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Purification and Properties of Glutathione Peroxidase from Human Liver[1)]

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Human liver glutathione peroxidase was purified to homogeneity by using acetone precipitation, ammonium sulfate precipitation, DEAE-cellulose column chromatography, Sephadex G-150 gel filtration, and ECTEOLA-cellulose, CM-Sephadex, and DEAE-Sephadex column chromatographies. Its homogeneity was confirmed by polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis. Human liver glutathione peroxidase contained 3.7 g-atoms of selenium per mole of enzyme. The molecular weights of the enzyme and subunit were 90000 and 23000, respectively. Glutathione peroxidase consists of 4 subunits with equal subunit molecular weight. The properties of human liver glutathione peroxidase were compared with those of human placental glutathione peroxidase which was purified by the same method. In an immunological study, placental and erythrocyte glutathione peroxidases both reacted with the antibody to human liver glutathione peroxidase.

Keywords——glutathione peroxidase; liver; purification; peroxidase; immunological similarity; enzyme inhibition

Introduction

Glutathione peroxidase (EC 1.11.1.9 GSH-Px), a selenium containing enzyme,[2)] can catalyze the reduction of many organic hydroperoxides as well as hydrogen peroxide. The enzyme was first discovered in erythrocytes[3)] as a hemoglobin anti-oxidant factor and was later identified in other tissues.[4)]

Concerning its physiological role, Cohen and Hochstein,[5)] and Jacob *et al.*[6)] studied GSH-Px as a scavenger of H_2O_2 generated in erythrocytes and maintained that the enzyme prevented membrane weakness induced by H_2O_2. The GSH-Px–H_2O_2 reducing system is presumed to link glutathione reductase and the glucose-6-phosphate dehydrogenase system.[5)] Lehninger *et al.*[7)] deduced from their observation of GSH-induced swelling of mitochondria, that GSH-Px is one of the anti-swelling factors. On the basis of the observation that mitochondrial swelling is accompanied with production of lipid hydroperoxides,[8)] Little and O'Brien[9)] and Christopherson[10)] found that GSH-Px can reduce lipid hydroperoxides and nucleic acid hydroperoxides. The effect of GSH-Px on physiological lipid peroxidation was ascribed to its action as a free radical inhibitor[11)] or an indirect preventive *via* the reduction of H_2O_2 and presumably any water-soluble organic hydroperoxides,[9,10)] but Burk *et al.*[12)] concluded that GSH-Px did not prevent lipid peroxidation, and that the enzyme which is responsible for lipid peroxidation prevention effect is glutathione *S*-transferase. Recently Ursini *et al.*[13)] discovered a new peroxidation-inhibiting protein (molecular weight, 20000) which exhibits GSH-Px activity in pig liver.

GSH-Px is a non heme peroxidase containing selenium as selenocysteine in the peptide chain,[14)] and the molecular weight has been reported as 95000 for the enzyme from human erythrocytes[15)] and as 83000 for that from bovine erythrocytes.[2)] GSH-Px has been purified from bovine,[16)] ovine,[17)] and human[15)] erythrocytes,human placenta,[18)] rat liver cytosol[19)] and rat liver mitochondria.[20)]

The characteristics of human GSH-Px have been described in comparison with those of

the erythrocyte and placental enzymes,[15,18] but the immunological and some other properties have not yet been reported. For the use of GSH-Px as a clinical indicator, clarification of the immunological characteristics is indispensable. The present paper describes the similarity of human liver GSH-Px to human erythrocyte and placental GSH-Px.

Materials and Methods

Materials——Human liver from an autopsy was used and this specimen was frozen at −30°C until used. Reduced glutathione was from Sigma Chemicals Co. Glutathione reductase was purchased from Boehringer Co.; cumene hydroperoxide and *tert*-butyl hydroperoxide were from Nakarai Chemicals Co.; DEAE-cellulose, Sephadex G-150, DEAE-Sephadex A-50, CM-Sephadex C-50 were purchased from Pharmacia Fine Chemicals and ECTEOLA-cellulose was from Brown Co. Marker protein for molecular weight determination, immunoglobulin G, bovine serum albumin, cytochrome c, ovalbumin, myoglobin, and alcohol dehydrogenase were purchased from Sigma Chemical Co. Other chemicals were of reagent grade or higher quality and were purchased locally.

Analytical Determinations——Glutathione peroxidase was assayed according to the coupled assay described by Awasthi *et al.*[18] A unit of activity is defined as that amount of enzyme that catalyzes the formation of 1 μmol of GSSG/min at 25°C.

Selenium was determined fluorometrically by the method of Watkinson.[21]

Protein was determined by the method of Lowry *et al.*[22] with bovine serum albumin as a standard and by measuring the absorbance at 280 nm.

Electrophoresis——Polyacrylamide gel electrophoresis was performed by the method of Davis.[23] The gels were stained for enzyme activity by the following method. The gels were soaked for 1 min in 0.1 M potassium phosphate buffer (pH 8.0) containing 0.02 M GSH and 0.01 M cumene hydroperoxide, then placed in 0.002 M INT [3-(*p*-iodophenyl)-2-(*p*-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride] solution. When red dye was identified, the gels were soaked in 7% acetic acid solution. Protein was stained with 1% Coomassie Blue. SDS-polyacrylamide gel electrophoresis was performed by the method of Weber and Osbonr.[24]

Enzyme Preparation——Human liver GSH-Px was purified according to the method described by Awasthi *et al.*[18] with minor modifications. All the steps were performed at 4°C and all the buffers contained 1.4 mM 2-mercaptoethanol to keep the enzyme in its reduced form.

Human liver (300 g) was cut and homogenized with a Polytron homogenizer in 500 ml of 5 mM potassium phosphate buffer (pH 6.8). The homogenate was centrifuged at 8000×*g* for 20 min and 1.5 volumes of cooled acetone were added to the supernatant. After being allowed to stand for 10 min, the mixture was centrifuged at 8000×*g* for 20 min and the resulting precipitate was suspended in a small volume of 5 mM potassium phosphate buffer (pH 6.8). The suspended solution was stirred for 1 h and centrifuged at 8000×*g* for 20 min, then the supernatant was dialyzed against 5 mM potassium phosphate buffer (pH 6.8). Solid ammonium sulfate was added to the dialyzed solution to give 10% saturation. The mixture was allowed to stand for 1 h and centrifuged at 8000×*g* for 20 min. The supernatant was made up to 50% ammonium sulfate saturation, allowed to stand for 1 h and centrifuged at 8000×*g* for 20 min. (During the ammonium sulfate fractionation, the mixture was kept at pH 6.8 by adding 8% ammonium hydroxide.) The precipitate was dissolved in a small volume of 5 mM potassium phosphate buffer (pH 6.8), and dialyzed against the same buffer.

The enzyme solution was passed through a DEAE-cellulose column (3×20 cm) equilibrated with 5 mM potassium phosphate buffer (pH 6.8). The column was washed with the same buffer and the enzyme was eluted with a linear gradient of NaCl (0—0.2 M). The enzyme eluted as a single peak. A large protein peak appeared just before the enzyme activity peak, so active fractions were collected and dialyzed against 5 mM potassium phosphate buffer (pH 7.2) containing 0.1 M NaCl.

The dialyzed enzyme was centrifuged to 3 ml in an Amicon ultrafiltration cell using a PM-10 membrane. The enzyme was applied to a Sephadex G-150 column (2.6×110 cm) equilibrated with the dialysis buffer and eluted with the same buffer.

The active fractions from Sephadex G-150 gel filtration were dialyzed against 5 mM potassium phosphate buffer (pH 6.8), and passed through an ECTEOLA-cellulose column (2×20 cm) equlibrated with the above buffer and eluted with the same buffer. The eluate was applied to a DEAE-Sephadex A-50 column (2×7 cm) equilibrated with 5 mM potassium phosphate buffer (pH 6.8). The column was washed with the same buffer and subsequently eluted with a linear gradient of NaCl (0—0.3 M).

The enzymatically-active fractions were collected and dialyzed against 5 mM potassium phosphate buffer (pH 6.5), then concentrated to 10 ml by the use of an Amicon ultrafiltration cell with a PM-10 membrane, and subjected to CM-Sephadex C-50 column chromatography. The column (2×15 cm) was equilibrated with 5 mM potassium phosphate buffer (pH 6.5). The enzyme was passed through the column and the eluate was immediately dialyzed against 5 mM potassium phosphate buffer (pH 6.8).

The dialyzed enzyme was applied to a DEAE-cellulose column (2×7 cm) equilibrated with the above buffer, and the column was washed with the same buffer. The enzyme was eluted with a linear gradient of

NaCl (0—0.2 M). The activity peak coincided with the protein peak.

Human placental GSH-Px was purified by the above method, and human erythrocyte GSH-Px was purified according to the method described by Awasthi *et al.*[15]

Anti-human Liver GSH-Px Antibody——A female rabbit was injected with 100 μg of the purified enzyme emulsified in Freund's complete adjuvant into the foot pads. The injection was repeated 4 times subcutaneously at two-week intervals. Antiserum was extracted and the antibody was purified by the method of Fahey.[25] Immunotitration studies were done by incubating 0.06 units of purified GSH-Px with the antiserum in 80 μl of 0.02 M potassium phosphate beffer (pH 8.0) overnight at 4°C. Then 20 μl of goat anti-(rabbit immunoglobulin G) serum was added and the mixture was further incubated overnight at 4°C. Subsequently, the reaction mixture was centrifuged at 3000×*g* for 20 min, and GSH-Px activity was measured in the supernatant.

Results

Purification of Human Liver Glutathione Peroxidase

Table I summarizes the results of the purification of human liver GSH-Px, and Fig. 1 shows the pattern of the second DEAE-cellulose column chromatography. Human liver GSH-Px was purified approximatery 3520-fold from the crude enzyme with a recovery of 8.3%. This specimen was subjected to polyacrylamide gel disc electrophoresis. As shown in Fig. 2-a, the active peak coincided with the protein peak. SDS-polyacrylamide gel electrophoresis was also performed in 7.5% gel, and a single protein band was observed as shown in Fig. 2-b.

Properties of Human Liver Glutathione Peroxidase

The molecular weight of the enzyme was determined by Sephadex G-200 gel filtration using five standard proteins. The results (Fig. 3-a) shows that the molecular weight of human

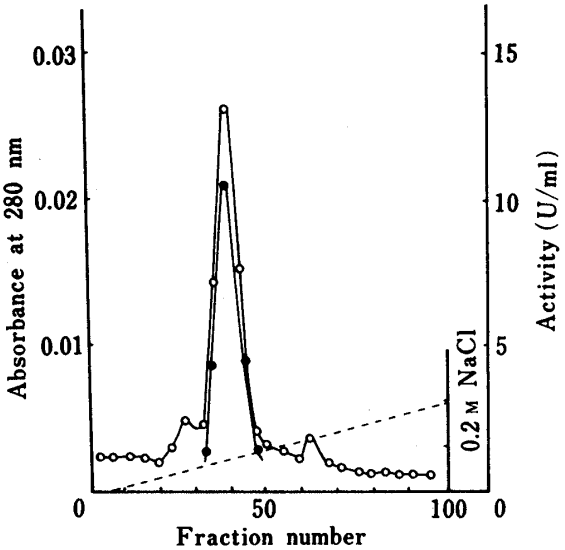

Fig. 1. Second DEAE-cellulose Column Chromatography of Human Liver Glutathione Peroxidase

A 2×7 cm column was used. Fractions of 4 ml were collected. —O—, absorbance at 280 nm. —●—, glutathione peroxidase activity.

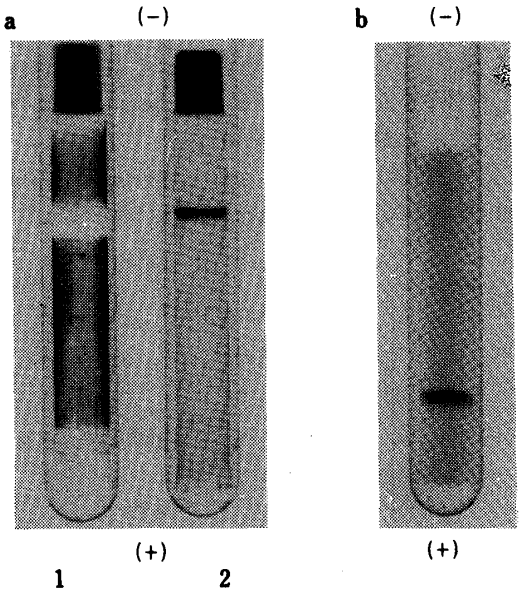

Fig. 2. The Electrophoretic Pattern of Human Liver Glutathione Peroxidase

a: Polyacrylamide gel disk electrophoresis of human liver glutathione peroxidase. Three μg of enzyme was applied to the gels. On 7.5% gels at pH 9.4, a current of 2 mA/gel for 1.5 h was applied to move the tracking dye, bromophenol blue, almost to the end of the 6 cm gel. The enzyme stain is described in the text.

b: SDS-polyacrylamide gel electrophoresis of human liver glutathione peroxidase. The gel and buffer contained 0.1% SDS. The sample was incubated at 100°C for 20 min in 0.01 M Sodium phosphate buffer (pH 7.0) containing 1% SDS and 1% 2-mercaptoethanol.

TABLE I. Purification of Human Liver Glutathione Peroxidase

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purificarion factor
Homogenate	31200	1610	0.052	1
Acetone treatment	7550	968	0.138	2.65
$(NH_4)_2SO_4$ fractionation (10—50% satn.)	3750	856	0.228	4.38
DEAE-cellulose column chromatography	768	551	0.720	13.8
Sephadex G-150 gel filtration	48.7	332	6.85	132
DEAE-Sephadex A-50 column chromatography	8.01	229	28.6	550
CM-Sephadex C-50 column chromatography	3.52	210	59.7	1150
Final DEAE-cellulose column chromatography	0.73	134	183	3520

Activity was assayed using *tert*-butyl hydroperoxide.
One unit of enzyme is that amount which oxidizes 1 μmol of GSH in 1 min.

liver GSH-Px is 90000. SDS-polyacrylamide gel electrophoresis was performed to estimate the molecular weight of the subunit of human liver GSH-Px (Fig. 3-b). The enzyme has a subunit molecular weight of 23000. Thus, human liver GSH-Px consists of four subunits with equal subunit molecular weight.

Selenium content was determined fluorometrically; the enzyme contained 3.7 g-atoms of selenium per mole of protein.

The pH optimum, pH stability, and heat stability of liver GSH-Px were investigated. The enzyme had an optimum pH at 8.5 and was stable over a pH range of 6.5—11.5. The enzyme retained 50% of the original activity after treatment at 45°C for 30 min in 50 mM potassium phosphate buffer (pH 7.0). It lost half of the original activity during storage for 3 months in 5 mM potassium phosphate buffer (pH 7.2) at 4°C. The K_m and V_{max} values are listed in Table II.

The effects of various reagents and divalent cations on the activities of liver and placental GSH-Px were investigated, and the results are shown in Table III. *N*-Bromosuccinimide and

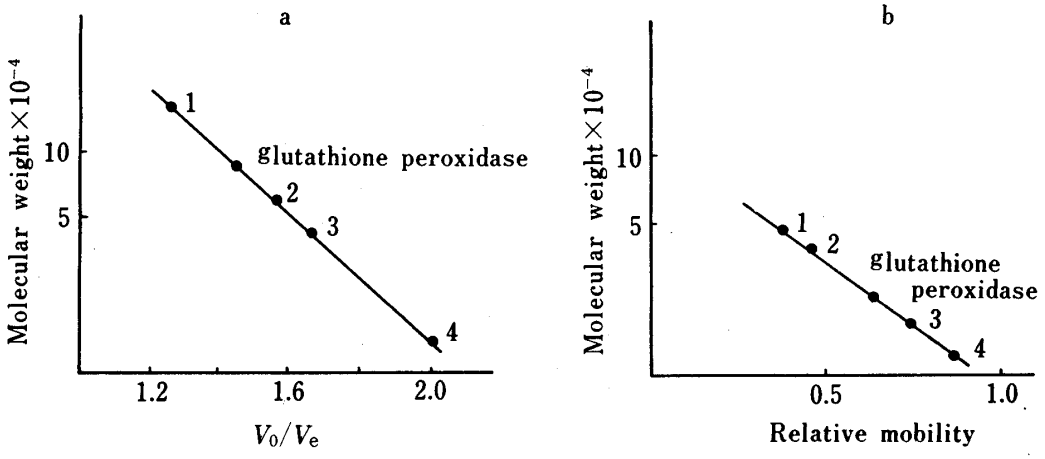

Fig. 3. Molecular Weight of Human Liver Glutathione Peroxidase

a: The enzyme was applied to a Sephadex G-200 column (2 × 110 cm) equilibrated with 5 mM potassium phosphate buffer (pH 7.2) containing 0.1 M NaCl. The calibration curve was obtained with immunoglobulin G (1), bovine serum albumin (2), ovalbumin (3), and cytochrome c (4).

b: Subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis. The gels and the buffer contained 0.1% SDS. The samples were incubated at 100°C for 10 min in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% SDS and 1% 2-mercaptoethanol. Marker proteins were ovalbumin (1), alcohol dehydrogenase (2), myoglobin (3), and cytochrome c (4).

TABLE II. Apparent K_m Values and Substrate Specificity of Human Liver Glutathione Peroxidase

Substrate	K_m	V_{max}
H_2O_2	83.3 μM	224 U/mg
Linolic acid hydroperoxide	63.5 μM	217 U/mg
tert-Butyl hydroperoxide	33.3 μM	183 U/mg
Cumene hydroperoxide	27.8 μM	137 U/mg
GSH	4.6 mM	

These K_m values were estimated from Lineweaver-Burk plots.
Four mM GSH was used to determine the K_m values of hydroperoxides, and 100 μM *tert*-butyl hydroperoxide was used to determine the apparent K_m value for GSH.

TABLE III. Effects of Various Metal Ions and Reagents on the Activities of Glutathione Peroxidase from Human Liver and Placenta

Metal salt	Remaining activity (%)		Reagents	Remaining activity (%)	
	Liver	Placenta		Liver	Placenta
None	100	100	None	100	100
$CoCl_2$	81	92	*o*-Phenanthroline	107	81
$NiCl_2$	107	100	PMSF	97	104
$CuCl_2$	110	104	PCMB	90	56
$CdCl_2$	116	92	Cysteine	97	95
$ZnCl_2$	107	100	NBS	19	17
$HgCl_2$	68	33	Iodoacetate	55	77
$MnCl_2$	26	33	KCN	103	83
$CaCl_2$	97	102			
$MgCl_2$	94	87			
$BaCl_2$	107	108			

PMSF, phenylmethyl sulfonylfluoride; PCMB, *p*-chloromercuribenzoate; NBS, *N*-bromosuccinimide. One half ml of incubation system containing 0.38 U of enzyme was incubated with 1 mM metal ions or reagents at pH 7.0 at 0℃ for 1 h. Each incubation mixture was dialyzed against 50 mM potassium phosphate buffer (pH 7.0) at 4℃ for 12 h, then the remaining activity was determined.

manganous ion inhibited the liver and placental enzymes by 80 and 70%, respectively. Hg^{2+} and *p*-chloromercuribenzoate affected the liver and placental enzymes differntly. Awasthi *et al.*[17] used another approach to study the effect of divalent cations on erythrocyte GSH-Px, *i.e.*, masking of the metal ions with EDTA after incubation with the enzyme. We also used this method and obtained results similar to those of Awasthi *et al.* (Hg^{2+}, Cu^{2+}, and Co^{2+} inhibited both enzymes strongly). However, when tested by the dialysis method, Cu^{2+} and Co^{2+} showed no effect on the activity of GSH-Px.

In immunotitration studies, liver GSH-Px, as well as placental GSH-Px and erythrocyte GSH-Px, was precipitated by the antibody raised against liver GSH-Px (Fig. 4). On double-immunodiffusion, a fused precipitin line for liver, placental and erythrocyt GSH-Px was observed.

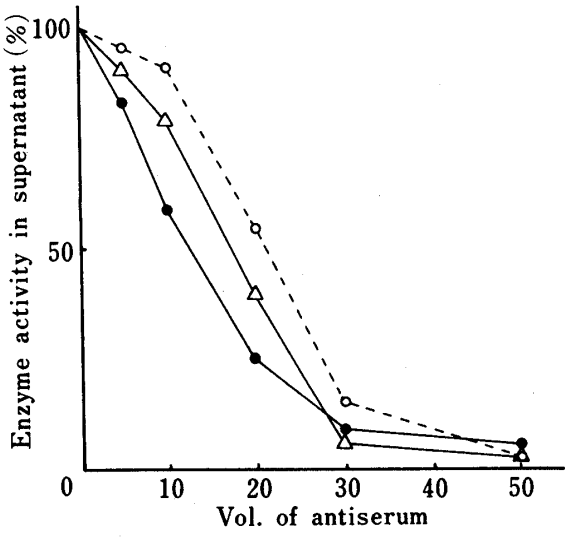

Fig. 4. Immunotitration of Glutathione Peroxidase
—●—, liver glutathione peroxidase.
—△—, placental glutathione peroxidase.
---○---, erythrocyte glutathione peroxidase.

Discussion

Human liver GSH-Px is an achromatic protein containing 3.7 g-atoms of selenium. The molecular weight was calculated to be 90000 from the results of gel filtration, and the enzyme could be dissociated into subunits with a molecular weight of 23000. Thus, human liver GSH-Px consists of four subunits having the same molecular weight. The similarity of human erythrocyte and placental GSH-Px has been reported by Awasthi *et al.*,[15,18] who gave the following data. For erythrocyte GSH-Px: molecular weight 95000, subunit molecular weight 23000, Se content 3.5 g-atoms per mole of enzyme, optimum pH 8.5, K_m for GSH 4.1 mM and for *tert*-butyl hydroperoxide 52 μM. For placental GSH-Px: molecular weight 85500 and subunit molecular weight 23000. The data obtained by us and by Awasthi *et al.* are generally in good agreement. Further, we found that human liver GSH-Px, human placental GSH-Px and human erythrocyte GSH-Px are immunologically similar.

In order to investigate the effect of metal ions on the enzyme activity, two different methods have been described. Firstly, the method in which metal ions are masked after incubation with the enzyme and subsequently the remaining activity is measured by coupled assay was described by Awasthi *et al.*[15] Secondly, a direct spectrophotometric assay that monitors the decrease of GS– concentration in ammonium buffer at 237 nm was described by Splittgerber and Tappel.[26] At a concentration of 1 mM metal ions, Hg^{2+}, Cu^{2+}, and Co^{2+} were potent inhibitors and Ni^{2+} was a fairly potent inhibitor of human liver and placental GSH-Px as determined by Awasthi's method, while with Tappel's method Co^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, and Hg^{2+} completely inhibited both enzyme activites. These two methods have some problems, such as interference by masking agent in the assay in Awasthi's method or the state of metal ions and ammonium ion complexes in Tappel's method. Therefore, the dialyzing method may be preferable. By this method,it has found that human liver and placental GSH-Px were irreversibly inactivated by *N*-bromosuccinimide and monoiodoacetate. Placental GSH-Px was more strongly inhibited by Hg^{2+} and *p*-chloromercuribenzoate than liver GSH-Px. It is possible that an -SH or -SeH group in active site, or a thiol group on the enzyme surface is more intimately involved in the activity of the placental enzyme than in that of the liver enzyme. The discrepancy of enzyme behavior with Cu^{2+} and Co^{2+} in all three methods suggests that GSH-Px activity may be reversibly controlled by these metal ions in the physiological state.

The K_m values for four different hydroperoxides reveal that human liver GSH-Px has high affinity for hydroperoxides having large carbon groups. This suggests that GSH-Px is physiologically important in the decomposition of long chain fatty acid hydroperoxides derived from membrane fatty acids. The K_m values for GSH were almost the same as the *in vivo* GSH concentration. The concentration of lipid hydroperoxides in the physiological state is much smaller than the K_m values for hydroperoxides, so decomposition of hydroperoxides by GSH-Px should be dependent on substrate concentration. We are now investigating this.

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