Selenium-Dependent Glutathione Peroxidase Protein and Activity: Immunological Investigations on Cellular and Plasma Enzymes

By Kazuhiko Takahashi and Harvey J. Cohen

Selenium-deficient humans and animals are known to be deficient in glutathione peroxidase (GSHPx) activity in their cells and plasma. To determine the relationship between enzyme activity and protein content, the enzyme was purified from human erythrocytes, and polyclonal antibodies were made against the purified protein in rabbits. These antibodies were found to be monospecific, noninhibitory, and capable of precipitating the enzymatic activity. All the GSHPx activity in erythrocytes and almost all the activity in neutrophils and platelets was precipitated by these antibodies. None of the plasma enzyme was precipitated by these antibodies, indicating that the plasma enzyme activity was attributable to a different selenium-dependent protein moiety. Utilizing a radioimmunoassay, we were able to demonstrate that there was a direct relationship between GSHPx activity and protein content in the erythrocytes of both normal and selenium-deficient individuals, and a similar relationship between control and selenium-deficient rat erythrocytes and liver cells. Thus, the ability to examine GSHPx as a protein resulted in two new observations concerning the selenium-dependent GSHPx. The first is that the plasma enzyme is antigenically distinct from the erythrocyte enzyme, and the second is that in the absence of selenium, there is a concomitant decrease in GSHPx protein.

G LUTATHIONE peroxidase (GSHPx) plays an important role in the detoxification of hydrogen peroxide, organic hydroperoxides, or lipid peroxides.1-3 Selenium (Se)-deficient animals and humans have decreased GSHPx activity.4-7 Because Se in the form of selenocysteine is present in the active site of this enzyme,8 it is not surprising that Se deficiency results in a decrease in enzymatic activity. However, it was not known whether the absence of Se could affect the GSHPx protein or whether apo-enzyme was present. To determine if there is a direct relationship between GSHPx activity and protein in Se deficiency, or if apo-protein exists, we purified the enzyme from erythrocytes and developed polyclonal, monospecific antibodies against the enzyme. Utilizing these antibodies in a radioimmunoassay, we were able to correlate protein content with enzymatic activity. We were also able to show that these antibodies cross-reacted with rat GSHPx and that Se-deficient rat liver as well as rat erythrocytes had a concomitant decrease in GSHPx protein content and activity. Finally, using this antibody, we were able to determine that the plasma GSHPx activity, although Se dependent, is not due to the same antigenic material as that found in erythrocytes, neutrophils, or platelets.

MATERIALS AND METHODS

Materials. Reduced glutathione (GSH), glutathione reductase (type IV, from baker’s yeast), bovine serum albumin (essentially fatty acid and globulin free), NADPH, Triton X-100, and t-butyl hydroperoxide (t-BuOOH) were purchased from Sigma Chemical Company, St Louis; Dextran T500, Ficoll-Paque, DEAE-Sephadex A-25, Sephadex G-200, and Sepharose 2B from Pharmacia Fine Chemicals, Piscataway, NJ; diethylaminoethanol (DEAE)-cellulose DE-52 and CM-cellulose CM-52 from Whatman, Clifton, NJ; hydrogen peroxide from Fisher Scientific, Pittsburgh; Bolton-Hunter reagent from New England Nuclear, Boston; and Freund’s complete adjuvant, rabbit IgG and goat anti-rabbit IgG antibody from Calbiochem, La Jolla, Calif. Other chemicals were of analytical grade and were obtained commercially.

Sources of cells. Samples of erythrocytes were obtained from normal and Se-deficient individuals as previously described.9 The erythrocytes from one of these individuals were used during the course of Se repletion.10 Control and deficient rats were fed diets as previously described10 for >3 months prior to analyses.

GSHPx activity. GSHPx activity using t-butyl hydroperoxide as a substrate was assayed by an adaptation of the method of Beutler.11 Both sample and reference cuvettes contained 0.1 mol/L of Tris-HCl, pH 8.0, 0.5 mmol/L of EDTA, 0.2 mmol/L of NaN3, 1 U of glutathione reductase, 2 mmol/L of GSH, and the appropriate amount of enzyme in 1 mL. The oxidation of NADPH by t-BuOOH (70 μmol/L), added to the sample cuvette only, was followed spectrophotometrically at 340 nm at 37 °C. An additional blank containing all components except the enzyme was determined to correct for nonenzymatic oxidation of GSH and NADPH by t-BuOOH.

GSHPx activity using hydrogen peroxide as a substrate was assayed as follows. Both sample and reference cuvettes contained 0.1 mol/L of sodium phosphate buffer, pH 7.0, 0.5 mmol/L of EDTA, 0.2 mmol/L of NADPH, 1 U of glutathione reductase, 1 mmol/L of NaN3, 2 mmol/L of GSH, and the appropriate amount of enzyme in 1 mL. The oxidation of NADPH by hydrogen peroxide (15 μmol/L), added to the sample cuvette only, was followed at 340 nm at 37 °C. An additional blank was measured as described above. One unit of enzyme activity is defined as the oxidation of one nmol of NADPH per minute.

Cell extracts and plasma preparation. To prepare plasma and erythrocytes, human or rat blood collected in heparin was centrifuged in a Triac Combination centrifuge for five minutes. The plasma was removed and assayed for GSHPx activity. Erythrocytes were washed three times in 0.154 mol/L of NaCl. To 0.1 mL of the washed packed erythrocytes, 1.9 mL of 1 mmol/L of potassium phosphate buffer, pH 7.5, was added. The resulting hemolysate was centrifuged at 14,000 g for 10 minutes to remove the erythrocyte membranes. The clarified hemolysate was assayed for hemoglobin and GSHPx activity.

Human granulocytes (PMNs) were prepared by dextran and Ficoll-Paque sedimentation from blood collected in acid-citrate-dextrose as described previously.12 Rat PMNs were obtained from

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their peritoneal cavities 18 hours after the intraperitoneal injection of casein as previously described. PMNs at a concentration of $1 \times 10^6$ cells/mL, were suspended in 0.34 mol/L of sucrose, 0.02 mol/L of Tris-HCl, pH 7.4, containing 0.1% Triton X-100, and placed in a sonicator bath for six to eight seconds. The lysates were centrifuged at 70,000 g for five minutes at 4 °C. The supernatant was centrifuged at 20,000 g for 20 minutes at 4 °C. The resulting cytosolic solutions were assayed for GSHPx activity.

Human platelets were prepared from platelet-rich plasma by gel filtration on Sepharose 2B as described previously. Platelets at a concentration of $3.5 \times 10^6$ cells/mL, were suspended in 0.34 mol/L of sucrose, 0.02 mol/L of Tris-HCl, pH 7.4, containing 0.1% Triton X-100, and placed in a sonicator bath for six to eight seconds. The lysates were centrifuged at 20,000 g for 20 minutes at 4 °C. The resulting cytosolic solutions were assayed for GSHPx activity.

Human liver obtained at autopsy, or rat liver, was rinsed with cold 0.154 mol/L of NaCl, blotted, weighed, and homogenized in 9 vol of 0.25 mol/L of sucrose, 0.02 mol/L of Tris-HCl, pH 7.4, in a glass Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 600 g for 10 minutes to remove whole cells and debris. This supernatant was centrifuged at 12,000 g for 20 minutes at 4 °C to pellet the mitochondria. The resulting cytosolic solutions were assayed for GSHPx activity.

**Purification of human erythrocyte GSHPx.** GSHPx was purified from human erythrocytes according to the methods of Awasthi and colleagues including ammonium sulfate fractionation, CM-cellulose CM-52, DEAE-cellulose DE-52, Sephadex G-200, and DEAE-Sephadex A-25 column chromatography.

**Gel electrophoresis.** Polyacrylamide gel electrophoresis (PAGE) (6% acrylamide gel) was performed by the method of Davis. Sodium dodecyl sulfate (SDS)-PAGE (10% acrylamide) was performed by the method of Laemmli. After electrophoresis, proteins were stained as described previously or eluted from gels.

**Immunization and isolation of antibody.** One hundred and fifty micrograms of purified GSHPx was mixed with Freund's complete adjuvant and injected intramuscularly in four sites on the back of a New Zealand white rabbit. This procedure was repeated weekly for 4 weeks. Serum was collected 5 weeks after the first immunization. Because rabbit serum itself contains GSHPx activity, the IgG fraction was purified free from GSHPx activity using ammonium sulfate precipitation followed by DEAE-cellulose DE-52 chromatography.

**Radioimmunoassay.** Radioimmunoassays were performed using the double antibody technique of Roberts and Parker with a slight modification. GSHPx purified from human erythrocytes was radio-iodinated using the Bolton-Hunter reaction. Unknown samples were assayed by competition with the antibody generated against purified GSHPx.

**Fig 1.** Purity of isolated glutathione peroxidase (GSHPx). (A) Diethylaminoethyl (DEAE)-Sephadex A-25 column chromatography as the final purification step for GSHPx. Partially purified fractions of GSHPx dialyzed against 5 mmol/L of potassium phosphate buffer, pH 8.0, containing 0.7 mmol/L of β-mercaptoethanol were applied to a 1.5 x 3.0 cm column of DEAE-Sephadex A-25 equilibrated with the same buffer. The column was washed with this buffer, and the proteins were subsequently eluted with a linear gradient of 0 to 100 mmol/L of NaCl. Protein was detected by measuring absorbance at 280 nm, and GSHPx activity was measured as described in the Materials and Methods section. The fractions under the bar were concentrated by the use of Amicon Centriflo. (B) Polyacrylamide gel electrophoresis (PAGE) of purified GSHPx. Twenty micrograms of purified enzyme were applied to each gel. After electrophoresis, protein was stained by the method of Burges and GSHPx activity was measured in 2 mm slices of the gel. (C) Sodium dodecyl sulfate (SDS)-PAGE of purified GSHPx. Twenty micrograms of purified enzyme were treated with 1% SDS and 1% β-mercaptoethanol and applied to the gels. After electrophoresis, protein was stained as described above. The mol wt was estimated using bovine serum albumin, with the heavy and light chains of human IgG and cytochrome as standards.
ples and standard samples (purified GSHPx) were incubated with 10 μL of rabbit anti-GSHPx IgG (0.65 mg/mL of the isolated IgG, with 1 mg/mL carrier rabbit normal IgG) for 15 minutes at 25°C followed by an additional 60-minute incubation at 25°C with ~1,500 cpm of 125I-GSHPx (20 μL). The reaction mixtures contained 50 mmol/L of Tris-HCl, pH 8.0, 2.5 mmol/L of EDTA, 0.2% NaN₃, and 0.2% bovine serum albumin in a total volume of 160 μL. After incubation, 40 μL of goat anti-rabbit IgG antibody was added at equivalence and incubated for 15 minutes at 25°C. The mixtures were then centrifuged at 10,000 g for 5 minutes at 4°C, and the radioactivity of resulting immune precipitates was determined. The amount of GSHPx in unknown samples was calculated from a standard curve obtained with purified GSHPx (1 to 100 ng).

**Protein and hemoglobin determination.** The protein concentration was measured by the method of Lowry et al, using bovine serum albumin as a reference protein. Hemoglobin concentration was determined with Drabkin’s reagents.

**RESULTS**

**Purity of human erythrocyte GSHPx.** Using the method previously described by Awasthi and colleagues, we purified human erythrocyte GSHPx to homogeneity. Unlike previously described results, we could not find two peaks of GSHPx activity when gel filtration on Sephadex G-200 was performed and found only a single peak of activity during the final purification step. The elution pattern of DEAE-Sephadex A-25 column chromatography is shown in Fig 1A. PAGE revealed a single protein stained band, shown in Fig 1B. Slicing the polyacrylamide gels into 2-mm slices, followed by elution of the slices into buffer resulted in the recovery of a single peak of GSHPx activity, which corresponded to the protein peak as shown in Fig 1B. SDS-PAGE of the purified enzyme showed a single band with mol wt of 22,000, as shown in Fig 1C. This is similar to the major GSHPx protein that was previously described. The specific activity of the purified enzyme was 224 units/mg.

**Properties of anti-GSHPx antibody.** Rabbit antibody against human erythrocyte GSHPx was prepared as described in the Methods section. Because rabbit serum contains GSHPx activity, the IgG fraction of rabbit serum was separated from GSHPx using ammonium sulfate precipitation followed by DEAE-cellulose DE-52 column chromatography. The resulting IgG fraction contained no GSHPx activity. Figure 2 shows a result of the Ouchterlony analysis of the antibody preparation. A single precipitin line between the antibody preparation and purified GSHPx was found. An identical single precipitin line was also found when the antibody reacted with the initial partially purified fractions during purification of GSHPx, and a zone of identity was present between the lines formed with the crude fractions and the purified enzyme, indicating that no major modifications of the enzyme occurred during purification. Thus, we had monospecific polyclonal antibodies against erythrocyte GSHPx. To determine the ability of these antibodies to inhibit enzymatic activity, we incubated various amounts of antibody with the purified enzyme and either directly measured enzyme activity or measured enzyme activity of the supernatant after centrifugation of the mixture at 100,000 g. As shown in Fig 3, the antibody did not inhibit GSHPx activity, but did precipitate the activity in a dose-dependent manner. Control rabbit IgG did not precipitate the enzymatic activity (data not shown). Thus, we had monospecific, polyclonal, precipitating antibodies that did not inhibit enzyme activity and therefore were not directed against the active site of the enzyme.

**Reactivity of antibody with cell lysates.** Figure 3 also shows the result of incubating increasing amounts of this antibody preparation with erythrocyte hemolysates followed by centrifugation on the GSHPx activity present in the supernatant. There was a decrease in supernatant GSHPx activity with increasing amounts of antibody. At the highest concentration of antibody used, no GSHPx activity was demonstrable in the resulting supernatant. This result confirms our previous results with Se-deficient erythrocytes, indicating that all of the GSHPx activity present in human erythrocytes is due to this Se-dependent GSHPx protein.

We then examined other human cells for reactivity of their GSHPx protein with this antibody. Table 1 shows that
has precipitated GSHPx activity from rat erythrocytes, diminished in Se-deficient individuals, the plasma enzyme and erythrocyte GSHPx activities were markedly increased. We therefore examined the ability of the anti-erythrocyte GSHPx antibody to precipitate the plasma enzyme. As can be seen in Table 2, the addition of purified erythrocyte GSHPx to plasma and the subsequent addition of antibody followed by centrifugation resulted in the precipitation of only that amount of enzyme activity contributed by erythrocyte GSHPx. Thus, there appear to be antigenic differences between the erythrocyte and plasma enzymes. The antibody preparation was also unable to precipitate rat plasma GSHPx activity.

**Relationship of enzyme activity to protein content.** Using a radioimmunoassay as described in the Methods section, we examined the relationship between protein content and GSHPx activity in erythrocytes from normal individuals and Se-deficient individuals, including one individual studied during repletion with Se. As shown in Fig 4, there is a direct relationship between GSHPx protein and GSHPx activity in human erythrocytes. Samples were obtained from five normal individuals (II), five Se-deficient individuals (8), and a Se-deficient individual during the course of Se repletion (0) as described previously. The hemolysates were assayed for hemoglobin content, GSHPx activity, and protein as described in the Materials and Methods section.

Table 1. Precipitation of Human Cellular GSHPx Activity by Rabbit Anti-GSHPx IgG

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Substrate</th>
<th>GSHPx Activity (x 10^-2 U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>- Ant. + Antibody Control</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>t-BuOOH</td>
<td>11.6 1.45 12.5</td>
</tr>
<tr>
<td>Platelets</td>
<td>t-BuOOH</td>
<td>8.09 0.26 3.2</td>
</tr>
<tr>
<td>Liver cytosol</td>
<td>t-BuOOH</td>
<td>2.91 0.90 30.9</td>
</tr>
<tr>
<td>Liver cytosol</td>
<td>H2O2</td>
<td>1.58 &lt;0.1 &lt;6</td>
</tr>
</tbody>
</table>

Samples were incubated at 25 °C for 60 minutes either with or without rabbit anti-GSHPx IgG (225 pg). The mixtures were centrifuged at 100,000 g for 30 minutes at 4 °C, and the resulting supernatants were assayed for GSHPx activity. Results are the average of duplicate determinations.

Table 2. Precipitation of Plasma GSHPx Activity by Rabbit Anti-GSHPx IgG

<table>
<thead>
<tr>
<th>Preparation</th>
<th>GSHPx Activity (x 10^-2 U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Ant. + Antibody</td>
</tr>
<tr>
<td>1 x purified</td>
<td>7.10</td>
</tr>
<tr>
<td>2 x purified</td>
<td>14.20</td>
</tr>
<tr>
<td>Plasma</td>
<td>5.00 5.20</td>
</tr>
<tr>
<td>Plasma + 1 x purified</td>
<td>12.90 5.47</td>
</tr>
</tbody>
</table>

Samples were incubated at 25 °C for 60 minutes either alone or with rabbit anti-GSHPx IgG (225 pg). The mixtures were centrifuged at 100,000 g for 30 minutes at 4 °C, and the resulting supernatants were assayed for GSHPx activity. Results are the average of duplicate determinations.

DISCUSSION

GSHPx, as found in mammalian cells, is a Se-dependent enzyme. The mechanism of action of this enzyme...
activity in these cells. Because the radioimmunoassay does
evidence that the Se GSHPx enzyme is the major GSHPx
of the GSHPx protein.

above criteria and therefore were used to examine the nature
antibodies were not directed against the active site that may
previously and assayed for hemoglobin (or protein) content, GSHPx
activity. and
previously'0 and assayed for hemoglobin (or protein) content, GSHPx
anti-GSHPx antibody precipitated -70% of the activity in
enzyme may be the liver. As demonstrated above, the
rabbits against human erythrocyte GSHPx satisfied all the
is part of the active site of GSHPx, it was important that the
protein. To measure protein content, we required the
development of polyclonal, monospecific antibodies that did
not inhibit enzyme activity. Polyclonal antibodies were
required rather than monoclonal antibodies in case the
monoclonal antibodies were directed against a determinant
not found in the Se-deficient protein. Monospecificity was
required to be able to measure only this protein. Because Se
cytoplasmic enzyme but from glutathione S-transferase,26 it
cytosol GSHPx assayed by t-BuOOH is not from a Se-
dependent enzyme but from glutathione S-transferase, it
does not appear that the liver cytosolic enzyme is the direct
source of the plasma GSHPx protein. It is still possible that
liver cytosol enzyme is modified for transport prior to
secretion in the plasma and that this modified protein may
not react with the antibody against native GSHPx. Zakowski
and Tappel described a liver mitochondrial Se-dependent
GSHPx with different properties from the cytosol enzyme.27
Although unlikely, it is possible that the plasma enzyme is
derived from this form of liver GSHPx. We are currently
investigating the nature of the plasma enzyme.

The absence of GSHPx protein in Se-deficient erythro-
cytes suggests that there may be a relationship between Se
and synthesis of GSHPx protein. These results are similar to
those obtained using Se-deficient rats.28 Sunde and Evenson
have found that the selenocysteine of rat liver GSHPx comes
from serine and is therefore due to either a cotranslational or
posttranslational modification.29 Hawkes and colleagues,
have demonstrated evidence for a specific seleno-
cysteine transfer RNA in rat liver.30 Thus, it is possible that
the absence of Se results in a deficiency of selenocysteine,
which might also result in the inability to translate the
message for GSHPx. Interpretation of the studies in erythro-
cytes are complicated by the inability of these cells to
synthesize protein. Thus, a deficiency of GSHPx protein in
cells may be due to instability of an abnormal protein
made in the absence of Se. The studies of rat liver GSHPx
described here are consistent with the view that even cells
capable of synthesizing protein may not synthesize GSHPx
in the absence of Se.

We have recently examined the relationship between
GSHPx protein and activity in the human promyelocytic cell
line HL-60 grown in the absence of Se and then supple-
mented with sodium selenate. We found a direct relationship
between GSHPx protein content and enzymatic activity.31
Thus, in a human cell line capable of synthesizing protein
and GSHPx specifically,32 there was an absence of the protein
in the Se-deficient state. The studies of rat liver described
here and the previously described studies on HL-
60 cells are consistent with the view that even cells capable
of synthesizing protein may not synthesize GSHPx in the
absence of Se. It is still possible that an abnormal protein is
made and very rapidly degraded.

The ability to study GSHPx as a protein in addition to
studying it as an enzyme, using polyclonal, monospecific,
noninhibitory precipitating antibodies, has resulted in two
important new pieces of information concerning Se-depen-
dent GSHPx. First, the plasma GSHPx enzyme is antigeni-
cally different from the erythrocyte GSHPx enzyme; second,
in the absence of Se, there is concomitant decrease in GSHPx
protein. The biochemistry and molecular biology of these
phenomena await further investigations.

ACKNOWLEDGMENT

The authors acknowledge the advice of Dr John Whitin and the
technical help of Patricia Liegey and William Ogle.

Table 3. Activity and Protein of Erythrocyte Hemolysate and
Liver Cytosol GSHPx From Control and Se-Deficient Rats

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>GSHPx Activity (% Control)</th>
<th>GSHPx Protein (% Control)</th>
<th>Specific Activity (U/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control erythrocytes</td>
<td>235*</td>
<td>425†</td>
<td>0.553</td>
</tr>
<tr>
<td>Se-deficient erythrocytes</td>
<td>13.8* (5.9)</td>
<td>25.9† (6.1)</td>
<td>0.533</td>
</tr>
<tr>
<td>Control liver cytosol</td>
<td>2.96†</td>
<td>4.69§</td>
<td>0.631</td>
</tr>
<tr>
<td>Se-deficient liver cytosol</td>
<td>0.046§ (1.6)</td>
<td>0.080§ (1.7)</td>
<td>0.575</td>
</tr>
</tbody>
</table>

Samples were obtained from control and Se deficient rats as described previously and assayed for hemoglobin (or protein) content, GSHPx activity, and GSHPx protein as described in the Materials and Methods section.

*Hemoglobin (U/g).
†Hemoglobin (µg/g).
‡Protein (U/mg).
§Protein (µg/mg).

requires the presence of selenocysteine in the active site.7
Both experimental and clinical Se deficiency result in
decreased enzyme activity.4-7 Without the ability to measure
specific protein content unequivocally, it was not possible to
distinguish between an inactive enzyme and the absence of
the protein. To measure protein content, we required the
development of polyclonal, monospecific antibodies that did
not inhibit enzyme activity. Polyclonal antibodies were
required rather than monoclonal antibodies in case the
monoclonal antibodies were directed against a determinant
not found in the Se-deficient protein. Monospecificity was
required to be able to measure only this protein. Because Se
is part of the active site of GSHPx, it was important that the
antibodies were not directed against the active site that may
be altered in the absence of Se. The antibodies made in the
rabbit against human erythrocyte GSHPx satisfied all the
above criteria and therefore were used to examine the nature
of the GSHPx protein.

The observation that this antibody preparation could
precipitate all the GSHPx activity from erythrocytes and
most of the enzyme activity from PMN and platelets is
evidence that the Se GSHPx enzyme is the major GSHPx
activity in these cells. Because the radioimmunoassay does
not require active enzyme in cell preparations, and because
there is a direct relationship between enzyme activity and
protein content in the hemolysates from normal individuals,
radioimmunoassay can be used to measure GSHPx in
samples that have been stored for prolonged periods of time.

The ability of this antibody to react with rat cellular
GSHPx demonstrates that the rat GSHPx protein is immu-
nologically similar to the human protein. The inability of
the rabbit antibody to react with human or rat plasma GSHPx
was an unexpected and intriguing finding. We had previously
suspected that the plasma enzyme activity was not related to
erthrocyte activity, but until these investigations were
performed, there was no evidence that it might have a
different antigenic structure. A logical source for the plasma
enzyme may be the liver. As demonstrated above, the
anti-GSHPx antibody precipitated ~70% of the activity in
human liver cytosol when assayed with t-BuOOH and almost
all the enzyme activity when assayed with hydrogen perox-
ide. Because the plasma enzyme has hydrogen peroxide-
dependent activity and because ~30% of human liver
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