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PURIFICATION OF MOUSE LIVER CYTOSOLIC EPOXIDE HYDROLASE

Sarjeet S. Gill

Division of Toxicology and Physiology, Department of Entomology,
University of California, Riverside, CA 92521

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Summary: Cytosolic epoxide hydrolase was purified from male Swiss mouse liver cytosol by passing it sequentially through CM-cellulose, DEAE-cellulose, phenylsepharose, DEAE-cellulose, and hydroxyapatite. The overall yield was ca. 10% with a 180-fold purification, and a final specific activity of 1.5 μmol/min/mg protein as monitored with trans-stilbene oxide. The purified epoxide hydrolase was apparently homogenous as determined by SDS-PAGE and it appears to exist as a dimer in its native form as evidenced by a monomeric molecular weight of 59,000 by SDS-PAGE and a reported native molecular weight of 130,000 by gel filtration.

Epoxidized compounds, both natural and anthropogenic in origin, are hydrolyzed in mammals by epoxide hydrolases (EC 3·3·2·3) (1). Because a variety of such epoxides are highly reactive, their hydrolysis by epoxide hydrolases may be important in providing protection to mammalian systems. Epoxide hydrolase activity is essentially ubiquitous in mammalian cell, and has been demonstrated to occur in microsomal (2), nuclear (3), cytosolic (4), and mitochondrial fractions (5,6). Epoxide hydrolase activity in the microsomal fraction is immunologically identical to that in the nuclear fraction (7) but it is immunologically distinct from that in the cytosolic fraction (8). Epoxide hydrolase activity in the cytosolic and mitochondrial fractions appears to be similar based on their substrate specificity, pH optima, inhibition characteristics and molecular weight (5,6).

A great deal of effort has been directed toward the microsomal epoxide hydrolase, which has been purified from common laboratory animals and man (9-12). In contrast, cytosolic epoxide hydrolase has only recently been purified from rabbit and human liver (13,14). However, most of our understanding of the cytosolic epoxide hydrolase has been from mouse liver (4,

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15-25). This report thus presents the successful purification of the cytosolic epoxide hydrolase from mouse liver.

MATERIALS AND METHODS

Chemicals: [^{3}H]-Trans and cis-stilbene oxides (TSO and CSO, respectively, 60 mCi/mmol) were synthesized by sodium borotritride reduction of desyl chloride followed by base treatment of the resulting chlorohydrin. The radioactive products were purified by thin-layer chromatography to >99% chemical purity and >97% geometrical purity. Cold TSO was obtained from Aldrich Chemical Company (Milwaukee, WI) while CSO was synthesized by m-chloroperoxybenzoic acid epoxidation of cis-stilbene and purified by crystallization. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were obtained from Whatman, Inc., Clifton, NJ. Phenylsepharose and hydroxyapatite were obtained from Pharmacia Fine Chemicals, Piscataway, NJ, and Clarkson Chemical Co., Williamsport, PA, respectively. Sodium dodecylsulfate (SDS), acrylamide and N,N-methylenebisacrylamide were obtained from Polysciences, Inc., Warrington, PA. All other chemicals used were of analytical grade or the best commercially available.

Enzyme Sources: Livers of male Swiss mice (30-40 g, 12-15 wk old) were removed, perfused and homogenized in 0.25M sucrose. The homogenate was centrifuged at 10,000 g for 10 min and the 10,000 g supernatant then centrifuged at 100,000 g for 60 min to give the microsomal pellet and cytosolic supernatant. The cytosolic fractions were stored at -76^{o}C for 1-2 weeks before use. Protein determinations were performed basically following Bradford (26) using bovine serum albumin, fraction V, as standard.

Enzyme Assay: Cytosolic epoxide hydrolase activity was monitored with TSO in 0.1M phosphate buffer at pH 7.4 using partition assays (16,21). Briefly TSO in 1 µL ethanol (final conc. 5 x 10^{-5}M) was incubated with 100 µL of enzyme solution for 10 min at 37^{o}C, after which the mixture was extracted with n-dodecane. The aqueous phase (50 µL) containing the product diol was quantitated by liquid scintillation counting (lsc). Microsomal epoxide hydrolase was similarly monitored using CSO as substrate in 0.1M Tris-HCl, pH 9.0 buffer. Glutathione transferase assays were performed with CSO as substrate in 0.1M sodium phosphate buffer, pH 7.4. The reaction mixture was, however, extracted with 1-hexanol, which removes the epoxide and metabolically formed product diol, if any, from the aqueous phase. Quantitation of the aqueous phase (50 µL) for GSH-conjugate was performed by lsc.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in discontinuous buffer system following Laemmli (27) with 0.75 mm, 7.5% acrylamide gels using 0.375M Tris, pH 8.8 containing 0.1% SDS. Gels were stained with Coomasie Brilliant Blue R-250, for visualization of protein bands. Molecular weights were determined by comparison with the mobility of protein markers of known molecular weight. Proteins markers used were bovine serum albumin, bovine liver catalase, ovalbumin, rabbit muscle aldolase, and lyzozyme.

Purification of cytosolic TSO hydrolase. All purification steps were performed at 4-5^{o}C. Cytosolic fractions previously stored were thawed and the pH adjusted to 6.0 with 5mM potassium phosphate buffer. The crude cytosol (1.6L) was applied at 3 mL/min to a CM 52 column (5 x 30 cm) that had been equilibrated with 5mM potassium phosphate pH 6.0 buffer containing 0.1mM EDTA and 0.1mM dithiothreitol (DTT) (buffer A). The column was washed with 2L of buffer A and then eluted with 2L of a linear gradient of 0-0.2M NaCl in buffer A. Cytosolic epoxide hydrolase activity which did not bind to CM cellulose was pooled. GSH transferase activity which appeared in two distinct peaks in NaCl gradient were stored for subsequent use.

The pooled CM-52 fraction containing cytosolic epoxide hydrolase was diluted with Tris-HCl buffer to give a final concentration of 20mM Tris-HCl, pH 7.6 containing 0.1mM EDTA and 0.1mM DTT (buffer B) in a total volume of 2L and then applied to a DE 52 column (5 x 44 cm) previously equilibrated with buffer B. The column was washed with 2L of buffer B and then eluted with a 4L linear gradient of 0-0.2M NaCl in buffer B. Fractions with cytosolic epoxide hydrolase activity were pooled.

The pooled DE-52 fraction was concentrated by ultrafiltration, adjusted with Tris-HCl and NaCl to give a final concentration of 100mM Tris-HCl, pH 7.6 with 0.1mM EDTA and 0.1mM DDT (buffer C) and 0.35M NaCl, and then applied to a phenylsepharose CL-4B column (2.5 x 35 cm) previously equilibrated with buffer C and 0.35M NaCl. The column was washed sequentially with 200 mL of buffer C and 0.35M NaCl, 200 ml gradient of 0.35-0M NaCl in buffer C, and a 2L gradient of buffer C-5mM Tris-HCl, pH 7.6 with 0.1mM EDTA and 0.1mM DTT (buffer D) containing 1% ethylene glycol. Subsequently the column was eluted with a 2L gradient of 1-10% of ethylene glycol in buffer D and finally with 1L of 0.4% Lubrol PX in the same buffer. Fractions containing cytosolic epoxide hydrolase were pooled, concentrated and dialyzed for 36 hr against 20 vol of 5mM potassium phosphate buffer, pH 7.7 containing 0.1mM EDTA and 0.1mM DTT (buffer E).

The dialyzed phenylsepharose fraction was applied to a DE-52 column (2.5 x 40 cm) previously equilibrated with buffer E. The column was washed with 400 mL of buffer E, then eluted with 1L of a linear gradient of 0-75mM NaCl in buffer E and finally eluted with 1L of 75mM NaCl in buffer E. Fractions containing cytosolic epoxide hydrolase were pooled, concentrated and dialyzed against 30 vol of 5mM potassium phosphate buffer, pH 7.25 containing 0.1mM EDTA and 0.1mM DTT (buffer F).

The dialyzed cytosolic epoxide hydrolase was then applied to a hydroxyapatite column (1.0 x 26 cm) previously equilibrated with buffer F. The column was washed with 150 mL of 20mM potassium phosphate (pH 7.25), then eluted with 500 mL of a linear gradient of 40-120mM potassium phosphate and then with 500 mL of 120mM potassium phosphate, all containing 0.1mM EDTA and 0.1mM DTT. The cytosolic epoxide hydrolase fraction from this step was homogenous as determined by SDS-PAGE.

RESULTS AND DISCUSSION

Male mice (12-15 weeks old) were chosen as the source for purification of cytosolic epoxide hydrolase because there is significant information on its charactization (18-20,24,25), and because male mice have high levels of cytosolic epoxide hydrolase (18). The large number (200) of mice used necessitated the storage of cytosol at -78°C until such time that a sufficient quantity was available. Mouse cytosolic epoxide hydrolase is stable for extended periods when stored at -78°C in 0.25M sucrose.

Use of CM-cellulose and DEAE-cellulose as the first two steps in the purification greatly aided in the purification procedure. In addition, use of CM-cellulose as the first step enabled the separation of GSH transferase, another cytosolic enzyme critical in the metabolism of epoxidized compounds.

Preliminary data indicates that two major peaks of GSH-transferase activity were obtained when the CM 52 column was eluted in a NaCl gradient and when assayed with CSO as substrate. Thus, the same cytosol can readily be used for purification of GSH transferase(s) and epoxide hydrolase. Unlike that observed with human cytosolic epoxide hydrolase (14), mouse cytosolic epoxide hydrolase did not precipitate at pH 6.0. Although there was a change in the buffer between CM-cellulose and DEAE-cellulose, it was not necessary to dialyze the CM 52 pool before application to the DE 52 column. However, the CM 52 pool was adjusted to pH 7.6 with Tris-HCl soon after elution and assaying of epoxide hydrolase activity. Use of the phenylsepharose step in purification was very useful because it afforded a 8-9X purification and could be used directly after DEAE-cellulose, without dialyzing the eluted fractions. Cytosolic epoxide hydrolase activity from the second DEAE-cellulose column was contaminated with two other protein bands, which were removed by the hydroxyapatite column. The recovery in the final purification step using hydroxyapatite was essentially quantitative, although only 85% of the epoxide hydrolase activity was in the peak fraction. The rest, consisting of pre- and post-peak fractions, were contaminated with one other protein as determined by SDS-PAGE.

The overall purification of cytosolic TSO hydrolase was 180 fold with a yield of ca. 10% (Table 1). The degree of purification needed to obtain an apparently homogenous preparation was significantly less than that required for rabbit and human, but the yield was significantly greater than that

TABLE 1
Purification of mouse liver cytosolic TSO hydrolase

Fraction	Protein, mg	Specific Activity nmol min^{-1} mg $protein^{-1}$	Total Activity nmol min^{-1}	Recovery	Purification factor
Cytosol	13000	8.17	106,000	100	1
CM-cellulose	8460	10.3	86,900	82	1.3
DEAE-cellulose	1900	29.1	55,300	52	3.6
Phenylsepharose	120	256	30,600	29	31
DEAE-cellulose	13.8	894	12,300	12	110
Hydroxyapatite	7.1	1490	10,500	9.8	182

obtained with procedures used for the purification of rabbit and human cytosolic epoxide hydrolases (13,14).

The cytosolic TSO epoxide hydrolase purified following the scheme in Table 1 was apparently homogenous as determined by SDS-PAGE (Fig. 1). Using protein markers, the molecular weight of mouse cytosolic epoxide hydrolase was approximately 59,000. This value compares favorably with estimates of 57,000 and 58,000 for rabbit and human cytosolic epoxide hydrolases, respectively (13,14). Mouse cytosolic epoxide hydrolase thus appears to exist as a dimer in its native form as evidenced by a reported native molecular weight of

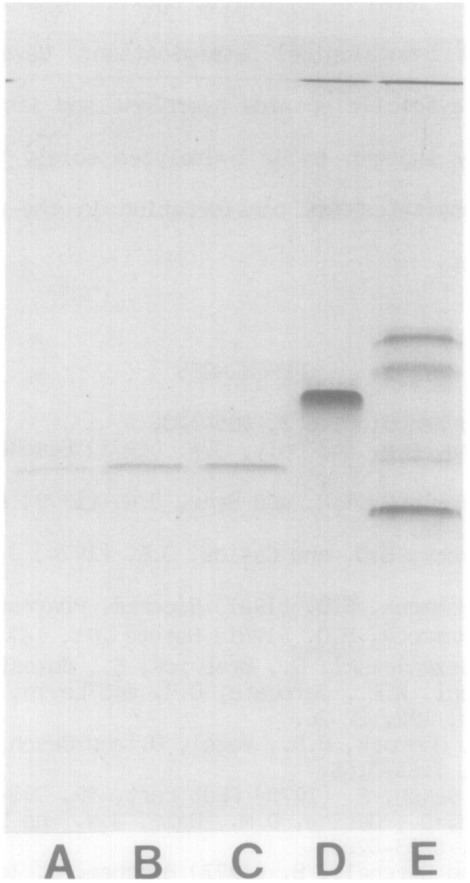

Figure 1. SDS-PAGE of cytosolic epoxide hydrolase performed according to Laemmli (27) in 0.75 mm gels which were visualized by Coomassie Brilliant Blue R-250 staining. The anode was at the top of the figure. A, B and C have 0.22, 0.43 and 0.86 µg of mouse liver cytosolic epoxide hydrolase, respectively. D has 5 µg of partially pure rat liver microsomal epoxide hydrolase. E contains 2 µg each of standard proteins, bovine serum albumin (M_r 66,000), bovine liver catalase (M_r 58,000), ovalbumin (M_r 45,000), rabbit muscle aldolase (M_r 40,000) and lyzozyme (M_r 14,300 - not visible in photograph but faintly visible in gel).

approximately 130,000 as estimated by gel filtration (18). There was no evidence that other oligomers of the cytosolic epoxide hydrolase exist in mouse cytosol as apparently observed with rabbit cytosolic epoxide hydrolase (13).

Based on the specific activities of purified mouse cytosolic epoxide hydrolase and that in crude cytosol, approximately 0.6% of cytosolic protein in Swiss mouse liver is the cytosolic epoxide hydrolase. Because cytosolic protein is approximately 3-4X that of microsomal protein in liver cells, the levels of cytosolic epoxide hydrolase and microsomal epoxide hydrolase apparently occur in similar amounts in mouse and rat liver, respectively. A better estimate of the level of cytosolic epoxide hydrolase in mouse cytosol, however, has to await immunological determination. Nevertheless, the relatively high levels of cytosolic epoxide hydrolase and its ability to hydrolyze substrates previously thought to be hydrolyzed solely by microsomal epoxide hydrolase (13), necessitates its consideration in the overall metabolism of epoxidized xenobiotics.

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