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Purification and properties of hyaluronidase from human liver

Differences from and similarities to the testicular enzyme

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Human liver hyaluronidase was purified to homogeneity by $(NH_4)_2SO_4$ fractionation, chromatography on hydroxyapatite and DEAE-cellulose, and preparative disc polyacrylamide-gel electrophoresis. The enzyme had a pH optimum of 3.8–4.0, a molecular weight (determined by gel filtration) of 76000, and a K_m of 0.05 mg/ml for purified human umbilical-cord hyaluronic acid. It generally resembled hyaluronidases studied in other tissues which are believed to be lysosomal, but shared a number of characteristics with a partially purified bovine testicular hyaluronidase. Neither enzyme exhibited inhibition by high concentrations of substrate, but both were competitively inhibited by dermatan sulphate and keratan sulphate. Both enzymes exhibited increased activity in the presence of albumin, probably owing to an increased susceptibility of substrate to enzyme action. The liver enzyme was inhibited by NaCl, but the testicular enzyme exhibited an increase in activity in the presence of the salt which was similar to the effect observed with albumin. The different response toward Cl^- ion appeared to be the most significant difference between the two enzymes.

Hyaluronidase (hyaluronate glycanohydrolase, EC 3.2.1.35) of apparent lysosomal origin has been detected in many mammalian tissues and organs. It has been purified and examined in detail only in rat liver (Aronson & Davidson, 1967*a*,*b*) and human placenta (Yamada *et al.*, 1977). Nevertheless, certain properties of the enzyme, such as the effect of NaCl on activity and the existence of substrate inhibition, remain unresolved. For instance, Aronson & Davidson (1967*b*) observed inhibition of rat liver hyaluronidase by high concentrations of substrate. NaCl could prevent this inhibition, but at low substrate concentrations the salt itself caused inhibition. Stevens *et al.* (1975) found no substrate inhibition of synovial-fluid hyaluronidase, but observed complete inhibition of enzyme activity in 0.3 M-NaCl, which they attributed to an ionic-strength effect. No substrate inhibition was exhibited by the purified enzyme from human placenta, but it was reported for crude hyaluronidases from rat liver (Hutterer, 1966) and kidney (Bollet *et al.*, 1963). In most studies of hyaluronidases the enzyme assays were performed in 0.15 M-NaCl.

Meyer (1971) classified hyaluronidases on the basis of their mode of action. He included the lysosomal enzyme, and the more widely studied hyaluronidase found in testes, in one of his three groups. Although these two types of hyaluronidases exhibit a number of common characteristics, they are generally believed to be different. However, the distinctions between them, if any, remain unclear.

In the present study hyaluronidase from human liver was purified to homogeneity and its properties were examined in an attempt to resolve the uncertainties associated with the characteristics of the lysosomal enzyme. These results were compared with those obtained with a partially purified preparation of the testicular-type enzyme in order to determine exactly what the differences between these two enzymes are.

Methods

Preparation of hyaluronic acid

The principal source of substrate used in this study was hyaluronic acid from human umbilical cord obtained from Miles. A preparation of bovine vitreous-humour hyaluronic acid (Sigma, grade IV) was also used. Both preparations were contaminated with small quantities of sulphated glycosaminoglycans (Gold, 1981), which were removed by taking advantage of the different affinities exhibited by the two kinds of glycosaminoglycans toward albumin as described by Gold (1980).

Purified samples of hyaluronic acid, dermatan sulphate and keratan sulphate were obtained from

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Enzyme assay

Degradation of hyaluronic acid by purified liver hyaluronidase was routinely measured by incubating enzyme with 125 μg of purified hyaluronic acid, 20–30 μg of crystalline bovine albumin (Sigma or Pentex) and 5 μmol of sodium acetate buffer, pH 4.0, in a final volume of 0.08–0.10 ml at 37°C. Mixtures for determination of enzyme activity during the purification procedure contained, in addition to the other components, 15 μmol of acetamide, which had no effect on hyaluronidase activity, but prevented degradation of oligosaccharides released by hyaluronidase by inhibiting *N*-acetylglucosaminidase, a persistent contaminant throughout the purification procedure. Degradation of chondroitin sulphate was measured as described above, except that chondroitin sulphate (Sigma, mixed isomers) was used in place of hyaluronic acid and the reaction was carried out at pH 4.1. The enzyme reaction was terminated in all instances by adding 0.10 ml of saturated sodium borate, followed by heating in a boiling-water bath for 3 min. The release of *N*-acetylglucosamine end groups was measured, after centrifugation to remove any particulate matter, by the procedure of Reissig *et al.* (1955).

Enzyme incubations of 60 min were employed in kinetic analyses. Experiments verified that enzyme activity progressed linearly over this length of time.

Testicular enzyme

A commercially available preparation of bovine testicular hyaluronidase (type IV, Sigma) was purified 8-fold by gel filtration on a column (1.8 cm × 60 cm) of Sephadex G-200 eluted with 50 mM-sodium phosphate buffer, pH 7.4. The enzyme still contained *N*-acetylglucosaminidase activity and was therefore assayed in the presence of acetamide. Incubations were performed in polypropylene tubes at pH 4.4, and 10 μmol of NaCl was included. The addition of albumin to assays, to make final protein concentrations about the same, avoided interference owing to differences in purity and therefore stability between the hyaluronidases studied.

Purification of human liver hyaluronidase

Human liver (150 g) was obtained at necropsy, homogenized in 0.25 M-sucrose (2.0 ml/g liver) and centrifuged at 10000 *g* for 15 min. Enzyme was precipitated from the resulting supernatant by adding solid $(NH_4)_2SO_4$ to 60% saturation, redissolved in water and dialysed for 24 h against 5 mM-phosphate buffer, pH 7.2. The enzyme solution was applied to a column (2.8 cm × 15 cm) of hydroxyapatite (Bio-gel HTP, DNA grade) equilibrated with

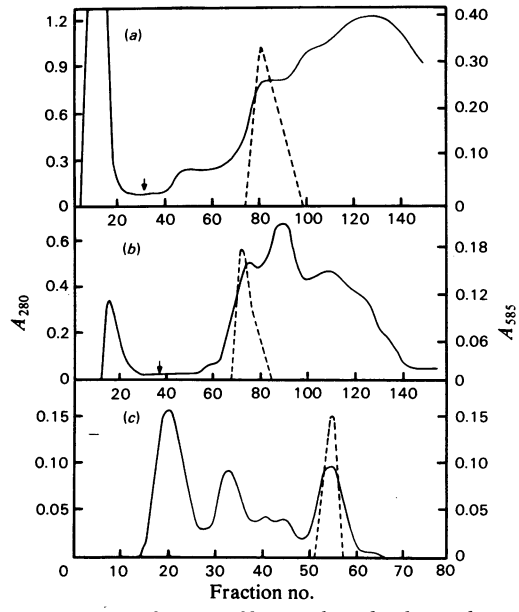

Fig. 1. *Purification of human liver hyaluronidase*
Enzyme was subjected to column chromatography on hydroxyapatite (*a*) and DEAE-cellulose (*b*) and preparative disc-gel electrophoresis on polyacrylamide (*c*). The appropriate eluting gradients in the column chromatographies were begun where indicated by the arrows. Fraction sizes: (*a*), 1.0 ml; (*b*), 0.6 ml; (*c*), 0.2 ml. Protein was monitored by recording A_{280} (——). Enzyme activity, plotted as the change in A_{585} (– – – –), was determined as described in the text.

5 mM-phosphate buffer containing 5 mM-NaCl. The column was washed with starting buffer, followed by a linear 5–80 mM-phosphate gradient (total vol. 120 ml) (Fig. 1*a*). The pH was maintained at 7.2. Fractions exhibiting enzyme activity were pooled, dialysed against water, concentrated by freeze-drying and dialysed against the 5 mM-phosphate/NaCl buffer. The enzyme was then applied to a column (2.0 × 6.0 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with the 5 mM-phosphate/NaCl buffer. The column was washed with starting buffer, followed by a linear 5–100 mM-NaCl gradient in 5 mM-phosphate (total vol. 80 ml) (Fig. 1*b*). Enzyme was recovered from fractions exhibiting enzyme activity as described above and subjected to preparative polyacrylamide-disc-gel electrophoresis. The enzyme was applied to a 7.5%-acrylamide gel of 150 mm^2 cross-sectional area. Electrophoresis was run at pH 8.5 at a constant voltage of 100 mV in a Canalco Prep-Disc apparatus (Miles) by using Canalco ultrapure electrophoresis reagents (Fig. 1*c*). Protein was determined by the procedure of Lowry *et al.* (1951).

Results and discussion

Purity of enzyme

The purified enzyme exhibited one band of protein after electrophoresis in 7%-polyacrylamide gels at pH 8.8, which corresponded to the location of enzyme activity extracted from pulverized 0.5 cm gel segments. A single band was also obtained after electrophoresis in 10%-polyacrylamide gels in the presence of sodium dodecyl sulphate at pH 7.0. The purification procedure is summarized in Table 1.

Properties of liver and testicular hyaluronidases

The pH optimum for degradation of hyaluronic acid by purified liver hyaluronidase was 3.8–4.0. It was not altered by the presence of bovine serum albumin, dermatan sulphate, keratan sulphate or NaCl. The pH optimum for the degradation of chondroitin sulphate was 4.0–4.2. The enzyme exhibited no activity at pH 4.8 or above. The liver enzyme therefore resembled the hyaluronidases present in other tissues, which are believed to be lysosomal In contrast, the activity of testicular hyaluronidase exhibited activity over a broader pH range, with an optimum around 4.4. These pH differences have historically been one of the principal distinctions between the two types of enzymes.

The molecular weight of the purified liver enzyme was estimated by gel filtration on a column of Sephadex G-200 calibrated with the following standards: ovalbumin (mol.wt. 45000), albumin (69000), transferrin (90000) and γ-globulin (160000). The molecular weight was calculated to be 76000, which was close to the 70000 found for the purified human placenta enzyme (Yamada *et al.*, 1977), but less than the 89000 reported for the purified rat liver enzyme (Aronson & Davidson, 1967*b*).

Table 1. *Summary of purification of human liver hyaluronidase*

Enzyme was purified from 150g of human liver. One unit of activity is the amount of enzyme that releases 1μmol of *N*-acetylglucosamine/min from purified hyaluronic acid. All procedures were carried out at 5°C.

Procedure	Specific activity (munits/mg)	Purification (fold)	Yield (%)
Tissue homogenate	0.027	1.0	100.0
$(NH_4)_2SO_4$ fractionation	0.120	4.3	96.0
Hydroxyapatite chromatography	1.10	39.6	82.0
DEAE-cellulose chromatography	5.50	203.0	44.0
Preparative gel electrophoresis	25.0	960.0	5.0

It has been reported that testicular hyaluronidase is adsorbed on to glass surfaces, thereby requiring the use of plastic or silicone-treated glass equipment (Rasmussen, 1954). In accord with that report, it was found that the activity of the testicular enzyme was higher in incubation tubes made of polystyrene or polypropylene than in tubes of glass. These differences were, however, largely abolished if albumin was included in the incubation medium. Liver hyaluronidase, on the other hand, exhibited no significant difference in activity in incubation tubes made of glass or polystyrene and only slightly higher activity in polypropylene tubes. The presence of albumin increased enzyme activity in all types of tubes to about the same value.

Effect of albumin on enzyme activity

The presence of albumin increased the rate of degradation of both hyaluronic acid and chondroitin sulphate by purified liver hyaluronidase (Fig. 2*a*). The enzyme exhibited little difference in activity whether albumin was added to incubations before or after preincubation for several hours, suggesting that albumin did not act by preventing inactivation of the enzyme during assay. The testicular enzyme also exhibited increased activity in the presence of albumin, but the effect was decreased as the concentration of NaCl approached the optimum (Fig. 2*a*). The effect was due, perhaps, to an interaction between albumin and hyaluronic acid (or chondroitin sulphate), which somehow increases the susceptibility of the substrate to enzyme action. Albumin has previously been shown to interact with hyaluronic acid, resulting in a conformational change in the structure of the protein (Gold, 1980). A similar type of structural change occurring in the hyaluronic acid could be neither confirmed nor disproved by the previous study.

Effect of NaCl

The testicular enzyme exhibited increased activity in the presence of NaCl (Fig. 2*b*). The activity was extremely low if both NaCl and albumin were excluded from the medium. It seems unlikely that NaCl would exert its effect by the same mechanism as did albumin. It has previously been shown that low concentrations of NaCl can disrupt the interaction between albumin and hyaluronic acid or chondroitin sulphate (Gold, 1980), but the nature of the interaction between the salt itself and the polysaccharide remains to be established. An explanation for the effect of NaCl may lie in the observation that, at low pH, intermolecular hyaluronic acid interactions may be disrupted as the ionic strength is increased (Welsh *et al.*, 1980). Alternatively, NaCl may exert its effect through its ability to enhance the development of tertiary and

higher structures of hyaluronic acid in solution (Morris *et al.*, 1980).

The activity of the lysosomal enzyme, in contrast, was markedly decreased, after an initial small increase, as the concentration of NaCl was increased (Fig. 2*b*). The small increase at low concentrations of NaCl may be attributable to the effects of a small amount of sulphated glycosaminoglycan still present or, perhaps, to the same mechanism proposed for the increase in activity of the testicular enzyme, namely an NaCl-induced alteration in the substrate. Higher concentrations of Cl^- inhibited enzyme activity through its apparent affinity for the active site of the enzyme.

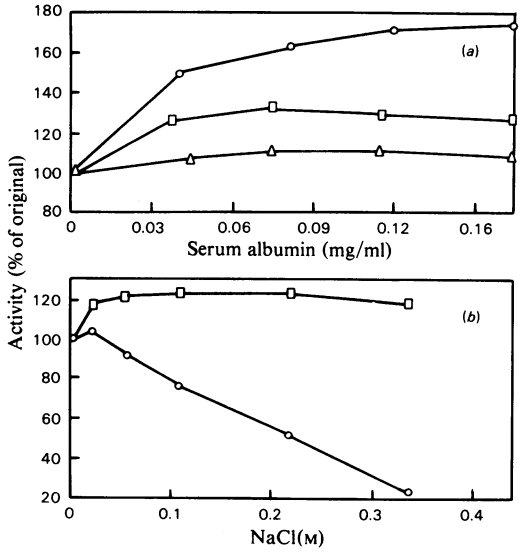

Fig. 2. *Effect of albumin and NaCl on purified human liver and partially purified bovine testicular hyaluronidases*

(*a*) The liver enzyme was assayed in the absence of NaCl (O) and the testicular enzyme in the presence of (□) 0.02 M- or (△) 0.06 M-NaCl. (*b*) Incubations of both liver (O) and testicular (□) enzymes included 50 μg of bovine serum albumin.

Analysis of hyaluronidase kinetics

Maximal velocity of the liver enzyme increased with increasing concentration of albumin (Fig. 3*a*). Substrate affinity was lowest in the absence of albumin. In experiments at various concentrations of NaCl, in the presence of a fixed concentration of albumin, Cl^- ion exhibited what appeared to be competitive inhibition, with a K_i calculated to be 0.15 M (Fig. 3*b*). The K_m for purified hyaluronic acid for the human liver enzyme was determined from these studies to be 0.05 mg/ml.

The maximal velocity of the testicular enzyme, in the absence of NaCl, also increased with increasing concentrations of albumin (Fig. 4*a*). Experiments carried out in various concentrations of NaCl, in the absence of albumin, demonstrated that increasing concentrations of NaCl produced effects that were quite similar to those observed with albumin (Fig. 4*b*). The K_m for purified hyaluronic acid for the testicular enzyme was found to be 0.55 mg/ml.

Dermatan sulphate and keratan sulphate were both found to be competitive inhibitors of the two enzymes (Fig. 5). Dermatan sulphate was the more potent inhibitor with K_i 0.06 mg/ml and 0.03 mg/ml for the liver and testicular enzyme respectively. The K_i for keratan sulphate was 0.08 mg/ml for the liver enzyme and 0.22 mg/ml for the testicular enzyme.

In the present study neither the liver nor the testicular enzyme was inhibited by high concentrations of substrate. The previous reports of substrate inhibition with the rat liver enzyme may be attributable to the substrates employed. This is quite

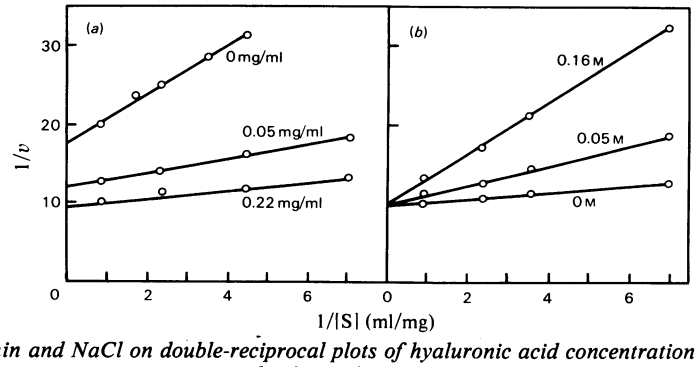

Fig. 3. *Effect of albumin and NaCl on double-reciprocal plots of hyaluronic acid concentration and purified human liver hyaluronidase activity*

Incubations included the indicated concentrations of albumin (*a*) or NaCl (*b*). Enzyme activity (v) is expressed as the change in A_{585}/h.

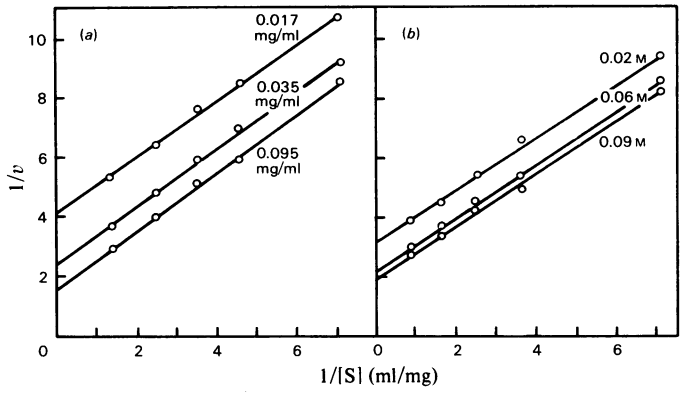

Fig. 4. *Effect of albumin and NaCl on double-reciprocal plots of hyaluronic acid and partially purified bovine testicular hyaluronidase activity*

Incubations included the indicated concentrations of albumin with no NaCl (*a*) or NaCl with no albumin (*b*). Enzyme activity (v) is expressed as the change in A_{585}/15 min.

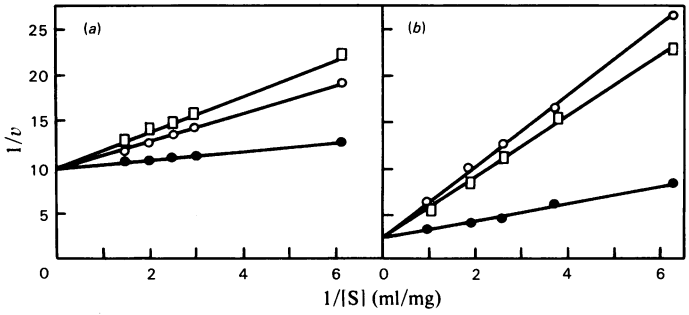

Fig. 5. *Effect of dermatan sulphate and keratan sulphate on double-reciprocal plots of hyaluronic acid concentration and hyaluronidase activity*

(*a*) Incubations of purified human liver enzyme included 0.5 mg of albumin/ml and 0.12 mg of dermatan sulphate/ml (○), 0.28 mg of keratan sulphate/ml (□) or no inhibitor (●). Enzyme activity is expressed as in Fig. 3. (*b*) Incubations of partially purified bovine testicular hyaluronidase included 0.5 mg of albumin/ml, with no NaCl, and 0.08 mg of dermatan sulphate/ml (○), 0.40 mg of keratan sulphate/ml (□) or no inhibitor (●). Enzyme activity is expressed as in Fig. 4.

clear in the study (Hutterer, 1966) which used a commercial preparation of hyaluronic acid which has been shown to contain a great deal of impurity (Gold, 1981). Aronson & Davidson (1976*b*), observing that NaCl decreased enzyme activity at low concentration of substrate but increased activity at higher concentrations of substrate, suggested that their substrate too contained some polyanionic inhibitor, which was responsible for the apparent substrate inhibition. It is clear that useful information about hyaluronidases can be obtained only if highly purified substrates are employed.

It might be noted that the two enzymes function in very different physiological environments. The testicular enzyme probably acts extracellularly, where it plays a role in the process of fertilization. In contrast, the lysosomal enzyme would find conditions of pH and NaCl concentration outside its cellular organelle highly unfavourable, although an extra-lysosomal role has been proposed for hyaluronidase in the degradation of cartilage matrix (Bollet, 1967). The most important distinction between the two enzymes, however, appears to be the different response to NaCl.

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