Purification and Properties of Human Erythrocyte Glutathione Peroxidase*

YOGESH C. AWASTHI, ERNEST BEUTLER, AND SATISH K. SRIVASTAVA†

From the Division of Medicine, City of Hope Medical Center, Duarte, California 91010, and the Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, Texas 77550

Glutathione peroxidase has been purified to homogeneity from human erythrocytes. The purification steps involved ammonium sulfate precipitation of hemolysate, CM-cellulose (CM-52), DEAE-cellulose (DE52), Sephadex G-200, and DEAE-Sephadex column chromatography. In the last step, i.e., DEAE-Sephadex A-25 column chromatography, the enzyme was eluted in a major peak and tailing fraction. The major peak was found to be homogeneous on polyacrylamide disc electrophoresis and designated as glutathione peroxidase A (GSH-Px A). The tail fraction, however, separated into two protein bands on polyacrylamide disc electrophoresis. One of the bands corresponded to GSH-Px A while the other band was slower moving and was designated as GSH-Px B. GSH-Px A and GSH-Px B had specific activity of 103 and 4 enzyme units per mg of protein, respectively. Antibodies raised against the homogeneous GSH-Px A have been found to cross-react with GSH-Px B. Both, GSH-Px A and B are selenoproteins. GSH-Px A has been found to contain 3.5 g atoms of selenium per mol of protein. Selenium content of GSH-Px B, however, could not be determined accurately due to insufficient material.

The molecular weight of GSH-Px A as determined by the sedimentation equilibrium method is 95,000 ± 3,000. On urea-sodium dodecyl sulfate-polyacrylamide disc electrophoresis GSH-Px A and B dissociate into single subunits. The molecular weight of the subunits of GSH-Px A is 23,000 and that of GSH-Px B is 47,000. Thus, it appears that GSH-Px A is a tetramer. Our results suggest that GSH-Px B is probably an altered form of the major component, GSH-Px A, or its precursor.

The properties of GSH-Px A have been studied. The isoelectric pH was found to be 4.9 and the optimum pH for enzyme activity was 8.5. The energy of activation was 8.2 kcal. The $K_m$ of the enzyme for GSH was 4.1 μM while the $K_m$ for $t$-butyl hydroperoxide was 52 μM. The effect of sulfhydryl reagents and the metal ions on the enzyme was also studied.

Glutathione peroxidase catalyzes the reduction of hydrogen peroxide by GSH. Mills and Randall (1, 2) first demonstrated the presence of glutathione peroxidase in erythrocytes, and in various mammalian tissues (3). Catalase has long been considered to be the major enzyme responsible for reducing hydrogen peroxide. However, Cohen and Hochstein (4) speculated that glutathione peroxidase may be the first line of defense against the oxidative damage by hydrogen peroxide or lipid peroxide produced in various cells of the body. They provided evidence that in erythrocytes it is glutathione peroxidase rather than catalase that protects the hemoglobin from oxidation to methemoglobin by hydrogen peroxide. Red cells are known to produce hydrogen peroxide by various mechanisms such as the reaction between ascorbic acid and oxyhemoglobin (2), and the decomposition of oxygen amon by superoxide dismutase (5).

Unlike catalase, glutathione peroxidase catalyzes the reduction of lipid peroxides as well as of $H_2O_2$ (6). Christophersen (7) identified the products formed by the reduction of lipid peroxide by glutathione peroxidase and it has been suggested that glutathione peroxidase may be able to break the autocatalytic chain reaction of lipid peroxidation protecting the membrane from oxidative damage.

Glutathione peroxidase has been purified partially from various tissues (8-10). Recently, Flohe and his group (11, 12) have purified and studied the properties of glutathione peroxidase from beef erythrocytes. Dietary selenium has been shown to exert a protective effect against the oxidative damage to rat erythrocyte membranes (13) and it has been demonstrated that glutathione peroxidase purified from sheep red blood cells is a selenoprotein containing about 4 atoms of selenium per mol of enzyme protein (14). We have purified from human erythrocytes two homogeneous selenoproteins having glutathione peroxidase activity and similar antigenic properties with different electrophoretic and chromatographic properties. The more anodal protein having most of the glutathione peroxidase

*This work was supported in part by United States Public Health Service Grant HL 07449.
†Send reprint request to Dr. Satish K. Srivastava, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, Tex. 77550.
activity has been designated as glutathione peroxidase A (GSH-Px A). The electrophoretically more cathodal protein, having only about 4% of GSH-Px A activity, has been designated as GSH-Px B.

**MATERIALS AND METHODS**

Oxidized and reduced glutathione, glutathione reductase (Type III yeast), and NADPH were purchased from Sigma Chemical Co., DEAE-cellulose (DE52) and CM-cellulose (CM-52) were purchased from Whatman, Sephadex G-200 and DEAE-Sephadex A-25 were purchased from Pharmacia Chemical Co., Uppsala, Sweden. t-Butyl hydroperoxide was purchased from Koch-Light and Co., Colnbrook, England.

Glutathione peroxidase was assayed in a 1-ml system containing potassium phosphate buffer, 0.1 M, pH 7.0; NADPH, 0.2 mM; glutathione, 0.5 mM; GSH-Px (0.03 g/100 ml potassium ferricyanide containing 0.01 g/100 ml potassium cyanide) prior to the removal of all hemoglobin, after which a 0.1% solution of glutathione peroxidase-free bovine serum albumin was used as diluent. An additional blank contained all the components except the enzyme. Protein was determined by Lowry's method (15).

Polyacrylamide gels were stained for the enzyme activity by using a solution consisting of phosphate buffer, 100 mM, pH 7.0; EDTA, 4 mM; reduced glutathione, 10 mM; t-butyl hydroperoxide, 0.1 M; GSH and glutathione reductase, 1.0 l.u./ml. The gels were incubated at 37°C for 30 min and photographed under long wave ultraviolet light. The enzyme stain appeared as a dark band (nonfluorescent) due to the utilization of NADPH.

An affinity resin consisting of GSH bound to Sepharose 4B, was made by first activating the Sepharose with cyanogen bromide at pH 11.0 (20°C) followed by attachment of an arm by treating with N-hexamethylene 1,6-diamino for 24 hours in dioxane (16). The resin was then washed with dioxane to remove unbound amine and suspended in water. The pH of the suspension was brought to 4.0 by adding 1 N HCl. GSH and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl were added to bring a final concentration of 40 mM and 0.9 g/100 ml, respectively. The mixture was stirred at 25°C for 24 hours. After the pH of the suspension was then adjusted to 5.0 with 1 N NaOH and the reaction was allowed to proceed for another 24 hours, at the end of which the resin was washed free of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl and unbound GSH.

Antiserum was produced by injecting into the foot pad of a rabbit 50 μg of homogeneous GSH-Px A solution in 0.5 ml of potassium phosphate buffer, 10 mM, pH 7.0, suspended in 0.5 ml of Freund's complete adjuvant. After 2 weeks a second dose was administered. The rabbit was bled after 1 month and the serum was separated. The antiserum was heat-inactivated at 56°C for 30 min and filtered through a Millipore filter and stored at 4°C under sterile conditions. For the double immunodiffusion studies, the agar plates were obtained from Hyland Laboratories.

**RESULTS**

**Purification of Glutathione Peroxidase**—Outdated human blood was centrifuged at 2000 × g for 15 min at 4°C. The supernatant and theuffy coat were removed by aspiration. Only blood containing the normal levels of GSH and glutathione peroxidase was used. About 1800 ml of packed red cells were washed two times with 7 to 8 volumes of phosphate-NaCl solution (potassium phosphate buffer, pH 7.0, 0.1 M, 1 part + NaCl, 0.145 part, 9 parts). A 15% hemolysate of washed red cells (v/v) was prepared in water containing 0.7 mM β-mercaptoethanol. The hemoglobin concentration of the hemolysate was then adjusted to 3 g/100 ml by the addition of water containing 0.7 mM β-mercaptoethanol. All the purification steps including centrifugations were performed at 4°C. The purification steps are given in Table I. The hemolysate was centrifuged for 30 min at 10,000 × g to remove the ghosts. Solid ammonium sulfate was added to the supernatant with constant stirring and the precipitate formed between 25% and 50% saturation of (NH₄)₂SO₄ was separated by centrifugation and suspended in 10 mM potassium phosphate buffer, pH 7.0, with 0.7 mM β-mercaptoethanol and dialyzed against the same buffer to remove the (NH₄)₂SO₄. The dialyzed solution was centrifuged at 15,000 × g for 30 min and the precipitate was discarded. The supernatant was then adjusted to 45% saturation of (NH₄)₂SO₄ and centrifuged. The pellet obtained was resuspended in potassium phosphate buffer, 10 mM, pH 6.0, containing 0.7 mM β-mercaptoethanol and dialyzed for 24 hours against three changes of 20 volumes each of the same buffer. The dialyzed material, 900 ml, was centrifuged at 15,000 × g for 30 min and the supernatant was then passed through a CM-52 column (40 × 2.5 cm) which was equilibrated with the dialyzing buffer at a flow rate of 30 ml/hour. The eluate contained most of the enzyme activity and almost all of the residual hemoglobin remained in the column.

The pooled eluate from the CM-52 column was concentrated to 200 ml using a PM-10 membrane in an Amicon ultrafiltration cell. This was dialyzed against 20 volumes of potassium phosphate buffer, 5 mM, pH 7.2, containing 0.7 mM β-mercaptoethanol overnight with two changes of dialyzing buffer. The dialyzed enzyme was passed through a DE52 column (2.6 × 40 cm) equilibrated with the dialyzing buffer at a flow rate of 30 ml/hour. The column was washed with 1 liter of the same buffer and then eluted with a 2 liter linear gradient of 0 to 100 mM NaCl. The enzyme was eluted between 45 to 70 mM salt concentration (Fig. 1). The fractions containing at least 25% of enzyme activity of the peak fraction were pooled and dialyzed against 10 mM potassium phosphate buffer, pH 7.0, containing 0.1 M (NH₄)₂SO₄ and 0.7 mM β-mercaptoethanol and were designated as DE52 fraction.

The DE52 fraction was concentrated to 100 ml using a PM-10 membrane in an Amicon ultrafiltration cell and dialyzed against potassium phosphate buffer, 10 mM, pH 6.0, containing (NH₄)₂SO₄, 100 mM. The enzyme was then passed through a Sephadex G-200 column (100 × 5 cm) equilibrated with the dialyzing buffer using an upward flow at a rate of 60 ml/hour. The enzyme activity appeared in a rather wide peak showing a shoulder just prior to the major peak (Fig. 2). The total peak fraction (inclusive of the shoulder) was pooled and the supernatant was then passed through a CM-52 column (40 × 2.5 cm) which was equilibrated with the dialyzing buffer at a flow rate of 30 ml/hour. The eluate contained most of the enzyme activity and almost all of the residual hemoglobin remained in the column.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Enzyme activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysate</td>
<td>4,905</td>
<td>613,500</td>
<td>0.008</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>First (NH₄)₂SO₄</td>
<td>3,840</td>
<td>32,544</td>
<td>0.117</td>
<td>17</td>
<td>78</td>
</tr>
<tr>
<td>Second (NH₄)₂SO₄</td>
<td>3,300</td>
<td>7,750</td>
<td>0.427</td>
<td>61.0</td>
<td>67</td>
</tr>
<tr>
<td>CM-52</td>
<td>2,808</td>
<td>1,527</td>
<td>1.84</td>
<td>261</td>
<td>57</td>
</tr>
<tr>
<td>DE52</td>
<td>2,100</td>
<td>312</td>
<td>6.73</td>
<td>957</td>
<td>42</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>1,160</td>
<td>46</td>
<td>25.22</td>
<td>3,145</td>
<td>23</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>102</td>
<td>0.99</td>
<td>103.03</td>
<td>14,715</td>
<td>2</td>
</tr>
</tbody>
</table>

*a One unit of enzyme brings about oxidation of 1 μmol of GSH to GSSG per min at 37°C.*
dialyzed against potassium phosphate buffer, 5 mM, pH 6.0, with 0.7 mM β-mercaptoethanol for 48 hours. It was concentrated to about 100 ml in an Amicon ultrafiltration cell and was then passed through a DEAE-Sephadex A-25 (dissolved to remove bound CO2) column (25 × 1 cm) equilibrated with the dialyzing buffer at a flow rate of 15 ml/hour. The column was washed with 100 ml of the equilibrating buffer and subsequently eluted with a 200-ml linear gradient of 0 to 100 mM NaCl. The major fraction of the bound enzyme was eluted between 10 to 20 mM NaCl solution while a significant amount of the enzyme activity was found trailing in the subsequent fractions (Fig. 3).

The peak fractions from DEAE-Sephadex A-25 column (Fig. 3) containing glutathione peroxidase activity were pooled, dialyzed against potassium phosphate buffer, 5 mM, pH 7.0, and subjected to polyacrylamide disc electrophoresis using a dual buffer system. The upper chamber buffer was Tris-glycine, 0.052 M, pH 8.9, and the lower chamber buffer was Tris-HCl, 0.1 M, pH 8.2. Ten to twenty micrograms of protein were applied to each gel. When the gels were stained for protein with Amido black or Coomassie brilliant blue R as described previously (17, 18), a single protein band was observed (Fig. 4). The nonfluorescent band of the enzyme activity corresponded to the protein band (Fig. 4). This enzyme was designated as GSH-Px A. However, the subsequent tail fractions from the DEAE-Sephadex A-25 column (from 50 to 100 ml, Fig. 3) eluted at higher salt concentration showed two protein bands on polyacrylamide disc electrophoresis (Fig. 5). The fast moving band having most of the enzyme activity correspond to the homogeneous GSH-Px A (Table I). The slow moving band, enzymatically less active was designated as GSH-Px B. The area corresponding to the proteins of both GSH-Px A and B were sliced from 11 gels and the enzymes were isolated by extracting the polyacrylamide gels with potassium phosphate buffer, 10 mM, pH 7.0, containing 0.7 mM β-mercaptoethanol. GSH-Px A and B thus isolated were again subjected to polyacrylamide disc electrophoresis. Single protein bands corresponding to the parent bands were observed (Fig. 5). The specific activity of GSH-Px A and B were found to be 103 and 4 units/mg of protein, respectively. The electrophoretically homogeneous preparation of GSH-Px B thus obtained was used in subsequent studies.

**Affinity Chromatography**—The tail fraction from DEAE-Sephadex A-25 column chromatography was dialyzed against phosphate buffer, 20 mM, pH 7.5, with 0.7 mM β-mercaptoethanol. A column (20 × 1.5 cm) of affinity resin was equilibrated with the same buffer. The enzyme solution was passed through
the column at a rate of 10 ml/hour and the column was washed with the equilibrating buffer. The enzyme was eluted with 100 mM NaCl in the same buffer. About 80% of the enzyme was recovered. However, the separation of GSH-Px A and B could not be achieved by this procedure. When a small sample of the freshly prepared red cell hemolysate was purified by affinity chromatography, a hemoglobin-free preparation of the enzyme with about 85-fold purification was obtained. However, this could not be used as the first step of purification from a large volume of enzyme solution because on a larger column the enzyme behaved differently and did not bind tightly to the resin.

**Molecular Weight Determination of GSH-Px A by Sedimentation Equilibrium Method**—The molecular weight of highly active GSH-Px A was determined by the sedimentation equilibrium method (19). In the sedimentation equilibrium centrifugation the logarithmic plot of fringe displacement with respect to radius gave a linear relation for protein solution with a correlation coefficient of over 0.998 indicating the homogeneous dispersion of protein. The molecular weight of the enzyme was calculated to be 95,000 ± 3,000 on an assumed value of partial specific volume of 0.725.

**Molecular Weight of Subunits of GSH-Px A and B**—Both GSH-Px A and B dissociated into single bands (Fig. 6) when subjected to sodium dodecyl sulfate-urea-polyacrylamide disc electrophoresis in Tris-borate-EDTA buffer containing β-mercaptoethanol (18). Aldolase, cytochrome c, bovine serum albumin, and human globin β chain were used as standards. The molecular weight of GSH-Px A and B subunits corresponded to 23,000 and 47,000, respectively.

**Kinetic and Other Properties of GSH-Px A**—The enzyme is very stable at pH 7.0 at 4°C. The enzyme lost only 15 to 20% activity when stored at 4°C at pH 7.0 for 4 months. The isoelectric pH of the enzyme determined by the isoelectric focusing was found to be 4.9. In the isoelectric focusing about 90% of the enzyme activity was lost. The enzyme was also very unstable at low pH. At pH 4.0 the enzyme irreversibly lost all the activity even in 20 min. The pH optimum of the enzyme was 8.5 and the energy of activation was 8.2 kcal. The K_m of the enzyme was 4.1 mm for glutathione and 52 μM for t-butyl hydroperoxide.

The effect of the sulfhydryl reagents and the divalent cations on the enzyme activity is given in Tables II and III. p-Hydroxymercuribenzoate and iodoacetate, 10 mM inhibited the enzyme activity by 75 to 96%. N-Ethylmaleimide brought about 50% inhibition of the enzyme activity. Copper and Hg^2+ completely inhibited the enzyme activity. Nickel, Co^2+, Zn^2+, Ca^2+, and Mg^2+ were also found to be inhibitory to the enzyme activity.

**Selenium Content of GSH-Px A and B**—The enzyme samples containing about 20 to 50 μg of protein were oxidized with HNO_3 and perchloric acid as described by Hoffman and Westerby (20) and allowed to react with 2,3-diaminophthalene. The mixture was extracted with cyclohexane and the fluorescence was measured using a Turner Fluorometer, model 110. Corning primary 7-60 and secondary filter No. 58 (Turner) were used. The selenium content of GSH-Px A was found to be 0.29%. Since the molecular weight of the protein is approximately 95,000 as evidenced by ultracentrifugation, it would account for about 3.5 atoms of selenium per mol of enzyme protein. GSH-Px B contained approximately 0.2% selenium. Since we could not determine accurately the protein content and the molecular weight of GSH-Px B because of lack of

---

**Fig. 4. Polyacrylamide disc electrophoresis of GSH-Px A.** The electrophoresis was carried out on 7.5% gels using a double buffer system, Tris-glycine buffer, 0.052 M, pH 8.3, in the upper chamber and Tris-HCl buffer, 0.1 M, pH 8.2, in the lower chamber. Twenty micrograms of protein were applied to each gel. Protein was stained by Amido black. The enzyme stain is described in the text. 1, stained for protein; 2, stained for enzyme activity.

**Fig. 5 (left).** Polyacrylamide disc electrophoresis of pooled fractions from 50 to 100 ml (Fig. 3) containing a mixture of GSH-Px A and B. The samples were subjected to polyacrylamide disc electrophoresis using the same system as for Fig. 4. One of the gels was stained for protein by Amido black and the zones of unstained gels corresponding to GSH-Px A and B were sliced and homogenized in 10 mM potassium phosphate buffer, pH 7.0. The samples were concentrated and resubjected to electrophoresis on polyacrylamide gels using the same conditions and stained for protein with Amido black. 1, re-electrophoresis of GSH-Px B isolated from the mixture of GSH-Px A and B; 2, mixture of GSH-Px A and B; 3, re-electrophoresis of GSH-Px A isolated from the mixture of GSH-Px A and B.

**Fig. 6 (right).** Urea-sodium dodecyl sulfate polyacrylamide disc electrophoresis of GSH-Px A and B. The enzyme preparations and the standard protein samples as described in the text were incubated at 37°C for 6 hours with 8 M urea containing 1% sodium dodecyl sulfate and 50 mM β-mercaptoethanol in nitrogen atmosphere; dialyzed overnight against 50 mM Tris-borate buffer, pH 7.4, containing 1% sodium dodecyl sulfate, 50 mM β-mercaptoethanol, and 12 mM EDTA. Gels, 7.5%, containing 1% sodium dodecyl sulfate and 50 mM β-mercaptoethanol were used. The electrophoresis was carried out in Tris-borate buffer, 50 mM, pH 7.4, containing 1% sodium dodecyl sulfate, 50 mM β-mercaptoethanol, and 12 mM EDTA. F, subunit of GSH-Px A, molecular weight, 23,000; S, subunit of GSH-Px B, molecular weight, 47,000.
TABLE II

Effect of divalent cations on activity of glutathione peroxidase

In a 1.0-ml system, potassium phosphate buffer, 0.1 M, pH 7.0; GSH, 2.0 mM; sodium azide, 4 mM; NADPH, 0.2 mM; and chloride salt of divalent cations, 5.0 mM, were incubated with 5 milliunits of GSH-Px A for 10 min at 37° followed by the addition of EDTA final concentration 10 mM and glutathione reductase, 1.0 unit. This was incubated for ten more minutes. The enzymatic reaction was started by the addition of t-butyl hydroperoxide, 1.0 mM. The per cent inhibition is presented as compared to controls without divalent cations.

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM</td>
<td>%</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>100</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>100</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>65</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>39</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>31</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>25</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>20</td>
</tr>
</tbody>
</table>

TABLE III

Inhibition of glutathione peroxidase by sulphydryl blocking reagents

In a 1.0-ml system, glutathione peroxide A (50 milliunits) was incubated with the sulphydryl reagents, at pH 7.4 for 1 hour at 4°, and the excess of sulphydryl reagents was removed by overnight dialysis against 10 mM phosphate buffer, pH 7.0, containing 0.7 mM β-mercaptoethanol. Enzyme activity was determined as described in the text. The per cent inhibition was compared to a dialyzed control without any sulphydryl reagent.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Final concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>10</td>
<td>96</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>Sodium arsenite</td>
<td>10</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

sufficient material, the exact number of selenium atoms per mol of GSH-Px B could not be calculated.

Immunological Studies—The antiserum produced in rabbits against a homogeneous preparation of human erythrocyte GSH-Px A obtained after DEAE-Sephadex A-25 step (Table I) showed a precipitin line in an agar double immunodiffusion plate both with crude hemolysate and with homogeneous GSH-Px A. The antibodies also showed precipitin line with homogeneous GSH-Px B (Fig. 7). This indicates that the antigenic properties of both GSH-Px A and B are similar.

To study the precipitation of glutathione peroxidase by the antiserum, 20 μl of hemolysate or purified GSH-Px A containing about 0.2 enzyme unit were incubated at 25° for 60 min with 20 μl of antiserum, and sufficient phosphate buffer, 10 mM, pH 7.0, to make a final volume to 0.2 ml. No loss of the enzyme activity was found prior to centrifugation. However, after centrifugation of the mixture at 27,000 × g for 30 min at 4° virtually no enzyme activity was recovered in the supernatant.

For the titration studies, 0.2 enzyme unit of homogeneous GSH-Px A (against which the antibodies were raised) was incubated with varying dilutions of the antiserum for 60 min at 25°. In another set of tubes approximately the same amount of protein from GSH-Px B was incubated with varying concentration of antiserum for 30 min at room temperature and at the end of this period 0.2 unit of GSH-Px A was added to incubation mixture. At the end of 60 min both sets of the incubation mixtures were centrifuged at 27,000 × g for 30 min at 4° and the supernatant was assayed for glutathione peroxidase activity. In those tubes in which only GSH-Px A was present, 7 μl of antiserum were needed to precipitate all the enzyme (Fig. 8). However, in those tubes in which the antiserum was incubated first with GSH-Px B and later GSH-Px A was added, about 10 μl of antiserum precipitated all the enzyme (Fig. 8). Thus, GSH-Px B also consumes the antibodies raised against GSH-Px A.

DISCUSSION

Glutathione peroxidase has been purified about 14,000-fold from human erythrocyte to homogeneity. In the initial stages of purification the enzyme was difficult to separate from hemoglobin by ion exchange chromatography. Although the isoelectric pH of the enzyme is 4.9 it does not bind to anion...
exchange resins like DEAE-cellulose (DE52) or ECTROLA (epichlorohydrin triethanolamine)-cellulose even at pH 7.4 in 10 mM phosphate buffer. However, after the removal of hemoglobin the enzyme easily binds to anion exchangers. This suggests a strong protein-protein interaction between hemoglobin and glutathione peroxidase. (NH₄)₂SO₄ precipitation of the hemolysate seems to be a very efficient way of removing hemoglobin. However, the concentration of hemoglobin in the hemolysate is fairly critical in this step. We found that when the concentration of hemoglobin was higher than 3 g/100 ml in the hemolysate, precipitation of considerable amounts of hemoglobin occurred along with the enzyme at 50% saturation of (NH₄)₂SO₄.

Although an enzymatically highly active homogeneous protein with a specific activity of 103 i.u./mg of protein was obtained and designated as GSH-Px A, there was another protein besides GSH-Px A in the tail fractions of DEAE-Sephadex A-25 column chromatography, the final step of purification, which we designated as GSH-Px B. On polyacrylamide disc electrophoresis, GSH-Px A moved faster than GSH-Px B. The specific activity per mg of protein of GSH-Px B was approximately 4% of that of GSH-Px A. The fraction containing both GSH-Px A and B was subjected to polyacrylamide disc electrophoresis and the protein from each band was extracted with 10 mM phosphate buffer, pH 7.0. After concentration of the protein by ultrafiltration the electrophoresis of both of these proteins on polyacrylamide disc showed single bands corresponding to original bands.

Both GSH-Px A and B showed precipitin lines with the antiserum raised against homogeneous GSH-Px A indicating antigenic similarity. In the titration studies, absorption of antibodies raised against GSH-Px A by GSH-Px B would further support that both GSH-Px A and B have common antigenic determinants. The enzyme-antibody complex was found to be enzymatically active, suggesting that the antigenic and the enzyme activity sites are at different locations on the enzyme molecule.

On polyacrylamide disc electrophoresis in sodium dodecyl sulfate-urea β-mercaptoethanol, GSH-Px A dissociated into a single band. The molecular weight of subunits was found to be 23,000. Based on the molecular weight determination of GSH-Px A by the sedimentation equilibrium method which showed the molecular weight to be 95,000 ± 3,000, the human red cell GSH-Px A is a tetramer. This is in agreement with the findings of Flohe et al. (11) for the bovine red cell enzyme. GSH-Px B has subunits of molecular weight of about 47,000 as determined by urea-sodium dodecyl sulfate-polyacrylamide disc electrophoresis. This corresponds to double the size of GSH-Px A subunits.

The total selenium content of GSH-Px A is 0.29% which corresponds to 3.5 atoms of selenium per mol of protein. This value appears to be in close agreement to the selenium content of the sheep red blood cell glutathione peroxidase (14) which has been reported to be 3.8 atoms/mol of protein. GSH-Px B has the selenium content of about 0.2%. This value is subject to a considerable uncertainty due to the difficulty in measuring the very low amount of protein in the sample. However, it is clear that like GSH-Px A, GSH-Px B is also a selenoprotein. Due to the lack of sufficient material, the molecular weight of GSH-Px B could not be determined.

It is possible that GSH-Px B is an altered form of the enzyme in which two subunits combine in such a way that they can no longer be dissociated by urea-sodium dodecyl sulfate treatment. On the other hand the possibility of GSH-Px B being the precursor of GSH-Px A cannot be ruled out.

REFERENCES
Purification and properties of human erythrocyte glutathione peroxidase.
Y C Awasthi, E Beutler and S K Srivastava


Access the most updated version of this article at http://www.jbc.org/content/250/13/5144

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/13/5144.full.html#ref-list-1