Selenium-containing 15-Mer Peptides with High Glutathione Peroxidase-like Activity* 

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Glutathione peroxidase (GPX) is one of the most crucial antioxidant enzymes in a variety of organisms. Here we described a new strategy for generating a novel GPX mimic by combination of a phage-displayed random 15-mer peptide library followed by computer-aided rational design and chemical mutation. The novel GPX mimic is a homodimer consisting of a 15-mer selenopeptide with an appropriate catalytic center, a specific binding site for substrates, and high catalytic efficiency. Its steady state kinetics was also studied, and the values of $k_{cat}/K_m$ for ROOH and $k_{cat}/K_m$ for H$_2$O$_2$ were found to be similar to that of native GPX and the highest among the existing GPX mimics. Moreover, the novel GPX mimic was confirmed to have a strong antioxidant ability to inhibit lipid peroxidation by measuring the content of malondialdehyde, cell viability, and lactate dehydrogenase activity. Importantly, the novel GPX mimic can penetrate into the cell membrane because of its small molecular size. These characteristics endue the novel mimic with potential perspective for pharmaceutical applications.

Glutathione peroxidase (GPX)$^{3}$ (EC 1.11.1.9) is a selenoenzyme that protects the biomembrane and other cellular components against oxidative damage (1–3). Mills discovered its activity in 1957 (4), and the stoichiometric amount of selenium was found in this enzyme in 1973 (5, 6). As shown in Reaction 1,

\[
\text{Glutathione peroxidase} \rightarrow \text{ROOH} + 2\text{GSH} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{GSSG}
\]

the enzyme catalyzes the reduction of a variety of hydroperoxides by using glutathione as the reducing substrate (7). ROOH is a hydroperoxide such as hydrogen peroxide (H$_2$O$_2$), cumene hydroperoxide (CuOOH), tert-butyl hydroperoxide, etc. GSSG is the oxidized form of GSH, which is reverted to GSH by glutathione reductase, and ROH is the corresponding alcohol or H$_2$O when the substrate is H$_2$O$_2$.

GPX is distributed extensively in cell, blood, and tissues, and its activity decreases when an organism suffers from diseases such as diabetes (8), Keshan disease (9), angiocardiopathy (10), cataracts (11), and other maladies; therefore, GPX is involved in many pathological conditions and is one of the most important antioxidant enzymes in living organisms. However, the therapeutic usage of the native GPX is limited because of its instability, its limited availability, and the fact that it is extremely difficult to prepare by using genetic engineering techniques because it contains selenocysteine encoded by the stop codon UGA. Thus, many artificial mimics of GPX have been made, but most of them have low GPX activity because they lack a substrate binding site. Among these mimics, the selenoorganic compound ebselen (2-phenyl-1, 2-benzisoselenazol-3(2H)-one) was first used for hydroperoxide-inactivating therapy. Ebselen has properties such as free radical and singlet oxygen quenching. It can protect against oxidative challenge in vitro in liposomes, microsomes, isolated cells, and organs (12). It has been demonstrated to have a potent inhibitory effect on inflammation in several animal models (13–15) and has been clinically shown to be effective for acute ischemic stroke without significant adverse effects (16). As a result, ebselen is currently approved in Japan to treat stroke patients despite its limitations, such as poor water solubility and low activity (0.99 units/μmol) as compared with native GPX.

Most of the existing GPX mimics have low GPX activities because they lack a substrate binding site. To make GPX mimics with higher activity and better specificity, we have synthesized a series of GPX mimics with a GSH binding site since the end of 1980s (17–24). They all display rather high activities and have been clinically shown to be effective for acute ischemic stroke without significant adverse effects. As a result, ebselen has been approved in Japan to treat stroke patients despite its limitations, such as poor water solubility and low activity. Nevertheless, some of them have large molecular sizes (>31 kDa), which hampered their pharmacological potentials.

Here we describe a low molecular weight novel GPX mimic with much greater catalytic activity than ebselen. We identified a series of GSH-binding peptides by affinity selection from phage-displayed random 15-mer peptide libraries against GSH, one of the substrates of GPX. We enable the peptide to possess GPX enzymatic activity by introducing the catalytic group, selenocysteine, into the peptides using chemical mutation. In addition, considering the nature of the double substrate reaction, we altered the peptide sequence to optimize its binding affinity for the second substrate, ROOH or H$_2$O$_2$. The resulting GPX mimic shows superior kinetics and antioxidant ability.
**15-Mer Selenopeptide Is a Novel GPX Mimic**

### Experimental Procedures

**Materials**—The phage strains K91 and K91Kan bearing random 15-mer peptides libraries were gifts from Professor George P. Smith from the Division of Biological Sciences, University of Missouri. GSH-agarose resin, reduced glutathione, NADPH, and glutathione reductase were products of Sigma. Culture medium (Dulbecco’s medium) and fetal bovine serum were obtained from Invitrogen and Hyclone (Logan, UT), respectively. All chemicals were of analytical or reagent grade.

**Scanning of a 15-Mer Phage Display Peptide Library and Analysis by Phage Enzyme-linked Immunosorbent Assay (ELISA)**—The phage library of random 15-mer peptides was incubated with GSH-agarose resin overnight at 4 °C. Specifically bound phage was eluted by Buffer A. The recovered phage was amplified in liquid nitrogen and finally freeze-dried to obtain the product of the mimic.

**Determination of GPX-like Activity and Kinetics**—The catalytic activity was determined by the method of Wilson et al. (30). The reaction was carried out at 37 °C in 700 μl of solution containing 50 mM sodium phosphate buffer (pH 7), 1 mM EDTA, 1 mM sodium azide, and 10 μM of the mimic. The reaction was initiated by the addition of 0.5 mM H2O2 or CuO2. The activity was determined by the decrease of NADPH absorption at 340 nm. Background absorption was run without mimic and subtracted.

**Culture of Epidermal Cells and Evaluation of Antioxidative Effect**—Epidermal cells were cultured as described in references (32, 33). Epidermal cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction as described in the references.

### Table I

| Peptide sequences | 15P1  |
|-------------------|-------|
| GPPRHNNVGYGRPAS  | ON    |
| GPPRHNNVGYGRPAS  | ON    |
| GPPRHNNVGYGRPAS  | ON    |
| GPPRHNNVGYGRPAS  | ON    |
| GPPRHNNVGYGRPAS  | ON    |
| GPPRHNNVGYGRPAS  | ON    |
| GPPRHNNVGYGRPAS  | ON    |
| GPPRHNNVGYGRPAS  | ON    |

### Table II

| Species | Substrate | GPX activity units/μmol |
|---------|-----------|-------------------------|
| 15SeP   | CuO2      | 1176                    |
| 15P1    | CuO2      | 570                     |
| 15SeP2  | H2O2      | 260                     |
| 15SeP3  | H2O2      | 190                     |
| 2-SeCD  | H2O2      | 7.4                     |
| 6-SeCD  | H2O2      | 4.2                     |
| 6-iSeCD | H2O2      | 13.5                    |
| Ebselen | H2O2      | 0.99                    |
| Native  | H2O2      | 5780                    |

### Table III

| Species Substrate | GPX activity units/μmol |
|-------------------|-------------------------|
| 15SeP             | 1176                    |
| 15P1              | 570                     |
| 15SeP2            | 260                     |
| 15SeP3            | 190                     |
| 2-SeCD            | 7.4                     |
| 6-SeCD            | 4.2                     |
| 6-iSeCD           | 13.5                    |
| Ebselen           | 0.99                    |
| Native (GPX, rabbit liver) | 5780 |
15-Mer Selenopeptide Is a Novel GPX Mimic

Results and Discussion

Synthesis and Characterization of the Selenopeptide—To find a peptide that can mimic the GPX activity, we first screened a 15-mer phage display peptide library using one of the GPX substrates, GSH. The phages with high affinities for GSH were selected according to the result of ELISA. The amino acid sequences of these peptides were determined by DNA sequencing. The amino acid sequences of these peptides are listed in Table I.

Sequence analysis revealed that the 12 peptides were rich in hydrophobic amino acid and had no negatively charged amino acids. No clear conservative motif was identified. Furthermore, alignment of the individual peptide sequence with the sequence of native GPX for GSH binding did not show any homology, although it remains possible that one or more of the peptides might be a structure mimic of a discontinuous or conformational epitope with the binding site for GSH. Among these peptides, 15P1 had the strongest ability to bind GSH and, thus, was chosen for synthesis and chemical modification to imitate GPX.

The synthetic route of selenopeptide is shown in Scheme 1. The selenohydryl group was introduced into the peptide by selective sulfonylation of the serine hydroxyl of the 15-mer peptide. The nucleophilic substitution by sodium hydroselenide gave selenopeptide 1, which was oxidized in air to form selenopeptide 2, named 15SeP1. The resulting compound, 15SeP1, contains a diselenium bridge (-Se-Se-) and two fragments of 15-mer peptides. To confirm this result, the selenium content of 15SeP1 was measured by the 5,5'-dithiobis(2-nitrobenzoic acid) method and found to be 1.97 ± 0.30 mol of selenium per mole of molecule. This indicates that 1 mol of molecule contains 2 mol of selenium. The introduction of SeH into 15P1 decreased the ability of 15P1 to bind GSH, as the association constant for the GSH of 15P1 was 7.97E+05, 10 times higher than that of 15SeP1 (7.263E+04) (See Table II).

The GPX-like Activity and the Steady State Kinetics—The GPX activities of 15SeP1 for the reduction of H2O2 or CuOOH were listed in Table III. The GPX activities of other mimics were included for comparison. The GPX activity of 15SeP1 for the reduction of H2O2 by GSH was found to be 190 units/μmol, significantly higher than those of ebselen, 2-SeCD, and 6-SeCD.

Steady state kinetics was observed for both H2O2 and GSH. The initial velocities for the reduction of H2O2 by GSH were determined as a function of substrate concentration at 37 °C and pH 7, varying one substrate concentration while the other was fixed. Double reciprocal plots of the initial velocity versus the concentration of substrates gave a family of parallel lines (Fig. 1), a result consistent with a ping-pong mechanism. This result demonstrated that the GPX mimic, 15SeP1, has the same catalytic mechanism as that of native GPX. The apparent kinetic parameters were calculated from the double-reciprocal plots and listed in Table IV. The pseudo first-order rate constant kcat and the apparent Michaelis constants KmH2O2 and KmGSH were found to be 2.62E+03 min⁻¹, 8.54E−02 mM, and 2.55E−02 mM, respectively; The apparent second-order rate constants kcat/KmH2O2 and kcat/KmGSH were 3.07E+07 M⁻¹ min⁻¹ and 1.03E+08 M⁻¹ min⁻¹, respectively.

Modification of the Selenopeptide 15SeP1—The selenopeptide was modified for optimal GPX activity and substrate specificity. Upon comparing the apparent second-order rate constants kcat/KmGSH and kcat/KmH2O2 of native GPX with those of 15SeP1 (Table IV), we found that there are no obvious differences in kcat/KmGSH values between GPX and 15SeP1, which indicate that the two have similar affinities to GSH. However, a significant difference exists in kcat/KmH2O2 values between

| Table IV | Kinetic parameters of GPX mimics |
|----------|--------------------------------|
| Species (Substrate) | \( K_{cat} \) (min⁻¹) | \( K_{mGSH} \) (μM) | \( K_{mH2O2}/K_{catGSH} \) | \( K_{cat}/K_{mGSH} \) | \( K_{cat}/K_{mH2O2}/(k_{cat}/K_{catGSH}) \) |
| 2-SeCD (H2O2) (19) | 16 | 8 | 0.321 | 2.0E−03 | 4.96E+04 |
| 15SeP1 (H2O2) | 2.92E+03 | 2.55E−02 | 8.54E−02 | 1.03E+08 | 3.07E+07 |
| 15SeP1 (CuOOH) | 2.11E+03 | 1.94E−02 | 5.24E−03 | 1.09E+08 | 4.03E+08 |
| 15SeP1 (H2O2) | 2.33E+03 | 1.87E−02 | 9.64E−03 | 1.25E+08 | 2.42E+08 |
| 15SeP1 (CuOOH) | 1.94E+03 | 1.26E−02 | 2.57E−04 | 1.54E+08 | 7.55E+09 |
| Se-4A (H2O2) (22) | 3.0E−03 | 4.16 | 2.80E−01 | 7.20E+05 | 1.10E+07 |
| Imprinted enzyme (H2O2) (23) | 7.88E+02 | 1.48 | 1.24 | E+08 | E+09 |
| Native GPX (H2O2) (25) | | | | | |
native GPX and 15SeP₁, indicating that the native GPX has more specific binding for the second substrate, H₂O₂, than does 15SeP₁. Because the GPX enzyme catalyzes a double substrate reaction, it is more reasonable to consider the binding for both substrates in the peptide GPX mimic. According to the active site structure of the native GPX, we proposed that the GPX mimic should include a hydrophobic environment, which is essential for the GSH and H₂O₂ (ROOH) binding, and that the GPX activity may therefore be enhanced upon increasing the binding affinity for the substrate, H₂O₂/ROOH. In doing this, the sequence of first four amino acids from the N terminus of 15P₁, Gly, Pro, Pro, and Pro, was changed to the sequence of Trp, Pro, Phe, and Leu. There are several reasons for this alteration, as follows: (i) these amino acids are highly hydrophobic; (ii) they appeared at a relatively high rate of frequency in the active central structure of native GPX (Fig. 2); (iii) the sequence of the four modified amino acids can form a structure that shares the most similarity with the active central structure of native GPX (Fig. 2) through computer modeling with Insight II modeling software; and (iv) these amino acids appeared at a relatively high rate of frequency in the sequences of 15-mer peptides screened out from the phage library (Table I). The altered peptide sequence, WPFLRHNVYGRPRAS, is named 15P.

The preparation of the selenopeptide 15SeP was same as that of 15SeP₁ in Scheme 1. The GPX catalytic efficiency of 15SeP was measured (Table III). We compared the GPX activities and kinetics of 15SeP with those of 15SeP₁ (Tables III and IV). The activity of 15SeP that catalyzed the reduction of H₂O₂ by GSH (260 units/μmol) is much higher than that of 15SeP₁ (190 units/μmol). More significantly, the activity of 15SeP was increased much more compared with that of 15SeP₁ when the substrate H₂O₂ was changed to CuOOH; the activity of 15SeP for the reduction of CuOOH by GSH (1176 units/μmol) is 4.5 times that of 15SeP for the reduction of H₂O₂ by GSH (260 units/μmol), whereas the activity of 15SeP₁ for the reduction of CuOOH by GSH (570 units/μmol) was only three times of that of 15SeP₁ for the reduction of H₂O₂ by GSH (190 units/μmol).

The association constant for 15SeP to GSH (3.151E⁻¹⁰⁴) was not greatly lower than that of 15SeP₁ (7.263E⁻¹⁰⁴) (Table II). This finding is consistent with the result that the association constant for 15P to GSH, 3.478E⁻¹⁰⁵, was close to that of 15P₁, 7.974E⁻⁰⁵. There was no obvious difference between 15SeP and 15SeP₁ when kcat/Km for GSH was compared. These results show that 15SeP and 15SeP₁ have similar substrate binding abilities toward GSH.

There is a 10-fold difference in the kcat/Km for Cu(II)O₂⁻ between 15SeP and 15SeP₁ (Table IV), indicating that 15SeP has more specific binding for H₂O₂ than does 15SeP₁. Thus, the selenopeptide 15SeP had very strong ability to bind not only to the substrate GSH but also to the substrate H₂O₂. Furthermore, the affinity of 15SeP for H₂O₂ was the highest among the existing GPX mimics and is close to that of native GPX. These data showed that the 15SeP obtained from the phage display peptide library had very high specificity for binding the first substrate, GSH, and was superior to Se-Fv (22) and the imprinting enzyme (23). In addition, 15SeP has a stronger activity to bind the hydrophobic hydroperoxide CuOOH. Its kcat/Km for Cu(II)O₂⁻ (3.55E⁻⁰⁹ M⁻¹ min⁻¹) is significantly higher than that of 15SeP₁ (3.03E⁻⁰⁸ M⁻¹ min⁻¹), indicating that 15SeP had a much stronger hydrophobic microenvironment for catalysis than does 15SeP₁ and, therefore, substrates with a strong hydrophobic property were suitable substrates for 15SeP.

The results for activity and kinetics measurements are consistent with our earlier hypothesis that 15SeP, with more hydrophobic microenvironment, should be easier for substrate
ROOH binding than 15SeP, which led to higher GPX activity.

Antioxidant Ability of the GPX Mimic Selenopeptide—The antioxidant effect of 15SeP is shown in Fig. 4. The culture of epidermal cells was treated with 1 mM H$_2$O$_2$, followed by treatment with or without protective agents. The MDA contents were measured to reflect the lipid peroxidation (32, 35), LDH activity was assayed to assess the cell leakage as a result of the peroxidation (36), and cell viability was also measured to evaluate the oxidative damage. MDA content, LDH leakage, and the decrease of the cell viability induced by H$_2$O$_2$ were measured and set as the standard for comparison (Fig. 4; *, p > 0.05). 15SeP significantly reduced the level of MDA, lowered the LDH activity, and increased the viability of cells (Fig. 4; *, p > 0.05). The increase of the MDA content and LDH leakage induced by H$_2$O$_2$ were also inhibited by ebselen at the same molar amount of 15SeP, but they were obviously higher than that of 15SeP group (Fig. 4; *, p < 0.05), and the cell viability was lower than that of 15SeP group (Fig. 4; #, p < 0.05). This indicates the antioxidant ability of 15SeP is superior to that of ebselen. At the same time, we also compared the antioxidant ability of 15SeP with that of another GPX mimic, 6-diSeCD. The GPX activity of 15SeP (260 units/μmol) is 19.3-fold higher than 6-diSeCD (13.5 units/μmol; see Table III). We found that the efficacy of 6-diSeCD to抗氧化ize in reducing MDA content and LDH activity and increasing cell viability was between that of 15SeP and ebselen, indicating that the antioxidant ability of 15SeP is also superior to that of 6-diSeCD. Taken together, the GPX activity is critical for a GPX mimic in clinical application. Although native GPX has high activity, it has many drawbacks for clinical application, such as poor stability, immunogenicity, and bigger molecular mass (~80 kDa), which limited its cellular accessibility. The activity of 15SeP is 22.2-fold lower than that of native GPX, but the former does not have the drawbacks of the latter. This finding gives 15SeP great pharmaceutical potential. In addition, as is also shown in Fig. 4, 1 μM 15SeP did not affect the viability of epidermal cells, indicating that 15SeP itself has very low toxicity to the cells.

More importantly, we found that the 15SeP can penetrate into cell membrane and increase the GPX activity in the cells (Fig. 5). As shown in Fig. 5, the GPX activity in epidermal cells, to which 1 unit/ml 15SeP was administered for 1 h, was only 0.26 units/ml, indicating that only 26% of 15SeP entered into the epidermal cells. The GPX activity in the cells was increased with time, indicating that more and more 15SeP molecules penetrate the cell membrane to enter the cells, and ~84% of 15SeP penetrated into epidermal cells within 10 h. Compared with the existing mimics, our new GPX mimic has rather high activity and specificity. It displayed strong antioxidant effect, low toxicity, better water solubility, and small size. These advantages indicate that 15SeP could be a more effective antioxidant in clinical application.

Because of the lack of GSH binding site, early GPX mimics...
usually have rather low activities. Take, for example, the well known GPX mimic dithiol; its activity is only 0.99 units/μmol. Wilson et al. (30) introduced a quaternary ammonium salt close to the diselenide bridge of a GPX mimic to improve its ability to bind GSH by electrostatic attraction to GPX. As a result, the activity of diphenyl diselenide compound can be increased up to 11 units/μmol. The key to imitating GPX is to generate affinity for GSH. Based on this idea, we have developed several selenium-containing catalytic antibodies such as Se-Fv (22) and Se-scFv (20). They had high catalytic activities, but their molecular sizes were large. To reduce the molecular size of the GPX mimic by taking advantage of the phage display technology, we created a new mimic, 15SeP, with the specific affinity for GSH. As a result, the binding to the first substrate, GSH, and also created the favored microenvironment for the second substrate, H2O2, aided by rational design. Data showed that the helpful microenvironment for the second substrate was equally important in order to generate a high activity of mimic. Our results indicate that recognition sites for two substrates should be considered at the same time for imitating the enzyme that catalyzes a double substrate reaction.

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