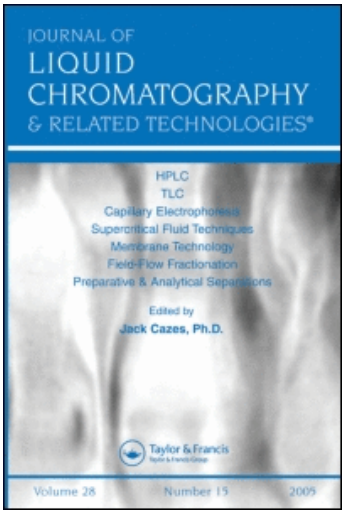

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RAPID, HIGH-YIELD PURIFICATION OF RAT LIVER GLUTATHIONE PEROXIDASE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Glutathione peroxidase (GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) was purified 3500-fold from rat liver with a yield of 42% using high performance liquid chromatography. The crucial purification step was size-exclusion chromatography on a Spherogel TSK-3000SW column, and the purified enzyme eluted as a single peak. The enzyme stained as a single band following SDS-gel electrophoresis. The molecular weight of the enzyme was estimated to be 105,000, and the subunit molecular weight determined by SDS-gel electrophoresis was 25,000. Polyacrylamide gel electrophoresis indicated five bands of protein with a broad band of enzymatic activity. Isoelectric focusing resulted in a peak of enzymatic activity at pH 6.9 with a shoulder at pH 7.3. The specific activity of the purified enzyme was 1,100 μmol of NADPH oxidized per minute per milligram of protein.

INTRODUCTION

Glutathione peroxidase (glutathione hydrogen peroxide oxidoreductase, EC 1.11.1.9) was first reported by Mills (1,2) to catalyze the breakdown of hydrogen peroxide in bovine erythrocytes using glutathione as the reducing agent. Since that time, glutathione peroxidase from several mammalian and avian species has been studied. The enzyme has been obtained in highly purified form from bovine erythrocytes (3), ovine erythrocytes (4), human erythrocytes (5), bovine lens (6), and rat liver (7). In all

these studies, the yields of enzymatic activity were low. Low yields necessitate large quantities of starting material to obtain sufficient glutathione peroxidase to use in other experiments. Only Flohé et al. (8) and Little et al. (9) reported specific activities greater than 400 units/mg of protein when the enzyme assays were done by the method described by Paglia and Valentine (10). Stults et al. (11) reported obtaining a specific activity of 278 units/mg of protein, and Stults (12) indicated that it was possible to achieve a specific activity of 800 units/mg of protein from rat liver. Nakumura et al. (7) reported a final specific activity for purified rat liver glutathione peroxidase of 35.2 units/mg of protein; this low value probably reflects the loss of enzymatic activity as a result of the purification procedures used. This paper reports the relatively high yield purification of rat liver glutathione peroxidase with high specific activity. High yield and high specific activities are both required for studies of physical properties of the enzyme or for the use of the enzyme as a biochemical tool.

MATERIALS AND METHODS

Sephadex G-100 and DEAE-Sephacel were obtained from Pharmacia Fine Chemicals; reduced glutathione, glutathione reductase, NADPH, α-amylase from B. subtilis, and bovine erythrocyte carbonic anhydrase from Sigma Chemical Co.; cumene hydroperoxide from Bio-Polymers, Inc.; SDS-gel electrophoresis standards from Bio-Rad Laboratories; and [^{75}Se]selenite (9 to 35 Ci/mmol) from New England Nuclear.

Fifteen male Sprague-Dawley rats (350-400 g) purchased from Simonsen Laboratories, Inc. were fed Ralston Purina Rat Chow. The rats were fasted for 24 hr prior to killing them by decapitation. The livers were removed and placed in 0.25 M sucrose at 4°C. All subsequent procedures were done at 4°C unless otherwise specified. Three of the fifteen rats were injected intraperitoneally with 50 μCi of [^{75}Se]selenite 3 days before they were killed. The livers from all fifteen rats were minced, washed twice with 0.25 M

sucrose, and homogenized 1:4 in 0.25 M sucrose, 11 mM glutathione for 30 sec in a Waring blendor. The homogenate was centrifuged at 13,000 X g for 30 min. The supernatant portion was adjusted to pH 7.6 with 100 mM Tris base, heated at 50°C for 45 min and cooled to 4°C. The cooled sample was made 52% acetone (-20°C) and centrifuged at 13,000 X g for 20 min. The acetone precipitate was resuspended in 10 mM Tris-HCl, 0.1 mM EDTA, 5 mM reduced glutathione (pH 7.6) and centrifuged at 27,000 X g for 30 min. The supernatant portion was chromatographed on a Sephadex G-100 column (5 X 100 cm) equilibrated with 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.6). The fractions that contained active glutathione peroxidase were pooled and made 7.1 mM in 2-mercaptoethanol. After maintaining the samples for 20 min at 4°C, 10-ml aliquots of DEAE-Sephacel equilibrated with 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.6) were added until there was no further change in the absorbance at 280 nm as monitored on small filtered aliquots. The resultant mixture was filtered and the DEAE-Sephacel was washed with 20 ml of buffer. The filtrate and the wash solution were pooled, concentrated in an Amicon concentrator using a PM-10 filter, and applied to a DEAE-Sephacel column (2.5 X 100 cm) equilibrated with 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6. The sample was eluted isocratically with the same buffer used to equilibrate the column, and the fractions that contained ^{75}Se were pooled. The pooled fractions were concentrated to a volume of approximately 1 ml using an Amicon concentrator with a PM-10 filter. The sample was injected as four 250-µl aliquots onto a Beckman HPLC model 322 with a Spherogel TSK-3000SW size-exclusion column (7.5 X 600 mm) equilibrated with 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0. After each set of four 250-µl injections, the fractions that contained ^{75}Se were pooled, concentrated in an Amicon concentrator, and rechromatographed. This procedure was repeated twice.

Glutathione peroxidase was assayed by a modification of the method of Paglia and Valentine (10) as described by Stults et al. (11). Protein in all samples was determined by a modified Lowry technique (13), except the fractions obtained in the final purifi-

cation steps on HPLC were assayed by measurement of A_{280}-A_{260} (14) and A_{224}-A_{214} (15,16). ^{75}Se was measured using a Packard model 5110 Auto-gamma counter with 30.6% efficiency.

Purified glutathione peroxidase obtained after the third chromatography on the HPLC was subjected to disc-gel electrophoresis according to the procedure of Davis (17). Samples of 80 µg of protein were applied to 7.5% gels that were stacked at pH 8.3 and electrophoresed at pH 9.5. SDS disc-gel electrophoresis was performed according to the method of Weber and Osborn (18) using 10% gels at pH 7.0. Gel isoelectric focusing was done by the method outlined in a Bio-Rad Technical Bulletin 1030. Coomassie blue stain was used to detect protein in the analytical and SDS gels, both of which were scanned at 540 nm with a Beckman DU spectrophotometer model 2400. The gels were frozen, sliced and counted for ^{75}Se radioactivity. An unstained analytical gel and an isoelectric focusing gel were frozen, sliced and assayed for enzymatic activity.

RESULTS

The data obtained from the purification of rat liver glutathione peroxidase are shown in Table 1. Sephadex G-100 chromatography resulted in an aggregated form of glutathione peroxidase. An aggregated form of the enzyme was first described by Stults et al. (11). Addition of 2-mercaptoethanol at 7 mM followed by a DEAE-Sephacel titration resulted in complete dissociation of the aggregated enzyme. This step resulted in a 5-fold purification of the enzyme and minimal loss of activity. Use of DEAE-Sephacel chromatography resulted in retardation of the enzyme by the gel and subsequent elution after elution of the major nonbound protein peak. Use of the 100-cm DEAE-Sephacel column resulted in a much better purification of the enzyme than previously reported (11). The fractionations to this point have been done a number of times with good reproducibility. For the four previous fractionations through the DEAE-Sephacel chromatography step, the specific activities were 52, 48, 50 and 54, and the corresponding yields

TABLE 1
Purification of Glutathione Peroxidase

Fraction	Total protein (mg)	Total activity (units X 10^{-3})[a]	Specific activity[b]	Yield (%)	Purification (-fold)
Homogenate	17 000	5.35	0.32	100	1
Supernatant 13,000 X g	6 853	5.31	0.78	99.3	2.4
Heat-treated 50°C	5 966	4.78	0.80	90.0	2.5
Acetone (52%) precipitate	3 075	4.40	1.4	82.2	4.4
Sephadex G-100	752	3.51	4.7	65.6	15
DEAE-titration	147	3.44	23	64.3	72
DEAE-Sephacel	54	2.87	54	53.7	170
TSK-HPLC[c]	2.0	2.24	1 100	41.8	3 560

[a] μmol of NADPH oxidized per minute.

[b] μmol of NADPH oxidized per minute per milligram of protein.

[c] Following the third chromatography.

were 62, 45, 53 and 51%, respectively. The use of a second DEAE-Sephacel column resulted in a 20-30% yield of glutathione peroxidase that was 30-50% pure. Use of three consecutive chromatographies on a TSK-3000 HPLC column (Fig. 1) separated glutathione peroxidase from other proteins to the limit of detection. Calibration of the TSK column was accomplished using five molecular weight standards. Chromatography of the enzyme on the calibrated column gave an apparent molecular weight for the enzyme of 105,000 $\pm$ 4000 (Fig. 2).

Analytical disc-gel electrophoresis resulted in five equally-spaced bands of protein with the middle band being predominant. Enzymatic activity was found in a wide band encompassing the 5 bands of protein. SDS disc-gel electrophoresis of the purified glutathione peroxidase resulted in a single coincident band of protein and ^{75}Se radioactivity. The subunit molecular weight of the enzyme based on comparison with six molecular-weight stand-

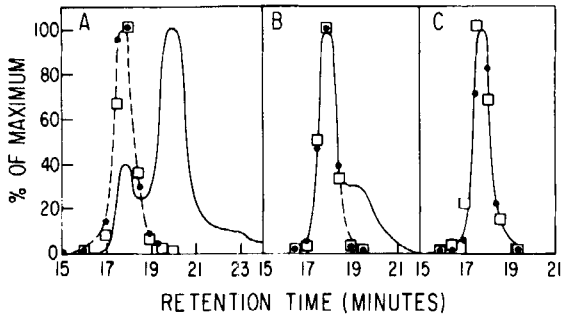

FIGURE 1. TSK-3000 HPLC size-exclusion chromatography of rat liver glutathione peroxidase. Concentrated glutathione peroxidase from DEAE-Sephacel chromatography was applied to a TSK-3000 size-exclusion column equilibrated with 10 mM sodium phosphate, 0.1 mM EDTA (pH 7.0) and resolved at 1.0 ml/min and 400 p.s.i. After the first chromatography (A), the active fractions were pooled and concentrated. Chromatography on the TSK-3000 column was repeated twice as shown in (B) and (C). ^{75}Se (□), enzymatic activity (●) and absorbance at 280 nm (—).

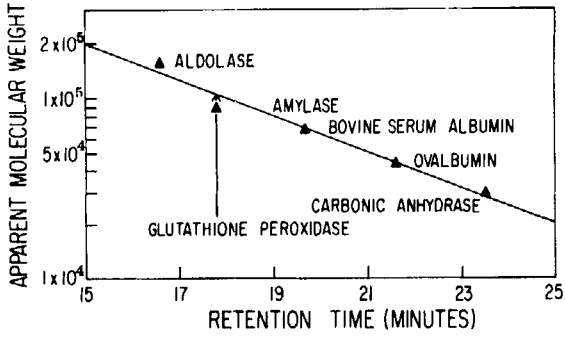

FIGURE 2. Estimation of the molecular weight of glutathione peroxidase by size-exclusion chromatography on a TSK-3000 HPLC column. Aliquots of 100 µg of each protein were injected in 50 µl of 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0.

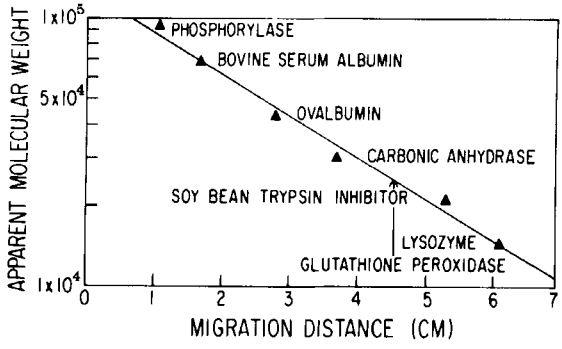

FIGURE 3. Estimation of the subunit molecular weight of glutathione peroxidase by SDS-polyacrylamide disc-gel electrophoresis. A solution that contained 2.5 μg of each standard and 80 μg of glutathione peroxidase was applied to a gel (5 X 100 mm).

ards (Bio-Rad low molecular weight standards) was 25,000 ± 2000 (Fig. 3). Disc-gel isoelectric focusing gave a coincidence of enzyme activity and ^{75}Se radioactivity in a single peak at pH 6.9 and a shoulder at pH 7.3.

DISCUSSION

The development of the purification procedure described started with that reported by Stults et al. (11). The choice of techniques used through the DEAE Sephacel chromatography step and the chosen sequence of the individual steps are based on results obtained by many purifications. The outlined procedure resulted in the purification of glutathione peroxidase with a yield higher than any yet reported and with a high specific activity surpassed only by that reported by Little et al. (9). The described HPLC procedure eliminates the problem of aggregated enzyme (11), and it further demonstrates the usefulness of HPLC for protein separation. The purification of the enzyme can be completed in three days, thus it can be used to provide a routine source of glutathione peroxidase.

The molecular weight of the rat liver glutathione peroxidase as estimated by size-exclusion chromatography is 105,000 ± 4000, which correlates well with a tetramer having an SDS-gel electrophoresis monomer size of 25,000 ± 2000. The observed molecular weight is significantly different from that reported for the rat liver enzyme (7,11), and it more closely matches the 96,000 molecular weight reported for the human erythrocyte enzyme (5).

The protein pattern obtained with analytical disc-gel electrophoresis and the multiple charge forms of glutathione peroxidase observed upon isoelectric focusing suggest the possibility that there are two isozymic forms of the monomer of glutathione peroxidase in rat liver. The two possible isozymes of glutathione peroxidase, "acidic" (pI = 6.9) and "basic" (pI = 7.3), could upon free hybridization, give five mixed enzymes. This possibility is consistent with the protein pattern seen on disc-gel electrophoresis.

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