Electrophoretic Heterogeneity of Bovine Seminal-Plasma 5'-Nucleotidase

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5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) has been shown to have a wide specificity for nucleoside 5'-monophosphates (Heppel & Hilmoe, 1951; Levin & Bodansky, 1966). Attempts have been made to resolve this enzyme into active components by electrophoresis in starch gel (Kowlessar, Pert, Haeffner & Slesinger, 1959; Bernsohn & Barron, 1964; Lisowski, 1966) and polyacrylamide gel (Barka, 1961), but in no case was evidence for multiple forms obtained. Moreover, Levin & Bodansky (1966), using a purified preparation from bovine semen, reported that precipitation with protamine sulphate and ammonium sulphate, denaturation with guanidine, freezing and thawing, starch-paste electrophoresis and chromatography on DEAE-cellulose did not resolve 5'-nucleotidase into isoenzymes. The enzyme from human serum has also been subjected to gel filtration on Sephadex G-200 (Moss, 1966), but again only one molecular form was reported. However, Center & Behal (1966) resolved bovine (calf) intestinal 5'-nucleotidase into three fractions on chromatography on DEAE-cellulose.

The present communication reports the resolution of 5'-nucleotidase of bovine seminal plasma into three active components by disk electrophoresis in polyacrylamide gels. An advantage of polyacrylamide-gel electrophoresis is that the architecture of the gel can be altered by varying the acrylamide and bisacrylamide concentrations, allowing selective sieving of protein molecules over a great size-range (Ornstein, 1964; Davis, 1964). Gels were prepared with concentrations of acrylamide and bisacrylamide varying from 5% and 0.2% to 10% and 0.6% respectively.

To inactivate the non-specific phosphatases present, the bovine seminal plasma was heated at 60° for 10 min., quickly cooled in an ice-water bath and centrifuged at 10000g for 10 min. The supernatant was taken.

Non-specific phosphatase and 5'-nucleotidase were assayed before and after heating. Non-specific phosphatase was assayed at pH 7.5 and pH 9.5 by determining the P_i liberated from 5 mM-sodium β -glycerophosphate. 5'-Nucleotidase was assayed by determining the P_i released from 5 mM-AMP at pH 7.5 and subtracting the value obtained for non-specific phosphatase. The assays were carried out at 37° in 80 mM-tris-HCl buffer in the presence of

10 mM-Mg²⁺, and P_i was determined by the method of Fiske & Subbarow (1925) as modified by Bartlett (1959).

Protein content was also determined before and after heating by the method of Lowry, Rosebrough, Farr & Randall (1951), but no detectable difference was found.

Before the heating, 1 ml. of undiluted seminal plasma containing 79.8 mg. of protein released 28.0 μ moles of P_i /min. from AMP and 0.97 μ mole of P_i /min. from β -glycerophosphate at pH 9.5. After the heating, 1 ml. of undiluted plasma released 26.7 μ moles of P_i /min. from AMP and 0.02 μ mole of P_i /min. from β -glycerophosphate at pH 9.5. Non-specific phosphatase activity at pH 7.5 was not detectable under these conditions. Therefore the heating procedure caused a slight loss of 5'-nucleotidase activity (4.7%) and a great decrease in non-specific phosphatase activity (98%).

The seminal plasma preparation was stored frozen at a dilution of 1:20 in either ion-free water or 50 mM-tris-HCl buffer, pH 7.0, and was further diluted to 1:100 before use, giving a protein concentration of about 800 μ g./ml. Immediately before electrophoresis, the seminal plasma preparation was mixed with an equal volume of 10% (w/v) sucrose and 0.5 ml. of this mixture was applied to the gel columns.

Electrophoresis was carried out by the procedure of Davis (1964), except for the inclusion of 0.015% $K_3Fe(CN)_6$ in the small-pore gels. Sample gels were not employed, the sample being layered under the buffer directly on to the large-pore gels. A current of 1 mA/gel was applied until the dye front (bromophenol blue) entered the small-pore gel, when the flow was increased to 2 mA/gel. After electrophoresis, the gels were removed from the glass tubes and first washed in several changes of cold (4°) ion-free water and then cold 80 mM-tris-HCl buffer at the pH at which incubation was to take place. Two enzyme reaction media were employed for this incubation: system A, 80 mM-tris-HCl, 20 mM-CaCl₂, 1 mM-substrate, final pH 9.2; system B, as system A but with final pH 7.5.

The substrates used were AMP and β -glycerophosphate, stock solutions of which were prepared and the required pH obtained with 0.1 N-HCl or 0.1 N-ONaH. For some incubations the media were modified by the inclusion of 10 mM-Mg²⁺. Gels were

Fig. 1. Diagrammatic representation of polyacrylamide-gel electropherograms of 5'-nucleotidase of bovine seminal plasma. All four gels were incubated with AMP as substrate. Gels (i) and (ii) were incubated at pH 9.2 and gels (iii) and (iv) at pH 7.5. For gels (i) and (iii) 10 mM-Mg²⁺ was present during incubation. The degree of hatching is roughly proportional to the density of lead sulphide precipitates.

incubated for 30 min. They were then immersed for 45 min. in 3 mM-Pb(NO₃)₂ in 80 mM-tris-maleate buffer, pH 7.0 (at 37°), to convert the calcium phosphate, precipitated during incubation at the sites of reaction, into lead phosphate. This was followed by washing in several changes of ion-free water for several hours at room temperature to remove non-precipitated lead. The gels were then immersed in 2% ammonium sulphide for 5 min. to convert the lead phosphate into visible lead sulphide.

The optimum conditions for separation and clarity of three components were obtained in 7.5% acrylamide with 0.6% bisacrylamide and a dye-front migration distance of 8–9 cm. Under these conditions components II and III were sharply defined, but component I was more diffuse (see Fig. 1).

Gels containing less than 7% acrylamide and 0.2% bisacrylamide failed to resolve the enzyme sharply into three components; 10% gels containing 0.2–0.4% bisacrylamide separated the components very sharply, but the slowest-moving component was often obscured by nucleotidase activity retained at the origin.

With reaction system A, with AMP as substrate, 5'-nucleotidase activity was localized at three sites in the gels. The fastest-migrating component (I) was much less dense and more diffuse than the intermediate (II) and slowest (III) ones. All three components were slightly activated by 10 mM-Mg²⁺. Gels incubated in media containing β-glycerophosphate showed no visible lead sulphide deposits.

With reaction system B, with AMP as substrate, the 5'-nucleotidase activity was again localized at three sites in the gels. However, components II and III gave less dense deposits than at pH 9.2, whereas component I was darker. At this pH 10 mM-Mg²⁺

did not appear to activate any component. Again β-glycerophosphate did not give a visible precipitate.

In view of the controversy about the existence of more than one species of 5'-nucleotidase in certain tissues (Segal & Brenner, 1960; Levin & Bodansky, 1966; Center & Behal, 1966; Song & Bodansky, 1966, 1967), the electrophoretic heterogeneity observed here is of interest. It seems unlikely that this heterogeneity resulted from the heat treatment, as at least two specific components were observed when non-heated enzyme preparations were used, although definition was poor owing to interference by non-specific components. The results probably suggest a true heterogeneity of the enzyme molecule. It is noteworthy that bovine (calf) intestinal 5'-nucleotidase was also resolved into three components chromatographically (Center & Behal, 1966).

The enzyme of bovine seminal plasma (Levin & Bodansky, 1966) and of other tissues (Song & Bodansky, 1966, 1967) exhibits a double pH optimum, at pH 7.5–8.0 and pH 9.1–9.3, in the presence of Mg²⁺ when AMP, GMP and IMP are substrates.

Our results show that there are differences between the three components with respect to the influence of pH, but the limited sensitivity of the method used renders quantitative determination difficult. However, it is possible that the double pH optimum previously reported is a reflection of the heterogeneity for which the present communication provides evidence.

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