*Biochemical Pharmacology*, Vol. 39, No. 2, pp. 293–300, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
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# PRODUCTION OF MONOSPECIFIC ANTISERUM TO A CYTOSOLIC EPOXIDE HYDROLASE FROM HUMAN LIVER

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(*Received* 10 *February* 1989; *accepted* 5 *July* 1989)

**Abstract**—A method for the purification to apparent homogeneity of cytosolic *trans*-stilbene oxide hydrolase from human liver is presented. The method employed ion exchange and gel filtration chromatography. From 50 g of human liver, 4.9 mg of homogenous enzyme protein was obtained. Although the enzyme had lost much of its catalytic activity during purification, it was nevertheless suitable for the preparation of antibodies to the enzyme. Only one immunogenic species was present in the antigen preparation, but some antibodies that were cross-reactive to sites on catalase were present in the antiserum. These catalase-specific antibodies were removed by immunoaffinity chromatography, and an IgG fraction that is monospecific to the cytosolic epoxide hydrolase was obtained. The usefulness of antibodies to this enzyme in immunoblotting experiments, following either sodium dodecyl sulfate–polyacrylamide gel electrophoresis or isoelectric focussing, as well as in enzyme-linked immunosorbent assays, is demonstrated.

Epoxide hydrolases have long been known to play an important role in protecting the cell from the toxic and carcinogenic effects of reactive epoxide intermediates derived from a wide variety of xenobiotic compounds [1–3]. It is becoming increasingly apparent that epoxide hydrolases also play a significant role in the regulation and deactivation of epoxides derived from endogenous compounds, whose toxicological and hormonal significance are beginning to be appreciated [4,5].

Several different epoxide hydrolases have been shown to exist in the cell. These enzymes can be distinguished by both structural and catalytic criteria. The epoxide hydrolase that has been studied most widely is found primarily in the endoplasmic reticulum; it catalyzes the hydrolysis of arene oxides and of other *cis*-disubstituted and monosubstituted oxiranes [1–3]. This form of epoxide hydrolase has been found in almost every organ and tissue investigated and is distributed throughout the animal kingdom [6,7]. The important role of this enzyme in the metabolism and detoxification of a large number of toxins and carcinogens has been well established [2,3]. This epoxide hydrolase actively metabolizes the model substrates styrene oxide and benzo[*a*]pyrene 4,5-oxide (BPO)†, and is referred to in this report as microsomal BPO hydrolase.

More recently, several additional epoxide hydrolases have been identified and partially characterized. These enzymes also hydrate a number of endogenous and xenobiotic epoxides, but have catalytic and immunoreactive properties that demonstrate their non-identity with microsomal BPO hydrolase. One such epoxide hydrolase is found primarily in the cytosol and in peroxisomes. It catalyzes the hydration of a number of olefinic epoxides that are poor substrates for the microsomal epoxide hydrolase, including *trans*-1,2-disubstituted styrene oxides and epoxides derived from unsaturated fatty acids [5,8–11]. This epoxide hydrolase has been shown to be different from microsomal BPO hydrolase by catalytic [12–14] and immunological criteria. This enzyme selectively hydrates *trans*-substituted styrene oxide analogs, including *trans*-stilbene oxide (TSO) [12, 16], and is referred to in this report as (cytosolic) TSO hydrolase.

Our laboratory has been interested in the characterization of multiple epoxide hydrolases in human tissues, with the ultimate goal of determining the degree of diversity of expression of these multiple enzyme forms in the human population. To this end we have purified previously the microsomal BPO hydrolase from human liver, and used antibodies to the enzyme to characterize its properties in human lung [17]. Similar studies characterizing the cytosolic TSO hydrolase in human lung and liver requires production of a specific antiserum to this protein derived from a human tissue source. Cytosolic TSO hydrolase has previously been isolated and purified from several mammalian species in a number of laboratories. Rabbit, rat and mouse liver cytosol have served as the source of purified cytosolic TSO hydrolase preparations in high yield [18–24]. Two methods have been reported for the purification of

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† Abbreviations: BPO, benzo[*a*]pyrene 4,5-oxide; TSO, *trans*-stilbene oxide;DEAE-cellulose, diethylaminoethyl-derivatized cellulose; ELISA, enzyme-linked immunosorbent assay; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; and IgG, immunoglobin G.

TSO hydrolase from human liver cytosol, one of which yields microgram quantities of the pure enzyme, which may not provide a sufficient amount of protein to be useful in the production of antiserum [25]. A more recently reported method has employed a synthetic ligand affinity chromatography column to purify TSO hydrolase from livers of a number of species, including human [26].

In this report we describe a method for the isolation of milligram amounts of electrophoretically pure TSO hydrolase from human liver cytosol. This technique has provided us with sufficient amounts of the pure enzyme to raise monospecific antiserum in rabbits in pursuit of our ongoing immunochemical studies of the diversity of epoxide hydrolases in the human population.

## MATERIALS AND METHODS

*Materials.* Unlabeled and tritiated *trans*-stilbene oxide were prepared as previously described [27]. DEAE-cellulose (DE 23) was obtained from Whatman, Inc. (Hillsboro, OR), and prepared according to the instructions of the manufacturers. Superose 12B was obtained from Pharmacia, Inc. (Piscataway, NJ). Chemicals for SDS–polyacrylamide gel electrophoresis were obtained from BioRad Laboratories (Richmond, CA). Preformed ampholyte-containing agarose isoelectric focussing plates (Isogel pH 3–10) were obtained from FMC Bioproducts (Rockland, ME). Second antibodies for ELISA and Western blotting assays (peroxidase- or phosphatase-labeled antirabbit IgG FAB fragment), and a prepackaged phosphatase substrate system (5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium) were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). 4-Chloro-1-naphthol was obtained from Sigma Chemical Co. (St Louis, MO).

*Enzyme preparation.* Human liver was obtained as surgical waste from exploratory biopsy for diagnosis of metastatic cancer. A 50-g specimen obtained from an adult male patient was quick frozen in liquid nitrogen within 30 min of removal, and maintained at $-70°$. This sample was later proved to be tumor-free by histological examination. It was homogenized in 50 mL of Buffer A (10 mM Tris, pH 8.0, 0.1 mM EDTA, 0.1 mM DTT), and subjected to ultracentrifugation. The supernatant fraction from an initial 20 min, 9000 *g* centrifugation was spun at 150,000 *g* for 1 hr. The resulting supernatant fraction was collected and designated as cytosol. The cytosol was applied at a flow rate of approximately 5 mL/min to a DEAE-cellulose column (2.6 × 28 cm) that had been equilibrated previously with Buffer A. The column was then washed using 500 mL of Buffer A, and retained proteins were eluted using a 2-L gradient of 0 to 300 mM NaCl in Buffer A. The absorbance at 280 nm was monitored, fractions of 15 mL were collected, and TSO hydrolase activity was measured in each fraction. The enzyme activity was measured by a radiometric assay, as previously described [27]. Fractions containing the highest activity were pooled and concentrated by pressure ultrafiltration. These fractions were dialyzed overnight against Buffer A and subjected to gel filtration on a Superose 12B column that had been equilibrated previously with Buffer A. Fractions were eluted with Buffer A at a flow rate of 1 mL/min and those fractions exhibiting absorbance at 280 nm were assayed for TSO hydrolase activity. Enzyme purity was analyzed during the procedure using standard SDS–polyacrylamide gel electrophoresis techniques [28]. Guinea pig liver TSO hydrolase was purified by reported methods [29], which are essentially the same as those used in these studies for purification of the human enzyme.

*Immunization.* The purified enzyme was used to immunize male adult New Zealand White rabbits. Protein (200 μg) was emulsified in 500 μL of Freund's complete adjuvant and injected at five intradermal sites. Four weeks later, a second series of injections was administered, using Freund's incomplete adjuvant as vehicle. Blood was drawn from the animal 1 week later, and the serum fraction was prepared. Serum prepared from blood drawn from the same animals prior to the first immunization was prepared and used as control serum.

*Immunoaffinity purification of antiserum.* An IgG-containing fraction was prepared from the polyvalent antiserum by DEAE-Affigel blue chromatography [15]. Catalase-binding antibodies were removed from it by affinity chromatography over a column containing catalase covalently bound to agarose. The catalase affinity column was prepared by coupling 175 mg of crystalline bovine catalase to 6.5 mL of activated agarose (Affigel 15), according to the instructions of the manufacturer, at a pH of 8.0 for 4 hr at 4°. The concentrated IgG fraction, equivalent to 55 mg of total protein, was then run over the affinity gel, and the unbound protein fraction was collected and concentrated.

*Western blotting.* Western blotting experiments were carried out using either SDS–polyacrylamide gel electrophoresis or isoelectric focussing as the first step. In the former case, previously described methods were used to separate proteins of human liver cytosol, human lung cytosol, or the purified enzyme preparation, and to transfer the separated proteins to nitrocellulose membranes [17]. The membranes were exposed to the unfractionated antiserum to the purified enzyme, and immunoreactive proteins were indicated using a peroxidase-linked second antibody and 4-chloro-1-naphthol as the chromogenic substrate. When isoelectric focussing was used as the first step, samples were applied to pre-formed, ampholyte-containing agarose gels according to the instructions of the manufacturer. Samples were focussed for 40 min with an initial power setting of 25 W. Because the gel could not be removed from its plastic backing, electroblotting onto nitrocellulose was not possible. However, overnight blotting of the gel under pressure at 4° led to efficient transfer of proteins from the gel and minimal band diffusion. Gels were fixed in 0.7% acetic acid for 15 min and a "sandwich" was prepared comprising a glass plate, the plastic-backed gel (face up), a piece of nitrocellulose, a piece of filter paper saturated with distilled water, a 2-inch stack of paper towels, and a second glass plate. A 5-kg weight was placed on the sandwich and it was left overnight at 4°. Coomassie blue staining of both blotted and pre-blotted gels

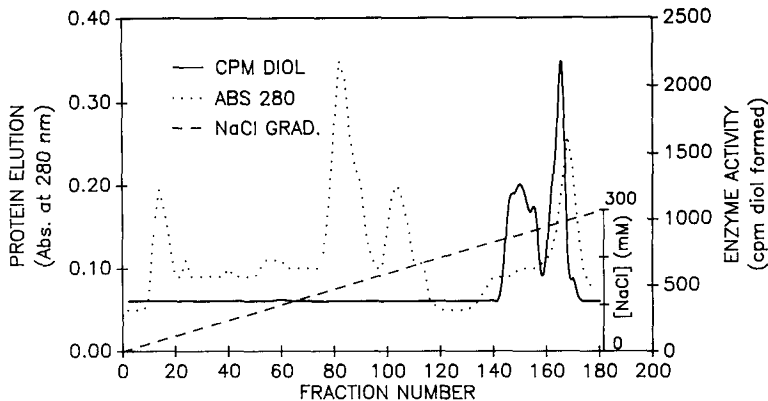


Fig.1. Elution pattern of TSO hydrolase activity from DEAE-cellulose. Fractions (15 mL) were collected and assayed for TSO hydrolase activity. The solid line shows enzyme activity (expressed as cpm product recovered in the assay) in each fraction. The dotted line shows the protein content (measured by monitoring the eluate at 280 nm) of each fraction. The dashed line shows the NaCl gradient for elution.

showed that proteins were uniformly transferred, and that at least 60–70% transfer efficiency occurred. Membranes were cut into vertical strips for further processing, to permit the use of minimal amounts of antibodies. The strips were exposed overnight at room temperature to the unfractionated anti-TSO hydrolase antiserum (1/200 dilution), or to monospecific anti-TSO hydrolase IgG (1/200 dilution of a 10 mg/mL solution), and blocked for 1 hr with a 3% gelatin solution. Immunoreactive proteins were detected using a phosphatase-linked goat anti-rabbit IgG second antibody (1/50,000 dilution for 1 hr at room temperature) and a pre-packaged phosphatase substrate system containing 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (30–45 min at room temperature).

*Other procedures.* ELISA assays for microsomal epoxide hydrolase have been described previously [17,30–32]. Previously reported conditions [17] were used in this study to quantitate immunoreactive proteins in either intact cytosol or in the final enzyme preparation. Amino acid analysis was performed by Dr M Rahima of the Department of Oral Biology, Northwestern University, using a single column Jeol 6AH amino acid analyzer with ninhydrin detection.

## RESULTS

Cytosol was prepared from the combined human liver samples and subjected to chromatography over DEAE-cellulose at pH 8.0. Figure 1 shows the elution pattern of total protein (measured as absorbance at 280 nm) and cytosolic epoxide hydrolase (measured as TSO hydrolase activity) from the column. It should be noted that no enzyme activity eluted with the void volume. Furthermore, the activity that was retained on the column did not elute until a fairly high (greater than 200 mM) concentration of NaCl was applied. Two peaks of enzyme activity were observed; these fractions were concentrated separately and applied to a Superose 12B gel filtration column. The first fraction, which contained a high amount of enzyme activity but little total protein, did not yield significant amounts of protein after gel filtration. The second fraction, which contained more enzyme activity, albeit at a lower specific activity due to the presence of a larger amount of total protein, yielded two major peaks absorbing at 280 nm upon gel filtration (Fig.2). The fraction whose peak corresponded to higher molecular weight proteins did not contain enzyme activity. The fraction corresponding to the second peak, which contained protein with a molecular weight calculated at 122 kD, did contain TSO hydrolase activity. A total yield of 4.9 mg of enzyme protein was recovered from the fraction corresponding to this absorbance peak. When this fraction was concentrated and subjected to SDS–polyacrylamide gel electrophoresis, a single band migrating at the 60 kD position was observed (Fig.3).

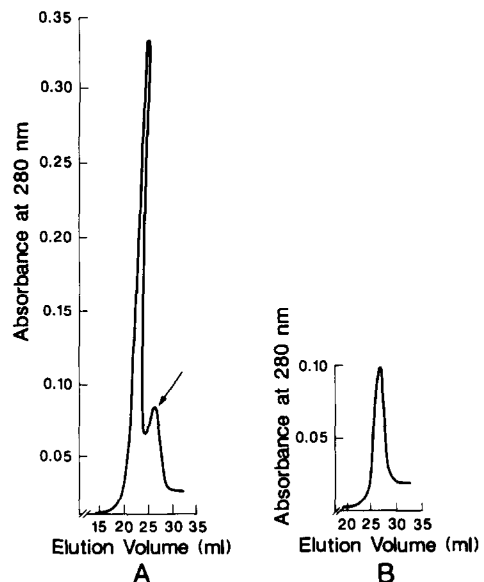


Fig. 2. Elution of TSO hydrolase activity from a Superose 12B gel filtration column. Absorbance at 280 nm was monitored, and two peaks were observed (A). Enzyme activity was only detected in the fraction corresponding to the second peak (designated by the arrow). Panel B shows rechromatography of this fraction. All of the recovered enzyme activity was found in the fraction corresponding to this absorbance peak.

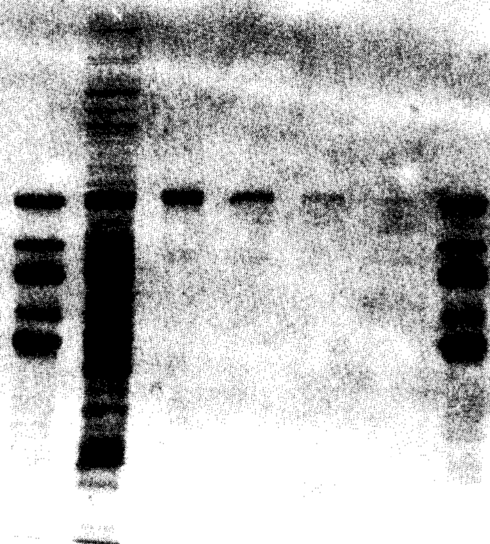

Fig.3. SDS–polyacrylamide gel electrophoresis of the purified enzyme preparation. Samples applied to the gel were (left to right): molecular weight standards, human liver cytosol, and purified enzyme (10, 5, 2.5, and 1.25 μg). The gel was stained with Coomassie blue dye and dried prior to photography. Molecular weight standards and reported subunit molecular weights were: catalase, 59 kD; glutamate dehydrogenase, 53 kD; ovalbumin, 45 kD; aldolase, 40 kD; and pepsin, 35 kD.

Table 1 shows the yield and specific activity of enzyme obtained after each step of purification. In the first step, DEAE-cellulose chromatography, approximately 30% of the enzyme was recovered in the two main enzyme-containing fractions shown in Fig.1. During the two gel filtration steps, much of the enzyme activity was lost. In fact, when the first enzyme-containing fraction from the DEAE-cellulose column was subjected to gel filtration, no enzyme activity was recovered (data not shown). Gel filtration of the second fraction produced a high yield of homogeneous enzyme protein which had a very low specific enzymatic activity. Thus, the low yield of total enzyme activity appears to be due to a loss of specific activity by the enzyme, rather than simply a low recovery of total enzyme protein. Although only 4.5% of the total enzyme activity was recovered, the total yield of 4.9 mg enzyme protein was more than sufficient for our purposes of antibody production.

The purified enzyme appeared to be homogeneous by the criterion of SDS–polyacrylamide gel electrophoresis (Fig.3). Further proof of the purity of the enzyme preparation was offered by Western blotting experiments. Figure 4 shows a Western blot obtained after SDS–polyacrylamide gel electrophoresis of human liver cytosol, human lung cytosol, and the pure enzyme preparation; the antibody used was the unfractionated antiserum raised to the final enzyme preparation. In each case, a single band, corresponding to a protein with a molecular weight of 60 kD, was seen. This demonstrates that the enzyme is found in both liver and lung, with subunits of apparently similar molecular weights. The pure enzyme produced antiserum that reacted with a single band on the gel; no additional immunogenic species were identified in the pure enzyme preparation. Figure 5 shows Western blots obtained after isoelectric focussing of either the pure enzyme preparation or of human liver cytosol. In the first two

Table 1. Purification of human liver cytosolic epoxide hydrolase

| Purification step | Protein (mg) | Activity (units*) | Specific activity (units/mg) | Yield (%) |
|---|---|---|---|---|
| Cytosol | 869 | 88,000 | 101 | 100 |
| DEAE-Cellulose | 32.5 | 26,500 | 810 | 30 |
| Gel filtration I | 16.9 | 15,700 | 930 | 18 |
| Gel filtration II | 4.9 | 4,000 | 816 | 4.5 |

* One unit catalyzes the formation of 1 pmol diphenylethanediol product/min.

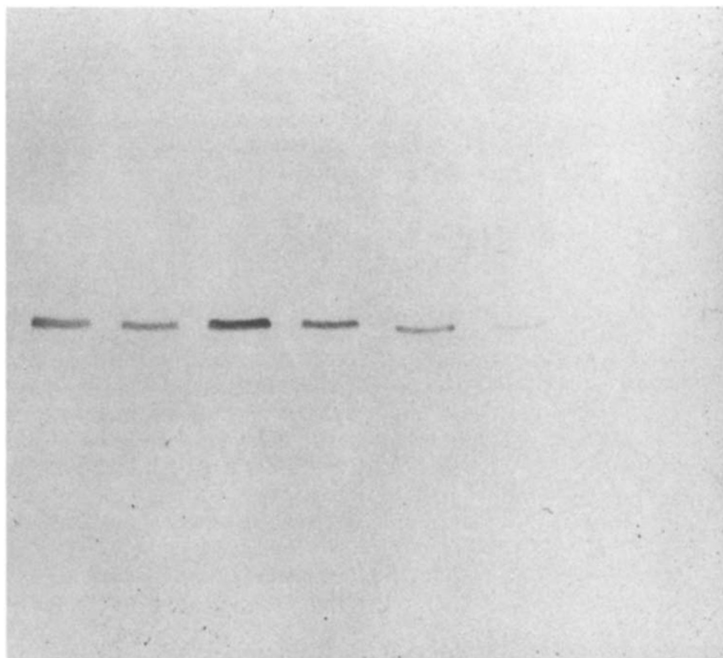

Fig.4. Western blot of TSO hydrolase-containing preparations. An SDS–polyacrylamide electrophoresis gel was prepared using the following samples (in lanes left to right): human liver cytosol (25 and 10 μg), human lung cytosol (100 and 50 μg), and purified human cytosolic epoxide hydrolase (5 and 2 μg). The gel was electroblotted onto nitrocellulose which was incubated with antiserum to human cytosolic epoxide hydrolase, and developed as described.

lanes on the left, the crude antiserum to the enzyme preparation was used to detect immunoreactive proteins on the nitrocellulose membranes. In the case of the pure enzyme preparation, a single band was seen, corresponding to a pI of 4.0. Reaction of the antiserum with a human liver cytosol preparation showed two additional immunoreactive bands. We have determined previously that these bands reflect a cross-reactivity of the antiserum with catalase, and that antiserum to TSO hydrolase cross-reacts with catalase [29]. However, this cross-reactivity is not due to an immunogenic catalase contaminant in the human liver TSO hydrolase preparation, as demonstrated by catalytic criteria (lack of catalase activity in the TSO hydrolase preparation [29]) or by the absence of a corresponding immunoreactive band in the purified enzyme preparation (second strip). After affinity chromatography, to remove catalase-reactive antibodies from the IgG fraction, the IgG fraction thus obtained appeared to be monospecific for TSO hydrolase, as shown by the two strips on the right side (reaction of the monospecific antibody with human liver cytosol, and reaction of the monospecific antibody with purified enzyme). Similar experiments using SDS–polyacrylamide gel electrophoresis prior to blotting would not be instructive, because the subunits of catalase and TSO hydrolase have very close molecular weights and, therefore, would not produce distinct bands.

The unfractionated antiserum was also used to develop conditions for an ELISA assay, whereby the amount of cytosolic epoxide hydrolase protein in a tissue sample can be measured directly without measuring enzyme activity. We employed assay conditions reported elsewhere [17], using 1.0 μg of purified TSO hydrolase per well, and anti-epoxide hydrolase antiserum dilutions of 1:1 up to 1:500. Figure 6 shows curves obtained from experiments using human antigen and anti-TSO hydrolase antiserum, as well as reaction of the same antiserum to an epoxide hydrolase preparation purified from guinea pig liver [29]. The antiserum to the human enzyme was highly specific for its own antigen at dilutions ranging from 1:5 to 1:50. It also cross-reacted with guinea pig enzyme, but much more weakly.

Table 2. Amino acid compositions of epoxide hydrolases

| Amino acid | Human cEH (residues/1000) | Human mEH |
|---|---|---|
| Lys | 84.7 | 76.9 |
| His | 24.6 | 32.0 |
| Arg | 43.7 | 44.9 |
| Asp | 104.5 | 66.2 |
| Thr | 52.2 | 38.5 |
| Ser | 56.8 | 66.2 |
| Glu | 138.6 | 94.0 |
| Pro | 64.5 | 55.6 |
| Gly | 55.9 | 74.8 |
| Ala | 106.5 | 53.4 |
| Cys | 29.7 | 8.5 |
| Val | 50.3 | 62.0 |
| Met | 6.8 | 36.3 |
| Ile | 17.7 | 53.4 |
| Leu | 89.1 | 102.6 |
| Tyr | 26.1 | 49.1 |
| Phe | 48.0 | 62.0 |

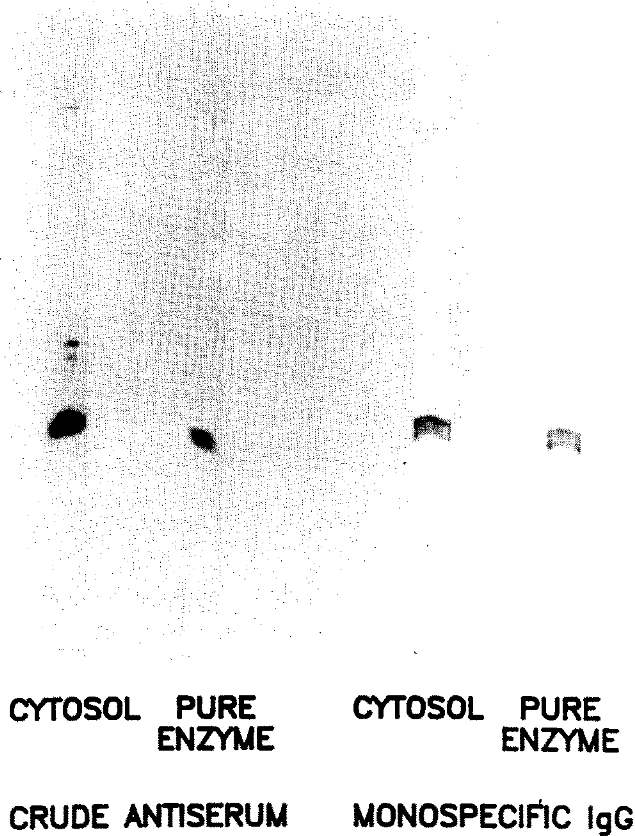


Fig.5. Immunoblots of TSO hydrolase-containing preparations after isoelectric focussing. Samples were subjected to isoelectric focussing, and the resulting gels were pressure-blotted onto nitrocellulose membranes. Membranes were cut into strips, and the strips were exposed to antibodies to TSO hydrolase. Antibody-protein binding was visualized as described. The following sample/antibody combinations are shown (from left to right): human liver cytosol/crude (unfractionated) antiserum; purified enzyme/crude (unfractionated) antiserum; human liver cytosol/affinity purified (monospecific) IgG; and purified enzyme/affinity purified (monospecific) IgG.

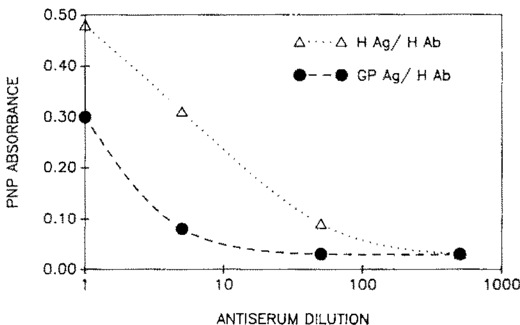


Fig.6. Enzyme-linked immunosorbent assay (ELISA) of TSO hydrolase using anti-TSO hydrolase antiserum. Purified human (△) or guinea pig (●) liver TSO hydrolase (1 μg) was coated onto microtiter wells and exposed to anti-human TSO hydrolase antiserum in the dilutions shown. Antibody binding was visualized using a phosphatase-linked second antibody, and is expressed on the ordinate as total absorbance of the *p*-nitrophenol product.

The amino acid composition of the human cytosolic epoxide hydrolase was determined and is shown in Table 2. For the sake of comparison, the amino acid composition of human microsomal epoxide hydrolase is included. As anticipated from immunochemical studies comparing the same two enzymes obtained from rodent liver [15], they had different structural properties reflecting different amino acid compositions.

## DISCUSSION

We present here a method for the purification of cytosolic epoxide hydrolase from human liver. The method provided enzyme protein in good yield and at high purity. Although several methods have been previously published describing purification of this enzyme in good yield from mouse, rat, and rabbit liver [18–24], methods for purification of the human liver enzyme [25, 26] provide small amounts of

enzyme protein, probably not sufficient for raising antiserum in rabbits. The method reported here is convenient and rapid, entailing only two chromatographic steps. Recovery of enzyme activity from the first step was good, with 30% of the total applied activity recovered. During the second step, gel filtration, recovery of enzyme protein was good, but much of the enzyme activity was lost. Previously published methods for purification of mouse cytosolic epoxide hydrolase have cited a marked susceptibility of the enzyme to oxidation during purification [19, 20]. We attempted to protect the enzyme by adding dithiothreitol to the buffer, and by saturating the buffer with nitrogen as suggested. Despite these precautions, much of the specific activity of the enzyme was lost upon gel filtration, even though enzyme activity in either the cytosol or in the preparation after DEAE-cellulose chromatography was stable for at least a week at 4°. It is possible that loss of enzyme activity may be due to removal of trace amounts of an endogenous antioxidant by gel filtration.

Although much of its catalytic activity was lost, the final enzyme preparation appeared to be of high purity and well suited for the preparation of antiserum. SDS–polyacrylamide gel electrophoresis showed a single band, with a molecular mass of 60 kD (Fig.3). The observed molecular weight by gel filtration of the intact enzyme (Fig.2) was 122 kD. Therefore, as observed previously for rat, rabbit and mouse [18–24], the intact human enzyme was a dimer comprising two similar or identical subunits of approximately 60 kD. Western blots of human liver and lung cytosol using the unfractionated antiserum also showed a single band at the 60 kD position (Fig.4). This was taken as further proof of the purity of the isolated enzyme, since any additional immunogenic contaminants in the enzyme preparation would be recognized by the antiserum as additional bands in the pattern of total cytosolic protein. This figure also demonstrates that the enzyme is found in lung cytosol, and that the enzyme in lung has similar subunit molecular weight. The question of monospecificity of the unfractionated antiserum and the affinity purified IgG fraction for epoxide hydrolase was determined by a second criterion as well, that of immunoblotting after isoelectric focussing. It is possible that immunogenic contaminants of identical molecular weight to the epoxide hydrolase subunits exist, and that these contaminants would not be detectable by Western blotting after SDS–polyacrylamide gel electrophoresis, since they would not produce a band distinguishable from that produced by the epoxide hydrolase. Therefore, a second Western blotting experiment was performed, using isoelectric focussing as the initial step. It is very unlikely that a contaminant would have not only the same molecular weight as the enzyme, but also the same isoelectric point. Using this second protocol, we also observed no contaminating immunogenic species in the purified enzyme preparation (Fig.5), but we did observe an additional cytosolic protein species (catalase [29]) that cross-reacted with the unfractionated antiserum. Removal of catalase-binding antibodies by affinity chromatography produced an IgG fraction that was monospecific; it recognized only TSO hydrolase among all cytosolic proteins (Fig.5). The ELISA assay results show that the unfractionated antiserum cross-reacted somewhat with a preparation of cytosolic epoxide hydrolase from guinea pig liver, but that it was much more specific to its own antigen. In this regard, it has been reported that antiserum to rat liver cytosolic epoxide hydrolase cross-reacts with homologous cytosolic enzymes in cytosols from mice, guinea pigs, hamsters and rabbits [24], but not with corresponding cytosolic enzymes from monkeys [24] or humans [33].

We have observed previously that a preparation of epoxide hydrolase from guinea pig liver cytosol contains significant amounts of contaminating catalase, which can be detected by catalase activity assays as well as by the presence of a small absorbance peak at 405 nm [29]. Likewise, similar small absorbance peaks in the 405 nm region, characteristic of possible hemoprotein contamination, in a preparation of purified mouse liver cytosolic epoxide hydrolase have been reported elsewhere [20]. However, our human liver cytosolic epoxide hydrolase preparation appeared to be catalase-free, as no catalase activity was detectable in the purified preparation, and no absorbance in the 400–420 nm region was seen (data not shown).

In summary, we have isolated cytosolic epoxide hydrolase from human liver and purified it to apparent homogeneity. Although the enzyme had lost much of its catalytic activity during the purification process, it was nevertheless useful for antiserum production. The toxicological and physiological roles of cytosolic epoxide hydrolase remain to be fully elucidated. The ability to produce a specific antiserum, using the antigen provided by this purification method, should prove useful in future biochemical characterization of this enzyme.

*Acknowledgements*—This work was supported by PHS Grants CA 34455 and CA 46129, as well as a Research Career Development Award (CA 01287) to T.M.G.

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