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ARYLAMIDASES OF RAT LIVER AND CHEMICALLY INDUCED HEPATOMAS

II. PREPARATION AND CHARACTERIZATION OF MONOSPECIFIC ANTISERA AGAINST TWO DISTINCT ARYLAMIDASE-ACTIVE ANTIGENS

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Summary

Monospecific antisera were prepared against the most prominent arylamidase (α-aminoacyl-peptide hydrolase (microsomal), EC 3.4.11.2) active antigen in plasma membranes (the plasma membrane arylamidase) and lysomal content (the lysosomal content arylamidase), respectively. Plasma membrane extract and lysosomal content were allowed to react in crossed immunoelectrophoresis against their homologous antisera. The electrophoretic plates were washed extensively, dried and subsequently stained for arylamidase activity. The particular immunoprecipitates were thus identified and could be excised to be used for immunizations.

The two resulting antisera precipitated the arylamidase used for immunization, but failed to be monospecific as they also precipitated additional antigens. These antisera with restricted specificity against some plasma membrane and lysosomal content antigens, respectively, were used to produce immunoprecipitates intended for new attempts to prepare monospecific antisera by a second cycle of immunizations. A monospecific antiserum against the plasma membrane arylamidase was thus obtained, while a third cycle of immunizations was needed to get a monospecific anti-lysosomal content antiserum.

The plasma membrane arylamidase showed ATPase activity also after precipitation with the monospecific antiserum, thus still retaining its characteristics as a multienzyme complex.

Introduction

We have earlier reported on the presence of several distinct arylamidase (α-aminoacyl-peptide hydrolase (microsomal), EC 3.4.11.2) active antigens

in rat liver plasma membranes, microsomes, lysosomal membranes and lysosomal content [1—4]. Recently, we have shown that all of these fractions have at least two such antigens in common [5]. One of these antigens is predominantly present in plasma membranes while the other shows the highest concentration in the lysosomal content. Thus, the latter antigen apparently exists in both a soluble and a membrane-bound form.

One interesting property of two of the at least three arylamidase active antigens present in plasma membranes is that they also show ATPase and NADH-neotetrazolium reductase activities [1,2]. Further studies on these associated enzymatic activities within certain antigens then made us consider them as multienzyme complexes that might be of physiological significance in the membrane [1,2]. One of the lysosomal membrane arylamidase active antigens also exhibited additional enzyme activity, but in this case it was acid phosphatase [4]. Arylamidase activities in microsomes or lysosomal content, however, were not found to be associated with other enzyme activities tested for [1,2,4].

For further characterization and closer subcellular localization of the different arylamidase active antigens of rat liver we have now produced monospecific antisera against the two common antigens mentioned above. This study describes the mode of production of these antisera and is a characterization of their monospecificity.

Materials and Methods

Subcellular fractions. Preparations were made from livers of female Sprague-Dawley rats starved overnight. Plasma membranes were isolated according to the method of Emmelot et al. [6]. The membranes were washed twice in 0.15 M NaCl in order to remove nonmembranous proteins. The soluble protein of lysosomal content was prepared as previously described [4] from secondary lysosomes isolated by means of Triton WR 1339 [7,8].

Antisera. Polyvalent antisera against plasma membranes and lysosomal content were prepared as previously described [1,4].

Solubilization. Plasma membrane antigens were solubilized at 4°C for 1 h with 1% (w/v) sodium deoxycholate and 0.5% (w/v) Lubrol W (cetylpolyoxyethylene condensate, I.C.I., Manchester, England) and unsolubilized material was spun down at 105 000 × *g* for 1 h.

Protein concentrations were determined according to the method of Lowry et al. [9] with bovine serum albumin (Armour Pharmaceutical Co., Ltd., Eastbourne, England) as standard.

Crossed immunoelectrophoresis. This was carried out according to the method of Clarke and Freeman [10] using 40 µl antigen extract adjusted to 10 mg protein/ml in each plate except when otherwise indicated. 10% (v/v) antiserum was used in 1% (w/v) agarose gels (Behringwerke AG, Marburg Lahn, W. Germany). The electrophoresis was run at 4 V/cm for 5 h (first dimension) and subsequently for 16—20 h (second dimension). After electrophoresis the plates were dried under several changes of filter paper.

Zymogram techniques. Arylamidase activity (EC 3.4.11.2) was detected as described by Nachlas et al. [11] using L-leucine 2-naphthalymide · HCl as sub-

strate and Fast Blue B salt as the staining reagent. Nucleoside di- or triphosphatase activity (EC 3.6.1.6) was demonstrated according to the method of Wachstein and Meisel [12] using ATP as substrate [13].

Protein staining of immunoprecipitates was made by means of Coomassie Brilliant Blue R as described by Chua and Bennoun [14].

All substrates and staining reagents were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Monospecific antisera. These were prepared by immunization of immunoprecipitates obtained in crossed immunoelectrophoresis.

Preparation of immunoprecipitates for injection. Antigen solutions (lysosomal content or detergent extract of plasma membranes) were resolved in crossed immunoelectrophoresis against polyvalent homologous antisera. After electrophoresis the plates were washed extensively in several changes of phosphate buffered saline for 48 h and then dried under filter paper. The zymogram staining for arylamidase activity [11] was applied to the dried plates and the staining was allowed to proceed until the active immunoprecipitates were distinctly red-coloured. Immediately after the staining procedure, when the agarose was still somewhat wet the immunoprecipitates to be used for immunization were excised from the plates by means of a scalpel. Only regions where the precipitates were seemingly uncontaminated by other precipitates were used. After excision the precipitates were stored at −20° C.

Immunization. Excised agarose pieces containing the immunoprecipitates were homogenized in phosphate buffered saline and the homogenate (about 1 ml) was emulsified in half its volume Freund's complete adjuvant (Difco Labs, Detroit, Mi., U.S.A.). Immunizations were made in rabbits by intramuscular injections in the hind legs with six immunoprecipitates each time. Injections were made at biweekly intervals at least three times, and the rabbits were bled 1--2 weeks after the last injection.

Results

Both plasma membrane extract and lysosomal content give a complex pattern of immunoprecipitates when reacted in crossed immunoelectrophoresis against their homologous polyvalent antisera (Figs. 1a and 2a) [1,4]. Three plasma membrane antigens exhibit arylamidase activity (Fig. 1b) while only two antigens with such activity are apparent in lysosomal content (Fig. 2b) [1,4]. Our aim was to raise monospecific antisera against the most prominent arylamidase active antigen in each of these immunoprecipitate patterns (arrows Fig. 1a, b and Fig. 2a, b). The particular antigens have earlier been shown to be nonidentical by immunological criteria and have been designated the plasma membrane arylamidase and the lysosomal content arylamidase, respectively [5].

The plasma membrane arylamidase. This antigen is of great interest as it is one of the previously described multienzyme complexes, exhibiting arylamidase, nucleoside di- and triphosphatase and NADH-neotetrazolium reductase activities [1,2]. The purification of this antigen is not easily accomplished by means of conventional chromatographic procedures as it is very heterogeneous both in regard to molecular weight and charge properties [15].

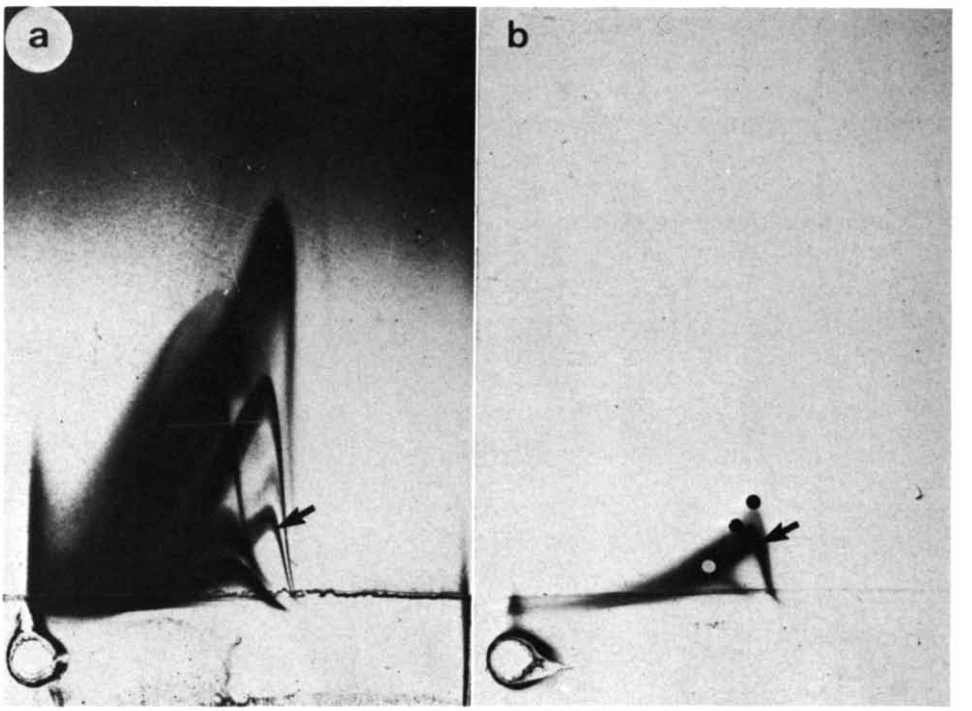

Fig. 1. Crossed immunoelectrophoresis of plasma membrane extract against anti-plasma membrane antiserum. The plates were stained for protein (a) and arylamidase activity (b). The arrow in (a) and (b) indicates the arylamidase-active antigen (the plasma membrane arylamidase) used for immunizations. For this purpose only the area around the arrow-tip was carefully excised so that obvious contamination by other immunoprecipitates was avoided. Dots in (b) indicate the three arylamidase-active immunoprecipitates detected.

As an immunoprecipitate in crossed immunoelectrophoresis the antigen is, however, obtained in a concentrated form separated from other plasma membrane components.

Only restricted regions of the immunoprecipitates were excised and used for immunizations in order to avoid contamination with other antigens in neighbour immunoprecipitates. The antiserum obtained after three injections with such precipitates was not able to form any visible precipitate when reacted against a plasma membrane extract in crossed immunoelectrophoresis. When the plates, however, were stained for arylamidase activity one distinct precipitate appeared, showing that we had achieved an immune response against the antigen injected. The antiserum failed to be monospecific as subsequent protein staining of the electrophoretic plate detected also another immunoprecipitate.

This antiserum, with restricted specificity against two plasma membrane antigens, was used to again produce immunoprecipitates of the plasma membrane arylamidase, to be used in a new attempt to raise a monospecific antiserum in another rabbit. After three injections with such precipitates the obtained antiserum precipitated only one antigen from plasma membrane extracts, as judged from protein staining in crossed immunoelectrophoresis

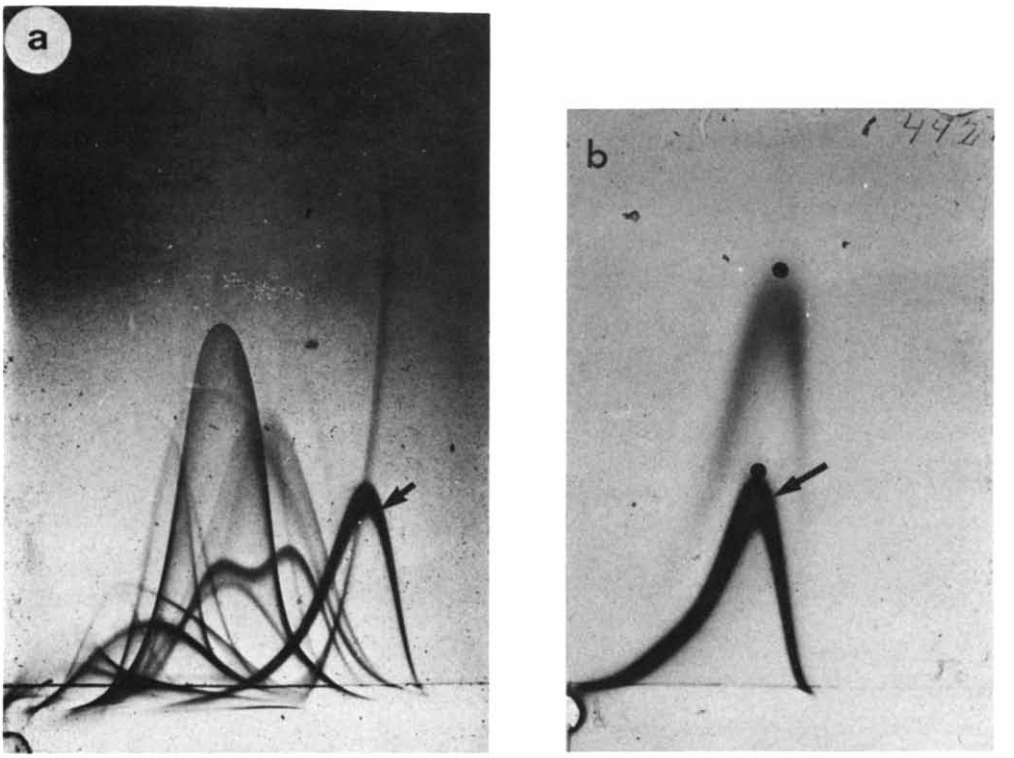

Fig. 2. Crossed immunoelectrophoresis of lysosomal content against the homologous antiserum. The plates were stained for protein (a) and arylamidase activity (b). The arrow in (a) and (b) indicates the arylamidase immunoprecipitate (the lysosomal content arylamidase) used for immunizations. The area around the arrow-tip was excised for this purpose and care was taken to avoid obvious contamination by neighbour precipitates. (a) contains 40 μl of the antigen extract and (b) 50 μl. The increased amount of antigen in (b) was necessary to make the staining of the upper precipitate clearly visible.

(Fig. 3a). This single antigen exhibited arylamidase activity (Fig. 3b) and was also shown to be ATPase active (Fig. 3c).

The lysosomal content arylamidase. In chromatography of lysosomal content on Sephadex G-200 columns this antigen is eluted together with the bulk of lysosomal content proteins in one main protein peak (Lando et al., unpublished). As the enzyme active antigen under study also in this case seemed difficult to purify by chromatographic or electrophoretic techniques the same rationale to accomplish purification by means of immunoprecipitation in crossed immunoelectrophoresis as mentioned above was used again. For this purpose electrophoresis was performed in which lysosomal content was reacted against its homologous polyvalent antiserum. Because of their soluble nature the antigens of lysosomal content give very distinct immunoprecipitates and, thus, they should be easy to excise without much apparent contamination by other precipitates. In spite of this, the antiserum obtained after three injections with immunoprecipitates containing the lysosomal content arylamidase (arrows Fig. 2a, b) gave at least four precipitates when reacted against lysosomal content. One of these antigens was arylamidase active, while no enzyme activity was detected in the others.

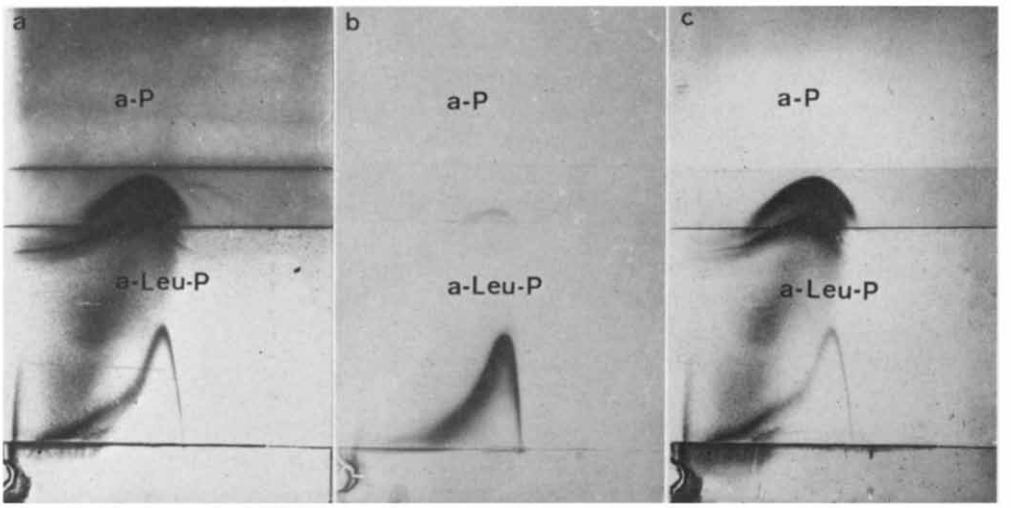

Fig. 3. Crossed immunoelectrophoresis with an intermediate gel. Plasma membrane extract was reacted against monospecific antiserum to the plasma membrane arylamidase (a-Leu-P in the lower part of the plates) and anti-plasma membrane antiserum (a-P in the upper part of the plates). The plates were stained for protein (a), arylamidase activity (b) and ATPase activity (c). All three stainings detect only one antigen precipitated by the monospecific antiserum. The rest of the plasma membrane antigens are precipitated by the polyvalent antiserum in the upper part of the plates.

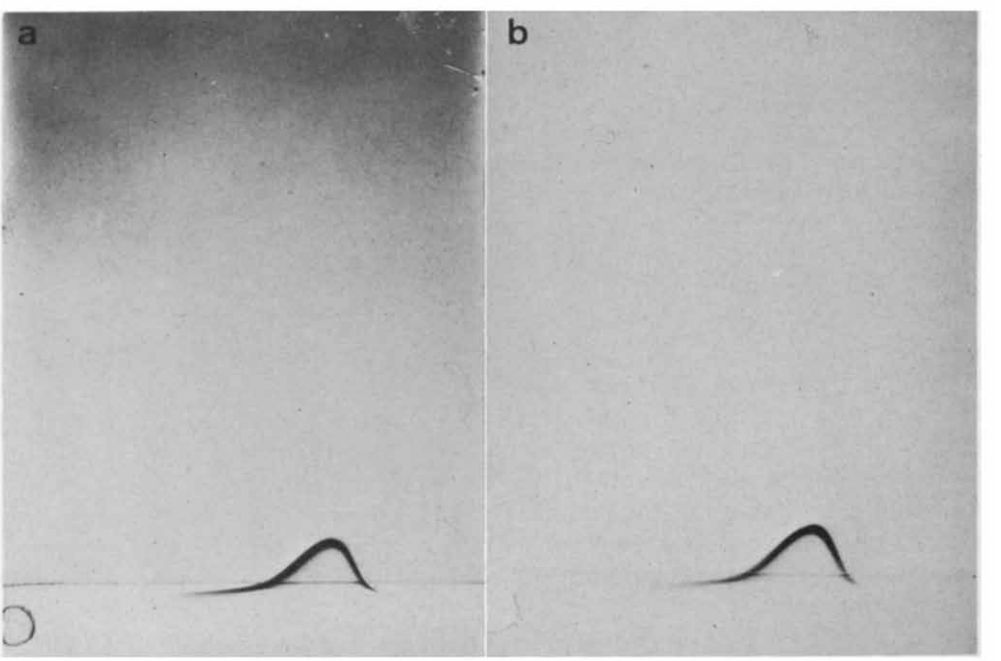

Fig. 4. Crossed immunoelectrophoresis of lysosomal content against monospecific antiserum to the lysosomal content arylamidase. The plates were stained for protein (a) and arylamidase activity (b). Only one precipitate is detected in both plates.

In a second attempt to rise a monospecific antiserum, the antiserum obtained from the first rabbit and having restricted specificity against four lysosomal content antigens was used for production of new arylamidase immunoprecipitates which were then immunized as above in another rabbit. The resulting antiserum precipitated three antigens from lysosomal content one of which was arylamidase active. By means of a third cycle of immunizations using the latter antiserum for immunoprecipitate production the intended monospecific antiserum was finally obtained. The antiserum precipitated one single antigen from lysosomal content. The antigen showed no other enzyme activity than arylamidase activity (Fig. 4a, b).

Discussion

The use of immunoprecipitates as antigen source for production of antisera with restricted or monospecific reactivity has been frequently practiced [16—22]. In early studies [16—18], immunoprecipitates obtained in double immunodiffusion tests [23] were employed for this purpose. Although double immunodiffusion is a relatively sensitive method the resolution is too poor for isolation of discrete immunoprecipitates from a complex mixture of antigens. Instead, Crowle et al. [19] devised the use of immunoprecipitates obtained in crossed immunoelectrophoresis for production of monspecific antisera. By means of this high-resolving technique complex antigen mixtures can be sufficiently separated and single immunoprecipitates can be excised in a relatively pure form. Monospecific antisera against a variety of antigens have been obtained by immunizations with such immunoprecipitates [19—22].

By means of a zymogram staining of dried electrophoretic plates we could identify the arylamidase active antigens in complex immunoprecipitate patterns of lysosomal content or plasma membrane extracts. As immunoprecipitated detergent-solubilized membrane antigens often are invisible in the wet agarose plates [15], a zymogram staining might be necessary for their detection. The dried agarose film becomes again somewhat wet after the staining procedure, which makes it easier to excise only the proper immunoprecipitate within narrow limits. The immunoprecipitates can thus be removed in one piece of thin agarose film.

In both cases studied we failed to obtain monospecific antisera after a first cycle of immunizations. The most probable explanation for this is that parts of other invisible immunoprecipitates were excised together with the arylamidase active ones and thus gave rise to unwanted antibodies. It could also be, however, that unrelated antigens which had been entrapped in the immunoprecipitates during electrophoresis still were present although the plates were extensively washed prior to excision of the precipitates. Finally, since we succeeded in raising monospecific antisera the failure in the two first attempts cannot be explained by some antigens having common determinants with the antigens immunized.

By the use of the antisera with restricted specificity against a few antigens different antibody/antigen ratios are obtained for the individual antigens than by means of the polyvalent antisera. Thus, the possibility of obtaining pure immunoprecipitates in a later cycle of immunizations is increased.

It should be pointed out that monospecificity is a relative concept which depends on what test-systems are used. In this study we define the antisera as monospecific if they precipitate only one of the antigens detectable in the complex antigen mixtures by means of crossed immunoelectrophoresis.

The physiological function of the arylamidases is not yet known. It has been suggested that they might be involved in amino acid transport functions or in the regulation of peptide hormone action at the cell surface [24]. Future use of the monospecific antisera against two of these enzymes for localization studies will make it possible to more precisely determine their subcellular distribution in the liver and in other tissues. Furthermore, the monospecific antibodies can be used in affinity columns for simple isolation of the antigens. The multienzyme complex, of which the plasma membrane arylamidase apparently is a constituent [1,2], should thus be possible to isolate. Such a purified fraction could then be used to resolve and clarify the peptide composition of a multienzyme complex by means of SDS polyacrylamide gel electrophoresis.

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