GLUTATHIONE PEROXIDASE ACTIVITY OF GLUTATHIONE-S-TRANSFERASES PURIFIED FROM RAT LIVER[1]

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Summary: Gel filtration chromatography demonstrated the presence of two peaks of glutathione peroxidase activity assayed with cumene hydroperoxide in the soluble fraction of rat liver, brain, kidney, and testis. The peak with an approximate molecular weight of 45,000 (GSH-Px II) was purified from rat liver labeled *in vivo* with $Na_2{}^{75}SeO_3$. Chromatography on DEAE-cellulose, Sephadex G-150, DEAE-cellulose, and CM-cellulose resulted in the co-purification of glutathione-S-transferase activity measured with 1-chloro-2,4-dinitrobenzene and glutathione peroxidase activity assayed with cumene hydroperoxide, and in the removal of all detectable ^{75}Se. Studies on GSH-Px II indicated that the apparent K_m for both cumene and t-butyl hydroperoxides was considerably higher than that for purified seleno-glutathione peroxidase. The V_{max} estimated with cumene hydroperoxide was only 1/300 of that determined for the selenoenzyme at pH 7.5 and with 1 mM GSH.

In 1957, Mills (1) discovered an enzyme in erythrocytes, glutathione peroxidase (GSH-Px) (EC 1.11.1.9), which catalyzed the reduction of hydrogen peroxide to water using GSH as the donor substrate. GSH-Px was later identified in other tissues and found to act on a variety of organic hydroperoxides (2), and in 1973 was found to contain 4 g-atoms of selenium per 84,000 molecular weight (MW) (3, 4). Recently an additional glutathione peroxidase activity (GSH-Px II) of approximately 45,000 MW was detected in rat brain and liver soluble fractions following gel filtration chromatography when assayed with cumene hydroperoxide (5, 6). GSH-Px II was not active with hydrogen peroxide (6) and does not appear to be a selenoenzyme, since it does not incorporate ^{75}Se *in vivo*[2] and persisted in severe Se deficiency (5, 6) in contrast to the selenoenzyme. Also, GSH-Px II shows zero-order dependence on GSH concentration (5) and is not inhibited by

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cyanide[2], whereas the selenoenzyme shows first-order dependence on GSH (7) and is inhibited by cyanide (8).

Glutathione-S-transferases (EC 2.5.1.18) are a family of proteins, MW 45,000, that bind hydrophobic substrates and catalyze the reaction of GSH with an electrophilic center in these substrates (9). This suggested that the glutathione peroxidase activity of GSH-Px II might be accounted for by the ability of GSH transferases to bind organic hydroperoxides and GSH in close proximity, thus promoting a nucleophilic attack on the electrophilic oxygen. This idea was supported by preliminary studies on GSH-Px II from rat testis[2] comparing kinetic behavior and effects of competitive inhibitors. The present paper describes the co-purification of GSH-Px II and GSH transferase activities from rat liver and indicates that the glutathione:cumene hydroperoxide oxidoreductase of 45,000 MW is identical with the GSH-S-transferases.

MATERIALS AND METHODS

Portions of liver and whole brain, testis, and kidney pooled from three adult male rats fed laboratory chow and injected with a tracer dose of $Na_2{}^{75}SeO_3$ (New England Nuclear, Boston, MA) 6 weeks earlier were homogenized with 3 volumes of buffered KCl (5) and centrifuged at 100,000 x g for 1 hr. The supernatant fraction was chromatographed on Sephadex G-75.

GSH transferases were isolated from a 7.5 g liver sample from a male rat, who had received $Na_2{}^{75}SeO_3$ 4 weeks earlier, according to the method of Habig et al. (10). A soluble fraction obtained by centrifuging the liver homogenate at 15,000 x g for 15 min. was chromatographed on DEAE-cellulose followed by: (1) chromatography on Sephadex G-150; (2) chromatography on DEAE-cellulose (DE-52, Whatman) using conditions identical to those of the first DEAE step (10 mM Tris-Cl at pH 8.0); (3) chromatography on CM-cellulose by the procedure of Habig et al. (10). Fractions were measured for ^{75}Se content in a well-type gamma counter (Nuclear-Chicago), for protein by the procedure of Lowry et al. (11) using bovine serum albumin as a standard, for GSH-S-transferase with 1 mM 1-chloro-2,4-dinitrobenzene according to Habig et al. (9), and for GSH-Px activity with 0.1 mM cumene hydroperoxide as described previously (8) except that the hydroperoxides were dissolved in 10% (v/v) ethanol, since high amounts of ethanol (>0.5%) were found to be inhibitory towards GSH-Px II.

RESULTS

Following Sephadex G-75 chromatography two peaks of GSH-Px activity were found in testis, liver, brain, and kidney (Fig. 1). The first peak was located in the void volume and was associated with the only ^{75}Se peak (not

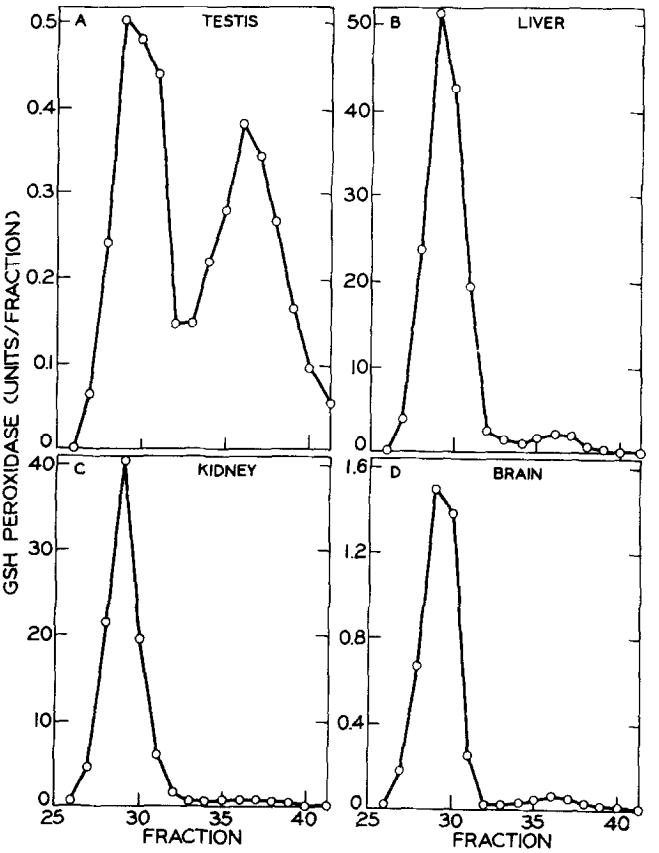

Figure 1. Sephadex G-75 chromatography and GSH-Px activity of the soluble fraction from various tissues. The column was 2.5 x 95 cm equilibrated in 0.1 M NaCl buffered with 0.01 M Tris-Cl pH 7.5. The soluble fraction of each tissue was prepared from a pool of 3 male rats and 6 ml was chromatographed: (A) testis, 57 mg protein; (B) liver, 233 mg; (C) kidney, 152 mg; (D) brain, 60 mg. Fractions (6 ml each) were assayed for GSH-Px activity with 0.1 mM cumene hydroperoxide as described in Methods.

shown). The second peak, GSH-Px II, eluted with proteins of approximately 45,000 MW. At the concentration of cumene hydroperoxide employed in these studies (0.1 mM), testis GSH-Px II accounted for over half the total GSH-Px activity chromatographed; for liver, brain, and kidney GSH-Px II was a minor component of the total activity. GSH-Px II was not detectable in plasma or red blood cells following gel filtration; however, red blood cells did contain a peak of GSH-S-transferase (MW 45,000) whose activity (0.002 units/mg) was less than 0.1% that of either testis or liver.

Table I

COPURIFICATION OF RAT LIVER GSH-S-TRANSFERASES AND GSH:CUMENE HYDROPEROXIDE OXIDOREDUCTASE

Purification Step	Total Protein (mg)	Total ^{75}Se (cpm)	Specific Activity GSH-S-Transferase[a] (μmoles min^{-1} mg^{-1})	Specific Activity GSH Peroxidase[b] (μmoles min^{-1} mg^{-1})
Liver Extract	544	59,744	0.68	1.3
DEAE-Cellulose	131	29,564	1.8	3.2
Sephadex G-150	23.8	2,055	4.9	1.2
DEAE-Cellulose	10.6	128	5.7	1.4
CM-Cellulose				
Fraction 33	0.5	-	20	3.8
Fraction 50	0.08	-	17	5.0

[a]Determined with 1 mM 1-chloro-2,4-dinitrobenzene as described in METHODS.

[b]Determined with 0.1 mM cumene hydroperoxide as described in METHODS.

Liver had the highest GSH-Px II activity of those tissues examined and was, therefore, used for further purification. Passage of the 15,000 x g liver supernatant fluid through the first DEAE cellulose column resulted in elimination of about half the total ^{75}Se (Table I). The eluted sample was concentrated by ultrafiltration (PM-10, Amicon Corp.) and chromatographed on Sephadex G-150 (Fig. 2). Two peaks of GSH-Px activity were found, the first associated with ^{75}Se and the second eluting in the position of ovalbumin (MW 45,000) determined previously in a calibration run. GSH transferase activity was found only in the fractions containing GSH-Px II. The GSH-Px II peak was pooled, concentrated by ultrafiltration, equilibrated against 10 mM Tris-chloride pH 8.0 and chromatographed on DEAE-cellulose (Fig. 2, insert). Only 0.2% of the original ^{75}Se was eluted from the second DEAE column (Table I). The pooled peak was concentrated by ultrafiltration and dialyzed against 10 mM potassium phosphate

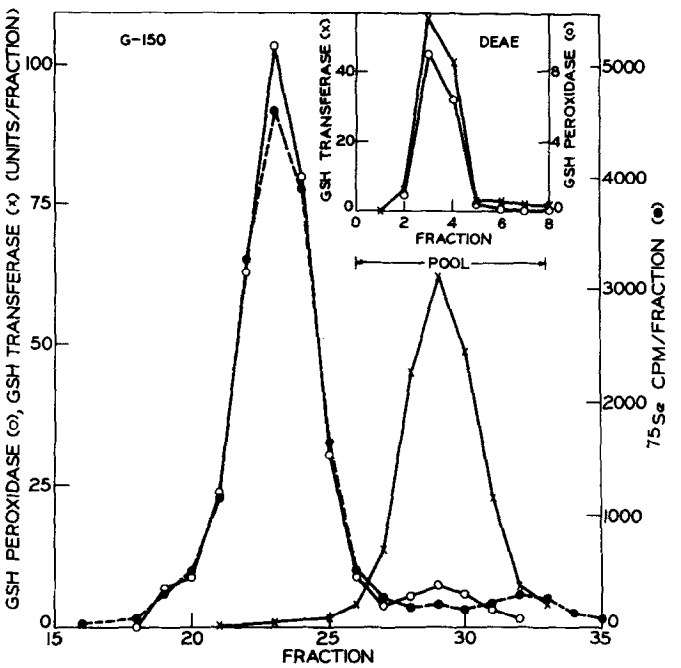

Figure 2. Sephadex G-150 chromatography of rat liver soluble fraction on a 2.5 x 93 cm column equilibrated in 0.1 M NaCl buffered with 0.01 M Tris-Cl pH 7.5. The sample which was applied had gone through a DEAE-cellulose step and had been concentrated (PM-10) to 9.5 ml containing 131 mg of protein, 29,564 cpm of ^{75}Se, 423 units of GSH-Px, and 235 units of GSH-S-transferase. Fractions (9.6 ml each) were analyzed for ^{75}Se, GSH-Px with 0.1 mM cumene hydroperoxide, and GSH-S-transferase with 1 mM 1-chloro-2,4-dinitrobenzene as described in Methods. The inset shows the elution of GSH-Px II and GSH-S-transferase after the G-150 peak had been pooled, concentrated, equilibrated and chromatographed on DEAE-cellulose (DE-52) 2.5 x 7 cm, 10 mM Tris-Cl pH 8.0.

pH 6.7 and chromatographed on CM-cellulose (Fig. 3). Four peaks of GSH transferase activity were eluted after the KCl gradient was begun (peaks 1, 2, 3, and 5) and three of these had GSH-Px activity (peaks 2, 3, and 5). A fourth peak of GSH-Px was also detected (peak 4); both activities were detected in those fractions which eluted before the KCl gradient was begun (Fig. 3). Table I summarizes the purification of GSH-Px II and GSH-S-transferase including the data for the most active fraction of peaks 3 and 5. Samples from peak 3 from this purification and an earlier preparation were used to determine the K_m for various substrates (Table II). K_m values determined for GSH-Px II from rat

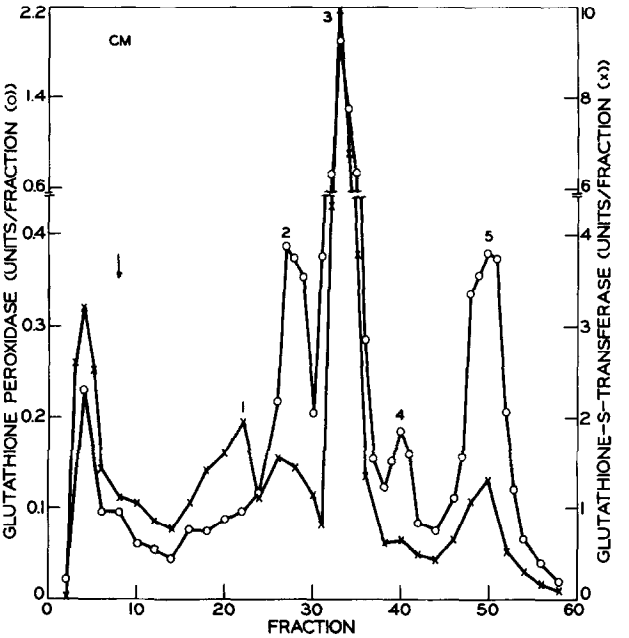

Figure 3. CM-cellulose chromatography of rat liver GSH-S-transferases on a 1.5 x 14 cm column equilibrated in 10 mM potassium phosphate pH 6.7. The pooled sample from the second DEAE-cellulose step was concentrated and equilibrated by dialysis against the elution buffer. The sample was 9.8 ml containing 10.6 mg of protein, 15 units of GSH-Px II and 60 units of GSH-S-transferase. The sample was pumped (14.9 ml/cm^{2}hr) onto the column and after washing with starting buffer a 500 ml linear gradient from 0-100 mM KCl in the phosphate buffer was begun (indicated by an arrow) and fractions (5.4 ml each) were analyzed for enzyme activity as described in Methods.

testis cytosol and for ^{75}Se-GSH-Px isolated from sheep blood are also included. For liver and testis, K_m values for cumene hydroperoxide were 0.18 mM and 0.3 mM respectively, while corresponding values were 5.4 mM and 2.0 mM for t-butyl hydroperoxide. These values are considerably higher than the apparent K_m determined for the selenoenzyme assayed under equivalent conditions. The liver and testis GSH-Px II samples yielded biphasic reciprocal plots when GSH was varied (not shown).

DISCUSSION

Partial purification of glutathione-S-transferases from rat liver by Habig et al. (10) yielded five peaks of activity eluting from CM-cellulose when assayed

Table II

K_m VALUES FOR VARIOUS SUBSTRATES OF GSH PEROXIDASE AND GSH-S-TRANSFERASE

Substrate	Km (mM) Rat Liver[a] CM-Peak 3	Rat Testes[b] G-150 Peak	Sheep Blood[c]
Cumene hydroperoxide[d]	0.19; 0.17	0.3	0.08
t-Butyl hydroperoxide[d]	5.0; 5.7	2.0	0.12
Glutathione[e]	0.03 0.1	0.04 0.2	-[f]
1-chloro-2,4-dinitrobenzene[d]	0.9; 0.3	0.07	-

[a]Values are from two separate purifications, GSH-Px 3.7 and 3.8 units/mg and GSH transferase 25 and 20 units/mg, respectively.

[b]Peak pooled from Sephadex G-150 was completely insensitive to 20 mM KCN.

[c]^{75}Se-GSH-Px partially purified according to Oh *et al.* (3); 104 U/µg Se.

[d]Determined with 1 mM GSH as described in METHODS.

[e]Cumene hydroperoxide (0.5 mM) was employed as the second substrate. The reciprocal plots were biphasic with a break at approximately 0.1 mM. K_m values were determined for both phases.

[f]Indeterminant (7).

with 1-chloro-2,4-dinitrobenzene. The isolation procedure used in the present study, which included 2 additional steps, resulted in the appearance of four activity peaks (Fig. 3 peaks 1, 2, 3 and 5). GSH-Px activity was associated with peaks 2, 3, and 5 as well as in peak 4, strongly suggesting that the GSH-Px activity was catalyzed by the glutathione-S-transferases; both activities were detected in the fractions preceding the KCl gradient. Although the identity of the peaks of coincident GSH-Px and GSH transferase activity has not been established, the GSH transferase profile appears similar to that reported previously (10, 12) so that peaks 2, 3 and 5 might correspond to glutathione-

S-transferase C, B(ligandin) and AA, respectively.[3] Recently, Hales and Neims (13) have fractionated liver GSH-S-transferases on CM-cellulose according to the method of Habig et al. and have also detected only four activity peaks after the KCl gradient was begun. Since the ratio of GSH-Px/GSH transferase following CM-cellulose is higher for peaks 2, 4 and 5 and lower for peak 3 and the peak eluting ahead of the gradient, it seems likely that the apparent GSH-Px activities are catalyzed by different transferases but this must be established with homogenous and ligand-free proteins.

It is not surprising that the glutathione-S-transferases can catalyze the reduction of hydroperoxides with GSH. They bind GSH and a wide variety of hydrophobic ligands in close proximity and have already been shown to catalyze GSSG formation from GSH when acting upon organic nitrates (14), and can reduce prostaglandin endoperoxides (15). It has been suggested that the general mechanism involves enhancement of the dissociation of GSH and nucleophilic attack of enzyme-bound GS^- on the electrophilic center (C, N, or S) of the second substrate (16). This may also be the case for the electrophilic oxygen in ROOH. Deprotonation of GSH to GS^- is not thought to be involved in catalysis by seleno-GSH-Px (7); it may be important in the non-enzymatic reaction.

We estimate the V_{max} (moles min^{-1}/mole enzyme) of peaks 3 or 5 (Fig. 3) with cumene hydroperoxide to be 400, which indicates that it is a relatively good substrate for the glutathione-S-transferases as compared to other substrates (12); the rate for purified Se-GSH-Px under equivalent conditions, however, is 300 times greater. Thus, even though the transferases are found in high concentration in liver (10) their contribution to removal of organic hydroperoxides when Se is adequate and the concentration of hydroperoxides

[3]Peak 3 might be a mixture of both transferases B and A since the specific activity determined with 1-chloro-2,4-dinitrobenzene for two separate purifications, 25 and 20 (Table II), is similar to the value of 22 calculated for a mixture of transferase B (specific activity 11) and A (specific activity 62) in their relative amounts found in liver, 1.0 and 0.22, respectively (12). The specific activity determined for peak 5 for the two purifications, was 12 and 17, respectively. Habig et al. (10) have reported a value of 14 for purified transferase AA.

is below 0.1 mM must be minimal compared to Se-GSH-Px (Figs. 1, 2). When cumene hydroperoxide was employed at 1.5 mM the liver GSH-Px II peak increased relative to the selenoenzyme (6), presumably because the transferases were saturated with hydroperoxide (Table II). In some tissues, testes for example, the contribution to organic hydroperoxide removal by glutathione-S-transferases may be quite significant. Other compounds such as the fatty acid hydroperoxides may be found to be substrates for glutathione-S-transferases.

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