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SINGLE-STEP PURIFICATION OF EPOXIDE HYDROLASE FROM RAT LIVER MICROSOMES USING MONOCLONAL-ANTIBODY CHROMATOGRAPHY

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Abstract—Monoclonal antibody to rat liver microsomal epoxide hydrolase has been obtained after immunization of mice with the enzyme prepared by conventional methods. Antibody from ascitic fluid was purified and coupled to CNBr-activated Sepharose 4B, to give a specific affinity column. Monoclonal-antibody affinity chromatography provided a rapid single-step method of purifying to homogeneity active epoxide hydrolase from crude solubilized microsomes. The techniques used offer an effective method for characterization of a non-inhibitory monoclonal antibody.

Isolation of hepatic membrane-bound drug-metabolizing enzymes is essential for examination of their function and interaction with other proteins in the membrane. A number of hepatic microsomal enzymes such as cytochrome P-450 and UDP glucuronyl transferase exist as multiple forms within the membrane, exhibiting individual substrate specificities [1, 2]. Purification of an individual form from a family of microsomal enzymes is extremely time-consuming and difficult because of the small quantities of protein involved and the similarity of the individual forms [2]. Immunoaffinity chromatography promises a method for rapid and specific isolation of individual forms of enzyme protein from crude extracts [3]. However, the high affinity of antibody for antigen requires rather drastic conditions to dissociate the immune complex and generally results in purification of an inactive enzyme [4]. The advent of hybridoma technology [3] has considerably improved the possibility for isolation of an extremely specific monoclonal antibody with selected properties appropriate to its proposed usage as an immunochemical. Thus an antibody with a lower than normal affinity for antigen can be selected for affinity chromatography, so that relatively mild conditions can be used for elution of the antigen from the immunoadsorbent.

We have assessed the practical approach to this problem by examining the possibility of purifying epoxide hydrolase (EC 3.3.2.3) by immunoaffinity chromatography. Epoxide hydrolase has been purified to homogeneity [5, 6] and a simple high-yield purification procedure has been adopted for routine work [7]. This report demonstrates the simplicity with which a membrane-bound enzyme may be isolated, following the selection of an appropriate, low-affinity monoclonal antibody.

MATERIALS AND METHODS

Isolation of a monoclonal antibody to epoxide hydrolase. Hepatic microsomal epoxide hydrolase was purified from male Wistar rats as previously described [7]. BALB/c mice (8 weeks old) received two subcutaneous injections, 4 weeks apart, of 30 μg epoxide hydratase in complete Freund's adjuvant.

A single intravenous injection of epoxide hydrolase was given 3 days before the spleens were removed for fusion with myeloma cell line X63-Ag8-653 by the method of Kennett *et al.* [8]. Hybrid cells were grown in RPMI 1640 medium (Flow Laboratories, Irvine, U.K.) containing HAT and 10% foetal calf serum. Positive cultures were detected by radioimmunassay.

Positive hybridomas were cloned several times by limiting dilution [9] and positive clones grown in 10% foetal calf serum in RPMI 1640 for several months without loss of antibody production. Cloned hybridoma cells ($1–2 \times 10^6$) were injected (i.p.) into pristane-primed BALB/c mice to obtain ascites fluid which contained high concentrations of monoclonal antibody.

A solid-phase radioimmunassay using 96-well flat-bottomed polyvinyl chloride microtitre plates (Dynatech Laboratories, Billinghurst, U.K.) was used to assay for monoclonal antibody. Wells were coated with crude preparations of epoxide hydrolase (25–60% ammonium sulphate fraction of Lubrol-solubilized microsomes) at 4° overnight. The remaining non-specific sites were covered with bovine serum albumin (BSA) by incubating the washed wells with 0.5% BSA in phosphate-buffered saline containing 10 mM sodium azide (PBS) and incubating at room temperature for 90 min. Then 100 μl aliquots of fluid from hybridoma cultures were added to the coated wells for a further 90 min at room temperature. The wells were washed 3 times with 0.5% BSA in PBS and antibodies bound to the wells were detected by incubation with ^{125}I-labelled sheep antimouse IgG (50,000 cpm) for 90 min at room temperature. The wells were washed 3 times with 0.5% BSA in PBS, dried and punched out of the plate. The individual wells were placed in plastic tubes and the radioactivity was measured in a gamma-counter.

Preparation of the monoclonal antibody–Sepharose column. The monoclonal antibody was purified

from ascites fluid by precipitation in the presence of 50% saturation ammonium sulphate followed by ion-exchange chromatography on DEAE-cellulose [DE52 (Whatman Ltd, Maidstone, U.K.)] equilibrated with 5 mM sodium phosphate, pH 7. The antibody was eluted from DEAE-cellulose with a linear gradient of 0–0.2 M NaCl in 5 mM sodium phosphate, pH 7. The concentrated purified antibody was bound to CNBr-activated Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) by the routine methods previously described [10].

Immunoaffinity purification of epoxide hydrolase. The affinity chromatography material was equilibrated with 25 mM potassium phosphate, 5 mM mercaptoethanol, 0.05% (w/v) Lubrol, pH 7.4 (buffer A). A Lubrol-soluble fraction of liver microsomes was freshly prepared [7] and gently mixed by inversion with antibody–Sepharose at 4° for 1 hr. The mixture was then packed in a glass column and washed with 10 volumes of buffer A followed by 10 volumes of 0.15 M NaCl in buffer A. Finally, epoxide hydrolase was eluted by 50 mM diethylamine in buffer A, pH 11.2 [11], and fractions (1.5 ml) were immediately neutralized by collection into tubes containing 0.5 ml of 2 M Tris–HCl, pH 7.

Analysis of epoxide hydrolase preparations. Epoxide hydrolase activity was measured by the method described by Oesch [12] using [7-^{3}H]styrene oxide (Radiochemical Centre, Amersham, U.K.) as substrate. One unit of enzyme activity is defined as the formation of 1 nmole styrene glycol/min. Protein concentrations were determined by the method of Bradford [13] using bovine serum albumin as standard.

SDS–polyacrylamide electrophoresis and staining of polypeptides was performed as described in Ref. 7.

RESULTS

Preparation and properties of a monoclonal antibody against epoxide hydrolase

Epoxide hydrolase purified as in Ref. 7 was used to immunize mice for the production of monoclonal antibodies. An indirect assay was developed to screen the antibody-producing hybridomas (see Materials and Methods). A hybridoma-secreting antibody to epoxide hydrolase was cloned by limiting dilution and is called EH.101. Monoclonal antibody exhibiting a high titre (10^{-5}–10^{-6}, see Fig. 1) against epoxide hydrolase was present in ascites fluid produced by injection of hybridoma EH.101 into pristane-primed mice.

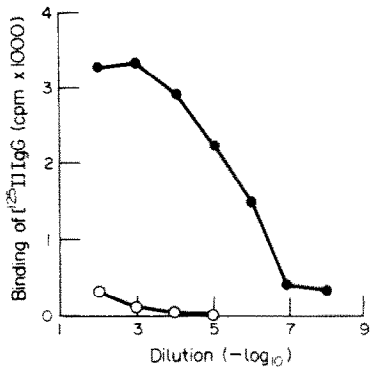

Fig. 1. Titration of EH.101 by solid-phase radioimmunoassay. Microtitre wells were coated with crude antigen preparation (0.1 mg/well). Ascites fluid produced by hybridoma EH.101 (●) and a nonspecific hybridoma (○) were diluted in PBS. Bound antibody was detected using ^{125}I-labelled sheep- anti-mouse IgG. (For details see Materials and Methods.)

Incubation of native rat liver microsomes or the Lubrol-soluble fraction [7] with ascites fluid did not result in any loss of epoxide hydrolase activity. Further, epoxide hydrolase activity was not precipitated from Lubrol-solubilized microsomes by incubation with ascites fluid alone or in conjunction with *Staphylococcus aureus* protein A containing 'ghosts' or sheep anti-mouse IgG.

Thus, an alternative method for characterization of this monoclonal antibody is required.

Purification of epoxide hydrolase by immunoaffinity chromatography

Antibody raised against purified epoxide hydrolase in rabbits [7, 14] was not a good ligand for use in immunoaffinity chromatography. When purified immunoglobulin G obtained from rabbit antiserum was attached to CNBr-activated Sepharose 4B, the immunoadsorbent specifically bound epoxide hydroase from a crude enzyme preparation [7] but active enzyme could not be eluted from this column material.

The monoclonal antibody from clone EH.101 was purified from ascites fluid as described in Materials and Methods, and covalently bound to CNBr-activated Sepharose 4B. The immunoadsorbent (1 ml packed Sepharose) was incubated with 0.5 ml of Lubrol-soluble supernatant (approximately 6.5 mg of protein) for 60 min at 4°. The material was then transferred to a small glass column and washed with buffer A, followed by 0.15 M NaCl in buffer A. Approximately 80% of the epoxide hydrolase activity remained bound to the column material (Table 1). Assay of aliquots of the column material at this stage revealed that epoxide hydrolase was immobilized in an active form. This active enzyme was eluted from the immunoadsorbent using 50 mM diethylamine in buffer A, pH 11.2, and the eluate immediately neutralized by collection into 2 M Tris–HCl, pH 7. Epoxide hydrolase eluted from the column exhibited activity towards styrene oxide as substrate. The final specific activity of the enzyme was about half that of normal values obtained by conventional purification methods and thus the apparent 20-fold purification compared to the Lubrol-soluble supernatant was lower than expected (Table 1) (see Discussion). Up to 100 μg of enzyme protein was released from the immunoadsorbant by this elution procedure with an apparent recovery of 20–37% of the bound activity. When Lubrol-soluble supernatant from rats pretreated with phenobarbitol was used as the source of enzyme, approximately 300 μg of enzyme protein was obtained by immunoaffinity chromatography.

Microsomal protein fractions applied to, and eluted from the antibody–Sepharose column were compared to homogeneously purified epoxide hydrolase

Table 1. Immunoaffinity purification of epoxide hydrolase from rat liver microsomes

Purification step	Protein (mg)	Specific activity (units/mg protein)	Total activity (units)	Yield (%)
Lubrol-soluble fraction applied	6.65	8.6	57.6	100
Antibody–Sepharose				
Activity bound	—	—	45.85 (32.8–54.6)	79.6
Activity specifically eluted	0.089 (0.071–0.108)	170 (129–196)	15.1 (11.3–21.3)	26.2

Data reported were obtained from three separate purification experiments using Lubrol-solubilized microsomes from an untreated male Wistar rat liver. The range of results between experiments is given in parentheses.

(prepared as in Ref. 7), by SDS–polyacrylamide gel electrophoresis. The results (Fig. 2) indicate that: (a) a polypeptide exhibiting the same electrophoretic mobility as epoxide hydrolase was removed from the Lubrol-soluble fraction of hepatic microsomes by the immunoadsorbent, and (b) a single polypeptide exhibiting the same molecular weight as purified epoxide hydrolase was eluted from the column by 50 mM diethylamine, pH 11.2. Examination by SDS-gel electrophoresis of the immunoadsorbent after elution with diethylamine buffer revealed that no epoxide hydrolase remained bound to the column. These results indicate that although epoxide hydrolase has been purified to apparent homogeneity, not all of the eluted enzyme is present in the active form (see Discussion).

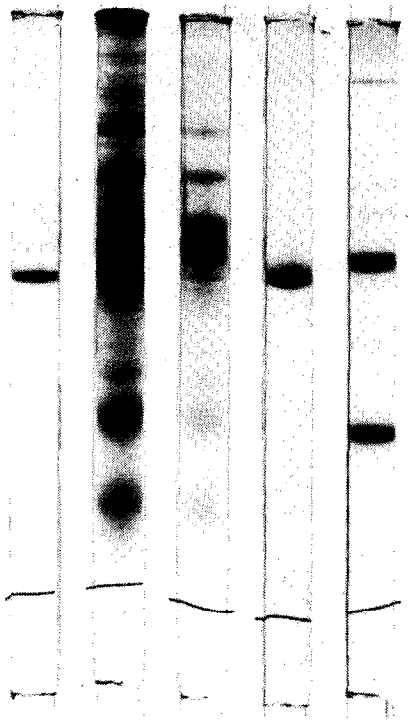

Fig. 2. SDS–polyacrylamide gel electrophoresis of immunoaffinity chromatography samples. (a) Homogeneously purified epoxide hydrolase (prepared as in Ref. 7). (b) Lubrol-soluble supernatant of rat liver microsomes. (c) Lubrol-soluble supernatant after passage through EH.101 antibody–Sepharose. (d) Epoxide hydrolase eluted from immunoaffinity column with 50 mM diethylamine, pH 11.2 (e) Sample of immunoadsorbent after elution of bound protein.

DISCUSSION

A monoclonal antibody has been prepared against rat liver microsomal epoxide hydrolase. This antibody was used to obtain homogeneously purified epoxide hydrolase by immunoaffinity chromatography of a crude extract within 6 hr of killing the rat.

The final specific activity of the apparently homogeneously purified enzyme was lower than that of the conventionally purified enzyme, presumably due to the fairly harsh conditions at high pH. The diethylamine elution buffer does not affect the epoxide hydrolase activity in the Lubrol-soluble fraction of microsomes, however the presence of numerous other proteins and lipids might afford some protection for the enzyme in the crude extract.

Epoxide hydrolase could also be eluted from the immunoabsorbent using 2 M $MgCl_2$ in buffer A, pH 4, but the high ionic strength of this elution buffer causes a greater inactivation of the enzyme.

Monoclonal antibody prepared against cytochrome P-448 bound to Sepharose has been previously used for selective adsorption of cytochrome P-448 [15]. The specificity of the monoclonal antibody was assessed by reconstitution of the immobilised cytochrome with NADPH–cytochrome P-450 reductase and dilauryl-α-phosphatidylcholine to produce an active monooxygenase system capable of oxidising benzo[*a*]pyrene [15]. This result and spectral data together with electrophoretic analysis indicated that monoclonal antibody had been obtained against cytochrome P-448. However, it was not possible to dissociate cytochrome P-448 from the immobilised antibody in a form which was active in a reconstituted monooxygenase system [16].

The immunoaffinity chromatography study reported here provides positive proof that our non-inhibitory, non-precipitating monoclonal antibody reacts specifically against hepatic epoxide hydrolase. Monoclonal antibody–Sepharose binds the enzyme in an active form and can therefore be used to detect epoxide hydrolase in various small quantities of tissues. Further, the proposed heterogeneity and polymorphism of hepatic microsomal epoxide hydrolase [17–19] can be investigated using this monoclonal antibody. Similar approaches are being used to phenotype cytochrome P-450 in human tissues [20].

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