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A Simple Method for Purification of Epoxide Hydratase from Rat Liver

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Rat liver epoxide hydratase was purified 460-fold to homogeneity by detergent solubilization and ion-exchange chromatography. The enzyme obtained in high yield (36%) exhibited a specific activity of 479 nmol of styrene glycol formed/min per mg of protein, with styrene oxide as substrate. Only one polypeptide-staining band, mol.wt. 49 500, was visible after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Hepatic microsomal epoxide hydratase (EC 4.2.1.63) is a pharmacologically important enzyme which catalyses the formation of *trans*-dihydrodiols from epoxide substrates. Reviews by Oesch (1973) and Jerina & Daly (1974) strongly implicate epoxide metabolites of polycyclic hydrocarbons as potent mutagenic and potentially carcinogenic agents. The primary role of epoxide hydratase appears to be the inactivation of potentially dangerous epoxide metabolites, although formation of the 7,8-dihydro-7,8-dihydroxy derivative of benzo[*a*]pyrene leads to production of the extremely carcinogenic metabolite 7,8-dihydro-7,8-dihydroxy-9,10-epoxybenzo[*a*]pyrene (Sims *et al.*, 1974).

Little is known about (a) the mechanisms regulating epoxide hydratase activity or (b) the relationship and interaction with other components of the microsomal membrane involved in xenobiotic metabolism, such as cytochrome *P*-450 and UDP-glucuronyltransferase. Therefore pure preparations of epoxide hydratase would be invaluable in the study of these problems and the mechanism of chemical carcinogenesis.

Pure epoxide hydratase has been obtained (Bentley & Oesch, 1975; Lu *et al.*, 1975). These published methods are technically difficult and produce low yields of enzyme. Therefore we have developed a simple high-yield procedure for purification of epoxide hydratase involving detergent solubilization and ion-exchange chromatography.

Materials and Methods

[7-³H]Styrene oxide was a kind gift from Professor F. Oesch, Section of Biochemical Pharmacology, Institute of Pharmacology, University of Mainz, D-65 Mainz, West Germany. Lubrol 12A-9 (a condensate of dodecyl alcohol with approx. 9.5 mol of ethylene oxide) was a kind gift from ICI Organics Division, Blackley, Manchester, U.K. DEAE-cellulose (DE-52 grade) and CM-cellulose (CM-52

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grade) were purchased from Whatman Biochemicals, Maidstone, Kent, U.K.

Protein standards, ovalbumin, carbonic anhydrase and myoglobin, used for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were purchased from Boehringer, Mannheim, West Germany. Bovine serum albumin and Coomassie Brilliant Blue protein stain were from Sigma Chemical Co., London S.W.6, U.K.

Assay of epoxide hydratase activity

Epoxide hydratase activity was determined by the method of Oesch (1974). One unit of enzyme activity is defined as 1 nmol of styrene glycol formed/min.

Protein concentrations were determined by the biuret method (see Layne, 1957) and the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Gel electrophoresis

Gel electrophoresis was performed by using 7.5% (w/v) polyacrylamide gels in the presence of 0.1% sodium dodecyl sulphate at 20°C, pH 7.2, and a constant 60 V (6 mA per gel). The proteins were stained in Coomassie Blue, as previously described (Weber & Osborn, 1969). Stained gel-electrophoresis patterns were scanned in a Joyce-Loebl Chromoscan 200 densitometer. Molecular weights were estimated by comparison of the mobility of the purified enzyme with standard proteins of known molecular weight (see Burchell, 1977).

Preparation of the liver microsomal fraction

A microsomal fraction was prepared from the livers of five male (200–250 g) Wistar rats as previously described (Burchell, 1977).

Results

Purification of epoxide hydratase

Liver microsomal pellets were resuspended in a volume of 1% Lubrol/0.2 M-potassium phosphate

buffer, pH7.0, equivalent to the volume of the 105000g supernatant, by gentle homogenization, and mixed for 20 min at 4°C. This preparation was further centrifuged at 105000g for 60 min. The supernatant contained more than 90% of the original microsomal epoxide hydratase activity and 82% of the original microsomal protein. The Lubrol supernatant was

then dialysed at 4°C overnight against volumes (about 5 litres) of 5mM-potassium phosphate/0.05% Lubrol buffer, pH7.4 (buffer A). If any slight precipitates were observed they were removed by centrifugation at 10000g for 10 min.

As previously reported, activation of epoxide hydratase occurs during solubilization. The overall

Table 1. *Purification of rat liver epoxide hydratase*

Complete data are reported for the purification of epoxide hydratase from five rat livers as described in the Materials and Methods section. One unit of enzyme activity represents 1 nmol of styrene glycol formed/min. Values in parentheses are the specific activities obtained at each stage during purification of epoxide hydratase from one rat liver and the final yield of enzyme activity in this case was 20% (0.7 mg of pure protein).

Purification step	Total protein (mg)	Specific activity (units/mg of protein)	Relative purification	Total activity (units)	Yield (%)
10000g supernatant	9541	1.04 (2.23)	1	9879	100
Lubrol-soluble supernatant	1475	9.32 (19.9)	9	13747	139
DEAE-cellulose eluate	57	149 (147)	143	8506	86
CM-cellulose eluate	7.6	479 (844)	461	3637	36.8

Fig. 1. *Gel electrophoresis of purified epoxide hydratase*

Disc electrophoresis was performed with cylindrical 7.5% polyacrylamide gels (9.5 cm × 0.6 cm) in the presence of 0.1% sodium dodecyl sulphate (see the Materials and Methods section). Samples of purified epoxide hydratase (specific activity 479 units/mg of protein): (a) 2.5 µg of protein; (b) 5 µg of protein. Gels were stained with 0.25% Coomassie Blue for 60 min and destained with acetic acid/methanol/water (7:5:43, by vol.). — and ———, Densitometric traces of gel-electrophoresis patterns of (a) and (c) (DEAE-cellulose eluate, 18 µg of protein) respectively.

yield of enzyme activity increased by approx. 40–50% (see Burchell *et al.*, 1976). The clear red non-diffusible material was divided into two equal volumes and each volume of material was applied to one of two identical DEAE-cellulose columns (40 cm × 3 cm) previously equilibrated with buffer A, pH 7.4. After elution with buffer A, pH 7.4, approx. 60% of epoxide hydratase activity applied to these columns was recovered in the first protein peak obtained. This chromatographic step resulted in a further 15-fold purification of the enzyme (see Table 1). The eluates from both columns were concentrated by vacuum dialysis, combined and dialysed at 4°C overnight against buffer A, adjusted to pH 6.5. The dialysed concentrate was applied to a CM-cellulose column (11 cm × 4 cm), previously equilibrated with buffer A, pH 6.5. Epoxide hydratase activity was eluted from the column with the same buffer A, pH 6.5.

A 36% yield of pure epoxide hydratase was obtained, exhibiting a specific activity of 479 units/mg of protein (see Table 1). The enzyme was also purified from a single rat liver (see Table 1); in this case 0.7 mg of pure enzyme protein was recovered, representing a 20% yield of epoxide hydratase activity exhibiting a specific activity of 844 units/mg of protein.

Purity of epoxide hydratase

Samples of purified epoxide hydratase were analysed by electrophoresis in the presence of sodium dodecyl sulphate (see the Materials and Methods section). Only one polypeptide-staining band was visible after gel electrophoresis, and, furthermore, densitometric tracing of these gel patterns showed one symmetrical peak of absorbing material, indicating the presence of only one polypeptide species (Fig. 1). By comparison of epoxide hydratase with protein standards of known molecular weight, the subunit mol.wt. was determined to be 49 500.

Specific antiserum produced in response to purified epoxide hydratase in New Zealand White/Lop rabbits was used to ascertain the purity of the enzyme. Ouchterlony double-diffusion analysis (Ouchterlony, 1949) of pure enzyme preparation, allowed to react with antiserum or purified γ -globulin fraction obtained from this antiserum, showed a single sharp precipitin line, with no spur formation. When the antiserum was mixed with purified epoxide hydratase in solution the enzyme was completely inactivated, within 1 h at 4°C, and subsequent centrifugation removed all the immunoreactive epoxide hydratase from the resulting supernatant. Thus the antiserum appears to bind specifically to epoxide hydratase. Furthermore purified immunoglobulin G fraction obtained from the antiserum, when attached to Sepharose 4B, binds only epoxide hydratase from a crude enzyme preparation (B. Burchell & A. Burchell, unpublished work), indicat-

ing that the antibody obtained is specific for only epoxide hydratase.

Discussion

Epoxide hydratase has been purified to homogeneity by a simple procedure. The number of steps required and complexity of the method has been decreased in comparison with previously published procedures (see Bentley & Oesch, 1975; Lu *et al.*, 1975). However, the properties of the pure enzyme compare favourably with the previous preparations. The subunit mol.wt. obtained, 49 500, agrees with the value reported by Bentley & Oesch (1975), but differs slightly from the value of 53 000–54 000 determined by Lu *et al.* (1975). However, Lu *et al.* (1975) isolated the liver enzyme from rats pretreated with phenobarbital, which may account for the discrepancy. The final specific activity of epoxide hydratase obtained (479 units/mg of protein) agrees with values previously reported. One enzyme preparation obtained from a single rat liver exhibited a much higher specific activity (844 units/mg of protein). However, as epoxide hydratase can be activated and presumably also inactivated during chromatography (see Bentley & Oesch, 1975; Burchell *et al.*, 1976), this result does not appear to be unusual. Furthermore a second preparation of pure epoxide hydratase from a single rat liver exhibited a specific activity of 560 units/mg of protein.

In conclusion, we have obtained a preparation of homogeneous epoxide hydratase in higher yields than previously reported, by a relatively simple procedure. This enzyme has the same properties as epoxide hydratase isolated by published procedures. Pure preparations of epoxide hydratase obtained by this method can be used to improve our understanding of chemically induced cytotoxicity and carcinogenesis.

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