IMMUNOLOGICAL STUDIES OF BOVINE AORTA LYSYL OXIDASE: EVIDENCE FOR TWO FORMS OF THE ENZYME

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SUMMARY: Evidence is presented that beef aorta contains two forms of lysyl oxidase which we have designated as lysyl oxidase A and B. The two forms of the enzyme can be separated by DEAE-cellulose chromatography. Immunogical tests show that lysyl oxidase A and B have distinct antigenic determinants. Immunoelectrophoresis at pH 8.6 showed that the aorta lysyl oxidase A and B differed in net charge. Antisera to pure lysyl oxidase A formed a precipitin line with lysyl oxidase A but did not react with lysyl oxidase B in the Ouchterlony double immunodifffusion test. These findings show that it will now be necessary to separate the two forms of enzymes for certain types of biochemical studies of lysyl oxidase.

Lysyl oxidase is a connective tissue enzyme which is required for the conversion of tropoelastin and tropocollagen to elastin and collagen. The enzyme converts certain lysine residues to allysine residues in the precursor form of the structural proteins. Thus far, lysyl oxidase has been isolated in a highly purified form from chicken aorta by Harris et al. (1) and from chicken cartilage by Narayanan et al. (2). From these reports, it would be concluded that there is only one form of lysyl oxidase. By careful immunological investigations of the enzyme during purification of bovine aorta lysyl oxidase, we have noted several forms of the enzyme and the present report is concerned with the immunological studies on the two forms of the enzyme.

MATERIALS AND METHODS

Pure bovine aorta lysyl oxidase. The procedure for the purification of these enzymes will be described elsewhere but followed the published procedure of Harris et al. (1). The preparations were shown to be

pure by SDS-disc electrophoresis as described by Weber and Osborn (3) as well as by immunological tests. Freund's complete adjuvent was purchased from Difco Labs. Coarse DEAE-cllulose was purchased from Sigma Chemical Co. and fines were filtered before use. Sepharose 4B was purchased from Pharmacia Fine Chemical Co. Soluble collagen was purchased from Sigma Chemical Co. Reagent grade chemicals were used throughout the present investigations.

Protein concentrations were determined from the absorbance of the solutions. The $E^{1\%}_{1cm}$ at 280 nm values adopted for lysyl oxidase (and other proteins) was 15.0.

Enzyme activity was determined by the tritium release from tritiated structural proteins as described by Pinnell and Martin (4).

Antibody preparations. Either crude tissue preparations purified lysyl oxidase A and B at a concentration of 6 mg/ml in Freund's complete adjuvent was injected into the foot pads and legs of the rabbit. The antibody fraction was isolated by ammonium sulfate precipitation (40% saturation), followed by reprecipitation at 33% saturation. The final precipitate was dissolved in 0.02 M borate buffered saline and dialyzed against the same buffer for two days.

Ouchterlony double immunodiffusion experiments. Double immunodiffusion was done in 1.2% pure agar in 0.02 M borate buffered saline, pH 8.0. Samples of 0.5 - 0.7% were applied into different wells. Reaction was allowed to proceed for a least two days.

Immunoelectrophoresis experiments. Electrophoresis was carried out with a voltage gradient of 6-8 V/cm for 1 hour in 1.2% pure agar, 0.03 M barbital buffer, pH 8.6. The plate was incubated for 16 hours at 37° C after application of the antisera.

RESULTS

Lysyl oxidase components in crude extracts of bovine aorta. Rabbit antisera to crude bovine aorta extracts were used in the experiments to be described. The result with the crude aorta extract is shown in

Fig. 1. which showed two precipitin lines by the Ouchterlony double immunodiffusion test. The addition of purified aorta lysyl oxidase A and B to wells on either side of the crude extract showed the following reactions. Purified aorta lysyl oxidase A produced a precipitin line of identity with the outer precipitin line of the crude sample and formed a line of non-identity with the inner precipitin line which coincided with the sample of purified aorta lysyl oxidase B except for the very slight formation of what appeared to be a spur. This spur is due to the presence of a nonlysyl oxidase component as can be surmised from results shown in Fig. 2.

Immunoelectrophoresis of purified samples of aorta lysyl oxidase A and B were run at the same time with the crude sample. As shown in Fig. 2 aorta lysyl oxidase A moves to the anode while lysyl oxidase B moves to the cathode. Thus, the two forms of the enzyme show a charge difference.

Separation of lysyl oxidase of DEAE-cellulose. During the purification of the bovine aorta lysyl oxidase, it was observed that two main lysyl oxidase components were separated. The chromatogram for the aorta is shown in Fig. 3. The two main lysyl oxidase components were eluted by 0.01 M potassium phosphate buffer, pH 7.4 (lysyl oxidase A) and 0.07 M phosphate buffer, pH 7.4 (lysyl oxidase B). The various fractions under each peak

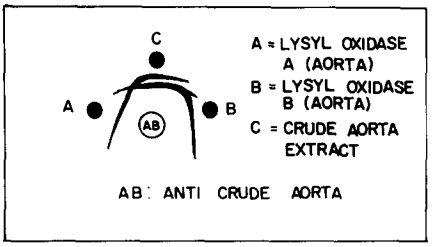

Figure 1. Ouchterlony double immunodiffusion experiments. Rabbit antisera to the crude aorta extract was added to the center well. Starting from the left, pure lysyl oxidase A (5 mg/ml) in 0.1 M phosphate buffer, pH 7.4, crude bovine aorta extract (15 mg/ml) and pure lysyl oxidase B (7 mg/ml) were added to the outer wells.

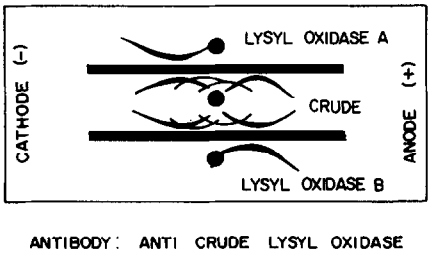

Figure 2. Immunoelectrophoresis experiments. From the top, wells contain lysyl oxidase A (5 mg/ml), crude aorta extract (15 mg/ml) and lysyl oxidase B (7 mg/ml) in 0.1 M phosphate buffer, pH 7.4. The troughs contained antisera to crude bovine aorta extract. See experimental section for details of the experiment.

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were assayed for enzyme activity and were analyzed by the immunoelectrophoretic procedure described previously and from the pattern, it was shown that the first form to be eluted contained lysyl oxidase A as shown in Fig. 3. The second component to be eluted was shown to contain lysyl oxidase B by immunoelectrophoresis. Thus, the immunological methods and enzymatic assay suggested where the two forms of the enzyme were eluted (and the presence of lysyl oxidase A and B in earlier fractions eluted may be due to the bound forms of the enzyme which require urea in order to free them. Rechromatography of pooled fractions III and IV were eluted with the same buffers as was used in the original chromatography of the enzyme fractions on DEAE-cllulose. The tritium assay of Pinnell and Martin showed the presence of lysyl oxidase in all of the fractions eluted although the procedure showed that the two main forms of the enzyme were eluted at 0.01 M and 0.07 M buffer.

Studies with anti-lysyl oxidase A. Immunochemical tests were run to see if the aorta lysyl oxidase A and B were identical or not. When purified lysyl oxidase A and B were examined by the Ouchterlony technique, a precipitin line was observed with lysyl oxidase A but no line was observed with lysyl oxidase B (Fig. 4). The results confirmed the non-immunological identity of lysyl oxidase A and B observed with anti-crude serum.

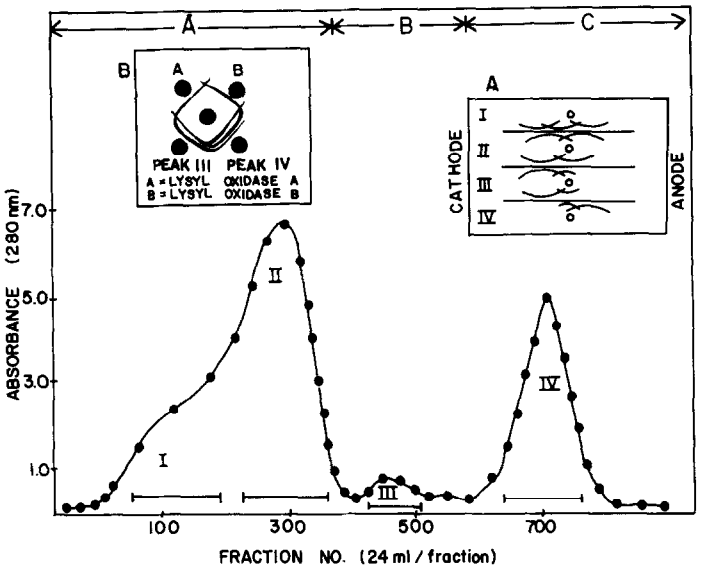

Figure 3. DEAE-cellulose chromatography of aorta extracts and immunological studies of the eluates. For the experiments,a DEAE-cellolose column (6.4 x 114 cm) was equilibrated with 0.01 M phosphate buffer, pH 7.4. Then water soluble extracts obtained by homogenizing 2,500 g of aorta in 0.01 M phosphate buffer, pH 7.4 was applied to the column and eluted with 0.01 M phosphate buffer (A) At the point indicated in the figure, the buffer was changed to 0.01 M phosphate buffer, pH 7.4-4 urea solution (B) and then to 0.07 M-4 M urea (C) all at 4°C. Eluted fractions showed by the solid bars were pooled and analyzed by both the Ouchterlony and immunoelectrophoresis tests to determine the form of lysyl oxidase eluted. Insert A shows immunoelectrophoresis of peaks III-IV and insert B shows the results of the Ouchterlony test.

DISCUSSION

Highly purified lysyl oxidase has been isolated recently by Harris et al. (1) from chicken aorta and Narayana et al. (2) from chicken cartilage. Thus far, the reports have not shown the presence of multiple forms of the enzyme. Harris et al noticed that the purified chicken aorta enzyme could be dissociated into two types of lysyl oxidase with different molecular weights by 8M urea or 0.1% sodium dodecyl sulfate (1). From this report it would seem that the enzyme has been dissociated into two different subunits.

In the present investigation, immunological methods have been used to show that there are two forms of lysyl oxidase present in bovine aorta. We

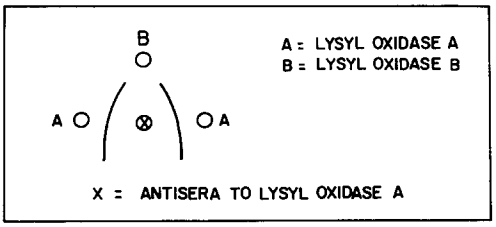

Figure 4. Ouchterlony double immunodiffusion experiments with antisera to pure lysyl oxidase A. Conditions as described in the legends to Fig. 1.

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have tentatively designated the two forms of the enzyme as lysyl oxidase A and B. The two forms of the enzyme are present in crude extracts of bovine aorta. We have followed the purification of the enzyme on DEAE-cellulose chromatography and have obtained immunochemical evidence that the form eluted by 0.01 M phosphate buffer-4M urea solution is lysyl oxidase A and the form eluted by 0.07 M phosphate buffer-4M urea solution is lysyl oxidase B.

When Ouchterlony tests were performed on the aorta lysyl oxidases A and B, it was observed that the two forms of the enzymes formed crossing precipitin lines. Thus, immunologically, they are different enzymes. Immunoelectrophoresis at pH 8.6 showed that lysyl oxidase A was basic while lysyl oxidase B was slightly acidic. Anti-lysyl oxidase A formed a precipitin line with pure lysyl oxidase A but no precipitin line was observed with purified lysyl oxidase B. The results confirmed the observation with crude antisera that lysyl oxidase A and B are immunochemically distinct enzymes.

From the literature and from the data found in this study, the following properties of lysyl oxidase A and B can be mentioned (1). Lysyl oxidase A has a molecular weight of about 59,000 and lysyl oxidase B has a molecular weight of about 61,000. The net charge of the two lysyl oxidases differ. Lysyl oxidase A is more positively charged than lysyl oxidase B. At pH 8.6,

lysyl oxidase A moved to the cathode while lysyl oxidase B moved slightly to the anode.

Data which is not shown in this paper have shown that lysyl oxidase A and B act on both tropocollagen and tropoelastin. It is not obvious at the present time why there are two forms of lysyl oxidase.

The preliminary immunochemical studies are being extended to the enzyme from other organs in the cow as well as to the enzyme from different animals.

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