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PURIFICATION AND PROPERTIES OF RAT LUNG SOLUBLE GLUTATHIONE PEROXIDASE

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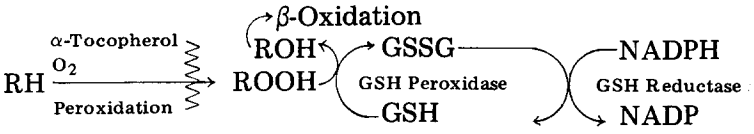
Summary

Gluthathione peroxidase (gluthatione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9) has been purified approximately 2700-fold from rat lung soluble fraction. The purified enzyme was shown to be homogeneous by sodium dodecyl sulfate/urea polyacrylamide gel electrphoresis. Selenium-75 tracer cochromatographed with the enzyme activity, indicating that rat lung soluble gluthathione peroxidase is a selenium enzyme. The enzyme had an approximate molecular weight of 80 000 and contained four identical subunits. The optimal activity of the enzyme was at between pH 8.8 and 9.1. The enzyme had general specificity toward hydroperoxides, and high specificity for reduced glutathione. The kinetic behavior of the purified lung soluble glutathione peroxidase followed a ping-pong-like mechanism; the enzyme first reduced the lipid hydroperoxide substrate to the corresponding hydroxy fatty acid, then was regenerated to the native form by reduced glutathione.

Introduction

Glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9) was first reported by Mills and Randle [1] to be in erythrocytes, and later it was reported to be in various mammalian tissues [2]. This enzyme catalyzes the reaction $2\ GSH + ROOH \rightarrow GSSG + ROH + H_2O$. Mills and Randle [3] partially purified glutathione peroxidase free from catalase activity by DEAE-cellulose column chromatography. Catalase has long been considered to be the major enzyme responsible for reducing hydrogen peroxide. However, Cohen and Hochstein [4] found that glutathione peroxidase rather than catalase protects erythrocyte hemoglobin from oxidation to methemoglobin by hydrogen peroxide. From these results they suggested that glutathione peroxidase may be the first line of defense against oxidative damage by hydrogen peroxide or lipid peroxides produced in various cells of the animal. Little and O'Brien [5] added more evidence to support this hypothesis by finding that

glutathione peroxidase decomposes lipid hydroperoxides in the liver cell, and thus it protects the cell from the deleterious effects of lipid peroxidation. Christophersen [6] identified the lipid products formed by the reduction of lipid peroxides by glutathione peroxidase, and he suggested that glutathione peroxidase may be able to break the autocatalytic chain reaction of lipid peroxidation. Chow and Tappel [7,8] suggested the following mechanism as being protective against lipid peroxidation in various tissues:



Most work done on glutathione peroxidase has been with the erythrocyte enzyme [9,10], and very little information has been reported on this enzyme in other tissues. Since lung tissue is most accessible to oxidant air pollutants, such as ozone and nitrogen dioxide, and since it is well-documented that these compounds can cause lipid peroxidation in lung tissue [11—13], it was a major objective to study the nature of glutathione peroxidase of rat lung.

Materials and Methods

Enzyme source. Male Sprague-Dawley rats (300—500 g) were killed by intraperitoneal injection of sodium pentobarbital 48 h after each rat had been injected with 50 μCi of ^{75}Se as $Na_2{}^{75}SeO_3$. Lung homogenate prepared in 250 mM sucrose and 1 mm EDTA in 5 mM Tris · HCl buffer (pH 7.6) was used as the starting material for purification of glutathione peroxidase.

Sources of reagents and column chromatographic materials. Bovine serum albumin, hemoglobin, cytochrome *c*, glutathione reductase (type III yeast), NADPH, reduced glutathione, and all sulfhydryl compounds were purchased from Sigma Chemical Co., St. Louis, Mo.; all peroxides except hydrogen peroxide were obtained from Polysciences, Rydal, Penn.; the hydrogen peroxide was purchased from Mallinckrodt Chemical Works, St. Louis, Mo.; $Na_2{}^{75}SeO_3$ was obtained from New England Nuclear, Gardena, Calif.; Sephadex G-100 and G-150, and Sepharose 4B were purchased from Pharmacia Fine Chemicals, Piscataway, N.J.; and DEAE Bio-Gel A was purchased from Bio-Rad Laboratories, Richmond, Calif.

γ-Scintillation counting. ^{75}Se radioactivity was measured with a Packard scintillation system converted to a γ-counting instrument with a Bicron NaI γ conversion vial. The counting efficiency was approximately 16%.

Enzyme assays and protein determination. Glutathione peroxidase activity was assayed by the procedure described by Little et al. [14] with slight modifications. The enzyme was coupled to NADPH via glutathione reductase and the rate of NADPH oxidation was measured spectrophotometrically at 340 nm. The reaction mixture consisted of 0.25 mM reduced glutathione, 0.2 mM cumene hydroperoxide, 0.12 mM NADPH, 1 unit of glutathione reductase (1 unit oxidizes 1 μmol NADPH per min), 0.091 mM EDTA, and 50 mM Tris · HCl buffer (pH 7.6). Unless otherwise stated, the enzyme assays were carried out at 37°C.

For some assays a second method was used. The glutathione peroxidase activity was assayed at 25°C with glutathione as hydrogen donor in a system that consisted of 50 mM Tris · HCl buffer (pH 7.6), 1 mM EDTA, 500 μM reduced sulfhydryl compound, 350 μM peroxide, and approx. 0.5 μg of enzyme protein in a final volume of 1.0 ml. The reaction was started by addition of the peroxide and was stopped by denaturation of the enzyme with 4 ml of 6.3% trichloroacetic acid. The reduced glutathione or other sulfhydryl compound was measured according to a modification of the procedure of Sedlack and Lindsay [15]. Protein was determined by both 280 nm absorbance and Miller's method [16].

Purification of glutathione peroxidase. A homogenate from the lungs of 30 rats was centrifuged at 12 000 × *g* for 20 min. The supernatant portion was filtered through glass wool and the filtrate was centrifuged at 100 000 × *g* for 30 min. The supernatant solution was subjected to an acid treatment by addition of reduced glutathione to a final concentration of 5 mM and a final acidity of pH 5.0. After incubation for 1 h at 4°C, the solution was centrifuged at 12 000 × *g* for 20 min. The supernatant portion was neutralized and heated at 50°C for 45 min. After centrifugation at 12 000 × *g* for 15 min, the supernatant portion was precipitated with 50% acetone at −20°C for 20 min. The pellet was redissolved in 30 ml of 10 mM Tris · HCl buffer (pH 7.6). After centrifugation at 12 000 × *g* for 10 min, the clear supernatant portion was applied to a Sephadex G-100 column (5 × 100 cm) and eluted with 10 mM Tris · HCl buffer (pH 7.6). The most active fractions were pooled and concentrated to about 10 ml by pressure filtration through an Amicon PM 30 ultrafiltration membrane. The concentrated sample was applied to a DEAE-Agarose column (2.5 × 40 cm). After application of the enzyme sample, the column was washed with 5 mM Tris · HCl buffer (pH 8.0), and the enzyme was eluted with a 0.0—0.2 M NaCl linear gradient in the same buffer. The most active fractions were pooled and concentrated as described above. The concentrated DEAE fraction was applied to a Sephadex G-150 column (2.5 × 100 cm). The elution procedure was identical with that used for the first Sephadex column. The most active fractions were again pooled and concentrated as described before. The pooled and concentrated Sephadex G-150 sample was then applied to the final column, a valine-agarose affinity column (1.25 × 30 cm). This column was prepared by addition of valine (200 mg in 50 ml of 0.1 M sodium borate, pH 9.5) to Sepharose 4B (50 ml) that had been activated by cyanogen bromide (5 g) by the method of Cuatrecasas [17]. After application of the enzyme sample, the column was washed with 1.5 M potassium phosphate buffer (pH 7.5). The enzyme was eluted with a 1.5—0.5 M phosphate gradient (250-ml total gradient). The pooled enzyme fraction was dialyzed against 10 mM Tris · HCl buffer (pH 7.6), lyophilized, redissolved in 10 mM Tris · HCl buffer, and stored at 4°C.

Sephadex-gel filtration for molecular weight estimation. The molecular weight of glutathione peroxidase was estimated by the modified method of Andrews [18]. A 1.25 × 150 cm Sephadex G-100 column was calibrated with α-amylase (97 000 daltons), hemoglobin (64 000 daltons), pepsin (35 000 daltons) and cytochrome *c* (12 000 daltons). Dextran Blue was used to determine the void volume (V_0).

Polyacrylamide disc-gel electrophoresis for subunit molecular weight deter-

mination and for homogeneity test. Sodium dodecyl sulfate/urea disc-gel electrophoresis was carried out according to the procedure of Bruening [19]. An aliquot of enzyme solution that contained 30—40 μg of protein in 25 μl was heated to boiling together with 25 mg of urea, 12.5 μl of mercaptoethanol, 4 μl of 2 M triethanolamine, and 250 μg of dithiothreitol for 10 min. After the sample had been cooled, 2 μl of 2 M HCl and 1 drop of 1 μg/ml phenol red were added to the sample. Electrophoresis was carried out in 0.06 M glycine/ethanolamine buffer (pH 9.4) that contained 0.1% sodium dodecyl sulfate, and with a constant current of 5 mA/tube for 6 h at room temperature. Bovine serum albumin, hemoglobin, and cytochrome *c* were used as standards. After electrophoresis, the gels were stained with Coomassie Blue and destained by the method of Fishbein [20].

Absorbance spectroscopy of glutathione peroxidase. Absorbance spectral data for glutathione peroxidase were obtained with a recording spectrophotometer (Cary model 118C) with 1-cm light path quartz cells.

Optimal pH for glutathione peroxidase activity. Tris · HCl of pH 7.2—9.0, at 37°C, were used. The purified enzyme preparation was mixed with the buffer solutions and the enzymatic activity was determined by both assay methods.

Substrate specificity of glutathione peroxidase. Specificity toward peroxides: 20 μl of 6.6 mM hydroperoxides and peroxides were added separately to assay systems that contained reduced glutathione as the sole sulfhydryl compound. The reaction rates were recorded and compared.

Specificity toward sulfhydryl compounds: 20 μl of 10 mM reduced sulfhydryl compounds were added to assay systems that contained cumene hydroperoxide as the sole peroxide. The reaction rates were recorded and compared.

Kinetic behavior of glutathione peroxidase. The procedure used in this study was based essentially on the method discussed by Cleland [21]. While holding the cumene hydroperoxide concentration constant, the concentration of the reduced glutathione was varied, and the initial rate of each reaction was determined. The same process was repeated with varying concentrations of cumene hydroperoxide. The data were plotted by a double reciprocal plot.

Results

Purification of glutathione peroxidase. The redissolved 50% acetone precipitate of the heat treated fraction was applied to a Sephadex G-100 column, and the glutathione peroxidase activity was eluted between the void volume protein peak (molecular weight greater than 100 000) and the second major protein peak where hemoglobin was visible. The enzymatically active fractions were pooled and concentrated as described in Materials and Methods. The pooled and concentrated Sephadex G-100 fraction was applied to a DEAE-agarose column and the elution pattern is shown in Fig. 1. The glutathione peroxidase was bound to the DEAE column but it eluted before the major protein peak. The fractions with the highest enzyme activity were colorless and had a specific activity 235 times higher than that of the starting homogenate. Fractions 24—35 from the DEAE-agarose fractionation were pooled and concentrated as described in Materials and Methods. The pooled and concentrated DEAE fraction was then applied to a Sephadex G-150 column. The elution pat-

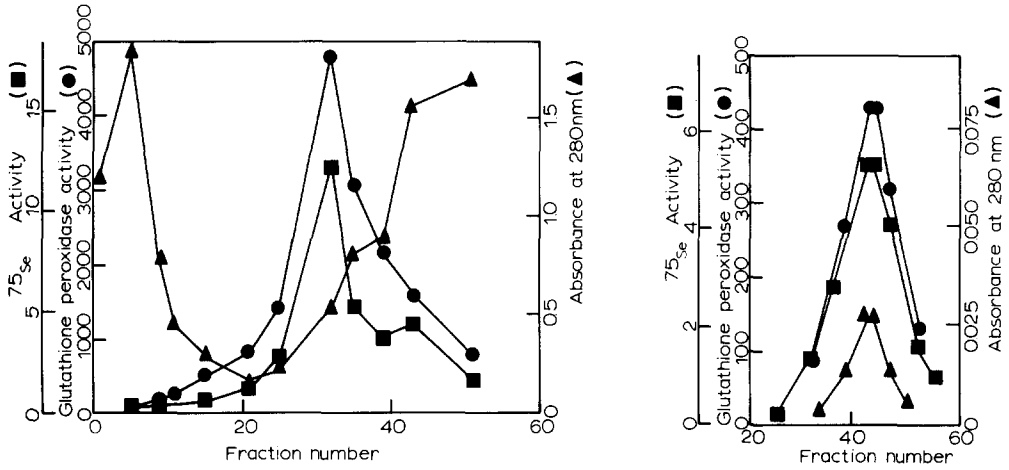


Fig. 1. DEAE-agarose column chromatograph of pooled and concentrated Fractions 26—33 from the Sephadex G-100 separation. Chromatography conditions are detailed under Materials and Methods. A linear gradient of 0—0.2 M NaCl was used to elute Fractions 1—60. The elution patterns for protein (▲), glutathione peroxidase activity (●) and radioactivity of ^{75}Se (■) are shown. Glutathione peroxidase activity is expressed in nmol NADPH oxidized/min per 0.1 ml of each fraction. Radioactivity is expressed as cpm × 10^{-3}.

Fig. 2. Valine-agarose column chromatograph of pooled and concentrated Fractions 34—39 from the Sephadex G-150 separation. Chromatography conditions are detailed under Materials and Methods. The elution patterns for protein (▲), glutathione peroxidase activity (●) and radioactivity of ^{75}Se (■) are shown. Glutathione peroxidase activity is expressed in nmol NADPH oxidized/min per 0.01 ml of each fraction. Radioactivity is expressed as cpm × 10^{-3}.

tern from this column was very similar to that from the first Sephadex column. The fractions with the highest enzyme activity were again pooled and concentrated as before. The pooled and concentrated Sephadex G-150 fraction was applied to the final valine-agarose affinity column. The elution pattern is shown in Fig. 2. Only one protein peak eluted from the valine column, and this peak was coincident with the enzyme activity peak and the radioactivity peak, which indicated that the enzyme was pure. The purified enzyme had a specific activity of 170 μmol NADPH oxidized/min per mg protein, which was 2746 times higher than the activity in the starting homogenate. Data from a typical fractionation are shown in Table I. This fractionation was repeated several times with similar results.

The purified glutathione peroxidase was fairly stable at 4°C. After 1 month, the enzyme still retained more than 50% of its original activity.

Molecular weight of glutathione peroxidase. A Sephadex G-100 column (1.25 × 150 cm) was calibrated with known protein standards, including α-amylase, hemoglobin, pepsin, and cytochrome *c*. There was an excellent linear correlation between the log molecular weight of the proteins and the ratio of V to V_0, where V_0 was the void volume of the column and V was the elution volume of the individual proteins. The molecular weight of glutathione peroxidase was estimated to be 84 000 ± 5000.

Subunit molecular weight determination and homogeneity test. Sodium dodecyl sulfate/urea polyacrylamide gel electrophoresis of the enzyme, pre-

TABLE I

PURIFICATION OF RAT LUNG SOLUBLE GLUTATHIONE PEROXIDASE

Details are described under Results.

Fraction	Total protein (mg)	Specific activity (μmol NADPH oxidized/ min per mg protein)	Yield (%)	Purification (-fold)
Homogenate	7700	0.063	100	1
Acid treated	6870	0.070	90	1
Heat treated	3100	0.125	84	2
50% Acetone precipitate	980	0.176	40	3
Sephadex G-100				
Fraction Nos. 26—33	70	1.66	26	26
DEAE Agarose				
Fraction Nos. 24—35	2.6	14.9	9	235
Sephadex G-150				
Fraction Nos. 34—39	0.23	80.8	5	1281
Valine-Agarose				
Fraction Nos. 36—46	0.11	173	4	2746

treated with 8 M urea and 1% 2-mercaptoethanol, showed a single dense band that corresponded to more than 90% of the total protein. No other band was observed, which indicated that the enzyme preparation was homogeneous. The molecular weight of the single band was estimated to be 20 000. From these results, together with the information on the molecular weight of the native enzyme, it can be concluded that rat lung soluble glutathione peroxidase contains four identical subunits with a molecular weight of 20 000 ± 1000.

Absorbance spectrum of glutathione peroxidase. In general, the absorbance spectrum of glutathione peroxidase throughout the visible and ultraviolet regions did not differ greatly from that of most non-heme proteins (Fig. 3). The broadness of the absorption peak in the 260—300 nm region was probably contributed by the selenium moiety of the enzyme.

pH optimum of glutathione peroxidase. When glutathione peroxidase activity was assayed at 37°C in various pH buffer solutions, the enzyme had maximum activity between pH 8.8 and 9.0. This study also indicated, strangely

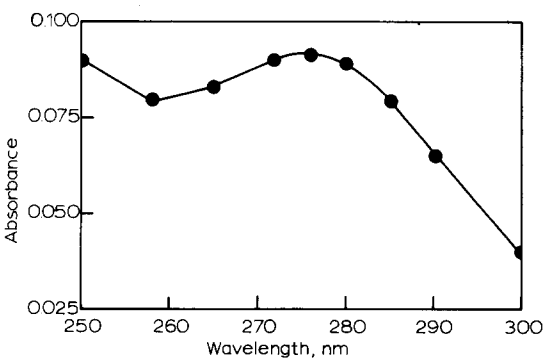


Fig. 3. Absorbance spectrum of rat lung soluble glutathione peroxidase in the 250—300 nm region. Experimental details are described under Materials and Methods.

TABLE II

SUBSTRATE SPECIFICITY OF GLUTATHIONE PEROXIDASE

Details are described under Materials and Methods.

Substrates	Relative activity
Hydroperoxides	
H_2O_2	100
Cumene	97
t-Butyl	84
Lauryl	77
Peroxides	
Di-*t*-butyl	5
Dicumene	5
Sulfhydryl compounds	
Glutathione	100
Mercaptoethanol	0
Dithiothreitol	10
Cysteine	6
N-Acetylcysteine	6
Homocysteine	5

enough, that glutathione peroxidase had very little activity (less than 20% of the activity at pH 8.8) at physiological pH, namely, pH 7.0.

Substrate specificity of glutathione peroxidase. The specificity of glutathione peroxidase toward peroxides and sulfhydryl compounds is given in Table II. Glutathione peroxidase had only general specificity toward hydroperoxides with the general structure of ROOH, and it had very little activity when ROOR was the sole peroxide substrate. Of all the sulfhydryl compounds tested, only glutathione was able to serve as substrate for the enzyme.

Kinetic behavior of glutathione peroxidase. Several methods are available to

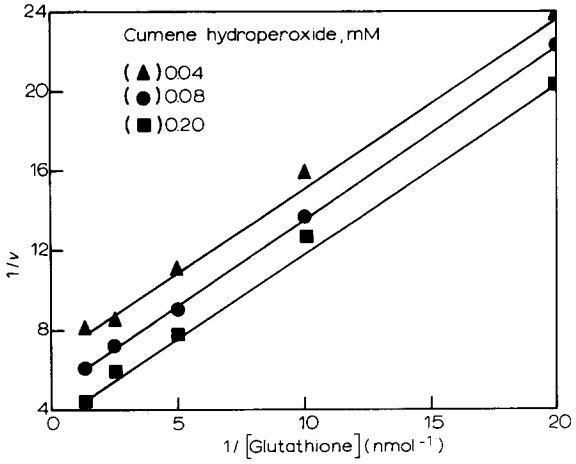


Fig. 4. Double reciprocal plot of glutathione peroxidase activity in various concentrations of both reduced glutathione and cumene hydroperoxide. Glutathione peroxidase activity is expressed in μmol NADPH oxidized/min/per 0.1 ml of enzyme solution. The experimental procedures are detailed under Materials and Methods.

distinguish ordered and random enzyme mechanisms. A ping-pong mechanism can be distinguished from a sequential mechanism by analysis of the initial rate data as described in Materials and Methods. Using double reciprocal plots of glutathione concentration vs. velocity, the enzyme systems that follow a sequential mechanism give lines that intersect to the left of the vertical axis, while those systems that follow the ping-pong mechanism give parallel lines. The series of parallel lines obtained with lung glutathione peroxidase (Fig. 4) indicate a ping-pong-like mechanism for the enzyme.

Discussion

During the course of enzyme purification, ^{75}Se tracer cochromatographed with the enzyme activity, as shown in Figs. 1—3. This evidence, together with the evidence that the selenium associated with the enzyme was nondialyzable, strongly indicates that selenium is an integral part of rat lung soluble glutathione peroxidase. Rotruck et al. [22] first suggested that selenium is an integral part of rat erythrocyte glutathione peroxidase. In support of the findings of Rotruck et al. [22], Smith et al. [23] found a linear correlation between dietary selenium and tissue glutathione peroxidase activities. Flöhé et al. [24] later reported that there were 4 selenium atoms per mol of erythrocyte glutathione peroxidase, or one selenium per enzyme subunit. Our study indicates that rat lung soluble glutathione peroxidase is also a selenium enzyme.

The ultraviolet absorption spectrum of glutathione peroxidase devoid of nucleotides and dialyzable cofactors has a broader peak than that of other proteins free of nucleotides or other cofactors [25]. Similar results were obtained with purified liver soluble glutathione peroxidase [26]. In general, the absorbance of proteins at 260—300 nm is contributed mainly by the sum of the aromatic amino acids and disulfide bridges, but the breadth of the absorbance exhibited by glutathione peroxidase cannot be accounted for by these factors alone. The unaccounted for absorbance was probably due to a selenium chromophore. Diethyl selenide is reported to have maximum absorbance at 250 nm, while diselenide (-Se-Se-) has maximum absorbance in the 310 nm region [27]. Based on the above information, the selenium chromophore of glutathione peroxidase could have the structure RSSeR′ or RSSeSR′.

In the purification procedure, a valine-agarose column was used. This type of column, which was first described by Rimerman et al. [28], operates primarily on the basis of the hydrophobic characteristics of the proteins. Glutathione peroxidase bound to the valine-agarose column at a high concentration of phosphate buffer, which tends to create a hydrophobic environment in the column, and this indicates that the enzyme has a hydrophobic region. This finding could explain how glutathione peroxidase protects cell membranes, which are composed mainly of polyunsaturated fatty acids, from lipid peroxidation. The hydrophobic region of the enzyme may enable it to gain access to the lipoperoxides in membranes, and to reduce them to the harmless corresponding hydroxy fatty acids, thus preventing further damage to the membranes.

Substrate activation by glutathione was reported by both Little et al. [14] and Flöhé et al. [9]. We were able to confirm this substrate activation with

lung glutathione peroxidase. No specific inhibitor has yet been found for glutathione peroxidase. Little et al. [14] reported that certain nucleotides inhibited enzyme activity. However, the inhibition caused by nucleotides is actually due to inhibition of glutathione reductase in the coupled assay (Chiu, D.T., Forstrom, J.W., Stults, F.H. and Tappel, A.L., unpublished).

From the general catalytic properties studied, there appeared to be no major differences between glutathione peroxidase derived from lung, liver, and erythrocyte tissues. However, two differences in physical properties, namely, molecular weight and the elution patterns from a DEAE column, indicate that isozymes of the enzyme exist, but a direct comparison must be made before further conclusions can be reached.

Acknowledgement

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