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PURIFICATION AND PROPERTIES OF RAT LIVER GLUTATHIONE PEROXIDASE

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SUMMARY

Glutathione peroxidase (GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) was purified 400-fold from rat liver by fractionation with $(NH_4)_2SO_4$ and chromatography with DEAE-cellulose, Sephadex G-200 and DEAE-Sephadex A-50 in the presence of 0.5 mM GSH. The purified enzyme was shown to be homogeneous in Sephadex chromatography and ultracentrifugation.

The molecular weight of the enzyme was estimated to be 78 500 from $s^\circ_{20,w}$ and $D^0_{20,w}$, 76 000 from sedimentation equilibrium measurement and 75 000–76 000 by thin-layer gel filtration. In the presence of 1.0% sodium dodecylsulfate, the molecular weight was reduced to 19 000 from polyacrylamide-gel electrophoresis, indicating that the native enzyme consists of four subunits with the same molecular size. The molecular weight of the subunit was also calculated to be 17 000 from amino acid composition. It was also found that the enzyme contained 4 atoms of selenium ion and 8 moles of free sulfhydryl groups per mole of protein. No absorption spectrum characteristic of heme or flavin was observed.

The enzyme activity was decreased gradually and significantly during the storage of the enzyme in the absence of dithiothreitol even at the frozen state, probably due to the denaturation of the enzyme.

INTRODUCTION

Mills [1, 2] first found glutathione (GSH) peroxidase (GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) in erythrocytes and obtained the enzyme preparation free from catalase activity by DEAE-cellulose column chromatography. Subsequently, Holmberg [3] purified the enzyme from bovine lens up to about 1400-fold in the presence of high concentration of GSH, but sufficient information on the physicochemical properties of the enzyme could not be obtained because of small amount of the enzyme purified. Flohe and co-workers [4–9] have reported various properties of the enzyme purified from erythrocytes together with the evidence that the enzyme consists of four subunits with an identical molecular weight of 21 000. Very recently they have demonstrated that the erythrocyte peroxidase is a seleno-enzyme, containing 4 atoms of selenium ion per mole of protein [10].

Although the physiological importance of GSH peroxidase in liver metabolism was stressed [11–14], only a partial purification of this enzyme has been performed in rat liver [15]. We have, therefore, attempted to purify the enzyme from rat liver.

This paper mainly deals with the purification procedure and physicochemical properties of the enzyme. The results obtained show that the rat liver GSH peroxidase also consists of four identical subunits, contains 4 atoms of selenium ion and 8 moles of cysteine residue per mole of protein.

EXPERIMENTAL

Materials

Female Moriyama rats weighing 150–200 g were killed by decaptation, and the livers were quickly removed and stored at −80 °C after washing with Hanks' solution [16] (without glucose and phenol red, pH 7.2) to remove contaminated erythrocytes.

The following chemicals were purchased from Boehringer Mannheim: GSH, NADPH, horse heart cytochrome *c*, bovine pancreas chymotrypsinogen A, bovine liver catalase and yeast glutathione reductase (spec. act. approx. 90 units/mg). Bovine serum albumin, hemoglobin and seleno-DL-methionine were products of Sigma Chemical Co., and the egg albumin of Nutritional Biochemicals Corporation. DEAE-Sephadex A-50 (capacity 3.5 ± 0.5 mequiv/g) and Sephadex G-200 superfine were obtained from Pharmacia, and DEAE-cellulose (capacity 0.89 ± 0.2 mequiv/g) from Brown. All inorganic salts and organic reagents were purchased from other commercial sources.

Enzyme assay

GSH peroxidase activity during purification was routinely assayed by following the oxidation of NADPH at 340 nm in the presence of glutathione reductase which catalyzed the reduction of GSSG formed by the peroxidase, in a Shimadzu QV-50 spectrophotometer. The standard reaction mixture was composed of 50 mM sodium phosphate buffer (pH 7.0), 0.16 mM NADPH, 1 mM NaN_3, 0.4 mM EDTA, 1 mM GSH, 0.2 mM H_2O_2, 4 μg of yeast glutathione reductase and 10 μl of an enzyme fraction in a total volume of 2 ml. The reaction was started by the addition of H_2O_2 and measured at 25 °C. The non-enzymatic oxidation of GSH was measured by using water instead of the enzyme fraction in the standard reaction mixture, and its reaction rate was subtracted from that of the former system in order to determine the true enzymatic activity. One unit of the enzyme was defined as the amount required to oxidize 1 μmole GSH/min which corresponded to 0.5 μmole NADPH oxidized/min. Protein concentration was estimated by the method of Lowry et al. [17] using crystalline bovine serum albumin as a standard.

Analytical methods

Ultracentrifugal analysis. Sedimentation velocity, diffusion and sedimentation equilibrium experiments were performed in a Beckman Spinco Model E analytical ultracentrifuge equipped with a RTIC temperature control system and a phase plate as a schlieren diaphragm. Diffusion coefficient was estimated according to the method of Ehrenberg [18]. For sedimentation equilibrium experiments a schlieren optical system was used with a 3-mm column in a filled Epon double sector cell rotating

12 590 rev./min at room temperature. The photographs were taken with five different phase plate angles.

Thin-layer gel filtration. For the estimation of the approximate molecular weight of enzyme the thin-layer gel filtration was employed according to the method of Radola [19] using Sephadex G-200 superfine and Pharmacia thin-layer chromatographic apparatus. A gel suspension in 50 mM sodium phosphate buffer (pH 7.0) was heated in boiling water for 3 h. After cooling the gel was coated with the thickness of 0.6 mm on a glass plate (20 cm × 20 cm). Each 5 μl of sample solution was charged on the gel layer equilibrated with the same buffer solution and the migration was carried out with a plate angle at 20 ° for 3 h at room temperature. Whatman 3MM filter paper was used to take a print from the gel. The migrated proteins were stained with 0.25% Coomassie Brilliant Blue R-250 in methanol–glacial acetic acid (9:1, v/v), and then the ratio of the migration distance of each protein to that of horse heart cytochrome *c* was calculated. Pancreatic chymotrypsinogen A, serum albumin, liver catalase and egg albumin were used as authentic samples.

Polyacrylamide-gel electrophoresis. Analysis for enzyme subunit was made in sodium dodecylsulfate–polyacrylamide-gel electrophoresis with a slight modification of the method described by Shapiro et al. [20]. Before electrophoresis the enzyme sample was incubated in 50 mM sodium phosphate buffer (pH 7.0) containing each 1% of sodium dodecylsulfate and 2-mercaptoethanol for 2 h at 50 °C. Electrophoresis was carried out in 0.1 M sodium phosphate buffer (pH 7.1) containing each 0.1% of sodium dodecylsulfate and 2-mercaptoethanol with a constant current of 5 mA/tube for 8 h, at room temperature. Horse heart cytochrome *c*, bovine serum albumin and egg albumin were used as standard substances.

Amino acid analysis. Enzyme samples were hydrolyzed in 5.7 M HCl for 24, 48 and 72 h in vacuo at 100 °C. Analyses were performed by the method of Spackman et al. [21] using a Beckman amino acid analyzer Model 120 B. Cysteine was measured as cyteic acid after the oxidation of the enzyme sample with performic acid [22], and tryptophan was determined spectrophotometrically [23].

Selenium content analysis. Microdetermination of selenium ion was performed by the method developed by Watkinson [24] with some modifications. Initially, 0.5 ml of the enzyme solution (0.5–1.0 mg protein/ml) was mixed with 2.5 ml of conc. HNO_3, allowed to stand overnight and strongly digested at 100 °C for 30 min. Thereafter, the sample solution was boiled for 15 min with 1 ml of $HClO_4$ (70%). Next, after the addition of each 1 ml of water and 10% HCl, the solution was incubated again in the boiling water bath for 5 min, and its pH was adjusted to 1.0 with 7 M NH_4OH. This acidified solution, containing selenium ions released from protein moiety, was diluted to 25 ml with 0.1 M HCl and incubated with 2.5 ml of 0.1% 2,3-diaminonaphthalene in 0.1 M HCl at 50 °C for 20 min in dark. Finally, the piazselenol complex formed was extracted with 5 ml of cyclohexane. The organic phase was washed twice with 15 ml of 0.1 M HCl. Selenium concentration was estimated from the fluorescence intensity of the complex formed at 520 nm under the 376 nm Xenon line for excitation in an Hitachi EPF 2 type fluorescence spectrometer using seleno-DL-methionine as a standard. When the heat treatments of the acidified sample described above were omitted, the formation of piazselenol complex from seleno-DL-methionine or sodium selenate was found to be negligible. On the contrary, the almost same level of selenium could be detected from potassium selenite with or without the heat treatments. A solution

of quinine (0.01 μg/ml) in 0.05 M H_2SO_4 was used to adjust the fluorometer for each determination. Selenium of 0.2 μg/ml could be determined accurately by this procedure. Before analysis the enzyme sample was dialyzed against 50 mM sodium phosphate buffer (pH 7.0) containing 10 mM EDTA for 2 days with 3 changes of external fluid.

Other analyses. Estimation of sulfhydryl groups of the enzyme protein was carried out spectrophotometrically according to the method of Boyer [25] using cysteine, GSH and egg albumin as standard substances. Before determination enzyme was dialyzed against 50 mM sodium phosphate buffer (pH 7.0) for 2 days with 5 changes of external fluid. Absorption spectrum of the purified enzyme was measured with a Cary Model 14 recording spectrophotometer. Analysis for non-heme iron in the enzyme was made with a Perkin–Elmer atomic absorption apparatus.

RESULTS AND DISCUSSION

Purification of GSH peroxidase

All purification procedures were performed at 4 °C.

Enzyme extraction. Rat liver (400 g) were homogenized with 3 vol. of Hanks' solution [16] (without glucose and phenol red, pH 7.2) in a Waring blendor for 1 min, and the homogenization was repeated in a glass–Teflon homogenizer. The supernatant fraction was obtained by centrifugation at 105 000 $\times$ *g* for 1 h. The fraction had a specific activity of about 0.18 μmole/min/mg protein.

$(NH_4)_2SO_4$ fractionation. The crude extract thus obtained was adjusted to 30% satn with $(NH_4)_2SO_4$. The precipitate formed was removed by centrifugation at 10 000 $\times$ *g* for 15 min and the resulting supernatant was made up to 50% satn of $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 10 000 $\times$ *g* for 15 min, dissolved in a small amount of 0.008 M sodium phosphate buffer solution containing 0.5 mM GSH and 0.25 mM EDTA, and dialyzed against 50 vol. of the same buffer solution with 3 changes of external fluid. When the precipitated enzyme was dissolved in a buffer solution without GSH, the enzyme activity was decreased to two-third of that with GSH. Further purification, therefore, was carried out with sodium phosphate buffer solution containing GSH. Dithiothreitol at concentration of 0.25–0.5 mM prevented the decrease in enzyme activity same as GSH did.

DEAE-cellulose chromatography. After removal of impurities precipitated during dialysis the dialyzate was applied to a column (5 cm $\times$ 40 cm) of DEAE-cellulose equilibrated with 0.01 M buffer solution. In this step no GSH peroxidase was adsorbed. The enzyme fraction passed through the column was collected by making up to 55% satn of $(NH_4)_2SO_4$. The precipitate obtained was dissolved in a minimum volume of 0.05 M buffer solution and dialyzed against 50 vol. of the same buffer solution with 3 changes of external fluid.

First DEAE-Sephadex A-50 chromatography. The dialyzed sample was charged on a column (4 cm $\times$ 30 cm) of DEAE-Sephadex A-50 equilibrated with 0.05 M buffer solution. Through this chromatography the enzyme fraction was separated from large amounts of impurities absorded on the column, and the eluate with the enzyme activity was concentrated by ultrafiltration.

Gel filtration on Sephadex G-200. The sample was then applied on a column (4 cm $\times$ 30 cm) of Sephadex G-200 equilibrated with 0.05 M buffer solution and the

run was made at an effluent rate of 0.5 ml/min. The activity emerged as a symmetrical peak after an elution of small amounts of contaminated protein with higher molecular weight.

2nd–5th DEAE Sephadex A-50 chromatography. The concentrated enzyme solution was loaded on a column (3 cm × 30 cm) of DEAE-Sephadex A-50 equilibrated with 0.05 M buffer solution. The column was developed in a linear gradient system made with each 500 ml of 0.05 and 0.25 M buffer solution, and 10 ml fractions were collected. Active fractions were combined and concentrated by ultrafiltration. The chromatography was repeated 3 times in the same manner as described above. On the fifth chromatography the enzyme was eluted at the buffer concentration between 0.085 and 0.115 M as a single sharp peak which had constant specific activity (approx. 70 units/mg of protein) throughout the active fractions (Fig. 1). The active fractions

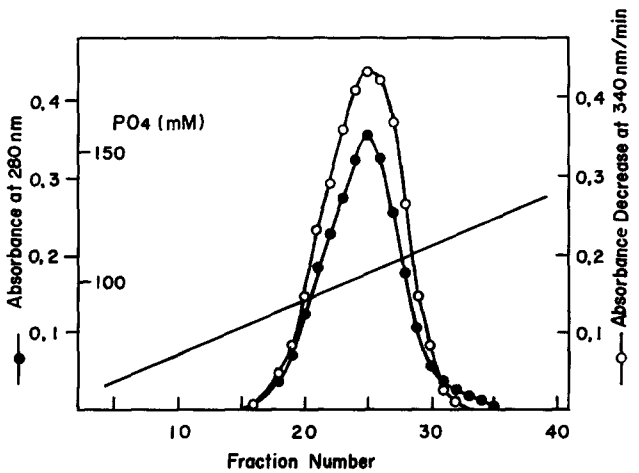

Fig. 1. Elution diagram of GSH peroxidase in 5th DEAE-Sephadex A-50 chromatography. The concentration of sodium phosphate buffer in eluate is shown in mM. Fraction volume: 10 ml.

combined were finally concentrated to 1.5 ml by ultrafiltrations. The preparation thus obtained was purified about 400-fold compared with the original crude extract. A typical result of the purification is summarized in Table I.

Molecular weight of the enzyme

When the freshly prepared enzyme was examined in the ultracentrifuge, it sedimented with a single symmetrical peak, indicating that it was homogeneous (Fig. 2). The sedimentation coefficients were almost independent of protein concentrations, and $s^{\circ}_{20,w}$ value of 4.85 ± 0.08 was obtained. A partial specific volume of 0.741 ml/g was estimated from amino acid analysis data described later. Diffusion coefficient was also calculated to be $5.76 \pm 0.09 \cdot 10^{-7}$ cm·s^{-1}. From these values an estimation of the molecular weight could be made at 78 500 ± 2500. Fig. 3 shows a typical result of the sedimentation equilibrium measurement. The molecular weight of 76 000 ± 1000 was obtained as an average value on three independent measurements. However, the schlieren plots versus the square of the radius of rotation gave a slightly curved line.

TABLE I

PURIFICATION OF RAT LIVER GSH PEROXIDASE

One unit of the enzyme was defined as the amount required to oxidize 1 μmole GSH/min in the presence of 1 mM GSH at 25 °C (pH 7.0). The units can also be expressed as logarithmic units [26] which may be calculated by the equation, unit = $(2.303/t)\cdot\log(C_0/C_t)$ (C_0: initial GSH concn, C_t: GSH concn in time t). One unit corresponds to about 1.05 logarithmic units according to this equation.

Step	Volume (ml)	Total protein (mg)	Spec. act. (units/mg protein)	Yield (%)
105 000 × *g* supernatant	1020	48 500	0.182	100
30–50% $(NH_4)_2SO_4$	112	9 830	0.624	69
DEAE-cellulose	212	3 080	1.266	44
1st DEAE-Sephadex A-50	118	516	7.14	42
Sephadex G-200	63	255	10.52	30
2nd DEAE-Sephadex A-50	104	108	18.26	22
3rd DEAE-Sephadex A-50	75.5	45.3	37.10	19
4th DEAE-Sephadex A-50	6.0	22.0	68.16	17
5th DEAE-Sephadex A-50	1.5	15.0	70.4	12

Furthermore, the approximate molecular weight was also estimated by thin-layer gel filtration using Sephadex G-200 superfine. From the migration distance of the enzyme relative to that of horse heart cytochrome *c*, the molecular weight of the enzyme was also estimated to be 75 000–76 000 (Fig. 4). The molecular weights of the enzyme obtained by three different analytical methods were not different significantly from each other. Consequently, it may be concluded that the molecular weight of rat liver GSH peroxidase is slightly smaller than that of the enzyme purified from erythrocytes [5]. The enzyme purified from bovine lens has a molecular weight of 96 000, which was estimated only from a pattern of the enzyme activity in a gel filtration, so that this value can not be compared directly with that of the rat liver enzyme.

Subunit of the enzyme

Sodium dodecylsulfate–polyacrylamide gel electrophoresis of the enzyme preincubated with each 1% of sodium dodecylsulfate and 2-mercaptoethanol at 50 °C for 2 h, resulted in a single dense band which corresponded to about 90% of the total protein and two faint bands migrated slower than the former, suggesting that most of

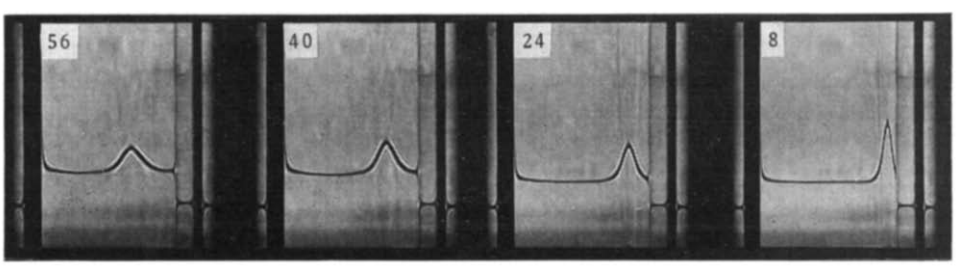

Fig. 2. Ultracentrifugal pattern of the purified GSH peroxidase. The protein concentration was 6.5 mg/ml in 50 mM sodium phosphate buffer (pH 7.0). The photographs were taken, from right to left, at 8, 24, 40 and 56 min after reaching 59 780 rev./min at 20 °C.

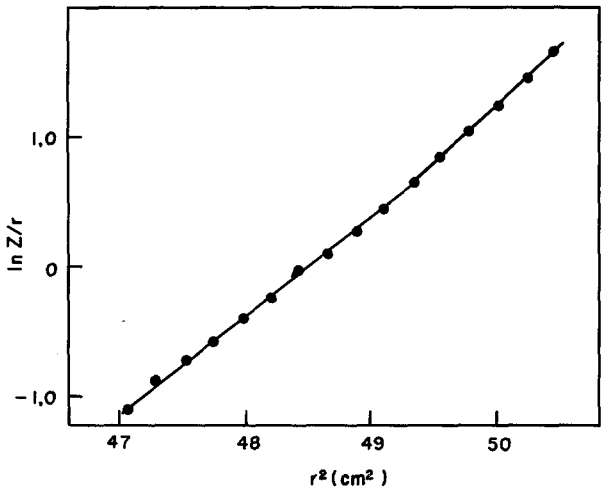

Fig. 3. Sedimentation equilibrium of GSH peroxidase in 50 mM sodium phosphate buffer (pH 7.0). The ordinate scale is the natural logarithm of the ratio of the vertical displacement to the distance from the axis of rotation, and the abscissa gives the square of the distance from the axis of rotation. Phase plate angle: 80°.

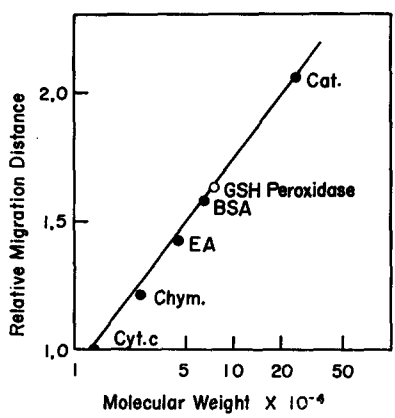

Fig. 4. Estimation of the molecular weight of GSH peroxidase by thin-layer gel filtration on a Sephadex G-200 superfine. Standard curve shows the relationship between the molecular weights of authentic proteins and their relative migration distance. Abbreviations: Cyt. c, horse heart cytochrome *c*; Chym, bovine pancreas chymotrypsinogen A; EA, egg albumin; BSA, bovine serum albumin; Cat, liver catalase.

the enzyme tested was dissociated to a subunit with the same molecular size. The molecular weight of the main component was estimated to be 19 000 (monomer) from its migration distance, and the other two faint components were found to be of 38 000 and 56 000 molecular weight, respectively (Fig. 5). These findings suggest that the rat liver enzyme consists of four subunits with the same molecular size which were also found in erythrocyte enzyme [5].

Table II shows the amino acid composition per subunit of the enzyme. The molecular weight of subunit was calculated to be 17 000 from amino acid composition.

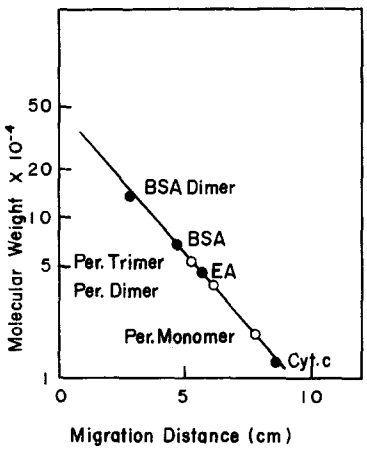

Fig. 5. Estimation of the molecular weight of GSH peroxidase-subunit by polyacrylamide-gel electrophoresis in the presence of 0.1% sodium dodecylsulfate. Each 50 μg of the enzyme protein and authentic samples was applied to a column (7 mm × 125 mm). Abbreviation as given in Fig. 4 was used.

TABLE II

AMINO ACID COMPOSITION OF SUBUNIT OF GSH PEROXIDASE

Amino acid	Molar ratio*	Residue per subunit**
Lys	0.0448	11
His	0.0164	4
Arg	0.0267	7
Trp***	—	(1)
Asp	0.0568	14
Thr	0.0310	8
Ser	0.0354	9
Glu	0.0606	15
Pro	0.0396	10
Gly	0.0530	13
Ala	0.0416	10
Cys§	—	(2)
Val	0.0439	11
Met	0.0124	3
Ile	0.0305	8
Leu	0.0576	15
Tyr	0.0189	5
Phe	0.0289	7

* Data is based on the average of three determinations.

** A value of 19 000 as the molecular weight of subunit was used to determine the nearest integer relative to methionine. This value was obtained by sodium dodecylsulfate–polyacrylamide-gel electrophoresis as described in the text.

*** Tryptophan was measured by spectrophotometrically [23].

§ Half-cystine was measured as cysteic acid after performic acid oxidation [22].

This value is not different significantly from that of 19 000 estimated from sodium dodecylsulfate–polyacrylamide gel electrophoresis. Therefore, the molecular weight of the enzyme calculated from the amino acid composition is about 10% smaller than those estimated from sedimentation experiments and gel filtration. The analysis also revealed two cysteine residues per subunit.

Selenium content of the enzyme

Table III shows that the content of selenium was 4.24 μg per mg enzyme, which corresponds to 4 atoms of selenium ion per mole of protein. This value agrees quite

TABLE III

DETERMINATION OF SELENIUM CONTENT OF GSH PEROXIDASE BY FLUOROMETRIC ANALYSIS

Experimental conditions used as described in text. The fluorescence intensities of each sample with (+) and without (−) heat treatments were measured. Selenium content of the enzyme was calculated using seleno-DL-methionine as a standard.

Sample	Heat treatments	Relative fluorescence intensity at 520 nm	Selenium content (μg/mg protein)
0.5 M phosphate buffer	+	31	
2 μg Se in seleno-DL-methionine	+	55	
	−	30.5	
0.5 mg GSH peroxidase	+	56.5	4.24
	−	32.5	0.25
0.5 mg GSH peroxidase + 1.0 mg bovine serum albumin	+	54	3.84
2 μg selenium in sodium selenate	+	77	
	−	33	
2 μg selenium in potassium selenite	+	94	
	−	90	

well with that reported Flohe et al. [10] who determined selenium in the erythrocyte enzyme by neutron activation analysis. It is also noticed in this table that when the heat treatment of the acidified sample solution was omitted, the formation of piazselenol complex from the enzyme as well as seleno-DL-methionine and sodium selenate was hardly detectable, suggesting that selenium in the enzyme may not exist as a form of selenite. From the results described above, it is apparent that GSH peroxidase purified from rat liver is the same seleno-enzyme as that from bovine erythrocyte [10].

Other properties of the enzyme

Free sulfhydryl groups of the enzyme were found to be 8 moles/mole of protein by the spectrophotometric titration with *p*-chloromercuribenzoate (Fig. 6). This result corresponds closely to the amino acid analysis data mentioned above.

On the other hand, Flohe et al. [7] described that the titratable amount of SH groups of the enzyme varied from 3 to 7.5 per mole of protein depending on the func-

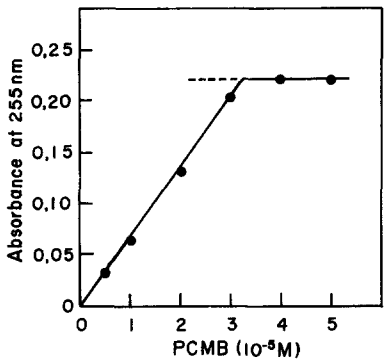

Fig. 6. Titration of free sulfhydryl groups in GSH peroxidase with *p*-chloromercuribenzoate (PCMB). Absorbance increases at 255 nm as a result of formation of the mercaptide complex were measured after the incubation of the reaction mixture for 15 min 37 °C. The reaction mixture was composed of 0.33 M sodium acetate buffer (pH 4.5), $4 \cdot 10^{-6}$ M enzyme protein and $5 \cdot 10^{-6}$–$5 \cdot 10^{-5}$ M of PCMB in a total volume of 3 ml.

tional state of the enzyme. The difference in free sulfhydryl groups observed by the two groups is unclear, probably being due to different pH values employed in assay conditions.

The absorption spectrum of the purified enzyme showed a peak at 280 nm with a small shoulder at 290–293 nm. No significant absorption was observed in the visible range. This suggests that the GSH peroxidase has no prosthetic group such as heme and flavin derivative. Furthermore, iron in the enzyme was found to be negligible (0.038 atom of iron per mole of protein).

Stability of the enzyme

The purified enzyme has a specific activity of 70 units/mg protein. The enzyme was markedly stabilized during storage in the presence of reducing agents such as dithiothreitol and GSH. No appreciable loss of the activity was detected even after 3 months storage in the presence of 1 mM dithiothreitol at the frozen state. On the other hand in the presence of 2 mM GSH, the initial enzyme activity was maintained for a week, though 15–22% of the activity was decreased over 1 month of the storage, probably due to the oxidation of GSH.

When either dithiothreitol or GSH was omitted, the activity was decreased gradually during the storage even at the frozen state and reduced to a level of 60–70% of the initial activity for 1 month. These findings may indicate that in the rat liver GSH peroxidase free sulfhydryl groups play an important role for the efficient operation of the peroxidation of GSH.

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