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MINI-REVIEW

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Microbial proteases in peptide synthesis: approaches and applications

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Abstract Enzymatic synthesis of peptides has attracted a great deal of attention in recent years. The proteases from bacterial, fungal, plant, and animal sources have been successfully applied to the synthesis of several small peptides, mainly dipeptides and tripeptides. Peptide bonds can be synthesized using proteases in either a thermodynamically controlled or a kinetically controlled manner. The development of new methods suitable for the large-scale production of biologically active peptides has been actively pursued over the last decade due to their bioactive nature as well as better understanding of their biological functions and properties. The aim of this study was to review the basic techniques of peptide synthesis and some advancement in biotechnological methods for their production.

Introduction

Under normal aqueous conditions, proteases catalyze the hydrolysis of peptide bond, but the reaction proceeds in reverse direction (i.e., synthesis of peptide bond) in water-restricted media (Fig. 1). The hydrolysis reaction is reversible in principle, and the studies of the heats of hydrolysis of peptide bonds indicate that the thermodynamic barriers to the reversal of hydrolysis can be allowed to proceed in the direction of peptide bond formation by any scavenging mechanism which can keep the concentration of the condensation product below its equilibrium concentration in the reaction mixture (Glass 1981).

Chemical peptide synthesis has several drawbacks (Gill et al. 1996): (a) racemization remains a problem during peptide bond formation; (b) protection of the side chain function of amino acids increases the costs of the substrates, which leads to loses in the yield during deprotection; (c) it is difficult to recycle the coupling reagent and acyl donor used to achieve rapid and complete acylation of the nucleophile; and (d) the toxic nature of solvents and coupling reagents when applied to food grade endproducts may lead to health and environment concerns. The enzymatic method of peptide synthesis offers several advantages over chemical methods (Morihara 1987; Bhalla et al. 1999). The advantages associated with the application of enzymes to the synthesis of peptides include the enantioselectivity, dispensation with side-chain protection, and the use of wild, nonhazardous reaction conditions (Cheetham 1994).

Enzymes are generally very labile catalysts, and therefore, the enzymatic reactions at industrial scale or enzyme reaction engineering are designed very carefully. While designing a practical enzyme reaction, it is important to study the effect of different variables which define the reaction medium (pH, temperature, presence of organic solvents) and the parameter of industrial interest such as activity/stability of catalyst, solubility of reactants, stability of reactants and products, yields of the product, etc. (Blanco et al. 1991).

Specificity of proteases

Proteases for peptide synthesis are selected on the basis of their specificity against amino acid residues on either side of the splitting point (Table 1). The proteases that are used for peptide synthesis include the majority of the commercially available serine, metallo, and aspartate endo- and exoproteases. Among proteases, carboxypeptidase Y is the only case exhibiting the esterase and amidase activity typical of serine proteases (Morihara 1987; Gill et al. 1996; Kasche 1996). The broad specificity of proteases restricts their application in peptide synthesis. The peptide product that accumulates during the reaction can be attacked by the protease simultaneously with the synthesis reaction (Schellenberger et al. 1991). Morihara and Oka (1981) reported a subtilisin with broad substrate specificity as a catalyst in peptide synthesis reactions in water. Kumar

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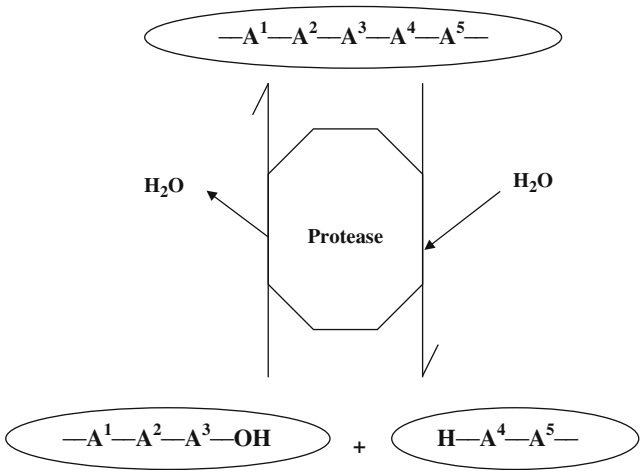

Fig. 1 Schematic representation of protease catalyzed reaction specific for A^3—A^4 peptide bond

et al. (2003) have evaluated the efficiency of a protease of *Bacillus* sp. APR-4 in various dipeptide syntheses.

Immobilized proteases in peptide synthesis

The immobilization is the most used strategy to improve the operational stability of biocatalysts, other benefits include better control of reaction, flexibility of reactor design, and facilitated product recovery without much loss of catalyst. Several approaches are being used to increase operational stability of enzymes, i.e., chemical modification of enzyme structure, derivatization, immobilization, crystallization, and medium engineering (Katchalsky-Katzir 1993). Out of these, immobilization is one of the most important techniques in the application of enzyme catalysis to synthetic reactions in organic solvents. The techniques of enzyme immobilization may be divided into five groups, i.e., (a) covalent attachment to solid support, (b) absorption on solid support, (c) entrapment in polymeric gel, (d) crosslinking with bifunctional reagents, and (e) encapsulation. Immobilized proteases are becoming increasingly popular for use in peptide synthesis due to their increased stability in organic solvents, the ease of separation of enzymes from the products, and their reusability (Wilson et al. 1994). Due to higher cost of purified proteases than the chemical reagents, the use of immobilized enzyme makes the process economically viable. Immobilized enzymes generally have the advantages that they are more stable than the enzymes in solutions containing the concentration of organic solvents (Morihara 1987; Kise et al. 1990). The direct effect of support on the catalytic properties of the enzyme was examined by studying the dependence of water content, pH, ionic strength, and reaction temperature on the yields of model dipeptide Boc-Phe-Val-Ome in ethyl acetate under thermodynamic control (Zhang et al. 1996). Some immobilized proteases used in the synthesis of peptides are shown in Table 2.

Organic solvents/water-restricted peptide synthesis

Many enzymes can function well in nearly anhydrous organic solvents, and very low amount of water is required for proteins to maintain their catalytically active conformation. This has led to widespread application of proteases in low water reaction mixtures (Dordick 1989; Khemlnitsky Yu et al. 1988; Klibanov 1990; Zaks and Klibanov 1988; Gill et al. 1996).

The applications of enzymatic reactions in water-restricted organic solvents have been reviewed by several authors (Klibanov 1986; Khemlnitsky Yu et al. 1988; Zaks and Russell 1988; Kise et al. 1990). There are numerous advantages in employing enzymes in organic solvents as opposed to aqueous solvents, such as increased solubility

Table 1 Site specificity of various proteases

Name of enzyme	Peptide bond cleaved
Serine proteases	↓
Trypsin	-A-A′- (-A=Lys, Arg, -A′=nonspecific)
Chymotrypsin	-A-A′- (-A=Trp, Phe, Leu, -A′= nonspecific)
Cathepsin	-A-A′- (-A= Phe, Leu, Trp, -A′= nonspecific)
Elastase	-A-A′- (-A=Ala, Ser, -A′= nonspecific)
Subtilisin	-A-A′- (-A= neutral, acidic amino acids preferred, -A′=nonspecific)
Carboxypeptidase Y	-A-A′- (nonspecific)
Proline-specific protease	-A-A′- (-A= Pro)
Cysteine proteases	
Papain	-A-A′- (-A=Arg, Lys, -A′= nonspecific)
Bromelain	-A-A′- (nonspecific)
Clostripain	-A-A′- (-A=Arg, -A′=pro preferred)
Cathepsin B	-A-A′- (substrate dependant)
Metal proteases	
Thermolysin	-A-A′- (-A=Leu, Phe, -A′=Leu, Phe, Val, Met, Ala, Ile)
Aspartic proteases	
Pepsin	-A-A′- (-A=Phe, Tyr, Leu, -A′=Trp, Phe, Tyr)
Cathepsin D	-A-A′- (-A=Phe, Leu, -A′= cannot be Val, Ala)

Table 2 Immobilized proteases used in peptide synthesis

Protease	Support used for immobilization	Peptide formed	Reference
Papain	QAE-Sephadex A-25, Trisacryl MCM	Boc-Arg-Glu-OBu^t	Zhang et al. 1995
Papain	Celite, CM-cellulose, QAE-Sephadex, egg white protein	Boc-Phe-Val-OMe	Zhang et al. 1996
Thermus proteinase	Pore glass beads	Bz-Ala-Tyr-NH_2	Wilson et al. 1994
Neutrase	Celite 545, Polyamide EP-700	Several N-α protected dipeptides with Z-Phe-Leu-NH_2 as a model	Clapes et al. 1997
Thermolysin	Amberlite XAD-7	Z-L-Tyr-Gly-Gly-L-Phe-L-Leu-Oet	Kimura et al. 1990a–c
α-Chymotrypsin	Porous chitosan beads	Ac-Tyr-GlyNH_2, Ac-Tyr-Ala-NH_2, Ac-Tyr-Leu-NH_2	Kise and Hayakawa 1991
Subtilisin Carlsberg	Celite, porous glass	Z-Ser-Phe-NH_2	Ferjancic et al. 1990
Subtilisin 72	Poly (vinyl alcohol)-cryogel	Z-Ala-Ala-Xaa-Phe-pNa	Bacheva et al. 2001
Thermolysine	Controlled pore glass derivatives, polyamide, celite, support	α-L-Asp-Phe-OMe	Rao et al. 1998
Subtilisin	Cryogel of polyvinyl alcohol	Z-Ala-Ala-Xaa-Yaa-pNa	Bacheva et al. 2003

of nonpolar substrates and shifting of thermodynamic equilibrium to favor synthesis over hydrolysis, reduction in undesirable side reactions, easy separation of product and enzyme recovery and reuse, and reduced risks of microbial contamination (Kise et al. 1990; Khemlnitsky Yu et al. 1988; Gill et al. 1996; Kise 1992).

Several model dipeptides and tripeptides have been synthesized in water-restricted media using free, immobilized, suspended, and chemically modified enzymes; and synthesis of several small oligopeptides has also been carried out (Gill et al. 1996; Clapes et al. 1997; Noritomi and Kise 1987; Kimura et al. 1990a–c; Wong et al. 1988; Barbas and Wong 1987). However, the peptide synthesis using organic solvents remains limited to the synthesis of model di- and tripeptides, and very little efforts have been done on the synthesis of bioactive oligopeptides as compared to conventional aqueous system (Jakubke et al. 1985; Morihara 1987). Use of nonaqueous system had some disadvantages in terms of reduction of enzyme activity, deleterious effects of hydrophobic solvents on enzyme activity, and stability (Deetz and Rozzell 1988; Lanne et al. 1987). The use of organic solvents in food sector imposes additional restriction on process design and engineering (Gill et al. 1996). Low activity of enzymes in nonaqueous solvents as compared to that in water can be largely overcome by crown ether treatment of enzymes, and crown ether-enhanced enzyme activity is retained even after removal of the crown by washing with a dry organic solvent (van Unen et al. 2002).

Investigations of Okazaki et al. (2000) revealed that the surfactant–subtilisin Carlsberg (STC) complex is a powerful biocatalyst for dipeptide synthesis because the STC complexes display a high catalytic activity in anhydrous hydrophilic organic solvents and did not require excess amount of water. Thus, the side (hydrolysis) reaction is effectively suppressed, and the yield of the dipeptide formed becomes considerably high.

Strategies used in peptide synthesis

Enzymatic peptide bond synthesis can be carried out either as an equilibrium-controlled or a kinetically controlled process (Kasche 1996). The scheme for kinetically controlled synthesis of Bz-Ala-Tyr-NH_2 (Wilson et al. 1994) using *Thermus* strain Rt 41A protease is illustrated in Fig. 2. Using an activated substrate (amino acid) in kinetically controlled process (I–III in Fig. 2), the protease

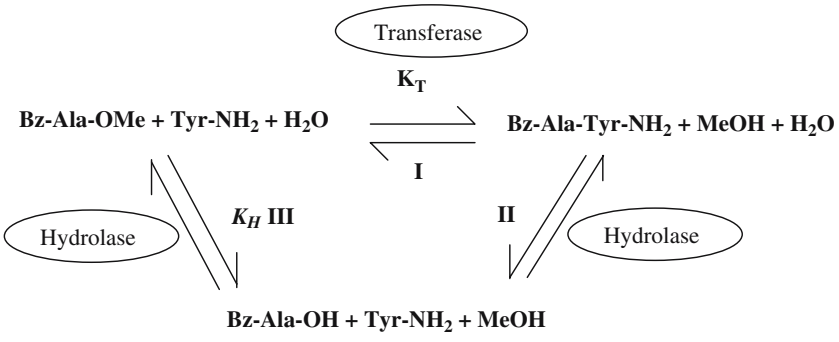

Fig. 2 *Thermus* strain Rt41A protease catalyzed kinetically controlled (I+II+III) and equilibrium-controlled (II) synthesis of dipeptide Bz-Ala-Tyr-NH_2

acts as a transferase and catalyzes the transfer of the acyl group to a nucleophile (amino acid). The hydrolysis of the activated substrate (reaction II) is competing with this reaction, and the product peptide can be hydrolyzed by protease as a substrate in reaction III. The yield of product depends on the apparent ratio of the transferase to hydrolase rate constant (K_T/K_H)app, and the rate with which the product peptide is hydrolyzed. Only proteases that form covalent acyl–enzyme intermediates can be used as catalysts in kinetically controlled peptide synthesis. They have values of (K_T/K_H)app in the range 10^2–10^4 (Kasche 1996). An industrial protease, alcalase has been reported for kinetically controlled peptide synthesis in ethanol solution (Chen et al. 1991). The precursor dipeptides of RGD (Arg-Gly-Asp), N-CBZ-Arg-Gly-NH_2, and CBZ-Gly-Asp-NH_2 were synthesized in reverse micellar system and organic solvent using several proteases in different forms under kinetic control (Chen et al. 1999).

In an equilibrium-controlled process, peptide bond synthesis is the reverse of hydrolysis (reaction II in Fig. 2). One of the serious problems with the enzymatic method is the low equilibrium yield of the synthesis. There are several ways to overcome this. Precipitation of the condensation product in the reaction mixture gives a high yield. The addition of water-miscible organic cosolvent to the reaction mixture is another way. Reaction in an aqueous/organic biphasic system shifts the equilibrium toward the product (Kimura et al. 1990b). Various methods that can be used to favor the reaction toward peptide synthesis in an equilibrium-controlled synthesis (Morihara 1987) are described in the following subsections.

Precipitation

This is the most popular method for enzymatic peptide synthesis. When certain soluble carboxyl and amine components are used as the starting materials (reactants), the equilibrium between dissolved and solid product favors formation of solid product by precipitation (reaction A in Fig. 3). As the soluble (reactive) product concentration in the reaction mixture is decreased, the apparent equilibrium is shifted toward synthesis. In precipitation reaction, the concentration of product is dependent on the concentration of the reactants. The reaction will be driven toward product formation, if the concentration of the reactants dictates an equilibrium concentration of the product at which significant precipitation occurs. To shift the equilibrium of the reaction to favor the synthesis, precipitation of the product is often required, and in these cases, the product yield is determined primarily by its solubility (Saltman et al. 1977; Oka and Morihara 1978).

Biphasic system

In biphasic system, the reaction takes place in water and water-immiscible organic solvent. In two-phase systems, the concentrations of substrates and products around enzymes can be easily controlled, and the low concentration of the product in the aqueous phase indicates that equilibrium in the aqueous phase is not reached until there are high concentrations of the product in the solvent. A general scheme for peptide synthesis using biphasic system is given in reaction B of Fig. 3. The excessive partitioning of substrates or products in aqueous phase of small volume often cause inhibition or inactivation of enzymes (Cassells and Halling 1989). The reaction mixture can be regarded as an emulsion of an enzyme aqueous solution in water-immiscible organic solvent in stirred condition because the water content in this system is usually 2–5%. As the enzyme is in the aqueous phase, the reactants dissolved in the organic phase will diffuse into the water and undergo enzyme-catalyzed peptide synthesis, and the end products will diffuse back into the organic phase. The system is suitable for the formation of water-insoluble products.

Homandberg et al. (1978) reported that the equilibrium constant (K_{Syn}) for the synthesis of dipeptide Cbz-Trp-Gly-NH_2 from Cbz-Trp-OH and Gly-NH_2 increased with increase in organic cosolvents, such as glycerol. Nilsson and

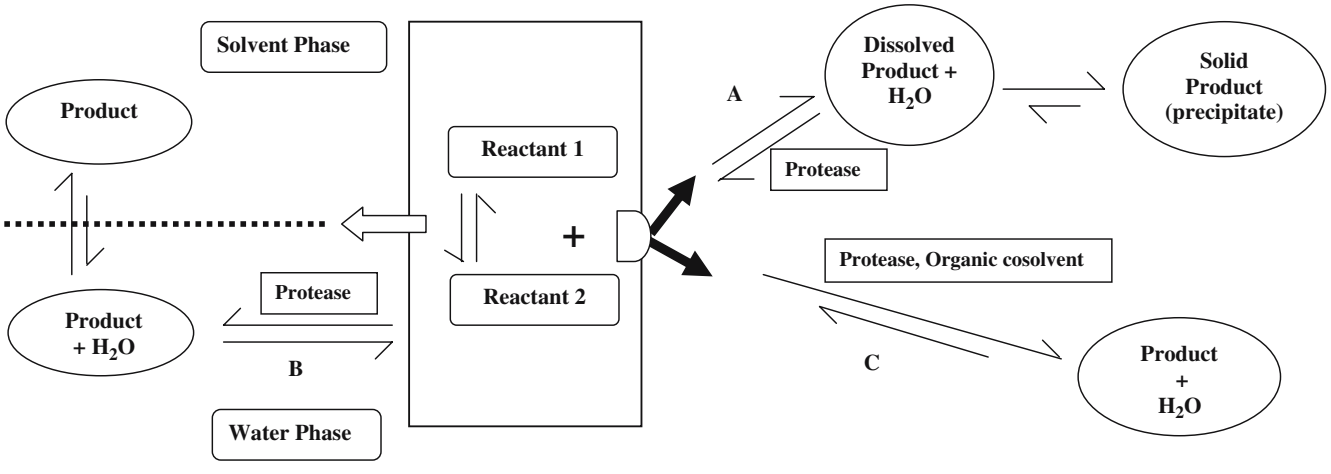

Fig. 3 A generalized reaction for precipitation (**A**), biphasic (**B**), and dissolved state (**C**) systems in equilibrium-controlled peptide synthesis

Mosbach (1984) reported peptide synthesis by substitution reactions of ester substrates, such as Ac-Phe-OMe, with Gly-NH_2 or Ala-NH_2 by the catalysis of immobilized chitosan in organic cosolvent systems (mostly 50% DMF). Martinek et al. (1981) theoretically evaluated the effects of the use of biphasic system on the equilibrium yield. The precursor of Leu-enkephalin, Z-L-Tyr-Gly-Gly-L-Phe-L-Leu-OEt, was synthesized from amino acid derivatives with three proteinases without the protection of the side chain of L-Tyr. First, Z-Gly-Gly-OBu^t and Z-L-Tyr-Gly-Gly-OBu^t were synthesized in a quite high yield, 83 and 99% in an aqueous/organic biphasic system by papain and α-chymotrypsin, respectively. Then, Z-L-Phe-L-Leu-OEt was synthesized by thermolysin from Z-L-Phe and L-Leu-OEt either in buffer or in a biphasic system; the yields were 95 and 100%, respectively (Kimura et al. 1990b).

Dissolved state system

The synthesis of water-soluble products (e.g., short peptides, high molecular weight peptides, proteins) cannot be carried out in precipitation and biphasic systems. In such cases, shifting the reaction toward synthesis requires either a high concentration of one reactant (mass action) or addition of a high concentration of water-miscible organic cosolvent (reaction C in Fig. 3), and the excess of one of the reactants drives the reaction toward product formation.

In serine protease catalyzed condensation reaction in water, acylation of enzyme is rate determining, and the peptide yield is an equilibrium control (Oka and Morihara 1978; Kasche 1996). The product yields at equilibrium are also the function of partition coefficient and aqueous/organic volume ratio (Martinek et al. 1981; Eaggers et al. 1989). The ratio of the initial rates of aminolysis and hydrolysis in peptide semisynthesis catalyzed by chymotrypsin and trypsin was found to depend nonlinearly on the concentration of the added nucleophile (Riechmann and Kasche 1985).

Under optimum conditions, the equilibrium yields of the peptide Cbz-Arg-Leu-NH_2 synthesized from Cbz-Arg and Leu-NH_2 using the *Pseudomonas aeruginosa* PST-01 protease were 71.6 and 87.7% in the presence of 50% (v/v) DMF and 60%(v/v) DMSO, respectively (Ogino et al. 1999). Oka and Morihara (1980) carried out the coupling between Cbz-Phe-OH and Leu-NH_2 using thermolysin under various experimental conditions, and maximum yield (80%) was obtained in reaction conditions containing 0.05 M each of carboxyl and amino components and 10 μm enzyme at pH 7.0 and 37°C for 5 h.

Biologically active peptides

Biologically active peptide constitutes a diverse group of peptides such as physiologically active peptides, antibiotic and antiviral agents, neuroactive peptides, enzyme regulators and inhibitors, hormonal peptides, and immunoactive peptides (Gill et al. 1996). In recent years, a large number of biologically active peptides have been isolated from bacterial, fungal, plant, and animal sources and characterized in some detail.

The enzymatic approach has been successfully applied to the synthesis of a variety of peptides, including aspartame, lysine sweet peptide, kyotorphin, angiotensin, the enkephalins, and dynorphin (Aso 1989; Clapes et al. 1989; Kullman 1982; Oyama et al. 1987; Takai et al. 1981; Fandino et al. 1994). A few of the proteases have been successfully applied to the synthesis of several small peptides of pharmaceutical and nutritional interests such as enkephalin (Kullman 1979; Kimura et al. 1990b), aspartame precursor (Nakanishi et al. 1990), and some nutritional dipeptides and tripeptides (Kimura et al. 1990c; Monter et al. 1991). Some small peptides can be continuously synthesized at the commercial scale by using efficient enzyme reactors (Herrmann et al. 1991; Serralheiro et al. 1994).

Peptides are nutritionally and physiologically different from free amino acids and proteins. Some peptides, such as enkephalin and angiotensin, have biological activity; some, such as aspartame, have tastes (Kimura et al. 1990a). Kimura et al. (1990b) have proposed various kinds of reactors to synthesize various peptides consisting of essential amino acids using papain, α-chymotrypsin, and thermolysin. Telios Pharmaceutical Co. has explored RGD (a tripeptide Arg-Gly-Asp) as a new drug for injury of heavy burns, dermal ulcer, etc. In addition, RGD tripeptide contains two hydrophilic amino acids (Arg and Asp) and a neutral one (Gly) (Chen et al. 1998). Various biologically active peptides synthesized using proteases are shown in Table 3.

Advances in peptide synthesis

Biologically active peptides have generally ranged from simple dipeptides to complex linear and cyclic structures. The application of enzymes to peptide synthesis, although offer several advantages, has still been discouraged by some unfavorable factors. At present, several technologies are being actively explored to overcome these and other difficulties. Some advances in peptide synthesis are discussed.

Modified biocatalysts

Protein engineering has been applied to tailor proteases and to improve the catalytic properties and stabilities of enzymes (Wells and Estell 1988), and chemical modification of physical and chemical properties of enzymes is another method for synthesis of peptides (Roig and Kennedy 1992). Subtilisin has been extensively studied, and considerable progress has been made in engineering subtilisin and its substrates for peptide bond formation in aqueous solution (Abrahamsen et al. 1991). In *P. aeruginosa* PST-01, a disulfide bond between Cys-30 and Cys-58 played an important role in the organic solvent stability of protease

Table 3 Biologically active peptides synthesized using proteases

Peptide	Peptide bond formed	Enzymes used	Reference
Aspartame	Asp-Phe	Thermolysin	Kuhn et al. 2002; Erbeldinger et al. 2001
Kyotorphin	Tyr-Arg	α-Chymotrypsin	Clapes et al. 1987
Nutritional peptide	Tyr-Trp-Val	α-Chymotrypsin, papain	Kimura et al. 1990a
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu	α-Chymotrypsin, papain	Kimura et al. 1990b
Dynorphin (1–8)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile	α-Chymotrypsin, trypsin, papain	Jorba et al. 1992
Cerulein	Asp-Tyr-Thr-Gly-Trp-Met-Asp-Phe	α-Chymotrypsin, papain, thermolysin, subtilisin	Takai et al. 1981
Sweet lysine peptide	Phe-Lys	α-Chymotrypsin	Aso 1989
Cholecystokinin	Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe	Thermolysin, α-chymotrypsin, papain	Kullmann 1982
RGD tripeptide	Boc-Arg-Gly-Oet	Alcalase, trypsin, papain, chymotrypsin	Chen et al. 1998
Skin growth promoter	Boc-Arg-Glu-Obt Boc-Arg-Gly-Oet	Papain	Zhang et al. 1995
Bovine γ-melanocyte stimulating hormone fragment (1–8)	Tyr-Val, Val-Met	α-Chymotrypsin, papain	Kullmann 1983a,b
Somatostatin	(-Ala-Gly-Cys-Lys-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-)[a]	Thermolysin, chymotrypsin	Bille et al. 1991
Vasopressin	Tyr-Phe Phe-Gln	Thermolysin chymotrypsin	Rizo and Gierasch 1992
Oxytocin	Cys-Tyr Tyr-Ile Pro-Leu Leu-Gly	Papain thermolysin chymotrypsin	Rizo and Gierasch 1992

[a]Peptide bond formation between first and last amino acid residue for cyclization of the oligopeptide somatostatin

(Ogino et al. 2001). The effect of a novel disulfide bond engineered in subtilisin E from *Bacillus* based on the structure of a thermophilic subtilisin-type serine protease aqualysin I was examined and suggested that an electrostatic interaction between Lys 170 and Glu 195 is important for catalysis and stability in subtilisin E (Takagi et al. 2000). Organic solvent stability of proteases can also be enhanced by chemical modifications, and the resultant biocatalysts are highly active and stable in polar solvents and display an increased resistance to thermal inactivation (Wang et al. 1992; Gill et al. 1996).

Substitution of native amino acids by fluoroalkyl analogs represents a new approach for the design of biologically active peptides with increased metabolic stability as well as defined secondary structure using commercially available proteases trypsin and α-chymotrypsin, which provides a powerful level for spectroscopic investigations (Thurst and Koksche 2003). Proteases have also been extensively applied to the modification and semisynthesis of proteins and large polypeptides (Gill et al. 1996). Modified proteases catalyze synthesis of a wide variety of peptides of various lengths and structures both in solution and on solid phase organic solvents. Pepsin sorbed on celite, a noncovalent complex of subtilisin with sodium dodecysulphate and subtilisin or thermolysin covalently immobilized on a cryogel of polyvinyl alcohol, was used as biocatalysts for peptide synthesis in polar organic solvents (Filippova and Lysogorskaia 2003).

Organofullerene derivatives have shown a great potential in a wide variety of biological activities such as DNA photocleavage, HIV protease inhibition, neuroprotection, and apoptosis. Incorporation of fullerene framework into peptides substantially modifies its original properties. Fullerene-based peptides have been found to substantially activate enzymes involved in the oxidative deamination of biogenic amines (Pantarotto et al. 2004). Solid phase peptide synthesis of the amphiphilic peptide Ac-(Leu-Arg-Leu) (3)-linker, which is modified at the C-terminus with 1,8-diamino-3,6-dioxaoctane as linker moiety was investigated by Wilking and Sewald (2004). A site directed mutagenesis in AprP, an alkaline protease from *Pseudomonas* sp. KFCC 10818, was carried out by replacing Gly199 and Phe236 residues with cysteine, and introduction of disulfide bond enhanced the thermostability and catalytic efficiency of the enzyme (Ko et al. 1996).

Several peptides/proteins, viz., bovine ribonuclease (Homandberg and Laskowski 1979), staphylococcal nuclease (Komoriya et al. 1980), human insulin (Morihara et al. 1980), and $[Gly^{\alpha 142}]$ -hemoglobin (Nagai et al. 1982), have been modified and semisynthesized using enzyme-catalyzed protein fragment condensation and site-specific modifications. A wild-type RNase and its variants were synthesized by replacing two active-site histidine residues singly or simultaneously with 4-fluorohistidine (Jackson et al. 1994) using protease.

A peptide-based label was developed for opioid receptor [Leu (5)] enkephalin and DTLET (Tyr-D-Thr-Gly-Phe-Leu-Thr) protagonists for delta receptors. The affinity labels were prepared using standard Fmoc-solid-phase peptide synthesis in conjunction with Fmoc-phe (p-NH Alloc) (Fmoc-flourenylmethoxycarbonyl) and selective modification of the p-amino group of this residue. The electrophilic isothiocyanate and bromoacetamide groups were introduced into the para-position of Phe (4); the corresponding free amine containing peptides were also prepared for comparison (Choi et al. 2003). A new N-protecting group, ethanesulfonylethoxycarbonyl (Esc), was designed to perform peptide synthesis in both aqueous and organic solvents. Esc amino acids were prepared by the reaction of Esc-Cl and amino acids. A more stable reagent ethanesufonylethyl-4-nitrophenyl carbonate (Esc-ONp) was used for preparation of Esc amino acids. Leu-enkephalin amide was synthesized using Esc amino acids by solid phase method in water on a polyethyleneglycol-grafted polystyrene resin (Hojo et al. 2004).

Stereochemically modified peptides

Racemization-free condensation of oligopeptide fragments using proteases has also been found eminently suitable as compared to chemical synthesis, which is accompanied by high level of racemization (Xaus et al. 1992; Hwang et al. 1993). Chiral resolution of arginine and Arg derivatives was demonstrated by Schug and Linder (2005) with electrospray ionization-tandem mass spectrometry (ESI-MS) using two methods, one based on the measurement of a competitive-dissociation-based branching ratio by the kinetic method (KM) and other based on the measurement of a chiral recognition ratio (CR). Incorporation of N-blocked Arg derivatives (Z-Arg and Boc-Arg) as chiral references provided chiral resolution greater than that previously reported for Arg enantiomers. Pure Arg is used as reference for discriminating enantiomers of these N-blocked Arg derivatives. Condensed-phase and gas-phase Ca (II) ion affinity relative to Arg is used qualitatively for acidic, basic, and neutral amino acids. These experiments demonstrated the applicability of, and difference between, the KM and CR methods for improved quantitative analysis of enantiomeric excess for Arg.

Barros et al. (2000) reported the synthesis of di- and tripeptides based on selectivity. The work is concerned with the effect of mass-transfer limitations on different kinds of selectivity: acyl donor, stereo, and nucleophile selectivity. Nucleophiles [Ala NH(2)] diffuses faster than the any donors employed. These factors are expected to give rise to concentration gradients inside porous biocatalyst particles higher for acyl donor than for nucleophile and explain why acyl donor selectivity and stereoselectivity are much more influenced by mass transfer limitations than nucleophile reactivity.

Substrate mimetics

Substrate mimetics are excellent tools for protease-mediated peptide synthesis that enable the coupling of peptides independently of the primary specificity of the enzyme without undesired cleavage of the newly formed peptide bonds. However, the synthetic utility of this beneficial approach is limited to reactions with nonspecific amino acid containing peptides, whereas the coupling of specific ones leads to unwanted cleavages due to the native proteolytic activity of the biocatalyst (Grunberg et al. 2000).

The replacement of peptide bond is an important segment in the synthesis of peptidomimetics, because this modification may result in the preparation of biologically active analogs with improved properties, especially regarding bioavailability and metabolical stability. The introduction of sulfonamide group increases polarity of a molecule, and the hydrogen bond donor properties as a sulfonamide N-H is more acidic than carboximide. Recent advances in the synthesis of building blocks for sulfonamidopeptides, such as alpha and beta-substituted aminoalkylsulfonates, and efficient methods for the formation of sulfonamide bond have enabled the preparation of large number of oligomers with potential applications in various fields (Obreza and Gobec 2004). Enzymatic peptide synthesis using eutectic mixtures of substrates offer an interesting alternative to conventional solvent-based media (Gill and Vulfson 1993, 1994), and a range of lyophilized and immobilized serine, cysteine, and metalloproteases were found to catalyze the synthesis of variety of neutral, acidic, and basic dipeptides in this mixture.

C-Terminal amidation and recombinant DNA technology

Despite the recent advances in oral delivery of therapeutic peptide hormones, the bioavailability of peptides with any of the current technologies is much lower than obtained by injection. Commercially viable peptide products have to face strong acidic gastric environment, high levels of intestinal proteolytic activity, and high intestinal permeability barrier (Woodley 1994; Lee 2002). Many bioactive peptides and over half of all peptides hormones must be amidated at their C-terminal carboxy group to exhibit full biological activity. In nature, the mechanism for C-terminal peptide amidation involves a posttranslational modification of the C-terminal glycine-extended peptide precursor catalyzed by a specific peptidylglycine α-amidating enzyme (Cerovsky and Kula 2001). Various recombinant protein production technologies have been developed. However, the production of peptides, and particularly of peptide hormones that require C-terminal amidation for biological activity, remains a significant challenge.

Recombinant DNA technology is preferably used in the synthesis of large peptides up to several hundred amino acids (Harford 1988). During the synthesis of short sequences, low expression efficiencies obtained and difficulties encountered in product extraction and recovery remain impractical using genetic engineering (Merkler 1994). The inability to incorporate unnatural amino acids is another drawback of genetic engineering during the synthesis of drug and pharmaceutical peptides (Harford 1988). Moreover, the application of recombinant DNA technology typically requires a long and expensive research and development phase (Gill et al. 1996), and usefulness of recombinant DNA technology for shorter peptides is yet to be established.

Recent applications in peptide synthesis

The multiple peptide synthesis approach originated as an immunological tool for epitope mapping, and recent developments such as the introduction of novel-grafted polymeric surfaces, new linkers, as well as novel cleavage formats have extended the scope of this application for modular grafted surfaces (Ede 2002).

The technologies enabling the creation of large scale, miniatured peptide on protein microarray are emerging. Gao et al. (2004) reviewed the concepts related to the synthesis reactions of photogenerated acids in the deprotection step of peptide synthesis or oligonucleotide synthesis and applications of high density peptide chips in antibody binding assays. Peptide chips provide versatile tools for probing antigen–antibody, protein–protein, and peptide–ligand interactions as basic components for miniaturization, automation, and system integration research and clinical diagnosis applications. Immune responses to cancer cells can be elucidated in vivo by administering synthetic peptides derived from proteins uniquely or overexpressed on tumor cells [tumor-activated antigens (TAAs)]. In contrast to peptides derived from an exogenous source (viral or bacterial), tumor peptides bind weakly to MHC class I molecules. The low binding affinity of these peptides makes them poor candidates for vaccination due to poor immunogenic response produced. To enhance antigen recognition, hence immunogenicity, peptide-binding affinity for MHC can be improved by modifying the TAAs (Lazoura and Apostolopoulos 2005), including the use of proteolytically tolerant peptides which incorporate non-natural amino acids, retro inversion, and cyclization to improve their bioavailability.

An aminopeptidase was identified and purified from spirochete *Borrelia burgdorferi*, which is deficient in pathways for amino acid synthesis. Thermophilic aminopeptidase of *B. burgdorferi* could play a role in supplying amino acids, and the enzyme could be involved in peptide and/or protein processing (Bertin et al. 2005). A new 4-(N,N′ dimethylamino)phthalimide-based environment-sensitive fluorescent building block for solid phase peptide synthesis has been synthesized and incorporated into peptides which show great potential for biological applications in sensing protein–protein interaction (Eugenio et al. 2004). United Biomedical, Inc. (UBI) has developed a set of core technologies for the discovery and production of synthetic peptide-based immunotherapeutics and vaccines. These technologies have led to products that stimulate functional site-directed antibody responses for therapeutic effects. UBI active therapies can be used to modulate physiological processes effective for the control of prostate cancer and allergy and growth promotion in swine (Wang and Walfield 2005).

Conclusion

Increasing health and safety regulations and growing demand for biologically active peptides have prompted an intensive search for biotechnological alternatives of chemical synthesis of oligopeptides of interest and their analogs. The search will continue for proteases, which show unique specificities that may lead to further development of the enzymatic method for peptide synthesis. A general approach to enzymatic peptide synthesis remains to be formulated despite the large number of studies carried out on the synthesis of model and bioactive peptides. Use of protected substrates in oligopeptide synthesis has led to a loss of continuity in the reaction sequence and a reduction in yield and productivity and thereby has limited the continuous synthesis of oligopeptides using enzymes. New strategies or methods are needed to develop and taken as a significant challenge to overcome the difficulties faced during C-terminal amidation for expression of complete biological activity by recombinant protein and peptide hormones, as well as for the use of recombinant DNA technology in shorter peptide synthesis.

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