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Straightforward development of stereoselective biocatalysts—from detergent to semisynthetic peroxidase seleno-subtilisin

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Abstract

The industrially produced serine protease subtilisin was chemically converted into a peroxidase by selective modification of the active site serine 221 into selenocystein. The synthesis of seleno-subtilisin was up-scaled to gram-scale and the semisynthetic peroxidase utilized for the enantioselective reduction of racemic alkyl aryl hydroperoxides. The enantiomeric distribution of the products was determined (up to 98% ee) and the enantioselectivity rationalized by comparison of the hydroperoxides to corresponding subtilisin substrates. Substrate affinity of several substituted 1-arylethyl hydroperoxides to seleno-subtilisin was reasonable in comparison to corresponding aryl boronic acid inhibitors of subtilisin. Kinetic studies of the semisynthetic seleno-subtilisin revealed a catalytic efficiency comparable to native horseradish peroxidase.

Keywords: Seleno-subtilisin; Subtilisin; Semisynthetic peroxidase; Hydroperoxide

1. Introduction

The challenge of designing new biocatalysts demands rational and efficient ways to create catalysts with desired catalytic activity and predictable regio- or stereoselectivity. Artificial enzymes such as synthetic macrocyclic systems, molecular aggregates or catalytic antibodies often proved to be inferior in terms of selectivity and catalytic efficiency compared to their natural archetypes [1]. As an alternative, semisynthetic enzymes feature the optimized molecular structures of native enzymes and provide appropriate active sites [2]. The semisynthetic peroxidase seleno-subtilisin combines the framework of the protease subtilisin and the peroxidase activity of the selenium containing glutathion peroxidase. Studying the synthesis and the reactions of seleno-subtilisin, the advantages of semisynthetic enzyme's concept is demonstrated.

2. Results and discussion

Subtilisin represents the most important commercially produced enzyme with a world market of more than 500 tons year^{-1}. Its main applica-

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tion is in detergent additives or in skin and leather processing. The serine protease consists of a single polypeptide chain of about 275 amino acid residues (27.3 kDa) and has no disulfide bonds. Subtilisin was converted by a three-step protocol into the semisynthetic peroxidase seleno-subtilisin [3]. After selective activation of Ser211 with phenylmethanesulfonyl fluoride, the resulting sulfonate was substituted by hydrogen selenide under strictly oxygen-free conditions. Finally, oxidation of the selenol enzyme by hydrogen peroxide yielded the seleninic acid form of seleno-subtilisin. For an up-scaled synthesis of the semisynthetic peroxidase, several subtilisin preparations were used as starting material. For example, 200 g of the industrially produced Maxatase, an encapsulated detergent additive, was converted into seleno-subtilisin with an overall yield of 20–25% according to the procedure outlined in Fig. 1 [4].

Seleno-subtilisin is a glutathion peroxidase mimic which catalyses the enantioselective reduction of hydroperoxides [5]. Enantioselectivity of this reaction was studied by kinetic resolution of five racemic alkyl aryl hydroperoxides (Fig. 2). The analysis of chiral products by multidimensional gas chromatography (MDGC) [7] revealed enantiomeric excesses up to 98% (Table 1). Due to the identical tertiary structure of subtilisin and seleno-subtilisin, the enzymes have comparable substrate binding properties. Thus, a rational screening for suitable peroxidase substrates featuring structural characteristics of known subtilisin substrates was enabled. The high affinity of the substilisin binding pockets to aromatic residues encouraged the screening of corresponding aromatic hydroperoxides. The enantioselective recognition of (*S*)-configured alkyl aryl hydroperoxides **1a–c** by seleno-subtilisin was comprehensible by subtilisin's preference for comparable (*S*)-alkyl aryl amines or alcohols (Fig. 3) [10,11].

A detailed investigation of seleno-subtilisin's substrate affinity revealed further analogies between the semisynthetic enzyme and its template. Boronic acids are irreversible inhibitors of the serine protease subtilisin. The influence of different substituted phenyl boronic acids on their affinity to the hydrophobic S_1 binding pocket was measured by the inhibition constant

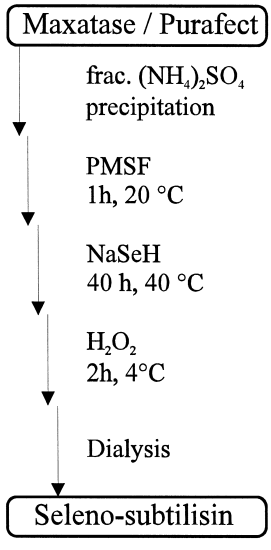

Fig. 1. Up-scaled synthesis of seleno-subtilisin. Maxatase® and Purafect® are encapsulated detergent additives and contain 7–8% (w/w) subtilisin. Sodium hydrogen selenide was freshly prepared by reduction of elementary selenium with sodium borohydride and used under strictly oxygen-free atmosphere.

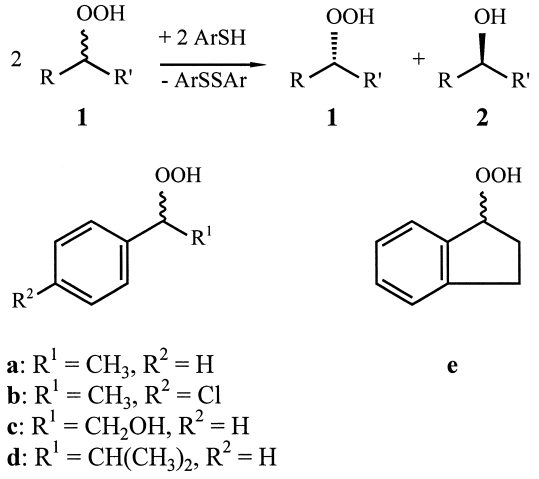

Fig. 2. Substrates for the kinetic resolution of racemic hydroperoxides. The compounds were synthesized in an acid-catalyzed perhydrolysis of corresponding alcohols or epoxides with 85% hydrogen peroxide [6].

Table 1
Enantioselectivity and kinetics of the reduction of racemic alkyl aryl hydroperoxides catalyzed by seleno-subtilisin

(*R*,*S*)-**1**	Seleno-subtilisin				HRP
	(*R*):(*S*)-**1**	(*R*):(*S*)-**2**	k_{cat} [min^{-1}]	k_{cat}/K_M [mM^{-1} min^{-1}]	k_{cat}/K_M [mM^{-1} min^{-1}]
a	76:24	20:80	2125	135	811
b	74:26	22:78	1723	287	110
c	1:99	99:1	2443	1150	39
d	30:70	71:29	1745	97	10
e	53:47	42:58	669	171	132

The apparent reversed enantioselectivity of **1c** is due to nomenclature. Reactions were performed in micro-scale according to literature [5]. Catalytic efficiency of the semisynthetic seleno-subtilisin was compared to native horseradish peroxidase HRP [8].

K_i [11]. Utilizing corresponding substituted phenyl residues of 1-phenylethyl hydroperoxides, a corresponding tendency in substrate affinity, expressed by the Michaelis–Menten constant K_M, was found (Table 2).

In artificial enzymes, unspecific substrate–catalyst interactions may result in low turnover numbers, product inhibition or even incomplete catalytic cycles [1]. However, the peptide framework of native enzymes has been optimized for millions of years and represents a most interesting building block for enzyme design. The catalytic properties of seleno-subtilisin are presented in Table 1. The comparison of the semisynthetic peroxidase to the native horseradish peroxidase demonstrated the high catalytic efficiency (k_{cat}/K_M) of seleno-subtilisin.

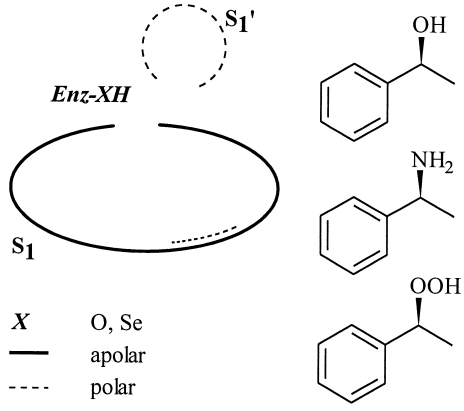

Fig. 3. Rationalizing the enantioselectivity of seleno-subtilisin. Due to the identical ternary structure of subtilisin and seleno-subtilisin [9], the enzymes prefer comparable enantiomers in esterification of alcohols [10], acylation of amines [11] and reduction of hydroperoxides.

Table 2
Rationalizing the substrate affinity of seleno-subtilisin by comparison of corresponding boronic acid inhibitors of subtilisin [12]

R: $B(OH)_2$ K_i [μM]	Inhibitor/ hydroperoxide	R: $CH(OOH)CH_3$ K_M [mM]
180	*p*-MeO-C_6H_4-R	30
100	C_6H_5-R	16
56	*p*-Cl-C_6H_4-R	6
23	*p*-Br-C_6H_4-R	4
7	*m*-Cl-C_6H_4-R	1.5

Michaelis–Menten constants K_M of hydroperoxides revealed a similar dependence on phenyl substituents like inhibition constant K_i of aryl boronic acids.

3. Conclusions

Seleno-subtilisin is the most promising example for a semisynthetic enzyme. For the first time,

– a semisynthetic enzyme is available in gram-scale;

– the enantioselectivity of a semisynthetic enzyme was rationalized based on its template;

– a semisynthetic enzyme revealed catalytic efficiency comparable to a native enzyme.

The capability of semisynthetic enzyme's concept was confirmed by seleno-subtilisin: Featuring established models for substrate binding of native enzymes and providing custom-designed synthetic catalytic activity, rational conclusions and predictions for tomorrow's stereoselective biocatalysts are feasible.

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References

[1] A.J. Kirby, Angew. Chem., Int. Ed. 35 (1996) 705.
[2] E.T. Kaiser, D.S. Lawrence, Science 226 (1984) 505.
[3] I.M. Bell, M.L. Fisher, Z.P. Wu, D. Hilvert, Biochemistry 32 (1993) 3754.
[4] D. Häring, B. Hubert, E. Schüler, P. Schreier, From detergent additive to semisynthetic peroxidase—simplified and up-scaled synthesis of seleno-subtilisin, Biotech. Bioeng. (1998) in press.
[5] D. Häring, M. Herderich, E. Schüler, B. Withopf, P. Schreier, Tetrahedron Asymmetry 8 (1997) 853.
[6] A.G. Davies, A.V. Foster, A.M. White, J. Chem. Soc. (1953) 1541.
[7] D. Häring, T. König, B. Withopf, M. Herderich, P. Schreier, J. High Resolut. Chromatogr. 20 (1997) 351, and cited literature therein.
[8] W. Adam, U. Hoch, M. Lazarus, C.R. Saha-Möller, P. Schreier, J. Am. Chem. Soc. 117 (1995) 11898.
[9] R. Syed, Z.-P. Wu, J.M. Hogle, D. Hilvert, Biochemistry 32 (1993) 66157.
[10] P.A. Fitzpatrick, A.M. Klibanov, J. Am. Chem. Soc. 113 (1991) 3166.
[11] H. Kitaguchi, P.A. Fitzpatrick, J.E. Huber, A.M. Klibanov, J. Am. Chem. Soc. 111 (1989) 3094.
[12] P. Seufer-Wasserthal, V. Martichonok, T.H. Keller, B. Chin, R. Martin, J.B. Jones, Bioorg. Med. Chem. 2 (1994) 35.