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PURIFICATION OF HUMAN SERUM HYALURONIDASE USING CHROMATOFOCUSING

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SUMMARY

A commerical chromatofocusing system was applied to Cohn's fraction III of human serum to purify hyaluronidase (E.C. 3.2.1.35.). The protein that eluted in the pH range 4.7–5.3 was pooled and precipitated by adding ammonium sulphate to 50% saturation. This sequence of fractionation purified hyaluronidase extensively by immunological criteria. It is shown that hyaluronidase is a population of enzymes displaying microheterogeneity. The commercial chromatofocusing system behaved as theoretically expected. The capacity of the gel is 3 mg per ml gel. Any overload will be trapped or precipitated in the gel. The gel is easy to handle and did not deteriorate on repeated use.

INTRODUCTION

Interest is growing in the distribution, organization, metabolism and functional aspects of proteoglycans and glycosaminoglycans. The analysis of these protein polysaccharides is at a quite an advanced stage, and employs chromatographic, enzymatic, spectophotometric and electrophoretic procedures. On the other hand, little has been done on elucidating the human hyaluronidases, which are the central catalytic enzymes in the metabolism of hyaluronic acid, chondroitin-4-sulphate and chondroitin-6-sulphate. There are several reasons for this. First, the catalytic activity is very low with the consequence that long reaction times are needed to obtain sufficient sensitivity in the enzymatic assays. Secondly, there are endogenic inhibitors in plasma and tissue extracts[1–4], which makes interpretation of enzymatic results very difficult. Finally, these difficulties impede exact localization of hyaluronidase in tissues.

The solution to some of these problems is to obtain monospecific antibodies to hyaluronidase. Purified hyaluronidase is required for this purpose. Only a few attempts have been made to obtain purified human serum or lysosomal hyaluronidase[5–7]. This paper describes an efficient procedure for purifying human serum hyaluronidase using alcohol fractionation, chromatofocusing and ammonium sulphate precipitation.

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MATERIALS

Chromatofocusing gel PBE 94, Polybuffers 96 and 74, Sephadex G-25, IEF-agarose, Pharmalyte 2.5–6.0 and isoelectric focusing standards were from Pharmacia (MEDA, Copenhagen, Denmark). Hyaluronic acid (H 1751) was from Sigma (St. Louis, MO, U.S.A.). Rabbit immunoglobulins raised against human serum (100 SF and 100 SG), transferrin (10-061) and immunoglobulin G (IgG) (10-090) were from DAKO-Immunoglobulins (Copenhagen, Denmark). All other reagents were the purest commercial products obtainable.

METHODS

Serum was obtained as a freeze dried product of Cohn's fraction III, method 6 (ref. 8). It was solubilized in 50 m*M* sodium acetate buffer pH 3.7 and dialyzed at 4°C for 2 days against 100 volumes of the same buffer with four equally spaced shifts. It was then centrifuged at 20,000 *g* for 1 h at 4°C and the precipitate discarded. The supernatant (Cohn III) was processed further as described below.

Chromatofocusing

A 10-ml volume of PBE 94 was equilibrated in 25 m*M* histidine hydrochloride buffer pH 6.0. It was packed on a glass column (1.6 × 5 cm) overlaid with 1 cm Sephadex G-25 and washed overnight in the histidine buffer. Cohn III was dialyzed in 10 m*M* histidine buffer pH 6.3 at 4°C for 2 days and centrifuged at 1500 *g* for 15 min. 5 ml (14 mg/ml) were applied to the column followed by 5 ml histidine buffer pH 6.0. The column was eluted with 300 ml Polybuffer 74 diluted 1:8 in degassed deionized water adjusted to pH 4.0. 10-ml fractions were collected.

On a preparative scale, 100 ml PBE 94 were equilibrated in 25 m*M* Tris–acetic acid pH 7.3, packed on a glass column (2.6 × 19 cm) overlaid with 1 cm Sephadex G-25 and washed overnight. Because the pH dropped in the cold to about 6.5, it was decided to wash the column with two volumes of Polybuffer 96 diluted 1:13 and adjusted to pH 6.0 with acetic acid. Cohn III was dialyzed against 25 m*M* Tris–acetic acid pH 7.3 for 2 days, centrifuged and 28 ml (26 mg/ml) were applied to the column. The sample was followed by 20 ml of Polybuffer 96 and the column was eluted with 1000 ml Polybuffer 74 as described above.

To test the capacity of PBE 94, 2 ml Cohn III (16 mg/ml) were run on a 10-ml column (1.6 × 5 cm) exactly as described in the previous paragraph.

The eluates were pooled and precipitated by 50% or 100% ammonium sulphate (see Results). Precipitates were resuspended in and dialyzed against 50 m*M* sodium acetate buffer pH 3.7.

Electrophoresis

Fused rocket* and crossed immunoelectrophoresis (CIE) were done essentially as described[9].

Isoelectric focusing was done exactly as described in the manual (Pharmacia) using IEF-agarose and Pharmalyte 4–6.5. One lane from the isoelectric focusing was used as a one-dimensional gel in CIE. 1% agarose gel in barbiturate buffer containing

* In the fused rocket procedure the samples from the PBE-columns are placed successively in two slightly displaced parallel rows of sample wells in an agarose gel. The proteins are allowed to diffuse out of the wells for 45 min. Finally the proteins are electrophoresed overnight (2 V/cm) into an antibody-containing gel (see *e.g.* Fig. 2A).

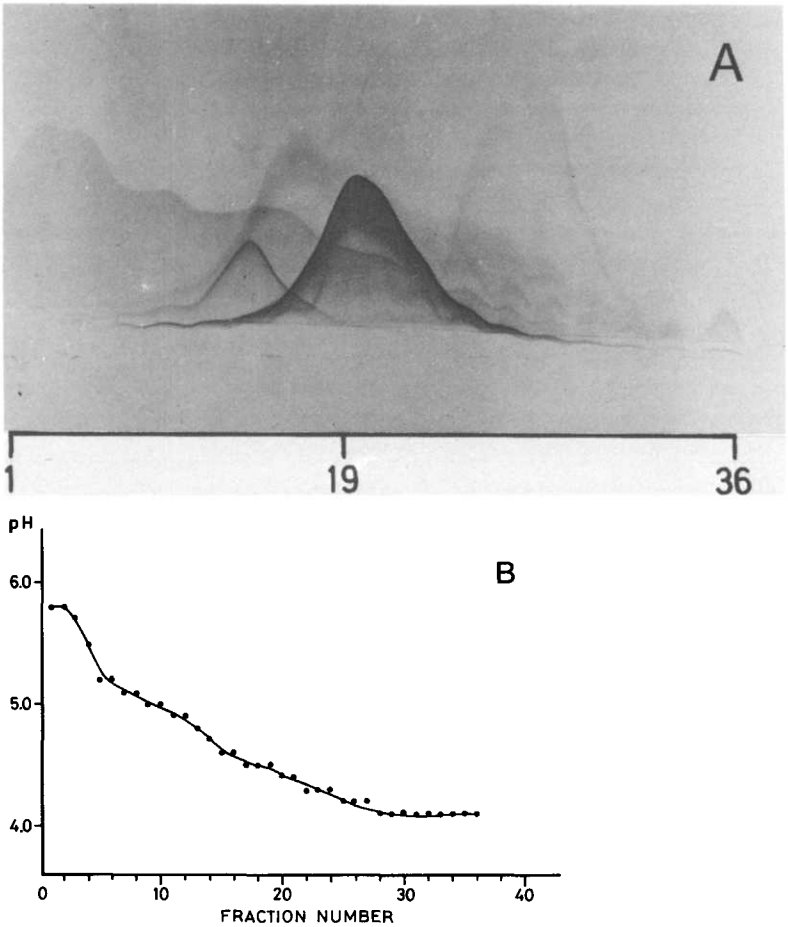

Fig. 1. Fused rocket of the eluates from 10-ml PBE 94 chromatofocusing in the pH range 6.0–4.0 (A). pH-profile from the same chromatofocusing (B). 10-ml Fractions were collected.

polyspecific antiserum (100 SG, DAKO) was cast on a clean glass plate. The IEF lane was transferred and laid parallel to but not in contact with the antibody containing-agarose gel. The sample lane was then moulded in place with 1 % agarose in barbiturate buffer. A potential of 2 V/cm was applied overnight, the cathode being at the IEF lane.

Enzyme assay

The optimum pH for hyaluronidase was found to be 3.7. The final reaction mixture contained 0.2 *M* acetate, 1.5 m*M* saccharolactone, 120 m*M* NaCl, 0.1 % bovine serum albumin, 225 μg hyaluronic acid and enzyme in a total volume of 250 μl. The reaction proceeds at 37°C for 20 h and was stopped by adding 50 μl of 0.86 *M* NaOH. N-Acetylglucosamine end-groups were determined as described previously[10]. One unit of activity was defined as 1 μmol liberated end-group per hour. Polybuffer did not influence the assay or enzymatic activity.

Protein was determined according to Layne[11].

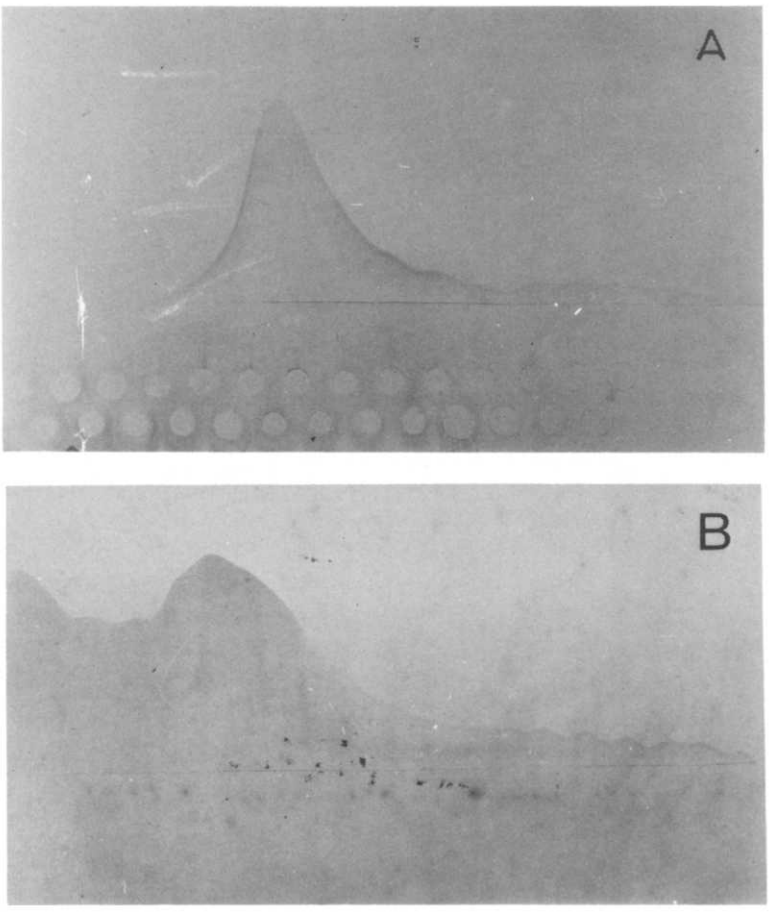

Fig. 2. Fused rocket of the eluates from the 100-ml PBE column. Monospecific antibodies against transferrin (A) and IgG (B) was used. Compare with Fig. 1.

RESULTS

A typical fused rocket immunoelectrophoresis of eluates from a PBE 94 column is shown in Fig. 1A, with the pH-profile in Fig. 1B. Compared with the CIE of isoelectric focusing (not shown), the apparent pH of elution of the proteins, *e.g.*, albumin, is lower than the real p*I* of the proteins. This is in accordance with the theory developed by Sluyterman and co-workers[12,13].

The same pattern emerged when the 100-ml column was used and when the starting buffer was changed from histidine hydrochloride to Tris–HCl. The manufacturers[14] recommend the histidine buffer for this pH range (4.0–6.0) but about 50% of the protein precipitated, without any purification of hyaluronidase. The buffer was therefore changed to Tris–HCl, which preserved almost all the protein.

Fig. 2 shows the fused rocket from a 100-ml PBE column using antibodies against human transferrin and Igb, respectively. These proteins elute in the expected

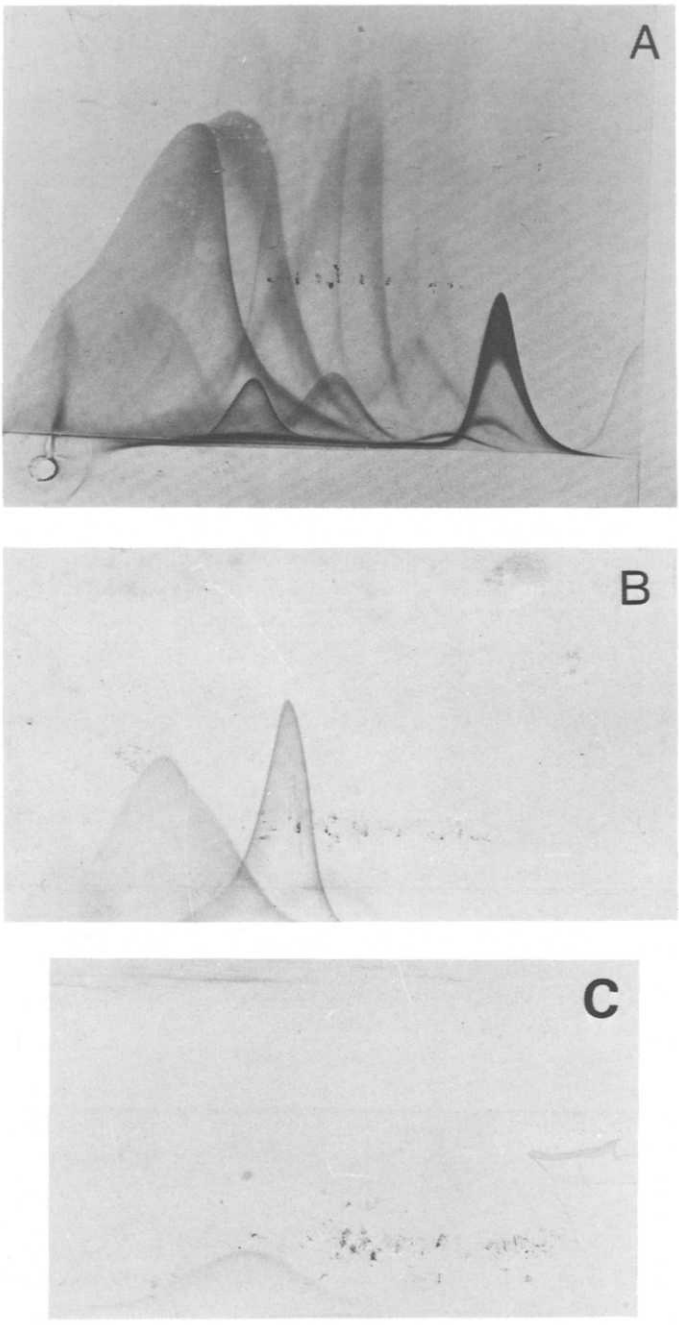

Fig. 3. One-dimensioned CIE using polyspecific antibodies against human serum: A, Cohn III; B, pooled fraction pH 4.7–5.3 from the 100-ml chromatofocusing column; C, the precipitate from 50% saturation with ammonium sulphate of the pool in B. As can be seen in C, only IgG and an unidentified precipitate with β-mobility were left behind.

TABLE I

ELUATES FROM THE 100-ml PBE COLUMN POOLED AND CONCENTRATED WITH AMMONIUM SULPHATE (SEE TEXT)

All fractions were dialyzed before they were analyzed.

Fraction	*Hyaluronidase (mU)*	*Protein (mg)*
Cohn III	1632	728
pH 5.4–6.0	4.5	48
pH 4.7–5.4		
50% Ammonium sulphate		
precipitate	207	44
supernatant	2	13
pH 4.0–4.7	16	8
1 *M* NaCl	456	302

pH ranges. The maximum capacity of PBE 94 was 3 mg per ml gel. If more protein is applied to the column it will partly be trapped in the column. It could be desorbed by 1 *M* NaCl. The absorption is non-specific.

Hyaluronidase could not be detected in the eluates, because of dilution. The fractions in the pH range 4.7–5.3 from the 100-ml PBE column were therefore pooled and solid ammonium sulphate was added to 50% saturation. The precipitate that developed after 16 h at 4°C was centrifuged at 1500 *g* and 4°C for 15 min, resuspended in 10 ml of 50 m*M* sodium acetate buffer pH 3.7 and dialyzed against the same buffer. Fig. 3B and 3C show the CIE of the pool and the precipitate, respectively. The purification achieved by these procedures is obvious using immunological criteria.

Table I shows the data from the 100-ml column. Only 13% of the hyaluronidase eluted in the expected pH range, 28% were trapped and the rest was lost or denatured. The fractions in the pH ranges 4.0–4.7 and 5.3–6.0 were pooled and saturated with solid ammonium sulphate. The precipitates and the supernatant from 50% ammonium sulphate precipitate described above were dialyzed against 50 m*M* acetate buffer pH 3.7. Practically no activity could be measured in these fractions. If 3 mg protein per ml gel were applied to the column, 40% of activity could be regained, but there was an obligatory loss of 60% activity.

DISCUSSION

Yamada *et al.*[5] partially purified hyaluronidase from human placenta using freezing, thawing, extraction, ammonium sulphate precipitation, ion exchange and gel filtration. They claimed to have an enzyme of 82% purity based on polyacrylamide gel electrophoresis (PAGE).

However, this is not a safe way to determine purity because the unspecific purification procedures cannot guarantee that only one protein, *e.g.*, hyaluronidase, is present in the main band of the PAGE. Besides, the identification of hyaluronidase in PAGE was indirect.

Nevertheless, this is the most pure human serum hyaluronidase which has so far been reported. In the present paper the use of chromatofocusing for purifying human serum hyaluronidase was investigated. A new commercial system from Pharmacia was explored. It is quite clear from Fig. 3A–C that extensive purification of

hyaluronidase based on immunological criteria was achieved. Tripling the antigen amount in the one dimension of CIE did not reveal new antigenic determinants in the preparation; *i.e.*, only three impurities are left behind after this sequence of separation techniques.

The exact pH at which hyaluronidase eluted in the chromatofocusing system could not be assessed because of dilution. However, all the hyaluronidase that eluted in the gradient was eluted in the pH region 4.75–5.34 in agreement with the p*I* of hyaluronidase[5]. No correction for observed p*I* was made according to Sluyterman and co-workers[12,13]. Theoretically, chromatofocusing should have a concentrating effect on a homogeneous protein. The dilution of hyaluronidase indicates fractionation into several hyaluronidases, reflecting the microheterogeneity of human serum hyaluronidase.

The 50% ammonium sulphate precipitate of the pH 4.7–5.3 fraction from chromatofocusing is a potential source for raising antibodies in rabbits and for production of radioactive tracers, to be used in a radioimmunoassay (RIA). The preparation is not pure and the antiserum is not monospecific, but preliminary experiments have shown that a combination of absorption of antibodies and gel chromatography of the iodinated antigen (tracer) will produce suitable tools for a RIA system and will be described in a future paper.

To the author's knowledge, the technique of chromatofocusing has not formerly been used to fractionate human serum proteins. The PBE gel performed well. It is quite easy to handle and no deterioration was observed by repeated use. It is essential for obtaining a smooth gradient and to reach the low pH range that the buffers are degassed. By adjusting the protein load to just below maximum capacity, up to 40% of enzyme activity could be regained in the pH region of the isoelectric point of hyaluronidase.

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