CROSSED IMMUNOELECTROFOCUSING IN COMBINATION WITH A ZYMOGRAM METHOD: STUDIES ON ESTERASE-ACTIVE ANTIGENS SOLUBILIZED FROM RAT LIVER MICROSOMES

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1. Introduction

Recently a simple and reproducible method for crossed immunoelectrofocusing was developed [1]. This procedure not only allows separation of proteins with very high resolution, but also concomitantly establishes their molecular identities by means of immunological criteria.

This study demonstrates the application of crossed immunoelectrofocusing in combination with a histochemical staining for non-specific esterase* to detergent-solubilized microsomal esterase-active antigens. The high resolving power of crossed immunoelectrofocusing and the high sensitivity of the zymogram method permitted the detection of microheterogeneities in distinct esterase-active antigens, which had been previously identified as single components by other electrophoretic techniques [2–5].

2. Materials and methods

Rat liver microsomes were isolated from female Sprague Dawley rats after overnight starvation [6]. The microsomes were washed twice in 0.9% (w/v) NaCl to remove adsorbed non-membraneous proteins. The membrane proteins were extracted either for 2 h at 4°C with 1% (w/v) sodium deoxycholate (Merck, Darmstadt, West Germany) and 0.5% (w/v) Lubrol W (cetylpolyoxyethylene condensate, ICI, Manchester, England) in distilled water or for 30 min at 37°C in 0.05 M Tris–HCl buffer, pH 8.5. After extraction unsolubilized material was sedimented by centrifugation at 105 000 *g* for 60 min. Protein concentrations in the samples were determined according to Lowry et al. with bovine serum albumin as the standard [7].

Isoelectric focusing was performed in thin-layer polyacrylamide gels [1,8] with a pH gradient ranging between 3 and 7 comprising ampholine (LKB-Produkter, Bromma, Sweden) pH ranges 3–6: 1% (w/v), 5–7: 1% (w/v), 3–10: 0.5% (w/v). Samples of microsomal extracts (30 μl, 10 mg protein/ml) were applied on to the gels on 50% cotton/50% cellulose fibre applicators (10 × 5 mm, Paratex II/80, Lohman KG, Fahr/Rein, West Germany).

Crossed immunoelectrofocusing was carried out according to Söderholm et al. [1]. Electrophoresis in the second dimension was run at 10 V/cm for 5 hours into an agarose gel (1% w/v, Miles Seravac, Miles Lab. Inc., Kankakee, USA) containing 10% (v/v) rabbit antiserum against rat liver microsomes, prepared as previously described [2,4]. After electrophoresis the plates were pressed and blotted with filter paper [1].

The non-specific esterase zymograms were developed with α-naphthyl propionate (Sigma Chemical Co., St. Louis, Mo., USA) as substrate and Fast Blue B salt (Merck, Darmstadt, West Germany) as the staining reagent [9].

* *Enzymes:* Non-specific esterase (EC 3.1.1.-).

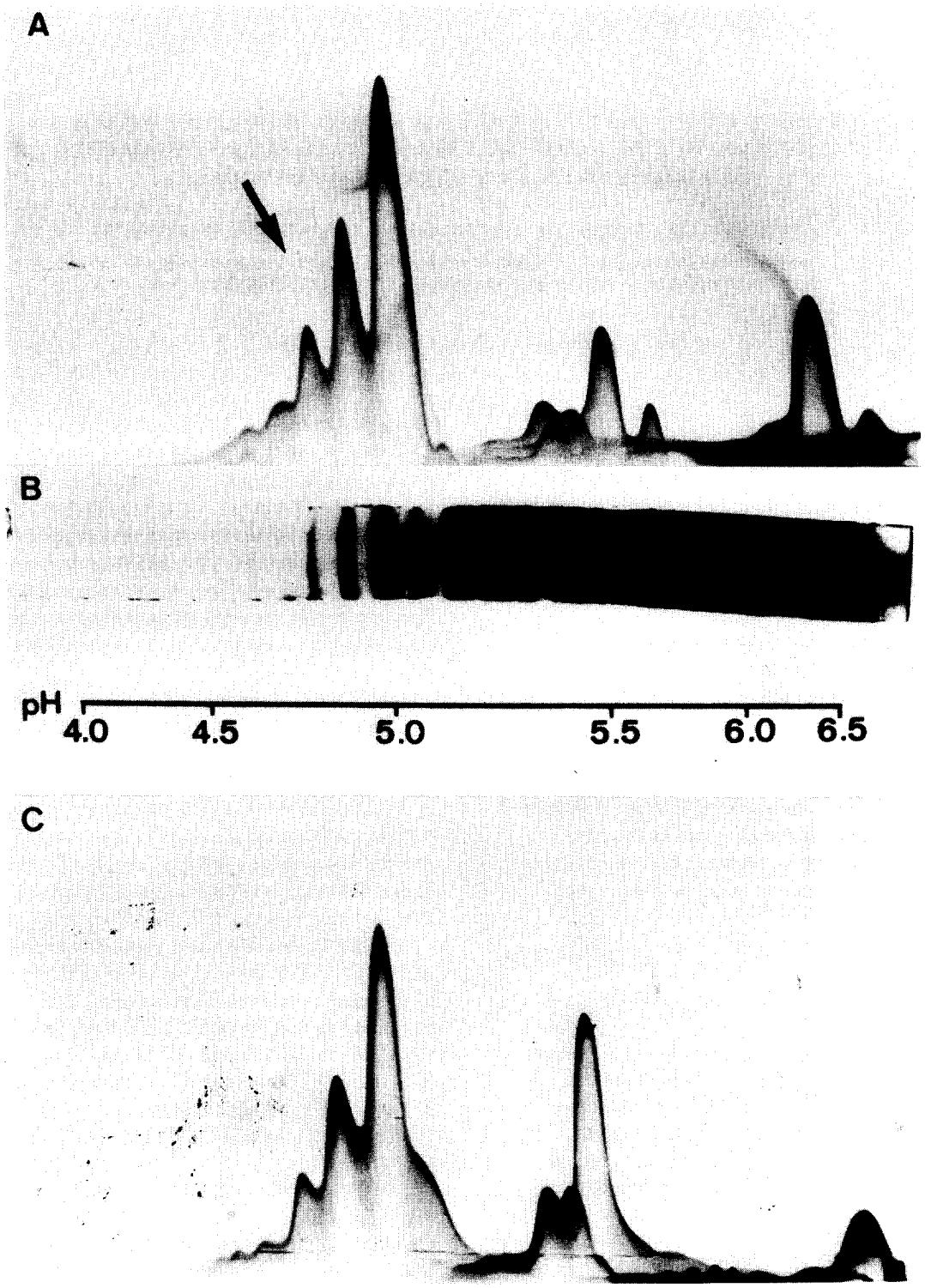

Fig.1

3. Results and discussion

Our laboratory has earlier reported on the polymorphism of rat liver microsomal antigens with esterase activity [2–5]. Thus, six non-specific esterase-active antigens were characterized using conventional immunoelectrophoresis [3]. By means of the high resolving power of crossed immunoelectrophoresis, none distinct microsomal antigens with this activity were identified [4,5]. This number of esterase-active antigens corresponds well with the results obtained when a microsomal extract was separated in polyacrylamide gel electrophoresis, where eight bands were revealed after staining for non-specific esterase [2].

When a detergent-extract of rat liver microsomes was subjected to gel electrofocusing followed by staining for non-specific esterase at least 20 separate bands with this activity were detected (fig.1B). The strongly stained area at the cathodal part of the gel (above pH 5.5) contained at least 8 distinct bands better resolved on a gel with a lower amount of extract or by using a shorter staining time. The majority of the microsomal non-specific esterase active components had their isoelectric points in the pH range 4.5 to 6.5.

After electrophoresis in the second dimension, into an antibody-containing agarose-gel no precipitates were visible in the wet plate. However, by applying the zymogram technique for non-specific esterase to the dried plates, at least five immunoprecipitates possessing distinct microheterogeneities were detected (fig.1A). The most dominating of these comprised several peaks with isoelectric points in the pH range 4.5 to 5.0. Each peak could be referred to a distinct esterase-active band in the isoelectric focusing gel. However, when these bands were precipitated with antibodies they formed a continuous precipitate indicating that they all comprised a single antigen displaying heterogeneity in charge. The strongly stained area (above pH 5.5) in the acrylamide gel, consisted of at least eight distinct esterase bands, but gave rise to only two distinct immunoprecipitates.

In the pH range 4.5–5.0 also another esterase active antigen gave a weakly stained immunoprecipitate (arrow, fig.1A). This corresponded to the phenobarbital inducible antigen earlier characterized and designated e_1 ([10], Raftell, Berzins and Blomberg, submitted for publication).

To investigate whether the detergents used for solubilization of the microsomes caused artifactual microheterogeneities of the esterase-active antigens, microsomes were solubilized in an alkaline buffer without detergent. In crossed immunoelectrofocusing, this detergent-free extract gave essentially the same pattern of esterase-active precipitates as the Lubroldeoxycholate extract. Although the concentration of some of the antigens differed between the two types of extracts, similar microheterogeneities were obtained and no distinct differences in isoelectric points were detected (fig.1C). Furthermore, the immunoprecipitate pattern was highly reproducible from one electrophoretic run to an other, and also for extracts from several microsomal preparations, suggesting that the esterase microheterogeneities seems to reflect differences in the composition of these membrane antigens rather than being artifactual.

It has been shown in some cases that on gel electrofocusing different banding-patterns were obtained with one sample, depending on the sample application site [11]. In our experiments no such differences in the banding-pattern were seen with the applicator used no matter where the sample was applied on the gel. However, in some

Fig.1. Crossed immunoelectrofocusing of rat liver microsomal extracts followed by staining for non-specific esterase with α-naphthyl propionate as substrate. The antigens were precipitated with a rabbit antiserum against rat liver microsomes (10%). (A) shows the pattern of esterase-active immunoprecipitates of a Lubrol-deoxycholate extract while (C) shows the corresponding pattern with an alkaline buffer extract. When the gel after isoelectric focusing was stained for non-specific esterase immediately after electrophoresis in the first dimension, at least 20 active bands were revealed testing either a Lubrol-deoxycholate extract (B) or an alkaline buffer extract. In (A) the arrow indicates a weakly stained precipitate corresponding to an antigen earlier characterized and designated e_1 ([10], Raftell, Berzins and Blomberg, submitted for publication).

cases non-specific staining or blurred bands were seen at the site of sample application.

The high resolution and reproducibility of the separated enzyme pattern makes electrofocusing combined with a zymogram method a useful tool for comparison of different methods used to solubilize membrane antigens and enzymes. Although a great variety of detergent-treatments and other methods have been widely used to solubilize membranes [12], very few comparative studies of these different methods have been reported. With crossed immunoelectrofocusing and other high-resolving separation techniques, such as crossed immunoelectrophoresis using electrophoresis in a polyacrylamide gradient in the first direction [13] it should be possible to reveal and study changes in conformation or charge properties and aggregations which might be induced by such treatments.

Furthermore, as the different immunoprecipitates get well separated by this method it is well suited for production of immunoprecipitates which can be used to rise specific antisera against individual membrane components [14,15]. For more extensive analysis of individual antigens in crossed immunoelectrofocusing it should be possible to obtain an even higher resolution by using such antisera with more restricted specificity in combination with more shallow pH-gradients.

It should also be possible to combine more sensitive methods for detection of immunoprecipitates in crossed immunoelectrofocusing as eg. autoradiographical detection of radioactively labelled antigens or anti-immunoglobulin antibodies [16,17], thus increasing the potential of this method. Detection of glycoprotein antigens with various lectins [18,19] is an other possible extention, which might give insight into the molecular basis of the microheterogeneities seen in many antigens.

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