NATUR WISSENSCHAFTEN REVIEW ARTICLES

Chemical Engineering of Enzymes: Altered Catalytic Activity, Predictable Selectivity and Exceptional Stability of the Semisynthetic Peroxidase Seleno-Subtilisin

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The increasing demand for enzymes as highly selective, mild, and environmentally benign catalysts is often limited by the lack of an enzyme with the desired catalytic activity or substrate selectivity and by their instability in biotechnological processes. The previous answers to these problems comprised genetically engineered enzymes and several classes of enzyme mimics. Here we describe the potential of chemical enzyme engineering: native enzymes can be modified by merely chemical means and basic equipment yielding so-called semisynthetic enzymes. Thus, the high substrate selectivity of the enzymatic peptide framework is combined with the catalytic versatility of a synthetic active site. We illustrate the potential of chemically engineered enzymes with the conception of the semisynthetic peroxidase seleno-subtilisin. First, the serine endoprotease subtilisin was crystallized and cross-linked with glutaraldehyde to give cross-linked enzyme crystals which were found to be insoluble in water or organic solvents and highly stable. Second, serine 221 in the active site (Enz-OH) was chemically converted into an oxidized derivative of selenocystein (Enz-SeO_2H). As a consequence, the former proteolytic enzyme gained peroxidase activity and catalyzed the selective reduction of hydroperoxides. Due to the identical binding sites of the semisynthetic peroxidase and the protease, the substrate selectivity of seleno-subtilisin was predictable in view of the well-known selectivity of subtilisin.

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Introduction

Today enzymes are widely accepted as valuable catalysts in synthetic chemistry, food chemistry, and many industrial processes (Drauz and Waldmann 1995). However, their "natural" properties such as catalytic activity, substrate selectivity, and process stability are often insufficient. Enzymes are not available for all reaction types; usually, a biocatalyst with complementary stereoselectivity is not at hand and the stability of enzymes towards organic solvents or physical influences is moderate. In the past, several methodologies have been developed to overcome these problems. Genetic engineering techniques taught us a great amount about the molecular dependence of overall stability (Damorsky 1998; Oxender and Fox 1987). However, fundamentally altered enzymatic properties such as new catalytic activities have hardly been achieved. Monoclonal catalytic antibodies have opened many opportunities in this field, but special expertise and high expenditure of energy is required to reach efficient and useful catalysts for asymmetric synthesis (Corey and Corey 1996; Wade and Scanlan 1997). Finally, efforts have been made to design synthetic enzyme mimics with desired activity and selectivity. Progress is being made continuously, but the breakthrough in terms of catalytic efficiency and enantioselectivity has not yet been achieved (Brady and Sanders 1997; Kirby 1996).

As an alternative, naturally occurring enzymes represent a valuable starting point for the development of new biocatalysts. The large number of available X-ray structures enables a rational design of enzymatic properties. Additionally, it is desirable to create new active sites which catalyze required transformations. The breakthrough in this major aspect of enzyme design was the chemical modification of enzymatic active sites yielding semisynthetic

enzymes (Kaiser and Lawrence 1984). This kind of "chemical engineering" (instead of genetic engineering) utilizes the well-known molecular framework of natural proteins for the introduction of appropriate catalytic centers, for example, cofactors, metal ions or functional groups. Although semisynthetic enzymes open unique opportunities in enzyme design, this technique has been used mainly as an analytical rather than a synthetic tool for several years. Here we summarize the development of a new generation of chemically engineered semisynthetic enzyme for organic synthesis combining altered catalytic activity, reasonable substrate selectivity and exceptional stability. We describe the way from the serine-protease subtilisin to the cross-linked crystals (CLC) of the semisynthetic peroxidase seleno-subtilisin.

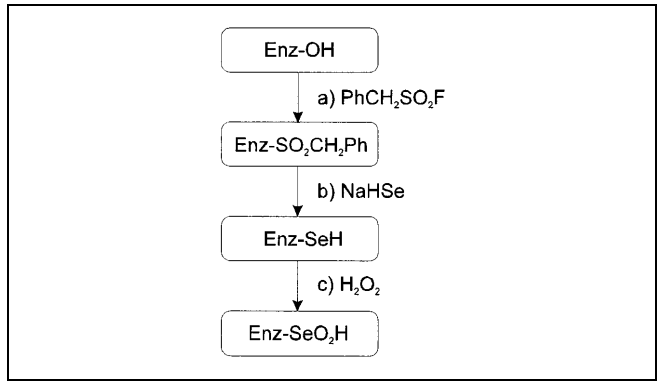


Fig. 1. Simplified and up-scaled chemical conversion of the protease subtilisin (Enz-OH) into the semisynthetic peroxidase seleno-subtilisin (Enz-SeO_2H; Häring and Schreier 1998a). *a*, 1 h at room temperature; *b*, 45 h at 40° C under argon atmosphere; *c*, 3 h at room temperature, followed by extensive dialysis

Altered Catalytic Activity

Subtilisins [3.4.21.14] are a family of closely related alkaline serine endoproteases secreted by a wide variety of *Bacillus* species. They catalyze the hydrolysis and formation of esters and amides. Especially subtilisin Carlsberg from *B. licheniformis* and subtilisin BPN' from *B. amyloliquefaciens* have been used extensively in industrial detergent formulations. Over 50 X-ray structure studies and extended substrate screenings delineate the binding of substrates and provide a sound basis for rationalizing kinetics and selectivity of subtilisin substrates (McPhalen and James 1988; Neidhardt and Petsko 1988). The specific role of individual active site residues on catalytic substrate binding, turnover, and enantioselectivity has been established (Bonneau et al. 1991; Wells et al. 1987). Almost every property has been altered by protein engineering, for example, stability at high temperature, in organic solvents or varying pH values (Takagi 1993; Wells and Estell 1988).

The protease subtilisin was converted by merely chemical means into the semisynthetic peroxidase seleno-subtilisin according to Fig. 1 (Wu and Hilvert 1990). The hydroxy group of serine 221 is the actual catalytic group of subtilisin embedded in a "catalytic triad" of asparagine 32, histidine 64, and serine 221. The close arrangement of these residues increases the acidity and nucleophilic character of serine 221 alcohol. For this reason, this residue can be specifically activated by addition of phenylmethanesulfonyl fluoride. Selenium was introduced into the active site by reaction of the activated serine 221 with sodium hydrogenselenide. In order to prepare a consistent enzyme preparation appropriate for storage, seleno-subtilisin was fully oxidized to the seleninic acid form by dialyzing against hydrogen peroxide. Excess hydrogen peroxide was removed by extensive dialysis against buffer. Finally, the aqueous solution was lyophilized yielding a white to light brown seleno-subtilisin powder. Using this simplified process the treatment of 10 g amounts of subtilisin gave seleno-subtilisin with reproducible yields of 20–25% (Häring and Schreier 1998a; Häring et al. 1998a).

As a result of introducing selenium into the active site of the former protease, the semisynthetic seleno-subtilisin catalyzes the reduction of hydroperoxides in the presence of thiols (Fig. 2; Wu and Hilvert 1990). The mechanism of the catalytic cycle is closely related to the native glutathione peroxidase, which is an important enzyme in fighting oxidative stress in mammals. X-ray studies reveal that the overall structure and substrate binding sites of subtilisin and seleno-subtilisin are identical (Syed et al. 1993).

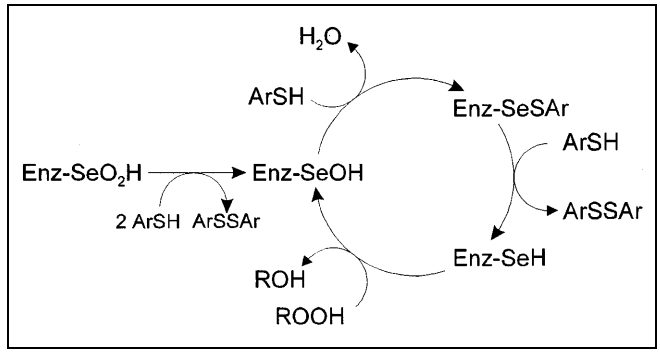
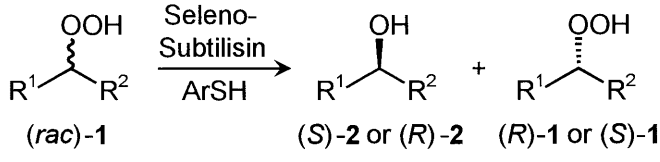


Fig. 2. Mechanism of the reduction of hydroperoxides (*ROOH*) in the presence of 5-thio-2-nitrobenzoic acid (*Ar-SH*) catalyzed by seleno-subtilisin (*Enz-SeO_2H*). (From Wu and Hilvert 1990)

Predictable Selectivity

The protease subtilisin has been used extensively in enantioselective hydrolysis and formation of esters and amides. Based on extended substrate screenings and X-ray studies, the enantioselectivity is well established and is described by a simple empirical binding model (Fig. 3; Kazlauskas and Weissfloch 1997). This detailed knowledge of subtilisin opened the unique possibility of rationalizing and even predicting the substrate selectivity of seleno-subtilisin, which has the same substrate binding pockets. This enabled a rational substrate screening for suitable peroxidase substrates featuring structural characteristics of known subtilisin substrates (Häring et al. 1997; Häring et al. 1998b).

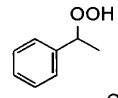
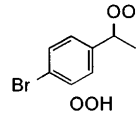
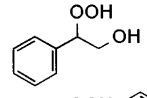
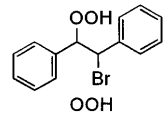
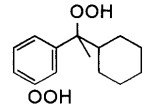
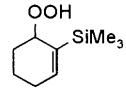
In order to evaluate the enantioselectivity of the seleno-subtilisin a series of structurally varying racemic hydroperoxides were synthesized. Theses substrates were subjected to a kinetic resolution catalyzed by the semisynthetic peroxidase according to Eq. 1 (Schüler et al. 1998):



The enantioselectivities and the catalytic efficiencies (k_{cat}/K_m) of the seleno-subtilisin catalyzed reactions are listed in Table 1. Hydroperoxides **1a–f** have similar structures reminiscent of known subtilisin substrates and fit well into the model for predicting the enantioselectivity (Fig. 3). The catalytic efficiency of the reactions is in the same order of magnitude as the native horseradish peroxidase. The reactions were up-scaled to semipreparative scale with 0.5 mmol substrate, which represented the first application of semisynthetic enzyme in asymmetric synthesis (Häring et al. 1998b).

The kinetic resolution of racemic hydroperoxides yields optically active hydroperoxides which are valuable enantioselective oxidants. They are of special interest in all reactions which were formerly performed with achiral hydroperoxides, for example, the Sharpless oxidation of allylic alcohols to epoxy alcohols (Katsuki and Sharpless 1980). The use of optically active hydroperoxides in such reactions would avoid the addition of chiral auxiliaries. In this domain the limiting point is the enantioselective production of the hydroperoxides, since only a few syntheses are available (e.g., chloroperoxidase, horseradish peroxidase, lipases; Adam et al. 1998) which yield hydroperoxides of the same absolute configuration in the cases of sterically unencumbered substrates only. Based on the model for predicting the enantioselectivity of subtilisin (Fig. 3), it was comprehensible that the semisynthetic peroxidase seleno-subtilisin yields the desired complementary absolute configuration (Häring et al. 1998b). Since the active site of seleno-subtilisin is located near the surface and is easily accessible for large substrates, even sterically demanding hydroperoxides such as **1d–f** are accepted for reduction (Schüler et al. 1998). Hence, the native and the semisynthetic peroxidases represent a valuable complementary toolbox for the synthesis of optically hydroperoxides.

Table 1. Enantioselectivity[a] and catalytic efficiency[b] (k_{cat}/K_m) of the kinetic resolution of the racemic hydroperoxides 1 catalyzed by seleno-subtilisin (Schüler et al. 1998; Häring et al. 1998b)

Hydroperoxide		Peroxide 1 ee (%)	Alcohol 2 ee (%)	k_{cat}/K_m (mM^{-1} min^{-1})
1a		52 (*R*) 46 (*R*)[c] 48 (*R*)[d]	60 (*S*) 58 (*S*)[c] 56 (*S*)[d]	135 71[c] –
1b		34 (*R*)	28 (*S*)	138
1c		99 (*S*) 94 (*S*)[c] 86 (*S*)[d]	99 (*R*) 86 (*R*)[c] 82 (*R*)[d]	1150 286[c] –
Erythro–**1d**		64[e]	90[e]	47500
1e		4[e]	4[e]	19
1f		80[e]	96[e]	97

[a] The conversion of the hydroperoxides was 50%. Analytical-scale reactions were carried out with 0.2 mM hydroperoxide 1, 0.2 mM 5-thio-2-nitrobenzoic acid, 1 mM EDTA, and 1 μM seleno-subtilisin Carlsberg in 3.0 ml of 0.1 M citric acid/NaOH buffer (pH 5.5). The stereochemical analysis was performed by multidimensional gas chromatography on chiral cyclodextrin columns (Schreier et al. 1995) and corrected for the nonenzymatic background reaction (5–15%)

[b] Measured at the fixed thiophenol concentration of 0.2 mM in 0.1 M citric acid/NaOH buffer (pH 5.5), 1 mM EDTA and 0.44 μM seleno-subtilisin Carlsberg. Initial rates were followed photometrically

[c] Seleno-subtilisin BPN'

[d] Products isolated from reactions at 0.5-mmol scale

[e] Configuration unknown

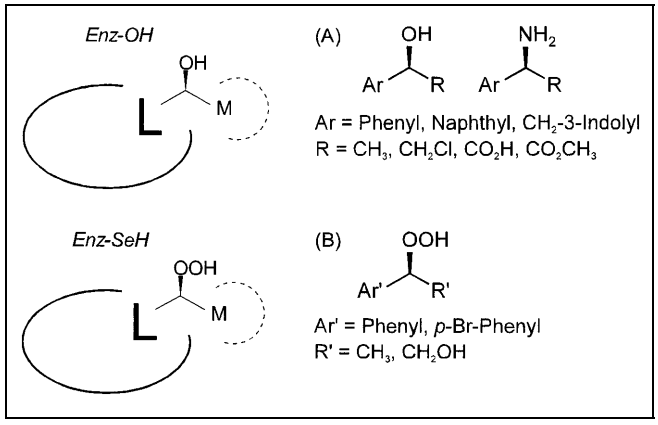


Fig. 3. Empirical model for predicting the enantioselectivity of subtilisin (*Enz-OH*; Kazlauskas and Weissfloch 1997) and seleno-subtilisin (*Enz-SeH*). *L*, Large, hydrophobic substituents; *M*, medium or polar substituents. Preferred enantiomers in *A* the subtilisin-catalyzed esterification or acylation of racemic alkyl aryl alcohols or amines, respectively, are compared to *B* the preferred enantiomers in the seleno-subtilisin-catalyzed reduction of alkyl aryl hydroperoxides

Exceptional Stability

The chemical modification of the enzymatic active site was used to alter the catalytic activity. However, there are also methods for the chemical modification of the enzymatic framework which resulted in an improved enzymatic stability. In 1964 Quiocho and Richards developed the concept of enzyme CLC. The prerequisite is a highly purified enzyme preparation which can be crystallized. In contrast to the large and difficult to obtain enzyme crystals which are necessary for X-ray studies, this method requires very small crystals (dimensions preferably below 0.1 mm). Their preparation is less difficult, and solvent or substrates can easily diffuse into the large channels and cavities of these microcrystals. In order to stabilize the crystal lattice the single enzyme molecules in a crystal are cross-linked with glutaraldehyde. Since the cross-linkage proceeds only via lysine residues on the enzyme surface, the tertiary structure is not perturbed (Fitzpatrick et al. 1994). Enzyme CLC are used for X-ray studies in organic solvents, in biotransformations, and as microporous material in chromatographic columns (Häring and Schreier 1999).

In order to demonstrate the potential of chemical enzyme engineering both the active site of subtilisin and its peptide framework were modified by simple chemical reactions (Häring and Schreier 1998b). First, subtilisin was subjected to batch crystallization yielding microcrystals (typically $100 \times 5 \times 5$ μm). Cross-linking of the crystalline structure with glutaraldehyde effected that the material was insoluble in water as well as in organic solvents. The second part of the subtilisin modification included the introduction of selenium into the crystals according to Fig. 1. The overall synthesis of CLC of the semisynthetic peroxidase seleno-subtilisin was achieved in gram scale. In contrast to other methods of enzyme development (e.g., genetic engineering or catalytic antibodies), this method needs only basic chemical equipment.

CLC seleno-subtilisin represents an immobilized biocatalyst which can easily be recycled by filtration or centrifugation (Häring and Schreier 1998b). This peroxidase catalyzed ten reaction cycles of the reduction of hydroperoxide **1c** without loss of catalytic activity or enantioselectivity (Fig. 4). The reaction was performed in preparative scale with up to 1.0 mmol hydroperoxide. The exceptional stability was confirmed by a 10-day treatment of CLC seleno-subtilisin with aqueous buffer at 60° C or with 50% acetone or dimethyl formamide at 40° C. While the non-cross-linked seleno-subtilisin lost its catalytic activity under these conditions after 2 days, the cross-linked peroxidase remained fully active for 10 days (Fig. 5).

Conclusions

In summary, we demonstrated that enzymes can be chemically engineered efficiently. The introduction

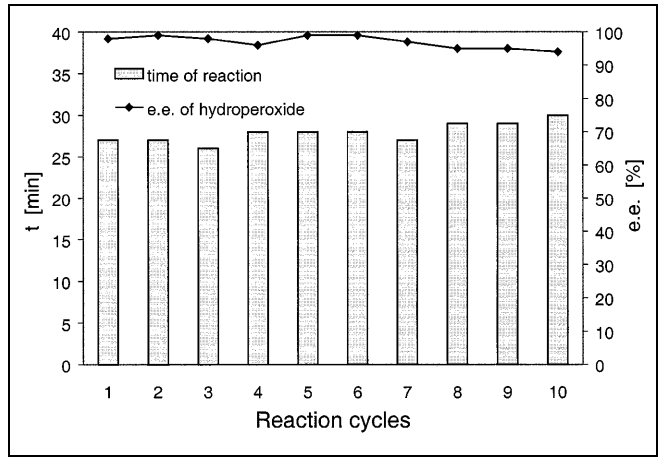


Fig. 4. Enantioselective catalysis and recycling of CLC seleno-subtilisin. In 325 ml 30 mM citric acid/NaOH buffer (pH 5.5) and 2 mM EDTA 3.8 μmol CLC seleno-subtilisin was preincubated with 0.06 mmol 2-nitro-5-thiobenzoic acid (TNB; 15 min); 0.33 mmol 2-hydroxy-1-phenylethyl hydroperoxide **1c** was added, and the reaction was started by slow addition of 0.33 mmol TNB. CLC seleno-subtilisin was recovered by centrifugation (1500 g). The hydroperoxide was extracted with diethyl ether and purified on a silica gel column. The e.e. of **1c** was determined by HPLC on a Daicel-Chiralcel OB-H

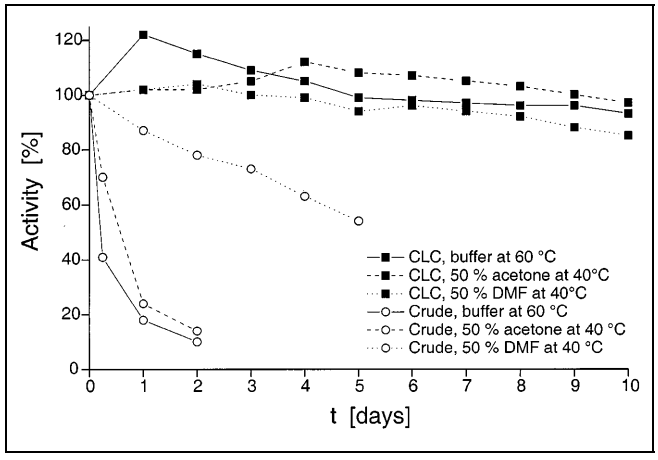


Fig. 5. Stability of amorphous and CLC of seleno-subtilisin in organic solvents or at high temperature. Peroxidase activity was measured photometrically, and the specific activities of crystalline (33 mU/mg) and amorphous (12 mU/mg) seleno-subtilisin were expressed as 100%

of a catalytically active group into a protein results in an altered catalytic activity. Utilizing a well-known protein framework, substrate selectivity may be transferred and predicted from the template to the semisynthetic enzyme. A new type of chemically engineered enzymes was designed by combining active site modification and crystal cross-linking. The advantages of semisynthetic enzymes are excellent and permit easy and reliable applications.

In recent decades several classes of enzyme mimics have been developed which attempt to reach the properties of native enzymes (Kirby 1996; Murakami et al. 1996). These studies deal with synthetic enzyme mimics, cyclodextrane or macrocycle derives systems, molecular aggregates, or catalytic antibodies. These constructs have only rarely reached the selectivity and efficiency of their native archetypes. However, semisynthetic enzymes that utilize the highly selective framework of native proteins and the catalytic power of chemically introduced groups represent a most promising concept. The next step would be to combine the chemical introduction of the new catalytic activity with site-directed mutagenesis. Thus, the points of attachment of the introduced group, the catalytic efficiency, and the substrate selectivity can be changed and improved (Kuang et al. 1997).

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 347 "Selective Reactions of Metal-Activated Molecules").

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