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Chromatographic distinction between hyaluronidases from human serum and ovine testes

Although activity in lowering the viscosity of hyaluronate[1] or in liberation of *N*-acetylglucosamine (GlcNHAc) end groups from hyaluronate[2] has been demonstrated in unfractionated extracts of a number of mammalian tissues and in blood and urine, relatively pure enzymes have been obtained only from testes (hyaluronate glycanohydrolase (EC 3.2.1.35)) and rat liver lysosomes[3].

The present paper describes chromatographic methods of distinguishing human serum hyaluronidase and ovine testicular hyaluronidase which may be applicable to other sources also.

Enzyme assays were done using potassium hyaluronate (Koch-Light and Co.) as substrate, incubation at pH 5.0 (testicular hyaluronidase), or 3.8 (serum hyaluronidase) and an estimation of GlcNHAc based on the method of BONNER AND CANTEY[4].

β-Glucuronidase and β-*N*-acetylglucosaminidase were assayed by the methods of LEVVY AND CONCHIE[5]. For the glucosaminidase assay one-tenth of all reagent volumes were used.

Protein was estimated by the method of LOWRY *et al.*[6], using bovine albumin powder as a standard.

In preparing columns of CM-cellulose (Schleicher and Schuell) or DEAE-cellulose (Sigma) the ion-exchanger was washed and then equilibrated with the same buffer used for the first elution stage. The material to be chromatographed was applied in the same buffer.

With commercial ovine testicular hyaluronidase (Koch-Light) on CM-cellulose and phosphate–citrate eluting buffers in the range 0.05–0.2 M and pH 4–6.65 it was found that the lower the equilibration pH at the beginning of the chromatogram the larger was the fraction of the total protein adsorbed and the stronger the binding of active material. However, at pH 5.0 and 4.0 the active material was more strongly adsorbed than the inactive. When the chromatogram was begun at pH 5.0 the most active effluent fraction contained 90% of the estimated activity applied to the column and the specific activity was 13 times the original material. These purification factors compare well with those obtained by other workers using DEAE-cellulose[7] and DEAE-Sephadex[8]. In these procedures the amounts of material applied were 50–100 times as much as in the present work.

Because of the dilute nature of the activity chromatograms with untreated human serum were difficult to interpret and rather variable. A preliminary ammonium sulphate fractionation was therefore used. The fraction precipitated at 4° by 35–50% saturation with ammonium sulphate contained 74–87% of the hyaluronidase activity. When a mixture of testicular hyaluronidase and this serum fraction was chromatographed on CM-cellulose (Fig. 1) the serum activity was clearly separated from the testicular activity by earlier elution. The separation of the two was followed by using the different ratios of activity at pH 3.8/activity at pH 5.0 which they exhibit (Fig. 2). For the fraction precipitated from serum with 35–50% ammonium sulphate the ratio was 3.0 whereas for the most active effluent from chromatograms of ovine testicular hyaluronidase alone it was 0.54. In Fig. 1 the ratio was 3.0 to 3.5 in the first protein

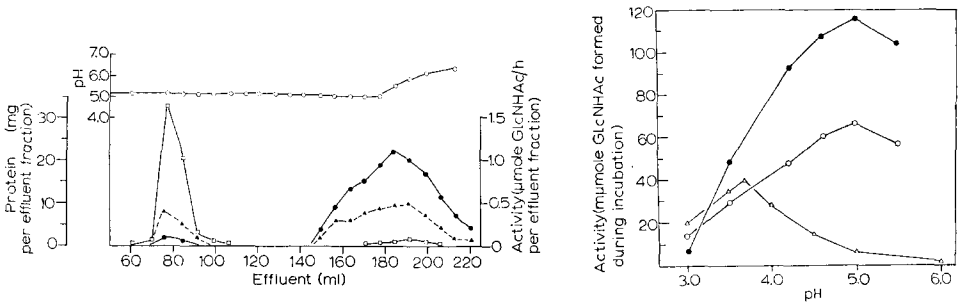

Fig. 1. Chromatography of the 35–50% ammonium sulphate precipitate from serum (70 mg) with testicular hyaluronidase (2.5 mg) on CM-cellulose (2.2 cm × 15 cm). Elution with a concave gradient formed by mixing 0.05 M phosphate–citrate buffer (pH 5.2) (250 ml) and 0.2 M phosphate–citrate (pH 6.65) (200 ml). ○—○, pH of effluent; □—□, protein (mg per effluent fraction); ▲— — ▲, activity at pH 3.8 (μmole GlcNHAc per h per effluent fraction); ●—●, activity at pH 5.0 (μmole GlcNHAc per h per effluent fraction).

Fig. 2. pH *versus* activity curves for ovine testicular and human serum hyaluronidases. ●—●, testicular hyaluronidase (active effluent from CM-cellulose chromatogram of Koch-Light material), 18 h incubation; ○—○, testicular hyaluronidase (active effluent from CM-cellulose chromatogram of Koch-Light material), 3 h incubation; △—△, active effluent from CM-cellulose chromatogram of 35–50% ammonium sulphate precipitate from serum, 3 h incubation.

peak. In the second protein peak the ratio was 0.7 in the first two active effluents and approx. 0.5 in the remainder. The glucuronidase activity of serum was found to be very low, with activities not exceeding 0.5 mμmole phenolphthalein or glucuronate liberated per h per 0.1 ml serum from phenolphthalein glucuronide. An activity of this order is too low to account for the liberation of GlcNHAc groups at the rate found for serum. The glucuronidase/hyaluronidase ratios of the testicular preparations used were found to be equally low. The β-acetylhexosaminidase activity of serum was found to be somewhat higher in relation to the hyaluronidase activity, but during purification of the hyaluronidase activity by ammonium sulphate and on CM-cellulose the

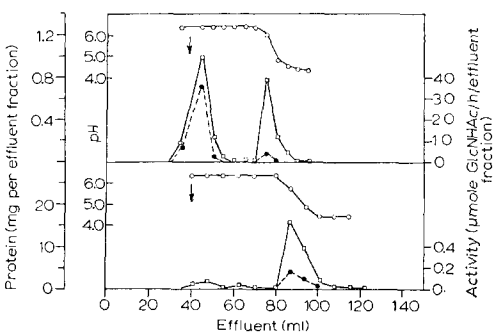

Fig. 3. Chromatography of testicular hyaluronidase (3.5 mg; upper graph) and the 35–50% ammonium sulphate precipitate from serum (70 mg; lower graph) on DEAE-cellulose (12 cm × 2.2 cm). Stepwise elution with (1) 40 ml 0.02 M phosphate–citrate buffer (pH 6.4), (2) 0.12 M phosphate–citrate (pH 4.6) containing 0.68 M NaCl and 1 mM EDTA (starting points shown by arrows). Both solutions contained 1 mM EDTA. ○—○, pH; □—□, protein (mg per effluent fraction); ●- - -●, activity at pH 3.8 (lower) or at pH 5.0 (upper) (μmole GlcHNAc per h per effluent fraction).

ratio of hyaluronidase/hexosaminidase activities was found to increase to a value approximately the same as that found for a commercial chromatographically purified testicular hyaluronidase (Worthington).

During chromatography on DEAE-cellulose (Fig. 3) the active material from testicular hyaluronidase was more weakly adsorbed than that from serum. Comparing this with the findings on CM-cellulose it appears that the active protein from serum has a lower positive charge under this range of conditions than the active protein from ovine testicular preparations. On the assumption that the same charge distribution is involved in the binding of enzyme to negatively charged hyaluronate molecules this would fit in with the finding that the serum fractions possess optimum activity around pH 3.7, whereas testicular material is more active at pH 5.0.

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A convenient titrant for α-chymotrypsin and trypsin

In order to determine the absolute concentration of active sites in a given enzyme solution, one requires a stoichiometric reaction which can be observed simply and accurately. Ideally, the titrant should react with active enzyme rapidly and irreversibly under conditions as close as possible to those of the kinetic determination for which the knowledge of enzyme concentration is required. Most of the reagents available for the enzymes trypsin and α-chymotrypsin[1,2] suffer from one or more of the following disadvantages: (i) dependence upon enzyme concentration, or pH, or reagent concentration; (ii) operation at pH values outside the normal range of kinetic experiments or in solutions containing organic solvents; (iii) lack of sensitivity (*i.e.*, requiring very concentrated enzyme solutions); or (iv) difficult and lengthy synthesis.