Journal of Chromatography, 426 (1988) 169–176
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4060

Note

Hydrophobic chromatography: a one-step method for the purification of human liver microsomal epoxide hydrolase

PHILIPPE H. BEAUNE*, THIERRY CRESTEIL, JEAN-PIERRE FLINOIS and JEAN-PAUL LEROUX

INSERM U 75, C H.U Necker, 156 Rue de Vaugirard, F 75730 Paris Cedex 15 (France)

(First received September 11th, 1987; revised manuscript received November 17th, 1987)

Liver microsomal epoxide hydrolase (EC 3.2.2.3) is involved in the biotransformation of epoxides produced from either xeno- or endobiotics [1–4]. The diols produced are generally detoxification products but in several instances, such as 9,10-epoxybenzopyrene-7,8-diol, they are very potent mutagens and possible carcinogens [5]. Moreover, microsomal epoxide hydrolase is an enzyme that can be induced by substances such as phenobarbital [6]. Therefore, in order to characterize and study the role and regulation of epoxide hydrolase, it is of importance to obtain a pure enzyme conveniently and in large amounts. Several groups have already purified epoxide hydrolase from human liver but its preparation necessitates several steps [1–3]. More recently, Kennedy and Burchell [7] have developed an immunoaffinity procedure to purify rat epoxide hydrolase, but this technique necessitates a monoclonal antibody which is not yet available for human epoxide hydrolase. As human liver is difficult to obtain in large amounts, it is also of interest to develop a method to purify epoxide hydrolase together with other drug-metabolizing enzymes from a unique liver sample. The first step in the purification of cytochrome P-450, cytochrome P-450 reductase and cytochrome b5 consists in a hydrophobic chromatography on Octyl Sepharose CL 4B. We demonstrate in this paper that this commercially available resin can be used to purify microsomal human liver epoxide hydrolase in a one-step procedure.

EXPERIMENTAL

Chemicals

Chemicals were of the highest quality available from Sigma (St. Louis, MO, U.S.A.), Merck (Darmstadt, F.R.G.), Fluka (Buchs, Switzerland) and Prolabo

0378-4347/88/$03.50

(Paris, France). [^{3}H]Benzo[*a*]pyrene-4,5-oxide was a generous gift from P. Dansette (CNRS, UA 400, Paris, France). [^{3}H]Styrene-7,8-oxide (200 mCi mmol $^{-1}$) was purchased from Amersham (Les Ulis, France). Octyl Sepharose CL 4B was purchased from Pharmacia (Uppsala, Sweden).

Samples

The animals used were female New Zealand rabbits purchased from CEGAV (Passais, France) and male Sprague–Dawley rats (150–200 g) from Iffa-Credo (Lyon, France). Rats were injected intraperitoneally daily for three days with phenobarbital (80 mg kg^{-1}) in isotonic sodium chloride solution. The rats were then starved overnight and killed by decapitation. Human liver was obtained from a renal transplantation donor; liver was removed within 30 min after circulatory arrest, frozen in dry-ice and stored at −80°C until use. No clinical information was available. The microsomes were prepared as described previously [8] and stored at −80°C until use.

Purification procedure

Microsomes were solubilized in 10 m*M* sodium phosphate buffer (pH 7.4) containing 1 m*M* EDTA, 500 m*M* sodium chloride, 20% (v/v) glycerol and 0.5% (w/v) sodium cholate as described previously [9] (buffer A). The amount of buffer was adjusted in order to add 1 mg cholate per milligram of protein. The solubilized microsomes were applied to an Octyl Sepharose CL 4B column (1 g of protein per 100 ml of Octyl Sepharose in a 50 cm×2.6 cm I.D. column). After loading, the column was washed with one column volume of buffer A. The different proteins were then successively eluted with the following buffers: buffer B = 10 m*M* sodium phosphate (pH 7.4)–20% (v/v) glycerol–0.5% (w/v) sodium cholate–0.2% Lubrol PX; buffer C = buffer B with 0.5% Lubrol PX; buffer D = buffer B with 1% Lubrol PX. Absorbances at 417 and 280 nm and epoxide hydrolase activity were monitored in order to identify the eluted proteins. Lubrol PX was used instead of Emulgen 913 as a detergent because it does not absorb at 280 nm.

The successive use of buffers A, B, C and D allowed four peaks, I, II, III and IV, respectively, to be eluted. The fractions corresponding to each peak were pooled, concentrated by ultrafiltration (Amicon PM 30) and for peaks II–IV lubrol was eliminated by a Biobeads SM_2 treatment as described [9]. Finally, they were dialysed overnight at 4°C against 10 m*M* Tris–acetate buffer (pH 7.4) containing 20% (v/v) glycerol. They were then used without further treatment for the measurement of protein, cytochrome b5 cytochrome P-450 and epoxide hydrolase activity.

Assays

Proteins were measured according to Lowry et al. [10] using bovine serum albumin as a standard. Cytochrome b5 and cytochrome P-450 were measured as described [11,12]. Epoxide hydrolase, using either benzopyrene-4,5-oxide or styrene-7,8-oxide as substrates, was measured according to Jerina et al. [13] and Guengerich [14], respectively.

Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [15], except that the concentration of acrylamide in the separating gel was increased to 9%, which gave a better separation in the 40 000–50 000 relative molecular weight region. A gradient system was not used.

"Western blots"

The method was adapted from Guengerich et al. [16]; briefly, after gel electrophoresis, proteins were electrotransferred to nitrocellulose, which was then saturated with 3% (w/v) serum albumin and 10% (v/v) foetal calf serum. Nitrocellulose was then incubated successively with antiserum anti-epoxide hydrolase and peroxidase-conjugated anti-rabbit immunoglobulins; peroxidase was finally stained with 4-chloro-1-naphtol as described [17].

Antibodies

Human epoxide hydrolase was purified as described by Guengerich and coworkers [1,2] and antibodies were obtained in female New Zealand rabbits as described [16]; these epoxide hydrolase and antibodies were used as references. After bleeding, blood was treated according to Guengerich et al. [16]. The antiserum was stored in aliquots at −20°C.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile of solubilized human liver microsomes loaded on an Octyl Sepharose CL 4B column. Four protein peaks were separated by the four buffers. Peak I corresponded to the non-adsorbed proteins; it contained mainly NADPH cytochrome c reductase, which could be further purified by affinity chromatography; the measurement of this enzyme has been described in another paper and could also contain haemoglobin, which is a frequent contam-

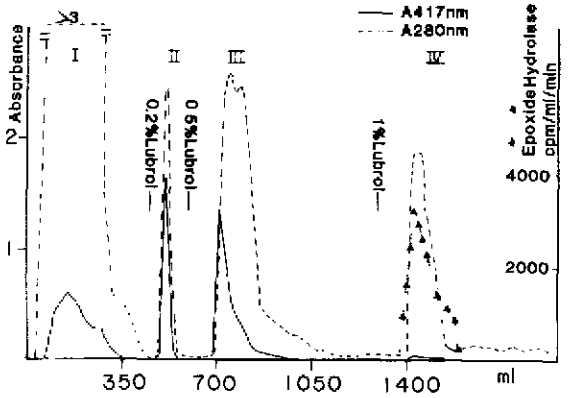

Fig. 1 Elution profile of human liver microsomes on an Octyl Sepharose CL 4B column. This experiment corresponds to that in Table I.

TABLE I

SEPARATION OF CYTOCHROME P-450, CYTOCHROME b5 AND EPOXIDE HYDROLASE FROM HUMAN LIVER MICROSOMES ON OCTYL SEPHAROSE CL 4B

The peaks correspond to those shown in Fig. 1.

Parameter	Microsomes	Peak I	Peak II	Peak III	Peak IV
Total protein (mg)	540	92	4.4	80	60
Cytochrome P-450					
Total nmol	270	0	0	150	0
Specific activity (nmol mg^{-1} protein)	0.5	0	0	1.9	0
Yield (%)	100	0	0	55	0
Cytochrome b5					
Total nmol	261	0	58	28	0
Specific activity (nmol mg^{-1} protein)	0.5	0	12 9	0.35	0
Yield (%)	100	0	22	10	0
Epoxide hydrolase (benzopyrene-4,5-oxide)					
Total activity (nmol min^{-1})	2054	221	15	351	1370
Specific activity (nmol min^{-1} mg^{-1} protein)	3.8	2.4	3.5	4.4	27 2
Yield (%)	100	10 8	0.7	17	67

inant of human liver microsomes [9]. Peak II represented mainly cytochrome b5 and a small amount of epoxide hydrolase; peak III contained the bulk of cytochrome P-450 with some epoxide hydrolase; finally, peak IV consisted of epoxide hydrolase, which was identified by its activity. Epoxide hydrolase activity and absorbance at 417 nm were not perfectly coincidental with the absorbance at 280 nm, this is due to the slight time lag between the detector and the collector. The results of the separation of human liver microsomal proteins on Octyl Sepharose CL 4B are summarized in Table I. Epoxide hydrolase was found in all peaks; however, peak IV represented the major fraction containing 67% of the total activity loaded on to the column. In this fraction the specific activity was increased seven-fold whereas in peaks I–III the specific activity of benzopyrene 4,5-oxide hydrolase remained unchanged.

For NADPH cytochrome c reductase and cytochromes b5 and P-450, the results were in agreement with those published previously [9,18]. In those studies, epoxide hydrolase activity was not measured and also the highest amount of detergent (1%) was not used. The yield of epoxide hydrolase might be further improved by using different detergent concentration steps, but the protocol described here has been chosen because it is also suitable for the purification of other drug-metabolizing enzymes, namely NADPH cytochrome c reductase, cytochrome b5 and cytochrome P-450.

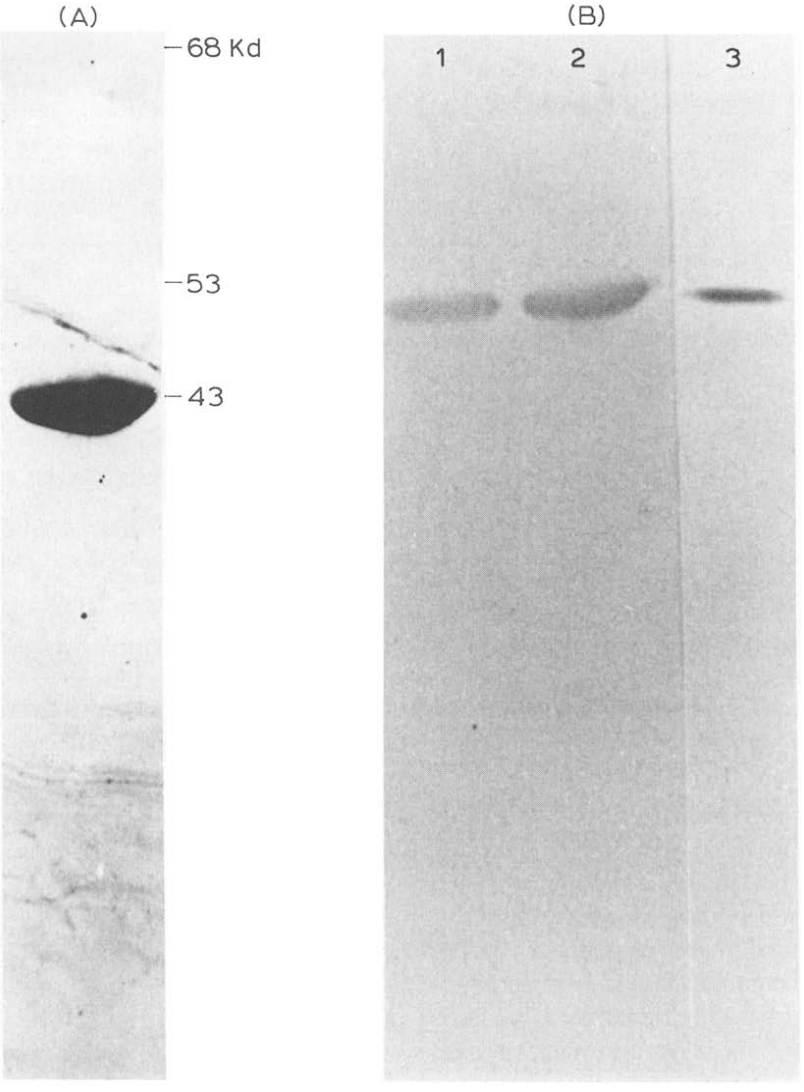

Fig. 2. (A) Sodium dodecyl sulphate polyacrylamide gel electrophoresis; 2 μg of peak IV corresponding to epoxide hydrolase was loaded. (B) "Western blots" of epoxide hydrolase. Peak IV is that described in Fig. 1 and Table I. Control epoxide hydrolase was purified as described [1,19]. Anti-epoxide hydrolase antiserum was used diluted 1:100. 1 = Epoxide hydrolase (control) (0.20 μg); 2 = human liver microsomes (9.0 μg); 3 = peak IV (0.22 μg).

In this study, we were interested in epoxide hydrolase, which was quickly resolved with high specific activity and yield. Therefore, it was interesting to monitor the purity of this enzyme further. Peak IV proteins were resolved by SDS/PAGE as a single band, suggesting that epoxide hydrolase might be pure at homogeneity (Fig. 2). No other protein band can be stained with silver nitrate even with the amount of protein loaded on to the gel. This result was obtained

TABLE II

PURIFICATION OF CYTOCHROME P-450, CYTOCHROME b5 AND EPOXIDE HYDROLASE IN LIVER MICROSOMES FROM PHENOBARBITAL-TREATED RATS ON OCTYL SEPHAROSE CL 4B

The peaks correspond to those shown in Fig. 1.

Parameter	Microsomes	Peak I	Peak II	Peak III	Peak IV
Total protein (mg)	560	155	28	125	60
Cytochrome P 450					
Total nmol	1120	0	0	360	0
Specific activity (nmol mg^{-1} protein)	2.0	0	0	2.9	0
Yield (%)	100	0	0	32	0
Cytochrome b5					
Total nmol	80	14	28.8	13.6	0
Specific activity (nmol mg^{-1} protein)	0.14	0.09	1.03	0.11	0
Yield (%)	100	4	36	17	0
Epoxide hydrolase (benzopyrene 4,5-oxide)					
Total activity (nmol min^{-1})	6166	226	37	776	3340
Specific activity (nmol min^{-1} mg^{-1} protein)	11.1	1.5	1.3	6.1	55.9
Yield (%)	100	3.7	0.6	13.4	56

three times with different liver samples, leading to preparations having the same degree of purity. However, we cannot rule out the possibility that another protein is co-purified during the detergent elution and co-migrates on to the SDS-PAGE together with epoxide hydrolase; however, the intensity of staining is similar to that for the reference enzyme (Fig. 2B).

The relative molecular mass was calculated to be 43 000, which is consistent with but slightly lower than those described previously [19]. The ratio of the specific activities obtained with benzopyrene-4,5-oxide and styrene-7,8-oxide (data not shown) indicated that the purified enzyme was similar to those previously purified, particularly by Wang et al. [19]. In order to confirm this point we compared by "Western blots" the immunochemical properties of the enzyme we had purified with an enzyme purified by the Guengerich's group [1,19]; Fig. 2B shows the immunochemical identity of the two preparations. Epoxide hydrolase (peak IV) has the same apparent molecular mass as the reference enzyme and is clearly recognized by anti-human epoxide hydrolase obtained against the reference enzyme. Moreover, the enzyme found in peak III appeared to be similar to epoxide hydrolase of peak IV (data not shown); however, epoxide hydrolase was not detected in peaks I and II.

This chromatographic procedure was tentatively used to purify rat liver epox-

ide hydrolase. We used rats treated with phenobarbital because they have an enzyme similar to that of untreated animals, but the specific content is higher. The results are shown in Table II and are very similar to those obtained in humans (yield and purification index), but gel electrophoresis indicated that our preparation was not totally homogeneous. The discrepancy between the specific activity and the purity of epoxide hydrolase in rats and humans might be due to partial elimination of detergent by Biobeads; it is possible that the presence of detergent inhibited the enzymatic activity. Therefore, a more efficient system for elimination of the detergent, e.g. using calcium phosphate gel, should be tested. Nevertheless, with a supplementary step such as the use of DEAE-cellulose, it will be easy to purify completely rat liver epoxide hydrolase and this kind of chromatography remains a powerful method for purifying drug-metabolizing enzymes from rat liver microsomes [20].

CONCLUSION

A simple procedure for purifying in good yield human liver microsomal epoxide hydrolase by means of affinity chromatography on Octyl Sepharose CL 4B has been developed. This kind of chromatography was used as the first step to purify NADPH cytochrome c reductase, cytochrome b5 and cytochrome P-450 from human liver microsomes. This method is a powerful means for the purification of at least four enzymes involved in drug metabolism.

ACKNOWLEDGEMENTS

The authors thank Dr. Frantz and Paris-Transplant for providing human liver samples and Miss Taieb for typing the manuscript.

REFERENCES

1 F.P. Guengerich, P. Wang, M.B. Mitchell and P.S. Mason, J Biol. Chem., 254 (1979) 12248.
2 F.P. Guengerich, P. Wang, P.S Mason and M.B. Mitchell, J. Biol. Chem., 254 (1979) 12255.
3 A.Y.H. Lu, P.E. Thomas, D.E. Ryan, D.M. Jerina and W. Levin, J Biol. Chem., 254 (1979) 5878
4 F. Oesch, C.W. Timms, C.H. Walker, T.M. Guenthner, A. Sparrow, T. Watabe and C.R. Wolf, Carcinogenesis, 5 (1984) 7
5 D.M. Jerina, Drug Metab. Dispos., 11 (1983) 1.
6 E. Bresnick, H Mukhtar, T.A. Stoming, P. Dansette and D.M. Jerina, Biochem. Pharmacol., 26 (1977) 891.
7 M.E. Kennedy and B.B. Burchell, Biochem. Pharmacol., 32 (1983) 2029.
8 P. Kremers, P.H. Beaune, T. Cresteil, J. de Graeve, S. Columelli, J.P. Leroux and J.E. Gielen, Eur. J. Biochem., 118 (1981) 599.
9 P.H. Beaune, P M. Dansette, J.P. Flinois, S. Columelli, D. Mansuy and J.P. Leroux, Biochem. Biophys. Res. Commun., 88 (1979) 826.
10 O.H. Lowry, N J. Rosebrough, A.L. Farr and S. Randall, J. Biol. Chem., 193 (1951) 265.
11 R.W Estabrook and J. Werringloer, Methods Enzymol., 52 (1978) 212.
12 R.A. Omura and R. Sato, J. Biol. Chem., 239 (1964) 2370.
13 D M Jerina, P.M. Dansette, A.Y H. Lu and W. Levin, Mol. Pharmacol., 13 (1977) 342.

14 F.P. Guengerich, in A.W. Hayes (Editor), Principles and Methods in Toxicology, Raven Press, New York, 1982, p. 609.
15 U.K. Laemmli, Nature (London), 227 (1970) 680.
16 F.P. Guengerich, P. Wang and N.K. Davidson, Biochemistry, 21 (1982) 1698.
17 A. Matus, R. Bernharot and P. Hugh-Jones, Proc. Natl. Acad. Sci. U.S.A., 78 (1982) 3010.
18 P.H. Beaune, J.P. Flinois, L. Kiffel, P. Kremers and J.P. Leroux, Biochim. Biophys. Acta, 840 (1985) 364.
19 P. Wang, J. Meijer and F.P. Guengerich, Biochemistry, 21 (1982) 5769.
20 E. Le Provost, J.P. Flinois, P.H. Beaune and J.P. Leroux, Biochem. Biophys. Res. Commun., 101 (1982) 545.