

Functional Mimicry of the Active Site of Glutathione Peroxidase by Glutathione Imprinted Selenium-Containing Protein

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For imitating the active site of antioxidant selenoenzyme glutathione peroxidase (GPx), an artificial enzyme selenosubtilisin was employed as a scaffold for reconstructing substrate glutathione (GSH) specific binding sites by a bioimprinting strategy. GSH was first covalently linked to selenosubtilisin to form a covalent complex GSH–selenosubtilisin through a Se–S bond, then the GSH molecule was used as a template to cast a complementary binding site for substrate GSH recognition. The bioimprinting procedure consists of unfolding the conformation of selenosubtilisin and fixing the new conformation of the complex GSH–selenosubtilisin. Thus a new specificity for naturally occurring GPx substrate GSH was obtained. This bioimprinting procedure facilitates the catalytic selenium moiety of the imprinted selenosubtilisin to match the reactive thiol group of GSH in the GSH binding site, which contributes to acceleration of the intramolecular catalysis. These imprinted selenium-containing proteins exhibited remarkable rate enhancement for the reduction of H₂O₂ by GSH. The average GPx activity was found to be 462 U/μmol, and it was approximately 100 times that for unimprinted selenosubtilisin. Compared with ebselen, a well-known GPx mimic, an activity enhancement of 500-fold was observed. Detailed steady-state kinetic studies demonstrated that the novel selenoenzyme followed a ping-pong mechanism similar to the naturally occurring GPx.

Introduction

Reactive oxygen species (ROS) are generated inside cells during normal metabolic activity. Excess production of ROS, however, may lead to oxidative stress, resulting in damage to many important biomacromolecules, that has been implicated in several human diseases. Mammalian cells possess elaborate defense mechanisms to detoxify ROS and maintain the balance between the production and destruction of ROS.^{1–5} Key steps involve the dismutation of superoxide to H₂O₂ and O₂ by superoxide dismutase (SOD) and reduction of H₂O₂ to H₂O and O₂ by glutathione peroxidase (GPx) and/or catalase. Enzymes such as SOD, GPx, catalase (CAT), and other antioxidative enzymes compose the enzymatic antioxidant system in an organism. GPx (EC 1.11.1.9), an important antioxidant selenoenzyme, can protect cell membranes and other cellular components from oxidative damage by scavenging hydroperoxides (ROOH) by glutathione (GSH). The tripeptide GSH evolved as the special substrate of this enzyme. In addition to H₂O₂, the enzyme can scavenge many other hydroperoxides such as phospholipid hydroperoxides, fatty acid hydroperoxides, cumene hydroperoxide, *tert*-butyl hydroperoxide, and cholesterol hydroperoxides. This enzyme is composed of four identical subunits; selenocysteine residue acts as its catalytic group in the active site in each subunit.⁶

Owing to its biological importance, considerable effort has been devoted to reproduce the properties of this selenoenzyme in recent years. Although genetic approaches for enzyme design

are currently in wide use, it is extremely difficult to produce selenium-containing protein by this method because selenocysteine is encoded by a UGA opal codon, which is usually a STOP codon. In this case, a chemical method or a combination of both chemical and biological methods displayed advantages for developing and constructing efficient GPx mimics.^{7–17} In recent years we devoted ourselves to a series of research projects regarding the imitation of this important selenoenzyme. An alternative strategy for designing an efficient enzyme model is chemical incorporation of catalytic groups into naturally existing or artificially generated substrate binding scaffolds. By using this concept, we have developed a variety of GPx mimics such as monoclonal antibodies, natural enzymes, cyclodextrins, and peptides.^{18–28} The main technologies developed in our group include monoclonal antibody technology, bioimprinting, chemical modification of nature enzymes, and chemical synthesis of peptides. The disquisitive work suggests that the general principle and strategies for constructing an enzyme model is that the catalytic efficiency of an enzyme mimic depends not only on catalytic groups but also on substrate recognition. In the active site of an enzyme, the substrate binding and the orientation of catalytic groups are responsible for efficient catalysis. Thus, for the creation of an efficient artificial enzyme, the affinity for the substrate must be reasonably high and the catalytic groups should be oriented to the substrate reactive moieties.

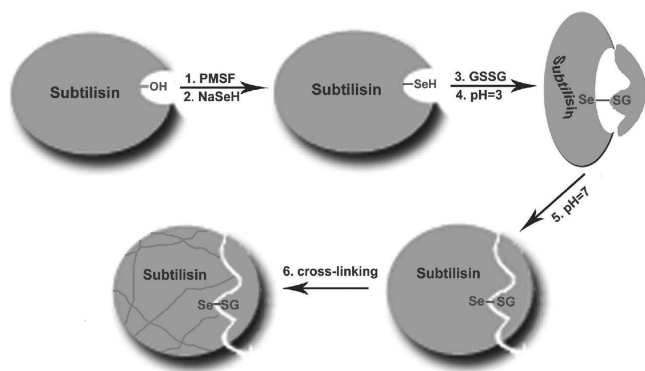
Molecular imprinting is an alternative method for making selective binding sites in synthetic polymer networks or on biopolymers (bioimprinting). Target molecules can be used as templates for imprinting cross-linked polymers. After removal of the template, the remaining polymer is more selective. As an important part of molecular imprinting, the bioimprinting

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Scheme 1



technique was demonstrated to be an efficient means for introducing a new binding site into proteins or other biological molecules in the presence of a template (e.g., a substrate or a transition state analogue).^{29–33} This technique has previously been used to create an artificial selenoenzyme with GPx-like activity in our group.²⁶ However, this technique makes it difficult to incorporate and orient catalytic selenium moieties into the imprinted binding site. In order to attain a higher catalytic efficiency, we used subtilisin Carlsberg as a scaffold and converted it to selenium-containing subtilisin by chemical modification. Thus the bioimprinted selenium-containing enzyme with a GSH binding site by reconstructing the active site of subtilisin was prepared (Scheme 1). The imprinted selenium-containing proteins exhibited remarkable GPx activity. Detailed steady-state kinetic studies demonstrated a similar mechanism to the naturally occurring enzymes.

Experimental Section

Materials. Subtilisin Carlsberg, phenylmethanesulfonyl fluoride (PMSF), sodium borohydride (NaBH_4), oxidized GSH (GSSG), reduced nicotinamide adenine dinucleotide phosphate (NADPH), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), 4-piperazinediethanesulfonic acid (PIPES), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma. Sephadex G-25 was obtained from Aldrich Chemical Co. All other chemicals were of the highest purity commercially available and were used without further purification.

Preparation of Selenosubtilisin. The preparation was carried out according to a method reported by Hilvert et al.³⁴ with a slight modification. NaHSe was synthesized according to the ref 35. The following buffers were used: buffer A (50 mM PIPES, 10 mM CaCl_2); buffer B (10 mM DTT, 1 mM CaCl_2 , 50 mM, pH7.0 PIPES); buffer C (10 mM DTT, 1 mM CaCl_2 , 50 mM, pH7.0 PIPES); buffer D (10 mM H_2O_2 , 1 mM CaCl_2 , 50 mM, pH7.0 PIPES buffer); buffer E (1 mM CaCl_2 , 10 mM, pH7.0 PIPES), and buffer F (10 mM PIPES, 10 mM CaCl_2).

Subtilisin Carlsberg (150 mg, 5.5 μmol) was solved in buffer A and phenylmethanesulfonyl fluoride (PMSF) (150 μL , 20 mg/mL, 17 μmol , CH_3CN) was added slowly at 25 $^\circ\text{C}$ for 1 h. The reaction mixture was bubbled under pure nitrogen gas for 20 min to remove O_2 and then NaHSe solution was added (500 μL , 1 M). Under the protection of pure nitrogen gas the mixture was kept for 36 h at 40 $^\circ\text{C}$, and then this mixture was oxidized in air for 8 h. After that, crude selenosubtilisin was purified by centrifugation and Sephadex G-25 column chromatography eluted with buffer A and was further purified by thiopropyl Sepharose 6B using buffer C as an eluent. The solution was concentrated and dialyzed against buffer D at 4 $^\circ\text{C}$ three times in 4 h. The solution was finally dialyzed three times against buffer E at 4 $^\circ\text{C}$ for 4 h and was finally lyophilized (20% yield).

Determination of the Concentration of Selenosubtilisin. The concentration of subtilisin or selenosubtilisin was determined by

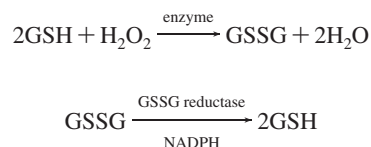
measuring the UV absorbance at 280 nm and pH 7.0, with an extinction coefficient of 23500 $\text{M}^{-1} \text{cm}^{-1}$.³⁶ SDS–polyacrylamide gel electrophoresis was performed on a Pharmacia gel system by standard procedures.

Determination of the Selenium Content of Selenosubtilisin. The selenium content of the product enzyme was determined by titrating the reduced protein with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) using the method reported by Hilvert et al. with a slight modification.³⁶ Selenosubtilisin in buffer F was treated with excess sodium borohydride (NaBH_4) under nitrogen for 20 min, the solution was carefully adjusted to pH 7.0 with degassed 1 M HCl and 1 M imidazole to quench the reducing agent, and 50 μL of the reduced sample was added to DTNB (950 μL , 200 μM) in PIPES (50 mM, pH 7.0). The concentration of enzyme-bound selenol was determined by the absorption of 3-carboxy-4-nitrobenzenethiolate (ArSH) at 410 nm ($\epsilon_{410} = 13600 \text{ M}^{-1} \text{cm}^{-1}$, pH 7.0).

Preparation of GSH–Selenosubtilisin. Selenosubtilisin with seleninic acid form (5 mg) was dissolved in buffer E, and NaBH_4 (1 mg) was added under pure nitrogen gas for 20 min to reduce the seleninic acid to the selenol group. The solution was carefully adjusted to pH 7.0 to quench the excessive reducing agent. GSSG (20 mL, 20 mM) in PIPES (50 mM, pH 7.0) was then added. GSH–selenosubtilisin was purified by Sephadex G-25 column chromatography using buffer E as an eluent.

Preparation of GSH-Imprinted Selenium-Containing Protein. The GSH–selenosubtilisin was dissolved in PIPES (10 mM, pH 7.0) and was carefully adjusted to pH 3.0 with 0.1 M HCl and stirred at 4 $^\circ\text{C}$ for 20 min under 300 revolutions/min using an electromagnetic stirrer. Then PIPES (10 mM, pH 7.0) was used to adjust to pH 7.0 100 μL of glutaraldehyde solution (1%) was added to cross-link the protein to fix the new conformation of the protein, finally freeze-dried the solution. As described in “Determination of the Selenium Content of Selenosubtilisin” above, the DTNB method was used to determine the yield of the bioimprinted enzyme.³⁷

The activity of Bioimprinted Enzyme. The GPx activity of the bioimprinted enzyme was determined by the GSH reductase–NADPH coupled assay.³⁸ The reaction was carried out at 37 $^\circ\text{C}$ in 500 μL of solution containing 50 mM, pH 7.0, PIPES, 1 mM EDTA, 10 mM CaCl_2 , 1 mM GSH, 1 U of GSH reductase, 0.5–5 μM of the bioimprinted enzyme. The mixture was preincubated for 7 min, and then 0.25 mM NADPH solution was added and incubated for 3 min at 37 $^\circ\text{C}$. The reaction was initiated by addition of 0.5 mM H_2O_2 . The activity was determined by the decrease of NADPH absorption at 340 nm ($\epsilon_{340} = 6220 \text{ M}^{-1} \text{cm}^{-1}$). Appropriate controls were run without enzyme mimic and were subtracted. The activity unit of the mimic is defined as the amount of the mimic that utilizes 1 μmol of NADPH/min. The activity is expressed in U/ μmol of enzyme mimic. The reactive route is



Kinetics of the Bioimprinted Enzyme. The kinetics was determined by the coupled enzymatic assay similar to the telluros subtilisin.³⁹ ArSH was prepared from DTNB by reduction with sodium borohydride in methanol. The initial reduction rate of hydrogen peroxide reduced by GSH in the presence of the bioimprinted enzyme was detected by UV absorption at 340 nm at 37 $^\circ\text{C}$ by the decreasing absorption of NADPH. One substrate concentration was varied while the other substrate concentration was kept constant. Each initial velocity was measured in triplicate and calculated from the first 5–10% of the reaction.

Results and Discussion

Synthesis of Selenosubtilisin. Following the first semisynthetic thiol subtilisin, Hilvert and co-workers developed a

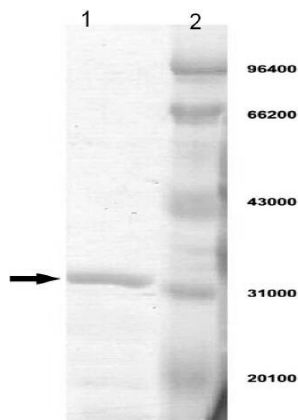


Figure 1. SDS–polyacrylamide gel electrophoresis of selenosubtilisin after affinity chromatography on thiopropyl Sepharose 6B under the pretreatment with SDS and β -mercaptoethanol: lane 1, selenosubtilisin; lane 2, marker proteins (from top to down) phosphorylase *b*, BSA, ovalbumin, carboanhydrase, the kunitztype soybean inhibitor.

methodology to introduce selenium into the binding pocket of subtilisin to yield selenosubtilisin.³⁶ Selenosubtilisin was synthesized according to a method reported by Hilvert et al. Ser221 was selectively activated with PMSF and was then substituted by NaHSe, the selenosubtilisin with seleninic acid form was obtained by the oxidation with H_2O_2 . The assay of SDS-PAGE indicated that the product is a single protein (Figure 1). The selenium content of selenosubtilisin was analyzed by DTNB titration under an inert atmosphere; 0.98 ± 0.3 selenium atom per selenosubtilisin was found.

Preparation and Characterization of Bioimprinted Selenoenzyme. GSH was first covalently linked to selenosubtilisin to form GSH–selenosubtilisin through the Se–S bond, then the GSH molecule was used as a template to cast a complementary binding site for GSH recognition. The bioimprinting procedure includes partly unfolding the conformation of the selenosubtilisin and fixing the new conformation of GSH–selenosubtilisin. Reduced-selenosubtilisin ($pI=7.8$) reacted with GSSG to form GSH–selenosubtilisin; thus GSH was introduced into the active site of selenosubtilisin. The solution was adjusted to pH 3.0 and stirred slowly in order to make selenosubtilisin partially denatured. The solution was then adjusted to pH 7.0. This procedure allowed the GSH residue in the selenosubtilisin active site to contact well with the protein and form a new conformation via electrostatic interactions, hydrogen bonds, hydrophobic interactions, and other means. A glutaraldehyde solution (1%) was added to selenosubtilisin solution with a low concentration to fix the new conformation of the protein by cross-linking the protein.

In the preparation of the imprinted selenosubtilisin, the stirring time has an important effect on the protein denaturation. The lower degree of denaturation led to a slight effect between amino acid residues of the protein and GSH and could not create the suitable GSH binding site. At the higher degree of denaturation, however, the GSH may be packed too closely and deeply in the protein which might prevent GSSG from forming. Therefore, partially denatured protein might create a suitable binding site for GSH, which could lead to bioimprinted enzyme with high enzyme activity. This hypothesis has been demonstrated by the activity determination of these imprinted systems. Under 300 revolutions/min the imprinted selenoenzyme exhibited quite different GPx activities with the changing stirring time (Figure 2). The activity of the bioimprinted enzyme was initially enhanced with the increased denaturation time and reached to maximal value of $597 \text{ U}/\mu\text{mol}$ for stirring for 20 min, but then the activity of selenosubtilisin gradually deceased.

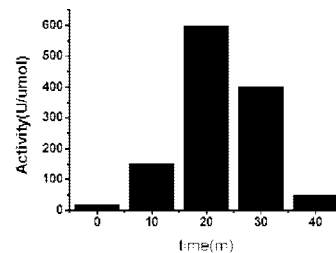


Figure 2. GPx activities of imprinted proteins vs denaturation time.

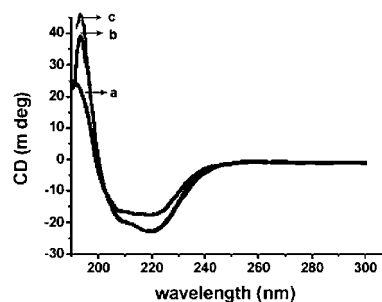


Figure 3. CD spectra of imprinted GSH–selenosubtilisin (a), subtilisin (b), and selenosubtilisin (c) monitored from 190 to 300 nm.

Table 1. The Comparison of Secondary Structure of Imprinted GSH–Selenosubtilisin and Subtilisin^a

protein	helix	beta	turn	random
subtilisin	29.1%	36.5%	5.9%	28.5%
imprinted GS–selenosubtilisin	26.2%	30.4%	6.7%	36.7%

^a The data in the table were obtained from the plots in Figure 3.

The change of the conformation of selenosubtilisin was detected by circular dichroism (CD) spectra of the imprinted GSH–selenosubtilisin and subtilisin (Figure 3). Selenosubtilisin gave CD spectra similar to that for subtilisin, indicating that the selenolation of subtilisin did not significantly affect the secondary structure (Figure 3).³⁶ However, the secondary structure was quite different between the native enzyme and the bioimprinted protein (Table 1). The proportion of α -helix and β -fold in the imprinted GSH–selenosubtilisin was 26.2% and 30.4%, respectively, but it was found to be 29.1% and 36.5% in subtilisin. The proportions of α -helix and β -fold imprinted GSH–selenosubtilisin were lower than that in subtilisin; in addition the proportion of random in imprinted GSH–selenosubtilisin was higher than that in subtilisin. The yield of imprinted GSH–selenosubtilisin was determined to be $96 \pm 3\%$ by the DTNB titration method; the titration results indicated that the form of selenosubtilisin was GSH–selenosubtilisin before imprinting.

GPx Activity of the Bioimprinted Enzymes. Selenosubtilisin combines the intrinsic chemistry of selenium to show GPx activity with the binding specificity of subtilisin for aromatic groups. The selenosubtilisin which had evolved to bind a specific substrate, was 70000 times more efficient than diphenyl diselenide (PhSeSePh) in catalyzing the reduction of *tert*-butyl hydroperoxide (*t*-BuOOH) by thiol substrate 3-carboxy-4-nitrobenzenethiol (ArSH);³⁶ however, it was incapable of reducing hydroperoxides by GSH. The selenosubtilisin that catalyzes GSH to reduce hydroperoxides is only several times more efficient than diphenyl diselenide (PhSeSePh) because there was no GSH binding site in active center of selenosubtilisin (Table 2).

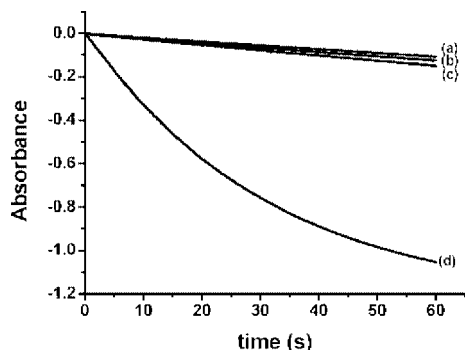
The binding site of selenosubtilisin was reconstructed by bioimprinting in order to obtain a new specificity for native GPx

Table 2. The Activity Comparison of Imprinted GSH–Selenosubtilisin, Ebselen, and Native GPx^a

catalyst	GPx activity (U/ μ mol)
ebselen ^b	0.99
selenosubtilisin	4.5
denatured selenosubtilisin	1.8
imprinted GS–selenosubtilisin 1	537.6
imprinted GS–selenosubtilisin 2	473.4
imprinted GS–selenosubtilisin 3	597.4
imprinted GS–selenosubtilisin 4	314.2
imprinted GS–selenosubtilisin 5	386.9
native GPx ^b	5780

^a The catalytic reduction of H₂O₂ (0.5 mM) by GSH (1 mM) was carried out in 50 mM, pH 7 PIPES buffer containing glutathione reductase (1 U), NADPH (0.25 mM) at 37 °C monitored at 340 nm for NADPH decrease.

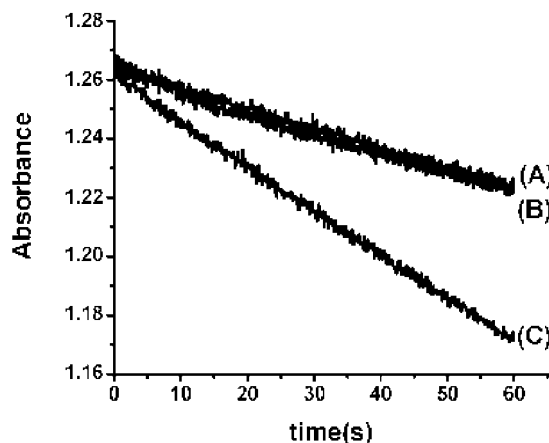
^b The data were obtained from ref 23.

**Figure 4.** Plots of absorbance vs time during the catalytic reduction of H₂O₂ (0.5 mM) by GSH (1 mM) in 50 mM, pH 7 PIPES buffer containing glutathione reductase (1 U), NADPH (0.25 mM) at 37 °C monitored at 340 nm for NADPH decrease. Catalysts: (a) none; (b) denatured selenosubtilisin; (c) selenosubtilisin; (d) imprinted selenosubtilisin.

substrate GSH. After imprinting, the GPx activity of selenosubtilisin for the reduction of hydroperoxides by GSH had a remarkable enhancement and was found to be 597 U/ μ mol. In comparison to nonimprinted selenosubtilisin (4.5 U/ μ mol), the bioimprinted enzyme had an activity enhancement of more than 130-fold. Under the same condition but without the imprinted molecule GSH, the denatured selenosubtilisin showed a little GPx activity of 1.8 U/ μ mol (Figure 4). The average activity of five samples was 462 U/ μ mol. Moreover, it was 100 times that of selenosubtilisin, and 500 times that of ebselen, the well-known GPx mimic.

This bioimprinting strategy facilitates the catalytic selenium moiety of the imprinted selenosubtilisin to match the thiol group of GSH well in GSH binding site, which is responsible for intramolecular catalysis. Although the activity has obviously increased, the bioimprinted enzyme displayed lower activity than the native enzyme.⁴⁰ At active sites of the native GPx, the sequences are very conserved, Arg-167, Arg-40, Gln-130, and Trp-148, and play an important role in the process of binding and catalysis. In addition, the salt bridge is composed of the carboxyl group of γ -Glu and Arg-167, and the carboxyl group of Arg-40, with hydrophobic amino acid residues (Phe, Trp, Asp), forms a hydrophobic cavity for thiol GSH binding. This active site enhances specific substrate recognition and the catalysis of the enzyme.⁴¹

Although the imprinted selenosubtilisin possesses the special GSH binding sites and its catalytic domain matched well with the substance, it has some difference from natural GPx. The GSH binding sites produced in this method are random, and it could not have the conserved amino acid sequences. So the GPx activity of imprinted protein molecule was not identical and

**Figure 5.** Plots of absorbance vs time during the catalytic reduction of H₂O₂ (250 μ M) by TNB (100 μ M) at pH 5.5 and 25 °C monitored at 410 nm for TNB decrease in the absence of catalyst (A) and in the presence of selenosubtilisin (C) and imprinted GS–selenosubtilisin (B).

lower than the native GPx. At the same time, we found that the imprinted selenosubtilisin had lost GPx activity completely when we used 3-carboxy-4-nitrobenzenethiol (TNB) as a thiol substance (Figure 5). This means that the active domain of subtilisin was changed completely and lost the specificity for the aromatic thiol group. Thus we reconstructed the active site of subtilisin successfully for recognizing the native GPx substrate GSH.

Kinetics of Imprinted Selenosubtilisin-Catalyzed Reaction. In order to realize the catalytic mechanism of the imprinted GSH–selenosubtilisin catalysis, we studied the kinetics of the enzyme-catalyzed reaction. The initial rates were monitored by measuring the concentration dependence on decrease of NADPH absorption at 340 nm. The initial rates were determined at different concentrations of one substrate while keeping the other constant. Rates were corrected for the background reaction between GSH and H₂O₂. Saturation kinetics were observed under each concentration of GSH and H₂O₂ (Figure 6), and the kinetics parameters under different concentrations of GSH and H₂O₂ are listed in Table 3.

A second-order rate constant did not change with the increase of other substrate concentration. The double-reciprocal plots of initial rates vs GSH and H₂O₂ concentration exhibited families of parallel lines, which fit a ping-pong mechanism in eq 1 (Figure 7). The catalytic mechanism is similar to subtilisin Carlsberg and native GPx. k_{\max} is a pseudo-first-order rate constant and $K_{\text{H}_2\text{O}_2}$ and K_{GSH} are the Michaelis constants for hydroperoxidase and GSH, respectively. Normally, the hydrophilic groups are exposed on the surface of the folded protein, with the hydrophobic groups hiding inside. However, there are many hydrophilic groups in GSH molecule, so when the partially denatured GSH–selenosubtilisin refolds, the electrostatic and hydrogen bond interactions led to the GSH molecule locating at the flat depression of the folded protein. Accordingly, GSH attacks the selenium–sulfur compound to form the disulfide in the catalytic process, and this makes the direct exposure of the selenium atom to H₂O₂, so the second-order rate constants of the bioimprinted enzyme toward H₂O₂ is 2 magnitudes larger than that of the selenosubtilisin.

$$\frac{V_0}{[E]} = \frac{K_{\max}[\text{GSH}][\text{H}_2\text{O}_2]}{K_{\text{GSH}}[\text{H}_2\text{O}_2] + K_{\text{H}_2\text{O}_2}[\text{GSH}] + [\text{GSH}][\text{H}_2\text{O}_2]} \quad (1)$$

Conclusion

The study extends our previous investigation on GPx-like mimics using bioimprinting.²⁶ Our strategy is different from

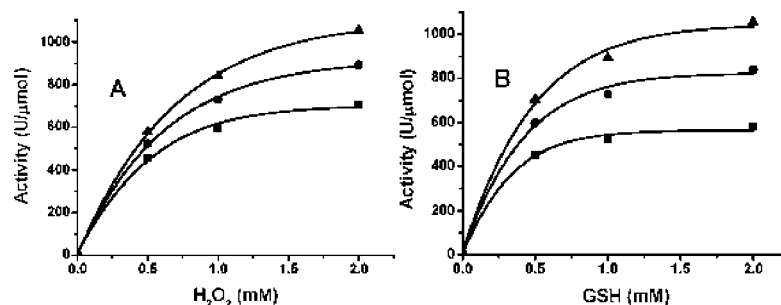


Figure 6. Plots of the activity vs substrate concentration for the reduction of H_2O_2 by GSH catalyzed by the imprinted selenosubtilisin at 37 °C and pH 7.0 (50 mM PIPES buffer, 1 mM EDTA). (A) Activity vs H_2O_2 obtained at various concentrations of the substrate GSH: (■) 0.5 mM, (●) 1.0 mM, and (▲) 2.0 mM. (B) Activity vs GSH obtained at various concentrations of the substrate H_2O_2 : (■) 0.5 mM, (●) 1.0 mM, and (▲) 2.0 mM.

Table 3. Kinetic Parameters for Peroxidase Reaction Catalyzed by the Imprinted Selenoenzyme*

[GSH] (mM)	k_{cat} (app) (min^{-1})	$K_{\text{H}_2\text{O}_2}$ (app) (mM)	$K_{\text{cat}}(\text{app})/K_{\text{H}_2\text{O}_2}(\text{app})$ ($\text{M}^{-1} \text{min}^{-1}$)
0.5	869 ± 50	0.45 ± 0.05	$(1.91 \pm 0.05) \times 10^6$
1.0	1170 ± 50	0.62 ± 0.08	$(1.89 \pm 0.05) \times 10^6$
2.0	1470 ± 90	0.77 ± 0.08	$(1.92 \pm 0.05) \times 10^6$

$[\text{H}_2\text{O}_2]$ (mM)	$k_{\text{cat}}(\text{app})$ (min^{-1})	$K_{\text{GSH}}(\text{app})$ (mM)	$k_{\text{cat}}(\text{app})/K_{\text{GSH}}(\text{app})$ ($\text{M}^{-1} \text{min}^{-1}$)
0.5	633 ± 50	0.20 ± 0.02	$(3.18 \pm 0.05) \times 10^6$
1.0	961 ± 50	0.30 ± 0.02	$(3.14 \pm 0.05) \times 10^6$
2.0	1250 ± 90	0.40 ± 0.03	$(3.15 \pm 0.05) \times 10^6$

* Reactions were carried out in 50 mM PIPES buffer, pH 7.0 at 37 °C, and followed by UV spectroscopy as described above. The data in the table were obtained from the plots in Figure 7.

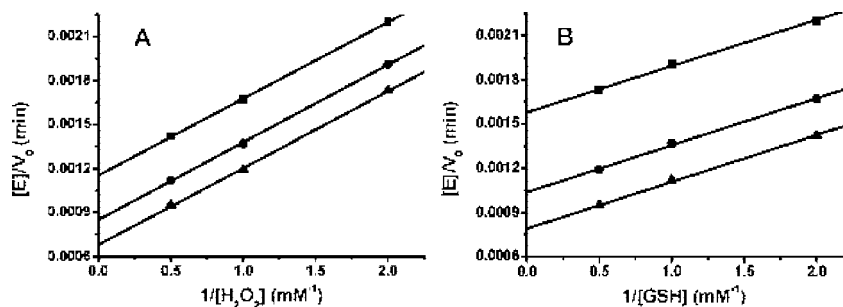


Figure 7. Line weaver–Burk plots for reduction of H_2O_2 by GSH catalyzed by 0.2 μM imprinted GSH–selenosubtilisin at 37 °C and pH 7.0 (50 mM PIPES buffer, 1 mM EDTA). (A) Primary plots $[\text{E}]/V_0$ vs $1/[\text{H}_2\text{O}_2]$ obtained at various concentrations of the substrate GSH: (■) 0.5 mM, (●) 1.0 mM, and (▲) 2.0 mM. (B) Primary plots $[\text{E}]/V_0$ vs $1/[\text{GSH}]$ obtained at various concentrations of the substrate H_2O_2 : (■) 0.5 mM, (●) 1.0 mM, and (▲) 2.0 mM.

the previous bioimprinting method: we use a covalent Se–S bond to link the template molecule GSH and subtilisin to form GSH–selenosubtilisin. Furthermore, by partial denaturation and renaturation, we create the bioimprinted selenium-containing enzyme with GSH binding site. This strategy makes the catalytic selenium moiety of the enzyme and the reactive thiol of the bound substrate GSH match very well, which facilitates the intramolecular catalysis. When the reduction of H_2O_2 is catalyzed using GSH as the reducing substrate, the bioimprinted enzyme increased the activity more than 100 times compared with nonimprinted selenosubtilisin, while it lost the ability to catalyze the reduction of aromatic substrate TNB. This indicates that we successfully reconstruct the binding site of selenosubtilisin via bioimprinting, which achieved the conversion of substrate specificity from aromatic group to GSH, and as a result, the new active site of the enzyme can recognize GSH better with losing binding specificity for aromatic groups. The bioimprinted selenium-containing enzyme exhibits much better GPx activity than the well-known GPx mimic ebselen (500 times higher), which reconfirms the assumption that recognition and substrate binding are the foundation of high catalytic activity of the enzymatic catalysis. The second-order rate constant of

the bioimprinted enzyme toward H_2O_2 is 2 magnitudes larger than that of the selenosubtilisin.

This strategy produces an appreciable change in the three-dimensional conformation of the peptide chain compared with the natural ones. The new binding conformation is rather complex and consists of a combination of electrostatic interactions, hydrogen bonds, hydrophobic interactions, and others. This bioimprinting strategy leads to a new form of three-dimensional, steric arrangement of the functional groups for the novel enzyme with GPx activity, which may give us valuable insights for the mechanism of GPx catalysis.

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