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THE PURIFICATION OF HYALURONATE LYASE* ON DEAE-SEPHADEX

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SUMMARY

Hyaluronate lyase of testicular origin has been obtained as an active, electrophoretically homogeneous enzymic preparation by means of the ion-exchanger DEAE-Sephadex (A-50 medium).

The conditions under which the crude preparation containing 4–5 electrophoretic fractions was obtained, the preparation of the ion-exchange column and the fractionation on this column of the crude product are described.

Under the experimental conditions (nature of the buffer, pH, molarity, ionic strength) the enzymically inactive proteins are adsorbed on the column, whereas the active, electrophoretically homogeneous, component is allowed to flow out at the very beginning of elution. Thus multiple gradient elution is unnecessary and the preparation of purified enzyme is greatly simplified.

INTRODUCTION

Various methods have been suggested for the purification of testicular hyaluronate lyase: some involve fractional salt and ethanol precipitation at temperatures below 0° (see ref. 1) while others combine salt precipitation with resin chromatography on Amberlite IRC-50 (see ref. 2) or Amberlite XE-64 (see ref. 3).

However, the preparation of a homogeneous enzyme remains to be achieved. The availability of such a preparation would be of great importance both for chemical study (determination of the relationship between chemical structure and enzymic activity) and for use in therapeutics.

In the following paragraphs a method is reported in which the purification of hyaluronate lyase is achieved by the use of chromatography on DEAE-Sephadex[4,5] (DEAE-Sephadex A-50 was kindly supplied by Pharmacia, Uppsala (Sweden)). By this means, an active and electrophoretically homogeneous preparation could be obtained under simple technical conditions.

Abbreviation: TRU, turbidity reducing units.

* The term hyaluronate lyase has recently been suggested in the Report of the Commission on Enzymes of the International Union of Biochemistry as a substitute for the term hyaluronidase (EC 4.2.99.1).

MATERIALS AND METHODS

Hyaluronic acid

The hyaluronic acid used as a substrate was prepared from umbilical cords by tryptic and peptic double digestion under standard technical conditions[6]. Proteins were removed by subsequent treatment with chloroform (Sevag's method). The product was stored in the lyophilized state. It meets the requirements of the British Pharmacopoeia and of the U.S. Pharmacopeia (see refs. 6 and 7).

Enzymic activity determination

Enzymic activity was determined according to the turbidimetric method of the W.H.O. (see ref. 8) for the testing of the international hyaluronidase standard. Activity is expressed in TRU/mg protein.

Testicular material

Freshly collected bovine testicles were decapsulated, chopped and immersed in acetone in which they were kept for a few days, the acetone being changed several times. The solvent was removed and the testicular fragments dried *in vacuo* at 40°. When dried in this way, the material may be stored for longer periods without significant losses of enzymic activity. The use of acetone-dried testicles as starting material for enzyme extraction offers the advantage of affording a crude extract which contains less inactive protein than extracts obtained from fresh glands. Thus, from dehydrated material a crude extract was obtained with an activity of 75.2 TRU/mg protein, whereas an extract obtained from fresh material had an activity of only 37.44 TRU/mg protein.

Preparation of the DEAE-Sephadex column

DEAE-Sephadex A-50 (medium) (100–250 mesh), exchange capacity 3.1 mequiv/g was used. DEAE-Sephadex powder was suspended in bidistilled water and the fine particles were removed. The sediment was washed on a Büchner filter with 0.5 M HCL, distilled water, 0.5 M NaOH and distilled water in that order. The gel was equilibrated with buffer and introduced into a 20 × 80 mm column, fitted with a sintered glass filter, with the usual precautions.

RESULTS

Hyaluronate lyase purification

The method of purification included the following steps: (a) Acetone-dried testicular material was passed through a meat grinder and extracted for 4 h at 4° with 0.1 N acetic acid–2 N hydrochloric acid (190:10) while stirring continuously. (b) The acid extract was centrifuged at 4° and the supernatant containing the crude enzyme was 30% saturated with ammonium sulphate. The inactive proteins precipitated at this stage were removed by centrifugation. (c) The ammonium sulphate concentration in the supernatant was raised to 60% saturation and the mixture was kept 20 h at 4° to complete precipitation of the enzymes (and inactive proteins). (d) The precipitate (preparation I) was collected by centrifugation and dialysed against bidistilled water at 4° until the sulphate had been removed. (e) The dialysate was treated with chloroform (2:1 v/v) and stirred mechanically for 15 min at 4°. (f)

The solution was again dialysed against distilled water for 24 h at 4°. (g) The dialysate was concentrated by dialysis against polyvinylpyrrolidone, distributed in vials and lyophilized. A white powder readily soluble in water and enzymically active was obtained (preparation II). (h) Chromatography on DEAE-Sephadex column, the column was equilibrated with 0.02 M phosphate buffer (pH 6.0; I 0.08). 2 ml of a solution of preparation II (containing 48 mg protein and 450 TRU) were placed on top of the column and washed into it with the buffer used for equilibration. The flow rate was 0.5 ml/min. Fractions of 5 ml were collected. In each fraction proteins were determined according to LOWRY's[9] modified method[10].

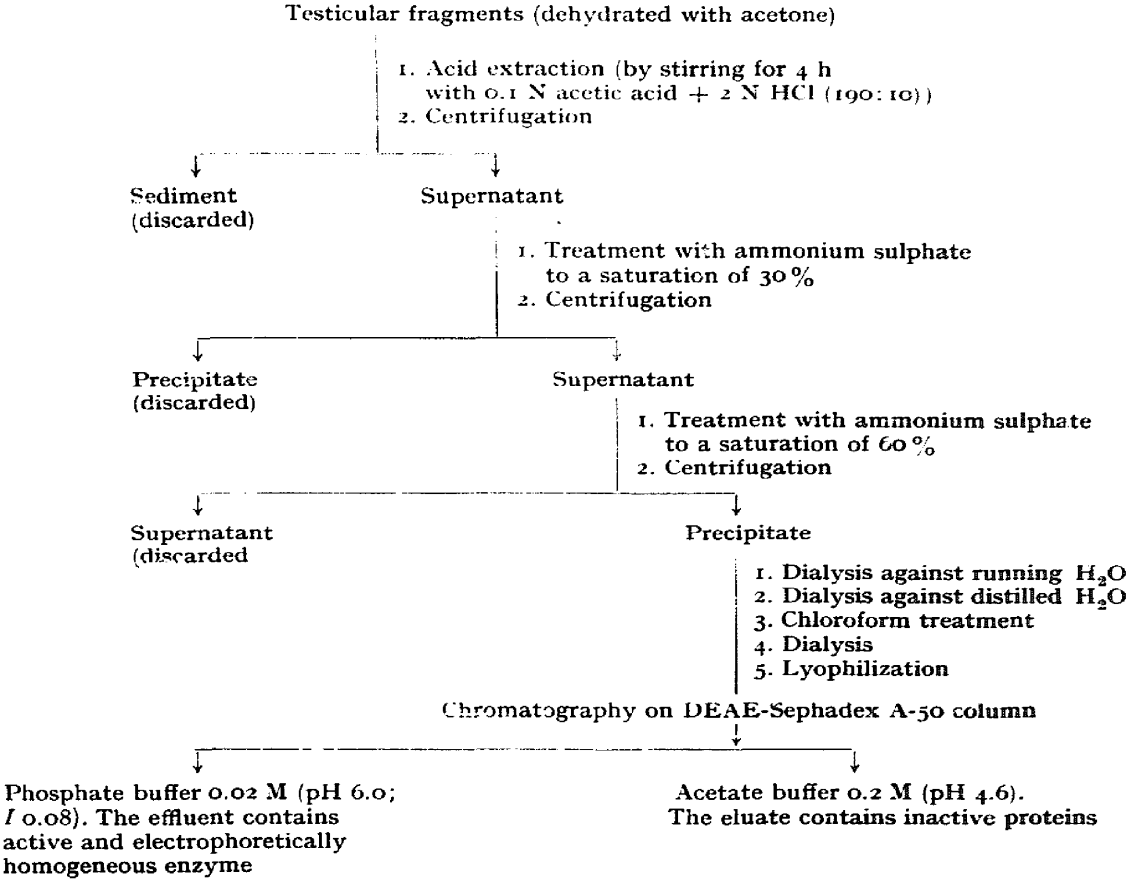

Fig. 1. Method of purification of hyaluronate lyase (All operations are carried out at 4°)

Chromatographic fractionation and preparation of the enzyme are summed up in Fig. 1. In our experimental conditions the active fraction was not adsorbed but passed very rapidly into the effluent. A first peak was recorded (Peak 1) at 20 ml, which coincided with the major peak of enzymic activity (Fig. 2).

Elution was continued with the same buffer solution and 5-ml fractions were collected until no proteins could be detected in the effluent (total of 100 ml). Sodium acetate buffer, 0.2 M (pH 4.6) was introduced into the column and the collection of 5-ml fractions was continued, each fraction being tested for protein. After collecting

about 30 ml the second major protein peak (Peak 2) was recorded. This protein displayed no enzymic activity. Elution with the same buffer solution was continued until no protein was demonstrable in the eluate. Elution was continued with 0.17 M NaCl followed by 0.38 M NaCl to yield traces of protein without enzymic activity (Eluates 160–180 ml (Fig. 2) and 300–400 ml (Fig. 3)).

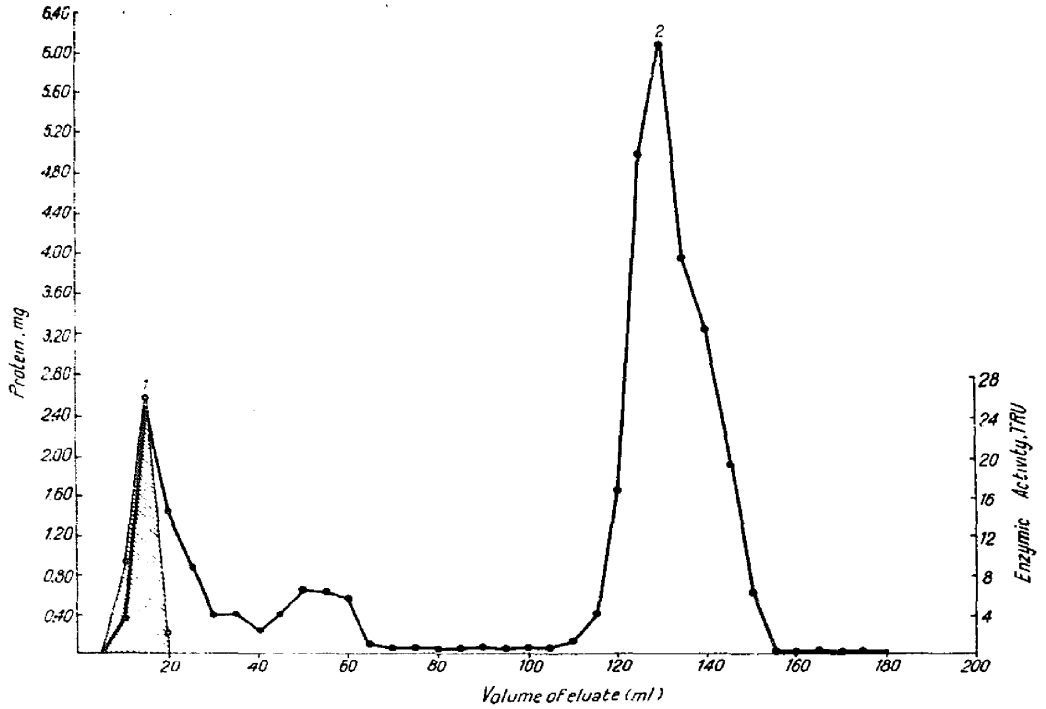

Fig. 2. Chromatography on DEAE-Sephadex. 5-ml fractions collected. Eluates 5–40 ml: 0.02 M phosphate buffer (pH 6.0; I 0.08). Enzymically active protein. The hatched area represents enzymic activity as determined in each 5-ml fraction. Eluates 40–180 ml: acetate buffer (pH 4.6). Enzymically inactive protein.

In some experiments, after elution with 0.02 M phosphate buffer, at I 0.08, another phosphate buffer, 0.05 M (pH 6.0; I 0.20) was used as an eluent, followed by the acetate buffer (pH 4.6). The second phosphate buffer (0.05 M) eluted some inactive protein (Fig. 3).

Enzymic activity was confined to fractions 5–20. The largest protein fraction was eluted by sodium acetate buffer (pH 4.6).

The homogeneity of the eluted fractions was checked by paper electrophoresis in phosphate buffer (pH 6.0; I 0.24) and veronal buffer (pH 8.0; I 0.05), after dialysis against polyvinylpyrrolidone.

Fig. 4 shows the electrophoretic patterns of the crude preparation before passage through the column (a) and of the fractions corresponding to the two peaks, (b) 0.02 M phosphate buffer eluate (Peak 1) and (c) 0.2 M acetate buffer eluate (Peak 2).

The fractions corresponding to enzymically active protein (Peak 1) consisted of single electrophoretic band close to the starting line (b) (Fig. 4), confirming the low mobility found by other authors[11].

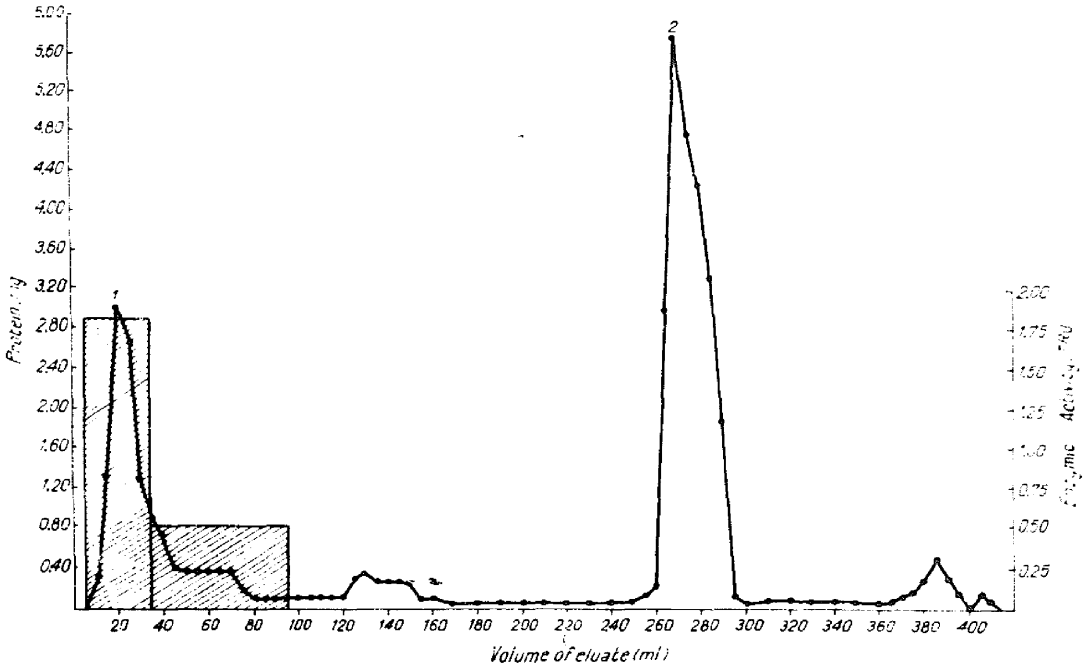

Fig. 3. Chromatography on DEAE-Sephadex. Eluates 5–95 ml represent the enzymically active protein eluted with 0.02 M phosphate buffer (pH 6.0; I 0.08). The hatched area corresponds to a mixture of the fractions eluted in these conditions and containing active enzyme. Eluates 95–360 ml: 0.05 M phosphate buffer (pH 6.0; I 0.20). Enzymically inactive protein. Eluates 360–400 ml: 0.38 M sodium chloride solution. Enzymically inactive protein.

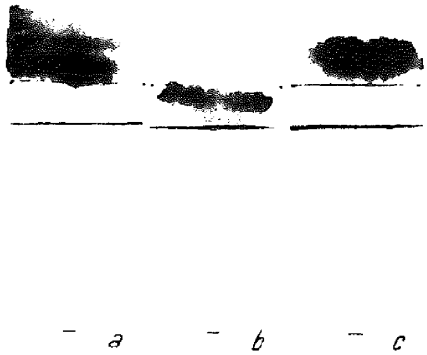

Fig. 4. Electrophoresis on Whatman paper No. 1. 0.06 M phosphate buffer (pH 6.0) (a) crude preparation (b) enzymically active fraction corresponding to Peak 1 (c) enzymically inactive protein corresponding to Peak 2 (Figs. 2 and 3).

The fractions corresponding to the peak of the major enzymically inactive protein (Peak 2) likewise displayed a single electrophoretic band which, however, had a higher mobility than the enzymically active protein (c) (Fig. 4)

Of 48 mg protein chromatographed 41,613 mg were recovered.

Of 450 TRU put on the column, 227 TRU were recovered after dialysis of the active effluent against distilled water and concentration against polyvinylpyrrolidone. Enzyme activity is not lost during passage through the column but on dialysis and concentration of the effluents. Since the lability increases with the degree of purity of the enzyme[13,14], it may be possible to circumvent this loss by the use of a specific stabilizer[6,12].

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