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PURIFICATION OF BULL SPERM HYALURONIDASE BY CONCANAVALIN-A AFFINITY CHROMATOGRAPHY

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Summary

A new method for obtaining highly purified hyaluronidase (hyaluronate glycanohydrolase EC 3.2.1.25) in high yield is described. Bull seminal plasma was fractionated with $(NH_4)_2SO_4$ and the 30 to 65% saturation fractions were applied to a DEAE-cellulose column. The first protein peak contained hyaluronidase, β-*N*-acetylglucosaminidase and β-glucuronidase. The latter two enzymes were separated by gel filtration on Sephadex G-200. The hyaluronidase was further purified by a Concanavalin-A Sepharose 4B affinity column. By gradient elution with α-methyl-D-glucoside a fraction which had a specific activity of 1998 units/mg protein (57 942 National Formulary Standard units/mg protein) was obtained. The highly purified enzyme showed one major protein band on acrylamide gel electrophoresis at pH 4.3. The purified hyaluronidase did not show any β-glucuronidase or β-*N*-acetylglucosaminidase activities. The percent yield of purified hyaluronidase calculated on the basis of total activity was ten times higher than by any previous method [Yang, C.H. and Srivastava, P.N. (1975) J. Biol. Chem. 250, 79—83].

Introduction

It is well established that sperm-acrosomal hyaluronidase is the enzyme a spermatozoon utilizes to penetrate the cumulus investment of the ovum. The inhibition of hyaluronidase by its isoantibodies blocks sperm penetration through the cumulus [1]. For immunological studies a large amount of pure enzyme is required. Hyaluronidase of varying degree of purity has been obtained from bull sperm acrosomal extracts [2] and from bull testes [3]. In fact, testicular hyaluronidase is the major source of the commercially available

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enzyme. By conventional methods of purification such as $(NH_4)_2SO_4$ fractionation, gel filtration and ion exchange chromatography hyaluronidase of the highest specific activity was obtained from bull seminal plasma [4]. Since, bull testicular, sperm acrosomal and seminal plasma hyaluronidase preparations are enzymatically and immunologically identical [2,4] bull seminal plasma appears to be a good and readily available source of this enzyme. No attempt appears to have been made to purifiy hyaluronidase by affinity chromatography. Recent reports show that Concanavalin-A has specific binding sites for α-D-glucosyl and sterically related residues of many polysaccharides and glycoproteins [5]. Concanavalin-A is now commercially available in an immobilized form as Concanavalin-A Sepharose for the isolation of glycoproteins.

This report described the initial separation of other glycosidases from hyaluronidase and further purification of the enzyme by Concanavalin-A Sepharose affinity chromatography, utilizing the fact that the enzyme is known to contain these carbohydrate residues [3].

Methods

Enzyme assays

Hyaluronidase activity was determined colorimetrically by a modification of the method of Aronson and Davidson [6], as described earlier [7]. One unit of enzyme is equivalent to 1 μmol of *N*-acetylglucosamine released per min. One unit of enzyme activity obtained by colorimetric assay is equal to 29.0 N.F.S. (National Formulary Standard) units.

β-Glucuronidase activity was measured by a modification of the method of Fishman et al. [8] using phenolphthalein-mono-β-glucuronide as substrate. The reaction was carried out in a total volume of 1 ml containing 0.1 ml of substrate (0.01 M, pH 7.0), 0.8 ml of sodium acetate buffer (0.1 M, pH 5.2) and 0.1 ml of the enzyme. After 1 h of incubation at 37°C, 5 ml of glycine · NaOH buffer (0.2 M, pH 10.4) was added. The absorbance of phenolphthalein color was read at 540 nm. One unit equals to the amount of enzyme which releases 1 μmole of phenolphthalein per min at 37°C under conditions where the assay is linear with time and protein.

β-*N*-Acetylglucosaminidase assay was carried out by the method of Tarentino and Maley [9]. One unit is equivalent to the amount of the enzyme that hydrolyzes 1 μmol of substrate per min at 37°C. Arylsulphatases activities were determined as described by Yang and Srivastava [10]. The hydrolysis of azocoll, a general substrate for proteinases, was determined by the method of Srivastava et al. [11]. The protein was determined by the method of Lowry et al. [12].

Fractionation of bull seminal plasma

Ejaculated bull semen obtained from the Artificial Breeders, Inc., stored at −20°C, was used as the starting material. After first centrifugation of semen (659 ml) at 3000 × *g* to remove sperm, the seminal plasma (494 ml; protein 76 mg/ml) was recentrifuged at 105 000 × *g* for 4 h. The supernatant (444 ml, protein 4.5 mg/ml) from second centrifugation was dialyzed against 0.01 M sodium phosphate buffer (pH 7.1) for 48 h with three changes of the buffer.

The solution after dialysis was brought to 30% saturation with solid $(NH_4)_2SO_4$ at 4°C and allowed to stand overnight. The supernatant from 30% saturation with $(NH_4)_2SO_4$ was brought to 65% saturation with $(NH_4)_2SO_4$, allowed to stand overnight at 4°C, centrifuged and the residue dissolved in glass distilled water and dialyzed against the same thoroughly to remove $(NH_4)_2SO_4$. The non-dialyzable solution was lyophilized and stored at −15°C.

DEAE-cellulose chromatography, Step I

The lyophilized material after $(NH_4)_2SO_4$ fractionation dissolved in sodium phosphate buffer (0.05 M, pH 7.3) was applied to a DEAE-cellulose column (2.5 × 35 cm) at 4°C pre-equilibrated with the buffer. The column was first eluted with 500 ml of the buffer and then with a stepwise gradient of 0.05 M to 0.05 M NaCl in the buffer at the rate of 25 ml/h. The tubes in the first peak indicated by arrows, were pooled and dialyzed against distilled water for 10 h. The non-dialyzable solution was freeze-dried.

Sephadex G-200 column chromatography, Step II

The freeze-dried material obtained from first peak of the DEAE-cellulose column (Step I) was dissolved in the sodium phosphate buffer (0.02 M) containing 0.15 M NaCl (pH 6.0) and applied to a Sephadex G-200 column (2.5 × 90 cm, V_t = 442 ml) pre-equilibrated with the buffer at 4°C. The void volume (V_o) of the column was 141 ml. The column was washed with 600 ml of the buffer at the rate of 17 ml/h. The hyaluronidase active fractions (4.7 ml/tube) were pooled and dialyzed against distilled water for 4 h. The non-dialyzable solution was freeze-dried and kept frozen.

Concanavalin-A Sepharose column, Step III

The freeze-dried hyaluronidase obtained from Step II was dissolved in 0.02 M Tris · HCl buffer (pH 6.9) containing 1.0 M NaCl, 10^{-3} M $MnCl_2$ and $CaCl_2$ (final pH 6.05) and applied to a Concanavalin-A Sepharose affinity column (0.84 × 22 cm) pre-equilibrated with the above buffer. Using 60 ml of the above buffer, the column was eluted at the rate of 13 ml/h and then developed with continuous gradient of 0 to 0.6 M of α-methyl-D-glucoside in 0.02 M Tris · HCl buffer (pH 6.9) containing 1 M NaCl. The third protein peak showing high constant hyaluronidase activity across the peak was pooled and concentrated by diaflo (Amicon, PM-10 filter) with successive addition of Tris · HCl buffer (0.02 M, pH 6.9) containing 1 M NaCl. The concentrated highly-purified hyaluronidase was kept frozen.

Acrylamide gel electrophoresis

Disc gel electrophoresis was performed at pH 4.3 and 5.3 (current density, 1.5 mA per gel, 3 h) on 7½% gel by the method of Brewer and Ashworth [13]. Methyl green was used as a tracking dye. The gels were stained with 1% amido black in 7% acetic acid and were destained with 10% acetic acid.

Results and Discussion

Bull sperm obtained by first centrifugation and the pellet of the second high speed centrifugation contained negligible amount of hyaluronidase com-

TABLE I

SUMMARY OF PURIFICATION OF BULL SPERM HYALURONIDASE

Step	Total protein (mg)	Total activity	Specific* activity	Relative activities	Yield
Bull seminal plasma 105000 × *g* super	1996	33932	17	1	100
$(NH_4)_2SO_4$ Fractionation	753	27861	37	2	82
DEAE-cellulose chromatography	168	21168	126	7	62
Sephadex G-200	24	9432	393	23	28
Con canavalin-A Sepharose	3.4	6793	1998	117	20

* 1 unit/mg protein = 29 N.F.S. units.

pared to the 105 000 × *g* supernatant. Table I shows the quantitative data on the purification. The percent yield of the final preparation calculated on the basis of total activity was ten times higher than by a previous method [4]. Four separate protein fractions were obtained by the DEAE-cellulose column (Step I). The first fraction eluted with buffer alone contained all three enzyme activities, i.e. hyaluronidase, β-*N*-acetylglucosaminidase and β-glucuronidase (Fig. 1). The first peak did not show any arylsuphatase and Azocoll hydrolysis activity. The hyaluronidase was separated from the other two enzymes by the gel filtration (Step II). As shown in Fig. 2, hyaluronidase activity was present in tubes 41 through 54 (first peak) and in tubes 55 through 68 (second peak). First peak also contained β-glucuronidase and β-*N*-acetylglucosaminidase activities. The second peak containing hyaluronidase was free from these two enzymes when tested on their respective synthetic substrates. The elution of the gel column with the buffer containing NaCl is essential to obtain separation of the first peak containing β-glucuronidase and β-*N*-acetylglucosaminidase and hyaluronidase and the second peak containing only hyaluronidase. It appears that NaCl prevents the protein-protein interaction between the enzymes.

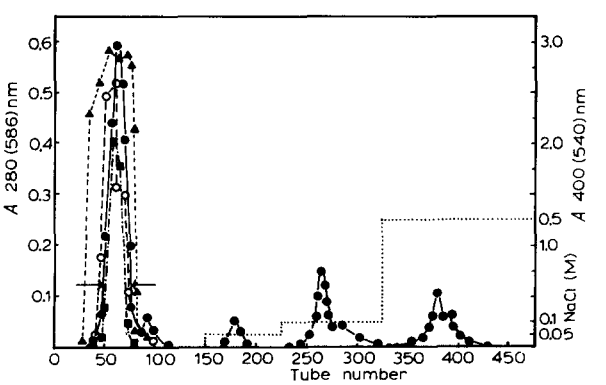

Fig. 1. Elution profile of active fractions from $(NH_4)_2SO_4$ fractionations by DEAE-cellulose column chromatography (Step I). The column was eluted with 0.05 M sodium phosphate buffer (pH 7.3) and developed with stepwise gradient of 0.05 M to 0.5 M NaCl. The column dimension was 2.5 × 38 cm and 5 ml fractions were collected. ●———●, protein; ▲- - - - -▲, β-*N*-acetyl-D-glucosaminidase; ■. — . —■, β-glucuronidase; ○— — —○, hyaluronidase.

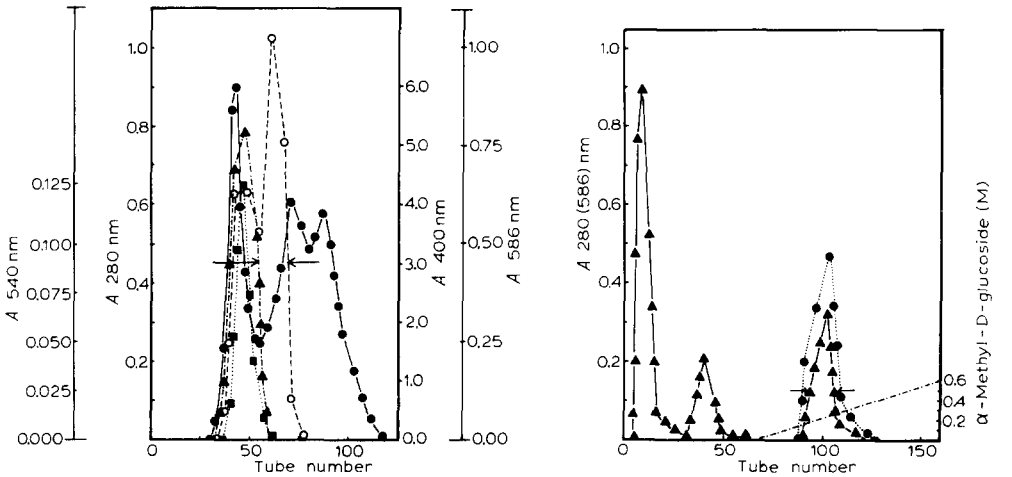

Fig. 2. Elution profile of Sephadex G-200 column chromatography (Step II). The column (2.5 × 90 cm) was developed with 0.02 M sodium phosphate buffer containing 0.15 M NaCl (pH 6.0) ●———●, protein; — — — —, hyaluronidase; ▲, — . — . —▲, β-*N*-acetylglucosaminidase; ■- - - - -■, β-glucuronidase.

Fig. 3. Elution profile of active fractions from Sephadex G-200 column by Concanavalin-A Sepharose affinity chromatography (Step III). The column (0.84 × 22 cm) was developed with 0.02 M Tris · HCl buffer (pH 6.9) containing 1.0 M NaCl, 10^{-3} M $MnCl_2$ and $CaCl_2$ and further eluted with gradient of 0 to 0.6 M of α-methyl-D-glucoside in 0.02 M Tris · HCl buffer (pH 6.9) containing 1.0 M NaCl. ▲———▲, proteins; ●- - - - -●, hyaluronidase.

Sephadex G-200 column chromatography in presence of NaCl is therefore essential to separate enzymes by size and is pre-requisite for Concanavalin-A Sepharose column. The assay of hyaluronidase in the first peak by the colorimetric method may be misleading as we found that highly purified β-*N*-acetylglucosaminidase reacts with hyaluronic acid to yield positive chromogen. Such interference in the colorimetric assay of hyaluronidase was also observed by Cashman et al. [14] if the enzyme preparation contained β-glucuronidase and β-*N*-acetylglucosaminidase. Therefore, the second peak of hyaluronidase, indicated by arrows, (Fig. 2) was further purified by Concanavalin-A chromatography (Step III). Hyaluronidase was eluted in a separate peak by a gradient of 0.2 M to 0.3 M of α-methyl-D-glucoside (Fig. 3).

The highly purified hyaluronidase showed no β-*N*-acetylglucosaminidase and β-glucuronidase activity. The purified hyaluronidase from Step III showed one major component with one possible minor band (Fig. 4). The protein which stayed at the origin appears to be aggregated hyaluronidase as most of higher molecular weight contaminants were removed by gel filtration. It is, therefore, reasonable to suggest that stacked protein (over 100 000 mol. wt) is the aggregated hyaluronidase. Gels run at two different pHs 4.3 and 5.3 to determine the purity gave identical results. At pH 7 and at alkaline pH pure hyaluronidase failed to migrate into the gels. The tracking dye was only faintly visible near the bottom of gels after destaining and, therefore, did not show up on the photograph. The migration pattern of the major band shown on gels was identical to that of sperm hyaluronidase reported earlier [4,7]. Thus, the major band represents hyaluronidase.

Fig. 4. Acrylamide gel electrophoresis of highly purified bull sperm hyaluronidase obtained by final Concanavalin-A Sepharose chromatography (Step III). The gels (7.5%) were run at pH 4.3 (A), and pH 5.3 (B) with 60 μg of protein each.

Borders and Raftery [3] reported that purified hyaluronidase contains 5% manosyl residue and this may then be the basis for affinity of hyaluronidase to Concanavalin-A. Finally, the method described in this paper appears to be the best means of obtaining high quantity of purified hyaluronidase.

Acknowledgements

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