Heparin–Sephadex Affinity Chromatography for Purification of Bull Seminal-Plasma Hyaluronidase

By Prakash N. SRIVASTAVA and Akhlaq A. FAROOQUI
*Reproduction Research Laboratories, Department of Biochemistry,
University of Georgia, Athens, GA 30602, U.S.A.*

(Received 23 February 1979)

Bull seminal-plasma hyaluronidase was purified 180-fold by chromatography on concanavalin A–Sephadex, heparin–Sephadex, Sephadex G-200 and Sephacryl S-200. With hyaluronic acid as the substrate, the specific activity and turnover number of purified hyaluronidase were $3.63 \mu\text{mol/min per mg}$ (104000 National Formulary units/mg of protein) and 214 min^{-1} (mol of product formed/mol of enzyme per min) respectively. Polyacrylamide-gel electrophoresis indicated that the purified enzyme migrated as a single band on 7.5 and 10 % (w/v) gels at pH 4.3 and 5.3. Bull seminal-plasma hyaluronidase was markedly inhibited by hydroxylamine, phenylhydrazine and semicarbazide. Purified hyaluronidase (1.25 munits; 1 unit = $1 \mu\text{mol}$ of *N*-acetylglucosamine liberated/min at 37°C) dispersed the cumulus clot of rabbit ova in 1 h at 22°C .

Hyaluronidase (hyaluronate 4-glycanohydrolase, EC 3.2.1.35) is an endoglycosaminidase that randomly acts on the $\beta 1 \rightarrow 4$ -*N*-acetylhexosaminide bonds of hyaluronic acid and chondroitin sulphates. The mammalian testes and semen are the richest sources of hyaluronidase, but this has been extensively purified from various mammalian tissues (Rasmussen, 1959; Soru & Ionescu-Stoian, 1965; Borders & Raftery, 1968; Zaneveld *et al.*, 1973). Several studies (Austin, 1960; Rogers & Morton, 1973; Talbot & Franklin, 1974) have indicated a relation between sperm capacitation and release of acrosomal hyaluronidase from spermatozoa. It is also well known that the spermatozoon utilizes hyaluronidase to penetrate the cumulus cell mass surrounding the ovum (McClean & Rowland, 1942; Fekete & Duran-Reynals, 1943). Metz (1972) has reported that hyaluronidase is an effective isoantigen in the female and is strongly inhibited by its isoantibodies. Russo & Metz (1974) have again shown that isoantisera obtained against rabbit semen inhibit fertilization *in vitro* by blocking penetration of cumulus and corona cells, and concluded that antibody inhibited sperm–egg interaction at the cumulus and/or corona-penetration step. Most recently, sheep immunized with highly purified ram hyaluronidase showed no decrease in fertility, although the animals exhibited circulating antibody to this enzyme (Morton, 1976, 1977).

Previous papers from our laboratory have described the purification of hyaluronidase from bull seminal plasma by multiple ion-exchange-column chromatography (Yang & Srivastava, 1975a) and

concanavalin–Sephadex chromatography (Yang & Srivastava, 1975b). The present paper describes the purification of bull seminal-plasma hyaluronidase by a new method using heparin–Sephadex affinity chromatography. Some properties of the homogeneous preparation of hyaluronidase are also presented.

Materials and Methods

Hyaluronic acid (grade III-P; potassium salt), *p*-dimethylaminobenzaldehyde and *N*-acetylhydrazide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sepharose 4B, Sephacryl S-200, Sephadex G-200 and concanavalin A–Sephadex were purchased from Pharmacia, Uppsala, Sweden. Acrylamide, *NN*-methylenebisacrylamide and cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) were obtained from Eastman Organic Chemicals, Rochester, NY, U.S.A. Heparin was purchased from Calbiochem, La Jolla, CA, U.S.A. All other chemicals were of analytical grade.

Bull semen was obtained from the Eastern Artificial Insemination Co., Ithaca, NY, U.S.A. It was kept frozen at -20°C until used.

Assay of hyaluronidase

The enzyme was assayed by the method of Aronson & Davidson (1967) as modified by Yang & Srivastava (1974). A unit of enzyme activity is defined as the amount liberating $1 \mu\text{mol}$ of *N*-acetylglucosamine in 1 min at 37°C . The specific activity is expressed as units/mg of protein. The protein was determined by

the method of Bradford (1976), with bovine serum albumin as the standard. The elution of protein from the chromatographic columns was monitored at A_{280} .

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out by the method of Brewer & Ashworth (1969) in buffers at pH 4.3 and 5.3 containing 7.5 and 10 % acrylamide and 0.14 % *NNN'*-tetramethylethylenediamine for 3 h at a current density of 2.0 mA/gel. Methyl Green was used as the tracking dye. The proteins were stained with 1 % Amido Black in 7 % (v/v) acetic acid and were destained by 10 % acetic acid.

Preparation of heparin-Sepharose 4B

Sepharose 4B (50 ml) was washed five times with 1 M- Na_2CO_3 and the excess of Na_2CO_3 was removed by draining. Heparin (100 mg) dissolved in 5 ml of 1 M- Na_2CO_3 was added to Sepharose 4B with constant shaking. Cyanuric chloride (1 g) suspended in 15 ml of acetonitrile was added to the above Sepharose 4B. The mixture was maintained at pH 11 with 1 M- Na_2CO_3 during shaking for 1 h at 65°C. The reaction mixture turned yellow during the reaction. After 1 h the mixture was filtered through a Buchner funnel, and washed with 500 ml of water/ethanol/trimethylamine (2:1:1, by vol.). Finally, the heparin-Sepharose 4B was washed with 0.05 M-sodium acetate buffer, pH 4.

Preparation of hyaluronidase

Bull seminal plasma was processed to the 65 % -satd. $(\text{NH}_4)_2\text{SO}_4$ fractionation step by the method of Yang & Srivastava (1975a). At this stage the enzyme had a specific activity of 0.016 $\mu\text{mol}/\text{min}$ per mg of protein. The freeze-dried powder from the 65 % -satd. $(\text{NH}_4)_2\text{SO}_4$ step (1.5 g) was dissolved in 50 ml of 0.02 M-Tris/HCl, pH 7.0, containing 1 M-NaCl, 1 mM- MnCl_2 and 1 mM- CaCl_2 . The pH of this solution was adjusted to 4.0 by the addition of 0.5 ml of 1 M-HCl and the solution was stirred at 5°C for 30 min. It was then centrifuged for 30 min at 15000g. The precipitate was discarded and the supernatant was dialysed for 24 h against 0.02 M-Tris/HCl buffer, pH 7.0, containing 1 M-NaCl, 1 mM- MnCl_2 and 1 mM- CaCl_2 . This supernatant was called the 'extract'.

Concanavalin A-Sepharose chromatography

The extract was applied at room temperature to a concanavalin A-Sepharose column (1 cm $\times$ 15 cm) equilibrated in 0.02 M-Tris/HCl buffer, pH 7.0, containing 1 M-NaCl, 1 mM- MnCl_2 and 1 mM- CaCl_2 and eluted with the same buffer at a flow rate of 6 ml/h. After 150 ml of the buffer had passed through the column, a linear gradient was applied formed from

100 ml of the starting buffer and 100 ml of the buffer containing 0.5 M-methyl α -D-mannoside, and the eluate was collected in 5 ml fractions. The enzyme appeared as a sharp peak as reported previously (Yang & Srivastava, 1975b). Active fractions were pooled and dialysed against 0.05 M-sodium acetate buffer, pH 4.0, for 24 h.

Heparin-Sepharose affinity chromatography

The dialysed concanavalin A-Sepharose fraction was then applied to a heparin-Sepharose 4B column (2 cm $\times$ 20 cm) equilibrated with 0.05 M-sodium acetate buffer pH 4.0. The column was washed with this buffer at a flow rate of 12 ml/h. The washing did not contain any enzyme and was discarded. The column was then eluted with a linear gradient of NaCl formed from 100 ml of the starting buffer and 100 ml of the buffer containing 0.5 M-NaCl; 5 ml fractions were collected. The active fractions were pooled, dialysed against water and freeze-dried.

Sephadex G-200 gel filtration

The freeze-dried powder was dissolved in 5 ml of 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl and applied to a Sephadex G-200 column (2.5 cm $\times$ 40 cm) that was equilibrated with the starting buffer. The column was eluted in 2.5 ml fractions with the same buffer at a flow rate of 12 ml/h. The enzyme appeared as a sharp symmetrical peak. The fractions that contained the enzyme were pooled and freeze-dried.

Sephacryl S-200 chromatography

The freeze-dried Sephadex G-200 fraction was dissolved in 2 ml of 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.1 M-NaCl, and applied to a Sephacryl S-200 column (1.5 cm $\times$ 30 cm) equilibrated with the same buffer. The column was washed with the buffer in 2 ml fractions at a flow rate of 10 ml/h. The fractions containing enzyme were pooled and freeze-dried.

Elution of hyaluronidase from heparin-Sepharose column by heparin and hyaluronic acid

A DEAE-cellulose fraction, as prepared by Yang & Srivastava (1975b), with specific activity 0.04 $\mu\text{mol}/\text{min}$ per mg, was subjected to heparin-Sepharose affinity chromatography in 0.02 M-sodium acetate buffer, pH 4.0. The column (1.2 cm $\times$ 10 cm) was first washed with this buffer at a flow rate of 12 ml/h and 2.5 ml fractions were collected. After 75 ml of the buffer had passed through the column, the elution was continued either with heparin (3 mg/ml) or hyaluronic acid (1 mg/ml) in the same buffer. Active fractions were pooled, freeze-dried and subjected to polyacrylamide-gel electrophoresis (Brewer & Ashworth, 1969).

Dispersal of cumulus clot by the homogeneous preparation of hyaluronidase

Freshly ovulated ova of the rabbit were obtained by flushing the fallopian tubes with 0.05M-Tris/HCl, pH7.0, containing 0.15M-NaCl. The cumulus clot containing ova was transferred to 5ml beakers containing 3.0ml of the buffer. Various amounts of purified hyaluronidase were added to the beakers and the ova were observed for the dispersal of cumulus cells of the clot at room temperature. The control cumulus masses were placed in 2.0ml of 0.05M-Tris/HCl, pH7.4, containing 0.15M-NaCl, and showed no evidence of dispersion after 24h at 22°C.

Results and Discussion

Bull seminal-plasma hyaluronidase was purified 180-fold (Table 1) with an overall recovery of 26 % by the above procedure. The purified enzyme has a turnover number of 214 min⁻¹ (mol of product formed/mol of enzyme per min) and a specific activity of 3.63 μmol/min per mg of protein, which is higher than reported previously (Borders & Raftery, 1968; Yang & Srivastava, 1975*a,b*). However, Morton (1977) reported that the purified ram semen hyaluronidase had a specific activity of 7.14 μmol/min per mg of protein. In previous papers from our laboratory it was erroneously reported that 1 colorimetric unit was equal to 29 National Formulary units (Yang & Srivastava, 1974, 1975*a,b*). This mistake was later corrected (Yang & Srivastava, 1976). Taking this correction into account, 1 colorimetric unit is equal to 29000 National Formulary units, so our purified enzyme preparation has 104000 National Formulary units/mg of protein. The retention of hyaluronidase on a concanavalin A-Sepharose column and its elution by methyl α-D-mannoside has been described by Yang & Srivastava (1975*b*). Balasubramanian *et al.*, (1976) confirmed the glycoprotein nature of testicular hyaluronidase and reported that the immobilized enzyme had only 30 % of the activity of

Fig. 1. Reaction sequence showing the coupling of heparin to Sepharose 4B by cyanuric chloride

The reaction was carried out at pH11 for 1h at 60°C. The experimental details are described in the Materials and Methods section.

the soluble enzyme. The elution of hyaluronidase from a concanavalin A-Sepharose column is temperature-dependent. Elution at room temperature is more effective than at 4°C. A similar phenomenon has been reported for *N*-acetylhexosaminidase

Table 1. Purification of hyaluronidase from bull seminal-plasma

	Total (mg)	Total activity units (μmol/min)	Specific activity (μmol/min per mg of protein)	Yield (%)
'Extract'	1500	30.0	0.02	100
Concanavalin A-Sepharose fraction	160	24.0	0.15	80
Heparin-Sepharose fraction	28	18.0	0.64	60
Sephadex G-200 fraction	4.2	12.0	2.85	40
Sephacryl S-200 fraction	2.2	8.0	3.63	26

Fig. 2. Heparin-Sepharose 4B affinity chromatography of bull seminal-plasma hyaluronidase. The experimental conditions are described in the Materials and Methods section. ---, A_{280} ; ●, enzyme activity; —, salt gradient.

Fig. 3. Sephadex G-200 chromatography of bull seminal-plasma hyaluronidase.

The experimental conditions and analysis of fractions for enzyme activity and protein are described in the Materials and Methods section. ---, A_{280} ; ●, enzyme activity.

(Brattain *et al.*, 1977) and acid phosphatase (Helwig *et al.*, 1978).

Cyanuric chloride has been successfully used for coupling enzymes to cellulose (Kay & Crook, 1957; Kay & Lilly, 1970; Smith & Lenhoff, 1974). In the present study, heparin, a well-known inhibitor of hyaluronidase (Yang & Srivastava, 1974; Bernfield *et al.*, 1961) was coupled to Sepharose 4B by the cyanuric chloride procedure. The proposed mechanism of this coupling is shown in Fig. 1. During the activation reaction the free hydroxy group of heparin reacts with cyanuric chloride to give a heparin-cyanuric chloride complex at alkaline pH. The hydroxy group of Sepharose 4B then reacts with a free chlorine atom to form heparin-monochlorotriazinyl-Sepharose 4B. The amount of heparin bound to Sepharose 4B was determined by measuring sulphate and hexosamine contents of the coupled gel (Iverius,

1971) and our procedure resulted in coupling of 0.8 mg of heparin/ml of wet Sepharose 4B.

The elution profile of bull seminal-plasma hyaluronidase from the heparin-Sepharose 4B column shows that about 80% of the protein is washed through, whereas the enzyme is completely retained in the column (Fig. 2). The enzyme appeared as a sharp peak when the column was eluted with the NaCl gradient.

The hyaluronidase is eluted from the Sephadex G-200 column as a single symmetrical peak away from the main protein peak (Fig. 3). The gel electrophoresis of the Sephadex G-200 fraction containing the enzyme showed a major protein band and some minor contaminants. Sephacryl S-200 chromatography of this Sephadex G-200 fraction resulted in complete removal of the minor contaminating proteins, and the purified enzyme moved as a single band on 7.5 and 10% polyacrylamide gels at pH 4.3 and 5.3 (Fig. 4). The R_f value of the bands was identical with that of the purified sperm hyaluronidase (Yang & Srivastava, 1974, 1975a,b). At alkaline pH, pure hyaluronidase failed to migrate into the gels. The purified hyaluronidase moved as a single band on SDS/polyacrylamide-gel electrophoresis (Yang & Srivastava, 1975b).

The elution of hyaluronidase from heparin-Sepharose can also be readily achieved either by heparin or by hyaluronic acid (Fig. 5). It is noteworthy that the elution with heparin resolves the enzyme into two discrete peaks of approximately equal activity. In a review, Morton (1977) reported that ram spermatozoal hyaluronidase can be separated into multiple forms by ion-exchange chromatography. The specific activity and recovery of heparin-eluted enzyme are 0.4 μ mol/min per mg and 26% respectively.

Heparin is a competitive inhibitor of the enzyme, as

Fig. 4. Polyacrylamide-gel electrophoresis of purified bull seminal-plasma hyaluronidase

The experimental conditions are described in the Materials and Methods section. (a) 7.5 % (w/v) polyacrylamide at pH 4.3; (b) 10 % polyacrylamide at pH 5.3.

the inhibition can be overcome by using increasing concentration of the substrate. Under optimal assay conditions, 0.25, 0.50 and 1.0 mg of heparin, with 1.2 mg of hyaluronic acid as the substrate, caused 48, 72 and 85 % inhibition respectively. When the concentration of hyaluronic acid was doubled, the inhibition was decreased to zero and the V_{max} approached that of the control. This also explains why hyaluronic acid is able to elute the enzyme from the column. The low specific activity and recovery of the enzyme are due to the presence of heparin. With hyaluronic acid, the enzyme is eluted as a sharp peak with a specific activity of $1.1 \mu\text{mol/min per mg}$ and 84 % recovery. The elution of hyaluronidase by heparin and hyaluronic acid from the heparin-Sepharose column establishes the true affinity of the absorbant. The binding of hyaluronidase, $pI 5.9$ (Stambaugh & Smith, 1974; Yamada *et al.*, 1977) to heparin-Sepharose is due to enzyme-inhibitor interaction. As heparin-Sepharose is a polyanionic

Fig. 5. Elution profile of hyaluronidase from heparin-Sepharose column with heparin and hyaluronic acid. The experimental conditions and analysis of fractions for enzyme activity and protein are described in the Materials and Methods section. ----, A_{280} ; ●, enzyme activity. (a) Heparin or (b) hyaluronic acid was added at the arrow.

Table 2. Effect of carbonyl reagents on bull seminal-plasma hyaluronidase

The enzyme (1.5 μg) was preincubated at 37°C for 10 min with indicated concentrations of carbonyl reagents in 0.05 M-sodium acetate buffer, pH 3.8, and the reaction was started by the addition of hyaluronic acid. The tubes were incubated for 30 min at 37°C . The control reaction mixture contained all components except carbonyl reagents.

Carbonyl reagent	Concn. (mM)	Product formed (nmol)
None (control)	0	123
Hydroxylamine	1	59
	2	25
	3	7
Phenylhydrazine	1	52
	2	47
	3	32
Semicarbazide	2.5	96
	5.0	80
	10.0	54

gel, it may interact with basic proteins like lysozyme ($pI=10.5$) Alderton *et al.*, 1945) and acrosin ($pI=10.2$) (Stambaugh & Smith, 1974). Both these proteins are retained on the heparin-Sepharose and can be eluted with a linear gradient of 0–1 M-NaCl, but not with

heparin or hyaluronic acid. This strongly suggests that, unlike hyaluronidase, the binding of lysozyme and acrosin to the heparin-Sepharose is mainly due to charge interactions. Polyacrylamide-gel electrophoresis of heparin- and hyaluronic acid-eluted enzyme showed at least two protein bands in each. The inert protein was removed on further purification.

Properties of bull seminal-plasma hyaluronidase

The inhibitory effect of Fe^{3+} , Cu^{2+} and Ag^+ on hyaluronidase is well known (Zaneveld *et al.*, 1973; Yang & Srivastava, 1975a). We also found that these metal ions strongly inhibit hyaluronidase activity. *p*-Chloromercuribenzoate and iodoacetate have no effect on the enzyme activity, suggesting that thiol groups are not involved in the reaction catalysed by hyaluronidase. Similar observations were also reported for partially purified hyaluronidase (Meyer, 1971).

Hydroxylamine, phenylhydrazine and semicarbazide produce considerable inhibition of hyaluronidase activity (Table 2). Hydroxylamine is the most effective inhibitor of hyaluronidase. Thus, at 3 mM, hydroxylamine produces 94 % inhibition, whereas with phenylhydrazine the corresponding value was 74 %. Semicarbazide at 2.5 mM causes 22 % inhibition of the enzyme activity. The inhibition of hyaluronidase by hydroxylamine develops rapidly with time (Fig. 6). In 5 min, 2 mM-hydroxylamine produces about 65 % inhibition of the enzyme activity, and after 30 min only 10 % of the enzyme activity was left. The mechanism of this inhibition is not known, but it is possible either that the enzyme is denatured by the carbonyl reagents or that carbonyl reagents react with hyal-

Fig. 6. Effect of hydroxylamine on hyaluronidase as a function of time

Hyaluronidase (1 μg) was preincubated with 2 mM-hydroxylamine and the reaction was started by the addition of hyaluronic acid. ●, Control; ○, in presence of hydroxylamine.

Fig. 7. Dispersal of cumulus clot by bull seminal-plasma hyaluronidase

At least four cumulus clots were taken in 3 ml of 0.05 M-Tris/HCl buffer, pH 7.0, containing 0.15 M-NaCl and incubated at room temperature (22°C) with purified hyaluronidase.

uronidase close to its active site and thus prevent the entry of hyaluronic acid into the active site owing to steric hindrance.

It is well accepted that hyaluronidase disperses the cumulus oophorus surrounding the ovum (McClean & Rowland, 1942; Fekete & Duran-Reynals, 1943). The homogeneous preparation of hyaluronidase at a concentration of 1.25 munits/ml disperses the cumulus clot in 58 min, and when the amount of the enzyme is increased to 2.5 munits/ml the dispersal time is decreased to 32 min, indicating that cumulus dispersal time is proportional to the amount of hyaluronidase added to the reaction mixture (Fig. 7). Similar results were also obtained by Talbot & Franklin (1974), who, when using a crude hyaluronidase preparation, reported that the cumulus dispersal time is linearly related to the hyaluronidase activity. On the basis of our data, 1 biological unit of hyaluronidase is defined as the amount of the enzyme that disperses the cumulus clot from ova in 1 h at room temperature and equals 1 munit of biochemical activity as determined by the colorimetric method.

The new method of purification of hyaluronidase as reported here should be useful in providing large quantities of homogeneous hyaluronidase for biological, immunological and structural studies.

This work was supported by U.S. Public Health Service grant no. 5R01 HD10871-02.

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