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# Efficient and enantioselective catalysis by the semisynthetic peroxidase seleno-subtilisin

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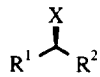
***Abstract:*** By selective modification of serine 221 in the active site, the preceding protease subtilisin carlsberg gained peroxidase activity which was subsequently utilized for the enantioselective reduction of hydroperoxides. Thus, semisynthetic seleno-subtilisin selectively catalyzed the kinetic resolution of four racemic alkyl aryl hydroperoxides *p*-$R^2$-$C_6H_4$-CH(OOH)$R_1$ ($R^1$=$CH_3$, $CH_2OH$, $CH(CH_3)_2$; $R^2$=H, Cl). The enantiomeric distribution of all products was determined and kinetic parameters were evaluated, indicating seleno-subtilisin as the first semisynthetic enzyme with catalytic efficiency comparable to native enzymes and good enantioselectivity. In order to facilitate further studies, structural motifs responsible for enantioselectivity and efficiency of catalysis were outlined in the discussion, thus providing a rational basis for the selection of suitable substrates. © 1997 Elsevier Science Ltd. All rights reserved.

Catalysis by artificial enzymes is one of the most challenging fields in bioorganic chemistry. Recent research has been focused on the application of various synthetic macrocyclic systems, molecular aggregates or catalytic antibodies[1]. However, as a distinct three-dimensional substrate binding site is prerequisite for selective transformation, the above mentioned man-made catalysts often proved to be inferior in terms of selectivity and catalytic efficiency compared to their natural archetypes[1].

As an alternative, biocatalysts obtained by site-directed mutagenesis[2] as well as semisynthetic enzymes[3] feature the optimized molecular structures of native enzymes and provide appropriate active sites. In the present work we introduce a concept based on application of the semisynthetic peroxidase seleno-subtilisin[4]. Demonstrating the potential of chemically-modified enzymes for asymmetric synthesis, seleno-subtilisin catalyzed the kinetic resolution of four racemic alkyl aryl hydroperoxides with an efficiency comparable to native peroxidases and an enantioselectivity assessable from known subtilisin substrates.

Enantiomerically enriched hydroperoxides are both potential stereoselective oxidants and attractive chiral building blocks difficult to obtain merely by organic synthesis[5]. In contrast, the recently established methods based on enzyme-catalyzed kinetic resolution of racemic hydroperoxides applying lipase[6], chloroperoxidase[7] and horseradish peroxidase[8] are restricted to sterically unhindered substrates and exclusively yielded the (*S*)-configured alkyl aryl hydroperoxides **1**.


(*S*)-**1**




(*S*)-**2**

$R^1$ = Phenyl, Naphthyl, 3-Indolyl, Cyclohexyl; $R^2$ = Me, Et; X = OH, $NH_2$

The peroxidase seleno-subtilisin was obtained by a three step protocol[9] from subtilisin [EC 3.4.21.62], a serine protease which is produced on an industrial scale. The native enzyme subtilisin accepts large substrates, e.g. proteins, and shows high enantioselectivity for (*S*)-configured alkyl aryl amines or alcohols **2** in acylation or esterification, respectively[10]. Extended studies on structure and binding sites of subtilisin opened unique possibilities to rationalize substrate recognition and selectivity

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**Table 1.** Enantiomeric distribution of the products from the kinetic resolution of racemic hydroperoxides (*RS*)-**3** catalyzed by seleno-subtilisin[11,13–15]

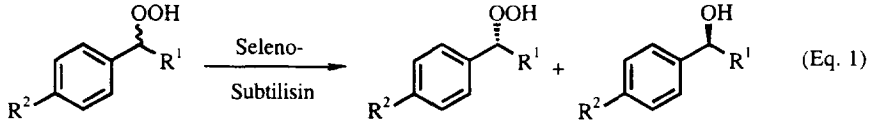
| (*RS*)-**3** | t [min] | (*R*) : (*S*)-**3** | (*R*) : (*S*)-**4** |
|---|---|---|---|
| **a** | 12 | 76 : 24 | 20 : 80 |
| **b** | 18 | 74 : 26 | 22 : 78 |
| **c** | 3 | 1 : 99 | 99 : 1 |
| **d** | 7 | 30 : 70 | 71 : 29 |

**Table 2.** Kinetic data of seleno-subtilisin[a]. for data with native horseradish peroxidase (HRP) cf. lit.[8]

| (*R*,*S*)-**3** | Seleno-Subtilisin | | | HRP |
|---|---|---|---|---|
| | $K_M$ [mM] | $k_{cat}$ [$min^{-1}$] | $k_{cat}/K_M$ [$mM^{-1}$ $min^{-1}$] | $k_{cat}/K_M$ [$mM^{-1}$ $min^{-1}$] |
| **a** | 15.7 | 2125 | 135 | 811 |
| **b** | 6.0 | 1723 | 287 | 110 |
| **c** | 2.1 | 2443 | 1150 | 39 |
| **d** | 18.0 | 1745 | 97 | 10 |

[a] Kinetic data were measured at 0.2 mM 5-thio-2-nitrobenzoic acid, 1 mM EDTA and 0.44 μM seleno-subtilisin in 0.1 M citric acid/NaOH buffer (pH=5.5; 25 °C) and followed photometrically at 410 nm ($\varepsilon_{TNB}$=12600 $M^{-1}cm^{-1}$). $K_M$ and $k_{cat}$ were calculated from initial velocities by the program DNRPEASY 3.55.

of the corresponding seleno-modified peroxidase. Hence we investigated the seleno-subtilisin catalyzed reduction of racemic hydroperoxides **3a–d** (Eq. 1) featuring structural motifs comparable to known subtilisin substrates. The enantiomeric ratios of the products **3** and **4** are summarized in Table 1. All alkyl aryl hydroperoxides showed an enrichment of the enantiomers which have not been accessible to date.




(Eq. 1)

(*RS*)-**3** (*R*)-**3** or (*S*)-**3** (*S*)-**4** or (*R*)-**4**

**a**: $R^1$ = Me, $R^2$ = H **c**: $R^1$ = $CH_2OH$, $R^2$ = H
**b**: $R^1$ = Me, $R^2$ = Cl **d**: $R^1$ = $CH(CH_3)_2$, $R^2$ = H

In artificial enzymes unspecific substrate–catalyst interactions may result in low turnover numbers and incomplete catalytic cycles[1], demonstrating the superiority of native enzymes, which have been optimized by natural selection processes lasting for millions of years. Thus, the catalytic properties of semisynthetic seleno-subtilisin were revealed by kinetic studies. For substrates **3a–d** individual catalytic efficiencies ($k_{cat}/K_M$) were in same order of magnitude as values obtained by reduction with native horseradish peroxidase (Table 2) proving the catalytic potential of this semisynthetic enzyme.

Encouraged by the well-known substrate–catalyst interactions of subtilisin, interpretation of seleno-subtilisin's kinetics and selectivity led to the identification of structural motifs providing a rational basis for the selection of suitable substrates: A large hydrophobic binding pocket $S_1$ (Leu126, Gly127, Ala152, Gly154, Asn155)[12] and a smaller, more polar binding pocket $S_1'$ (Tyr217, His64, Asn155,

Asn218)[12a] are located near the active site. This arrangement apparently counts responsible for both the high enantioselectivity and affinity (as deduced from the $K_M$ value) of **3c**. Introduction of an isopropyl group in **3d** led to an inversion of enantioselectivity and deterioration of the kinetic data because occupation of $S_1'$ with a sterically encumbered isopropyl substituent was difficult[10b]. The affinity and efficiency of *para*-chlorine substituted **3b** was doubled in comparison to **3a** due to a polar region on the bottom of $S_1$ (peptide bonds of Tyr167, Ala129, Gly128)[12], which facilitated an improved alignment of this residue.

As conclusion, we demonstrated a first promising application of semisynthetic enzymes in asymmetric catalysis. This concept utilizes well defined apo-enzymes as molecular framework, thus minimizing the synthetic work required for obtaining the custom-designed catalyst. In addition, established models of the relevant substrate binding sites facilitate identification of structural motifs required for efficiency and enantioselectivity of catalysis.

## Acknowledgements

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11. Seleno-subtilisin was synthesized according to lit.[13] from subtilisin carlsberg (Sigma protease type VIII) in 100 mg scale and its concentration was measured by active site titration[13]. Hydroperoxides **3a–d** were synthesized with 85% $H_2O_2$[14](CAUTION: Precautions against explosion must be taken). Kinetic resolution of racemic **3** was accomplished in 3.0 ml 0.1 M citric acid/NaOH buffer (pH 5.5) with 0.2 mM hydroperoxide, 0.2 mM 5-thio-2-nitrobenzoic acid, 1 mM EDTA and 1 μM seleno-subtilisin. Reaction progress was followed at 410 nm and non-enzymatic background reaction (10–15%) was subtracted. The conversion of the hydroperoxides was 50%. Extraction with diethyl ether stopped the reaction and alcohol was separated from hydroperoxide by preparative TLC on silica gel (diethyl ether/pentane 4/1). Hydroperoxides were reduced to the corresponding alcohols by $PPh_3$ in diethyl ether. Analysis of chiral alcohols was achieved by multidimensional HRGC on cyclodextrin phases[15]. Absolute configurations were determined in comparison to

reference compounds synthesized by HRP-catalyzed reactions[8]. The enantiomeric ratios were corrected by the amount of background reaction uncovering pure enzymatic selectivity.

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