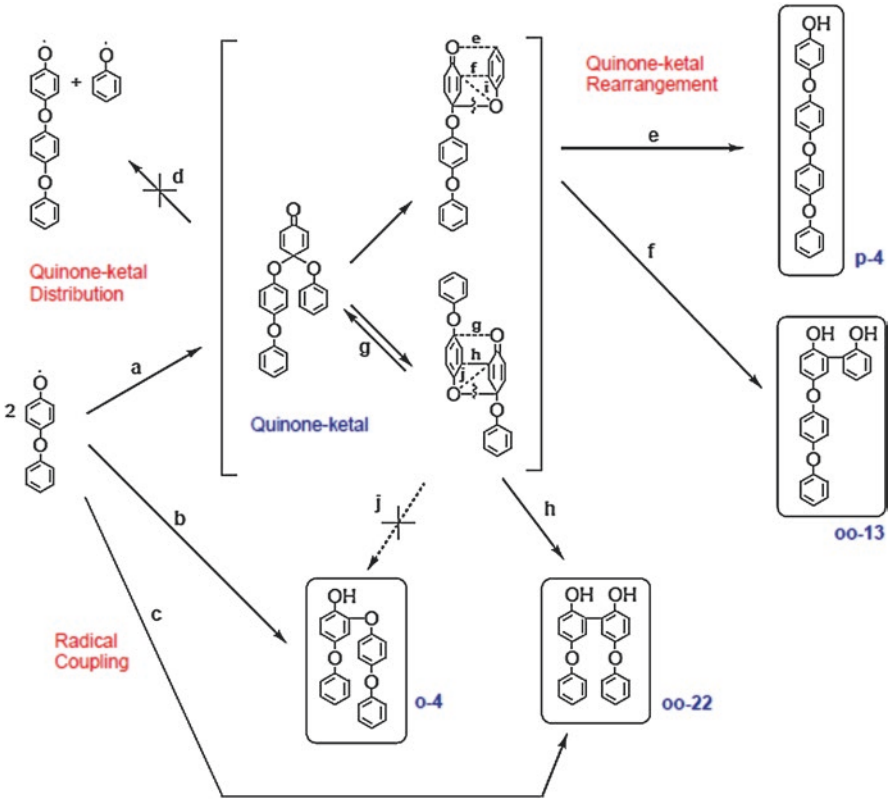
followed by abstraction of hydrogen atoms from phenols to give free phenoxy radical **5**. Therefore, this catalytic cycle should involve the free radical coupling with the formation of C–C linkages, although production of **2** from complex **8** also takes place.

HRP and the model complex Fe(salen) catalysts cause almost the same results, generating the radically active oxygen species and the resulting coupling of free phenoxy radicals (Scheme 10.3). However, tyrosinase and the model complexes show completely different behaviors, which can be explained as follows: although there are two mechanisms in oxygenation of a phenol by tyrosinase (Scheme 10.4), μ-η^2:η^2-peroxo-dicopper(II) complex forms and reacts with only one molecule of PPL in both cases. The following each route in *ortho*-oxygenation of the phenol affords the *o*-quinone, which eventually leads to the dark brown melanin. For the model complexes (Scheme 10.11), on the other hand, μ-η^2:η^2-peroxo-dicopper(II) complex reacts with two molecules of PPL to give two molecules of phenoxo-copper(II) complex, leading to PPO via the "radical-controlled" coupling. Tyrosinase possesses two functions: the formation of μ-η^2:η^2-peroxo-dicopper(II) complex and the oxygenation of phenols. The tyrosinase-model catalysts mimic the former function only and thereby enables "radical-controlled" oxidative polymerization.

The reaction mechanism of oxidative coupling of PPL to produce dimers is speculated as follows (Scheme 10.12), in which the controlled radical is simply expressed as the free radical without the catalyst part. First, two molecules of PPL are oxidized, and the following two phenoxy radicals are coupled to each other (radical coupling). Since radical coupling does not occur on the 4-phenoxy group of PPL, three reaction routes (**a**, **b**, and **c**) can take place, giving a quinone-ketal intermediate, o-4, and oo-22, respectively. From the quinone-ketal, the redistribution path (quinone-ketal redistribution, **d**) was omitted from no detection of phenol and the trimer, and therefore, the rearrangement path (quinone-ketal rearrangement) was proposed in the oxidative coupling of PPL [37]. On cleavage of the ketal C–O bond, synchronous bond formation occurs to p-4 by **e**, to oo-13 by **f**, and to oo-22 by **h**, although that by **g** regenerates the quinone-ketal.

In the radical-controlled oxidative coupling of PPL, the entire coupling takes place from controlled radicals. The steric effect of the catalyst moiety suppresses o-4 formation (**b**) and inhibits oo-22 formation (**c**), regioselectively generating the quinone-ketal intermediate (**a**). Also, almost no detection of oo-13 as well as oo-22 indicates that the catalyst must remain in contact with the quinone-ketal intermediate. In the rearrangement, bond formation at the *o*-position to oo-13 and oo-22 via routes **f** and **h**, respectively, is prohibited, and hence, regioselective production of p-4 via route **e** dominantly occurs. Consequently, in radical-controlled oxidative coupling, the tyrosinase-model catalysts can control regioselectivity not only in the radical coupling step but also more strongly in the quinone-ketal rearrangement step [42].

In a similar way of dimerization, oxidative coupling from controlled radicals of PPL and p-4 will give a trimeric quinone-ketal intermediate, followed by the rearrangement to afford linear C–O trimer (six oxyphenylene units). Further oxidative couplings between controlled radicals generated from PPL, dimer, trimer, and higher oligomers lead to linear PPO.



Scheme 10.12 Proposed reaction mechanism for oxidative dimerization of PPL

10.3.1.3 Oxidative Polymerization of Other Phenols by Tyrosinase-Model Catalysts

The substituent effect of phenol monomers on the reaction rate of oxidative polymerization catalyzed by the Cu(L^{iPr}) complex under O_2 was investigated in comparison with that by the conventional Cu/*N,N,N′,N′*-tetramethylethylenediamine (Cu(tmed)) complex [43]. With the Cu(tmed) catalyst, the order of the reaction rates was phenol, 3-methylphenol (3-MeP), 2-methylphenol (2-MeP), 2,5-dimethylphenol (2,5-Me_2P), and 2,6-Me_2P. This order agrees with that of the O–H homolytic bond dissociation energies of the monomers, because the Cu(tmed) catalyst forms bis(μ-oxo) dicopper(III) complex reacting with phenols to give free radicals. On the other hand, for the Cu(L^{iPr}) catalyst, the reaction rates of dimethylphenols were smaller than those of methylphenols, and as to dimethylphenols, that of 2,6-Me_2P was slower than that of 2,5-Me_2P. These data indicate that in the Cu(L^{iPr}) catalysis, the substituents at the *o*-positions of phenols hinder the formation of controlled radicals.

In the oxidative polymerization of phenol, the Cu(L^{iPr}) catalyst showed high selectivity for C–O coupling at the *p*-position, but it did not completely exclude the formation of C–C coupling at the *p*-position [41]. The resulting polymer consisted mainly of a 1,4-phenylene oxide unit but contained a slight amount of C–C coupling structures leading to no crystallinity. However, the temperature of 10% weight loss (T_{d10}) for the poly(phenylene oxide) (~500 °C) was much higher than that for the polyphenol obtained by HRP-catalyzed polymerization (<400 °C). The plot of monomer conversion vs. polymer yield for phenol showed a linear relationship like a chain growth mechanism, while PPL followed a typical stepwise growth mechanism (Fig. 10.13). This observation for phenol was assigned to the much higher reactivities of the dimers and oligomers than that of the monomer, as often seen in oxidative polymerization. Until the monomer is almost consumed, the reaction mixture consists mainly of the monomer and the polymer similarly to chain reaction mechanism, and hence, such behavior was named as reactive intermediate polycondensation [44].

The oxidative polymerization of 2-MeP and 3-MeP by the use of Cu(L^{iPr}) catalyst regioselectively produced soluble poly(phenylene oxide)s (Scheme 10.13a and b). The polymer obtained from 2-MeP consisted mainly of a 2-methyl-1,4-phenylene oxide unit with M_n of 3800 [45]. The polymer resulting from 3-MeP contained a 3-methyl-1,6-oxyphenylene units or 3-methyl-1,2-oxyphenylene units [46], but the M_n value was relatively high (ca. 40,000). Both the polymers from 2-MeP and 3-MeP showed highly thermal stability with T_{d10} of 437 and 414 °C, respectively.

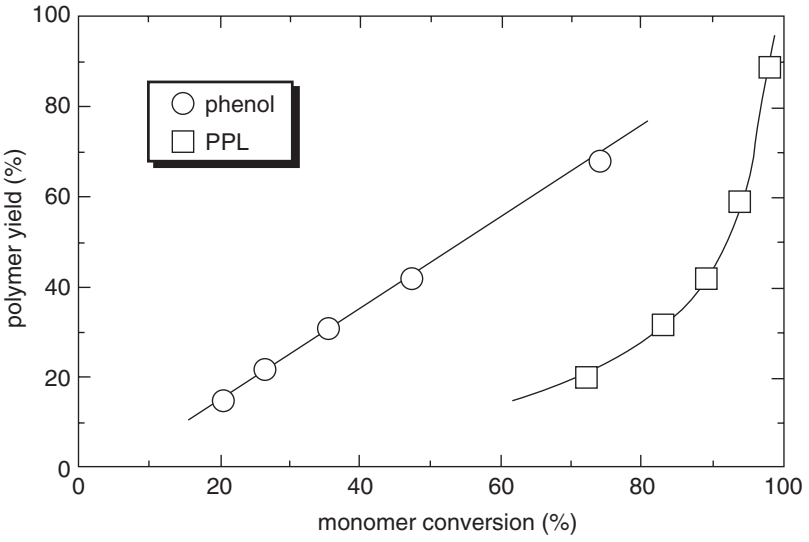
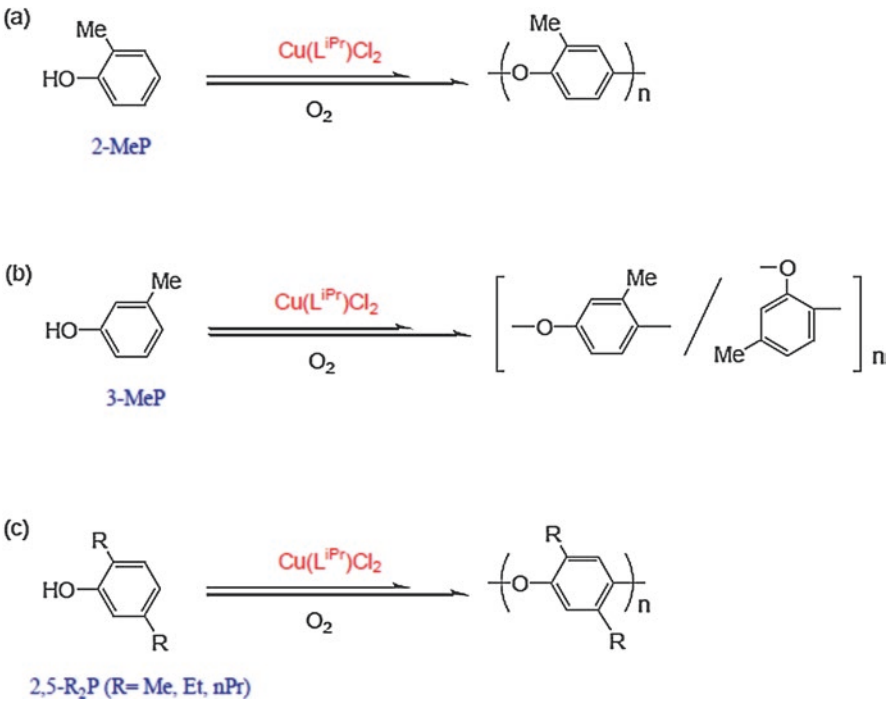


Fig. 10.13 Polymer yield vs. monomer conversion in oxidative polymerization of phenol (circle) and PPL (square). (Reprinted with permission from Ref. [41]. Copyright ©2005 Wiley periodicals, Inc)



Scheme 10.13 Cu(L^{iPr})-catalyzed oxidative polymerization of alkyl-substituted phenols

In the Cu(L^{iPr})-catalyzed oxidative polymerization of alkyl-substituted phenols, the polymers obtained from 2,5-dialkylphenols (2,5-R_2P: R = methyl (Me), ethyl (Et), and *n*-propyl (nPr)) are worthy of special mention (Scheme 10.13c) [47]. The oxidative polymerization of 2,5-Me_2P by using Cu(L^{iPr}) Cl_2 in toluene under O_2 at 40 °C produced a white polymer in a 78% yield (entry 1 in Table 10.3). The polymer had the M_n and M_w of 3900 and 19,300, respectively, and from the NMR spectrum, it was identified as poly(2,5-dimethyl-1,4-phenylene oxide) (P-2,5-Me_2P). In the DSC traces, P-2,5-Me_2P had not only an endothermic peak in the first heating but an exothermic peak in the cooling and an endothermic peak in the second heating. The melting temperatures of the highest peaks in the first and second (T_{m1} and T_{m2}) were 308 and 303 °C, respectively, and the crystallization temperature in the cooling (T_c) was 242 °C. A new polymer P-2,5-Me_2P with heat-reversible crystallinity was synthesized for the first time. The isomeric polymer P-2,6-Me_2P as synthesized showed a melting point at 237 °C of T_{m1} [48], but once the crystal part had been totally melted, recrystallization never occurred even by slow cooling or annealing. Since thermoplastics are practically used by melt-molding, P-2,6-Me_2P is accepted as an amorphous plastic, and however, P-2,5-Me_2P would be considered as a crystalline plastic.

The Cu(L^{iPr})-catalyzed oxidative polymerization of 2,5-Et_2P and 2,5-nPr_2P also produced white polymers with M_n of 9900 and 8500, respectively (entries 2 and 3).

Table 10.3 Cu(L^{iPr})-catalyzed oxidative polymerization of 2,5-R_2P[a]

Entry	R	Time	Conv.[b]	Yield[c]	M_n	M_w	T_{m1}[d]	T_c[d]	T_{m2}[d]
		(h)	(%)	(%)			(°C)	(°C)	(°C)
1	Me	24	99	78	3900	19,300	308	242	303
2	Et	24	97	77	9900	23,100	252	Nd[e]	Nd[e]
3	nPr	72	100	92	8500	32,200	301	247	276

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[a]Polymerization of 2,5-R2P by Cu(L^{iPr})Cl2 and 2,6-diphenylpyridine in toluene at 40 °C under dioxygen (1 atm)

[b]Conversion of monomers

[c]Yield of polymers

[d]Peak-top temperatures in the first heating (Tm1), in the cooling (Tc), and in the second heating (Tm2) of DSC

[e]Not detected

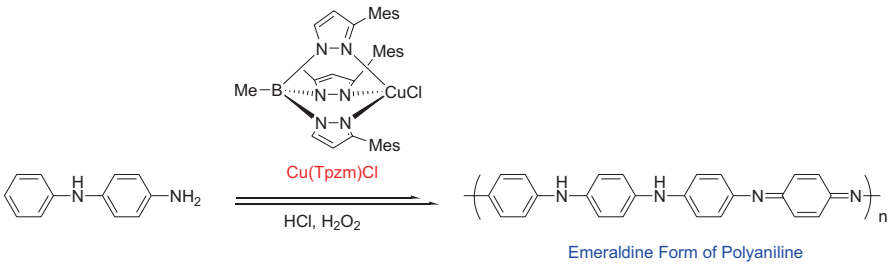
The polymer from 2,5-nPr_2P was identified as poly(2,5-di-*n*-propyl-1,4-phenylene oxide) showing heat-reversible crystallinity with T_{m2} at 276 °C. The polymer from 2,5-Et_2P was also poly(2,5-diethyl-1,4-phenylene oxide); however, the polymer did not show detectable T_{m2}. Therefore, whether or not recrystallization for poly(alkylated phenylene oxide)s occurs after melting is governed by both the position and group of their alkyl substituents.

It was also reported that the Cu(L^{iPr})-catalyzed oxidative polymerization of 2,6-F_2P under O_2 yielded soluble but noncrystalline P-2,6-F_2P with M_n of 13,000 [49], which property is deferent from the polymer by Fe(salen) catalysis described in 10.2.2. Since the Cu(L^{iPr}) complex is difficult to become planar due to the rigid trigonal structure of Cu-N_3 moiety, the Cu(II) state has high oxidation potential enough to oxidize 2,6-difluorophenol. Therefore, Cu-N_3 complexes as tyrosinase-model catalysts show not only high selectivity but also high reactivity in oxidative polymerization of phenols with O_2.

A tris(pyrazolyl)borate copper complex, Cu(Tpzm)Cl (Scheme 10.14), polymerized aniline dimer, *N*-(4-aminophenyl)aniline, with H_2O_2 in addition of HCl in CH_3CN/H_2O to produce an emeraldine base form of polyaniline in a 50% yield [50]. The emeraldine form was acidified to afford the emeraldine salt form with conductivity of 2.0×10^{-4} S/cm. The use of $CuCl_2$ in place of Cu(Tpzm)Cl as the catalyst gave the polyaniline containing benzoquinones, benzidines, or cross-linked structures.

10.3.1.4 Dinuclear Cu Complexes with Dinucleating Ligands

A dicopper complex with a dinucleating ligand (Cu_2(L1)) (Fig. 10.14a) was reported to form the μ-η^2:η^2-peroxo-dicopper(II) complex reversibly [51], also producing crystalline PPO by oxidative polymerization of PPL [52]. In comparison of the dicopper complex with a monocopper complex with a mononucleating ligand (Cu(L2)) (Fig. 10.14b), the coupling selectivity was almost the same; however, the



Scheme 10.14 Cu(Tpzm)-catalyzed oxidative polymerization of aniline dimer

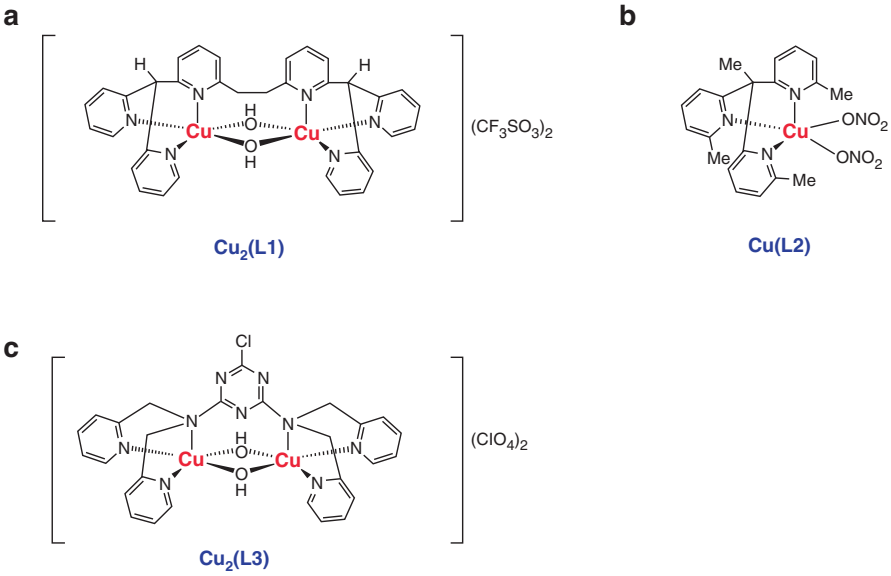


Fig. 10.14 Complexes (**a**) and (**c**): two dicopper complexes with dinucleating ligands. Complex (**b**): a monocopper complex to be compared with (**a**)

reaction rate toward the catalyst amount significantly changed. The initial reaction rate for Cu_2(L1) was independent on the catalyst amount in the range of small quantities, whereas the reaction rate decreased with a decrease in the amount of Cu(L2). These data suggest that the catalyst amount can be reduced by the use of the dicopper/dinucleating ligand complex catalyst.

To mimic the oxidation of catechol by tyrosinase, a dicopper complex possessing a dinucleating triazine-based ligand (Cu_2(L3)) (Fig. 10.14c) was subjected to the reaction with dopamine [53]. The Cu_2(L3)-catalyzed oxidation of dopamine with O_2 was carried out in MeOH/H_2O at 25 °C, and then, the initial colorless solution turned deep blue, followed by generating a dark brown precipitate like melanin. It is proposed that dopamine binds the Cu_2(L3) complex to give the semiquinone intermediate, and the subsequent polymerization produces poly(dopamine).

10.3.2 Multinuclear Cu Complex Catalysts

In oxidative polymerization of phenols, various multinuclear Cu complex catalysts having advantages for multielectron redox reactions are proposed, because complete reduction of O_2 and H_2O_2 needs four and two electrons, respectively. For such purpose, two discrete multinuclear Cu complexes were synthesized by the reaction of *N,N′*-bis(pyridin-3-yl)-2,6-pyridinedicarboxamide (H_2L4) with $CuSO_4•5H_2O$ and $Cu(OCOCH_3)_2$: a decanuclear $[Cu_{10}(H_2L4)_5(SO_4)_8(\mu_3\text{-}OH)_4(H_2O)_2]$ complex and a hexanuclear $[Cu_6(L4)_4(OCOCH_3)_4]$ complex, respectively [54]. These complexes were applied to the heterogeneous and recyclable catalysts for oxidative polymerization of 2,6-Me_2P in CH_3CN/CH_3OH under O_2, producing P-2,6-Me_2P with its selectivity toward 3,3',5,5'-tetramethyldiphenoquinone (DPQ) of max. 96%.

Multinuclear Cu complexes by using dendrimer ligands were demonstrated as the catalysts for oxidative polymerization of phenols. A dendritic phenylazomethine of the fourth generation (DPAG4) has a perfectly monodispersed molecular weight and structure with imine nitrogens, which can coordinate to metal and act as a weak base (Fig. 10.15a) [55]. The oxidative polymerization of 2,6-F_2P catalyzed by $2CuCl_2$-DPAG4 and $4CuCl_2$-DPAG4 under O_2 was achieved without additive bases to produce P-2,6-F_2P in 71% and 82% yields, respectively. The weak basicity of DPAG4 is one of the reasons for the strong oxidizing power of these Cu complexes, which can oxidatively polymerize 2,6-F_2P.

Amine-terminated poly(amidoamine) dendrimer of the third generation (PAMAG3) reacted with 11 equivalents of $CuCl_2$ to afford a multinuclear Cu complex (Cu(II)-PAMAMG3 in Fig. 10.15b) [56]. Oxidative polymerization of 2,6-Me_2P catalyzed by Cu(II)-PAMAMG3 was performed under O_2 in H_2O, producing P-2,6-Me_2P with M_w of 8600 in a 95% yield. The catalytic activity of Cu(II)-PAMAMG3 was higher than that of a Cu(II) complex with ethylenediamine-*N,N,N′,N′*-tetraacetic acid.

Metal organic frameworks (MOFs) consist of metal ions and organic ligands, regarded as multinuclear complexes with regulated porosities. Cu-containing MOFs with 1,1′-(1,5-pentanediyl)bis-1*H*-benzimidazole) (pbbm) were synthesized: $[Cu(SO_4)(pbbm)]_n$ (Fig. 10.16a) and $\{[Cu(CH_3CO_2)_2(pbbm)]CH_3OH\}_n$ [57]. The $[Cu(SO_4)(pbbm)]_n$ complex features 1-D double-chain framework by the single crystal X-ray diffraction. These complexes catalyzed oxidative polymerization of 2,6-Me_2P with H_2O_2 as oxidant and $NaOCH_3$ as co-catalyst in toluene/methanol at room temperature. The conversions of 2,6-Me_2P are 85% for $[Cu(SO_4)(pbbm)]_n$ and 90% for $\{[Cu(CH_3CO_2)_2(pbbm)]CH_3OH\}_n$, and for the both, the selectivity for P-2,6-Me_2P was almost up to 90%.

Hydrothermal synthesis from a mixture of $Cu(NO_3)_2$, 4,4′-bis(1,2,4-triazol-1-ylmethyl)biphenyl (btmb), and benzenetricarboxylic acid (HBTC) in H_2O at 130 °C produced $[Cu(btmb)(HBTC)]_n$ [58]. For the obtained MOF, $HBTC^{3-}$ anions coordinate to three Cu(II) centers to furnish a 2D honeycomb network (Fig. 10.16b), and the Cu(II) centers are connected by two *exo*-N atoms of triazole units in btmb to generate a 3D framework (Fig. 10.16c). The oxidative polymerization of 2,6-Me_2P

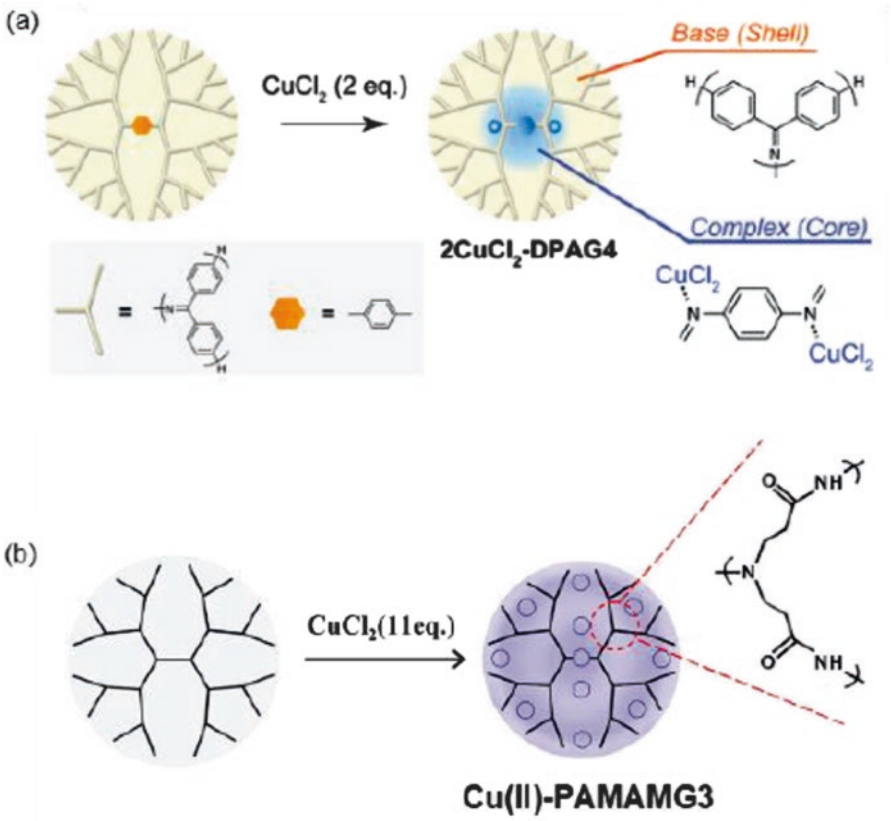


Fig. 10.15 (**a**) A dinuclear Cu complex with DPAG4. (Reprinted with permission from Ref. [55]. Copyright © 2007 American Chemical Society). (**b**) A multinuclear Cu complex with PAMAMG3. (Reprinted with permission from Ref. [56]. Copyright © 2010 American Chemical Society)

catalyzed by $[Cu(btmb)(HBTC)]_n$ with H_2O_2 and NaOH in H_2O produced P-2,6-Me_2P in a 88% yield.

By the way, in the oxidative coupling of 2,6-Me_2P, P-2,6-Me_2P is the C–O coupling product and DPQ is the C–C coupling one. Why conventional catalysts can control the C–O coupling of 2,6-Me_2P has been a most important question since the discovery of P-2,6-Me_2P [59]. Three possible reaction mechanisms for the C–O coupling selectivity have been proposed as follows: (i) coupling of free phenoxy radicals, (ii) coupling of controlled phenoxy radicals, and (iii) coupling of phenoxonium cation with the phenol (Fig. 10.17).

In the coupling of 2,6-Me_2P with Ag_2CO_3 as the oxidant in toluene, the addition of excess *n*-pentyl amine or acetic acid showed the product ratio of P-2,6-Me_2P/DPQ of >99/0 or 0/100, respectively. Moreover, the oxidative coupling of 2,6-Me_2P in alkaline water produced P-2,6-Me_2P in the max. 96% yield, in which the free phenoxy radical was observed by ESR [60]. These data indicate that, in the coupling of the free phenoxy radicals ((i) in Fig. 10.17), basic conditions would mainly lead to the C–O coupling, and acidic conditions would favor the C–C coupling. Consequently,

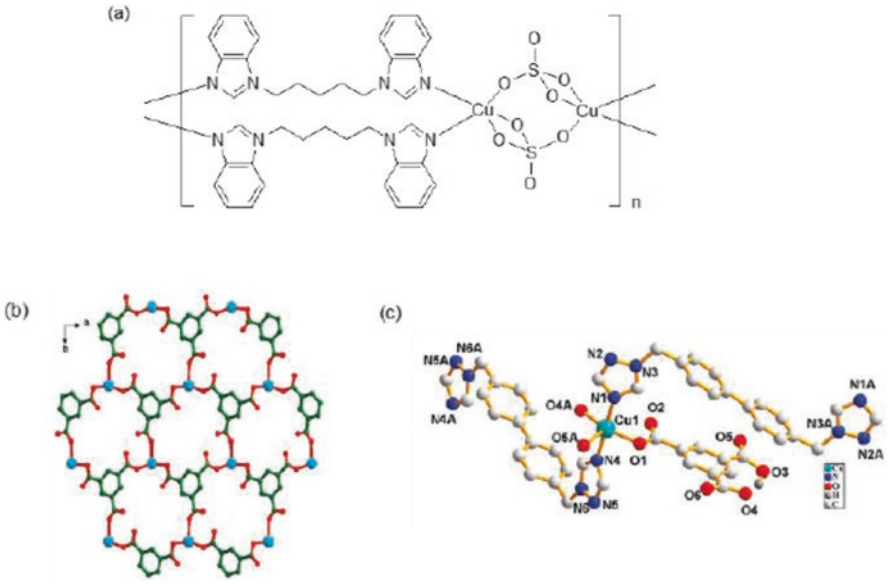


Fig. 10.16 Two Cu-containing MOFs: a 2-D MOF (**a**) and a 3-D MOF possessing a honeycomb network from $HBTC^{3-}$ anions and Cu(II) centers (**b**), which are connected by btmb ligands to generate a 3-D framework (**c**). (Reprinted with permission from Ref. [58]. Copyright © 2011 American Chemical Society)

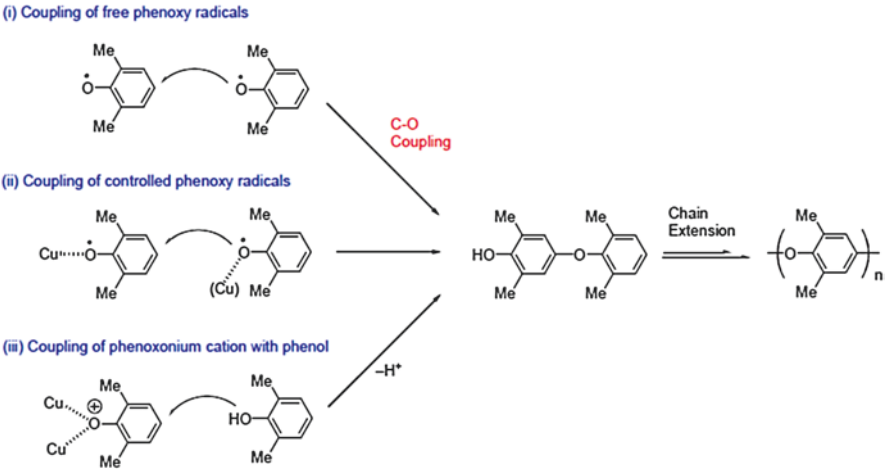


Fig. 10.17 Three proposed reaction mechanisms for C–O coupling in oxidative polymerization of 2,6-Me_2P. (Reprinted with permission from Ref. [59]. Copyright © 2003 Elsevier Science Ltd)

it seems that the C–O coupling of 2,6-Me_2P results from the free radicals in basic conditions, not necessarily from the controlled radicals, and therefore, even the conventional catalysts to form free phenoxy radicals can produce P-2,6-Me_2P.

10.4 Conclusion

Enzyme-model complexes have been studied not only to clarify the structures and reactivities of the metal-containing enzymes but also to utilize their functions for new technologies in organic synthesis. The Fe and Cu complexes for peroxidase, oxygenase, and oxidase models are applied to oxidative polymerization of phenols, anilines, and pyrroles with H_2O_2 and O_2. Although the enzyme-catalyzed oxidative polymerization has some merits of mild reaction conditions, high coupling selectivity, and nontoxic natural catalysts, their model complex catalysts have the following advantages:

- Their costs are generally much lower, because they can be prepared by chemical mass production and easy purification.
- Various reaction conditions like non-neutral conditions are applicable, and the solubility or reactivity is easily adjustable by changing their substituents.
- Bulky monomers such as cardanol are available, and ultrahigh-molecular-weight polymers like poly(*m*-cresol) can be obtained.
- It is possible to express unique functions that have not seen in enzymes: typically, tyrosinase-model complex catalysts achieve *para*-selective oxidative polymerization of phenols, although tyrosinase catalyzes *ortho*-selective monooxygenation.

The obtained poly(aromatic)s such as polyphenols, poly(phenylene oxide)s, polyanilines, and polypyrroles possess excellent characteristics in terms of mechanical strength, heat-resistance, and electric property, thereby becoming advanced materials in the frontier fields. Hence, oxidative polymerization by enzyme-model complex catalysts would provide a new synthetic method for poly(aromatic)s in terms of green polymer chemistry.

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Chapter 11
Synthesis of Vinyl Polymers via Enzymatic Oxidative Polymerisation

W. Zhang and F. Hollmann

Abstract Enzymatic methods for the polymerisation of vinyl monomers are presented and critically discussed. Vinyl monomers can be polymerised initiated by enzyme-catalysed radical formation. The most widely used initiators for this purpose are β-diketo compounds, which can be transformed into the corresponding radicals via peroxidase- or laccase-catalysed oxidation. For this, peroxidases use hydrogen peroxide as oxidant, while laccases rely on molecular oxygen. Both enzyme classes comprise specific advantages and disadvantages that are discussed in this chapter. Also, parameters to control the polymer properties are introduced and discussed.

Keywords Polymerisation of vinyl monomers · Laccase · Peroxidase · Biocatalysis

11.1 Introduction

Polymers obtained from vinyl monomers represent an important class of plastics with widespread applications. The most predominant mechanism for their synthesis relies on radical initiation followed by radical chain propagation and termination yielding the final product.

Next to the classical radical chain initiators, enzymatic radical initiation has received growing interest (mostly from the academic world) as possibly milder and more benign alternative.

In this chapter we will outline the current mechanistic understanding of the most important enzyme-initiated vinyl polymerisation reactions, present some recent application examples and discuss the advantages and drawbacks of these methods compared to the current chemical state of the art.

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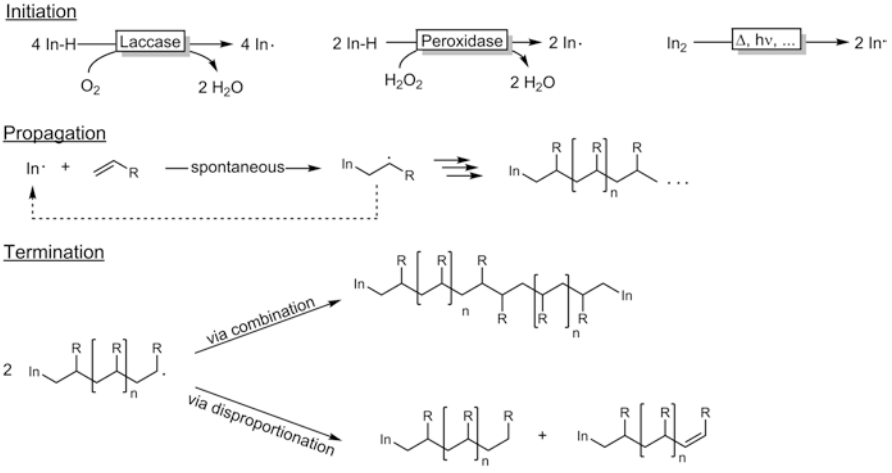
11.2 General Topics

The term 'enzymatic polymerisation of vinyl monomers', which is frequently found in the literature, is somewhat misleading as it suggests the biocatalyst being involved in the actual polymerisation reaction. In fact, the biocatalysts discussed in this chapter exclusively catalyse the first step of the polymerisation reaction (i.e. the initiation reaction), while the polymer formation occurs spontaneously. Hence, classical benefits of biocatalysis such as stereoselectivity [1] cannot be expected from this sort of polymerisation reactions. In essence, the course of an enzyme initiated polymerisation differs from a 'classical' chemical polymerisation reaction only in the initiation reaction (Scheme 11.1).

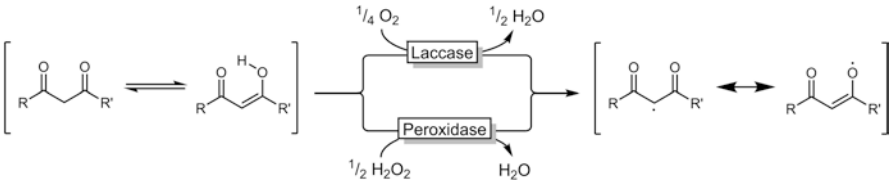
11.2.1 Mechanism of Enzyme-Initiated Polymerisations

Laccases and peroxidases are the enzymes most widely used for the enzyme-initiated polymerisation of vinyl monomers [2–4]. Their 'natural' substrates are phenolic (and related) compounds, and the enzymes catalyse a H-atom abstraction yielding reactive radical compounds. Therefore, laccases and peroxidases are also widespread used in the polymerisation of phenolics (Chaps. 9 and 10).

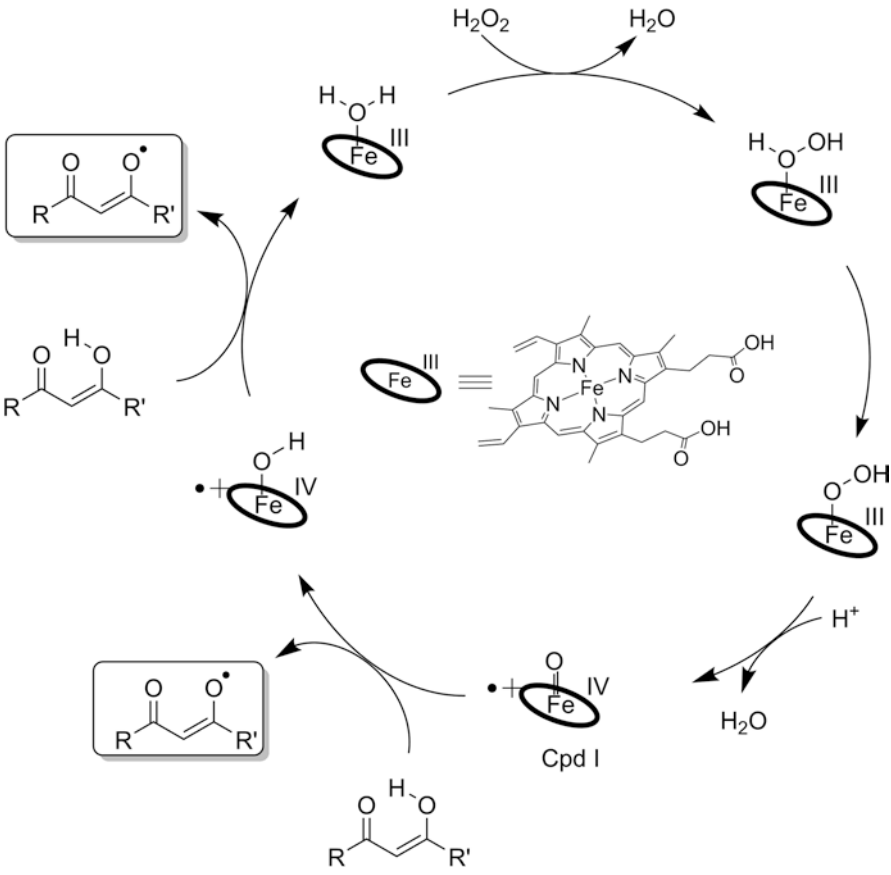
Next to phenols, laccases and peroxidases also mediate H-atom abstraction reactions from other activated starting materials, especially from β-diketo compounds (Scheme 11.2). The resulting radicals function as radical initiators (In•) for the polymerisation of vinyl monomers as discussed throughout this chapter.



Scheme 11.1 Essential steps of the radical polymerisation of vinyl compounds consisting of (1) initiation, (2) propagation and (3) termination. 'Classical chemical' and enzymatic polymerisations differ mostly in the first step (chain initiation)

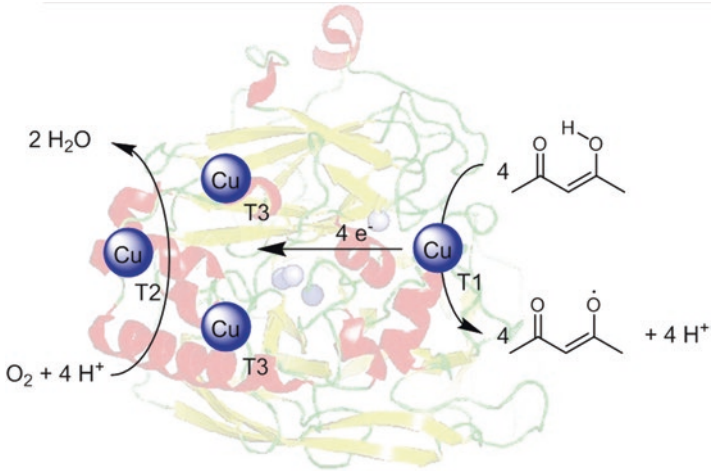


Scheme 11.2 Laccase- or peroxidase-catalysed H-atom abstraction from a β-diketone substrate

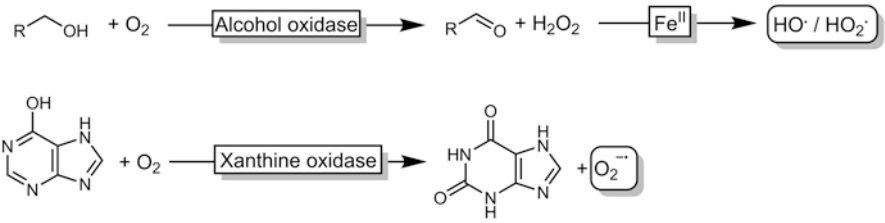


Scheme 11.3 Catalytic mechanism of the peroxidase-catalysed H-atom abstraction of β-diketones

The catalytic mechanisms of peroxidases and laccases differ considerably. *Peroxidases* are generally heme-dependent enzymes, which in the presence of hydrogen peroxide (or other organic peroxides) form a highly oxidised (formal) Fe^{V}-oxo-species (compound I). Compound I is best described as oxyferryl (Fe^{IV}) embedded in a porphyrin radical cation [5]. Compound I performs two successive H-atom abstraction reactions from activated substrates (phenols or β-diketones) forming two radical initiators (Scheme 11.3).



Scheme 11.4 Simplified reaction scheme of laccase-catalysed oxidation of β-diketones



Scheme 11.5 Miscellaneous enzymatic systems to generate polymerisation initiators

Laccases also catalyse H-atom abstraction reactions from substrates very similar to those of the aforementioned peroxidases. In contrast, however, laccases utilise molecular oxygen instead of hydrogen peroxide as oxidant for this reaction. Laccases contain four copper ions (which is why they are also called blue-copper oxidases) classified as T1, T2 and T3 [6, 7]. Generally speaking, the T1 Cu ion performs four successive single-electron oxidation steps on the starting material transferring the reducing equivalents to the T2/T3-Cu ions. O_2 reduction occurs in the T2/T3 cluster (which also very tightly binds the intermediate, partially reduced oxygen species, Scheme 11.4).

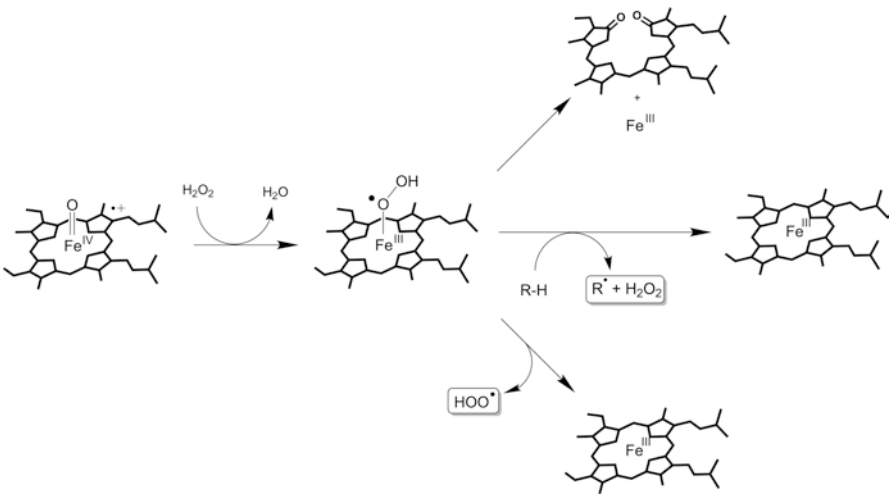
Next to the predominant peroxidases and laccases, also a few other enzymatic systems to generate polymerisation initiators are worth mentioning here. Alcohol oxidases catalyse the aerobic oxidation of alcohols to the corresponding carbonyl groups yielding hydrogen peroxide as by-product. In the presence of Fe^{II} ions, the latter can initiate Fenton-like reactions with reactive oxygen species (ROS) as initiators (Scheme 11.5) [8]. Similarly, xanthine oxidase can be used for the generation of ROS; in contrast to alcohol oxidases, this enzyme generates superoxide directly (Scheme 11.5) [8].

11.2.2 *Factors Influencing the Outcome of Enzyme-Initiated Polymerisation of Vinyl Monomers*

As for every radical polymerisation reaction, the yield and properties of the final product largely depend on the ratio of radical initiator to the monomer and the presence of possible chain growth inhibitors.

The in situ concentration of the active initiator radical can be influenced by parameters such as the enzyme concentration (its activity, respectively). Lalot and coworkers have investigated the effect of enzyme and initiator concentration on the polymer size of the HRP-initiated polymerisation of acrylamide (AAm, Scheme 11.7) [9]. These authors confirmed that a lower in situ concentration of the active initiator molecule (Acac radical) favours high molecular weights. This concentration directly correlates (increases) with the concentration of Acac and HRP. Qualitatively, the same trend was also found for the laccase-initiated polymerisation [10]. Overall, controlling the in situ concentration of the initiator radical via overall initiator concentration and/or enzyme concentration is a very good handle to control the polymer weight of the final product.

Also the oxidant concentration can play an important role in the polymerisation reaction but needs careful adjustment. In the case of peroxidases, H_2O_2 should not be applied in too high concentrations as H_2O_2 also is an efficient inactivator of the heme-enzymes [11]. The exact mechanism is not defined yet, and probably different inactivation pathways exist (Scheme 11.6), but it is clear that high in situ concentrations of H_2O_2 should be avoided.



Scheme 11.6 Different pathways of inactivation of heme enzymes by H_2O_2. Both oxidative destruction of the heme prosthetic group and formation of reactive oxygen species (highlighted) leading to enzyme inactivation are discussed

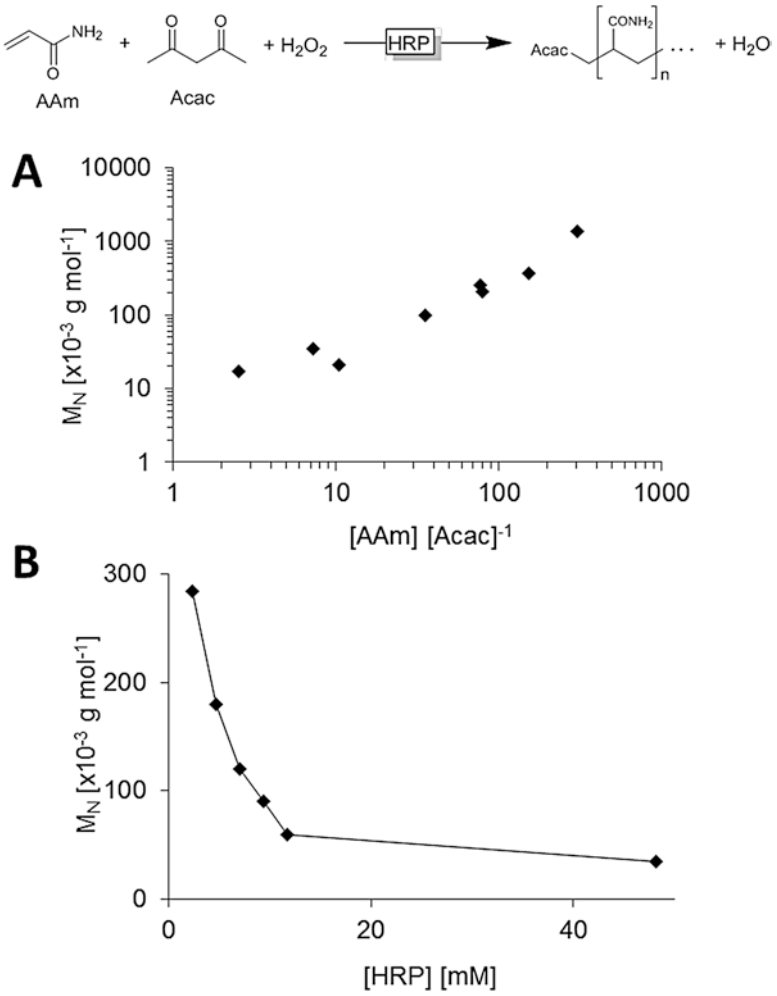
Therefore, H_2O_2 often is added several times in small portions to minimise H_2O_2-caused inactivation. More elegantly, some in situ H_2O_2 generation systems have been developed in the past years, which may be applicable to use peroxidases more efficiently in polymerisation processes [12–20].

For laccases, O_2 serves as oxidant to initiate the polymerisation reaction. The issue with O_2 is that it also is an efficient radical scavenger inhibiting the polymerisation reaction. Therefore, also in the case of laccases (though not for enzyme stability reasons), the oxidant concentration needs to be carefully controlled [10]. This is also true for peroxidase reactions as also here trace O_2 amounts can significantly impair the polymerisation reaction. Very recently an efficient measure to reduce the O_2 content simply by adding glucose/glucose-oxidase to the reaction mixture was proposed by Stevens and coworkers [21, 22] (Scheme 11.7).

It is generally assumed that the enol form of the β-diketo compound (more phenol-like) represents the actual substrate for the laccase- or peroxidase-catalysed H-atom abstraction [23]. Hence, factors influencing the keto-enol equilibrium will influence the in situ concentration of the actual enol substrate. Using more alkaline pH values is a double-edged measure; on the one hand, higher pH values favour higher enol concentrations, while on the other hand, the pH optima of laccases and peroxidases are more in the slightly acidic range [24]. Another possibility is to engineer the β-diketo compound itself and favour the enol content through steric and/or electronic variations. It should, however, be kept in mind that both factors may interfere with the acceptance of the β-diketo compound by the enzyme (especially in case of sterically demanding starting materials) or with the polymer-initiation activity of the resulting radical (particularly in case of using electronegative substituents to increase the enol content). Kaplan and coworkers systematically investigated the influence of the initiator molecule on the polymer properties for the horseradish peroxidase (HRP)-catalysed polymerisation of styrene [25] and acrylamide [26] (Table 11.1) impressively demonstrating the influence the initiator can have on the conversion as well as on polymer properties such as molecular weight (M_W) and polydispersity (PD).

Ideally, the initiator molecule would be circumvented at all. This would not only eliminate its cost contribution but would also be favourable from an enzyme activity point of view (many initiators exhibit solvent-like properties and can – in too high concentrations – inactivate the biocatalyst). Early reports claiming initiator-free enzyme-initiated polymerisation [27, 28] could not be reproduced by others [10, 26, 29].

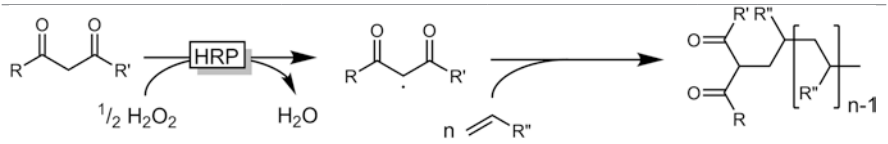
Finally, also the solvent can have a significant influence on the polymerisation reaction. Especially if hydrophobic monomers are used, their solubility in the mostly aqueous reaction mixtures can be an issue. Polar organic solvents can be used to increase the monomer solubility [25, 30]. But frequently the presence of water-mixable cosolvents impairs the stability of the biocatalyst used. An alternative to increasing the water solubility of the monomers is to use a biphasic reaction

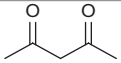
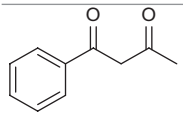
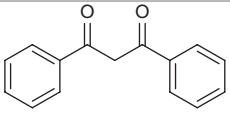
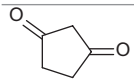
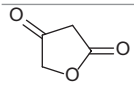
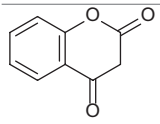
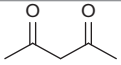
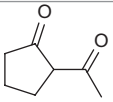
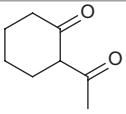
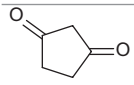
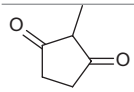


Scheme 11.7 Influence of initiator (Acac, **a**) and enzyme (HRP, **b**) concentration on the polymer weight of the HRP-initiated polymerisation of acrylamide

mixture containing an aqueous reaction mixture with the biocatalyst and a hydrophobic organic phase composed of the monomer in high concentrations (ideally neat). Such emulsion polymerisations have been investigated especially for styrenes [29, 31, 32]. Even better than a biphasic system would be to use neat reaction conditions without any cosolvent whatsoever. For this, immobilised preparations of the biocatalyst are required [33–41]. Another interesting approach is to solubilise the hydrophilic enzymes in organic media by coating them with surfactants [42].

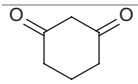
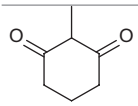
Table 11.1 Influence of the initiator molecule on the performance of HRP-initiated polymerisation reactions



Initiator	Yield [%]	M_W [g mol^{-1}]	PD [–]
Styrene polymerisation			
	16.7	26,900	2.07
	14.1	80,100	1.96
	14.4	96,500	2.16
	59.4	67,600	1.98
	41.1	50,900	2.22
	14.5	57,200	1.64
Acrylamide polymerisation			
	93	124,000	2.5
	76	5100	4.4
	84	56,300	2.9
	78	84,500	2.7
	38	10,500	3.9

(continued)

Table 11.1 (continued)

	72	27,000	3.3
	86	9800	3.9

11.3 Selected Examples

In recent years the number of reported examples for enzyme-initiated vinyl polymerisations has been growing steadily. Scheme 11.8 gives a representative overview over some of the literature examples.

Graft polymerisation is receiving increasing attention especially using HRP as catalyst. For example modifying starch with (poly)acrylamide [55], (poly) methyl acrylate [56] or (poly)butyl acrylate [57] has been reported (Scheme 11.9) [58]. As grafting mechanism, H-atom abstraction from a starch-OH-group by HRP-generated Acac has been proposed.

Another interesting grafting approach has been reported with silica surfaces using laccases [59] or HRP [60]. In the latter case, for example, SiO_2 particles were first covered with the initiator (Acac) followed by HRP-initiated grafting of acrylamide onto the SiO_2 particle (Scheme 11.10).

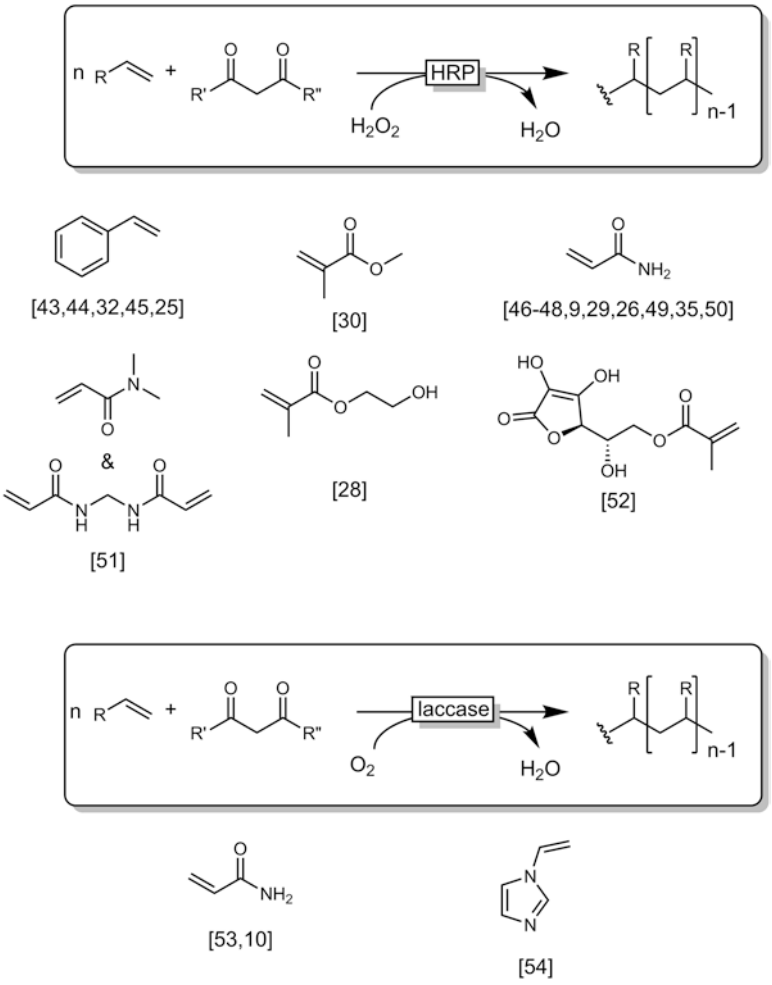
Also lignin represents an attractive target to graft polymers onto. Interestingly, this appears to be a laccase domain [61–67].

Cross-linking of chitosan using laccases was used to self-immobilise the enzyme [68].

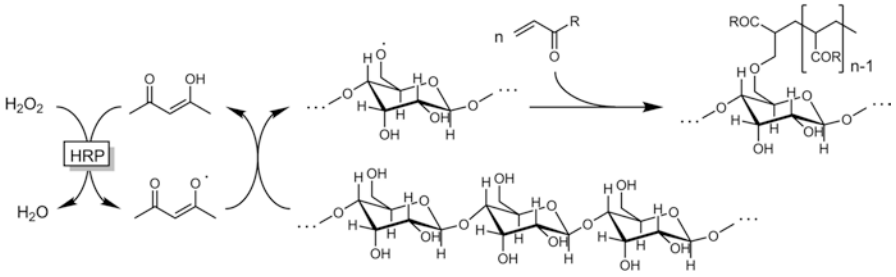
In polymer chemistry, the so-called reversible deactivation radical polymerisation (RDRP) is very much in focus now due to its power to control the molecular weight and the polydispersity of the polymer products. Also in enzyme-initiated polymerisations, RDRP is being used more frequently [54, 69, 70].

11.4 Conclusions

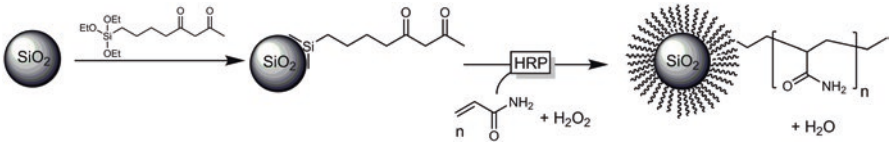
The use of enzymes to initiate radical polymerisation reactions is enjoying steadily growing interest. Partially, this may be due to the fact that enzymatic reactions are generally perceived to be more environmentally benign than ‘chemical’ reactions. A quantitative study comparing the environmental impact of both, however, is lacking so far. It should be kept in mind that not only the actual reaction (conditions) determines the environmental impact but also factors such as catalyst’s preparation and downstream processing to obtain the desired product. Hence, perceived advantages such as mild reaction conditions or the use of water as solvent may well turn out to be less important than thought or maybe even counterproductive.



Scheme 11.8 Selected examples of horseradish peroxidase- (HRP) or laccase-initiated vinyl polymerisations



Scheme 11.9 Proposed mechanism for the HRP-Acac-initiated acrylate grafting on starch



Scheme 11.10 Surface-initiated enzymatic polymerisation using HRP

Nevertheless, enzyme-initiated polymerisation remains an active and dynamic field of research, and some exciting new developments may be expected in the future.

Today, the peroxidase from horseradish is by far the most popular biocatalyst in use, which is somewhat astonishing considering that the number of available peroxidases/peroxygenases and laccases is steadily increasing [71]. Though it is not expected that new enzymes will have a significant impact on the polymer structure, it may well be that increased activity and/or stability may contribute to the economic feasibility of these processes.

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Chapter 12
Enzymatic Modification of Polymers

H. N. Cheng

Abstract In polymer applications and development, it is often necessary to modify an existing polymer structure in order to impart special end-use properties. Whereas chemical modification methods are most commonly practiced, sometimes enzyme-catalyzed modifications may be desirable because of the specificity of the reactions, reduction in the by-products produced, milder reaction conditions, and more benign environmental impact. A number of enzyme-catalyzed reactions are reviewed in this paper, covering primarily biobased materials like polysaccharides, proteins, triglycerides, and lignin. The enzymes used include mostly hydrolases, oxidoreductases, and transferases, with occasional involvement of lyases and isomerases. The types of reactions are diverse and include polymer hydrolysis and degradation, polymerization, oxidation, glycosylation, cross-linking, and transformation of functional groups. Because biopolymers are agro-based and occur abundantly in nature, they are often available in large quantities and amenable to enzymatic reactions. As such, the combination of biopolymers and enzymes represents a good product development opportunity and a useful tool for postharvest agricultural technology and green polymer chemistry.

Keywords Biopolymers · Enzymes · Functionalization · Hydrolysis · Lignin · Modification · Polymers · Polysaccharide · Protein · Triglyceride

12.1 Overview

In view of current interest in green polymer chemistry, reduction in the dependence of petroleum-based raw materials, and environmental stewardship, agro-based materials and enzyme-related technologies have become increasingly emphasized in polymer research and product development. Indeed, the use of enzymes for polymer modification reactions is now fairly well established and often encountered.

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A huge body of literature has been accumulated on numerous reactions and processes, including many books [1–7] and reviews [8–14]. A detailed literature search of Chemical Abstracts for "enzymatic modification" showed for the 2008–2018 time period 301 papers on polysaccharides, 6350 papers on proteins, 472 papers on lignin, 377 papers on triglycerides, and 7455 on synthetic polymers. In order to keep this chapter within a reasonable size, only a selected coverage is being made for the period 2012–2018, with the emphases on enzyme-catalyzed reactions that can be used for research and development (R&D) and potential industrial applications. Such reactions include polymer hydrolysis; addition, removal, or modification of functional groups; cross-linking; and (to a lesser degree) polymer synthesis.

12.1.1 Enzyme Categories

Enzymes are commonly classified into six categories, known as Enzyme Commission (EC) numbers: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Table 12.1) [15]. For polymer hydrolysis, modifications, and syntheses, three enzyme categories (hydrolases, oxidoreductases, and transferases) are most often utilized. The other three categories are helpful but less often observed for the topics covered in this review.

12.1.2 Hydrolases (EC 3)

Thus far in the literature, hydrolases have appeared more frequently in polymer studies than any other enzyme types. In addition to its natural function of hydrolyzing the substrates, hydrolases have been used extensively to carry out polymerizations and polymer modification reactions. Many hydrolases are non-specific and can accommodate a range of substrates. It is also helpful that many hydrolases are commercially available at reasonable prices.

Polymerization Reactions One successful application of hydrolases is to catalyze the synthesis of oligosaccharides and polysaccharides. This approach has been

Table 12.1 Uses of enzymes in synthesis, modification, and degradation of conventional polymers

Enzyme category	EC number	Polymer hydrolyses	Polymer modifications	Polymer syntheses
Oxidoreductase	1	√	√	√
Transferase	2	√	√	
Hydrolase	3	√	√	√
Lyase	4			√
Isomerase	5	√	√	
Ligase	6			

pioneered by Kobayashi et al. and summarized in several excellent reviews [8, 10, 16–18]. A somewhat different approach is to use glycosidases to prepare oligosaccharides [19–22].

Another very active approach is the synthesis of polyesters, polylactones, and polycarbonates. Lipases have been the most commonly used enzymes for this purpose, and a large number of polymers have been made. Particularly outstanding is *Candida antarctica* lipase B immobilized on acrylic resin (also known as CALB or Novozym® 435). This large body of literature has been reported and reviewed by a number of workers [23–34].

In addition, lipases and proteases have been utilized to make polyamides. Polyamides over 10 KDa have been enzymatically synthesized for water-soluble polyamides [35–37] or made through ring-opening polymerization of lactams [38–40]. Proteases have been used to prepare oligopeptides with controlled sequences and the effects of reaction parameters studied [41, 42].

An interesting development is silicon bioscience [43–46], where some proteins (called "silicateins") can act both as catalysts and as macromolecular templates in vitro, directing the condensation of silica and polysiloxanes from silicon alkoxides at neutral pH. The use of a lipid-coated lipase to catalyze the oligomerization of diethoxydimethylsilane (DEDMS) has been reported by Nishino et al. [47].

Polymer Modification Reactions This is a large area of endeavor where various hydrolases have been employed to insert or remove functional groups on substrate polymers or to convert one functionality to another. A good overview with selected illustrations has been given by Puskas et al. [48]. Some examples from the literature include CALB-catalyzed end-functionalization of many synthetic polymers [48, 49], lipase-catalyzed syntheses of fatty acid diester of poly(ethylene glycol) [50], lipase-catalyzed derivatization of silicone-containing polymers [51], and preparation of glycosilicone conjugates [52].

In the polysaccharide area, some examples include protease-catalyzed acylation of polysaccharides [53], papain-catalyzed amidation of pectin [53], hydrolase-catalyzed amidation of carboxymethyl cellulose [50], enzymatic syntheses of fatty acid esters of cationic guar [50], and modification of starch [54]. More detailed information is given in Sect. 12.2.

Hydrolysis and Degradation In nature, hydrolases are usually designed for hydrolysis and degradation of substrate molecules. Hydrolases can be used for molecular weight reduction, cleavage of branch chains, breakdown of polymeric matrices, and other applications. Some examples in the polysaccharide area include cellulolytic enzymes for biomass conversion [55], proteases for the degradation of guar gum [53], cellulase for viscosity reduction of xanthan gum [53], and beta-D-galactosidase for pectin hydrolysis [53]. More examples can be found in Sect. 12.2.1.

Examples in the polyester area include the use of specific depolymerases for the degradation of poly(lactic acid-co-hydroxybutyrate) [56], recombinant cutinases for

polyester degradation [57], cutinases for poly(ethylene terephthalate) hydrolysis [58], protease- and lipase-type depolymerases for poly(lactic acid) [59], and lipases for the degradation and recycling of poly(butyl adipate) and poly(butylene succinate) [60]. Other hydrolases for polymer degradation and hydrolysis include Alcalase® protease for the hydrolysis of end-terminated esters in polyamide [61] and nitrilase (and nitrile hydratase) for the bioconversion of nitriles to carboxylic acids [62–65]. Two reviews mostly on the enzymatic hydrolysis of synthetic polymers have appeared in 2008 and 2003 [66, 67].

12.1.3 Oxidoreductases (EC 1)

Oxidoreductases tend to be more specific with respect to their substrates. Some oxidoreductases require the concurrent action of cofactors and some do not. Because cofactors require extra cost and more skill in handling them, the reactions obviating the use of cofactors are preferred. However, in appropriate cases oxidoreductases requiring cofactors can still be useful.

Polymer Syntheses This is potentially a very fruitful area for enzymes in polymer science. A lot of papers have been published using oxidoreductase for the polymerization of phenols and anilines. Thus, polyphenols [68–73], poly(phenylene oxide) [74, 75], and electrically conducting polymers [76–78] have been made in this way. A different and very productive approach is to engage oxidoreductases in the free-radical polymerization of vinyl monomers [79–86].

Polymer Modifications Some oxidoreductases have been used in specific ways to convert one functionality to another in a biopolymer. A well-known example is galactose oxidase that oxidizes only the C6 alcohol on galactose to an aldehyde [87–89]. Thus, this reaction has been used on galactomannans (such as guar and locust bean gum) [87–89]. Another example is tyrosinase, which catalyzes the oxidation of phenolic compounds into quinones. It has been used to functionalize chitosan [90, 91] or to graft proteins onto chitosan [92].

An interesting oxidation reaction involves lipase (formally a hydrolase), which catalyzes the conversion of a carboxylic acid to a peracid in the presence of H_2O_2 [93, 94]. The resulting peracid can then carry out polymer modifications, such as the epoxidation of polybutadiene [95] and oxidation of hydroxyethylcellulose [96].

Polymer Hydrolysis and Degradation For pulp and paper industry, a significant application of oxidoreductase is "biobleaching," which refers to the enzyme-catalyzed removal of lignin from wood pulp [97–99]. Four oxidoreductases have been studied: laccase (with concurrent use of a mediator and oxygen), lignin peroxidase, manganese peroxidase, and versatile peroxidase (in combination with H_2O_2).

Presumably the enzymatic system oxidizes the lignin, such that it is more easily removed later in the pulping process. More information is given in Sect. 12.5.

12.1.4 Transferases (EC 2)

A transferase is an enzyme that catalyzes the transfer of a specific functional group from one molecule to another. Cofactors are sometimes needed for the processes. Thus far, quite a few reactions involving transferases have been used for polymer syntheses and modifications.

Polymer Syntheses Many articles describe the application of transferases for polymer syntheses. One example is the use of glycosyltransferase for the synthesis of oligosaccharides and polysaccharides, where the cofactors are recycled [100–104]. Wang et al. [100] have reviewed several approaches relating to glycosyltransferases. DeAngelis [105] described two methods using recombinant *Pasteurella multocida* synthase to generate glycosaminoglycans (GAG). These have been utilized to make GAG via immobilized mutant enzyme reactors [106]. Another approach is to take advantage of biopathway engineering to design different carbohydrate polymers [107].

Other transferases of interest include dextransucrase, which catalyzes the formation of dextran and some oligosaccharides [108–111]. Yet another transferase reaction entails the use of potato starch phosphorylase in the synthesis of low-molecular-weight amylose [112, 113]. Glycogen phosphorylases have been employed in a two-enzyme tandem reaction to produce artificial starches with different levels of branches [114].

Polymer Modifications Glycosyltransferases have also been utilized for glycan chain modifications, especially at outer or terminal positions [22]. Transglutaminases are acyl transfer enzymes that catalyze the condensation of glutamine and lysine residues of proteins [115, 116]. A calcium-independent microbial transglutaminase has been reported by Payne et al. [92] to cross-link the protein in gelatin-chitosan blends.

12.1.5 Lyases (EC 4)

A lyase is an enzyme that catalyzes the breaking of various chemical bonds by means other than hydrolysis and oxidation. It often forms a new double bond or a new ring structure. In practical terms, lyases are alternatives to hydrolases in degrading polymers. In the polysaccharide area, a number of lyases are known and sometimes used, e.g., pectin lyase, pectate lyase, xanthan lyase, alginate lyase, hyaluronate lyase, and heparin lyase.

12.1.6 Isomerases (EC 5)

An isomerase converts a molecule from one isomer to another, often involving bond breakage and reformation. For example, alanine racemase catalyzes the conversion of L-alanine to D-alanine, and glucose isomerase converts glucose to fructose. In polymer science, isomerases are less often used. Some examples in polysaccharides include the use of epimerases to convert mannuronate to guluronate in alginates [117–120] and to carry out C5 epimerization of oxidized konjac glucomannan [121] and oxidized galactomannan [122].

12.1.7 Ligases (EC 6)

DNA ligase is involved in DNA synthesis during replication. Other ligases are also useful in various biochemical processes. For conventional polymer synthesis or modification, the use of this category of enzymes is rather rare.

12.2 Enzymatic Modification of Polysaccharides

Polysaccharides are polymeric carbohydrates found in nature that consist of monosaccharides attached together by glycosidic bonds. Some of them, like starch and glycogen, are important for food and nutrition. Many of them (e.g., cellulose, hemicellulose, starch, chitin, guar, xanthan, carrageenan, and alginate) are industrially relevant materials, used as thickeners, gelling agents, stabilizers, interfacial agents, flocculants, and encapsulants in a variety of applications [123–126]. Yet, the polysaccharides found in nature often do not have the optimal properties needed for specific applications, and chemical modifications are needed in order to produce desirable and competitive commercial products [127–129]. Particularly successful are chemically modified cellulose and starch, with a range of derivative products available. For example, commercially available cellulosic derivatives include cellulose acetate, carboxymethyl cellulose (CMC), hydroxyethyl cellulose (HEC), and methyl cellulose (MC). Modified starches include cationic starch, hydroxyethyl starch, starch acetate, and starch phosphate.

It is well known that enzymatic reactions can complement the chemical modification reactions in suitable cases [130–133]. Examples of useful enzyme-catalyzed reactions of polysaccharides include (1) molecular weight reduction, (2) addition of charge, (3) addition of polar group, (4) hydrophobic modification, and (5) formation of reactive oligomers. For illustration, some enzyme-catalyzed modification reactions are shown below.

12.2.1 Molecular Weight Reduction

Through the use of an appropriate hydrolase, the molecular weight of a polysaccharide can be reduced [133]. An example of an advantageous reduction of molecular weight is shown for "biostable" cellulosic derivatives [134, 135], where CMC or HEC can be deliberately treated with a cellulase enzyme to hydrolyze substrate sites that are susceptible to enzymatic action. The resulting cellulosic products are then resistant toward microbial degradation.

In the recent literature, there has been some interest in degrading the molecular weight of glycosaminoglycans, particularly hyaluronic acid in order to yield new bioactive compounds. Thus, in one study, many enzymes and microbes were screened for their ability to degrade a marine exopolysaccharide comprising mostly hyaluronic acid, and one candidate was found to be particularly suitable [136]. Moreover, the degradation of hyaluronic acid by hyaluronidase was studied with a protein nanopore [137].

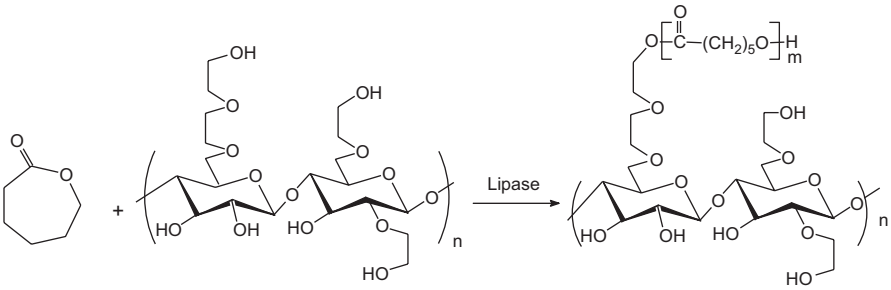
Another example of a functional product is low-molecular-weight guar, which can be used as a dietary fiber and bioactive substance [138, 139]. The molecular weight reduction can be done chemically or enzymatically [140, 141]. In an earlier study [142], the molecular weight reduction of guar was examined combinatorially with four enzymes (lipase, hemicellulase, pectinase, and protease). The hemicellulose/protease combination gave the greatest molecular weight reduction. That study pointed out the importance of protein (up to 7% in guar) in contributing toward the viscosity of guar.

12.2.2 Addition of a Polar Substituent

The nature of a substituent on a polysaccharide can significantly change the properties of the polysaccharide. This is why so many cellulose and starch derivatives have been made. Whereas most of the derivatizing reactions have been conducted through chemical means, there have been some enzymatic reactions. A simple example is the reaction involving lactose and β-galactosidase to insert a galactose moiety either at the chain ends of an oligosaccharide [143] or at the end of ethylene oxide units in HEC [144]. Another fruitful approach is to enzymatically graft a polymer onto a polysaccharide, as shown in the two examples below.

In the first example, HEC, when deposited as a film, could be grafted by caprolactone in bulk, using lipases derived from porcine pancreas (Scheme 12.1) [145]. The product had a degree of substitution between 0.10 and 0.32 on the anhydroglucose basis. The reaction demonstrates that lipase-catalyzed ring-opening polymerization can be employed to graft hydrophobic polyesters onto hydrophilic cellulose-based polymers.

The second example is the chemoenzymatic synthesis of amylose-grafted cellulose [146]. A maltoheptaose was chemically introduced to the amine-functionalized



Scheme 12.1 Modification of hydroxyethyl cellulose (HEC) via graft copolymerization of ε-caprolactone by a lipase catalyst

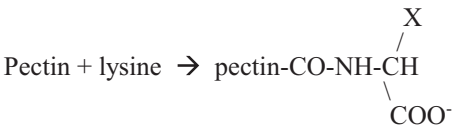
cellulose by reductive amination to produce the maltoheptaose-grafted cellulose. Then, the phosphorylase-catalyzed enzymatic polymerization of glucose 1-phosphate from the graft-chain ends on the cellulose derivative was performed, giving the amylose-grafted cellulose. The obtained material was shown to form gels and films [146].

$$\text{cellulose} + \text{maltoheptaose} \xrightarrow{\text{chemical}} \text{cellulose}\ (\text{glc})_7 \xrightarrow[\text{phosphorylase}]{\text{glucose 1 phosphate}} \text{amylose grafted cellulose}$$

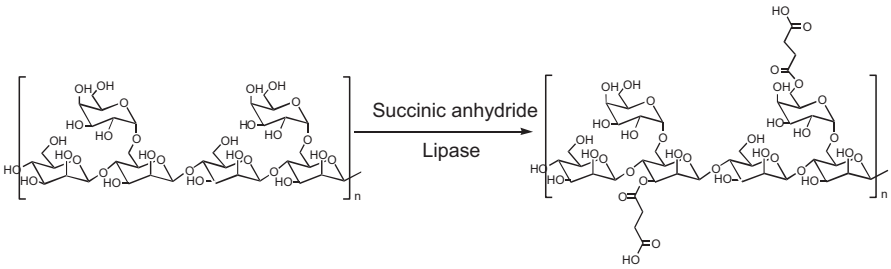
12.2.3 Addition of Charge

Sometimes it is useful to introduce an electrical charge to a polysaccharide to impart special properties. An example of the addition of an anionic group is shown by the lipase-catalyzed reaction of an anhydride (e.g., succinic and maleic) [147, 148]. Thus, lipase AK (*Pseudomonas* sp., from Amano) was found to have excellent activity for this reaction on guar, giving succinated guar (Scheme 12.2). The same reaction was also reported for HEC [147].

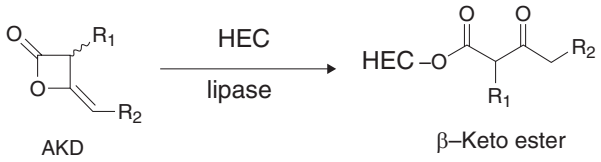
The enzyme-catalyzed addition of a cationic functionality to a polysaccharide is less common. An example is the papain-catalyzed amidation of high methoxy pectin with lysine and other diamines as shown below [149].



where X is $-(CH_2)_4-NH_2$ for lysine but can also be $-CH_2-(CH_2)_5-NH_2$, $-CH_2-(CH_2)_2$-imidazole, or $-CH_2-(CH_2)_2$-guanidine [149]. The active enzyme appeared to



Scheme 12.2 Modification of guar by succinic anhydride catalyzed by lipase



Scheme 12.3 Lipased-catalyzed modification of hydroxyethyl cellulose (HEC) with alkyl ketene dimer (AKD)

be a minor component in papain. The products were reported to show enhanced viscosity and gel-forming properties.

Another example of the addition of a cationic functionality was shown for the protease-catalyzed reaction of CMC with 1,6-hexamethylenediamine to form an amide with a free amine end group [148].

12.2.4 Hydrophobic Modification

A more commonly encountered reaction is hydrophobic modification. Because polysaccharides are usually water-soluble, a hydrophobic moiety on the polysaccharide tends to associate with each other to increase the viscosity of the polysaccharide solution at low shear rates [150]. These hydrophobically modified polysaccharides can often be used as surfactants or rheology modifiers. Whereas this reaction is mostly done chemically, an enzyme has been used in selected cases to facilitate the reaction or to provide additional hydrophobic structures. An example of the lipase-catalyzed reaction of alkyl ketene dimer with HEC is shown in Scheme 12.3 [151], where $R_1 = R_2 = C_{14}H_{29}$, for a ketene dimer derived from palmitic acid. Although the reaction can be done without an enzyme at higher temperature, the use of the enzyme increases the yield and decreases the reaction temperature. This enzyme-catalyzed reaction has also been carried out on starch [152].

Another example is the hydrophobic modification of HEC, using lipase and vinyl stearate [153]. The stearoyl functionality forms an ester with the –OH of HEC with the loss of acetaldehyde, thereby providing a hydrophobic substituent on HEC.

12.2.5 Formation of Reactive Functionalities

Enzymatic reactions can be used to convert the functionality of one substituent on a polysaccharide to another. A good example is the conversion of C6 alcohol on a galactose to an aldehyde through galactose oxidase [87–89, 154–156]. Another reaction is the addition of an acrylate functionality onto HEC with vinyl acrylate and a lipase. The acrylate functionality on HEC can then be reacted further to form additional derivatives [153].

12.3 Enzymatic Modification of Proteins

Enzymes are proteins themselves, and thus one may expect the use of enzymes in protein modification to be very productive. Indeed in living systems, a lot of proteins are modified in physiological processes. However, from the point of view of industrial product development, the largest applications of enzymatic modification of proteins happen in the food arena. As in other polymers, sometimes a given protein may not have the optimal properties needed for an application, and protein modifications are needed. In the food area, chemical modifications have to be done with care to ensure that the reagents used are nontoxic (or else fully reacted or removed after the reactions), the reaction products are nonhazardous, and all by-products involved in the reactions are compatible with FDA regulations for food use. In contrast, the enzymes are more specific in their reactions (thereby generating less by-products) and usually involve milder experimental conditions (such as lower temperature and aqueous solvents), and many enzymes are nontoxic themselves, such that the FDA requirements can be more easily met.

An excellent early review on this topic was provided by Whitaker [157]. More recent reviews included Filice et al. [158], Kumar et al. [159], Panyam and Kilara [160], and Chobert et al. [161]. For the enzymatic reactions on food proteins, the most common reactions are hydrolysis and cross-linking. Other modifications have been used with less frequency, such as phosphorylation/dephosphorylation, glycosylation, and oxidation/reduction. The following review was based on a Chemical Abstracts search of enzymatic modifications of food proteins covering the years 2012–2018.

12.3.1 Hydrolysis

For food applications, hydrolysis is the most common way of modifying proteins. Since 2012 at least 27 papers and reviews have appeared on enzymatic hydrolysis of food proteins. These papers can be grouped into four categories. In the first category, 21 papers deal with enzymatic hydrolysates of proteins in order to improve

their properties, to enhance their use in normal or clinical nutrition, and to produce bioactive substances or functional food ingredients. A wide range of food proteins were enzymatically hydrolyzed, including soy proteins [162, 163], sesame proteins [164–166], milk proteins [167–171], fish proteins [172, 173], egg proteins [174], canola meal protein [175], sunflower protein [176], flaxseed protein [177], rice bran protein [178], wheat protein [179], and pea protein [180, 181]. The properties being studied included emulsification, foaming, antioxidant, and bioactivity. Canola meal protein [175], milk protein [167], and sunflower protein [176] were specifically subjected to enzymatic hydrolysis in order to improve their poor water solubility. The enzymes used included mostly proteases, e.g., Alcalase®, papain, pepsin, chymotrypsin, trypsin, neutrase®, bromelain, and Flavourzyme®.

The second category of papers in the enzyme hydrolysis area deals with encapsulation. Thus, soy protein was degraded with Alcalase® in order to make oil-in-water emulsions that encapsulated tocopherol [182]. Sunflower protein was also enzymatically hydrolyzed to form microparticles and emulsions that encapsulated tocopherol [183]. Hydrolysis was combined with enzymatic cross-linking for soy and sunflower protein [184]. In all three cases, oil retention decreased with hydrolysis but increased with fatty acid acylation.

The third category consists of publications that use enzyme hydrolysis to reduce allergenicity of the protein. Thus, whey protein was treated with trypsin [185] and chymotrypsin and bromelain in combination with high hydrostatic pressure [186] in order to develop specialty products like infant formulas, geriatric products, diet foods, and high-energy food supplements.

There are also some papers that carried out enzymatic hydrolysis of proteins to gather fundamental information, e.g., use of Alcalase® and Flavourzyme® to degrade potato protein to get amino acid composition [187] and degradation of casein to determine its chemical structure and molecular weight distribution [188].

12.3.2 Cross-Linking

In the past 5–6 years, there have been at least 23 papers on enzyme-modified food proteins through cross-linking. Most of them used transglutaminase (TG) although tyrosinase and laccase were sometimes involved as well. In an excellent paper [189], the reaction conditions for TG, *T. hirsuta* laccase, and two types of tyrosinase (*A. bisporus* and *T. reesei*) were investigated. In raw milk, *T. reesei* tyrosinase was the only enzyme that induced intermolecular protein cross-linking. After heat treatment of milk, both TG and *T. reesei* tyrosinase were able to form covalently linked oligomers. β-Casein was the most readily cross-linked protein. Susceptibility of the whey proteins to enzymatic modification was restricted due to their compact globular structure. After heat treatment, which partially unfolded the whey protein molecules, both TG and *T. reesei* tyrosinase were capable of cross-linking whey proteins, whereas *A. bisporus* tyrosinase and laccase were inefficient [189].

Additionally, two other publications also studied the reaction parameters for tyrosinase [190] and laccase [191].

Most of the protein cross-linking papers deal with improved functional properties achieved, particularly with TG. In general, cross-linking affects solubility, gelation, emulsification, foaming, viscosity, and water-holding properties of the protein. Two reviews cover these effects well, one dealing with proteins in general [192] and one with dairy proteins [193]. Eight more papers deal with improvements in the properties of specific proteins with TG treatment. These include milk proteins [194], casein [195], chicken proteins [196, 197], oat and faba bean protein [198], pea protein [199], peanut protein [200], and fish protein [201]. Four papers studied the composite proteins made through cross-linking of two different proteins and their functional properties, e.g., casein/gelatin [202], and soy protein/gelatin [203–205]. Although the papers cited in this paragraph all deal with TG, the paper on oat and faba bean protein [198] also looked at the effect of tyrosinase on the colloidal and foaming properties, and one paper on soy protein/gelatin composite [204] studied the combined effect of TG and trypsin on the functional properties. A separate paper assessed the use of glucose oxidase to cross-link pork myofibrillar protein [206]. Glucose oxidase catalyzes the oxidation of glucose into gluconic acid and H_2O_2, which can degrade to the hydroxy radical. When applied to pork myofibrillar protein, firmer and more elastic gels were obtained by the enzymatic route than by the Fenton reagent at comparable H_2O_2 levels [206].

Two papers deal with a different use of cross-linking in food proteins. A review [207] indicated that gluten-free flours lack the viscoelastic network required to resist gas production and expansion during baking. Enzymatic cross-linking of gluten-free flours was proposed for creating protein aggregates that mimic gluten functionality but also for modifying proteins to improve their functionalities. Another paper was published on gluten-free amadumbe flour [208], where laccase was used; rheological data confirmed the presence of cross-linking due to protein and polysaccharide reactions.

One paper on wheat protein [209] pointed out that TG could decrease the immunoreactivity of the protein. Even greater decrease was observed for the combined TG/hydrolase treatments. Another paper on TG treatment of whey protein [210] noted that protein cross-linking and denaturation could change the rate of digestion of the resulting food and may be useful for people with special digestive needs.

One paper studied films made with soy protein and TG [211]. The mechanical properties of the films were measured. It was pointed out that such a system may be a suitable bioplastic.

12.3.3 Phosphorylation/Dephosphorylation

In nature the phosphorylation of protein is involved in many cellular processes, such as signal transduction, gene expression, cell cycle, cytoskeletal regulation, and apoptosis [212, 213]. Since the early work by Burnett and Kennedy [214],

numerous enzymes are known that carry out phosphorylation and dephosphorylation [157, 212]. For the purpose of in vitro synthesis of industrial proteins, the kinase reaction seems to be preferred. For example, two milk proteins, β-casein and α-lactalbumin, were compared as substrates for casein kinase from bovine mammary gland [215]. Soybean proteins were enzymatically phosphorylated with the catalytic subunit of cAMP-dependent protein kinase [216, 217]. Some of the functional properties of the phosphorylated soy protein were also reported [218, 219]. A 2010 review on the phosphorylation of food proteins by both chemical and enzymatic methods has more details [220].

A good example of both phosphorylation and dephosphorylation is given for genetically engineered spider silk (though not a food protein) [221]. In that publication, the protein was phosphorylated with cyclic AMP-dependent kinase and dephosphorylated with calf intestinal alkaline phosphatase.

12.3.4 Glycosylation

The Maillard reaction is well known in food chemistry, where heating of amino acids and reducing sugars brings about a coupling reaction that gives food its brown color and distinctive flavor. The same reaction can be used to glycosylate proteins [222–224]. However, this chemical reaction is non-specific, requires high temperatures, and often generates undesirable colored or other by-products. In contrast, enzymatic glycosylation reactions are usually more specific and can be carried out at lower temperatures. Indeed, in physiology, protein glycosylation is an important posttranslational modification that enhances the functional diversity of proteins and influences their biological activity. Many elaborate enzymatic glycosylation routes have been identified in a host of organisms. Several reviews have appeared on these physiological pathways [157, 225, 226] and their mechanisms [227]. It was pointed out [157] that some of these pathways (often involving glycosyltransferases) can function in vitro to glycosylate proteins. Nevertheless, in practical terms, these reactions tend to be more difficult to handle and probably less amenable to popular usage.

Two recent developments are worthy of note. In one approach [228], tyrosinase was used to convert tyrosine residues in gelatin to o-quinone moieties, which then underwent nonenzymatic reactions with nucleophilic amino groups of chitosan to furnish in vitro conjugation of gelatin to chitosan. In a variation of this approach [229], protein-oligosaccharide conjugates were produced by laccase and tyrosinase through coupling of the tyrosine side chains of α-casein and phenolic acids of hydrolyzed oat spelt xylan. The second approach used transglutaminase (TG) to conjugate an amino-containing saccharide with soy protein [230–232], caseinate [233–235], fish gelatin [236], and actomyosin [237]. Thus far, glucosamine and chitosan (up to 5 kDa in molecular weight) have been found to be suitable saccharides for this reaction. Enhanced emulsification, rheology, and solubility were reported for the glycosylated proteins [230, 234, 237]. Further development of these synthetic methodologies is expected in the future.

12.3.5 Redox Reactions

Like all polymers, proteins are susceptible to oxidative reactions. In fact, the normal aging process involves an increase in the accumulation of damaged polymers, and the buildup of oxidized proteins is regarded as a hallmark of cellular aging [238, 239]. Proteins can undergo oxidation in many different ways [240]. As regards enzyme-catalyzed oxidations, the greatest occurrence in the literature is the use of oxidative enzymes to carry out protein cross-linking reactions (as shown in Sect. 12.3.2) and conjugation of casein with oat spelt xylan (Sect. 12.3.4). Specific enzymatic oxidation reactions may be useful in selected applications, e.g., horseradish peroxidase- or lactoperoxide-catalyzed iodination of proteins at tyrosine residues [157], but these uses are relatively infrequent.

12.4 Enzymatic Modification of Fats and Oils

Fats and oils are used extensively for food and industrial applications. Although many chemical modifications are known and practiced, enzymes offer specific advantages, such as chemo-, regio-, and stereoselectivities and mild reaction conditions. A lot of work has been done, and this area has been often reviewed [241–249].

For convenience, the enzymes used for lipids can be grouped in four categories: (1) lipases, (2) enzymes for oxy-functionalization, (3) phospholipases, and (4) special enzymes obtained by protein engineering.

12.4.1 Lipases Used for Lipid Modifications

The lipases are the most commonly used enzymes for lipids. The lipase reactions can be separated into five types [247]: hydrolysis, esterification, alcoholysis, acidolysis, and transesterification. A large number of lipases from different microorganisms are commercially available. Immobilized lipases are also available, the most well known being Novozym® 435 from *Candida antarctica* B lipase. These enzymes have been used extensively to make triglycerides, diglycerides, monoglycerides, and structured lipids, as documented in the reviews [241–249].

12.4.2 Enzymes for Oxy-functionalization

These include P450 monooxygenases, hydratase, hydroxylase, lipoxygenase, and diol synthase [241, 250]. Cytochromes P450 (CYPs) are hemoproteins that are found widely in different organisms [251]. In the P450 monooxygenase reaction,

one atom of oxygen is inserted into the aliphatic position of an organic substrate (RH), while the other oxygen atom is reduced to water:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

For example, a saturated fatty acid can be converted into a ω-hydroxy fatty acid in this way [251, 252].

The fatty acid double-bond hydratase catalyzes the addition of water to the cis double bond in fatty acids to form a hydroxy fatty acid. It works with a cofactor like FAD. Thus, oleic acid is converted to 10-hydroxystearic acid and linoleic acid to 10-hydroxy-9-cis-octadecenoic acid [253, 254]. The enzyme 12-hydroxylase converts oleic acid into 12-hydroxyoleic acid (ricinoleic acid) [255]. Diol synthase converts an olefin in fatty acid to a diol [250, 256]; for example, 9-hexadecenoic acid was converted to 9,10-dihydroxyhexadecanoic acid [256].

Lipoxygenases are enzymes under the family of dioxygenases, which catalyze the synthesis of hydroperoxy fatty acids of polyunsaturated fatty acids (PUFAs) having one or more cis,cis-pentadiene units by insertion of molecular oxygen [257, 258].

12.4.3 Phospholipases

A phospholipase is an enzyme that hydrolyzes phospholipids into fatty acids and other lipophilic substances. Several types of phospholipases are known that catalyze reactions on different bonds of the glycerophospholipid [259]. The major use of these enzymes is to remove phospholipids from natural fats and oils, a process known as degumming. These enzymes have also found applications in food areas [260].

12.4.4 Special Enzymes Obtained by Protein Engineering

A large number of enzymes have been subjected to protein engineering in order to improve their attributes or properties. A major accomplishment [261] was the creation of a lipase that was highly selective for trans- and saturated fatty acids when applied to partially hydrogenated vegetable oil. Those fatty acids, identified as a major risk factor for human health, can then be removed by selective enzyme hydrolysis. Another useful modification of lipase was the chain-length selectivity; thus the medium-chain fatty acids can be selectively hydrolyzed [262]. In yet another modification, the stability of a *R. oryzae* lipase toward oxidation was improved [263, 264]. These developments have been given in more detail in a review written by Bornscheuer [243].

Another interesting development is the use of the yeast *Candida tropicalis* that was engineered with a select cytochrome P450 that converted methyl tetradecanoate

to 14-hydroxytetradecanoic acid with high yield and purity [265]. This ω-hydroxy fatty acid was then made into a polyester through chemical means (titanium-catalyzed polymerization) [266].

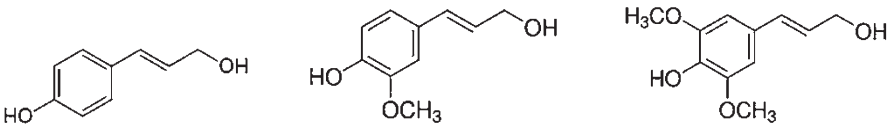
12.5 Enzymatic Degradation of Lignin

Lignin is one of the major components of woody tissue, together with cellulose and hemicellulose. It is an irregular, randomly cross-linked, heterogeneous polymer of three phenylpropanoid monomers: *p*-coumaryl alcohol (I), coniferyl alcohol (II), and sinapyl alcohol (III) (Scheme 12.4). The polymer contains both C-O-C (ether) and C-C linkages, the proportions of each varying depending on wood type. Most of the hydroxy group on the ring is methoxylated. In wood, the lignin polymer also forms covalent bonds with hemicellulose and probably cellulose.

Only a few enzymes are known to modify lignin. The ones that are most well-known are the oxidative enzymes, such as the heme-containing glycoproteins, lignin peroxidase (LiP), manganese peroxide (MnP), and versatile peroxidase (VP), and the copper-containing glycoprotein laccase. The hemoproteins (LiP, MnP, and VP) require H_2O_2 for their action, whereas laccase needs oxygen. The activity of all four enzymes (particularly laccase) can be enhanced with mediators. These enzymatic systems have been extensively studied [267–270]. They are used particularly in the pulp and paper industry in order to bleach pulp or to optimize the use of plant biomass.

Some recent development can be noted here. In an interesting paper [271], lignin was reacted with laccase and glucosamine or the tripeptide glycyl-tyrosyl-glycine in acetone/water mixture. IR and NMR data suggested that glucosamine and the tripeptide were somehow grafted onto the lignin. The detailed structures of the products and the mechanism were yet to be deciphered.

One of the active areas of research involves the mediators for laccase. In one paper [272] some phenolic compounds that seemed to be natural laccase mediators were studied; these natural phenolic compounds are eco-friendly and may facilitate the use of laccase in biorefining. In another paper [273], the mechanism of action for mediators was studied with ESR; the results suggested that electron shuttling by mediators was not a significant contributor to enhanced laccase oxidation of lignin. Yet another paper [274] addressed the question whether laccase can directly cata-



I II III

Scheme 12.4 Structures of *p*-coumaryl alcohol (I), coniferyl alcohol (II), and sinapyl alcohol (III)

lyze modification of lignin via catalytic bond cleavage. (Laccase is known to catalyze polymerization of lignin.) The authors concluded that bond cleavage indeed takes place for low-molecular-weight lignin model compounds, but for lignin itself, the laccase-mediator combination is needed.

In a review article, Gonzalo et al. [267] noted that several bacterial enzymes might be involved in lignin degradation, including bacterial laccases, glutathione-dependent β-etherases, superoxide dismutases, catalase-peroxidases, and bacterial dioxygenases. Additional delignification enzymes were discussed by Pollegioni et al. [268], such as β-etherases, demethylases, peroxidases, mycelium-associated dehydrogenase, and other oxidoreductases. The use and advantages of immobilized ligninolytic enzymes were delineated in a review article [275]. Laccase engineering, including both rational design and directed evolution, were reviewed in another publication [276].

12.6 Perspectives

It is clear from the above review that enzymatic modifications of polymers constitute an important and vibrant area of research and development. This is a multidisciplinary field, requiring the expertise in polymer chemistry (for polymer handling, polymer properties, and structure/property relationships), organic chemistry (for synthesis and workup procedures), biochemistry (for enzymology), and chemical engineering (for process development). Thus, a multidisciplinary team is often needed for an optimal research and development endeavor.

Several current trends favor the continued growth of this field. The first trend is the increasing popularity of green polymer chemistry. Indeed, the materials covered in this chapter (e.g., polysaccharides, proteins, triglycerides, and lignin) are all bio-based, degradable, renewable, and sustainable. They can be recycled if needed so that the resulting products would not produce polymer waste and cause disposal problems. The enzymes are themselves proteins, usually nontoxic, and easily disposable. The enzymatic reactions are often done in water, thereby minimizing the hazards associated with organic solvents. The enzymes are frequently specific in their reactivity and produce less by-products relative to chemical reactions. The enzymatic reactions usually entail lower temperatures, which minimize the energy usage. Thus, the use of enzymes is fully compatible with green chemistry.

The second trend is the continued development of gene technologies. Indeed, new enzymes can be (and have been) developed as needed for specific reactions. Improved enzymes can be obtained to enhance a specific functionality or to meet the demands of a given process. In the future, many advances in enzyme technologies are expected, and these should be helpful as researchers seek new or improved ways to modify the polymers or to optimize their processes.

An inherent advantage of agro-based materials like polysaccharides, proteins, oils/fats, and lignin is their availability in large quantities and their relatively low cost. As natural polymers, they are often amenable to enzymatic action. Thus, the

combination of agro-based materials and enzyme reactions should be a powerful platform for the development of green polymeric products and a useful tool for postharvest agricultural technology.

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Concluding Remarks

As seen above in the 12 chapters, fundamental and important aspects of *Enzymatic Polymerization towards Green Polymer Chemistry* are presented. In particular, enzymatic syntheses of polysaccharides, polyesters, polypeptides, poly(aromatic)s, and vinyl polymers, as well as enzymatic polymer modifications, are comprehensively covered in an up-to-date manner.

Enzymatic polymerizations involve several important characteristics; they are related to, e.g., catalyst enzymes, catalytic efficiency, new catalysis, reaction conditions, high reaction selectivities, starting materials, and product polymers. (1) Catalyst enzymes are natural renewable products and environmentally benign without showing toxicity. Immobilized enzymes can be recovered and repeatedly used. (2) Enzymatic reactions normally show high catalytic efficiency (high turnover number). (3) New enzymatic catalysis enabled for the first time to synthesize natural polysaccharides with complicated structure such as cellulose, chitin, hyaluronic acid, and chondroitin, which were not possible to prepare via conventional methods. (4) Enzymatic polymerizations proceed under mild reaction conditions, which allow to use not only an organic solvent but also a green solvent, like water, supercritical carbon dioxide, and an ionic liquid. Enzymatic catalysis induced a "dehydration polymerization in water" to produce polyesters, which is a new finding in organic chemistry. (5) The reactions exhibit characteristics of enzymatic catalysis and high reaction selectivities such as chemo-, regio-, stereo-, enantio-, and chiroselectivities, producing minimal by-products. It is normally very hard to control these selectivities with conventional chemical catalysts. (6) Enzymatic polymerizations allow to employ many renewable bio-based materials (biomass) as starting substrates in place of fossil-based raw materials. (7) Product polymers from these substrates are nontoxic and biodegradable in many cases, which are benign to nature.

Green polymer chemistry is brought about by the above mentioned characteristics of enzymatic polymerizations. The reactions produced actually a variety of value-added functional polymers via green processes. These polymers are expected

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as practically employed for daily necessaries, biomaterials, drug delivery systems, pharmaceuticals, and other application areas. As typical examples, phosphorylase-catalyzed synthesized amylose is currently used mainly for chiral-separation column carrier, which is prepared and sold by Glico Nutrition Co. and PS·Biotec Inc. (see Chap. 3). Another event happened for the enzymatic polymerization; Kaneka Co. built a pilot-scale plant in 2011 for production of polyhydroxyalkanoates (PHAs), polyesters that can be used as bio-based and biodegradable plastics (see Chap. 7). Enzymatic polymer modifications are widely conducted in industry area, like processes of "biobleaching" and degumming, and many such examples for various polymers are mentioned in Chap. 12.

The importance of green polymer chemistry is stressed here again. Due to the earth warming climate partly because of the increased carbon dioxide emission, we suffer big disasters more often recently than before. The other environmental crisis recently pointed out is that large and small plastic trashes or particles (micro-plastics ≤ 5 mm size) are found in many areas on the earth, in particular, not only in the sea but also on the seashore. This situation gives big damages to the ocean ecology, bringing about losing many lives due to their harmful actions, which eventually affect the human beings. Micro-plastics and other plastic trashes are from the traditional polymers like polyethylene, poly(ethylene terephthalate), nylon, etc. which are mainly originated from disposable plastic goods such as shopping bags, straws, dishes, cups, bottles, and others including the thrown-away. It is said that these plastics reach to around 8 million tons/year in the world and 85% of ocean pollutants are due to them. To prevent these problems because of such environmental pollutions, EU countries, some states in the USA, and some other countries have decided not to use and/or not to produce the disposable plastic goods, e.g., typically disposable straw. This issue was also seriously discussed at the G7 Summits, Quebec, Canada, in June 2018; it becomes recently one of the most important trends worldwide to reduce plastic wastes.

In addition, it is to be noted that public great attention on the 17 Sustainable Development Goals (SDGs) of the 2030 Agenda for Sustainable Development, which was adopted by United Nations in September 2015, has recently grown. SDGs have 17 goals, and green polymer chemistry provides a good example for achieving SDGs.

Now, enzymatic polymerization shall enable to mitigate or even exclude such a serious problem by pursuing green polymer chemistry as observed above. The polymerization produces biodegradable and nontoxic plastics, e.g., some polyesters, from renewable starting materials, and bio-based materials, not from the diminishing fossil resources. These polymers prepared in such processes can be employed in place of the traditional polymers, yet not becoming an origin of pollutants. This direction utilizing the enzymatic polymerization for producing environmentally benign plastics in a green process is a definitely right way to contribute for maintaining the sustainable society for the future.

We believe this book will serve the readers for gaining helpful information, good ideas, and right direction to their further/future activities. We also hope that the

readers will utilize these information not only for maintaining the sustainable society but also for realization of the better society in the future.

Finally, we express our deep thanks to all the chapter authors, who willingly accepted our invitation to write the chapter manuscript. Further, we are much thankful to Springer Co. and the staffs, who kindly invited us to edit this book *Enzymatic Polymerization towards Green Polymer Chemistry* for the Springer book series *Green Chemistry and Sustainable Technology*. It is very fortunate for us to do this, because the edition is just timely.

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