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MINI-REVIEW

Post-production modification of industrial enzymes

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Abstract Industry has an increasing interest in the use of enzymes as environmentally friendly, highly efficient, and specific bio-catalysts. Enzymes have primarily evolved to function in aqueous environments at ambient temperature and pressure. These conditions however do not always correspond with industrial processes or applications, and only a small portion of all known enzymes are therefore suitable for industrial use. Protein engineering can sometimes be applied to convey more desirable properties to enzymes, such as increased stability, but is limited to the 20 naturally occurring amino acids or homologs thereof. Using post-production modification, which has the potential to combine desirable properties from the enzyme and the conjugated compounds, enzymes can be modified with both natural and synthetic molecules. This offers access to a myriad of possibilities for tuning the properties of enzymes. At this moment, however, the effects of post-production modification cannot yet be reliably predicted. The increasing number of applications will improve this so that the potential of this technology can be fully exploited. This review will focus on post-production modification of enzymes and its use and opportunities in industry.

Keywords Post-production modification · Enzymes · Industrial applications · Pegylation · Glycoconjugation

Introduction

Enzymes are exceptional catalysts; they are capable of catalyzing a great variety of chemical transformations, and do so with high stereoselectivity, regioselectivity, and chemoselectivity, producing almost exclusively the desired product without unwanted side-products. As full conversion of reactants is not desired for every application, enzyme activity can be fine-tuned to fit the needs of the process. In addition, they function at ambient pressure and temperature, in contrast to most chemical syntheses. Better understanding of enzymes and improved production technologies has led to an increase in their application in industry (Iyer and Ananthanarayan 2008; Van den Berg et al. 2012). Enzymes are utilized in various industrial sectors such as the detergent, paper, food and pharmaceutical industry (Kirk et al. 2002). The demands of these industries with regard to enzyme performance, substrate specificity, stability, solubility, pharmacokinetic and pharmacodynamics properties are diverse and tuning of enzymes to fit the needs of a specific process is often desired. Protein engineering is often used to improve enzymes (Recktenwald et al. 1993), and while this is a powerful method, it is limited to the use of the 20 natural amino acids. Recently, it became possible to incorporate non-natural amino acids into enzymes (Kwon and Lim 2013; Schoffelen et al. 2008; Van Deventer et al. 2011), which can change the properties of the enzyme; however, this method is limited to incorporation of small, amino acid like groups. Furthermore, this approach often leads to a decrease in expression efficiency, which is undesirable for industrial applications. In nature, enzymes are often modified post-translational by addition of glycan chains (Spiro 2002), farnesyl groups (Novelli and D'Apice 2012), phosphate groups (Tarrant and Cole 2009), formyl groups (Baslé et al. 2010; Mader et al. 2013) or acetyl groups (Arif et al. 2010). These modifications can target enzymes to specific cellular compartments, activate or stabilize the protein. The engineering of post-translational modification (PTM) can be used to convey different properties on enzymes, like introduction of carbohydrate binding modules (CBM), glycosylation sequences or insertion of purification

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tags. As with protein engineering, modifications are limited to naturally occurring substances and processes; in addition, the modification of proteins during production can have a (negative) impact on the expression and purification efficiency. Alternatively, modification of enzymes can be done after expression and purification, i.e., post-production modification (PPM). In PPM, the enzyme can be modified with a selection of natural and synthetic substances with the goal to improve its properties to better suit the needs of the specific application. This review will focus on PPM and its use in industry. Crosslinking and immobilization strategies are often employed to increase stability, activity, and solvent tolerance of enzymes. These strategies have been extensively reviewed in other articles (Sheldon and van Pelt 2013; DiCosimo et al. 2013; Hanefeld et al. 2009; Franssen et al. 2013; Liese and Hilterhaus 2013), and will not be discussed here.

Modification methods

Chemical modification

Not all amino acids are equally suited to serve as an attachment point for modification. For this, the amino acids need to be situated on the surface of the enzyme, and contain a reactive group. Lysine, cysteine, glutamic acid, aspartic acid, serine, tyrosine, tryptophan residues can all be modified (Canalle et al. 2010), although not all are typically used. The majority of chemical modifications are performed using either lysine or cysteine (Hackenberger and Schwarzer 2008; Sletten and Bertozzi 2009; Davis 2003).

The ε-amine group of the lysine is reactive under physiological and alkaline conditions, and a wide variety of chemical methods are available for the modification of the lysine side chain (Fig. 1a) (Sletten and Bertozzi 2009). Different methods tend to be preferred for the attachment of different moieties to the enzymes. A more detailed discussion of specific methods can be found in the section commonly applied modification moieties. As multiple lysines are present on the surface of almost all enzymes, lysine modification can readily be applied to almost all proteins. Due to the presence of multiple lysines which can differ in reactivity towards the chemicals used, modification almost invariably results in differences in the modification degree and positions.

Like the ε-amine group of the lysine, the thiol headgroup of cysteine is also readily modified with a variety of methods (Fig. 1b) (Sletten and Bertozzi 2009; Hackenberger and Schwarzer 2008; Bernardes et al. 2008). In contrast to lysine, cysteine residues are mostly found in the interior of enzymes, and often bound to a second cysteine forming a sulfur bridge. They do offer the potential for site-selective modification, and can be introduced at a specific suitable position through protein engineering.

Glutamic and aspartic acid, which contain a carboxylic acid on their side chain, can be coupled to amine-containing molecules using peptide coupling strategies, usually using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as an activating agent.

Serine, tyrosine, and tryptophan are rarely used for enzyme modification. They are usually not present on the surface of enzymes, and require less standard coupling strategies (Sletten and Bertozzi 2009; McFarland et al. 2008; Romanini and Francis 2008; Tilley and Francis 2006; Canalle et al. 2010). Only a small portion of all enzymes can thus be modified this way.

In addition, the N-termini of proteins can be selectively modified, using the small pK_a difference between the N-terminal α-amine group and the ε-amine groups of lysines (Dixon 1984; Witus et al. 2013).

Enzymatic modification

The vast majority of post-production modifications are currently carried out using chemical modification strategies. Enzymatic modification methods are emerging; they offer a biocompatible route to protein modification, typically with high site specificity and without the use of toxic chemicals or creation of toxic waste products. However, they usually cannot be applied directly to any protein, as they generally require specific recognition sequences or residues. Several enzyme classes have the ability to modify other enzymes in vivo, a review by Heck et al. (2013) provides an overview. One of the main industrial applications is protein crosslinking through oxidative enzymes, peptidases, and transglutaminase for food products (Heck et al. 2013). Here, we will limit the discussion to protein decorating enzymes, mainly through five enzyme types: transglutaminase, sortase A, protein farnesyltransferase (PFTase), endoglycosidase H (EndoH), and peptide-*N*-glycosidase F (PNGaseF).

Transglutaminase (Canalle et al. 2010) links a glutamyl to a primary amine-containing group like a lysyl (Lorand and Graham 2003) (Fig. 2a). It is mainly used in industry to "glue" pieces of meat or fish together, but it also has applications in dairy- and wheat-based products (Yokoyama et al. 2004). By forming crosslinks between the proteins, new structures can be obtained as well as coatings to preserve and stabilize food products. Transglutaminase can also be used to couple non-protein compounds containing a primary amine to a glutaminyl on an enzyme. This strategy has for example been used to couple polyethylene glycol (PEG) chains to several proteins (Fontana et al. 2008; Sato 2002) and trypsin to cyclodextrins (Villalonga et al. 2003).

Transglutaminase also has deamidation activity (Boros et al. 2006; Fleckenstein et al. 2002), which could be used to lower the stability of enzymes. Site-directed mutagenesis studies modifying the asparagine or glutamine residues have

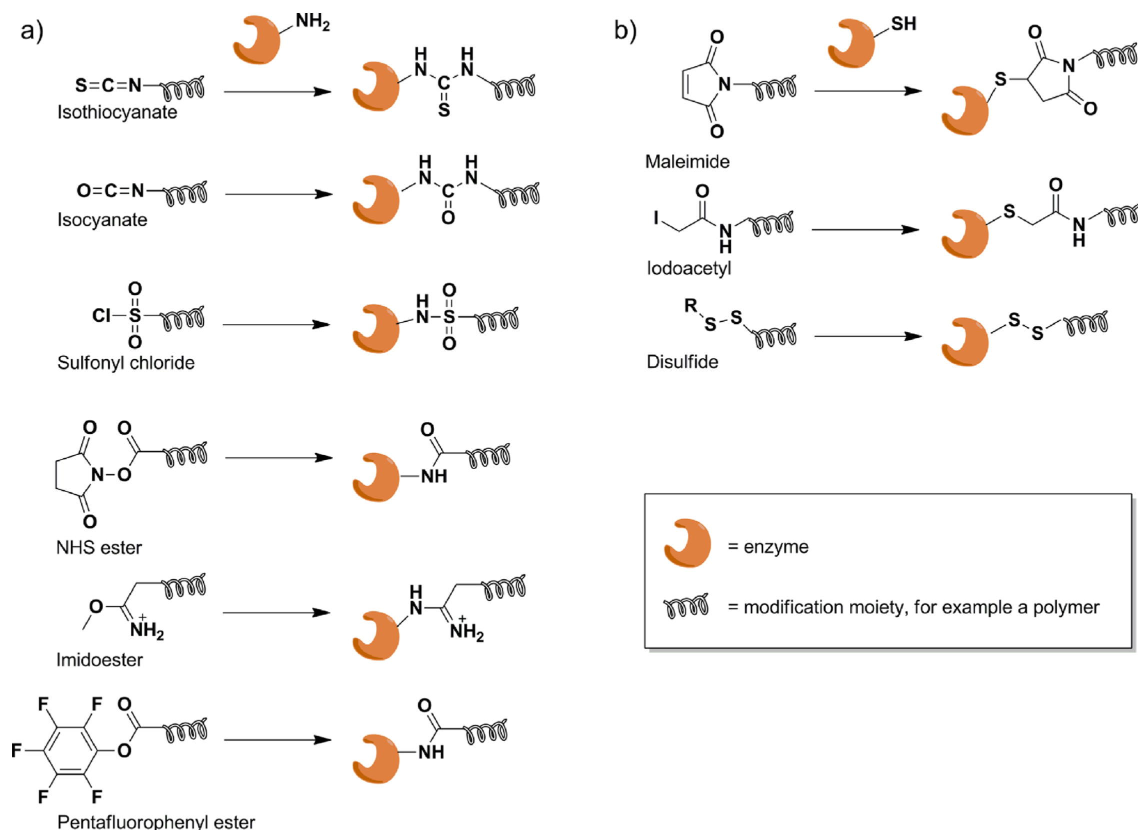

Fig. 1 Commonly used methods for modification of the lysine (**a**) and cysteine (**b**) side chains

shown that deamidation usually lowers the (thermo)stability of enzymes (Chen et al. 1994; Ahern et al. 1987; Tomazic and Klibanov 1988). Enzymatic or chemical deamidation might therefore be expected to have similar effects on enzyme stability. This could be of use in the food industry, where enzymes typically have to be inactivated at relatively low temperatures. Care must be taken however, as along with a decrease in stability, often also a decrease in activity is observed (Ahern et al. 1987; Zale and Klibanov 1986; DeLuna et al. 2005). Chemical deamidation is relatively easy through acid or base hydrolysis. However, strong alkaline or acidic treatments can result in unwanted protein degradation. Alternatively, milder alkali or acid treatments can be used in combination with higher temperatures, although the elevated temperature could also result in protein degradation. Enzymatic deamidation would allow for mild reaction conditions. In addition to transglutaminase, which can deamidate proteins in absence of primary amines and at low pH, also peptidoglutaminase (Pgase) (Hamada 1994) and protein glutaminase (PG), which converts glutamine into glutamate (Miwa et al. 2010; Yie et al. 2006; Yamaguchi and Yokoe 2000), can be used for the deamidation of proteins.

While transglutaminase is quite aspecific, Sortase A mediates the ligation between an acceptor molecule containing a LPXTG motif and a donor molecule containing an N-terminal oligoglycin motif (Wu and Guo 2012; Spirig et al. 2011). The glycine and all amino acids on the C-terminal side of the LPXTG motif are replaced by the substrate molecule (Fig. 2b). The substrate molecule preferably contains multiple glycines, although conjugation with a single glycine tagged substrate molecule (Proft 2010) or aminomethylene groups have also been reported (Samantaray et al. 2008) albeit with reduced efficiency. The LPXTG motif can be introduced using protein engineering, or chemical synthesis, both requiring an extra modification step before ligation to the substrate. It has been shown that a large number of different chemical entities, like carbohydrates (Samantaray et al. 2008), small organic molecules (Maximilian et al. 2007), lipids (Antos et al. 2008), and synthetic polymers (Popp et al. 2011) can serve as substrates, as long as they contain C-terminal glycines or aminomethylene groups. When incorporating both a LPXTG motif and an oligoglycine sequence in the same enzyme, it is possible to circularize the enzyme (Antos et al. 2009), which can have a stabilizing effect on the enzyme, due to reduced N or C-terminal protease reactivity and a decreased flexibility of the enzyme. Sortase A can also transfer the LPXTG motif from the donor molecule to the ε-amino group of substrate molecules, thereby forming branched oligomers (Dasgupta et al. 2011). While a peptidase like Sortase A depends on a specific target sequence and is able to recognize this at various positions in a protein, other protease dependent methods are developed to specifically target the free amine at the N-

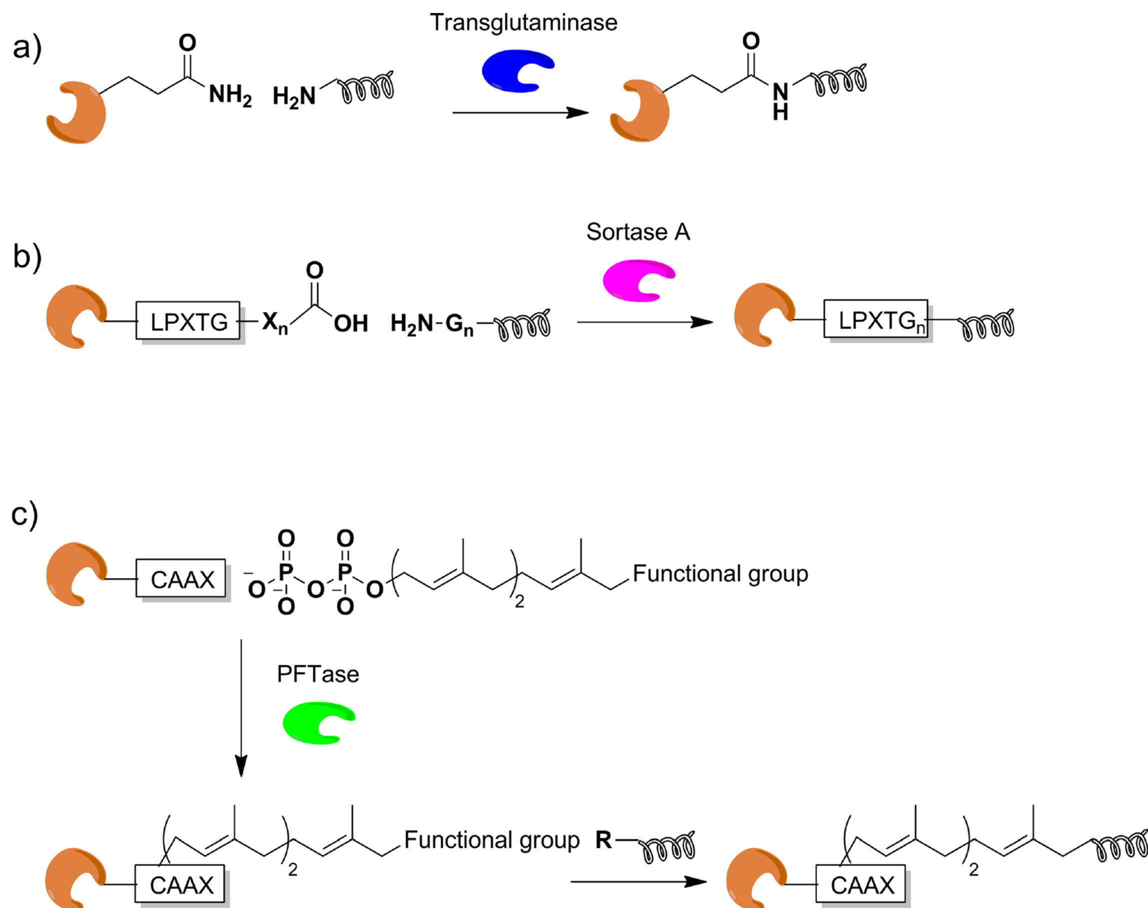

Fig. 2 Schematic representation of enzyme modification using transglutaminase (**a**), sortase A (**b**) and PFTase (**c**)

terminus. To this end, IgA-Protease was employed to specifically modify IgA's through controlled reversed proteolysis in water (Lewinska et al. 2004).

In a similar fashion, PFTase can be used to introduce a farnesyl group in proteins (Rashidian et al. 2012). PFTase catalyzes the reaction of a farnesyl group to a cysteine, when that cysteine is followed by two aliphatic amino acids and M, S, Q, A, or C, a so-called CAAX box. If this sequence is not present in the enzyme it can be introduced at the C-terminus, where the AAX sequence can be removed by treating the enzyme with carboxypeptidase after the farnesylation (Fig. 2c). By using farnesyl groups that contain a specific functional group, the farnesyl group can serve as a handle to site-specifically attach other moieties such as fluorescent labels or PEG.

Eukaryotes, as well as some industrially less relevant prokaryotes, are able to glycosylate enzymes (Messner 2004). This can serve diverse functions before as well as after expression of the enzyme (reviewed in Varki 1993). One of the functions of glycosylation is stabilization of the enzyme; consequently, many (secreted) enzymes are glycosylated. While for many applications increased stability and activity are desired, this is not true for all applications. For instance in food applications, where heat inactivation of enzymes at low temperatures is sometimes necessary to prevent ingestion of active enzymes, or incomplete conversion of the substrate is needed in order to obtain the desired food properties.

While deglycosylation can be achieved by removal of the glycosylation sequences from the encoding DNA sequence, this is not always the preferred method, as the glycan chains might have a significant function during protein expression. EndoH and PNGaseF are two enzymes that can remove the glycan chains from glycoproteins (Koide and Muramatsu 1974; Lee et al. 1986), although they have a different mode of action; i.e., EndoH cleaves the bond between two *N*-acetylglucosamine units linked to an asparagine, leaving only one unit linked to the amino acid, while PNGaseF breaks the bond between most types of glycosyl units and asparagine, and deamidates the remaining asparagine to an aspartic acid. As the functions of carbohydrate chains on proteins are diverse, deglycosylation can result either in drastic property changes of the enzyme or in no change at all (Ritter et al. 2013). The effects are thus difficult to predict, depending on the glycosylation site (Chen et al. 1994) and highly enzyme specific.

Deglycosylation of human liver α-L-fucosidase by PNGaseF did not result in changes in the catalytic properties

of the enzyme, but did lead to decreased activity at acid pH, and to decreased thermostability (Piesecki and Alhadeff 1992).

Heterologous expression of enzymes can lead to differences in glycosylation pattern and the extent of glycosylation. This can influence the performance of enzymes, as demonstrated for Cel7A, a cellulase, which showed significantly increased *N*-glycosylation upon heterologous expression. This contributed to a decreased enzymatic activity, which could be partially restored by PNGaseF deglycosylation (Jeoh et al. 2008).

Commonly applied modification moieties

PEGylation

One of the most frequently applied post-production modification is PEGylation. The covalent attachment of polyethylene glycol (PEG) molecules to the surface of proteins can convey among other attributes, increased solubility in organic solvents (Koops et al. 1999; Quintanilla-Guerrero et al. 2008; Mabrouk 1997), thermostability (López-Cruz et al. 2006; He et al. 2000; Zhang et al. 2001), increased pharmacokinetic and pharmacodynamics properties (Milton Harris and Chess 2003).

PEG is a synthetic polymer for which a wide range of molecular masses are commercially available. It is very hygroscopic and binds two to three water molecules per monomer unit in aqueous solutions. In combination with a very flexible polymer backbone, this leads to high hydrodynamic volumes of PEG in aqueous solvents. Since it is a synthetic polymer, it is inherently polydisperse, with polydispersity values ranging from 1.01 for low MW polymers to 1.2 for high molecular weight polymers (Veronese 2001). For protein conjugation purposes, mostly the monomethoxylated variant of PEG (mPEG) is used, of which only the hydroxyl end can be functionalized. This prevents crosslinking of proteins, which could occur using PEG diol. Unfortunately, up to 10 % PEG diol can be present in mPEG (Dust et al. 1990). This can be removed by creating carboxymethyl functionalized PEG (CM-PEG) (Fig. 3), subsequently the mono and disubstituted variants can be separated using ion-exchange chromatography.

PEGylation methods

A variety of modification methods has been developed for coupling of ε-amino groups of lysine and the α-amino group of the N-terminus with PEG chains. To couple PEG to amino groups on the protein, it is necessary to activate the PEG molecule. A range of activating agents can be used for this (Fig. 4) (Milton Harris and Chess 2003; Roberts et al. 2002), each with their own advantages and disadvantages (Milton Harris and Chess 2003; Adamczak and Krishna 2004; Zhang et al. 2009; Bianchi et al. 1993; Canalle et al. 2010; Veronese and Pasut 2005).

If the positive charge on lysine is important for biological functionality, then alkylating PEGs can be used (Fig. 4j–l), preserving the positive charge. PEG-aldehyde can react with amino groups on the protein to form a Schiff base, which after cyanoborohydride reduction gives a permanent linkage. Unfortunately, this reaction is slow, up to a day can be necessary to functionalize enzymes, which is a disadvantage for more labile enzymes. In addition, cyanoborohydride is toxic. In pharmaceutical or food applications this is highly undesirable, but for biocatalysis applications this can be acceptable. Alternatively, tresyl chloride (Heiss 1995; Khan et al. 1992) can be used (Fig. 4j); although this often results in ill-defined reaction products (Roberts et al. 2002). A more frequently used activating agent is trichlorotriazine (Abuchowski et al. 1977a; b). The activated PEG (Fig. 4g) can react with several amino acid reactive groups, displacing one of the chlorides. Reactions with the remaining less reactive chloride can lead to the formation of cross-linked products. To overcome this problem, Matsushima et al. (1980) developed a trichlorotriazine functionalized with two PEG molecules (Fig. 4h), thus creating a branched PEG moiety. The only remaining chloride has a lower reactivity, thus resulting in a more selective modification of lysines and cysteines. Coupling with trichlorotriazine is fast and easy, it gives stable products, but the reaction intermediate is also toxic.

Acylated PEGs result in the removal of the positive charge of lysines (Fig. 4a–i). To this end, PEGs can be activated as hydroxysuccidimidyl esters (OSu), chloroformates (Veronese et al. 1985), or pentafluorophenyl esters (Abello et al. 2008). The reaction conditions are generally mild. Variants with higher stability against hydrolysis are propionic or butanoic acid derivatives (Fig. 5) (Harris and Kozlowski 1997). The higher stability is the result of the increased distance between the active ester and PEG by the alkane spacer.

Due to the prevalence of lysines in proteins, reaction with PEG usually results in a high degree of modification, but also in heterogeneous products with varying number of PEG chains attached to proteins at different positions. While for biocatalysis applications a high degree of modification is often important, for pharmaceutical applications, reproducibility is highly important and therefore the formation of isomers due to the modification of different lysines is undesirable. Modification techniques that are more site-selective have been developed (González and Vaillard 2013; Veronese and Pasut 2005). By lowering the pH during reaction of PEG with the protein, the more reactive α-amino group of the N-terminus can be preferentially targeted (Gaudriault and Vincent 1992; Kinstler et al. 2002; Hu and Sebald 2011). Thiol groups can also be targeted, using vinylsulfones, maleimides, and iodoacetamides. Since

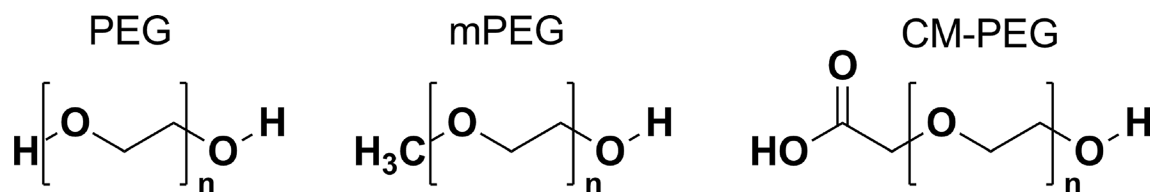

Fig. 3 Structures of PEG, mPEG, and CM-PEG

the latter two can also react with lysines, the reaction conditions have to be optimized to prevent this.

Alternatively, enzymatic PEGylation can be applied to introduce site-selective modifications. Transglutaminase catalyzes the acyl transfer between the carboxamide group of glutamine and the amino group of activated PEG-modules. Even more sophisticated single, targeted PEGylation is being explored by engineering *Escherichia coli* to efficiently produce proteins with add-on site-specific glycan (GalNAc) residue(s). PPM can target the glycan as site for PEGylation using sialyltransferase enzyme (Wright 2013).

Quantification of number of PEG molecules bound

Determining the amount of PEG bound to proteins is not trivial, as PEG is non-fluorescent, does not react easily and has different hydrodynamic properties from proteins making size exclusion difficult to quantify. Usually, the amount of bound PEG is derived from the decrease of free and detectable amine groups on the surface of proteins. This method is not completely reliable, as it is based on colorimetric or fluorimetric assays, which have an intrinsic variability. Additionally, the protein concentration needs to be separately determined.

SDS-PAGE might also be used, but this method is limited to low MW conjugates, as PEG hampers gel penetration, which together with the polydisperse nature of PEG leads to smearing of the bands, hindering quantification. Mass spectrometry, mostly MALDI or ESI-TOF can also be used for analysis (Gioacchini et al. 1997), because the product can be separated based on mass. In combination with endoproteinase digestion, this can also reveal the locations of the modifications, provided the different isoforms have been separated prior to analysis. In the absence of an internal standard, these methods are not quantitative however, and the percentage of

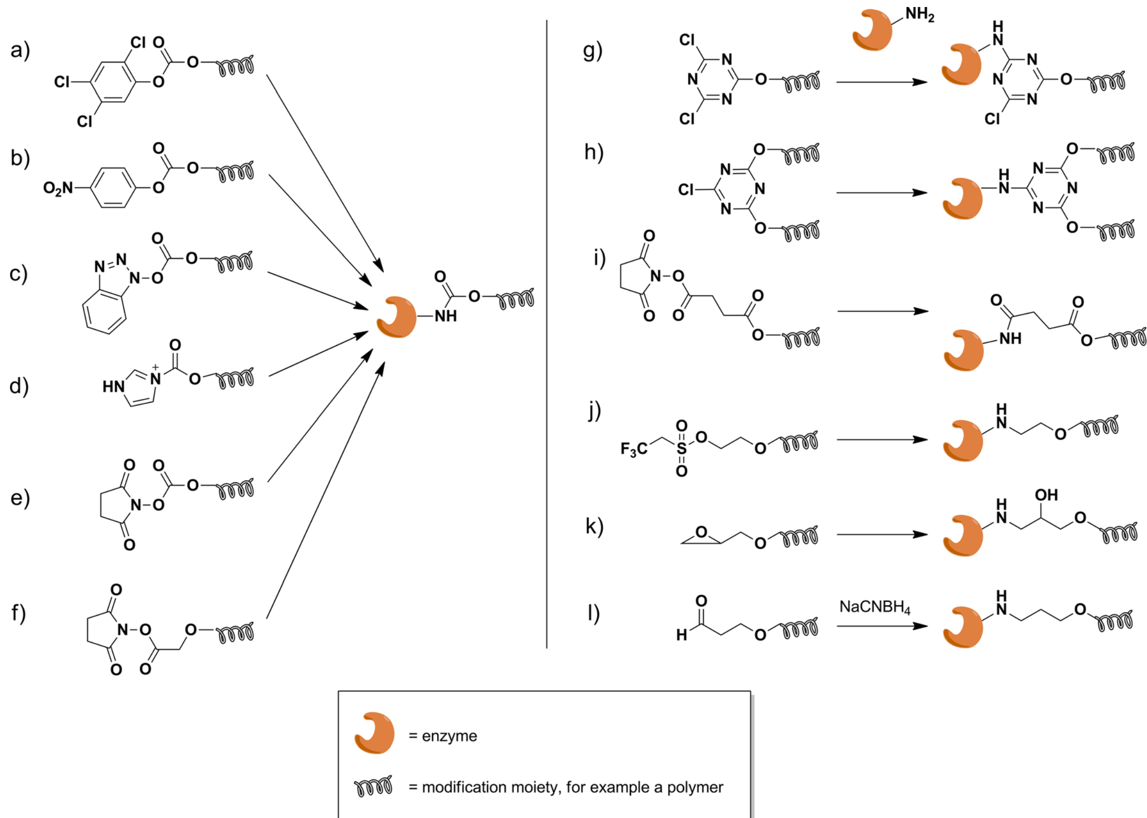

Fig. 4 Methods of PEG coupling to primary amines on proteins. **a** PEG trichlorophenyl carbonate; **b** PEG *p*-nitrophenylcarbonate; **c** PEG benzotriazole carbonate; **d** PEG carbonylimidazole; **e** PEG succinimidyl carbonate; **f** PEG succinimidyl ester; **g** PEG dichlorotriazine; **h** PEG_2 chlorotriazine; **i** PEG succinate succinimidyl diester; **j** PEG tresylate; **k** PEG epoxide; **l** PEG propanal

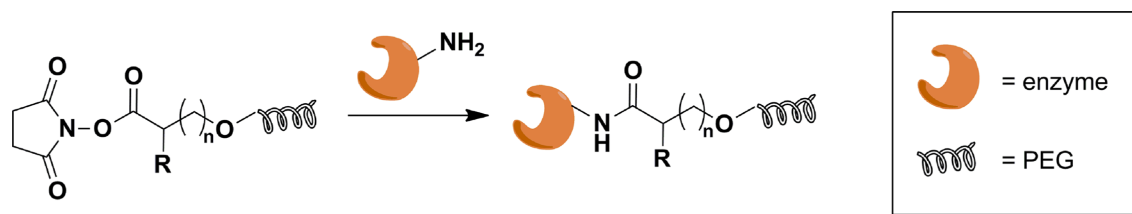

Fig. 5 Coupling of propionic or butanoic acid derivatives of PEG to primary amines on a protein

modified/unmodified can thus not be determined. The most quantitative method involves introducing a norleucine spacer between PEG and protein (Sartore et al. 1991). After separating the protein from unreacted PEG, hydrolysis of the protein-PEG conjugate and subsequent determination of the norleucine content gives the average modification degree. As this method increases the costs of the procedure, it might be more applicable on labscale rather than on industrial scale.

Applications

In biocatalysis, PEGylation of enzymes is applied to enhance solubility and stability in organic solvents, increase thermal and pH stability, and improve activity.

Increased solubility of enzymes in organic solvents has several benefits. It enables enzymatic reactions with hydrophobic substrates and/or products, that would not be (or poorly) soluble under aqueous conditions. The reduced presence of water can shift the equilibrium to favor synthesis over hydrolysis reactions, thus enabling the synthesis of polymers. This has been successfully applied to peptide synthesis by chymotrypsin (Rolland-Fulcrand et al. 1994). Similarly, side reactions for which water is needed can be reduced, and an enzymatic catalysis step can more easily be incorporated in a chemical synthesis pathway. The absence of water also eliminates microbial growth (Grant et al. 2004). The amphiphilic nature of PEG makes enzymes more soluble in organic solvents upon PEGylation (Kwon et al. 1999; Iyer and Ananthanarayan 2008; Polizzi et al. 2007; Koops et al. 1999; Pina et al. 1989; Quintanilla-Guerrero et al. 2008; Takahashi et al. 1984; Matsushima et al. 1984). Since PEG is very hygroscopic, it also provides the enzymes with a surrounding water layer and protects the enzymes from stripping of their water layer by polar organic solvents. It has been shown that water bound to enzymes is essential for their activity (Zaks and Klibanov 1988).

In addition to aiding solubilization of enzymes in organic solvents, PEG has also been shown to increase solubility of subtilisin and lipases from *Candida rugosa* in ionic solvents (Moniruzzaman et al. 2010).

The increased solubility of enzymes in organic solvents is a general effect of PEGylation. Other effects, like enhanced or decreased enzymatic activity, are to a large extent unpredictable. It is therefore necessary to optimize the degree of modification and length of the PEG chains for each enzyme and application (Gaertner and Puigserver 1992; Zhang et al. 2009). To retain or even enhance activity, it is important that amino acids essential for enzyme activity or stability are not modified with PEG. Residues with such an essential role are often linked via hydrogen or ion bonds with other residues, which decrease their reactivity and the likelihood of PEG functionalization. This mechanism however, does not always prevent functionalization of essential lysines or other residues, and thus contributes to the diverse effects of PEGylation on enzymatic activity. PEGylation of residues near the active site can limit binding of substrate, thereby reducing activity.

Thermostability is often desirable because it increases the operational stability of an enzyme at a defined temperature. It also enables the performance of reactions at higher temperatures, increasing reaction speed. As a rule of thumb, a two-fold increase in reaction rate is expected with a 10 °C increase in temperature (van't Hoff's rule). In addition, a higher thermostability increases the lifetime of the enzyme, when compared to less thermostable enzymes stored or used at the same temperature. PEGylation has been shown to increase thermal stability in among others: subtilisin (Yang et al. 1996), horseradish peroxidase (Garcia et al. 1998), trypsin (Zhang et al. 1999; Gaertner and Puigserver 1992), lipases (Basri et al. 1995; Hernáiz et al. 1999), penicillin G acylase (Kazan and Erarslan 1997), cellulase (Zhang et al. 2009), peroxidase (Quintanilla-Guerrero et al. 2008).

The stability increase by PEGylation is likely caused by either decreased charge repulsion on the surface, due to modification of the lysines or by the amphiphilic nature of PEG (Quintanilla-Guerrero et al. 2008). The hydrophilic regions of bound PEG connect with the hydrophilic parts of the protein surface, resulting in an organized shield of water molecules, while the hydrophobic part of PEG is bound to the hydrophobic parts on the protein surface, producing a shell-like structure, increasing the rigidity of the protein and decreasing the unfolding rate of the active site.

A second theory is that the increased thermal stability is caused by steric hindrance of the PEG chain, resulting in reduced autolysis and an increase in the number of hydrogen bonds, resulting in decreased thermal denaturation (Zhang et al. 2001; He et al. 2000). In general, a higher degree of modification with PEG seems to result in a higher thermal stability (Hernáiz et al. 1999; Zhang et al. 2009). Also the

molecular weight of the PEG is important, however the optimum usually is with PEG molecules that neither have a very low molecular weight nor a very high molecular weight, but the exact optimum is dependent on the specific enzyme.

PEGylation is successfully used in the pharmaceutical industry, and several commercial therapeutical proteins are on the market (Table 1). Various excellent reviews have covered this in depth (Milton Harris and Chess 2003; Veronese and Pasut 2005; Pasut and Veronese 2012; Knop et al. 2010; Kling 2013; Veronese and Mero 2008), thus we will only provide a short overview here.

Similarly as for non-pharmaceutical applications, PEGylation of therapeutic proteins will often improve the pH stability and thermostability of the proteins. More important for pharmaceutical application is the positive influence of PEGylation on the pharmacokinetic and pharmacodynamic properties of the therapeutic proteins. Due to the increased size of PEGylated proteins, the renal clearance of especially smaller therapeutic proteins is lowered (Milton Harris and Chess 2003; Pasut and Veronese 2012). The amphiphilic nature of PEG can increase water-solubility of the proteins, and protein aggregation is lowered due to repulsion between PEGylated surfaces (Basu et al. 2006; Hinds et al. 2000). The half-life of the proteins is improved and immunogenicity is reduced (Pasut and Veronese 2009; Caliceti and Veronese 2003), leading to decreases in adverse side effects and uptake by cells of the immune system (Knop et al. 2010). Although the in vivo activity of PEGylated proteins is often decreased, this is balanced by the positive effects, resulting in an improved clinical effect (Milton Harris and Chess 2003).

Although PEG is considered non-toxic, there are concerns that with an increasing amount of PEGylated drugs, side effects might become noticeable, like accumulation in the body, degradation under stress, and interaction with the immune system (Knop et al. 2010; Caliceti and Veronese 2003; Schellekens et al. 2013; Garay et al. 2012). For pharmaceutical products, reproducibility and low batch-to-batch variation are extremely important. The so-called first generation PEGylation techniques based on modification of lysines with relatively low molecular weight linear mPEG have many drawbacks, such as mixtures of isomers, unstable linkages, sometimes toxic coupling reagents, small hydrodynamic radii, incomplete shielding of the protein immunogenic sites, and potential crosslinking of proteins due to the use of diol contaminated mPEG (Milton Harris and Chess 2003; Pasut and Veronese 2012; Kozlowski and Milton Harris 2001). Second-generation PEGylation techniques (Milton Harris and Chess 2003; Roberts et al. 2002) like site-specific coupling to a cysteine residue—introduced on the surface of the protein by engineering—is one of the solutions to isomeric mixtures. Branched PEGs improve shielding and results in a higher hydrodynamic volume as compared with linear PEG of the same molecular weight.

Glycoconjugation

It is estimated that about 50 % of all enzymes are glycosylated in nature (Apweiler et al. 1999) suggesting this can be beneficial. Glycosylation plays a role in targeting of enzymes, and influences activity, solubility and stability (Varki 1993; Shental-Bechor and Levy 2008; Dwek 1996). Glycoconjugation, the non-enzymatic modification of enzymes with natural or synthetic (poly)saccharides is different from glycosylation in some aspects. N-glycosylation only occurs at specific consensus sequences and the many different types of glycosylation often result in complex glycosylation patterns, which can serve as targeting signals. Each glycan chain attached to enzymes and

Table 1 PEGylated pharmaceuticals on the market

Trade name	Company	Enzyme/peptide	Disease	Approval year
Adagen	Enzon	Adenosine deaminase (enzyme)	Severe combined immunodeficiency disease (SCID)	1990
oncaspar	Rhone-Poulenc Rorer and Enzon	L-asparaginase (enzyme)	Leukemia	1994
PEG-intron	Schering-Plough	Interferon-α2b (protein)	Hepatitis C	2000
Pegasys	Hofmann-La Roche	Interferon-α2a (protein)	Hepatitis C	2001
Neulasta	Amgen Inc.	GCFS (protein)	Neutropenia	2002
Somavert	Pfizer	Growth hormone receptor antagonist (protein)	Acromegaly	2002
Macugen,	Pfizer	Anti-VEGF aptamer (oligonucleotide)	Age-related macular degeneration	2004
Mircera	Roche	Erytropoietin (protein)	Anemia caused by chronic kidney disease	2007
Cimzia	UCB Pharma	Anti-TNFα Fab′ (antibody)	Rheumatoid arthritis and Crohn’s disease	2008
Krystexxa	Savient	Uricase (enzyme)	Gout	2010
Omontys[a]	Affymax	Synthetic peptide	Anemia caused by chronic kidney disease	2012

[a] Recalled from market due to fatal anaphylactic reactions in a small percentage of patients

proteins during post-translational modification in the cell is coupled to only one amino acid residue on the protein, while modification with polysaccharides often results in multipoint attachment of the chain to the protein.

Glycoconjugation of enzymes is often used to increase stability of enzymes (Mislovicová et al. 2006; Gonera et al. 2004; Villalonga et al. 1999; 2000; 2003; Rajalakshmi and Sundaram 1995), most notably thermal stability (Gonera et al. 2004; De La Casa et al. 2002; Venkatesh et al. 2005). This was clearly illustrated by the heterologous expression of different *Pseudomonas aeruginosa* elastase variants in *Pichia pastoris*. Removing one or more of the three glycosylation sites leads not only to a significant reduction in expression, but also to decreased stability for the hydrolytic (at 70 °C) as well as the synthetic (at 50 %*v*/*v* organic solvent) reactions (Han et al. 2014). There are several mechanisms through which glycoconjugation could improve stability. The addition of glycan chains to the surface of the enzyme increases the overall rigidity of the enzyme, thereby preventing unfolding. Intramolecular crosslinks caused by multipoint attachment of glycans have a similar effect, preventing unfolding by impairing the conformational freedom of the enzyme (Venkatesh et al. 2005). Electrostatic repulsion between the polyanionic glycans can prevent aggregation of the enzymes, which often plays an important role in thermal inactivation (Moskvichyov et al. 1986). Furthermore, salt bridges between the enzyme and the glycan can contribute to maintaining the active conformation (Perutz 1978).

Methods of glycoconjugation

The two most frequently used strategies to couple a polysaccharide to an enzyme are reductive alkylation and coupling using carbodiimides (Fig. 6). In reductive alkylation, the polysaccharide is first oxidized using $NaIO_4$; this requires the presence of two adjacent hydroxyl groups. The amino groups on the enzyme can then react with the formed aldehydes, resulting in Schiff base formation. Reduction of the Schiff base gives a stable amino linkage between the enzyme and the polysaccharide. Reduction is commonly carried out with $NaBH_4$ (Villalonga et al. 2000; Villalonga et al. 1999; De La Casa et al. 2002) or $NaCNBH_4$ (Mislovicová et al. 2006; Sundaram and Venkatesh 1998). Sodium borohydride is a stronger reducing agent than $NaCNBH_4$. When added during the coupling reaction, it will therefore reduce the oxidized hydroxyl groups, leading to a decrease in the modification degree. For this reason, it is usually added after the coupling reaction. $NaCNBH_4$ is a milder reducing agent and can be added during the coupling reaction, however, due to the cyanide group, it is toxic. Siddiqui and Cavicchioli (2005), have successfully used borane-pyridine complex, a mild non-toxic reducing agent as an alternative to the borohydrides.

Another commonly employed strategy is the use of a carbodiimide to couple a carboxy group and a primary amine group. Some polysaccharides, like carboxymethylcellulose (CMC) contain carboxyl groups, which can be coupled to lysines on the enzymes (Matos et al. 2012). Alternatively, polysaccharides can be functionalized with NH_2 groups (Hernández et al. 2006; Fernández et al. 2005).

Chemoenzymatic coupling methods have also been developed; transglutaminase can be used to couple polysaccharides functionalized with a diamine, thus resembling a lysine side chain, to glutamine residues on the surface of enzymes (Villalonga et al. 2003). A different method consists of two steps: in the first step, the enzyme is functionalized with sucrose molecules that have been activated with cyanogen bromide; in the second step the glycosidic chain is enzymatically lengthened using fructosyltransferase from *A. niger* (Longo and Combes 1995; Longo and Combes 1997).

Applications

Proteases are important enzymes in diverse industries, such as the pharmaceutical, detergent, and food. Several glycoconjugation strategies have been described to improve the properties of trypsin. For use in the detergent industry, increased stability of trypsin in the presence of detergents is desirable. Trypsin was modified with CMC (Villalonga et al. 2000), which resulted in a high degree of intramolecular cross linking and increased stability, extended pH range and higher affinity for the substrate. Modification with cyclodextrins also

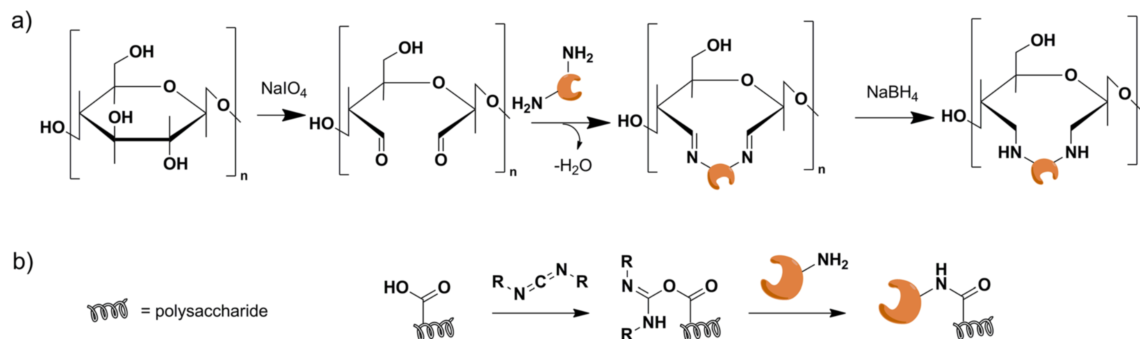

Fig. 6 Chemical coupling of polysaccharides to proteins. **a** coupling by reductive alkylation. **b** coupling using carbodiimides as an activating agent

resulted in increased stability, and also in a higher specific activity as well as a lower K_m (Fernández et al. 2002).

As modified cyclodextrins are expensive, which limit their industrial applicability, less expensive dextrans for conjugation with trypsin were explored (Hernández et al. 2006). The dextrans were monoactivated, thereby preventing crosslinking. Thermal stability was improved, as well as the autolytic resistance to degradation. Since autolytic degradation is the main inactivation mechanism for proteolytic enzymes, this leads to increased operational stability.

Other enzymes that have been stabilized using glycoconjugation include Penicillin G acylase (PGA) (Mislovicová et al. 2006; Masárová et al. 2001), α-amylase (Villalonga et al. 1999; Srimathi and Jayaraman 2005) and glucose oxidase (Matos et al. 2012). PGA is used for the production of 6-aminopenicillanic acid (6-APA), an important building block in the production of semi-synthetic penicillins. Upon modification with different dextrans, the stability and thermostability were improved slightly, which could lead to reducing the frequency of redosing in the factory (Mislovicová et al. 2006).

CALB, the cold-adapted *Candida antartica lipase B*, was reacted with dextran, ficoll and inulin (Siddiqui and Cavicchioli 2005). Modification with dextran resulted in an increase in thermal stability from 18 to 168 min at 70 °C, and a 65 % higher specific activity. In general, a higher thermal stability is related to a higher specific activity, indicating that activity does not necessarily have to be sacrificed to obtain better thermo stability.

Modification with polysialic acid (PSA) can have similar effects as modification with PEG (Gregoriadis et al. 2005). PSA is even less immunogenic than PEG, as there are no known receptors for PSA in the body (Gregoriadis et al. 1993; Crocker and Varki 2001). In contrast to PEG, which is either excreted or stored in the body (Caliceti and Veronese 2003), PSA is biodegradable, with non-toxic degradation products. Polysialation has been shown to increase blood circulation times and stability for asparaginase (Fernandes and Gregoriadis 1997; Fernandes and Gregoriadis 2001), superoxide dismutase (Wu et al. 2010), insulin (Jain et al. 2003), and an antibody fragment (Constantinou et al. 2008).

Modification with other polymers

In addition to PEG, other synthetic polymers have been used to modify enzymes. POA-MAA is a copolymer of polyoxyalylene and maleic anhydride. The ratio of propylene oxide to ethylene oxide (the monomer of PEG) determines the hydrophobicity of the polymer, which can thus be varied. The enzyme is coupled to the copolymer via reaction with the maleic anhydride monomers. POA-MAA has been used for the modification of cellulases, used for the saccharification of cellulose, an important step in the production of bioethanol. During saccharification, a large portion of the cellulases has been shown to adsorb to the substrate, which is presumed to have a negative impact on final conversion rates. Cellulases modified with different variants of POA-MAA showed lower activity, but also less adsorption to the substrate, such that the overall glucose yield was increased compared to native cellulase. The more hydrophilic POA-MAA gave the best increase in glucose yield (Park et al. 2002; Park and Kajiuchi 1995). POA-MAA has also been used in the modification of laccase (Shin-Ya et al. 2005), an enzyme with a broad range of industrial applications, including the removal of phenolic compounds in wastewater, whitening of paper pulp and dehazing of beer (Xu 2005). Modification with POA-MAA resulted in an increased thermal stability and increased activity.

Modification using small molecules

Modification with small molecules does not significantly increase the size of the enzyme or form a physical shield from the environment, like PEGylation or glycosylation can. Modification with small molecules are typically applied to neutralize or invert charges on the enzyme surface, adding more bulky headgroups on amino acids or a crosslink in the case of bi-functional reagents. Like PEGylation and glycosylation, modification with small molecules can have varying effects, depending on the nature of the small molecule, the modification degree, and the enzyme used. Thermostabilization is the most common objective for modification using small molecules.

Modification methods

Various methods are used to introduce small molecules on the surface of proteins, such as reaction with anhydrides, succinimates or acids. Modification with anhydrides is a relatively simple process where the anhydride is added stepwise to the enzyme at a slight alkaline pH. During the reaction, acid is released from the anhydride, and the pH is kept constant by addition of a suitable amount of a base like NaOH (Miland et al. 1996; Khajeh et al. 2001).

Applications

Functionalization with a monocarboxylic anhydride like citraconic anhydride will replace the positive charge of lysine with one negative charge, while functionalization with dicarboxylic anhydrides like trimellitic anhydride result in the replacement by two negative charges (Fig. 7). By varying the anhydride, one can tune the charge of the modified enzyme.

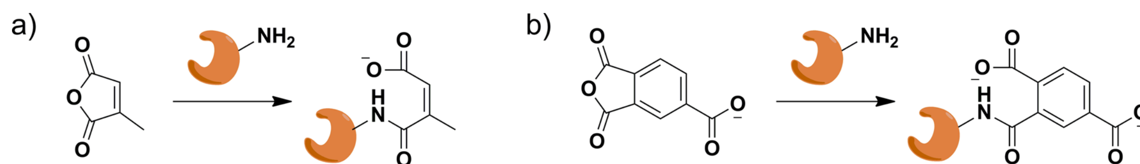

Fig. 7 Reaction of a monocarboxylic anhydride, citraconic acid **a** and a dicarboxylic anhydride, trimellitic anhydride **b** with a primary amine of an enzyme

Modification of horseradish peroxidase (HRP) with both mono- and dicarboxylic anhydrides showed that while below 65 °C both types of anhydrides provided thermostabilization, above 65 °C only modification with mono carboxylic anhydrides resulted in significant thermostabilization (Hassani 2012a). This might be explained by a balance between stabilization through hydrophilization of the surface, which is most effectively achieved by the use of dicarboxylic acids, and destabilization due to an increase in electrostatic repulsion. It appears that at lower temperatures the stabilizing effect of a hydrophilization is most important, where at higher temperatures the charge repulsion becomes more important. This effect was even more pronounced when HRP was modified with pyromellitic dianhydride, replacing the positive charge of the modified lysine groups with three negative charges from the carboxylic acid groups of the dianhydride (Hassani 2012b). Below T_m (50–65 °C), the thermostability of the modified HRP was increased, while it decreased above T_m (70–80 °C), confirming the balance between stabilizing and destabilizing interactions as hypothesized above. This dual behavior could be useful in food applications, where often it is required to inactivate the enzyme in the final product, but good stability during processing is beneficial.

Modification of HRP with phthalic anhydride resulted in an increased stability in aqueous buffer (Liu et al. 2002) and organic solvents (Song et al. 2005). In addition, the thermal stability in aqueous solutions at 50 °C was increased 10-fold, and thermal stability in tetrahydrofuran (THF), acetonitrile (ACN) and methanol at 30 and 60 °C was also increased. Modification of HRP using different bis-succinimides yielded 6–23-fold increase in thermal stability at 75 °C in aqueous buffer, while modification with bis-imidates hardly increased thermal stability (Ryan et al. 1994). In contrast to modification with bis-succinimides, modification with bis-imidates does not result in the neutralization of the positive charge of lysine, as it does not introduce a negative charge. Similarly, modification with acetic anhydride does not result in addition of a negative charge.

Papain, a cysteine protease, was also modified with different acid anhydrides (Szabó et al. 2009). This stabilized the enzymes in aqueous protic ethanol and polar ACN, but not in aprotic THF. Papain modified with acetic or propionic anhydrides, which do not introduce a negative charge, showed a greater increase in activity and stability than when modified with citraconic, maleic or succinic acid anhydrides, which do introduce a negative charge. Papain could be used for the removal of proteinacous stains from clothing, which is usually achieved by the addition of serine proteases to detergents. Papain has a broad substrate range and a high thermal stability, but is not stable under alkaline conditions and in the presence of detergents. Modification of papain with succinic anhydride shifts the pH optimum to more alkaline pH and greatly improves activity of papain in detergents, enhancing the detergent compatible protease spectrum (Khaparde and Singhal 2001).

Reduction of the positive-to-negative charged sites in α-chymotrypsin by modification with acetic or succinic anhydrides resulted in an increase of activity and stability in the ionic solvent [BMIM][Cl]. The decreased amount of positive charges reduces anion binding which is thought to have a stabilizing effect (Nordwald and Kaar 2013).

Conclusions

Nature has provided us with a myriad of highly efficient and selective enzymes. Use of these catalysts instead of the more traditional chemical ones, leads to more environmentally friendly processes, new pharmaceutical products and could allow the synthesis of novel products that are very hard or even impossible to produce with other methods. However, at this moment, only a fraction of the enzymes available to us are used for industrial purposes. One of the main reasons for this is that industrial process conditions and requirements are very different from the conditions and requirements in the host organism for which the enzymes were evolved. There is therefore a need for methodologies to adapt enzymes to the demands of the industry. In principle, two strategies can be applied, the first is incorporating modifications at the level of protein synthesis. In vivo synthesis can be steered through protein engineering, a powerful tool but can only partly help, as it is restricted to changing the amino acid sequence or incorporation of amino acid homologs. In vitro synthesis with artificial amino acids or peptide segment condensation intrinsically offers more possibilities to adapt the enzymes to the requirements (Miyazawa et al. 2002), but predicting the outcome is still rather difficult.

As shown in this review, enzymes can be modified post-production with a wide variety of both natural and synthetic compounds. This has the potential to combine the strengths and benefits of synthetic and natural molecules with the exceptional

Table 2 Effects of different types of enzyme modification as found in the literature. *Black* indicates a negative effect; *gray* a positive effect (i.e., increased temperature stability, increased pH stability, pH optimum shift towards the alkaline range, increased enzymatic activity, increased activity and stability in organic solvents, and increased resistance to chaotropic agents). *Dotted fill* indicates that no change in that property was found, *no fill* indicates that the effect is not mentioned in the article

modification class	modification moiety details	Enzyme	Temperature stability	pH stability	pH optimum shift	Activity	Solvent tolerance	Denaturation	reference
anhydride		α-amylase	gray			gray			(Khajeh et al. 2001)
		HRP	gray		dotted	gray			(Liu et al. 2002)
		Laccase	gray		gray	gray			(Xiong et al. 2011)
		Papain	gray		gray				(Sangeetha and Abraham 2006,Xue et al. 2009)
		Stem bromelain	gray		gray			gray	(Xue et al. 2010)
		Chloroperoxidase	gray			gray	gray		(Liu and Wang 2007)
		HRP	gray						(Ugarova et al. 1979,Hassani 2012a,Hassani 2012b,Ryan et al. 1994)
		Lipase					dotted		(Bianchi et al. 1993)
		Papain				gray	dotted		(Khaparde and Singhal 2001)
		Papain				gray	gray		(Szabó et al. 2009)
		HRP				gray	gray		(Song et al. 2005)
		α-Chymotrypsin				gray	gray	gray	(Kotormán et al. 2009)
Glyco conjugation	Various	Penicillin G acylase	gray	gray			gray		(Mislovicová et al. 2006)
	APA	HRP	gray	gray		black			(Gonera et al. 2004)
	CMC	Trypsin	gray	gray		black	gray	gray	(Villalonga et al. 2000)
	CMC	α-amylase	gray	gray		black		gray	(Villalonga et al. 1999)
	CMC-CD	Glucose oxidase	gray		gray	black		gray	(Matos et al. 2012)
	cyclodextran	Trypsin	gray	gray		gray			(Villalonga et al. 2003)
	cyclodextran	α-Chymotrypsin	gray			dotted			(Fernández et al. 2005)
	dextran	Papain				black			(Fukal et al. 1983)
	dextran	Lipases	gray						(De La Casa et al. 2002)
	dextran	Trypsin	gray			dotted			(Hernández et al. 2006)
	dextran	Cold adapted lipase B	gray			gray			(Siddiqui and Cavicchioli 2005)
	dextran	Trypsin	gray			gray		gray	(Fernández et al. 2002)
	Mannan	Penicillin G acylase	gray	gray		dotted			(Masárová et al. 2001)
	polymeric sucrose	Trypsin	gray			dotted			(Venkatesh et al. 2005)
	polymeric sucrose	Papain	gray			black			(Rajalakshmi and Sundaram 1995)
	polymeric sucrose	α-Chymotrypsin	gray			black			(Sundaram and Venkatesh 1998)
	polysialic acid	CuZn superoxide dismutase	gray	gray		gray		gray	(Wu et al. 2010)
deglycosylation		Acid phosphatase	black	black		dotted		black	(Barbarić et al. 1984)
		α-L-fucosidase	black			dotted			(Piesecki and Alhadeff 1992)
PEG		Trypsin	gray						(Zhang et al. 2001,He et al. 2000,Zhang et al. 1999)
		Cellulase	gray		gray				(Zhang et al. 2007)
		Trypsin	gray			gray		gray	(Gaertner and Puigserver 1992)
		Lipase	gray				gray		(Polizzi et al. 2007)
		Turnip peroxidase	gray			gray	gray		(Quintanilla-Guerrero et al. 2008)
		Protease, subtilisin, HRP	gray	gray					(Yang et al. 1996,Garcia et al. 1998)
		Cellulase	gray	gray		black			(Zhang et al. 2009)
		Penicillin G acylase	gray			black			(Kazan and Erarslan 1997)
		Lipase	gray			gray	gray	gray	(Basri et al. 1995)
		Elastase				gray			(Besson et al. 1995)
		Lipase				gray		gray	(Hernáiz et al. 1999)
		Lipase	dotted			gray	gray		(Koops et al. 1999)
		Subtilisin				gray	gray		(Kwon et al. 1999)
		HRP, chymostrypsin				black	gray		(Takahashi et al. 1984,Pina et al. 1989)
		Chymotrypsin				dotted	gray		(Matsushima et al. 1984)
		Papain				dotted	gray		(Iyer and Ananthanarayan 2008)
		Cellulase	gray	gray					(Park and Park 2001)
other polymers	PEG-MA	Cellulase	gray	gray		black			(Kajiuchi and Park 1992)
	POA-MAA	Cellulase	gray	gray		gray	gray		(Park and Kajiuchi 1995)
	POA-MA	Laccase	gray			gray			(Shin-Ya et al. 2005)
	POA-MA	Cellulase				gray			(Park et al. 2002)
	polyfenol complexation	Protease	gray						(Poh and Abdul Majid 2011)
	polyols	HRP	gray						(Hassani et al. 2006)
	PVP	Asparaginase			gray			gray	(Qian et al. 1997)

qualities of enzymes. Conjugation of an enzyme with other molecules does however not automatically combine the strengths of the two compounds. More often than not, the desired outcome is not achieved. Molecules that provide increased stability to one enzyme can have a different effect on the properties of another enzyme. To complicate matters even further, the type of molecule, site and degree of modification, and in the case of polymers, the molecular weight and amount of branching of the polymer, all have a profound influence on the resulting properties of the enzyme. There are only a few general observations that can be made from all literature at hand (Table 2). For each reported modification that resulted in an improved property, there are likely many non-reported attempts that did not result in any change, or led to a decrease of functionality. From the successful attempts, glycoconjugation often appears to result in increased temperature stability. PEGylation also regularly has this effect, next to an increased tolerance for organic solvents. In the pharmaceutical industry, PEGylation is regularly applied because it tends to lead to improved pharmacokinetic and pharmacodynamics properties of pharmaceutical products.

Variability in enzyme properties after conjugation can also occur within one experiment, as modification is usually not site-selective, resulting in several isomers. Fortunately, new modification techniques are still being developed, and the emergence of more site-selective methods could improve upon this. Over the years, the developed modification techniques have become milder, more biocompatible, and more specific. This will help unlock the full potential of post-production modification of enzymes, and aid in the increased use of enzymes in industrial applications.

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