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Thiol-targeted introduction of selenocysteine to polypeptides for synthesis of glutathione peroxidase mimics

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Because the seleno-L-cysteine (SeCys or Sec) insertion into selenoproteins occurs by a specific translational control process, it is quite difficult to express the SeCys-containing polypeptides even by the state-of-the-art genetic engineering techniques. In this paper, we describe a convenient synthetic method for the selective introduction of a SeCys derivative to polypeptides under physiological conditions. One SeCys residue in the seleno-L-cystine (SeCys–Se–Se–SeCys) methyl ester was first substituted with the Boc-protected penicillamine (Pen) methyl ester to form selenenylsulfide (SeCys–Se–S–Pen), an intermediate in the cellular glutathione peroxidase (GPx) catalytic cycle. Subsequently, the SeCys-Pen was coupled with the thiol-specific $N$-carboxymethylmaleimide through the $\alpha$-amino group of the SeCys {[(2-(N-maleimidyl)-1-oxo-ethyl-SeCys-methyl-Se-yl]-S-Pen methyl ester, MOE-SeCys-Pen}. The use of the MOE-SeCys-Pen allowed the selective introduction of the SeCys moiety to human serum albumin by alkylation of the thiol at its cysteine34, which generated the GPx-like activity responsible for the selenium atom in the MOE-SeCys-Pen. Consequently, this synthetic method will allow generating SeCys-containing artificial polypeptides with a GPx-like activity.
Introduction

The importance of selenium as an essential trace element in higher organisms is now well recognized. Oxidative stress is caused by an imbalance between the production of reactive oxygen species and the biological system’s ability to detoxify these reactive intermediates. Selenium-dependent glutathione peroxidases (GPxs) are the enzymes that can catalyze the reduction of \(\text{H}_2\text{O}_2\) and organic hydroperoxides using reduced glutathione (GSH) (\(\text{R--OOH} + 2\text{GSH} \rightarrow \text{R--OH} + \text{GSSG} + \text{H}_2\text{O}\)). These enzymes contain the seleno-L-cysteine (SeCys) residue, the 21st amino acid, in their catalytic centers. The SeCys residue is genetically coded by the UGA codon that is normally a signal for the polypeptide chain termination. The biosynthesis of SeCys to be incorporated in response to the UGA codon is distinctive from the twenty standard amino acids. Translation of the SeCys residue is a complicated mechanism which is controlled by the specific stem-loop SeCys insertion sequence (SECIS) located the downstream of the UGA codon and four execution elements [SeCys synthase (selA), an elongation factor (selB), seryl-tRNASeCys (selC), and selenophosphate synthase (selD)]. Because the SeCys insertion into the selenoproteins occurs by such a specific translational control process, it is quite difficult to express the SeCys-containing proteins even by the state-of-the-art genetic engineering techniques.

To understand the catalytic mechanism of natural GPxs and/or apply its peroxidase activity to medicine, a number of organoselenium compounds with low molecular masses (\(\leq 0.5 \text{ kDa}\)), e.g., ebselen, has been synthesized. Most of them have been previously known to be not yet comparable with the natural GPxs regarding their peroxidase activity. The X-ray structural analyses of the natural GPxs revealed a common structure of functionally important amino acid residues (e.g., glutamine 80 and tryptophan 154 in human GPx-1)
around the SeCys, namely a “catalytic triad”.\textsuperscript{19, 20} The efficient GPx activity appears to require the supporting roles of some amino acid residues within its polypeptide chain, something that seems difficult to achieve with low molecular mass organoselenium compounds. Indeed, the seleno-L-cystine [SeCyst, SeCys in the oxidized form] exhibits quite low GPx-like activity in the aqueous medium. Thus, higher-order structures of certain macromolecular scaffolds could possibly provide a means for obtaining a better chemical stability of the selenium atom and hence an improved GPx-like activity. We have reported a nano-structured GPx mimic using the polysaccharide pullulan conjugated with seleno-L-cystine, which improves the chemical stability of selenium atoms and hence increases the GPx-like activity.\textsuperscript{21} One of the potential scaffold candidates for such a purpose is a polypeptide material with a distinctive three-dimensional structure. Actually, several polypeptide-based SeCys-containing materials have been synthesized by which the selenation of polypeptides is performed by chemically converting the hydroxyl group of the serine residue into the active selenol using sodium hydroselenide (NaHSe).\textsuperscript{22, 23} However, the selenols are quite unlikely to be selectively introduced into the serine residues, because the selenation of hydroxyl groups from threonine and tyrosine residues also unavoidably occurs.

The purpose of this study is to develop a convenient and secure method for synthesizing structurally defined SeCys-containing polypeptides through their free thiols. In this study, human serum albumin (HSA) was used as a model scaffold polypeptide for the cysteine thiol-targeted SeCys introduction. HSA, a 585-residue single polypeptide, is monomeric globular, but contains three structurally similar $\alpha$-helical domains; each domain can be further divided into sub-domains A and B, which contain six and four $\alpha$-helices, respectively.\textsuperscript{24–26}
The most important structural feature is that it contains the single free thiol at Cys34 and the other 34 Cys residues intramolecularly form disulfide linkages. A synthetic method for the selective introduction of the SeCys moiety by alkylation of the thiol at the Cys34 of HSA using its maleimidyl (a thiol-specific group) derivatives was investigated.

**Experimental**

**Materials and instruments**

Seleno-L-cystine (SeCyst) and human serum albumin [HSA; purity, 98.9 %; the number of thiol groups determined by 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, Sigma Co., Ltd., St. Louis, MO) method, 0.31 ± 0.03 per HSA molecule] were obtained from Sigma Co., Ltd. L-Penicillamine (Pen) and L-cystine (Cyst) were from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Nacalai Tesque, Inc. (Kyoto, Japan), respectively. N-carboxymethylmaleimide (NCMM) was prepared from the condensation reaction of glycine and maleic anhydride. 2,3-Diaminonaphtharene (DAN) and the reduced glutathione (GSH) were obtained from Tokyo Chemical Ind. Co., Ltd. A selenium standard solution (1 g L\(^{-1}\) as Se(IV)O\(_2\) in 0.5 M nitric acid) used for the selenium determination was from Kanto Chemical Co., Inc. (Tokyo, Japan). Nicotinamide adenine dinucleotide phosphate in the reduced form (NADPH) and glutathione reductase (GR) were from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). \( \text{H}_2\text{O}_2 \), used for the substrate to determine the GPx-like activity, was obtained from Nacalai Tesque, Inc. Water, used for the preparations of the SeCys derivatives-HSA conjugates and their GPx-like activity measurements, was generated by a Milli-Q Biocel system (Millipore Corp., Billerica, MA). All other chemicals were of
commercial reagent or special grades and used as received.

Proton nuclear magnetic resonance (NMR) spectra were obtained on a Gemini 300 spectrometer (Varian Assoc., Palo Alto, CA) with TMS as an internal standard. Fourier transform infrared spectra (FTIR) were measured by the attenuated total reflectance (ATR) method using a FT/IR 4200 spectrometer and an ATR PRO410-S (JASCO Corp., Tokyo, Japan). A FP-6600 (JASCO Corp.) and a J725 (JASCO Corp.) were used for the measurements of fluorescence and circular dichroism spectra, respectively.

**Synthesis of bis[2-(N-maleimidyl)-1-oxo-ethyl]-seleno-L-cystine methyl ester (MOE-SeCyst)**

A mixture of SeCyst (30 mg) and p-toluenesulfonic acid (300 mg) dissolved in 10 mL of absolute methanol was refluxed for 40 h. After removal of the alcohol, the resulting solid was left for 24 h in diethylether. The obtained solid materials were washed several times with diethylether, and dried *in vacuo*. The obtained solid material was left in diethyl ether for another 24 h. The SeCyst methyl ester (17.5 mg) was allowed to react with N-carboxymethylmaleimide (15.5 mg, NCMM) in 5 mL of acetonitrile in the presence of O-(benzotriazole-1-yl)-N, N', N'-tetramethyluronium tetrafluoroborate (32.1 mg, TBTU) and triethylamine (15 μL, TEA) at room temperature. After the solvent evaporation, the resulting mixture was chromatographed under the following conditions: separation column, COSMOSIL 5C18-AR-II (φ2 × 25 cm, Nacalai Tesque, Inc.); mobile phase, 40 %(v/v) acetonitrile; flow rate, 5 mL min⁻¹; detection, UV 210 nm. The peak assigned to MOE-SeCyst was fractionated, and then followed by solvent evaporation (yield: 62.9 %).

$^1$H NMR (300 MHz, CDCl₃): $\delta$ 3.42–3.45 (t, 2H, $J = 5.1$ Hz), 3.78 (s, 3H), 4.29–4.31 (d, 2H,
$J = 4.2 \text{ Hz}$, 4.91–4.94 (m, 1H), 6.80 (s, 2H), 6.87–6.90 (d, 1H, $J = 7.5 \text{ Hz}$). FTIR (cm$^{-1}$): 1662 (imide C=O stretching), 1713 (amide C=O stretching), 1740 (ester C=O stretching).

MALDI TOF-MS (positive ion mode): calcd for C$_{20}$H$_{22}$N$_4$O$_{10}^{80}$Se$_2$ m/z 638.5, found 638.5.

**Synthesis of [2-(N-maleimidyl)-1-oxo-ethyl-seleno-L-cysteine-methyl-Se-yl]-S-L-penicillamine methyl ester (MOE-SeCys-Pen)**

A mixture of the SeCyst methyl ester (17.5 mg) and Boc-L-penicillamine methyl ester (12 mg, Boc-Pen-Me) dissolved in 3 mL of acetonitrile containing TEA (15 μL) was stirred for 1 h at room temperature. After the addition of NCMM (15.5 mg), the mixture was stirred for another 24 h in the presence of TBTU (32.1 mg) and then subjected to silica gel chromatographic separation using a 9:1 mixture by volume of CHCl$_3$ and CH$_3$OH as the mobile phase. The NCMM adduct was further treated with 2 mL of trifluoroacetic acid to remove the Boc group. After removal of the TFA by evaporation, the resulting mixture was left in 30 mL of diethyl ether for several hours to precipitate the solid materials. The obtained solid material was chromatographically separated under similar conditions as for MOE-SeCyst using 40 %(v/v) methanol in water containing 0.1 %(v/v) TFA as the mobile phase. The peak assigned to MOE-SeCys-Pen was fractionated, then followed by solvent evaporation (yield: 54.8 %). $^1$H NMR (300 MHz, CD$_3$OD): $\delta$ 1.49–1.52 (d, 6H, $J = 6.9 \text{ Hz}$), 3.16–3.50 (m, 2H), 3.75 (s, 3H), 3.87 (s, 3H), 4.10 (s, 1H), 4.22–4.23 (d, 2H, $J = 1.8 \text{ Hz}$), 4.80 (m, 1H), 6.90 (s, 2H). FTIR (cm$^{-1}$): 1671 (imide C=O stretching), 1714 (amide C=O stretching), 1741 (ester C=O stretching). MALDI TOF-MS (positive ion mode): calcd for C$_{16}$H$_{22}$N$_5$O$_7$S$_8^{80}$Se m/z 481.5, found 481.4.
Synthesis of cysteine analogues MOE-Cyst and MOE-Cys-Pen

As the control materials, the cysteine analogues MOE-Cyst and MOE-Cys-Pen, which are substituted by cysteine in place of SeCys of MOE-SeCyst and MOE-SeCys-Pen, were synthesized by procedures similar to that already described. Yield: 55.3 % for MOE-Cyst and 25.5 % for MOE-Cys-Pen. \(^1\)H NMR (300 MHz): MOE-Cyst (CDCl\(_3\)), \(\delta\) 3.22–3.23 (d, 2H, \(J = 4.8\) Hz), 3.78 (s, 3H), 4.29–4.31 (d, 2H, \(J = 6.6\) Hz), 4.86–4.88 (m, 1H), 6.80 (s, 2H), 6.94–6.97 (d, 1H, \(J = 7.5\) Hz); MOE-Cys-Pen (CD\(_3\)OD), \(\delta\) 1.44–1.52 (d, 6H, \(J = 20.7\) Hz), 3.10–3.20 (m, 2H), 3.75 (s, 3H), 3.87 (s, 3H), 4.17 (s, 1H), 4.23–4.24 (d, 2H, \(J = 2.1\) Hz), 4.80 (m, 1H), 6.90 (s, 2H). FTIR (cm\(^{-1}\)): MOE-Cyst, 1663 (imide C=O stretching), 1714 (amide C=O stretching), 1740 (ester C=O stretching); MOE-Cys-Pen, 1670 (imide C=O stretching), 1706 (amide C=O stretching), 1741 (ester C=O stretching). MALDI TOF-MS (positive ion mode): MOE-Cyst, calcd for C\(_{20}\)H\(_{22}\)N\(_4\)O\(_{10}\)S\(_2\) m/z 542.6, found 542.6; MOE-Cys-Pen, calcd for C\(_{16}\)H\(_{22}\)N\(_3\)O\(_7\)S\(_2\) m/z 433.5, found 433.4.

Conjugation of MOE-SeCyst and MOE-SeCys-Pen to HSA

Before the conjugation with MOE-SeCyst and MOE-SeCys-Pen, HSA was treated with 5-fold molar dithiothreitol (DTT) in 10 mM phosphate buffer (pH 7) with stirring for 1.5 h at room temperature. The resulting solution was ultrafiltered using an Amicon Ultra-4 Ultracel-10K Centrifugal Filter (nominal molecular mass cutoff, 10 kDa, regenerated cellulose membrane) to remove DDT, followed by lyophilization. The number of thiols determined by the DTNB method was 1.00 ± 0.00 per HSA molecule. The DTT-treated HSA (30 mg) dissolved in 3 mL of 10 mM phosphate buffer (pH 7) was combined with MOE-SeCyst (or
MOE-SeCys-Pen) (1 mg) dissolved in 1 mL of 10 % (v/v) acetonitrile (or methanol)-containing 10 mM phosphate buffer (pH 7). The mixture was allowed to react for 1 h with stirring at 35 °C. After the addition of the appropriate volume of deionized water, the resulting mixture was subjected to the ultrafiltration to remove any of the unreacted MOE-SeCyst or MOE-SeCys-Pen, followed by lyophilization (fluffy white materials, yield: 80 % for MOE-SeCyst-HSA; 85 % for MOE-SeCys-Pen-HSA). All the conjugates were stored in a desiccator at ambient temperature until used. The Cys analogues, MOE-Cyst and MOE-Cys-Pen, were also conjugated to HSA using the same procedure.

**Determination of selenium and HSA contents**

The selenium contents in the conjugates were fluorometrically determined using DAN after digestion by a 1 : 5 mixture by volume of HClO₄ and HNO₃. The HSA content was spectrophotometrically determined by Lowry’s method using bovine serum albumin as the reference.

**MALDI-TOF mass spectrometry of HSA derivatives**

The conjugate sample solutions were digested with trypsin (0.2 mg mL⁻¹ Milli-Q water) at 37 °C for 1 h. A matrix solution was prepared by dissolving a large excess amount of sinapinic acid (Fluka, Buchs, Switzerland) in 34 % (v/v) acetonitrile and 0.067 % (v/v) TFA. The digested sample solutions were mixed with the matrix solution in a one to three ratio by volume, and an aliquot was applied to an AnchorChip target (Bruker Daltonics, Inc., Billerica, MA) that was loaded with a sinapinic acid matrix thin layer. Mass spectra were
acquired in the linear positive ion mode using an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Inc.). Each spectrum was produced by accumulating data from 50 consecutive laser shots. Molecular mass calibration was carried out using the #206355 Protein Calibration Standard that is composed of insulin, ubiquitin I, cytochrome C and myoglobin (Bruker Daltonics, Inc.).

**Determination of GPx-like activity**\(^{15,28}\)

The solutions of HSA conjugates (final selenium or HSA concentration: 5 μM) were sequentially combined with ethylenediaminetetraacetic acid (1 mM), sodium azide (1 mM), glutathione reductase solution (1 unit), reduced glutathione solution (1 mM), and NADPH solution (0.375 mM) in 10 mM phosphate buffer (pH 7). The reaction was initiated by the addition of an \( \text{H}_2\text{O}_2 \) solution (0.5 mM). Absorbance at 340 nm due to the NADPH was recorded every 10 s just after mixing by inversion. The GPx-like activity was calculated using the following equation [eq (1)] as μmoles NADPH oxidized per minute, where \( \Delta A_{\text{SMP}} \) is the decrease in absorbance at 340 nm of the sample solutions between 10 and 190 SeCys after addition of the substrates, \( \Delta A_{\text{BLK}} \) is the decrease in absorbance at 340 nm per minute of the solutions without the conjugates, \( \varepsilon \) mM is the molar extinction coefficient for a 1 mM NADPH solution [6.22 mM\(^{-1}\) cm\(^{-1}\)], and \( c \) is final selenium concentration (μM).

\[
\text{GPx-like activity} = \left( \Delta A_{\text{SMP}} - \Delta A_{\text{BLK}} \right) \times 1000 \times \varepsilon^{-1} (\text{mM}^{-1} \text{cm}^{-1}) \quad (1)
\]

**Results and discussion**

In this study, seleno-L-cystine [SeCyst, seleno-L-cysteine (SeCys) in the oxidized form] was
used as the source material for the introduction of SeCys to the proteinous scaffolds. As control materials, the cysteine analogues, which are substituted by sulfur in place of the selenium in SeCys, were also synthesized by similar procedures using L-cystine [Cyst, L-cysteine (Cys) in the oxidized form] as the starting material. The α-carboxyl groups of SeCyst were esterified with methanol, and the SeCyst methyl ester (SeCyst-Me) was then coupled with a thiol-reactive maleimide moiety through its α-amino groups (MOE-SeCyst, Scheme 1A). Subsequently, the MOE-SeCyst was allowed to react with HSA in 10 mM phosphate buffer (pH 7). Because nearly 70 % of the Cys34 thiol in the commercially available HSA is oxidized to sulfenic acid (Cys34–SOH), sulfinic acid (Cys34–SO₂H) and/or the disulfide dimer (HSA-Cys34–S–S–Cys34-HSA), the received HSA was treated with dithiothreitol before the conjugation with MOE-SeCyst.

The resulting MOE-SeCyst-HSA conjugate was analyzed by MALDI TOF mass spectrometry after the tryptic digestion. A Cys34-containing fragment before the conjugation with the MOE-SeCyst was detected at 12,995 m/z (Fig. 1A), which was identified by the characteristic molecular mass gain (m/z = 125) after the reaction of HSA with a thiol-specific N-ethylmaleimide (Fig. 1B). The digested fragment from the MOE-SeCyst-HSA conjugate gave a peak at 13,327 m/z (Fig. 1C), resulting in an increase in m/z by 332 in comparison to the unconjugated one. Such an increase in the molecular mass corresponded to approximately half of that of the MOE-SeCyst. Meanwhile, the same Cys34-containing fragment from the cysteine analogue MOE-Cyst-HSA conjugate provided an increase in m/z by 553, which was in good agreement with the molecular mass of the MOE-Cyst (Fig. 1D). Because the MOE-SeCyst involves two chemically equivalent maleimide moieties at both ends, the reaction with the thiol at Cys34 of HSA was expected to
generate two kinds of molecular species; i.e., 1 : 1 and 1 : 2 molar ratios of the MOE-SeCyst to HSA. However, neither of them appeared in the mass spectrum of the MOE-SeCyst-HSA conjugate. Another possible mode of reaction is the thiol exchange between the diselenide (−Se−Se−) of the MOE-SeCyst and the thiol at Cys34 of HSA. Consequently, the mass spectral data were most probably thought to generate a conjugate with SeCys through a selenenylsulfide linkage from the reaction of the MOE-SeCyst with HSA (−Se−S−, Fig. 2A).

In general, nucleophilic thiol attack at the selenium atom is both kinetically and thermodynamically more favorable than at the sulfur one. In the synthetic pathway using the diselenide-containing SeCyst derivative, the thiol at Cys34 of HSA was favorably reacted with the diselenide by thiol exchange rather that by alkylation with maleimide. When such a conjugate is subjected to the GPx-like activity measurement, the SeCys moiety is thought to easily detach from HSA by the nucleophilic substitution of GSH to the selenium for transformation into a low-molecular mass species that is not expected to have an improved activity. The substitution (alkylation) of selenium in SeCyst by the protective groups for the thiol and selenol (e.g., benzyl and trityl groups) may be an effective way to overcome such a reaction diversity. However, polypeptidic materials with defined three-dimensional structures restrict the reaction conditions to be used for the deprotection.

Taken all these considerations together, we designed a SeCys-maleimide derivative involving a selenenylsulfide bond in place of the diselenide of SeCyst (MOE-SeCys-Pen, Scheme 1B). A selenenylsulfide intermediate in the cellular GPx catalytic cycle can be cleaved by cosubstrate glutathione that is also used for the GPx-like activity measurement. However, the selenenylsulfide linkage is intrinsically less stable than the diselenide ones. Thus, the Boc-L-penicillamine methyl ester (Boc-Pen-Me) was used as a thiol exchange
reagent with the diselenide to form a selenenylsulfide linkage between SeCys and Pen. Pen is structurally similar to Cys, which possesses two methyl groups at the $\beta$ carbon atom of Cys. The substitution of Cys by Pen remarkably improves the chemical stability of labile selenium compounds (e.g., $R-S-\text{Se}-S-R'$) and nitrogen oxide due to steric constrain by the two methyl groups. In this synthetic pathway, the SeCyst-Me was reacted with $\text{Boc}$-Pen-Me to form the selenenylsulfide linkage, and subsequently with NCMM to allow coupling with the maleimide moiety. Finally, the $\text{Boc}$ protection at the $\alpha$-carboxyl group of Pen was removed by treatment with trifluoroacetic acid, followed by conjugation with HSA (Fig. 2B).

The Cys34-containing fragment from the MOE-SeCys-Pen-HSA conjugate gave rise to a peak at 13,472 $m/z$ (Fig. 1F), which was 477 $m/z$ greater than that for the unconjugated HSA. Such a change in the molecular mass was almost equal to that of the MOE-SeCys-Pen (480). A similar trend in the molecular mass change was observed for its cysteine analogue, MOE-Cys-Pen-HSA (Fig. 1E). Consequently, the MALDI TOF-MS measurements revealed that the MOE-SeCys-Pen was attached to the thiol at the Cys34 of HSA by alkylation with its maleimide moiety.

To clarify the reaction specificity, the selenium content in the MOE-SeCys-Pen-HSA was fluorometrically determined by the DAN method after wet digestion. No selenium was detected from the DTT-treated HSA and the MOE-Cys-Pen-HSA. The selenium content of the MOE-SeCys-Pen-HSA was 1.21 mg g$^{-1}$-HSA, which was in good agreement with the calculated value for the conjugate that contains one selenium atom per HSA molecule (1.19 mg g$^{-1}$-HSA). In addition, no remaining thiols were detected from the MOE-SeCys-Pen-HSA conjugate, as was determined by the DTNB method. These analysis data indicate that the MOE-SeCys-Pen moiety can be almost qualitatively introduced through
the thiol at the Cys34 of HSA.

To examine the three-dimensional structural changes in the scaffold HSA molecule due to the MOE-SeCys-Pen conjugation, the intrinsic tryptophan fluorescence and circular dichroism (CD) spectra ranging from 200 to 300 nm of the MOE-SeCys-Pen-HSA conjugates were measured. HSA involves 30 α-helix structures and the single tryptophan residue (Trp214) in the middle of the 12th α-helix. The fluorescence of its indole chromophore is highly sensitive to environment, making it a choice for monitoring the HSA conformation. The sulfur atom of the Cys34 is toward the interior of the HSA molecule and surrounded by the side chains of Pro35, His39, Val77 and Tyr84, which prevent the thiol from coupling with any external counterparts. Therefore, alkylation of the thiol may result in a change in the polypeptide backbone conformation that brings the thiol toward the exterior of this molecule.

The introduction of MOE-SeCys-Pen somewhat weakened the intensity of the tryptophan fluorescence without any accompanying peak wavelength shift, which was almost identical to that of the NEM-adduct (Fig. 3A). The CD spectrum of MOE-SeCys-Pen-HSA was nearly the same as those of the DTT-treated HSA used as the starting material and the NEM-adduct (Fig. 3B). From these CD spectral data, the α-helix contents were calculated to be 81 % for the MOE-SeCys-Pen-HSA (α-strand, 0 %; random coil, 20 %) and 79 % for the DTT-treated HSA (β-strand, 0 %; random coil, 19 %) using the k2d program. These spectroscopic observations suggest a change in the orientation of the α-helices in HSA responsible for the alkylation of the Cys34 thiol. Consequently, no remarkable structural changes in the HSA scaffold appear to occur due to the introduction of the MOE-SeCys-Pen.

The GPx-like activity of the MOE-SeCys-Pen-HSA was measured using H$_2$O$_2$ as a substrate (Table 1). Both the DTT-treated HSA and the MOE-Cys-Pen-HSA, the
sulfur-substituent in place of selenium in the MOE-SeCys-Pen-HSA, did not show any GPx-like activity. On the other hand, the MOE-SeCys-Pen-HSA gave rise to the GPx-like activity, which is responsible for the SeCys moiety being introduced in a model macromolecular scaffold, HSA. The observed activity of the conjugate was only slightly higher than that of ebselen for \( \text{H}_2\text{O}_2 \) (0.99 \( \mu \text{mol Se}^{-1} \text{ min}^{-1} \)). To improve the GPx-like activity of the MOE-SeCys-Pen-HSA, we are now designing some MOE-SeCys-Pen derivatives that can stabilize the selenium atom, like the catalytic triad of the native GPxs. The structures of the polypeptides also appear to be one of the key factors governing the GPx-like activity of the conjugates. Thus, instead of HSA, other polypeptides or genetically engineered ones that are substituted by the Cys moiety in place of specific amino acids may be effective for further improvement of the GPx-like activity.

In summary, we have described a synthetic method for the selective introduction of SeCys to polypeptides under physiological conditions without losing their higher structures. To avoid any undesired reaction of the diselenide with the thiols of the polypeptides, one SeCys moiety in SeCyst (SeCys–Se–Se–SeCys) was first substituted with Pen to form selenenylsulfide (SeCys–Se–S–Pen). Subsequently, the SeCys-Pen was coupled with the thiol-specific maleimide moiety through the \( \alpha \)-amino group of the SeCys in the MOE-SeCys-Pen. The use of the MOE-SeCys-Pen allowed the selective introduction of the SeCys moiety to polypeptides by thiol alkylation, which generated the GPx-like activity responsible for the selenium atom in the SeCys moiety. Consequently, this synthetic method will allow generating artificial polypeptides with a GPx-like activity.
References

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<td>HSA</td>
<td>&lt; 0.00</td>
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<tr>
<td>MOE-Cys-Pen-HSA</td>
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ᵃ The values are mean ± standard deviation for three or more separate samples.
Scheme 1. Synthetic pathways of MOE-SeCyst (A) and MOE-SeCys-Pen (B). NCMM, N-carboxymethylmaleimide; TBTU, O-(benzotriazole-1-yl)-N, N’, N’-tetramethyluronium tetrafluoroborate; TEA, triethylamine; TFA, trifluoroacetic acid; p-TSA, p-toluensulfonic acid.
Scheme 1. Synthetic pathways of MOE-SeCyst (A) and MOE-SeCys-Pen (B). NCMM, N-carboxymethyl maleimide; TBTU, O-(benzotriazole-1-yl)-N, N, N', N'-tetramethyluronium tetrafluoroborate; TEA, triethylamine; TFA, trifluoroacetic acid; p-TSA, p-toluensulfonic acid.
Fig. 1 Mass spectra of the HSA conjugates. (A), HSA, *12996 m/z; (B), NEM adduct, *13121 m/z; (C), MOE-Cyst- HSA, *13549 m/z; (D), MOE-SeCyst- HSA, *13327 m/z; (E), MOE-Cys-Pen-HSA, *13430 m/z; (F), MOE-SeCys-Pen-HSA, *13472 m/z. Asterisk-labeled peaks indicate the Cys34-containing fragment and its derivatives. Dashed lines in panels (B)–(F) express m/z of the free Cys34-containing fragment in panel (A).
Fig. 2 Structures of the SeCys derivative-containing HSA conjugates generated. (A), MOE-SeCyst-HSA; (B), MOE-SeCys-Pen-HSA.
Fig. 3 Tryptophan fluorescence (A) and circular dichroism (B) spectra of MOE-SeCys-Pen-HSA. Dashed lines, DDT-treated HSA; broken lines, NEM-treated HSA; solid lines, MOE-SeCys-Pen-HSA. Sample concentrations, 0.5 mg HSA mL$^{-1}$ for fluorescence measurement (A) and 0.25 mg HSA mL$^{-1}$ for circular dichroism measurement (B). Fluorescence spectra were obtained using excitation wavelength of 292 nm. Medium, 10 mM phosphate buffer (pH 7). Both fluorescence and circular dichroism spectra of DDT-treated HSA were identical to those of non-treated one.
A convenient synthetic method is developed for the selective introduction of a SeCys derivative to polypeptides under physiological conditions. SeCys-containing artificial polypeptides with GPx-like activity are generated.