A rapid purification of bovine testicular hyaluronidase by chromatography on dermatan sulphate-substituted 1,6-diaminohexane–Sephadex 4B

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The binding of bovine testicular hyaluronidase to AH-Sephadex (1,6-diaminohexane–Sephadex) gels substituted with (1) dermatan sulphate, (2) desulphated dermatan sulphate, (3) heparin and (4) de-*N/O*-sulphated, re-*N*-acetylated heparin was investigated. Hyaluronidase was found to bind to (1) and (3), but not (2) and (4). On the basis of these observations a preparative scheme for the purification of testicular hyaluronidase was developed. This consisted of two steps: (i) chromatography on dermatan sulphate-substituted AH-Sephadex 4B; (ii) chromatography on acetylated AH-Sephadex 4B. This procedure gave hyaluronidase with a specific activity of 19.1 units ($\mu\text{mol}/\text{min}$)/mg in high yield. Polyacrylamide-gel electrophoresis at pH 4.3 revealed two components, both possessing hyaluronidase activity. Sodium dodecyl sulphate polyacrylamide-gel electrophoresis likewise revealed two close bands with approximate molecular weights of 61 000 and 67 200.

Testicular hyaluronidase (EC 3.2.1.35) is an endo- β -*N*-acetylhexosaminidase which degrades the glycosaminoglycans hyaluronic acid, chondroitin, chondroitin 4- and 6-sulphates and, to a variable extent, depending on the source, dermatan sulphate. Several studies resulting in highly purified enzyme preparations have been reported (Borders & Raftery, 1968; Yang & Srivastava, 1975; Srivastava & Farooqui, 1979). The last paper described the first use of an affinity matrix (heparin–Sephadex).

Hyaluronidase has found widespread clinical usage, especially in aiding the local dispersal of drugs. It has also proved to be an invaluable analytical tool in connective-tissue research. A promising application in the treatment of myocardial infarction has been proposed (Maroko & Braunwald, 1973; Maclean *et al.*, 1976).

Many studies have shown that certain glycosaminoglycans, namely heparin and some preparations of dermatan sulphate, are inhibitors of hyaluronidase (Hadidian *et al.*, 1957; Houck, 1957). The aim of the present study has been to investigate the interactions between hyaluronidase and a number of possible inhibitors, ultimately with a view to devising a purification scheme based on affinity chromatography.

Abbreviations used: AH-Sephadex 4B, 1,6-diaminohexane–Sephadex 4B; SDS, sodium dodecyl sulphate.

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Experimental

Materials

Potassium hyaluronate from human umbilical cords (batch C20 D) was purchased from Miles Laboratories (Slough, Berks., U.K.) and further purified by the cetylpyridinium chloride fractionation method of Scott (1960). Heparin from pig intestinal mucosa, pig mucosal residues from heparin manufacture and bovine testicular hyaluronidase (0.083 unit/mg) were obtained by courtesy of Dr. W. E. Lewis, Glaxo Operations Ltd., Runcorn, Cheshire, U.K. Chondroitinase AC (EC 4.2.2.5) and chondroitinase ABC (EC 4.2.2.4) were obtained from Seikagaku Kogyo Co., Tokyo, Japan. World Health Organisation (W.H.O.) International Standard of Hyaluronidase was obtained from the National Institute for Biological Standards and Control, Hampstead, London N.W.3, U.K. 1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride, *p*-dimethylaminobenzaldehyde and bovine serum albumin fraction V were purchased from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Cetylpyridinium chloride, PAGE Blue G90, Kenacid Blue R and 2,4,6-trinitrobenzenesulphonic acid were purchased from BDH Chemicals (Speke, Liverpool, U.K.). Sephadex G-200 and AH-Sephadex 4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of analytical grade.

Preparation of dermatan sulphate

A sample (100g) of residues from heparin manufacture dissolved in 0.5 M-acetic acid/5% (w/v) calcium acetate was fractionated by ethanol precipitation. The 18–36% (v/v)-ethanol precipitate was further purified by two successive copper precipitations (Cifonelli *et al.*, 1958) to give an iduronate-rich dermatan sulphate. Copper was removed by dialysis against 1 M-NaCl/5 mM-EDTA, then distilled water and finally passage through Amberlite monobed MB3 resin.

Preparation of desulphated dermatan sulphate and de-N/O-sulphated, re-N-acetylated heparin

Dermatan sulphate was solvolytically desulphated by the method of Nagasawa *et al.* (1977). Heparin was de-N-sulphated by the method of Inoue & Nagasawa (1976). The resulting free amino groups were acetylated with an excess of acetic anhydride in 0.25 M-sodium phosphate buffer, pH 7.5 at 0°C (Riordan & Vallee, 1972). Amino-group substitution was monitored by using trinitrobenzenesulphonic acid (Satake *et al.*, 1960). Finally, de-O-sulphation was performed by the method of Nagasawa *et al.* (1977).

Analytical methods

Total hexuronic acid was determined by the orcinol (Brown, 1946) and carbazole (Dische, 1947) methods. Amino acid and hexosamine analyses of glycosaminoglycans were performed on a LKB 4101 Autoanalyser (LKB Biochrom Ltd., Cambridge, U.K.) after hydrolysis in 4 M-HCl at 100°C for 10 h under a nitrogen atmosphere. Sulphate content was monitored by the method of Stone & Bradley (1967).

Chondroitinase-AC or -ABC digestions of dermatan sulphate were performed in 1 ml of 0.1 M-Tris/HCl/0.1 M-sodium acetate, pH 7.3, with the addition of 0.1 unit of either enzyme. After incubation at 37°C for 6 h, a further 0.1 unit was added and incubation was continued for a further 9 h. Digested samples were chromatographed on a Sephadex G-200 column in 1 M-NaCl. The void volume and total volume were determined with Dextran Blue and D-glucuronolactone respectively.

Preparation of glycan-substituted AH-Sepharose gels

Dermatan sulphate, desulphated dermatan sulphate and desulphated, re-N-acetylated heparin were dissolved in 0.1 M-sodium acetate buffer, pH 5.4, containing 0.15 M-NaCl, and digested with approx. 0.06–0.09 unit of bovine testicular hyaluronidase (3.9 units/mg prepared in our laboratory) per mg of glycan, at 37°C for 24 h. Samples of the digests were chromatographed on a Sephadex G-200 column in

1 M-NaCl. The digests were boiled and centrifuged (1500g, 10 min) to remove protein. Polymeric glycosaminoglycans were precipitated with 0.3 M-calcium acetate/0.5 M-acetic acid and 50% (v/v) ethanol (Meyer *et al.*, 1956).

The three treated glycosaminoglycans, plus heparin, were coupled via their carboxy groups to AH-Sepharose 4B by using 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride and the procedure recommended by the manufacturer (Pharmacia). After exhaustive washing of the gels, the unsubstituted amino groups were blocked by acetylation with an excess of acetic anhydride (Riordan & Vallee, 1972).

The amounts of glycosaminoglycan bound were determined by hexosamine analysis of the gels, after hydrolysis in 4 M-HCl at 100°C for 10 h under nitrogen. The vacuum-dried hydrolysates were taken up in 0.35 M-sodium citrate buffer, pH 5.28, filtered through a Millipore membrane (0.22 µm pore size) and then analysed on a LKB 4101 Autoanalyser by using a one-step elution with 0.35 M-sodium citrate buffer, pH 5.28.

Assay of hyaluronidase

Hyaluronidase activity was determined colorimetrically by a modification of the method of Aronson & Davidson (1967). The reaction mixture consisted of 0.4 ml of sodium hyaluronate (1 mg/ml) in 0.1 M-sodium acetate buffer/0.15 M-NaCl, pH 5.4, to which was added 0.1 ml of enzyme solution. After incubation at 37°C for 15 min, N-acetylglucosamine end-group colour was developed by the method of Reissig *et al.* (1955).

The modification by Doak & Zahler (1979), using a pH 3.8 incubation in the presence of bovine serum albumin (1 mg/ml), was used for assaying highly purified enzyme and for the determination of specific activities. One unit of enzyme activity (IUPAC) is defined as the liberation of 1 µmol of N-acetylglucosamine end-groups/min at 37°C. It was determined that one such unit is equivalent to 3185 international units (W.H.O.) by comparison with the W.H.O. International Standard of Hyaluronidase (Humphrey, 1957). Protein was determined by the methods of Sedmak & Grossberg (1977) and Lowry *et al.* (1951), with bovine serum albumin as a standard.

Plastic vessels and columns and silicone-treated glassware were used for all enzyme operations to minimize the inactivation of hyaluronidase by adsorption on glass surfaces (Rasmussen, 1954).

Chromatography of hyaluronidase on glycan-substituted AH-Sepharose gels

The four glycan-substituted gels and acetylated AH-Sepharose 4B (a control gel) were packed into plastic columns. Crude hyaluronidase was

chromatographed on the gels at low ionic strength, pH4 and/or pH6. Bound protein was generally eluted with a linear concentration gradient of NaCl. Fractions were analysed for hyaluronidase activity and protein (by the A_{280}). NaCl concentrations were monitored with a conductivity meter (model MC-1, Mark V; Electronic Switchgear London Ltd., Hitchin, Herts., U.K.).

All experiments were performed at 4°C. Further details are given in the legends to the Figures.

Purification of hyaluronidase

(1) *Chromatography on dermatan sulphate-substituted AH-Sepharose 4B*. Crude hyaluronidase (600mg) was dissolved in 30ml of 20mM-sodium acetate, adjusted to pH6 with 1M-NaOH and centrifuged at 1500g for 10min. The supernatant was applied to a dermatan sulphate-AH-Sepharose 4B column (1.8 cm × 6.5 cm) equilibrated with 20mM-sodium acetate buffer, pH6, and eluted with the same buffer at a flow rate of 9.6 ml/h; 4.8 ml fractions were collected. The column was then eluted with 20mM-sodium acetate buffer, pH4, followed by a linear 0–1M-NaCl gradient (total volume 100ml) in the same pH4.0 buffer (3 ml fractions were collected).

Hyaluronidase-active fractions were pooled, dialysed against 20mM-sodium acetate buffer, pH6.0, and then concentrated by ultrafiltration over a Diaflo PM10 membrane (Amicon, High Wycombe, Bucks., U.K.) in a Chemlab C50 ultrafiltration cell (Chemlab Instruments, Hornchurch, Essex, U.K.).

(2) *Chromatography on acetylated AH-Sepharose 4B*. The concentrated hyaluronidase fraction was centrifuged at 1500g for 10min. The supernatant was applied to an acetylated AH-Sepharose 4B column (1.8 cm × 6.0 cm) equilibrated with 20mM-sodium acetate buffer, pH6.0, and eluted with the same buffer at a flow rate of 9 ml/h; 4.5 ml fractions were collected. The column was washed free of bound protein with 1M-NaCl in pH6 buffer. The hyaluronidase-active fractions were pooled and concentrated by dialysis against buffered poly(ethylene glycol) (30 mg/ml).

Polyacrylamide-gel electrophoresis

This was performed by the method of Brewer & Ashworth (1969), with 7.5% acrylamide gels at pH4.3 and 8.9. Both upper and lower gels were polymerized by using riboflavin and light from a Shandon 'Photopol' fluorescent lamp (Shandon Southern Products Ltd., Runcorn, Cheshire, U.K.). Methyl Green and Bromophenol Blue were used as respective marker dyes. Gels were stained for protein with 0.5% (w/v) Kenacid Blue R in 45% (v/v) methanol and 10% (v/v) acetic acid, and then destained in 7.5% (v/v) acetic acid/5% (v/v)

methanol. Enzyme activity was located by slicing unstained gels into 2 mm lengths and elution of the individual slices overnight at 4°C in 0.5 ml of 0.1M-sodium acetate buffer, pH3.8, containing 0.15M-NaCl and 5 mg of bovine serum albumin/ml. Then 0.1 ml samples were assayed for hyaluronidase activity. A duplicate gel was stained for protein.

Polyacrylamide-gel electrophoresis in the presence of SDS was performed by the method of Weber & Osborn (1969) in 10%-acrylamide gels. Bovine serum albumin fraction V (mol.wt. 68 000), catalase (bovine liver, mol.wt. 58 000), ovalbumin (mol.wt. 43 000), carbonic anhydrase (bovine erythrocytes, mol.wt. 29 000), haemoglobin (bovine, mol.wt. 15 500) and lysozyme (egg white, mol.wt. 14 300) were used as molecular-weight markers.

Results

Preparation of dermatan sulphate

Two copper precipitations of the 36% (v/v)-ethanol fraction yielded 945 mg of a dermatan sulphate containing less than 1% of the total hexosamine as *N*-acetylglucosamine. The 'carbazole/orcinol' ratio of 0.295 is higher than the value of 0.23 expected for pure L-iduronic acid (Hoffman *et al.*, 1956), indicating the presence of D-glucuronic acid. It can be estimated that the polymer contains approx. 95% L-iduronic acid and 5% D-glucuronic acid (Fransson *et al.*, 1968). Table 1 summarizes the analyses of the material. Chromatography on Sephadex G-200 reveals a broad profile, with K_{av} 0.2 (Fig. 1a). Treatment with chondroitinase ABC resulted in complete degradation, whereas chondroitinase AC treatment seems to indicate a possible minor contaminant. This could be chondroitin sulphate (which would be removed by hyaluronidase treatment), or possibly accessible chondroitin sulphate-like sequences in the dermatan sulphate chain. On digestion with testicular hyaluronidase, the elution profile was broadened (K_{av} 0.28), indicating a very limited degradation, but with no detectable production of small oligosaccharides.

Table 1. *Characteristics of dermatan sulphate preparation*
See the text for experimental details. For the determination of the 'carbazole/orcinol' ratio, the orcinol sample was heated for 20 min.

Analyses	
Uronic acid (%)	40.1
Hexosamine (%)	19.5
'Carbazole/orcinol' ratio	0.295
GalN/GlcN ratio	112.3
GalN/Ser ratio	42.5
$\bar{M}_n$	21 700

Fig. 1. *Sephadex G-200 chromatography of dermatan sulphate and heparin derivatives*

(a) Dermatan sulphate (●) and hyaluronidase-treated dermatan sulphate (▲). Column size, $0.6 \text{ cm} \times 51.5 \text{ cm}$; eluent, 1 M-NaCl ; flow rate, 0.7 ml/h ; fraction volume 0.35 ml . (b) Desulphated dermatan sulphate (●) and hyaluronidase-treated desulphated dermatan sulphate (▲). Column size, $0.6 \text{ cm} \times 59 \text{ cm}$; eluent, 1 M-NaCl ; flow rate, 0.78 ml/h ; fraction volume, 0.43 ml . (c) Heparin (●) and de-*N/O*-sulphated, re-*N*-acetylated heparin (▲). Column size, $0.6 \text{ cm} \times 59 \text{ cm}$; eluent, 1 M-NaCl ; flow rate, 0.78 ml/h ; fraction volume, 0.43 ml . Fractions were analysed for uronic acid by the orcinol method. V_0 (void volume) and V_t (total column volume) were determined with Dextran Blue and D-glucuronolactone respectively.

Desulphated dermatan sulphate

Analysis of the sulphate content by Acridine Orange binding (Stone & Bradley, 1967) gave data consistent with appreciable loss of sulphate. Chromatography on Sephadex G-200 revealed a K_{av} of 0.43 (Fig. 1b), indicating loss of sulphate and/or limited depolymerization. Hyaluronidase treatment resulted in a peak with K_{av} 0.61, probably indicating a similar extent of limited susceptibility to that of the parent dermatan sulphate.

De-*N/O*-sulphated, re-*N*-acetylated heparin

Sulphate analysis indicated that appreciable desulphation had occurred. An estimated 98% of free amino groups were re-*N*-acetylated. Chromatography on Sephadex G-200 revealed a near-symmetrical peak (Fig. 1c) with K_{av} 0.36 (cf. K_{av} 0.32 for heparin). Treatment with hyaluronidase resulted in no observable degradation.

Chromatography on glycosaminoglycan-substituted AH-Sepharose gels

Hexosamine analysis estimated the coupling yields for the four substituted gels as: dermatan sulphate, $1.6 \mu\text{mol}$ of disaccharide/ml of swollen gel; desulphated dermatan sulphate, $0.12 \mu\text{mol/ml}$; heparin, $3.4 \mu\text{mol/ml}$; de-*N/O*-sulphated, re-*N*-acetylated heparin, $1.2 \mu\text{mol/ml}$.

Hyaluronidase bound to heparin-substituted gel in pH 7 buffer containing 0.15 M-NaCl , and was eluted with 0.2 – 0.25 M-NaCl (Fig. 2a). In comparison, gel substituted with de-*N/O*-sulphated, re-*N*-acetylated heparin did not bind a significant amount of enzyme at either pH 4 or 6 (results not shown). The very small amount of enzyme that did bind was eluted with 0.05 – 0.1 M-NaCl .

Dermatan sulphate-substituted gel bound hyaluronidase at pH 6, which could be eluted with approx. 0.15 M-NaCl (Fig. 2b). At pH 4 the enzyme was bound more strongly, requiring 0.2 M-NaCl for elution, but the overall capacity of the gel for enzyme appeared to be decreased (Fig. 2c). Desulphated dermatan sulphate-substituted gel did not bind hyaluronidase at pH 6 (results not shown).

Interestingly, acetylated AH-Sepharose 4B, although not binding hyaluronidase, did bind a significant amount of protein at pH 6.

Purification of hyaluronidase

(1) *Chromatography on dermatan sulphate-substituted AH-Sepharose 4B*. Hyaluronidase bound to the column at pH 6. Elution with pH 4 buffer removed a large proportion of the bound protein, after which the hyaluronidase was eluted with the NaCl gradient (Fig. 3a). The fractions indicated by horizontal arrows were pooled, dialysed and concentrated to give a solution containing 7 mg of enzyme with an activity of 6.25 units/mg .

Fig. 2. Chromatography of crude hyaluronidase on glycan-substituted AH-Sepharose 4B gels (a) Heparin-substituted AH-Sepharose 4B. Column size 1.4 cm $\times$ 16 cm; loading, 100 mg of crude enzyme; eluent, 50 mM-Tris/HCl buffer, pH 7.0, containing 0.15 M-NaCl, followed by a NaCl gradient (0.15–0.5 M in pH 7.0 buffer); flow rate, 5 ml/h. (b) Dermatan sulphate-substituted AH-Sepharose 4B. Column size, 1.8 cm $\times$ 6.5 cm; loading, 250 mg of crude enzyme; eluent, 20 mM-sodium acetate buffer, pH 6.0, followed by a NaCl gradient (0–1 M in pH 6.0 buffer); flow rate, 11.3 ml/h; fraction volume, 5.65 ml. (c) Dermatan sulphate-substituted AH-Sepharose 4B. Column size, 1.8 cm $\times$ 6.5 cm; loading, 250 mg of crude enzyme; eluent, 20 mM-sodium acetate buffer, pH 4.0, followed by a NaCl gradient (0–0.5 M in pH 4.0 buffer); flow rate, 11.3 ml/h; fraction volume, 5.65 ml. Analyses: protein A_{280} (●) or $\mu\text{g/ml}$ (O); hyaluronidase activity (▲); NaCl gradient (—).

Table 2. Summary of purification of testicular hyaluronidase

Protein was determined by the method of Sedmak & Grossberg (1977) in step 2 and by that of Lowry *et al.* (1951) in step 3.

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	Yield (%)
(1) Crude enzyme	600	49.8	0.083	1	100
(2) Chromatography on dermatan sulphate–AH-Sepharose 4B	7	43.75	6.25	75.3	88
(3) Chromatography on acetylated AH-Sepharose 4B	1.47	28.1	19.1	230	56

Fig. 3. Purification of bovine testicular hyaluronidase

(a) Chromatography on dermatan sulphate-substituted AH-Sepharose 4B; (b) chromatography on acetylated AH-Sepharose 4B. Full details are described in the Experimental section. Analyses: protein, A_{280} (●) or $\mu\text{g/ml}$ (○); hyaluronidase activity (▲); NaCl gradient (—). Arrows indicate pooled enzyme fractions.

(2) *Chromatography on acetylated AH-Sepharose 4B.* The majority of the protein from Step 1 bound to the column at pH 6, while the hyaluronidase was eluted straight through (Fig. 3b). The pooled wash-through fractions yielded 1.47 mg of enzyme, with a specific activity of 19.1 units/mg. The overall purification was 230-fold, with a yield of 56% (Table 2).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis of purified enzyme at pH 4.3 consistently revealed two bands. Detection of hyaluronidase activity by slicing up an unstained gel reveals a broad band of activity, which can be resolved by a longer electrophoresis time into two bands corresponding to the stained protein bands on a duplicate gel (Fig. 4). On electrophoresis at pH 8.9 the purified enzyme failed to migrate into the gel.

SDS/polyacrylamide-gel electrophoresis showed two close protein bands corresponding to apparent molecular weights of 61 000 and 67 200. A value of

Fig. 4. Polyacrylamide-gel electrophoresis of purified bovine testicular hyaluronidase at pH 4.3

Full details are described in the Experimental section. (a) Diagrammatic representation of gel stained for protein. (b) Duplicate gel from which protein was eluted and assayed for hyaluronidase activity.

61000–62000 is the currently accepted one for testicular hyaluronidase (Borders & Raftery, 1968; Yang & Srivastava, 1974).

Discussion

The limited susceptibility of both the dermatan sulphate and desulphated dermatan sulphate preparations to digestion by testicular hyaluronidase would indicate that resistance is due primarily to the preponderance of L-iduronic acid residues. Similarly, the presence of α -hexosaminidic linkages seems to explain the complete resistance of de-*N/O*-sulphated, re-*N*-acetylated heparin to hyaluronidase.

The binding of hyaluronidase to gels substituted with dermatan sulphate or heparin was to be expected from their known competitive inhibition of hyaluronidase action (Houck, 1957). The binding to heparin appears to be the slightly stronger of the two, as measured by the relative concentrations of NaCl required for elution of the enzyme. Binding is effectively abolished after desulphation of both these glycosaminoglycans. Binding thus appears to be dependent on the extent of sulphation.

The dermatan sulphate-substituted gel formed the basis of a two-step purification scheme which gave enzyme with a specific activity of 19.1 units/mg in a high yield. It is noteworthy that the Coomassie Blue-based protein assay of Sedmak & Grossberg (1977) indicated only 0.72 mg of protein in the purified preparation (i.e. a specific activity of 39.2 units/mg), relative to a bovine serum albumin calibration curve. This is only 49% of the protein (1.47 mg) indicated by the assay of Lowry *et al.* (1951). This variability of response to different proteins is a known deficiency of Coomassie Blue-based assays (Pierce & Suelter, 1977; Van Kley & Hale, 1977). This purification compares with that of Srivastava & Farooqui (1979), using a four-step procedure based on heparin–Sephadex chromatography, which gave enzyme of 104000 W.H.O. international units/mg (protein determined with Coomassie Blue!) in a yield of 26%.

The presence of two enzymically active bands on polyacrylamide-gel electrophoresis at pH 4.3 would seem to indicate two isoenzymes. Isoenzymes have been reported from bovine (Vikha *et al.*, 1971) and other mammalian testes. Whether this result reflects the composition *in vivo*, or is a result of limited degradation by proteinases or glycosidases during extraction and purification, is not clear. It is likely that these two active bands correspond to the two bands obtained on SDS/polyacrylamide-gel electrophoresis. It is known that glycoproteins can behave aberrantly on SDS/polyacrylamide gels (Segrest & Jackson, 1972), and any change in the carbohydrate moiety (e.g. by glycosidase action) could have a significant effect on the protein's mobility.

Even small changes in the amino acid composition can result in larger changes in mobility than would be expected from the corresponding molecular-weight change (e.g. de Jong *et al.*, 1978). Gorham (1974) has similarly obtained complex patterns on SDS/polyacrylamide-gel electrophoresis from otherwise highly purified hyaluronidase.

In conclusion, the scheme reported here can provide for the preparation of highly purified bovine testicular hyaluronidase in fewer stages and with a higher yield than previously reported methods.

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