Selenoprotein P in Human Plasma as an Extracellular Phospholipid Hydroperoxide Glutathione Peroxidase

ISOLATION AND ENZYMATIC CHARACTERIZATION OF HUMAN SELENOPROTEIN P*

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Selenoprotein P is an extracellular protein containing presumably 10 selenocysteines that are encoded by the UGA stop codon in the open reading frame of the mRNA. The function of selenoprotein P is currently unknown, although several indirect lines of evidence suggest that selenoprotein P is a free radical scavenger. We first developed a conventional procedure to isolate selenoprotein P from human plasma. Next, we investigated the reactivities of selenoprotein P against various hydroperoxides in the presence of glutathione. Although selenoprotein P reduces neither hydrogen peroxide nor tertiary butyl hydroperoxide, it does reduce phospholipid hydroperoxide such as 1-palmitoyl-2-(13-hydroperoxy-cis-9,trans-11-octadecadienoyl)-3-phosphatidylcholine hydroperoxide. Kinetic analysis demonstrated a tert-unii ping-pong mechanism, similar to those described for classical glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase. Not only glutathione, but also dithiothreitol, mercaptoethanol, cysteine, and homocysteine, were effective as reducing substances, as in the case of phospholipid hydroperoxide glutathione peroxidase. These results show that selenoprotein P functions as a phospholipid hydroperoxide glutathione peroxidase in extracellular fluids.

Several proteins from bacteria and animals contain selenocysteine in their primary structure (1). Each of the cDNA clones of these selenoproteins contains one TGA codon, which corresponds to UGA in mRNA, in the open reading frame (1, 2). This UGA, known formerly only as a stop codon, encodes and presumably 10 selenocysteine that are encoded by the UGA stop codon in the open reading frame of its mRNA (20, 21). The deduced amino acid sequence of SeP is reported to have no similarities with se-

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‡ The abbreviations used are: GPx, glutathione peroxidase; GSH, reduced glutathione; GPx, cellular glutathione peroxidase; PH-GPx, phospholipid hydroperoxide glutathione peroxidase; GGPx, extracellular glutathione peroxidase; GI-GPx, gastrointestinal glutathione peroxidase; SeP, selenoprotein P; Ni-NTA, nickel-nitrilotriacetic acid; PLPC, 1-palmitoyl-2-linoleoyl-3-phosphatidylethanolamine; PLPC-OOH, 1-palmitoyl-2-linoleoyl-3-phosphatidylcholine hydroperoxide; HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry.

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Enzymatic Characterization of Human Selenoprotein P

EXPERIMENTAL PROCEDURES

**Chemicals**—Heparin-Sepharose CL-6B and Q-Sepharose Fast Flow were obtained from Amersham Pharmacia Biotech, Uppsala, Sweden; nickel-nitritotriacetic acid (Ni-NTA)-agarose was from Qiagen Inc., Chatsworth, CA.; diisopropyl fluorophosphate was from Kishida Chemical Co., Osaka, Japan; polyethylene glycol (number 4,000), tertiary butyl hydroperoxide, cumene hydroperoxide, and hydrogen peroxide were from Nacalai, Kyoto, Japan; 1-palmitoyl-2-linoleoyl-3-phosphatidylcholine (PLPC), GSH, and GSH reductase were from Sigma; and soybean lipoxidase was from Biozyme Laboratories Ltd, Blaenavon, Great Britain. For high performance liquid chromatography (HPLC), distilled water was further purified by Organo Puric model-S water purifier. Amino acid analysis was performed with a Waters Co. (Osaka, Japan). Methanol and 2-propanol were distilled before use. Human out-dated frozen plasma was kindly donated from Hokkaido Red Cross Blood Center. Other chemicals were of the highest quality commercially available.

**Selenoprotein P Purification**—All purification procedures were conducted at 4 °C, and diisopropyl fluorophosphate (2 ms) was added to each pooled fraction to avoid the proteolytic cleavage of SeP. Human plasma (1,000 ml) was mixed with 50 g of polyethylene glycol under stirring. After 1 h, the precipitate was removed by centrifugation at 8,000 rpm for 10 min. The supernatant was applied to heparin-Sepharose CL-6B column (50 ml) equilibrated with 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.5 mM EDTA, at a flow rate of 100 ml/h. After washing with the equilibrated buffer, the bound proteins were eluted by linear gradient increase of NaCl concentration with each 500 ml of 0.15 and 0.6 M NaCl in the buffer. Among three selenium-containing proteins in human plasma, only SeP reportedly bound to heparin-Sepharose. So, we detected SeP by measuring selenium contents after the heparin-Sepharose fractionation. The fractions containing SeP were pooled and diluted with 6 volumes of 20 mM Tris-HCl, pH 8.0, and directly applied to a column (20 ml) of G-Sepharose Fast Flow equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.06 M NaCl, at a flow rate of 30 ml/h. The bound proteins were eluted by linear gradient increase of NaCl concentration with each 150 ml of 0.06 and 0.3 M NaCl in the buffer. The fractions containing SeP were pooled, and imidazole and NaCl were added to the pooled fractions at a final concentration of 20 mM and 1 M, respectively. The fractions were applied to a column (1 ml) of Ni-NTA-agarose equilibrated with 20 mM Tris-HCl, pH 8.0, containing 20 mM imidazole and 1 M NaCl, at a flow rate of 30 ml/h. After washing with the equilibrated buffer, the bound proteins were eluted with 20 mM Tris-HCl, pH 8.0, containing 250 mM imidazole and 1 M NaCl. For further study, imidazole and NaCl were removed by passing through PD-10 gel filtration column equilibrated with the desirered buffer.

**Selenium Assay**—Levels of selenium in plasma and in column fractions was determined according to the fluorometric method of Bayfield and Romalis (24).

**Protein Assay**—Protein concentrations of samples at each chromatographic step were determined using absorbance at 280 nm. Protein content of purified preparation was determined using a protein assay kit (Bio-Rad) with bovine immunoglobulin G as a standard. To determine the selenocysteine content per mole of SeP, quantitative amino acid analysis was applied.

**Amino Acid Analysis**—The protein was dialyzed against distilled water and hydrolyzed in 6 M HCl for 24, 48, or 72 h or in 3 M mercaptoethanesulfonic acid for 24 h. After removal of solvent in vacuo, amino acid analysis was performed on a Pico-Tag system (Water, Millipore Corp.).

**Amino Acid Sequence Analysis**—After electrophoresis, proteins in the gel were transferred to a Pro Blot membrane (Applied Biosystems) in 20 mM Tris containing 150 mM glycine and 20% methanol electrically. The proteins on the membrane were stained with 0.1% Coomassie Brilliant Blue R-250 in 1% acetic acid and 40% methanol for 1 min, and then the membrane was destained with 50% methanol. The protein bands were cut off and their NH₂-terminal amino acid sequences were analyzed with a 473A Protein Sequencer (Applied Biosystems).

**Preparation of Monoclonal Antibody and Immunoprecipitation**—A method to prepare hybridomas producing rat monoclonal antibodies was used (26, 27). The enlarged medial iliac lymph nodes from rats immunized with hind footpads via footpad injection were used. Freud’s complete adjuvant were used for cell fusion, followed by hybridization, cloning, and establishment of hybridomas. Eleven hybridomas producing specific monoclonal antibodies against human SeP were obtained. Details will be described elsewhere. One monoclonal antibody (BD1, IgG2a, κ) was used for immunoprecipitation study. The BD1 antibody was coupled to agarose using cyanogen bromide (28). After the addition of increasing amounts of BD1-agarose to the purified SeP preparation, the enzyme activities of the supernatant were measured as described below.

**Preparation and Purification of 1-Palmitoyl-2-(13-hydroperoxy-cis-9,trans-11-octadecadienoyl) Phosphatidylethanolamine (PLPC-OOH)—PLPC-OOH was prepared from PLPC by oxidation with lipoxidase (29, 30).**

The reaction mixture, containing 3 mM sodium deoxycholate and 0.13 mM PLPC in 500 ml of 0.2 M Tris buffer (pH 8.5) was incubated with 50 mg soybean lipoxidase at room temperature for 4 h, under continuous stirring. PLPC-OOH was extracted from the reaction mixture with ethyl acetate. The ethyl acetate extract after evaporation under reduced pressure was dissolved in methanol and subjected to preparative HPLC on an ODS column with a mobile phase of acetonitrile/methanol/water (75:25:4) to obtain PLPC-OOH. The PLPC-OOH was dissolved in methanol and stored at −15 °C. The concentrations of the hydroperoxides were determined by a fluorimetric method (31, 32), using cumene hydroperoxide as standard for calibration. PLPC-OOH was confirmed by fast atom bombardment mass spectrometry (FAB-MS). The deprotonated molecular ion of PLPC-OOH was observed at m/z 788 (negative FAB-MS). PLPC-OOH was reduced to PLPC-OH by the addition of sodium borohydride. PLPC-OH was the only product of the reduction and was isolated from the reduction product by preparative HPLC on an ODS column with a mobile phase of methanol/water (91:9). The deprotonated molecular ion of PLPC-OOH was observed at m/z 772 (negative FAB-MS).

**Enzyme Assay—GPx activities were examined by following the oxidation of NADPH in the presence of GSH reductase, which catalyzes the reduction of oxidized GSH formed by GPx (33) with a slight modification. Both samples and reference cuvettes contained 0.1 M Tris-HCl, pH 8.0, and directly applied to a column (20 ml) of Q-Sepharose Fast Flow equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.06 M NaCl, at a flow rate of 30 ml/h. The bound proteins were eluted by linear gradient increase of NaCl concentration with each 150 ml of 0.06 and 0.3 M NaCl in the buffer. The fractions containing SeP were pooled, and imidazole and NaCl were added to the pooled fractions at a final concentration of 20 mM and 1 M, respectively. The fractions were applied to a column (1 ml) of Ni-NTA-agarose equilibrated with 20 mM Tris-HCl, pH 8.0, containing 20 mM imidazole and 1 M NaCl, at a flow rate of 30 ml/h. After washing with the equilibrated buffer, the bound proteins were eluted with 20 mM Tris-HCl, pH 8.0, containing 250 mM imidazole and 1 M NaCl. For further study, imidazole and NaCl were removed by passing through PD-10 gel filtration column equilibrated with the desirered buffer.**

**Enzyme Assay—Gpx activities were examined by following the oxidation of NADPH in the presence of GSH reductase, which catalyzes the reduction of oxidized GSH formed by GPx (33) with a slight modification. Both samples and reference cuvettes contained 0.1 M Tris-HCl, pH 8.0, 0.2 mM NADPH, 0.5 mM EDTA, 2 mM GSH, and 1 unit of GSH reductase in a total volume of 1 ml. An aliquot of enzyme was added to the sample cuvette only. The reaction mixture was preincubated at 37 °C for 2 min, after which the reaction was started by the addition of peroxide in both cuvettes. In the case of phospholipid hydroperoxide, Triton X-100 and deoxycholate were added to the reaction mixture at an appropriate concentration. The oxidation of NADPH was followed at 37 °C. To confirme the reduction of oxidized NADPH by PLPC-OOH, an aliquot of PLPC-OH was also added. Incubations were done under appropriate conditions, a 9-fold volume of ice-cold 2-propanol was added to the reaction mixture. An aliquot of the reaction mixture was directly injected into the HPLC system. The HPLC conditions were basically those of Bao et al. (34). An ODS column (5 μm, 4.6 μm × 250 mm, TSK ODS-80Ts, Tosoh Co., Tokyo, Japan) was used for analysis and determination. The mobile phase was acetonitrile/methanol/water (75:25:4), containing 10 mM sodium chloride, and the flow rate was 1.5 ml/min. The UV peaks of phospholipids were monitored at 235 nm. The temperature of the column was maintained at 40 °C in a column oven.

**Kinetic Analysis**—Kinetic analysis was carried out following the time progress curves of substrate consumption (NADPH oxidation) in the coupled test with GPx as described previously (35). The substrate concentration and the reaction rate, at each time interval (typically from 1 to 3 min) thus obtained were then fitted to the Dalziel equation indicating a ping-pong mechanism as in the case of GPx, eGPx, and PH-GPx.

The rate constants k₁ and k⁻¹ were calculated from the Dalziel coefficients, as described previously (35, 36). The first step is the oxidation of selenol anion (E-Se⁻) by PLPC-OH to yield a selenenic acid derivative (E-SeOH) and PLPC-OH. The selenenic acid derivative is then oxidized by two molecules of GSH to yield a selenol anion.
We detected little activity (less than 0.02 units of droperoxides by coupled enzymatic assay with GSH reductase. SeP to catalyze the GSH-dependent reduction of various products. Furthermore, the rate (2.03 × 10^(-4) C unit/mg of protein) was confirmed by FAB-MS, as described under “Experimental Procedures.” The time course of the formation of PLPC-OH and the absolute values (60 nmol) of total PLPC-OH elution were 10.6 and 11.4 min, respectively. The identity of the two peaks was confirmed by HPLC analysis of reaction products. Fig. 2A shows elution profiles of untreated PLPC-OH, hydroxy derivative from PLPC-OH (PLPC-OH), and PLPC-OH treated with SeP for 5 min, respectively. The retention times of PLPC-OH and PLPC-OH treated with SeP for 5 min, respectively. The percent of conversion of PLPC-OOH to PLPC-OH by SeP was equal to that determined by coupling assay (2.23 units of conversion of PLPC-OOH to PLPC-OH by SeP was equal to the formation of PLPC-OH as a substrate. As shown in Fig. 2B, SeP caused an immediate and rapid decrease of PLPC-OOH to PLPC-OH. There was good agreement between the results of amino acid analysis and selenium analysis in Table II. The determined values of amino acid residues in purified SeP were in good agreement with the predicted values for the full-length SeP except for the number of selenocysteine residues, indicating the presence of 6.3 selenocysteines for a polypeptide mass of 41 kDa (as estimated from the sum of amino acid compositions). The NH2-terminal amino acid sequence (E-S-Q-D-Q-S-S-L-C-K-Q-P-P-A-W-S) obtained for purified SeP confirmed the sequence predicted from SeP cDNA (21).

Reduction of PLPC-OOH by SeP—We measured the ability of SeP to catalyze the GSH-dependent reduction of various hydroperoxides by coupled enzymatic assay with GSH reductase. We detected little activity (less than 0.02 μmol/min/mg of protein) using hydrogen peroxide or t-butyldihydroperoxide. This value is about 3 orders of magnitude lower than that of cGPx (37). Next, we examined the reducing activity using PLPC-OOH as a substrate. As shown in Fig. 2A, SeP caused an immediate and rapid decrease of A₅₉₀, suggesting that PLPC-OOH is reduced by SeP. There was good agreement between PLPC-OOH values (60 nmol) calculated from these measurements and the absolute values (60 nmol) of total PLPC-OOH determined independently by the iodometric assay. Reducibility of PLPC-OOH was also studied by HPLC analysis of reaction products. Fig. 2B shows elution profiles of untreated PLPC-OOH, hydroxy derivative from PLPC-OOH (PLPC-OH), and PLPC-OH treated with SeP for 5 min, respectively. The retention times of PLPC-OH and PLPC-OH elution were 10.6 and 11.4 min, respectively. The identity of the two peaks was confirmed by FAB-MS, as described under “Experimental Procedures.” The time course of the formation of PLPC-OH and the loss of PLPC-OOH by SeP in the presence of GSH is shown in Fig. 2C. The loss of substrate was equal to the formation of product. Furthermore, the rate (2.03 × 10^(-4) C unit/mg of protein) of conversion of PLPC-OOH to PLPC-OH by SeP was equal to that determined by coupling assay (2.23 μmol/min/mg of protein).

To confirm that SeP is responsible for the enzyme activity, an immunoprecipitation assay was conducted. After addition of various amounts of BD1 (rat monoclonal antibody against human SeP)-agarose (1.2 mg/ml agarose) to the purified SeP preparation (25 μg per ml), the enzyme activities of the supernatant were measured. When 10% (v/v) of BD1-agarose was added, less than 5% activity was observed in the supernatant.

Enzymatic Characterization of SeP—To determine the optimal conditions for SeP enzymatic activity, the effect of detergent concentration was studied. In the absence of detergent, no reducing activity of SeP was observed toward PLPC-OOH in

Enzymatic Characterization of Human Selenoprotein P

**TABLE I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Selenium</th>
<th>SeP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Recovery&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Purification&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Plasma</td>
<td>57,000</td>
<td>137</td>
<td>(5.0)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>5% polyethylene glycol supernatant</td>
<td>35,700</td>
<td>109</td>
<td>(4.0)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80</td>
</tr>
<tr>
<td>Heparin-Sepharose column</td>
<td>58</td>
<td>23</td>
<td>1.9</td>
<td>38</td>
</tr>
<tr>
<td>Q-Sepharose column</td>
<td>2.8</td>
<td>14</td>
<td>1.2</td>
<td>24</td>
</tr>
<tr>
<td>Ni-NTA-agarose column</td>
<td>0.70&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.9</td>
<td>0.8</td>
<td>16</td>
</tr>
</tbody>
</table>

<sup>a</sup> SeP is postulated to have 6.3 selenocysteines and a molecular mass of 41 kDa.
<sup>b</sup> Protein determined by a Bio-Rad protein assay kit, using bovine immunoglobulin G as a standard.
<sup>c</sup> ND, not determined.
<sup>d</sup> STP is postulated to have 6.3 selenocysteines and a molecular mass of 41 kDa.

![Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified selenoprotein P. 3 μg of purified selenoprotein P was run on a 10% gel under nonreducing conditions (A) and reducing conditions (B). The gel was stained with Coomassie Brilliant Blue R-250. Molecular mass standard locations are shown on the left.](http://www.jbc.org/)

![Table II](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Determined&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Deduced&lt;sup&gt;f&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Asp + Asn</td>
<td>33.2</td>
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</tr>
<tr>
<td>Glu + Gln</td>
<td>49.4</td>
<td>49</td>
</tr>
<tr>
<td>Cys</td>
<td>ND&lt;sup&gt;g&lt;/sup&gt;</td>
<td>16</td>
</tr>
<tr>
<td>Sec&lt;sup&gt;h&lt;/sup&gt;</td>
<td>6.3&lt;sup&gt;i&lt;/sup&gt;</td>
<td>10</td>
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<tr>
<td>Ser</td>
<td>34.6&lt;sup&gt;j&lt;/sup&gt;</td>
<td>32</td>
</tr>
<tr>
<td>Gly</td>
<td>18.3</td>
<td>15</td>
</tr>
<tr>
<td>His</td>
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<tr>
<td>Arg</td>
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<tr>
<td>Thr</td>
<td>15.4&lt;sup&lt;k&lt;/sup&gt;</td>
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</tr>
<tr>
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<td>Ile</td>
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<td>Leu</td>
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<td>Phe</td>
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<tr>
<td>Trp</td>
<td>2.4&lt;sup&lt;d&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>Lys</td>
<td>22.2</td>
<td>26</td>
</tr>
</tbody>
</table>

<sup>e</sup> Calculated from values extrapolated to 0 h based upon samples of 24-, 48-, and 72-h hydrolysate.
<sup>f</sup> Calculated from values extrapolated to 0 h based upon samples of 24-, 48-, and 72-h hydrolysate.
<sup>g</sup> Calculated from amino acid analysis and selenium analysis as described under “Experimental Procedures.”
<sup>h</sup> Calculated from values extrapolated to 0 h based upon samples of 24-, 48-, and 72-h hydrolysate.
<sup>i</sup> Calculated from values extrapolated to 0 h based upon samples of 24-, 48-, and 72-h hydrolysate.
<sup>j</sup> Calculated from values extrapolated to 0 h based upon samples of 24-, 48-, and 72-h hydrolysate.
<sup>k</sup> Calculated from values extrapolated to 0 h based upon samples of 24-, 48-, and 72-h hydrolysate.
<sup<l</sup> Calculated from values extrapolated to 0 h based upon samples of 24-, 48-, and 72-h hydrolysate.
dispersed form. The addition of Triton X-100 stimulated the enzyme activity of SeP (Fig. 3A). Maximum activity was found at 0.025% Triton X-100. The further addition of 0.3 mM deoxycholate to the assay mixture produced an increase in activity of 50%. As shown in Fig. 3B, the addition of deoxycholate alone little enhanced SeP activity. In the presence of 0.025% Triton X-100, a maximum activity was observed from 0.3 to 1.0 mM deoxycholate. Thus, the enzyme activity of SeP in the following experiments was assayed in the presence of 0.025% Triton X-100 and 0.3 mM deoxycholate. Under this standard condition, the specific activity of SeP against PLPC-OOH is 2.03 μmol/min/mg of protein.

To investigate the enzymatic nature of SeP, the kinetics of SeP for PLPC-OOH were studied following the time progress curves of substrate consumption (NADPH oxidation) in the coupled assay with GSH reductase (35, 36). Linear Lineweaver-Burk plots were obtained from reactions rate and substrate concentration values measured from progress curves (Fig. 4). Different GSH concentrations led to parallel lines, indicating a ping-pong mechanism as in the case of cGPx (38), eGPx (7), and PH-GPx (35). This allowed the distinct measurement of $k_1$ and $k_{-1}$ for the oxidative reaction and the sum of the two reductive steps of the peroxidatic reaction, respectively. The $k_1$ and $k_{-1}$ values calculated are 2,360 mM$^{-1}$ min$^{-1}$ and 29.3, respectively.

As PH-GPx is reported to reduce peroxides using thiols besides GSH as a reductant (4, 34), the thiol specificity of SeP was assayed by measuring the reduction of PLPC-OOH by HPLC assay. As shown in Table III, dithiothreitol, mercaptoethanol, cysteine, and homocysteine were also effective as a reducing substance.

**DISCUSSION**

Burk and his groups reported the purification of SeP from human and rat plasma (18, 19), using immunoaffinity chromatography. Although Eberle and Haas purified human SeP without immunoaffinity chromatography, their final preparation reportedly contained some contaminants (39). Until now, no one has succeeded in purifying SeP to homogeneity using conventional means. To investigate the structure and function of this distinctive selenoprotein, we first established the procedure for isolation of SeP. Prior to the column chromatography, polyethylene glycol was added to the plasma to remove some coagulation factors and macromolecular weight proteins. SeP was separated from the two other selenium-containing proteins, eGPx and albumin, by heparin-Sepharose chromatography. After Q-Sepharose chromatography, we selected immobilized metal chelate affinity chromatography. This column was generally used for the purification of recombinant proteins, which contain six consecutive histidine residues at their amino terminus. SeP has two histidine-rich domains; one of which includes a run of four histidines followed by a lysine, histidine, lysine for a total of seven consecutive basic amino acid residues.

As expected, SeP binds strongly to the resin and is eluted by competition with imidazole, a histidine analogue. During the purification, diisopropylfluorophosphate, one of the most potent serine-protease inhibitors, was added to each fraction. If diisopropyl fluorophosphate was not added, the recovery of SeP was very low, suggesting that a proteolysis of SeP occurs in the course of purification without diisopropyl fluorophosphate.

The purified SeP yielded a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amino acid composition and amino-terminal amino acid sequence of the
Fig. 3. Effect of Triton X-100 and deoxycholate on the activity of selenoprotein P. The activity of selenoprotein P toward PLPC-OOH was measured as described under “Experimental Procedures” in the presence of various concentrations of Triton X-100 and 0.3 mM DOC (A) or DOC and 0.025% Triton X-100 (B). The amount of enzyme used was 5 μg.

FIG. 4. Lineweaver-Burk plot of the activity of selenoprotein P on PLPC-OOH. The plots were traced through analysis of single progression curves. The GSH concentration was 1 mM (○), 1.5 mM (●), or 2 mM (□).

Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Selenoprotein P</th>
<th>PH-GPxd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% GSH activity</td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>100</td>
<td>100d</td>
</tr>
<tr>
<td>1,4-Dithiothreitol</td>
<td>126</td>
<td>327</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>49</td>
<td>52</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>81</td>
<td>10</td>
</tr>
<tr>
<td>DL-Homocysteine</td>
<td>54</td>
<td>NDd</td>
</tr>
</tbody>
</table>

a Values are from Roveri et al. (4).
b 2.03 μmol/min/mg of protein using PLPC-OOH as a substrate.
c 12.5 μmol/min/mg of protein using hydrogen peroxide as a substrate.
d ND, not determined.

The enzymatic characterization of human selenoprotein P is similar to those postulated for the deduced polypeptide (21), confirming the homogeneity of our final preparation. As shown in Table I, 800 μg of SeP was isolated from 1 liter of plasma. Multiple forms of rat SeP are reportedly present in plasma (40). As we only measured selenium contents to detect SeP, it is probable that only the major isofrom of SeP was detected, and other isoforms of SeP were missed. Only 6.3 mol of selenium was found in the selenium and quantitative amino acid analysis of purified SeP, even though 10 mol was predicted. Under the same analysis conditions, 1.1 mol of selenium was detected in the purified eGPx preparation, as expected. This discrepancy was also observed in a full-length form of SeP purified from rat plasma (41). Further structure analysis of purified SeP will be necessary to understand this difference.

Selenoproteins with known enzymatic activity are redox enzymes and contain selenocysteine in their active sites. The function of SeP is unknown. Its appearance in plasma correlates with protection against free radical injury of the liver by diquat, suggesting that SeP plays an important role in antioxidative defense (22, 23). Previous reports show that SeP does not reduce hydrogen peroxide in the presence of GSH (42). As we could prepare a highly purified SeP without denaturing conditions, it seems worth reexamining the GPx-like activity of SeP. We confirmed that SeP lacks the enzymatic activity of cGPx. Next, we investigated the PH-GPx activity of SeP. Using two different assays, coupled assay and HPLC assay, it is shown that SeP reduces the same amounts of PLPC-OOH in the presence of GSH. The conversion rate (2.03 μmol/min/mg of protein) of PLPC-OOH to PLPC-OH by SeP, directly determined by HPLC, is equal to that indirectly determined by coupling assay, in which PH-GPx activity is coupled to the oxidation of NADPH by GSH reductase. Immunoprecipitation experiments with monoclonal antibody against SeP rules out the possibility of contamination by another enzyme in our final preparation. As it is impossible to measure PH-GPx activity of plasma, it is unclear whether or not SeP alone is responsible for PH-GPx activity of plasma. Further studies to confirm this hypothesis are currently in progress.

In the presence of 0.025% Triton X-100 and 0.3 mM deoxycholate, the highest PH-GPx activity of SeP was observed. The addition of Triton X-100 to the assay mixture resulted in the transformation of the lipid bilayer into an optically clear solution of mixed micelles. This suggested that SeP catalyzes a reaction at the lipid-water interface, as is the case of PH-GPx (35). Deoxycholate reportedly can stimulate PH-GPx activity in the presence of Triton X-100 (30). The same stimulatory effect by deoxycholate was observed in our experiments using SeP.

A difference in peroxide specificity in the GPx family was reported. cGPx reduces hydrogen peroxide and t-butyldihydroperoxide, but does not reduce phospholipid hydroperoxide. PH-GPx reduces phospholipid hydroperoxide and is also reactive against hydrogen peroxide (4, 5). eGPx reduces hydrogen peroxide and t-butyldihydroperoxide and shows some reactivity against phospholipid hydroperoxides (7, 8). We found that SeP reduces phospholipid hydroperoxide and has no activity against hydrogen peroxide and t-butyldihydroperoxide. The peroxide specificity of SeP was more similar to that of PH-GPx than to that of cGPx or eGPx. Approximately 100-fold lower k between 1 and k between 2 values were observed in human SeP as compared with pig PH-GPx (35), suggesting that SeP is less reactive to PLPC-OOH, an artificial substrate, than is PH-GPx. Further studies to understand the reactivities of SeP against various hydroperoxides in physiological conditions must be done.

Previous reports indicated that PH-GPx, unlike cGPx, did not exhibit a strict requirement for glutathione as reducing agent. Using SeP as enzyme, we observed that 1,4-dithiothre-
itol has a stronger activity than glutathione. Other thiols, including 2-mercaptoethanol, cysteine, and homocysteine, also serve as reductant, as is already known in the case of PH-GPx (4, 34).

In conclusion, we isolated SeP from human plasma and characterized the enzymatic nature of the purified preparation. Immunoprecipitation with monoclonal antibodies against SeP results in the almost total loss of PH-GPx-like activity in the supernatant of the purified preparation. This rules out the possibility of contamination by another enzyme in our final preparation. Although SeP can reduce PLPC-OOH, it is approximately 100 times slower than the cellular counterpart, PH-GPx. It is currently unknown whether SeP is able to react with certain phospholipid hydroperoxides under physiological conditions. Using selenium-deficient rats supplemented with certain phospholipid hydroperoxides under physiological conditions. Using selenium-deficient rats supplemented with selenium, it was demonstrated that SeP appearance correlated with disappearance of diquat-induced lipid peroxidation (22). Furthermore, SeP was reportedly associated with protection against oxidant injury from GSH depletion in the selenium-deficient rat (23). These in vitro studies and our in vitro results described above strongly suggest that SeP serves to protect the plasma membrane from oxidative damage in the presence of GSH. Even though the GSH concentration in plasma is low, it is released continuously from the cells (43), and it may represent a significant source of reductant for SeP enzymatic activity.

SeP and eGPx are located in the extracellular fluids, and cGPx, PH-GPx, and GPx-GI are found in the cytosol. These enzymes exhibit different substrate specificities and collaborate to protect the biological molecules from oxidative stress inside and outside the cells, respectively.

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REFERENCES

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