THE PURIFICATION OF TESTICULAR HYALURONIDASE BY CHROMATOGRAPHY ON A MIXED COLUMN

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In a previous paper[1] the authors reported a method for obtaining a highly purified testicular hyaluronidase whereby in the last step of the purification procedure chromatography on diethylaminoethyl (DEAE)-Sephadex A-50 (medium) was used. As the method described involved quite a number of preliminary stages, we tried to avoid some of them by introducing a chromatographic procedure which could realize the desalting and the purification steps under simpler conditions and with greater efficacy. This was achieved by utilising a single column containing two layers, one consisting of the desalting gel and the other of the ion exchanger.

MATERIALS AND METHODS

The extract of the crude preparation of the hyaluronidase from the testicular material, the method of determination of the enzymic activity, and the preparation of the hyaluronic acid used as substrate were described in detail in our previous publication[1].

Preparation of the mixed chromatographic column

A 400 × 42 mm plastic column with a sintered filter was carefully packed up to a height of 150 mm with a DEAE-Sephadex A-50 (medium) suspension in phosphate buffer (pH 6.0, 0.02 M, $\mu = 0.08$)[1]. After the completion of the packing, in a vertical position, of the DEAE-Sephadex on to its upper, perfectly horizontal layer, a suspension of Sephadex G-75 (or G-100) in the same buffer is introduced, taking care not to disturb the surface. This gel layer is left to settle up to a height of 250 mm. The mixed column therefore consists of two layers, one above the other, with a total height of 400 mm, permitting a continuous flow during chromatography on two different gel suspensions. The upper layer (Sephadex G-75 or G-100) achieves the desalting stage by gel filtration[2], while the bottom layer carries out the purification by ion exchange (DEAE-Sephadex A-50*)[1].

RESULTS

The entire process of purification includes the following stages:

(a) Acetone-dried testicular material is 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.

* Supplied by Pharmacia, Uppsala, Sweden.

(b) The acid extract (protein: 10.6 mg/ml; activity: 2.40 turbidity reducing units (TRU) per mg protein) is centrifuged at 4° and the supernatant containing the crude enzyme is 30% saturated with ammonium sulphate and the precipitate discarded after centrifugation.

(c) The ammonium sulphate concentration in the supernatant is raised to 60% saturation and the mixture kept for 20 h at 4°.

(d) The precipitate obtained by 60% saturation is collected by centrifugation and, after solubilization in the same phosphate buffer as is used for the equilibration of the gels (pH 6.0, 0.02 *M*, μ = 0.08), is submitted to the next step of purification.

(e) Chromatography on the mixed column (effected at 4°). The enzymically active protein solution (40 ml containing 13 mg protein/ml, with an activity of 57 TRU/mg protein and 7.6 g ammonium sulphate) was placed on the top of the column and introduced into it at intervals corresponding to a flow rate of 0.25 ml/min. Fractions of 5 ml were collected. The first 240 ml were free of protein and ammonium sulphate (tested by the Nessler reagent and also by barium chloride). The next 100 ml of effluent, collected at the same rate (0.25 ml/min), in 5 ml fractions, contained the enzymically active protein corresponding to 0.46 mg protein/ml and an activity of 12.751 TRU/mg protein, representing therefore a high degree of purity. The next 130 ml of effluent contained the largest amount of protein, but with very little enzyme activity (37 TRU/mg protein), the bulk of enzyme activity being confined to frac-

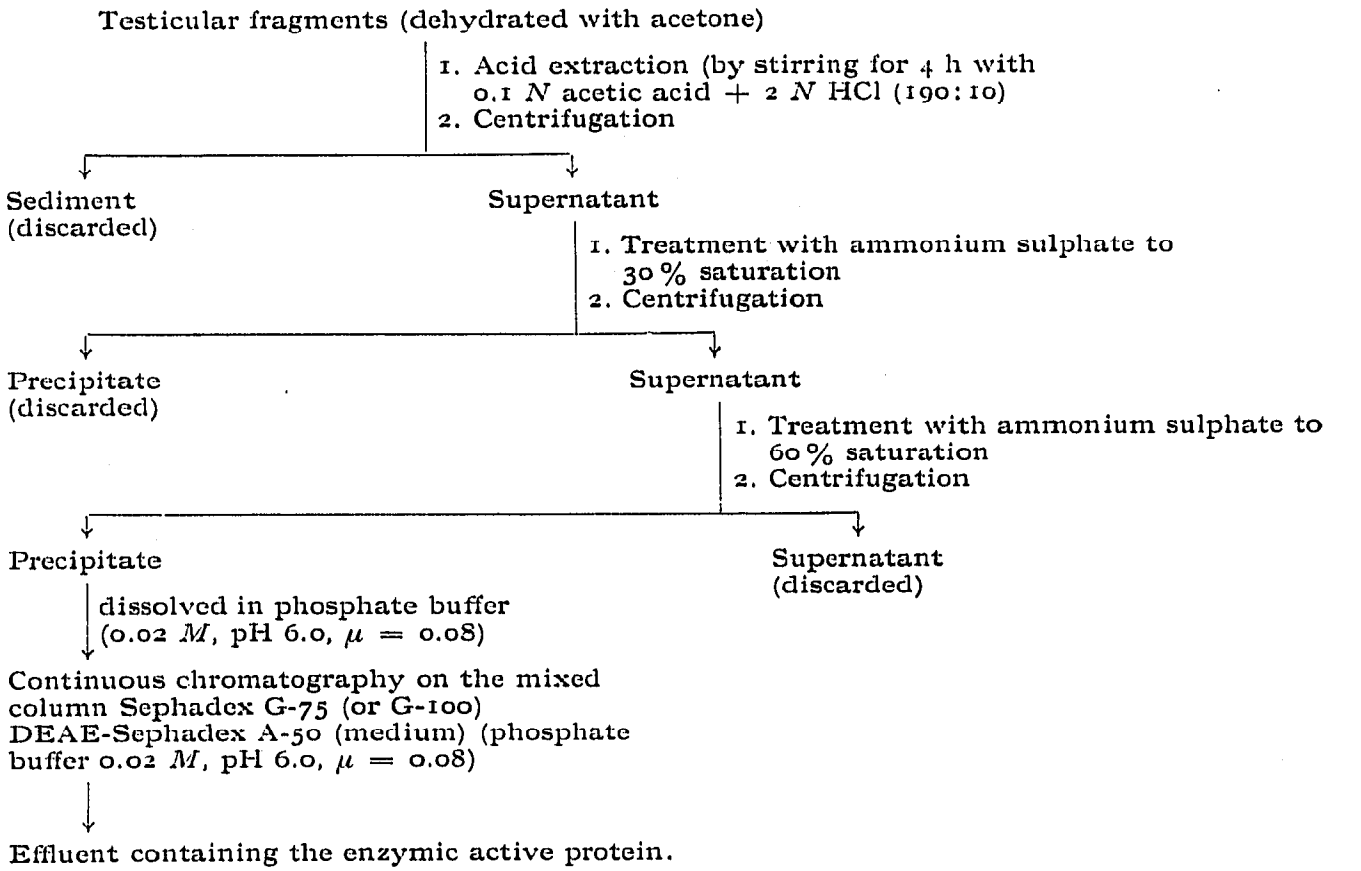

Fig. 1. Scheme of purification procedure for testicular hyaluronidase (all operations are carried out at 4°).

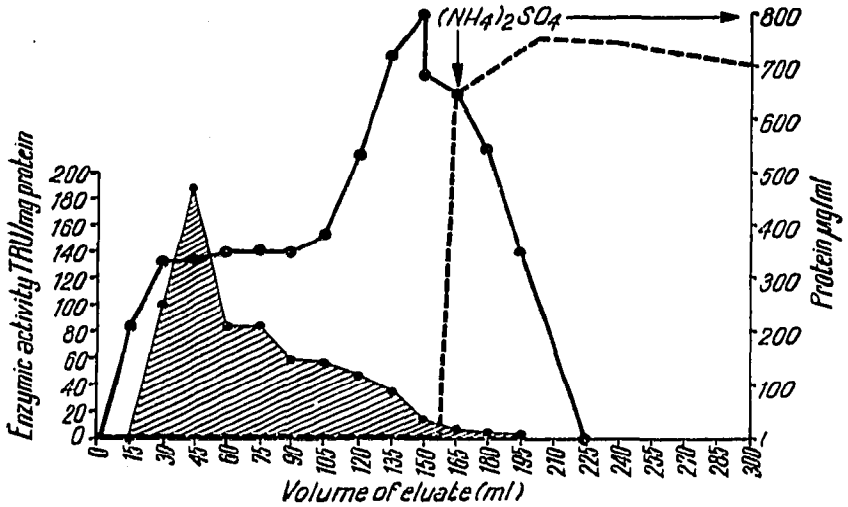

Fig. 2. Chromatography of testicular hyaluronidase on a Sephadex G-75–DEAE-Sephadex A-50 mixed column, 5 ml fractions collected (0.25 ml/min). Eluant: phosphate buffer (pH 6.0, 0.02 *M*, μ = 0.08). The hatched area corresponds to the fractions containing the active enzyme, the —·—·—· area corresponds to the fractions containing the inactive protein, and the — — — area corresponds to the zone of the $(NH_4)_2SO_4$ effluent.

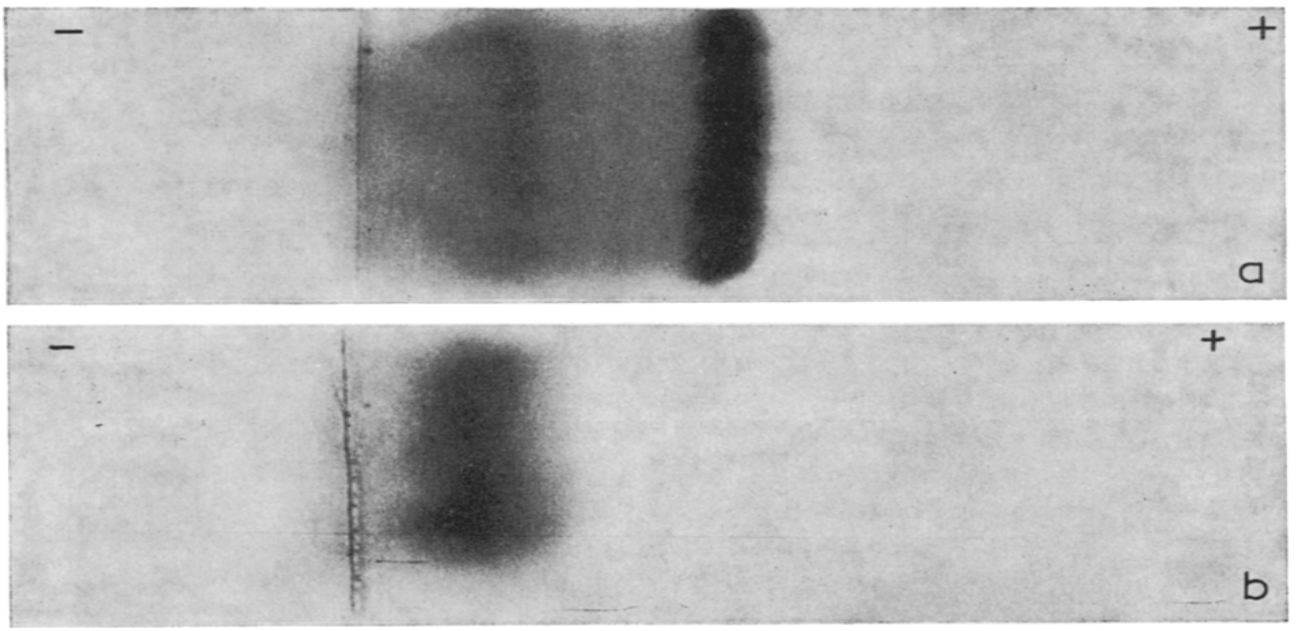

Fig. 3. Electrophoresis on Whatman No. 1 paper (0.06 *M* phosphate buffer, pH 6.0). (a) The crude preparation introduced into the column; (b) the eluates of the enzymically active fractions.

tions 1–20 (100 ml). The presence of ammonium sulphate could not be detected, either in the fractions containing the enzyme or in the fractions containing the inactive protein; it appears only in the subsequent fractions. The desalting process is thus efficient; the ammonium sulphate (7.6 g contained in the precipitate obtained at 60% saturation) remains absorbed on the column until the enzymically active proteins and the bulk of the inactive proteins have been completely eluted (reaction for ammonium ion with the Nessler reagent, for sulphate ions with the barium reagent). It was noted that the penetration of the ammonium sulphate into the DEAE-Sephadex layer has a shortening effect on it, but does not disturb the purification process. The purification stages described are summed up in Fig. 1. A representative experiment of a chromatographic separation on the mixed column where the desalting and purification of the crude testicular hyalyronidase is carried out in one step is illustrated in Fig. 2. The enzymically active protein is electrophoretically homogeneous (Fig. 3).

SUMMARY

A chromatographic method which achieves the desalting and purification of a crude hyaluronidase preparation (prepared by 60% ammonium sulphate saturation) in a single step by use of a mixed chromatographic column is described. The mixed column (400 × 42 mm) consists of a DEAE-Sephadex A-50 (medium) layer (150 mm in height), on top of which there is placed a Sephadex G-75 (or G-100) layer (250 mm in height); both layers are equilibrated with a phosphate buffer (pH 6.0, 0.02 *M*, $\mu = 0.08$). During the elution of the hyaluronidase with the same buffer, the inactive proteins and the ammonium sulphate were retained on the column; therefore an efficient purification of the enzyme can be achieved. The method described gives a good yield and a highly purified product under simplified experimental conditions.

REFERENCES

1 E. SORU AND F. IONESCU-STOIAN, *Biochim. Biophys. Acta*, 69 (1963) 538.
2 P. FLODIN, *Dextran Gels and their Applications in Gel Filtrations*, Pharmacia, Uppsala, 1962.