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Rapid and sensitive enzyme-linked immunosorbent assay for the microsomal epoxide hydrolase

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Abstract

A rapid and sensitive indirect enzyme-linked immunosorbent assay (ELISA) was developed for microsomal epoxide hydrolase of rat liver. The assay, which is easily and readily performed, is significantly more sensitive than most enzymatic epoxide hydrolase assays routinely used and electroimmunoassays previously developed. The limit of sensitivity of the ELISA is between 2–5 ng of microsomal epoxide hydrolase. Using the ELISA microsomal epoxide hydrolases of mouse and rat liver were shown to be antigenically very similar, while microsomal epoxide hydrolases of guinea pig, monkey and human liver are antigenically distinct from those of rat and mouse. The ELISA developed here is capable of detecting microsomal epoxide hydrolase of rat and mouse liver even when significant enzymatic activity is lost. These results indicate that the antigenic sites recognized by the antibodies used are distinct from the catalytic site of the epoxide hydrolase. Approximately 1.9% of rat microsomal protein was quantified as microsomal epoxide hydrolase by the ELISA. Low levels of microsomal epoxide hydrolase were also detected in rat liver cytosol (~0.02% of the cytosolic protein) demonstrating that microsomal epoxide hydrolase is not totally membrane bound or that an immunologically related protein occurs in the cytosol of normal rat liver. The ELISA developed here will be valuable in investigating further the role of microsomal epoxide hydrolase.

Introduction

Epoxide hydrolases (EC 3.3.2.3) catalyze the enzymatic hydration of a variety of epoxidized compounds, both endogenous and exogenous, to 1,2-diols (1). In mammalian liver epoxide hydrolases are either membrane bound, as in the endoplasmic reticulum, and in nuclear and plasma membranes, or are present in the soluble form in cell cytosol, and the mitochondrial matrix or intermembrane space (2–7).

The epoxide hydrolases may also be of significance as markers for hepatocarcinogenesis. Farber and co-workers (8–11) demonstrated that an antigen exists in the cytosol of preneoplastic and neoplastic liver tissue, which is not observed in normal tissue. Subsequent work has demonstrated that this preneoplastic antigen is identical with microsomal epoxide hydrolase (12–14). In normal rat liver this epoxide hydrolase is largely membrane bound. However, in preneoplastic and neoplastic tissue, some of the microsomal epoxide hydrolase may be released and observed in the cytosolic fraction of rat liver (12,14). This release may reflect a looser association of the enzyme with preneoplastic microsomes as compared to control microsomes (14,15).

Epoxide hydrolases are routinely monitored by enzymatic activity assays (2,16, and references cited therein). These assays, while they are extremely useful, need an enzymatically active protein, relatively high rates of substrate hydration and/or the availability of radiolabeled substrates with high specific activity. Enzymatic assays are also limited in that the levels detected will vary depending on the specificity of the substrate utilized for the assay.

While the problems with enzymatic assays indicated above can be overcome by a variety of procedures, such assays do not measure the actual levels of the epoxide hydrolase but rather its catalytic activity. In contrast, assays based on the immunoreactivity of epoxide hydrolase provide a direct indication of the actual levels of the protein. Electroimmunoassays have been developed for the detection of microsomal epoxide hydrolase, both in microsomes and in cytosol of rat liver (14,17). Because electroimmunoassays are not particularly sensitive and can be laborious, attempts were made to develop other immunoassays with much greater sensitivity and ease of use. This report presents the successful development of a sensitive enzyme-linked immunosorbent assay (ELISA)* for microsomal epoxide hydrolase or a preneoplastic antigen.

Materials and Methods

Reagents

Alkaline phosphatase-conjugated goat anti-rabbit IgG and alkaline phosphatase-conjugated rabbit anti-goat IgG were obtained from Miles Laboratory (Elkhart, IN). Lubrol PX and bovine serum albumin, fraction V were obtained from Sigma Chemical Co. (St. Louis, MO).

Enzyme preparation

Rats (Sprague-Dawley, 180–200 g, male) and mice (Swiss-Webster, 25–30 g, male) were obtained from Simonsen Laboratories (Gilroy, CA), while guinea pigs (Hartley, 200–250 g, male) were obtained from a local breeder. The animals were killed either by cervical dislocation or by decapitation; livers were removed, rinsed and perfused with 0.25 M sucrose, and homogenized in 0.25 M sucrose with a Potter-Elvehjem homogenizer to give a 20% (w/v) homogenate. The homogenate was centrifuged for 10 min at 10 000 *g* and then at 100 000 *g* for 60 min to give the microsomal pellet, which was resuspended in sodium phosphate, pH 7.4, 0.08 M buffer and recentrifuged at 100 000 *g* for 60 min to obtain washed microsomes. Microsomes from fresh monkey and human hepatic tissues, obtained from surgical biopsies, were similarly prepared. Dilutions were made in appropriate buffers prior to use. Protein levels were determined according to Bradford (18) with minor modifications using bovine serum albumin (fraction V) as standard.

Purification of antigen and preparation of antisera

Rat microsomal epoxide hydrolase was purified from adult male Sprague-Dawley rats (200–300 g) induced with 400 mg/kg *trans*-stilbene oxide administered 96, 72 and 48 h before sacrifice following published methods (19). The purified epoxide hydrolase was apparently homogeneous as determined

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*Abbreviations: ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline.

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by SDS-polyacrylamide gel electrophoresis and Ouchterlony double diffusion analysis. To raise antibodies to the rat microsomal epoxide hydrolase, 100 μg of the purified microsomal epoxide hydrolase suspended in complete Freund's adjuvant (1 ml) was injected subcutaneously into New Zealand white rabbits. Alternatively, 400 μg of the purified microsomal epoxide hydrolase suspended in complete Freund's adjuvant (2.5 ml) was injected subcutaneously into goats. Booster injections given 5–6 weeks later, using the same amounts of microsomal epoxide hydrolase were also administered in complete Freund's adjuvant. Two weeks after the booster injections, the goats and rabbits were bled and the serum separated. For storage and transport, the serum was lyophilized. Appropriate amounts of serum and antigen were dissolved in 0.1 M phosphate buffer, pH 7.4, and diluted further to working solutions in appropriate buffers before use.

ELISA

The ELISA was run according to Voller, *et al.* (20) with minor modifications. All reactions were performed in polystyrene cuvettes (Gilford Instruments, Oberlin, OH). Purified microsomal epoxide hydrolase used as a coating antigen was diluted in 0.1 M sodium carbonate buffer pH 9.6 containing 0.02% NaN_3 and added to each well (0.3 ng/250 μl). The plates were kept overnight at 4°C and then washed three times with phosphate buffered saline, pH 7.7 containing 0.05% Tween 20 and 0.02% NaN_3 (PBS-Tween). Either rabbit or goat anti-rat microsomal epoxide hydrolase serum (250 μl) diluted in PBS-Tween was added to the cuvettes which after an incubation of 2 h were washed three times with PBS-Tween. Subsequently, either goat anti-rabbit IgG-alkaline phosphatase or rabbit anti-goat IgG-alkaline phosphatase (250 μl, 1/2500 dilution in PBS-Tween) was added, and incubated for a further 2 h. After three washes, p-nitrophenyl phosphate (250 μl, 1 mg/ml) in 10% diethanolamine buffer, pH 9.8 was utilized to monitor enzyme activity. The reaction was stopped after 20–30 min with 50 μl of 3 N NaOH and the absorbance measured in a Gilford EIA reader at 405 nm. For inhibition assays, one ml of either rabbit or goat anti-rat microsomal epoxide hydrolase serum (1/20 000) was added to each of several tubes containing increasing amounts of purified rat microsomal epoxide hydrolase or microsomes from rat, mouse, guinea pig, human, or monkey in PBS-Tween containing 0.2% Lubrol PX. After an overnight incubation, these solutions (250 μl) were added to antigen coated cuvettes followed by goat anti-rabbit IgG-alkaline phosphatase or rabbit anti-goat IgG-alkaline phosphatase. The assays were then run as described above. Maximum and background absorbance were determined by adding control solutions without antigen or without antibody, respectively.

Enzymatic assays

Microsomal epoxide hydrolase activity was monitored with [^{3}H]*cis*-stilbene oxide (60 mCi/mmol) briefly as follows (16). To a microsomal suspension (100 μl) in 0.1 M Tris-HCl buffer, pH 9.0 preincubated for 1 min at 37°C, *cis*-stilbene oxide (5 x 10^{-5} M or 2.4 x 10^{-6} M when carrier free radiolabel, 15 Ci/mmol, was used) was added in ethanol (1 μl). After incubation for 10 min at 37°C the incubation mixture was extracted with n-dodecane (200 μl). Following centrifugation the aqueous phase (50 μl) was removed and quantified by liquid scintillation counting.

Results

Varying concentrations of purified microsomal epoxide hydrolase absorbed to polystyrene cuvettes were used to determine the optimum dilution of both goat and rabbit antiserum. A 1/20 000 dilution of both goat and rabbit anti-sera was found to give workable absorbance values with incubations of 20–30 min. The absorbance at 405 nm was proportional to the concentration of the epoxide hydrolase over the range of 10–100 ng/ml for both anti-sera. Rabbit anti-serum (1/1000 dilution) to the endotoxin of *Bacillus thuringiensis* serotype H-3a,3b did not react in the ELISA procedure described. Goat and rabbit anti-sera gave very similar inhibition curves with purified microsomal epoxide hydrolase (Figure 1). The inhibition curves were linear over an epoxide hydrolase concentration of 20–70 ng/ml with rabbit antiserum and from 10–80 ng/ml with goat anti-serum. Detection limits were 5 and 2 ng/ml for rabbit anti-serum and goat anti-serum, respectively.

For inhibition assays using microsomes from rat or mouse it was found that a higher inhibition (~10%) was observed with solubilized microsomes than with unsolubilized microsomes. However, with the addition of 0.2% Lubrol PX in anti-sera dilutions there was no difference in the inhibition curves obtained between solubilized or unsolubilized microsomes. Microsomes from rat and mouse strongly inhibited the binding of rat microsomal epoxide hydrolase. A maximal inhibition of 85–90% (Figure 2) was obtained with rabbit anti-serum. This inhibition level can be increased to 95–98% with higher levels of Lubrol PX (0.5–1.0%). However, slightly higher background levels are observed under these conditions. While the maximal inhibition obtained with rabbit anti-serum was the same with rat and mouse microsomes, rat and mouse microsomes gave 80 and 70% maximal inhibitions, respectively, if goat anti-serum was used (Figure 3). The inhibition curves obtained from either rat or mouse microsomes were similar in slope to those obtained with purified epoxide hydrolase. Microsomes from guinea pig liver inhibited only about 20% of the binding with either goat or rabbit anti-serum (Figures 2 and 3). There was even less inhibition of binding by human and monkey microsomes. The cytosol of rat and mouse liver also inhibited the binding of rabbit anti-rat microsomal epoxide hydrolase. Inhibition (50%) of the binding was observed with 81.2 and 191.2 μg protein/ml for mouse and rat cytosol, respectively, indicating that some microsomal epoxide hydrolase is possibly present in the soluble form in rat and mouse cytosol. Cytosol from

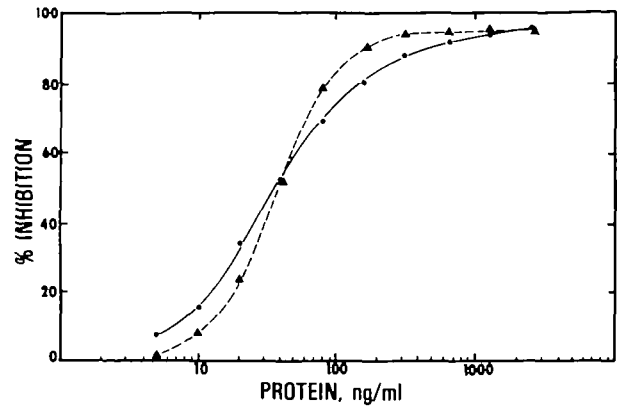

Fig. 1. Inhibition of binding of rabbit and goat anti-rat liver microsomal epoxide hydrolase by purified rat microsomal epoxide hydrolase. Rabbit antiserum (▲- - - -▲) and goat antiserum (●——●).

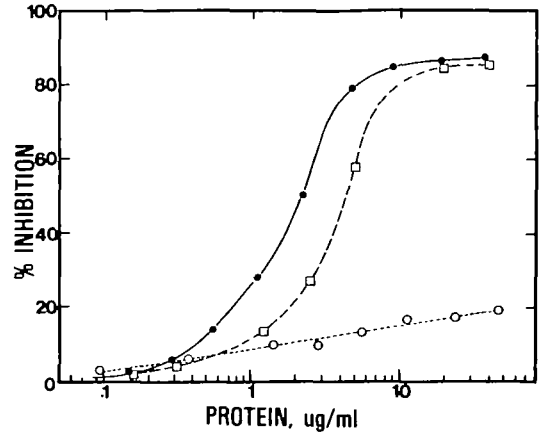

Fig. 2. Inhibition of binding of rabbit anti-rat liver microsomal epoxide hydrolase by rat, mouse and guinea pig microsomes. Rat (●——●), mouse (□- - - -□), and guinea pig (○——○).

human and monkey liver did not show any inhibition of binding up to 150 μg/ml of cytosolic protein. For rat, ~1.9% of the microsomal protein is microsomal epoxide hydrolase, while ~0.02% of the cytosolic protein is the soluble form of microsomal epoxide hydrolase.

The ELISA assay was compared with enzymatic activity assays for detection sensitivity. For routine epoxide hydrolase activity assays 2–5 μg of rat liver microsomal protein was necessary to obtain measurable rates with *cis*-stilbene oxide as substrate (16). With carrier free *cis*-stilbene oxide, 0.2–0.5 μg of protein was sufficient to obtain measurable rates with a standard 10 min assay as described. The ELISA developed here could detect microsomal epoxide hydrolase in 0.1 μg of microsomal protein, although linearity was observed with 0.2–1.2 μg of microsomal protein.

The effect of prolonged storage at 4°C in 0.1 M sodium phosphate buffer, pH 7.4 on enzymatic activity and immunoreactivity of rat and mouse liver microsomes was determined (Table I). While enzymatic activity steadily declined with time for both rat and mouse liver microsomes, their immunoreactivity was maintained, or even somewhat increased as observed with mouse. After an incubation at 4°C for 49 days only 37 and 30% of rat and mouse epoxide hydrolase enzymatic activity, respectively, is recovered, while 105 and 128% of the rat and mouse epoxide hydrolase immunogenic activity was observed after the same period.

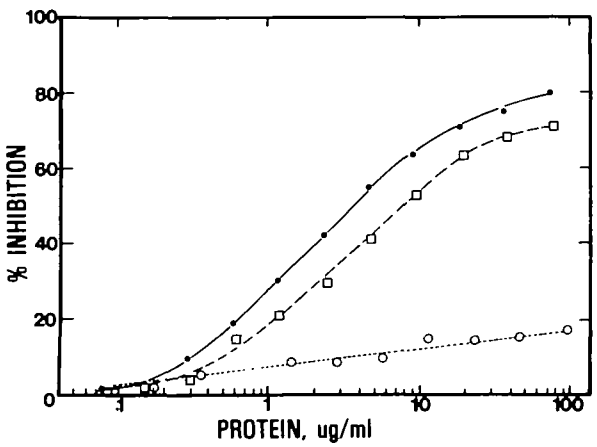

Fig. 3. Inhibition of binding of goat anti-rat liver microsomal epoxide hydrolase by rat, mouse and guinea pig microsomes. Rat (●——●), mouse (□- - - -□), and guinea pig (○——○).

Table I. Effect of storage at 4°C on enzymatic and immunogenic activity of the microsomal epoxide hydrolase of rat liver[a].

Days incubation	Epoxide hydrolase activity (nmol/min/mg protein)		ELISA, I_{50} μg/ml	
	Rat	Mouse	Rat	Mouse
0	4.6	3.7	2.0	4.1
8	3.3 (72)	1.7 (46)	1.7 (118)	3.3 (124)
49	1.7 (37)	1.1 (30)	1.9 (105)	3.2 (128)

[a]Values in parentheses are percent of 0 time activity. Epoxide hydrolase activity is expressed as nmol of *cis*-stilbene oxide hydrolase/min/mg protein. Immunogenic activity is expressed as μg/ml of rat microsomal epoxide hydrolase equivalents.

Discussion

A sensitive and rapid indirect ELISA was developed for the detection and quantification of microsomal epoxide hydrolase, also known as a preneoplastic antigen. The assay is easily performed and the quantity of the antigen is proportional over a wide range to the absorbance at 405 nm resulting from the formation of p-nitrophenol. With rabbit and goat anti-sera, the limit of sensitivity of the indirect assay is between 2–5 ng of the purified microsomal epoxide hydrolase or 0.1–0.3 μg of microsomal protein. The ELISA is significantly more sensitive than most routinely used enzymatic assays (16, and references cited therein). However, enzymatic assays utilizing carrier-free radiolabeled *cis*-stilbene oxide can be equally sensitive as the ELISA.

The ELISA developed here is significantly more sensitive than the electroimmunoassays developed previously (14,17), which had detectability limits of ~250 ng. The sensitivity of the ELISA can further be increased by replacing the colorimetric end-point used with either fluorometric or radiometric determinations (21). Even with the assay procedures described here, sensitivity was increased by 2–4-fold if we used a modified double antibody sandwich ELISA (20), using both rabbit and goat anti-rat microsomal epoxide hydrolase sera. As with most solid phase immunoassays several hundred ELISA's can be performed per man day. In this laboratory a standard deviation of <3% was observed within runs and <5% between runs on different days.

While the purified rat microsomal epoxide hydrolase inhibits most of the binding of rat microsomal epoxide hydrolase to rabbit or goat anti-sera, rat and mouse microsomes do not show the same level of inhibition under the assay conditions used. This lower level of inhibition can be due to the lack of total solubilization of microsomes or the formation of microsomal epoxide hydrolase aggregates, because the inhibition of binding by rat microsomes can be increased with higher levels of Lubrol PX. In the absence of total solubilization of microsomes or the presence of aggregates, it is possible that some antigenic sites recognized by either rabbit or goat antibodies are covered.

The amount of microsomal epoxide hydrolase in rat liver microsomal protein detected by the ELISA is very similar to values reported previously (14,17,22). The rat liver microsomal epoxide hydrolase is also antigenically very similar to the mouse liver microsomal epoxide hydrolase. Although the rat has significantly higher epoxide hydrolase catalytic activity/mg microsomal protein than the mouse (1,16), the level of microsomal epoxide hydrolase in microsomes is not very different in the two species as determined by ELISA using anti-rat microsomal epoxide hydrolase antibodies and when expressed in terms of the rat microsomal epoxide hydrolase. Guinea pig, monkey and human liver microsomes, however, have epoxide hydrolases which are antigenically distinct from those of the mouse and rat. These data provide further evidence that microsomal epoxide hydrolases in the various species are different proteins, even though they behave very similar enzymatically. Similar observations have been made by a number of other investigators (23–25).

The ELISA is capable of detecting microsomal epoxide hydrolase even when significant enzymatic activity is destroyed (Table I). The possibly greater stability of the antigenic site(s) on the enzyme in comparison to the catalytic site(s) may explain the differences in enzymatic and immunogenic activity with time. These data provide further evidence

that most of the antigenic determinants of the microsomal epoxide hydrolase are probably distinct from its catalytic site(s). Levin and co-workers (24) similarly reported that microsomal epoxide hydrolase antibodies do not inhibit the catalytic site. However, it has been previously demonstrated (23) that some of the enzymatic activity can be inhibited with antibodies, although inhibitory antibodies are difficult to obtain.

In many earlier studies no epoxide hydrolase activity was detectable in the cytosol of normal rats when typical substrates for the microsomal epoxide hydrolase were used. However, significant activity was observed in the cytosolic fraction of hyperplastic nodules and hepatomas which may, however, represent dissociation from the microsomal membrane rather than genuine cytosolic location (8 – 15). The present study essentially supports these results, however, small amounts of microsomal epoxide hydrolase are observed in cytosol just as small amounts of an enzyme protein closely related to or identical with cytosolic hydrolase are observed in the microsomes (26,27). The fact that epoxide hydrolase activity for typical substrates of the microsomal epoxide hydrolase was not detectable in the cytosol in previous studies is in line with the increased sensitivity of the present procedure. Slow enzymatic hydration of *cis*-stilbene oxide, a selective substrate for the microsomal epoxide hydrolase, by the cytosol also supports these results (16). It is unlikely that the immunogenic activity observed in the cytosol is due to the cytosolic epoxide hydrolase, since it was earlier shown to be immunologically distinct from that of the microsomal form by precipitation assays (28).

Thus, the ELISA provides a simple, rapid and sensitive means of detecting low levels of microsomal epoxide hydrolase. It may be possible with this increased sensitivity to investigate further the physiological role of the enzyme or its role in hepatocarcinogenesis and hepatotoxicity. Certainly the ELISA will be a valuable tool in investigating the molecular biology of epoxide hydrolase induction.

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