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Selenopeptides

Antioxidative Glutathione Peroxidase Activity of Selenoglutathione**

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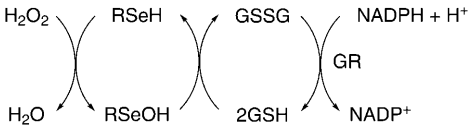
Selenoglutathione (GSeH),[1] a tripeptide comprising L-γ-glutamic acid (Glu), L-selenocysteine (Sec), and glycine (Gly), is a selenium analogue of glutathione (GSH), a biologically important redox substrate. This unique selenopeptide is attracting increasing attention as it was recently demonstrated that the oxidized form, that is, GSeSeG (**1**), is useful for oxidative folding of disulfide (SS)-containing proteins, such as bovine pancreatic ribonuclease A (RNase A) and trypsin inhibitor (BPTI).[2] Interestingly, diselenide **1** can even catalyze the folding reactions.[3] Due to the structural similarity to oxidized glutathione (GSSG), it is also a possible substrate for glutathione reductase (GR),[2] an enzyme that catalyzes reduction of GSSG with reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Thus, GSeH and GSeSeG (**1**) are promising targets to explore their unique biological functions. We recently succeeded in synthesizing a Sec derivative, *N*-(9-fluorenylmethoxycarbonyl)-*Se*-(*p*-methoxyphenylmethyl)-L-selenocysteine (Fmoc-Sec(MPM)-OH, **2**) using L-cystine as a starting material.[4] As an extension of our research, various Sec-containing peptides have now been synthesized from **2** by application of solid-phase peptide synthesis (SPPS), and their catalytic activity as an antioxidant has been investigated by employing the enzymatic assay of glutathione peroxidase (GPx), a well-known antioxidant selenoenzyme having a Sec residue at the redox-active site.[5]

The Sec-containing peptides[6] targeted in this study involve **1** and oxidized dimers of tri- and pentapeptides (**3** and **4**, respectively) having an amino acid sequence of the GPx active site. These peptides were synthesized by SPPS using Fmoc-Gly Alko-PEG resin or Fmoc-Gly or Fmoc-Thr(*t*Bu) Wang resin as a polymer support, by following the normal Fmoc methodology. The synthesized peptides were cleaved from the polymer support by using Reagent K[7] and reacted with trifluoroacetic acid (TFA) and 2,2′-dithiobis(5-nitropyridine) (DTNP)[8] to promote deprotection of the MPM group. The crude products were purified by reversed-phase (RP) HPLC using a Mightysil RP-18 column with a solvent gradient of acetonitrile (10–25% in H_2O), and fully characterized by MALDI-TOF mass spectrometry (MS) and amino acid analysis.

GPx-like antioxidant activity of the synthesized peptides was first assayed according to the reaction shown in Scheme 1.[9] In this assay, the reduction rate of hydrogen peroxide (H_2O_2) was monitored by a decrease in the UV



Scheme 1. Catalytic cycle of the GSH-coupled GPx activity assay for a selenium catalyst.

absorbance at 340 nm resulting from NADPH, which was added to the assay solution to reduce GSSG (a counter-product of the H_2O_2 reduction) to GSH in the presence of GR. Diselenides would enter the catalytic cycle either through oxidation to the corresponding selenenic acid (RSeOH) and seleninic acid ($RSeO_2H$), or by reduction to the selenol (RSeH). A recent investigation on the GPx cycle of selenocystine ($[H_2N\text{-Sec-OH}]_2$, **5**) revealed that the oxidation path is more feasible than the reduction path.[10]

Initial velocities for the reduction of H_2O_2 were measured in the presence of a catalytic amount of various diselenide compounds. The results are summarized in Table 1. When GSeSeG (**1**) was applied as a catalyst, the initial velocity for the reduction of H_2O_2 ($> 210\ \mu M\ min^{-1}$) was largest among the tested diselenides including diphenyl diselenide (PhSeSePh), a commonly used standard material for the GPx assay,[11] thus indicating the prominent antioxidant activity. It is also notable that the catalytic activity decreases in the order **5** > **3** > **4**, as amino acids are added to both sides of the Sec residue. The trend suggests that the local amino acid sequence at the GPx active site would not be essential for enhancement of the antioxidant activity.[12]

The high GPx activity of **1** can be ascribed to several causes. One possible reason is that **1** participates in the reaction cycle shown in Scheme 2 because **1** is also a possible substrate for GR.[2] The presence of this bypass cycle was indeed confirmed by the distinct GPx activity observed in the

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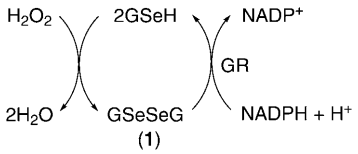
Table 1: Antioxidative GPx activity of selenopeptides and related compounds.

Catalysts	Initial velocities for the reduction of H_2O_2 [μM min^{-1}]		
	GSH[a]	Without GSH[b]	DTT^{red}[c]
$[H_2N$-γ-Glu-Sec-Gly-$OH]_2$ (**1**)	>210[d] (24±3)	>170[d] (7.5±0.7)	2000±100 (330±10)
$[H_2N$-Leu-Sec-Gly-$OH]_2$ (**3**)	44±6	1.3±0.2	1500±100 (290±10)
$[H_2N$-Ser-Leu-Sec-Gly-Thr-$OH]_2$ (**4**)	39±3	0.44±0.12	1600±100 (300±20)
$[H_2N$-Sec-$OH]_2$ (**5**)	112±10 (12±2)	0.35±0.12	1400±100 (240±10)
PhSeSePh	118±8 (15±1)	–	1200±100 (160±10)
None	10±2	0.39±0.12	110±10

[a] Reaction conditions: $[GSH]_0$ = 4 mM, $[H_2O_2]_0$ = 0.25 mM, $[NADPH]_0$ = 0.3 mM, [GR] = 4 units mL^{-1}, and [catalyst] = 50 μM in pH 7.4 phosphate buffer at room temperature. The values in parentheses were obtained at [catalyst] = 1 μM. [b] The reaction conditions were the same as [a], but GSH was not added. The values in parentheses were obtained at [catalyst] = 1 μM. [c] Reaction conditions: $[DTT^{red}]_0$ = 10 mM, $[H_2O_2]_0$ = 2 mM, and [catalyst] = 20 μM in pH 7.0 phosphate buffer at 25 °C. The values in parentheses were obtained at [catalyst] = 2 μM. [d] Exact values could not be determined because of the high activity.



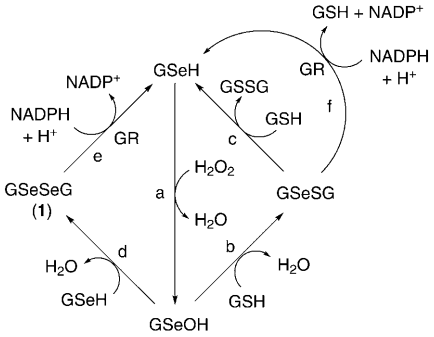
Scheme 2. Catalytic cycle of the GPx activity assay for GSeSeG (**1**) in the absence of GSH.

absence of GSH (>170 μM min^{-1}): under such conditions, no catalytic activity was observed for **4** and **5** although a slight activity was observed for **3**. Diselenide **1** would be regenerated by the rapid coupling reaction between GSeOH and GSeH in the absence of GSH. The bypass cycle (Scheme 2) should enhance the GPx activity of **1** to a significant extent, but it must not be enough to explain the prominent antioxidant activity exhibited by **1** as the distinct activity (24 μM min^{-1}) was still observed when the catalyst concentration was decreased from 50 to 1 μM: under such conditions, formation of diselenide **1** would be less feasible.

In the GPx cycle, three active intermediates, namely RSeH, RSeOH, and RSeSG, interconvert through a ping-pong mechanism, and the reduction process, RSeSG + GSH → RSeH + GSSG, is considered to be the rate-determining step because of the slow reaction rate relative to the other two processes.[13] When **1** was applied as a catalyst, a selenenylsulfide intermediate GSeSG, which is a structural analogue to GSSG and GSeSeG (**1**), was produced. Therefore, GR may also catalyze the reduction of GSeSG to GSeH and GSH by using NADPH as a reducing cofactor. The presence of this process can be another reason for the high GPx activity of **1**.

However, when the GPx assay was carried out by using dithiothreitol (DTT^{red}) as a redox couple[14] instead of GSH (see Table 1), it appeared that **1** has an intrinsically high antioxidant activity. Since GR is not present in the DTT-coupled assay, only the main catalytic cycle with a ping-pong mechanism can take place. In spite of this, the initial reaction velocity was still larger than those observed for **5** and PhSeSePh.

The entire reaction mechanism for the GPx activity of **1** is shown in Scheme 3. GSeSeG (**1**) would be activated either by reduction (process e) or oxidation with H_2O_2 (corresponding to a reverse process d). Processes a–c constitute a main GPx cycle, in which process c is the rate-determining step. When an insufficient amount of GSH is present in the solution, another cycle of processes a, d, and e and a bypass process (f) would rescue the reaction. The bypass cycle should become more important at a higher concentration of **1**. These processes are effective only for GSeH as a catalyst because of the substrate specificity of GR. In the real assay solution, all processes should proceed competitively.



Scheme 3. Entire reaction mechanism for the antioxidative GPx activity of GSeSeG (**1**).

The reaction mechanism shown in Scheme 3 was reasonably supported by the following HPLC analysis. When **1** was reacted with NADPH in the presence of GR, GSeH was produced as the sole selenium product (Figure 1 A).[15] GSeH was then oxidized with H_2O_2 to $GSeO_2H$, which appeared at almost the same retention time as **1**. $GSeO_2H$ would be formed through highly reactive GSeOH, which could not be detected by HPLC under any conditions. The direct oxidation of **1** with H_2O_2 also produced $GSeO_2H$ though the reaction was slow. On the other hand, when GSeH was reacted with H_2O_2 in the presence of GSH, a new peak, which corresponds to GSeSG, was clearly observed (Figure 1 B). GSeSG and $GSeO_2H$ were characterized by MS analysis, whereas GSeH was oxidized to **1** during the fraction collection and subsequent lyophilization.

In the GPx assay of **1**, variable biological functions of GSeH as an effective antioxidant and a GSH substitute have been demonstrated. The change in the amino acid sequence seems to play an important role in deciding the antioxidant activity. Replacing the Leu moiety in **3** with γ-Glu in **1** enhanced the antioxidant activity significantly in the GSH- and DTT^{red}-coupled assays (see Table 1). On the other hand, Hilvert and co-workers[2] reported that **1** is a better reagent than GSSG for oxidative protein folding, even though the Se–Se bond of **1** is thermodynamically more stable than the S–S

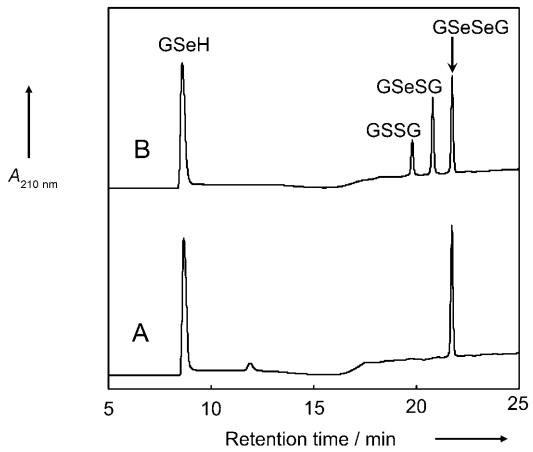


Figure 1. Chromatograms from RP-HPLC. A) Reaction mixture of GSeSeG (**1**; 0.11 mM) and NADPH (0.32 mM) in the presence of GR (4 U mL^{-1}) at room temperature in a pH 7.0 phosphate buffer solution. The reaction time was 10 min. B) Results 4 min after addition of GSH (3.3 equiv) and H_2O_2 (3.3 equiv) to the reaction mixture of (A). See the Experimental Section for details of the HPLC analysis conditions.

bond of GSSG. Taking these interesting features into account, GSeH would possess unique biological properties, which are probably related to the amino acid sequence. On the basis of the GPx activities obtained in the present work, we propose that the carboxylate of the zwitterionic γ-Glu residue (H_3N^+-CHR-COO^-) is important for regulation of the redox reactivity.[16]

Experimental Section

Procedure for SPPS of GSeSeG: The resin (1.28 g) attaching the Fmoc-protected Gly (0.23 mmol g^{-1}) was reacted with 20% piperidine and then **2** (100 mg, 0.20 mmol) activated with 1M *N*-hydroxybenzotriazole (HOBt) in *N*-methyl-2-pyrrolidone (NMP; 0.25 mL) and 1M dicyclohexylcarbodiimide (DCC) in NMP (0.25 mL) for 30 min. Subsequently, the resin was reacted with 20% piperidine and then Fmoc-γ-Glu(O*t*Bu)-OH (280 mg, 0.66 mmol) activated with 1M HOBt in NMP (0.50 mL) and 1M DCC in NMP (0.50 mL) for 30 min. After the reaction with 20% piperidine, the obtained resin (1.32 g) was treated with Reagent K[7] and then TFA and DTNP. The liberated peptide was purified by RP-HPLC. The yield of isolated GSeSeG (**1**) was 9% based on the resin employed. Tripeptide **3** and pentapeptide **4** were synthesized in a similar manner in approximately 3% yields. HPLC, MALDI-TOF-MS, and amino acid analysis data for **1**, **3**, and **4** are available in the Supporting Information.

Typical procedure for GSH-coupled GPx assay: A portion (300 μL) of a test solution (2.0 mL) containing NADPH (2.0 μmol), GSH (27.0 μmol), and GR (26.7 U) in a pH 7.4 phosphate/6 mM EDTA buffer solution was mixed with phosphate buffer solution (630 μL) containing a selenium catalyst: the concentration of the catalyst was regulated so that the final concentration was 1–50 μM. The reaction was initiated by addition of H_2O_2 (70 μL, 3.6 mM) to the mixture. The reaction progress was monitored by the absorption change at 340 nm resulting from consumption of NADPH.

Typical procedure for DTT-coupled GPx assay: A test solution (10 μL) containing DTT^{red} (1.0M) in a pH 7.0 phosphate buffer solution was mixed with a phosphate buffer solution (920 μL) containing a selenium catalyst at 25°C: the concentration of the catalyst was regulated so that the final concentration was 2–20 μM. The reaction was initiated by addition of H_2O_2 (70 μL, 28.6 mM) to the mixture. The reaction progress was monitored by the absorption change at 310 nm resulting from formation of DTT^{ox}.

Characterization of the intermediates: Compound **1** (0.11 mM) and NADPH (0.32 mM) were reacted at room temperature in a pH 7.0 phosphate buffer solution in the presence of GR (4 U mL^{-1}). After 10 min, GSH (3.3 equiv) and H_2O_2 (3.3 equiv) were sequentially added to the reaction mixture. Aliquots (90 μL) were taken from the reaction mixture and 1M HCl (10 μL) was added to quench the reaction. The products were analyzed by RP-HPLC using a Tosoh ODS-100V (4.6 mm × 150 mm) column at 35°C at a flow rate of 0.7 mL min^{-1}. A solvent gradient was applied (solvent A, 0.12% TFA in H_2O; solvent B, 0.12% TFA in acetonitrile; 10 min after sample injection, the %B was increased from 0 to 23.3% in 20 min). All solvents were rigorously bubbled with nitrogen before use. Fractionated GSeH and GSeSG were collected, lyophilized, and dissolved in 0.1% TFA in H_2O. The samples were analyzed by ESI(+)-TOF-MS on a Jeol JMS-T100LP mass spectrometer with an Agilent 1200 Series HPLC system. In the meantime, the mixture of **1** and H_2O_2 (15 equiv) was incubated at 25°C for 1 h, and the reaction products were analyzed by atmospheric-pressure chemical ionization(+)-TOF-MS on the same mass spectrometer. $GSeO_2H$ was observed as a main product.

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