# Semisynthetic Enzymes in Asymmetric Synthesis: Enantioselective Reduction of Racemic Hydroperoxides Catalyzed by Seleno-Subtilisin†

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The serine protease subtilisin was chemically converted into the peroxidase-active seleno-subtilisin. This semisynthetic enzyme catalyzes the enantioselective reduction of racemic hydroperoxides in the presence of thiophenols to yield optically active hydroperoxides and alcohols on the semipreparative scale. The kinetic parameters and enantioselectivities of seleno-subtilisin-catalyzed reduction of various chiral hydroperoxides were determined. The catalytic efficiency of this semisynthetic enzyme is comparable to that of the native horseradish peroxidase. The sense in the enantioselectivity of the seleno-subtilisin is opposite to the natural enzymes previously used in the synthesis of optically active hydroperoxides. Consequently, the semisynthetic enzyme seleno-subtilisin complements the naturally available peroxidases for the asymmetric synthesis of both enantiomers.

## Introduction

Enantiomerically pure hydroperoxides have a high potential as chiral oxidants;[1] however, their general and convenient preparation still demands intensive research efforts.[2,3] The enantioselective synthesis by purely chemical means has so far been restricted to sugar[1a] and furyl hydroperoxides.[1d] Best results have been achieved by enzymatic methods with lipase,[2a] lipoxygenase,[2b] chloroperoxidase (CPO),[2c] and horseradish peroxidase (HRP).[3] Especially our efficient kinetic resolution with HRP has made available a broad structural variety of optically active hydroperoxides. Despite the success, all these enzymes prefer the same absolute configuration of the chiral hydroperoxides, and their application is limited to sterically unencumbered substrates.

As a challenging proposition to overcome these shortcomings, we focused our interest on semisynthetic enzymes with designed properties. In this strategy of biocatalyst design a known enzyme is chemically modified at a specific site to introduce the desired catalytic activity with conservation of the selectivity of the peptide framework.[4] To date, the preparative application of semisynthetic enzymes in asymmetric catalysis has been limited to only few examples, either at the lower micromolar scale or under noncatalytic conditions.[5]

Previously, it has been reported[6] that the specific chemical modification of serine 221 into selenocystein at the active site of subtilisin imparts this protease with seleno-peroxidase activity. The semisynthetic peroxidase seleno-subtilisin with the peptide framework of the serine protease subtilisin catalyzes the reduction of hydrogen peroxide, *tert*-butyl hydroperoxide, and cumol hydroperoxide in the presence of 5-mercapto-2-nitrobenzoic acid (Figure 1).[6] Our preliminary study[7a] with chiral organic hydroperoxides revealed that seleno-subtilisin reduces racemic hydroperoxides enantioselectively and established this semisynthetic enzyme as the first one with catalytic efficiency and enantioselectivity comparable to the native enzymes. From the well-known regio- and stereoselectivity of the subtilisin template,[8] the affinity and enantioselectivity of seleno-subtilisin for chiral hydroperoxides may be rationalized and even predicted.[7b]

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† Dedicated to Professor C. -H. Brieskorn on the occasion of his 85th birthday.

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§ Institute of Organic Chemistry.

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(4) Kaiser, E. T. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 913.

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(8) (a) Takagi, H. *Int. J. Biochem.* **1993**, *25*, 307. (b) Kazlauskas, R. J.; Weissfloch, A. N. E. *J. Mol. Catal. B: Enzymol.* **1997**, *3*, 65. (c) Moree, W. J.; Sears, P.; Kawashiro, K.; Witte, K.; Wong, C.-H. *J. Am. Chem. Soc.* **1997**, *119*, 3942. (d) Fitzpatrick, P. A.; Klibanov, A. M. *J. Am. Chem. Soc.* **1991**, *113*, 3166.

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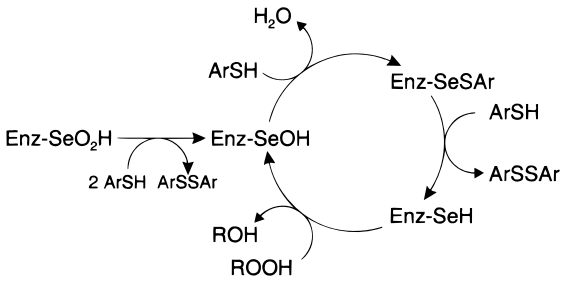
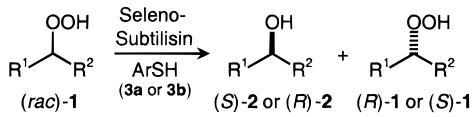



**Figure 1.** Proposed mechanism of the seleno-subtilisin-catalyzed reduction of hydroperoxides (ROOH) in the presence of thiophenols (ArSH; refs 6, 10).

**Scheme 1**




The essential prerequisite for the preparative application of seleno-subtilisin was its convenient and upscaled synthesis, which was the first preparation in gram-scale of a semisynthetic enzyme.[7c]

In the present work, we have investigated in detail the catalytic efficiency and the enantioselectivity of seleno-subtilisin. The relevance of this semisynthetic peroxidase for asymmetric synthesis has been demonstrated on a structurally varied set of organic hydroperoxides.

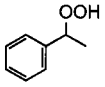
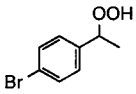
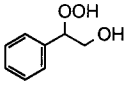
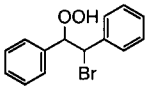
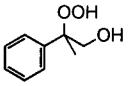
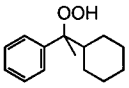
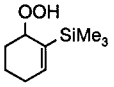
## Results and Discussion

The seleno-subtilisin, a glutathione peroxidase mimic, catalyzes the reduction of chiral organic hydroperoxides in the presence of thiophenols under kinetic resolution (Scheme 1). To assess the catalytic efficiency of this enzymatic transformation, the kinetic parameters $K_m$ (Michaelis affinity constant) and $k_{cat}$ (turnover number) were determined for the reduction of various hydroperoxides **1** in the presence of 5-mercapto-2-nitrobenzoic acid (TNB, **3a**). As the data in Table 1 show, both the $K_m$ and $k_{cat}$ values depend strongly on the steric and electronic effects of the substituents. For example, substrates with both a hydrophobic and a polar residue in the hydroperoxide display increased affinity to seleno-subtilisin [compare the $K_m$ values for **1a** and **1c** or for **1e** and cumol hydroperoxide ($K_m$ > 100 mM[6b])].

Sterically encumbered hydroperoxides are poor substrates for native peroxidases. Since the active site of seleno-subtilisin is located in a groove on the surface of the former endo-protease, it is more readily accessible for large substrates.[8b,c] This was demonstrated by the kinetic parameters of the sterically encumbered hydroperoxides **1d**−**f** and **1h**; especially the α-bromo hydroperoxide **1d** revealed an excellent $k_{cat}$ value. In general, the turnover numbers and the catalytic efficiency ($k_{cat}/K_m$) of the semisynthetic peroxidase seleno-subtilisin are similar to those of the native horseradish peroxidase (for HRP kinetics cf. ref 3b).

In addition to the above-discussed seleno-subtilisin derived from the subtilisin Carlsberg variant (from *Bacillus licheniformis*), the catalytic potential of the subtilisin BPN′ variant (from *B. amyloliquefaciens*) as peroxidase template was studied. As exemplified with the hydroperoxides **1a** and **1c**, the affinity constants were similar for both seleno-subtilisin variants (Table 1). Since the decrease in the $k_{cat}$ values was significant, the seleno-subtilisin BPN′ was not further employed for synthetic applications.

**Table 1. Kinetic Parameters for Seleno-Subtilisin-Catalyzed Kinetic Resolution of Hydroperoxides 1 in the Presence of 5-Mercapto-2-nitrobenzoic Acid (3a)[a]**

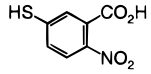
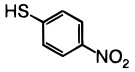
| hydroperoxide | | $K_m$ [mM] | $k_{cat}$ [$min^{-1}$] | $k_{cat}/K_m$ [$mM^{-1}$ $min^{-1}$] |
|---|---|---|---|---|
| **1a** |  | 15.7<br>14.3[b] | 2125<br>1020[b] | 135<br>71[b] |
| **1b** |  | 4.3 | 592 | 138 |
| **1c** |  | 2.1<br>3.0[b] | 2443<br>840[b] | 1150<br>280[b] |
| *erythro*-**1d** |  | 0.07 | 3322 | 47500 |
| **1e** |  | 9.3 | 905 | 96 |
| **1f** |  | 1.8 | 33 | 19 |
| **1g** |  | 29.1 | 981 | 34 |
| **1h** |  | 8.5 | 820 | 97 |
| *threo*-**1i** |  | 5.3 | 449 | 84 |
| *threo*-**1j** |  | 12.2 | 643 | 53 |

[a] Measured at the fixed thiophenol **3a** 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 by the consumption of **3a**. [b] Seleno-subtilisin BPN′.

Beside screening numerous chiral hydroperoxides for seleno-subtilisin catalysis, we tested chiral thiols with respect to their substrate acceptance. Neither 1-phenylethanethiol nor 1-indanethiol were oxidized by seleno-subtilisin. However, the thiophenol **3a** may be replaced by the commercially available 4-nitrothiophenol (**3b**) without loss of enantioselectivity in the reduction of the hydroperoxides. Comparison of the kinetic data reveals that thiophenol **3b** displays even a higher catalytic efficiency than **3a** (Table 2). Unfortunately, the application of **3b** for synthetic purposes is limited in view of its cumbersome workup (**3b** is soluble in aqueous and organic solvents and difficult to separate).

Once the kinetic parameters of seleno-subtilisin had been determined, the enantioselectivity of the semisynthetic peroxidase for various hydroperoxides was evaluated. In Table 3, the reaction times and the enantiose-

**Table 2. Kinetic Parameters for Seleno-Subtilisin-Catalyzed Oxidation of Thiophenols 3 in the Presence of 1-Phenylethyl Hydroperoxide (1a)[a]**

| | Thiophenol | $K_m$ [mM] | $k_{cat}$ [$min^{-1}$] | $k_{cat}/K_m$ [$mM^{-1}\ min^{-1}$] |
|---|---|---|---|---|
| **3a** |  | 0.031 | 282 | 9030 |
| **3b** |  | 0.011 | 510 | 47700 |

[a] Measured at the fixed concentration of 5.0 mM 1-phenylethyl hydroperoxide (**1a**) in 2.0 mL 0.1 M citric acid/NaOH buffer (pH 5.5), 1 mM EDTA, and 0.44 $\mu$M seleno-subtilisin Carlsberg. Initial rates were determined photometrically by following the consumption of thiophenol **3a** ($\epsilon_{410}$ = 12600 $M^{-1}\ cm^{-1}$) at 410 nm or **3b** ($\epsilon_{410}$ = 13830 $M^{-1}\ cm^{-1}$) at 410 nm and 20 °C.

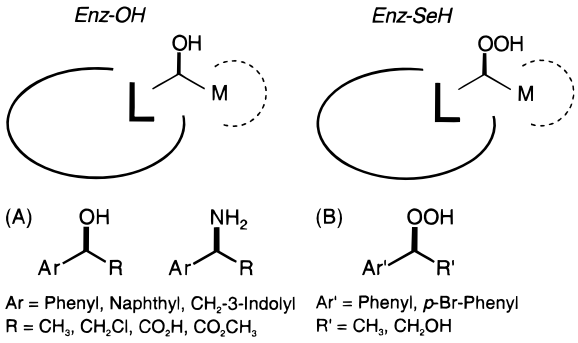


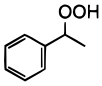
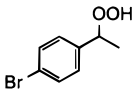
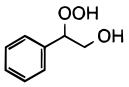
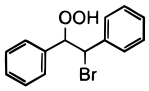
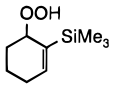
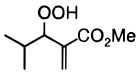

**Figure 2.** Empirical rule that predicts the enantiopreference of subtilisin (Enz-OH) and seleno-subtilisin (Enz-SeH).[8b,d] L: Large, hydrophobic residues; M: medium or polar groups. Some examples of preferred enantiomers in (A) the subtilisin-catalyzed esterification or acylation of racemic alkyl aryl alcohols or amines, respectively, are compared to (B) the preferred enantiomers in the seleno-subtilisin-catalyzed reduction of alkyl aryl hydroperoxides.

lectivities of the kinetic resolution of racemic hydroperoxides **1a**−**d**,**h**−**k** are listed. The screening of suitable hydroperoxide substrates was performed on the analytical scale (0.6 $\mu$mol) in order to minimize consumption of chemicals and time. Nevertheless, the seleno-subtilisin-catalyzed biotransformations may be upscaled to 0.5 mmol, as exemplified for hydroperoxides **1a** and **1c**. The enantiomeric distribution of the hydroperoxides was determined by HPLC analysis on chiral phases for the semipreparative transformations or, after reduction with triphenylphosphine to the corresponding alcohols, by multidimensional gas chromatography (MDGC) on chiral cyclodextrin columns[9] for the analytical transformations.

The enantioselectivity of the protease subtilisin has already been studied in detail.[8b] Figure 2 shows an empirical binding model and some examples of the preferred enantiomers in the transesterification of alkyl aryl alcohols or in the acylation of alkyl arylamines. The enantioselectivity reached frequently up to 99% ee depending on the substrate geometry and the solvent. Since the enzymatic backbone of the subtilisin and the semisynthetic seleno-subtilisin are identical,[10] the enantioselective substrate recognition is comparable in both enzymes. Accordingly, the structurally related enantiomers

(9) Schreier, P.; Bernreuther, A.; Huffer, M. *Analysis of Chiral Organic Molecules*; W. de Gruyter: Berlin, 1995.

**Table 3. Enantioselectivities of the Kinetic Resolution of the Racemic Hydroperoxides 1 Catalyzed by Seleno-Subtilisin[a]**

| hydroperoxide | | time[b] [min] | peroxide **1** ee [%] | alcohol **2** ee [%] |
|---|---|---|---|---|
| **1a** |  | 12<br>16<br>36 | 52 (*R*)<br>46 (*R*)[c]<br>48 (*R*)[d] | 60 (*S*)<br>58 (*S*)[c]<br>56 (*S*)[d] |
| **1b** |  | 8 | 34 (*R*) | 28 (*S*) |
| **1c** |  | 3<br>7<br>22 | 99 (*S*)<br>94 (*S*)[c]<br>86 (*S*)[d] | 99 (*R*)<br>86 (*R*)[c]<br>82 (*R*)[d] |
| *erythro*-**1d** |  | 0.4 | 64[e] | 90[e] |
| **1h** |  | 13 | 80[e] | 96[e] |
| *threo*-**1i** |  | 9 | 14 (*S*,*S*) | 30 (*R*,*R*) |
| *threo*-**1j** |  | 16 | 22 (*R*,*R*) | 38 (*S*,*S*) |
| **1k** |  | 13 | 60[e] | 44[e] |

[a] The conversion of the hydroperoxides was 50%. Analytical-scale reactions were carried out with 0.2 mM hydroperoxide **1**, 0.2 mM thiophenol **3a**, 1 mM EDTA, and 1 $\mu$M seleno-subtilisin Carlsberg in 3.0 mL of 0.1 M citric acid/NaOH buffer (pH 5.5). The products were extracted with ethyl ether, and the alcohol **2** was separated from the hydroperoxide **1** by TLC on silica gel (pentane/ethyl ether). The hydroperoxides were reduced to the corresponding alcohols by triphenylphosphine in ethyl ether (10 min at room temperature) followed by TLC to separate the alcohol from the excess of $PPh_3$. The stereochemical analysis of the alcohols was performed by multidimensional gas chromatography on chiral cyclodextrin columns[9] and corrected for the nonenzymatic background reaction (5−15%). [b] Reaction time for 50% conversion of the hydroperoxide. [c] For seleno-subtilisin BPN′ instead of seleno-subtilisin Carlsberg. [d] Products isolated from reactions at 0.5-mmol scale (for details cf. Experimental Section). [e] Configuration unknown.

of the hydroperoxides **1a**−**c** were also reduced preferably by seleno-subtilisin, as confirmed by the enantioselectivities in Table 3. The enantioselectivity of the seleno-subtilisin Carlsberg and its BPN′ variant are comparable, as shown by the hydroperoxides **1a** and **1c**. This is reasonable by the similar geometry of the subtrate binding sites in both enzyme variants.

Although the tertiary hydroperoxides **1e**,**f** were accepted by seleno-subtilisin (cf. turnover numbers and $K_m$ values in Table 1), they were reduced unselectively (not included in Table 3). Unfavorable steric interactions of these tertiary hydroperoxides prevent enantiomeric differentiation. Also the 3-cyclohexenyl hydroperoxide (**1g**) was reduced by seleno-subtilisin, but unselectively (not included in Table 3). Evidently, for this secondary hy-

droperoxide the two substituents (ethano vs etheno) are sterically too similar to provide enantiomeric discrimination.

For preparative purposes more significant, the sense of the enantioselectivity of seleno-subtilisin for all hydroperoxides in Table 3 is opposite to that exhibited in the reduction catalyzed by the native peroxidases HRP[3] or CPO[2c] and in the lipase-catalyzed acylation of hydroperoxides.[2a] Thus, for the first time both enantiomers of a particular chiral secondary hydroperoxide are in principle accessible in optically active form.

The enantioselectivity of the seleno-subtilisin-catalyzed reduction of the racemic hydroperoxides **1** is strongly influenced by the structure of the substrate. In case of the alkyl aryl hydroperoxides **1a**−**c**, the highest selectivity was observed for the α-hydroxy derivative **1c**. Its composition of functional groups—a hydrophobic phenyl residue and a polar hydroxy function—provides an optimal fit at the active site and hence a high enantioselectivity. The α-bromo hydroperoxide **1d** constitutes a similar substrate, and good enantioselectivity was observed despite its sterically encumbered structure. Although the aliphatic hydroperoxides **1i**,**j** have a α-hydroxy group, as polar functionality, they lack a large hydrophobic phenyl group, and consequently a lower enantioselectivity ensues.

In summary, seleno-subtilisin catalyzes the enantioselective reduction of a wide variety of functionalized racemic hydroperoxides. The kinetic data reveal that this semisynthetic peroxidase is in its catalytic efficiency comparable to the HRP and CPO. For preparative purposes advantageous, the seleno-subtilisin exhibits the opposite sense in the enantioselectivity compared to the native peroxidases. Contrary to native enzymes, the substrate affinity and selectivity of seleno-subtilisin may be inferred from the well-known active-site structure of the protease subtilisin. Thus, we have demonstrated for the first time that semisynthetic enzymes may complement optimally the set of naturally available biocatalysts for enantioselective synthesis. Future biocatalytic transformations with seleno-subtilisin are promoted by the highly stable and reusable cross-linked microcrystals of this semisynthetic peroxidase.[11]

## Experimental Section

**Materials and Methods.** Seleno-subtilisin Carlsberg was synthesized in 5-g quantities according to a simplified and upscaled three-step sequence.[7c] The source of the subtilisin Carlsberg preparation (Subtilisin A, Novo Nordisk, Denmark; Chirazyme P-1, Boehringer Mannheim, Germany, or Maxatase P440.000, Genencor Int., The Netherlands) had no influence on the enantioselectivity. Seleno-subtilisin BPN′ was prepared from subtilisin BPN′ (protease type XXVII, Sigma) in 0.5-g amounts.[7c] Hydroperoxides **1** are known in optically active form, and the racemates were prepared according to literature.[3,12] 5-Mercapto-2-nitrobenzoic acid (**3a**) was obtained by reduction of the corresponding disulfide with 2-mercaptoethanol.[13] Enantiomer distribution of the alcohols was determined by a multidimensional HRGC, as reported previously.[7b,12f]

**Determination of Kinetic Parameters.** Peroxidase kinetics were measured photometrically by monitoring the decrease of thiophenol **3a** concentration ($\epsilon_{410}$ = 12600 $M^{-1}$ $cm^{-1}$; pH 5.5) at 25 °C. The reaction was performed with 0.2 mM **3a**, 1 mM EDTA, 0.44 μM seleno-subtilisin and varying hydroperoxide concentrations in 0.1 M citric acid/NaOH buffer (pH 5.5) at a total volume of 2.0 mL. Fresh stock solutions of hydroperoxide in buffer (100 mM) and **3a** in ethanol (20 mM) were prepared immediately before the experiments. Prior to reaction, the seleninic acid form of seleno-subtilisin was incubated 15 min with thiophenol **3a** in buffer to produce the catalytically active selenol in situ. The reactions were immediately initiated by addition of the hydroperoxide **1**. Each reaction was repeated without enzyme, and the enzymatic reaction was subsequently corrected for the nonenzymatic background reduction of the hydroperoxide by the thiophenol. The $K_m$ and $k_{cat}$ values were calculated from the initial velocities with the program DNRPEASY 3.55.[14]

**General Procedure for the Semipreparative Kinetic Resolution of Hydroperoxides with Seleno-Subtilisin.** Prior to reaction, seleno-subtilisin Carlsberg (5 μmol) was preincubated for 15 min in 500 mL 50 mM citric acid/NaOH buffer (pH 5.5) which contained 2 mM EDTA and 150 μM thiophenol **3a**.[15] Subsequently, 0.5 mmol of racemic hydroperoxide **1** was added and the reaction started by slow addition of equimolar amounts of **3a** (dissolved in 5 mL of ethanol). The mixture was extracted with ethyl ether (5 × 100 mL) and dried over $Na_2SO_4$. The solvent was removed at 25 °C and 500 mbar (hydroperoxides should not be heated above 30 °C!), and the products were isolated by silica gel chromatography (pentane/ethyl ether) to yield 40−43% each of the hydroperoxide and alcohol. The enantiomeric excess of the hydroperoxides **1** and the alcohols **2** was determined by HPLC analysis on a Chiralcel OD-H or OB-H column (9:1 isohexane−isopropyl alcohol; 0.6 mL/min flow rate).

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