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MUCOPOLYSACCHARIDE-DEGRADING ENZYMES FROM THE LIVER OF THE SQUID, *OMMASTREPHES SLOANI PACIFICUS*

I. HYALURONIDASE

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SUMMARY

1. Liver extracts of the squid, *Ommastrephes sloani pacificus*, contain mucopolysaccharide-degrading enzymes: hyaluronidase (hyaluronate glycanohydrolase, EC 3.2.1.35), β-*N*-acetylhexosaminidase (EC 3.2.1.30); β-glucuronidase (EC 3.2.1.31) and chondrosulphatase (EC 3.1.6.4).

2. The squid hyaluronidase which was partially purified by gel chromatography on Sephadex G-150 showed the maximum activity at pH 4.5–5.0 and the temperature of 37°. The activity was completely inhibited by Cu^{2+} and Fe^{3+}.

3. Hyaluronate was the best substrate of the squid hyaluronidase and several sulphated mucopolysaccharides were also degraded. The digestion products of hyaluronate were identical with those obtained by bovine testicular hyaluronidase digestion, indicating that the squid liver hyaluronidase is an endo-β-*N*-acetylhexosaminidase.

4. This seems to be the first example of a testicular type hyaluronidase found in invertebrate tissues.

INTRODUCTION

Hyaluronidases (hyaluronate glycanohydrolase, EC 3.2.1.35) are known to be widely distributed in various animal tissues[1] and bacteria[2], and to degrade hyaluronic acid to yield oligosaccharides by several different mechanisms. However, there has been no report in reference to the hyaluronidase from the tissues of molluscs, although they contain a variety of glycosidases[3,4] and sulphatases[5–7]. Previously it was reported that liver extracts of a marine mollusc, the squid *Ommastrephes sloani pacificus*, liberate inorganic sulphate from chondroitin sulphate; chondrosulphatase (EC 3.1.6.4) activity was present[8]. This study demonstrates the presence in the squid liver of three other mucopolysaccharide-degrading enzymes, hyaluronidase, β-*N*-acetylhexosaminidase (EC 3.2.1.30) and β-glucuronidase (EC 3.2.1.31), and describes the partial purification and characterization of the hyaluronidase.

EXPERIMENTAL

Materials

Sodium hyaluronate was prepared from shark skin by cetylpyridinium chloride fractionation[9]. This preparation gave only one spot by electrophoresis on a cellulose acetate strip with pyridine–acetic acid at pH 3.5. Chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate were obtained from Seikagaku Kogyo, Ltd., Tokyo, Japan. Chondroitin[10] and chondroitin sulphate E (refs. 11, 12) were isolated from squid skin and cartilage, respectively. Heparin was purchased from Daiichi Pure Chemicals, Ltd., Tokyo, and *p*-nitrophenyl β-D-glucuronide from Junsei Chemicals, Ltd., Tokyo. *p*-Nitrophenyl β-*N*-acetyl-D-glucosaminide was synthesized by the method of LEABACK[13].

The bovine testicular hyaluronidase was a commercial preparation (Sigma Chemical Co. Ltd.) assaying at 280 National Formulary (NF) units/mg. *N*-Acetylhyalobiuronic acid was prepared by *N*-acetylation[14] of hyalobiuronic acid which was obtained from hyaluronate according to the method of DAVIDSON AND MEYER[15]. Hyaluronate tetra-, hexa- and octasaccharides were prepared by the method of FLODIN *et al.*[16].

The Sephadex column (K25/100) and flow adaptor for gel chromatography were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Analytical procedures

The ferricyanide method of SCHALES AND SCHALES[17] was modified for the determination of enzymically liberated reducing sugar, to obtain alkaline conditions in the presence of acidic buffers. The modified reagent was prepared by dissolving 1.65 g of potassium ferricyanide in 1 l of 0.5 M NaOH. The procedure followed was the same as that of the original method. By using the modified method 2–70 μg of glucose could be quantitatively determined in 0.2 M sodium acetate buffer of pH 3–6 and 0.1 M sodium phosphate buffer of pH 6–8.

The protein content was determined by the method of LOWRY *et al.*[18] with bovine serum albumin as a standard.

Enzyme assays

Incubations were carried out at 37° in 0.1 M sodium acetate buffer (pH 5.0), unless otherwise indicated. Control determinations were run in which enzyme and substrate were separately incubated.

For hyaluronidase assay, 150 μg of sodium hyaluronate was incubated with enzyme for 15 h in a final volume of 0.5 ml and the increase in reducing sugar was measured as glucose by the modified method described above. Hyaluronidase activity was also assayed by determination of reducing terminal *N*-acetylglucosamine liberated, according to the method of REISSIG *et al.*[19].

β-*N*-Acetylhexosaminidase and β-glucuronidase activities were assayed by the determination of *p*-nitrophenol released from *p*-nitrophenyl β-*N*-acetylglucosaminide and *p*-nitrophenyl β-glucuronide, respectively. Incubation mixtures of a final volume of 0.5 ml contained 100 μg of each substrate and enzyme. After incubation for 15 min for β-*N*-acetylhexosaminidase and 30 min for β-glucuronidase, 2.0 ml of 0.2 M Na_2CO_3 was added and the absorbance at 400 nm was measured using *p*-nitrophenol as the standard.

Chondrosulphatase assay was carried out as described previously[8].

Paper chromatography

The reaction products of enzymic digestion were chromatographed by a descending method on Toyo No. 51 paper in *n*-butanol–acetic acid–water (44:16:40, by vol.) for 48 h. Sugars were detected by staining with alkaline $AgNO_3$ (ref. 20) and also with Morgan–Elson reagent[21]. Unsaturated compounds on the paper chromatogram were detected by viewing under ultraviolet light with a Super-Light LS-D1 (2537 Å).

Extraction of crude enzyme preparation from squid liver

All procedures were performed at 0–5°. The fresh squids, *Ommastrephes sloani pacificus*, were obtained from a fish market and their livers were excised. The internal materials of the liver were placed in cold acetone, stirred and centrifuged for 10 min at 2500 × *g* to remove the supernatant. Acetone was added to the residue and this procedure was repeated until the supernatant became pale yellow. The residue finally obtained was dried in a desiccator and the powder (10 g) was homogenized with 10 vol. of 0.1 M sodium acetate buffer (pH 7.0) in a Potter–Elvehjem homogenizer. The homogenate was centrifuged for 30 min at 9500 × *g* and the supernatant was adjusted to pH 4.5 with 1.0 M acetic acid. After standing in a refrigerator overnight, the precipitate was removed by centrifuging for 30 min at 9500 × *g* and to the supernatant $(NH_4)_2SO_4$ was added to 70% saturation. After standing in a refrigerator overnight the resulting precipitate was dissolved in 60 ml of 0.1 M sodium acetate buffer (pH 5.0) and aliquots (10–20 ml) of this solution were passed through a column of Sephadex G-25 (2.5 cm × 30 cm) which had been equilibrated with 0.1 M sodium acetate buffer (pH 5.0). The column was eluted with the same buffer and the protein-containing, $(NH_4)_2SO_4$-free fraction was used as the crude enzyme preparation.

RESULTS

Degradation of hyaluronate and chondroitin 4-sulphate by crude enzyme preparation

Hyaluronate and chondroitin 4-sulphate were each incubated with the crude enzyme preparation from squid liver, and the release of reducing sugar and inorganic sulphate with time was measured. As shown in Fig. 1, hyaluronate was degraded by this preparation to release about 40% of reducing sugar as glucose after a 48-h incubation, and the value of reducing sugar reached nearly 80% after prolonged incubation (194 h). Chondroitin 4-sulphate was degraded more slowly than hyaluronate (Fig. 1) and released inorganic sulphate at the same time (Fig. 2). In the presence of 0.05 M NaF, which is known to be an inhibitor of bacterial chondrosulphatase[22], no release of inorganic sulphate from chondroitin 4-sulphate was observed (Fig. 2), nevertheless reducing sugar was released at the same rate as in the absence of inhibitor (Fig. 1).

To identify degradation products, the reaction mixtures of hyaluronate after 48-h and 194-h incubations were boiled for 1 min and centrifuged. The supernatants were applied to a column of Sephadex G-10 (1.2 cm × 100 cm) and eluted with water to be desalted. The fractions which gave a positive test for carbazole reaction[23] were collected and chromatographed on Toyo No. 51 paper. The degradation products obtained from hyaluronate after a 48-h incubation with squid enzyme preparation showed several spots on staining with alkaline silver nitrate. The two faster-moving

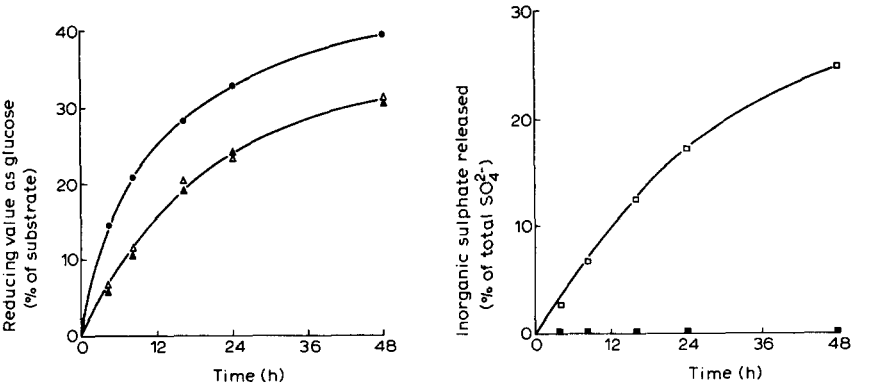

Fig. 1. Release of reducing sugar from hyaluronate and chondroitin 4-sulphate by crude enzyme preparation of squid liver. Substrates (each 2.5 mg) were incubated with 0.1 ml of enzyme in a final volume of 1 ml; at indicated intervals 0.1 ml of the reaction mixtures was removed to measure the reducing sugar released. ●, hyaluronate; △, chondroitin-4-sulphate; ▲, chondroitin 4-sulphate in the presence of 0.05 M NaF.

Fig. 2. Release of inorganic sulphate from chondroitin 4-sulphate by crude enzyme preparation of squid liver. Each tube contained 2.5 mg of chondroitin 4-sulphate and 0.1 ml of enzyme in a final volume of 1 ml. After incubation for the indicated time in the presence of 0.05 M NaF (■) or not (□), 0.25 ml of 3% cetylpyridinium chloride solution was added to the reaction mixtures and the supernatant (0.5 ml) was subjected to sulphate assay.

spots corresponded to *N*-acetylglucosamine and glucuronic acid, and the slower spots have nearly the same mobilities as those of oligosaccharides obtained from hyaluronate by testicular hyaluronidase digestion. The major products of a 194-h incubation were *N*-acetylglucosamine and glucuronic acid; practically no oligosaccharides were detected.

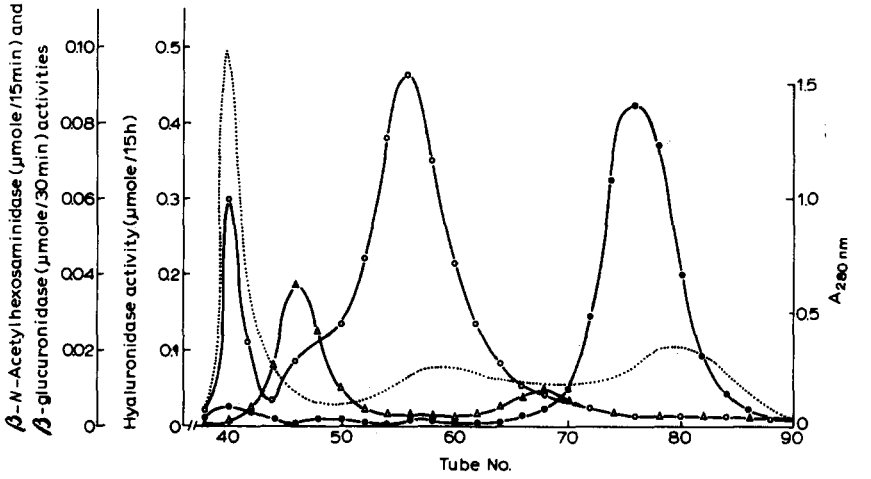

Fig. 3. Gel chromatography of the squid liver enzyme on Sephadex G-150. Column size was 2.5 cm × 100 cm. The crude enzyme solution (10 ml) was applied on the column and eluted upwards with 0.1 M NaCl. Fractions of 5 ml each were collected. Hyaluronidase activity (●) assayed for 0.1 ml of eluate was expressed as μmole glucose released in 15 h, β-*N*-acetylhexosaminidase activity (○) for 0.05 ml as μmoles *p*-nitrophenol released in 15 min and β-glucuronidase activity (△) for 0.1 ml as μmole *p*-nitrophenol released in 30 min. The absorbance at 280 nm (·····) was also measured.

These observations suggest that the crude enzyme preparation from squid liver contains hyaluronidase, β-*N*-acetylhexosaminidase and β-glucuronidase besides chondrosulphatase described previously[8].

Partial purification of squid liver hyaluronidase

The squid liver hyaluronidase was partially purified by gel chromatography. Crude enzyme solution (10 ml) was applied on a column of Sephadex G-150 (2.5 cm × 100 cm) which had been equilibrated with 0.1 M NaCl. The column was eluted upwards with the same solution at a flow rate of 20 ml/h and fractions of 5 ml each were collected. Aliquots were assayed for hyaluronidase, β-*N*-acetylhexosaminidase and β-glucuronidase activities, and the absorbance of the eluates at 280 nm was measured for protein content. The results are shown in Fig. 3. Hyaluronidase was eluted as a single peak and mostly separated from the other two enzymes. The hyaluronidase-active fraction was pooled and refractionated on the same column of Sephadex G-150. The elution pattern is shown in Fig. 4. The hyaluronidase-active fraction was pooled and used as the partially purified enzyme.

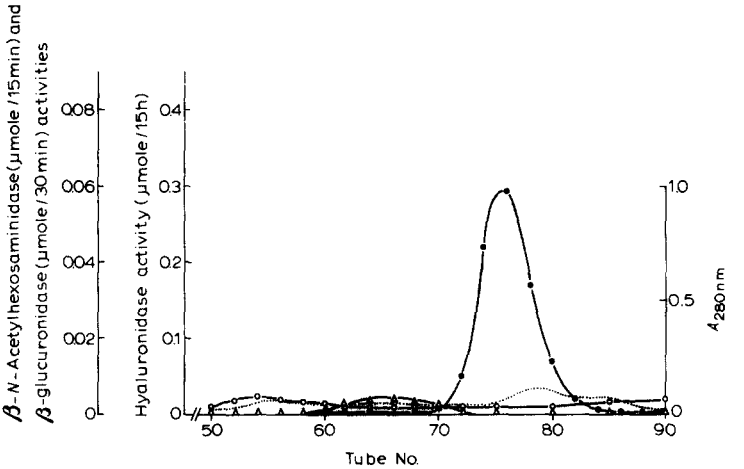

Fig. 4. Second gel chromatography of the hyaluronidase fraction on Sephadex G-150. The hyaluronidase-active fractions (tubes 70–84) obtained in the first gel chromatography (Fig. 3) were freeze-dried and the white powder was dissolved in 10 ml of water. After dialysis to remove excess NaCl, the solution was refractionated on the same column. Procedure and assay systems were the same as Fig. 3.

The procedure for purifying squid hyaluronidase is summarized in Table I. The purified preparation was almost free of β-*N*-acetylhexosaminidase and β-glucuronidase. About 25-fold purification of the squid hyaluronidase over the activity in the homogenate was indicated, but the actual purification may have attained a higher value than that indicated here, because the hyaluronidase activity in the homogenate was apparently over-estimated owing to the presence of β-*N*-acetylhexosaminidase and β-glucuronidase.

The partially purified hyaluronidase was stored at 4° or −20° without appreciable loss of activity for several weeks.

TABLE I

PARTIAL PURIFICATION OF SQUID LIVER HYALURONIDASE

Incubations were carried out at 37° in 0.1 M sodium acetate buffer (pH 5.0). Reaction mixtures contained 150 μg of hyaluronate and appropriate amounts of enzyme preparation in a final volume of 0.5 ml, and after 15 h the release of reducing sugar was measured as glucose. Specific activity is expressed as μmoles glucose released in 15 h per mg of protein.

Step	*Vol. (ml)*	*Activity (μmoles glucose released/ml after 15 h)*	*Protein (mg/ml)*	*Specific activity (μmoles/mg)*	*Purity*
Homogenate	90	59.3*	19.6	3.0	1
Crude preparation	54	90.3*	8.8	10.3	3.4
1st Sephadex G-150	75	10.2	0.24	42.5	14.2
2nd Sephadex G-150	45	6.5	0.085	76.5	25.5

* This value is not indicating accurate activity because of the presence of β-*N*-acetylhexosaminidase and β-glucuronidase.

Properties of squid liver hyaluronidase

The effects of pH on the squid hyaluronidase are shown in Fig. 5. The maximum activity was obtained with hyaluronate at pH 4.5–5.0 in sodium acetate buffer. The optimum temperature of this enzyme was 37° as shown in Fig. 6.

As shown in Table II, the squid hyaluronidase activity was completely inhi-

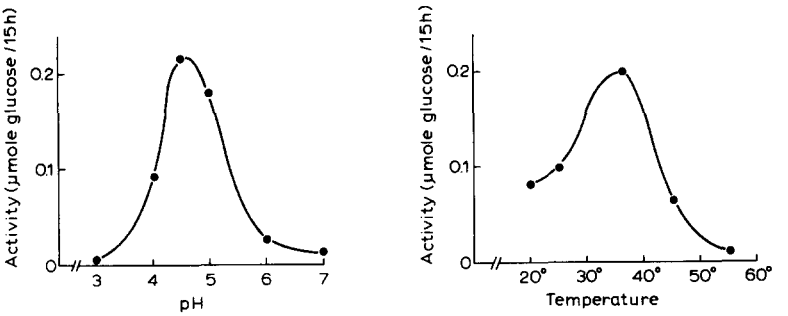

Fig. 5. Effect of pH on the activity of squid liver hyaluronidase. Assays were performed at 37° in 0.1 M sodium acetate buffer at indicated pH. Incubation mixtures contained 150 μg of hyaluronate and 0.1 ml of the partially purified squid liver hyaluronidase in a final volume of 0.5 ml. After 15 h reducing sugar liberated was measured as glucose.

Fig. 6. Effect of incubation temperature on the activity of squid liver hyaluronidase. Assays were performed in 0.1 M sodium acetate buffer (pH 5.0) at the specified temperature. Substrate and enzyme concentrations as in Fig. 5.

bited by 0.01 M $CuSO_4$ and $FeCl_3$. Dermatan sulphate and heparin also inhibited the activity, but NaF and Na_3PO_4, which are known to be general inhibitors of chondrosulphatase[22], had little effect. The effect of the mucopolysaccharides appears to be one of competitive inhibition, as is seen in other hyaluronidases[1]. An activating effect of NaCl on the activity was not observed.

Several mucopolysaccharides, as substrate, were each incubated with the par-

TABLE II

EFFECTS OF SEVERAL COMPOUNDS ON PARTIALLY PURIFIED SQUID LIVER HYALURONIDASE

Incubations were carried out at 37° in 0.1 M sodium acetate buffer (pH 5.0) containing several compounds to final concentrations indicated in the table. Substrate and enzyme concentrations as in Fig. 5.

Compound	*Concentration*	*Relative activity (%)*
None		100
NaCl	0.15 M	97
$MgCl_2$	0.01 M	139
$BaCl_2$	0.01 M	138
$CuSO_4$	0.01 M	0
$FeCl_3$	0.01 M	0
Na_3PO_4	0.01 M	86
NaF	0.01 M	97
Dermatan sulphate	200 μg/ml	46
Heparin	200 μg/ml	2

tially purified hyaluronidase, and aliquots of the reaction mixtures were taken at intervals to measure the reducing value as glucose. As shown in Fig. 7, hyaluronate was the best substrate, and chondroitin, chondroitin 4-sulphate, chondroitin 6-sulphate and chondroitin sulphate E were also degraded. In the hyaluronate incubation, the release of reducing terminal *N*-acetylglucosamine residues was determined at a rate comparable to that of reducing sugar, indicating that the hexosaminidic bonds of hyaluronate were degraded. Commercial dermatan sulphate from pig skin was slightly degraded with this enzyme, but heparin was completely resistant.

The reaction products obtained from hyaluronate by digestion with squid liver hyaluronidase were identified by paper chromatography. The digests gave mainly 4 spots whose mobilities corresponded to *N*-acetylhyalobiuronic acid ($R_{glucuronic\ acid}$, 0.88), hyaluronate tetrasaccharide ($R_{glucuronic\ acid}$, 0.49), hexasaccharide ($R_{glucuronic\ acid}$, 0.32) and octasaccharide ($R_{glucuronic\ acid}$, 0.19). All the spots of oligosaccharides

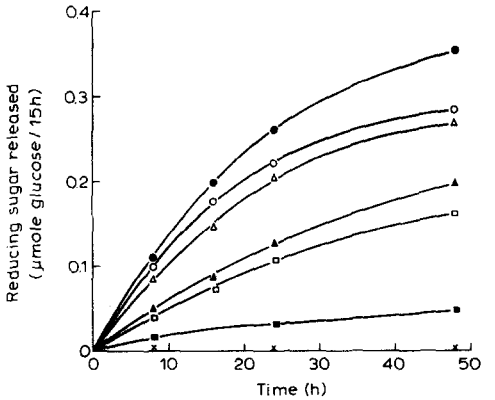

Fig. 7. Substrate specificity of squid liver hyaluronidase. Assays were performed at 37° in 0.1 M sodium acetate buffer (pH 5.0). Substrate and enzyme concentrations as in Fig. 5. ●, hyaluronate; ○, chondroitin; △, chondroitin 4-sulphate; ▲, chondroitin 6-sulphate; □, chondroitin sulphate E; ■, dermatan sulphate; ×, heparin.

gave positive reactions with Morgan–Elson spray as well as with alkaline $AgNO_3$. No ultraviolet absorption was detectable by viewing the paper chromatogram under an ultraviolet light and furthermore the increase in absorption at 235 nm was not observed during the digestion. These results indicate that each oligosaccharide carries an *N*-acetylhexosamine in the reducing end and no unsaturated uronic acid; the squid hyaluronidase is not an eliminase but an endo-*N*-acetylhexosaminidase which hydrolyzes the β-1,4-glucosaminidic bond of hyaluronate in the manner of bovine testicular hyaluronidase.

DISCUSSION

Liver extracts of the squid, *Ommastrephes sloani pacificus*, degraded hyaluronate to yield oligosaccharides, *N*-acetylglucosamine and glucuronic acid, and chondroitin 4-sulphate to release inorganic sulphate in addition to reducing sugars. These observations suggest that the squid liver contains the mucopolysaccharide-degrading enzymes, hyaluronidase, β-*N*-acetylhexosaminidase, β-glucuronidase and chondrosulphatase. These enzymes appear to participate, in this animal, in the degradation of mucopolysaccharides including chondroitin and chondroitin sulphate E which were isolated previously from skin[10] and cartilage[11], respectively.

The squid hyaluronidase described in this paper shows properties very similar to those of bovine testicular hyaluronidase. Animal hyaluronidases which have been well-characterized[1] are those from various mammalian tissues, particularly testis, tadpole tail fin, venoms of snake and bee, and leech head. All these enzymes except leech hyaluronidase act on hyaluronic acid as endo-β-hexosaminidases. On the other hand, the leech enzyme, which is an example of a hyaluronidase from invertebrate tissues, is endo-β-glucuronidase[24,25]. The hyaluronidases from venoms of snake[26,27] and bee[28,29] appear to resemble the testicular hyaluronidase, but their properties have not been sufficiently characterized. Recently, SILBERT AND DELUCA[30] reported that the tadpole hyaluronidase degraded chondroitin 4-sulphate as well as hyaluronic acid, but that other sulphated mucopolysaccharides were completely resistant. Thus hyaluronidases which occur in various sources differ from each other in several properties. This diversity of the hyaluronidases may be derived not only from the differences among animal species but also from the functions of the enzyme and the kinds of substrate mucopolysaccharides in various tissues. It is further interesting that all the animal hyaluronidases demonstrated hitherto including this of the present study, are glycohydrolases rather than the eliminases that have been found in bacterial sources. Recently, OHYA AND KANEKO[31] obtained a novel hyaluronidase from *Streptomyces hyalurolyticus*. This enzyme was an eliminase and acted on hyaluronic acid only to yield unsaturated tetra- and hexasaccharides.

The presence of chondrosulphatase was demonstrated previously in the squid liver[8]. However, even if chondrosulphatase activity was completely inhibited by 0.05 M NaF, chondroitin 4-sulphate was degraded by the squid liver extracts at the same rate as that found in the absence of inhibitor (Fig. 1). Furthermore, chondrosulphatase was separately eluted from hyaluronidase by Sephadex G-150 gel chromatography under the same conditions as that used in the present study (Y. KAWAI AND K. ANNO, unpublished), suggesting that the squid hyaluronidase acts on intact chondroitin sulphates.

In the experiments on substrate specificity, a slight increase of reducing value was observed in the incubation of commercial dermatan sulphate from pig skin with the squid hyaluronidase. However, this sample was not degraded after it had been exhaustively digested with testicular hyaluronidase. Consequently, the release of reducing sugar shown in our data appears to result from the degradation of a glucuronate portion which has been reported to be present in the pig skin dermatan sulphate chain making a hybrid structure[32], and the squid hyaluronidase may not act on "pure" dermatan sulphate.

It is still uncertain whether the squid hyaluronidase has transglycosylation activity which was reported in the hyaluronidase preparations from bovine testis[26,33] and snake venom[26]. A study to resolve this problem is now proceeding.

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