— Regular Article —

Preparation of Oligoselenodiglutathiones and Their Suppressive Effects on Oxidative DNA Damage Induced by Catechol and Copper

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We previously reported that novel polyselenodithiols are produced along with selenotrisulfide when a thiol (penicillamine) is reacted with selenite (H_2SeO_3). Here, we report the production of oligoselenodiglutathiones and their suppressive effects on oxidative DNA damage. Oligoselenodiglutathiones were produced by exceeding the conventional reaction ratio of [H_2SeO_3]/[glutathione (GSH)] = 1/4. In liquid chromatography-mass spectrometry (LC/MS) analysis, the observed isotope patterns showed good agreement with the calculated isotope patterns assuming that two, three, or four selenium (Se) atoms were incorporated in the molecules. Based on ^{1}H NMR and MS data, the structures of oligoselenodiglutathiones were assumed to have a common symmetrical structure that was centered by linearly bound Se atoms "wedged" in the disulfide bond of two GSH molecules. At 8 μM, selenodiglutathione (GSSeSG) and diselenodiglutathione ($GSSe_2SG$) showed 80% suppression of the formation of 8-oxo-7,8-dihydroxy-2′-deoxyguanosine (in calf thymus DNA) induced by carcinogenic catechol and copper. The suppressive effects of GSSeSG and $GSSe_2SG$ were around threefold higher than that of glutathione disulfide at the same concentration, and the suppressive effect was not observed for H_2SeO_3 or GSH. Thus, formation of oligoselenodiglutathiones is important for Se or GSH to exert its protective effects on biomolecules from oxidative damage.

Key words —— oligoselenodiglutathione, oxidative DNA damage, selenotrisulfide, glutathione, selenium, antioxidative

INTRODUCTION

Selenium (Se) is an important micronutrient for human health with respect to antioxidation, anticarcinogenesis, reproduction, and maintenance of the endocrine and immune systems.[1] The nutritional essentiality of Se as a component of glutathione peroxidases was recognized in the early 1970s.[2] Many studies have been carried out since then, but the details of the biological functions of Se are incompletely understood.

Se is taken up in inorganic and organic forms (*e.g.*, selenocysteine, and selenomethionine).[3] Inorganic Se is proposed to be absorbed mainly as selenite (H_2SeO_3) and is reduced by the primary endogenous thiol glutathione (GSH) to produce selenodiglutathione (GSSeSG).[4,5] In *ex vivo* experiments on the absorption of H_2SeO_3, selenotrisulfides such as GSSeSG, selenocysteineglutathione, and selenodicysteine were detected in perfusates of the intestinal lumen of rats.[6] Production of selenotrisulfide has been shown to occur during the reaction of thiol compounds with H_2SeO_3 in a molar ratio of 4 : 1 under acidic conditions accompanying equimolar disulfide formation.[7] In cells, the Se in GSSeSG is believed to be reduced to selenide.[8,9] Selenide and ATP form selenophosphate, which is a Se donor required for synthesizing selenocysteinyl-tRNA.[10] The twenty-first amino acid selenocysteine is incorporated via translation into antioxidative enzymes, such as glutathione peroxidase and thioredoxin reductase.[1,11] Thus, GSSeSG is believed to be an important metabolic intermediate that causes the biological effects of Se.[12]

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In our previous study of selenotrisulfide production using penicillamine (PenSH), we observed the production of novel polyselenodipenicillamines ($PenSSe_{2-4}SPen$) along with selenotrisulfide (PenSSeSPen) by increasing the molar ratio of H_2SeO_3 to thiol.[13] In addition, we found that PenSSeSPen protects DNA from oxidative damage.[14] In the present study, we investigated if oligoselenodiglutathiones ($GSSe_nSG$, $n \geq 2$) are produced from GSH, which is a key player in Se metabolism, and if GSSeSG or $GSSe_nSG$ suppress the oxidative DNA damage that is induced by carcinogenic catechol and copper(II) [Cu(II)].

MATERIALS AND METHODS

Materials —— GSH, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA), β-nicotinamide adenine dinucleotide reduced form (NADH), copper(II) chloride dihydrate ($CuCl_2 \cdot 2\,H_2O$), Se dioxide (SeO_2), and deuterium oxide (D_2O) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glutathione disulfide (GSSG), calf thymus DNA, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), and 2′-deoxyguanosine (dG) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). Nuclease P1, calf intestine alkaline phosphatase (CIP), ethylenediaminetetraacetic acid, and catechol were obtained from Yamasa Shoyu (Choshi, Japan), Roche Diagnostics (Mannheim, Germany), Dojindo Laboratories (Kumamoto, Japan), and Tokyo Kasei Kogyo, Ltd. (Tokyo, Japan), respectively.

Preparation of GSSeSG and $GSSe_{2-4}SG$ —— $GSSe_{1-4}SG$ were prepared based on a previous report.[15] Briefly, 10 mM GSH was reacted with 40 mM H_2SeO_3, which was prepared by dissolving SeO_2 in distilled water in 400 μl of 2.5 mM HCl. GSSeSG and $GSSe_2SG$ were purified using preparative high performance liquid chromatography (HPLC, LC-10 series; Shimadzu, Kyoto, Japan) that was equipped with an octadecylsilane (ODS) column [Develosil C-18, 250 × 20 mm internal diameter (i.d.), 5-μm pore size; Nomura Chemical, Seto, Japan] under the following conditions: gradient program, linear gradient elution from 0.05% formic acid to 100% methanol for 65 min; flow rate, 4.5 ml/min; and column temperature, 40°C. Isolated GSSeSG and $GSSe_2SG$ were identified based on a previously reported method[13] by HPLC (LC-10 series, Shimadzu) and liquid chromatography-mass spectrometry (LC/MS analysis equipped with an Agilent 1100 Series HPLC system; Agilent Technologies Japan Ltd., Tokyo, Japan) coupled to an LCQ DECA XP ion trap mass spectrometer (Thermo Fisher Scientific, Yokohama, Japan) under the following HPLC conditions: ODS column (Develosil C-18, 50 × 4.6 mm i.d., 3-μm pore size, Nomura Chemical), linear gradient elution similar in composition to that of the isolating condition for 15 min at a flow rate of 0.8 ml/min monitoring at 265 nm. The structures of GSSeSG and $GSSe_2SG$ were analyzed by ^1H NMR[13] using a JEOL ECP500 spectrometer (Jeol Ltd., Tokyo, Japan). GSSeSG: ^1H NMR (500 MHz, D_2O) δ: 4.78 (1 H, dd, J = 8.9, 4.8 Hz), 4.01 (2 H, s), 3.85 (1 H, t, J = 6.4 Hz), 3.53 (1 H, dd, J = 14.4, 4.8 Hz), 3.32 (1 H, dd, J = 14.4, 8.9 Hz), 2.63–2.53 (2 H, m), 2.24–2.14 (2 H, m). $GSSe_2SG$: ^1H NMR (500 MHz, D_2O) δ: 4.78 (1 H, dd, J = 9.2, 5.0 Hz), 4.00 (2 H, s), 3.84 (1 H, t, J = 6.9 Hz), 3.57 (1 H, dd, J = 14.7, 5.0 Hz), 3.32 (1 H, dd, J = 14.7, 9.2 Hz), 2.62–2.52 (2 H, m), 2.22–2.16 (2 H, m). The molar concentrations of GSSeSG and $GSSe_2SG$ were calculated from the integral value based on the methyl proton value of 10 mM acetic acid (δ 2.10).

Measurement of Formation of 8-oxodG —— Calf thymus DNA (100 μM base) was incubated with 40 μM catechol, 20 μM $CuCl_2$, and 100 μM NADH in 0.4 ml of 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA for 1 hr at 37°C. After incubation, 10 μl of 4 mM DTPA, 40 μl of 3 M sodium acetate (pH 7.0), and 1 ml of 100% (v/v) ethanol were added, and the reaction mixtures were centrifuged at 14000 × *g* for 12 min at 4°C. After removing the supernatant, 500 μl of 70% (v/v) ethanol was added and recentrifuged at 14000 × *g* for 2 min at 4°C. The remaining ethanol was removed by centrifugal concentration under reduced pressure. DNA dissolved in 64 μl of distilled water was denatured at 90°C for 5 min and was immediately chilled on ice-cold water. Then, 20 μl of acetate buffer (pH 5.0) and 5 μl of 0.67 u nuclease P1 were added and incubated for 30 min at 37°C. Eleven microliters of 1 M Tris buffer (pH 7.5) containing 1.3 u CIP was added and incubated for 1 hr at 37°C. The formation of 8-oxodG in the reaction mixture was measured using an HPLC (LC-10 series, Shimadzu) equipped with an electrochemical detector (Coulochem II, ESA Inc., Chelmsford, MA, U.S.A.). The HPLC conditions were: column, ODS-80Ts (TSK-gel, 150 × 4.6 mm i.d.; Tosoh, Tokyo, Japan); column temperature, 25°C; flow rate, 1 ml/min; mobile

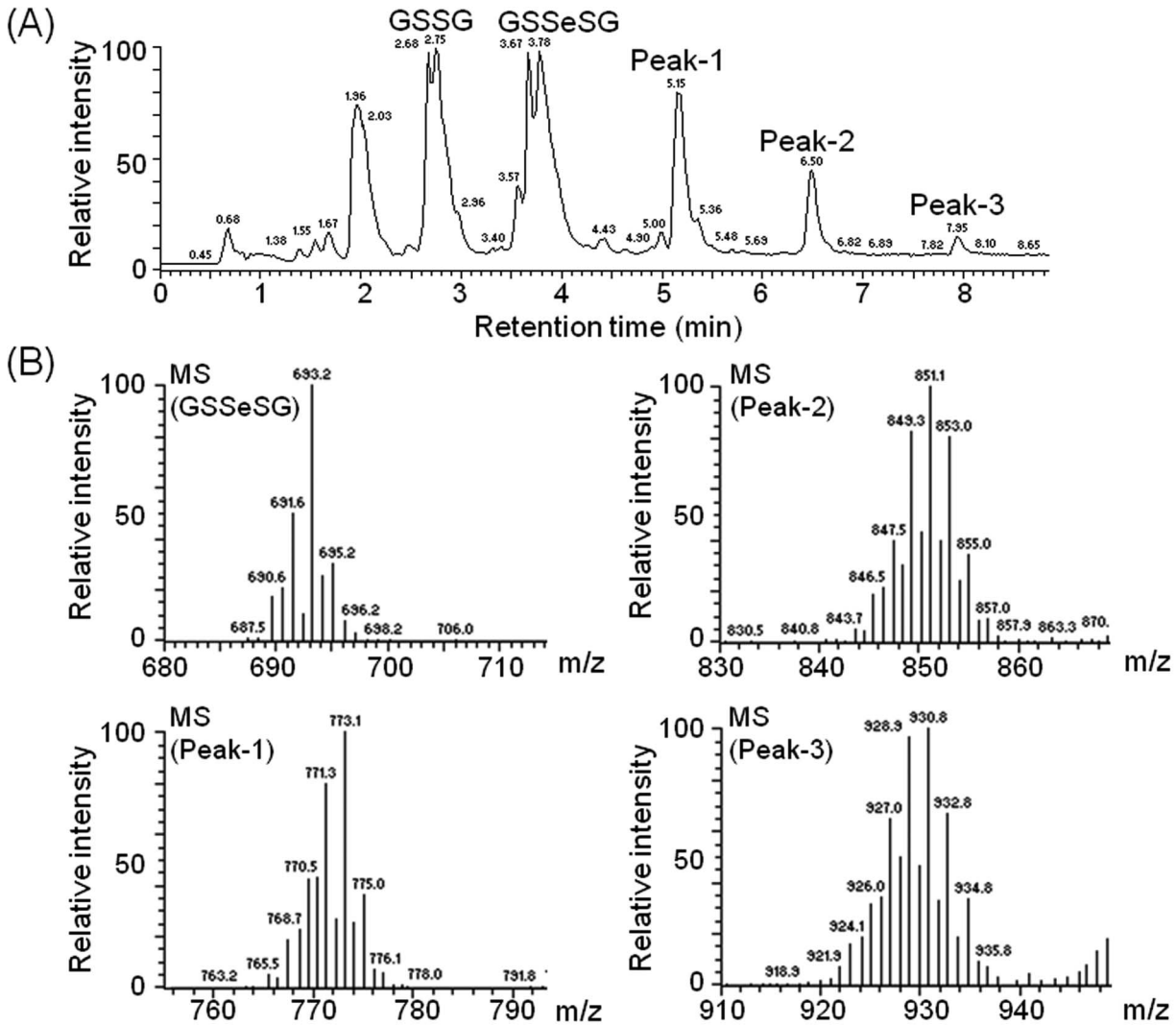

Fig. 1. Total Ion Chromatogram (TIC) and MS Spectra
(A) Reaction mixture of H_2SeO_3 and GSH; (B) GSSeSG, peak-1, -2, and -3.

phase, 8% methanol/10 mM sodium phosphate. Detection of dG was undertaken at 254 nm of UV absorption. The detection conditions for 8-oxodG were: guard cell, 400 mV; E, 150 mV; R, 100 μA; filter, 2; output, 1 V (channel 1); E, 300 mV; R, 200 nA; filter, 10; and output, 1 V (channel 2). GSH, H_2SeO_3, GSSeSG, or $GSSe_2SG$ was added before the addition of $CuCl_2$ to the reaction mixture.

RESULTS

Preparation of $GSSe_{2-4}SG$ by the Reaction of GSH with H_2SeO_3

When the molar ratio of H_2SeO_3 to GSH was increased compared with the conventional molar ratio of 4 : 1, three unknown peaks (designated peak-1, -2 and -3 in Fig. 1A) were clearly detected. The above-mentioned phenomenon was also observed in our previous study using PenSH after the elution of GSSG and GSSeSG on the total ion chromatogram of the LC/MS analysis of the reaction mixture. The m/z value (645) of the principal isotope ion of the peak eluted in 1.96 min was consistent with that of diglutathione ether, which is based on the analogy that the ether product (PenSOSPen) was eluted before the elution of disulfide when PenSH was used in our previous study. The mass spectra of peak-1, -2, and -3 gave $[M+H]^+$ ions at m/z 773, 853, and 933, respectively, corresponding to the addition of 80 (Se), 160 (2Se), and 240 (3Se) atomic mass units of GSSeSG (m/z 693, Fig. 1B). The observed isotope patterns were in good agreement with the calculated isotope patterns, which assumed

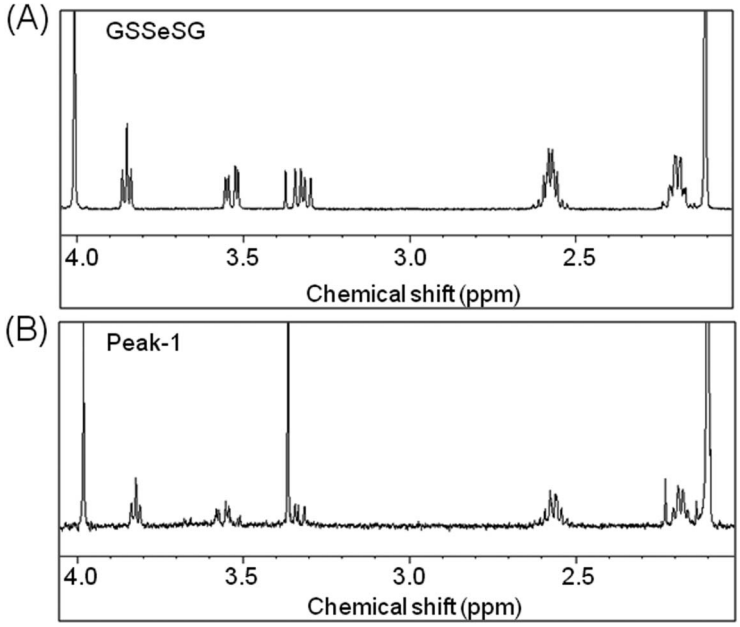

Fig. 2. ^{1}H NMR Spectra
(A) GSSeSG and (B) peak-1.

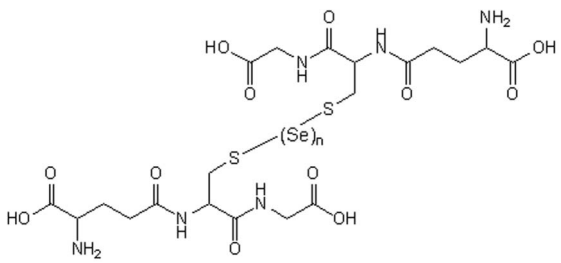

Fig. 3. Structures of Oligoselenodiglutathiones

that two, three, and four Se atoms were incorporated. In ^{1}H NMR analysis, the signals of methylene protons adjacent to each sulfur atom (-S-*CH*$_2$-) in GSSeSG shifted by around 0.28 ppm lower magnetic field than those observed in GSSG (Fig. 2A). Moreover, the signals of -S-*CH*$_2$- in peak-1 shifted by around 0.02 ppm lower magnetic field than those in GSSeSG (Fig. 2B). Additionally, based on the mass number and isotope patterns of peak-1, it was suggested that peak-1 was GSSe$_2$SG, in which the two Se atoms were supposed to be arranged linearly between sulfur atoms (Fig. 3). Based on the previously reported results that the observed isotope patterns in LC/MS analysis were consistent with those of polyselenodipenicillamines (PenSSe$_n$SPen, n = 3 or 4), although the proton signals of peak-2 and -3 were below the detection limit for ^{1}H NMR due to their small yields, peak-2 and -3 were supposed to be GSSe$_n$SG (n = 3 or 4, respectively).

Suppressive Effects of GSSeSG and GSSe$_2$SG on Catechol- and Copper-dependent Formation of 8-oxodG

To evaluate the antioxidative effects of GSSe$_n$SG, we investigated the suppressive effects of isolated GSSeSG and GSSe$_2$SG on 8-oxodG formation (in calf thymus DNA) induced by carcinogenic catechol and Cu(II). Under the buffer conditions applied for the DNA-damaging reaction, more than 80% of GSSeSG and GSSe$_2$SG were stably remained after 1 hr based on HPLC analysis (data not shown). H_2SeO_3 and GSH did not suppress 8-oxodG formation (Fig. 4). GSSG dose-dependently decreased 8-oxodG formation: 8-oxodG formation was decreased by 30, 60, and 80% in the presence of 8, 16, and 32 μM GSSG, respectively. Conversely, GSSeSG and GSSe$_2$SG suppressed 8-oxodG formation by 80% even at the lowest concentration tested (8 μM). Thus, the suppressive effect of GSSG on the oxidative DNA damage was considerably increased by forming GSSe$_n$SG, in which linearly bound Se atoms are "wedged" in the disulfide bond of two GSH molecules.

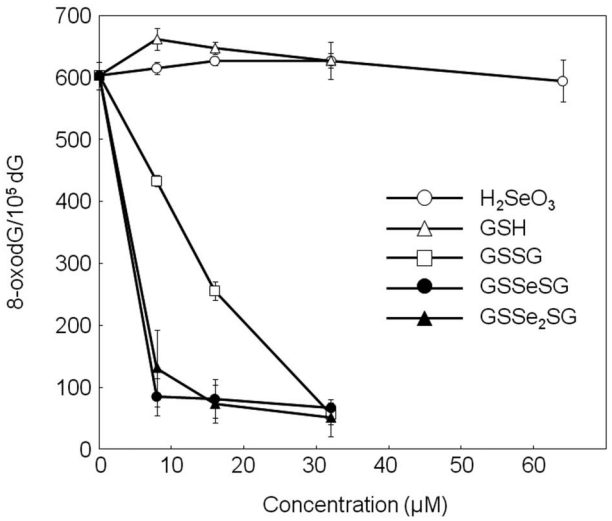

Fig. 4. Suppressive Effects of GSSG, GSSeSG, and $GSSe_2SG$ on 8-oxodG Formation (in Calf Thymus DNA) Induced by Catechol and Cu(II)
Data are mean ± S.D. (n = 3).

DISCUSSION

The present study showed the production of oligoselenodithiols $GSSe_nSG$ when GSH is reacted with H_2SeO_3. Our previous study using PenSH suggested that penicillamine selenopersulfide (PenSSeH) is involved in the production of $PenSSe_{2-4}SPen$.[13] Glutathione selenopersulfide (GSSeH) can be formed during the reduction of GSSeSG using excess GSH or glutathione reductase under physiological conditions.[16] Therefore, $GSSe_nSG$ can be formed in the human body.

In the present study, we demonstrated that GSSeSG and $GSSe_2SG$ suppressed 8-oxodG formation (in calf thymus DNA) induced by catechol and Cu(II) more effectively than GSSG. The latter would arrest the redox reaction of catechol and Cu(II) according to its ability to form complexes with Cu(II).[17] The above is also possible for GSSeSG and $GSSe_2SG$ to prevent DNA damage. Additional mechanisms must underlie the suppression of 8-oxodG formation by GSSeSG and $GSSe_2SG$. We previously proposed sequestration or oxidation of Cu(I) as a mechanism for PenSSeSPen to suppress Cu(II)-mediated oxidative DNA damage.[14] The same effects on Cu(I) could occur in GSSeSG and $GSSe_2SG$. Hydrogen peroxide is involved in the DNA damage induced by catechol and Cu(II),[18] but it was not the target of GSSeSG or $GSSe_2SG$ because PenSSeSPen is not decomposed in the presence of hydrogen peroxide (unpublished data). Hydrogen peroxide was formed spontaneously from the superoxide anion radical, thus, scavenging of the latter is also possible as a suppression mechanism for GSSeSG or $GSSe_2SG$. Therefore, the 8-oxodG formation induced by catechol and Cu(II) can be suppressed by GSSeSG and $GSSe_2SG$ more effectively than GSSG through not only complex formation with Cu(II) but also sequestration or oxidation of Cu(I) or the superoxide anion radical. One report suggests that GSSeSG has DNA-damaging activity.[19] However, in that report, DNA damage was observed under conditions in which the GSH concentration was 100-times higher than that of GSSeSG. GSH can induce metal-mediated oxidative DNA damage.[20] Therefore, in the presence of excess GSH, it would be difficult to prove that GSSeSG was involved in the DNA damage. GSH, known as an antioxidant, also promotes the oxidative stress in the reaction with Cu(II),[21] which is consistent with our present results (Fig. 4).

H_2SeO_3 suppresses the oxidative DNA damage *in vivo*,[22–24] but there is no such suppressive effect in our *in vitro* study. Both GSSeSG (a metabolic intermediate of H_2SeO_3) and $GSSe_2SG$ suppressed DNA damage in the present study. Therefore, they may be involved in the *in vivo* suppression of DNA damage by H_2SeO_3.[22–24]

In general, organic Se compounds are less toxic than inorganic Se.[3] Moreover, organic Se compounds are readily taken up and retained in the human body.[25,26] Therefore, GSSeSG and $GSSe_2SG$ are expected to be less toxic and effective suppressors of oxidative stress.

The present study suggests that the formation of $GSSe_nSG$ is important for Se or GSH to exert its protective effects on oxidative damage.

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