Chondrosulphatase of Squid Liver

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Chondrosulphatase, which hydrolyses the ester sulphate of chondroitin sulphate, was first found in bacteria (Neuberg & Rubin, 1914). Many studies have since been reported on bacterial chondrosulphatase, especially that of *Proteus vulgaris* (Dodgson *et al.*, 1957; Dodgson & Lloyd, 1957), and separation of two types of sulphatases, chondro-4-sulphatase and chondro-6-sulphatase, which act on disaccharide sulphates, was achieved by Yamagata *et al.* (1968).

In animal sources, it was found that liver extracts of the marine gastropod *Charonia lampas* contain a chondrosulphatase in addition to other sulphatases (Soda & Egami, 1938), and the viscera of *Patella vulgata* contain an enzyme that liberates inorganic sulphate from chondroitin sulphates and sulphated oligosaccharides (Lloyd & Fielder, 1968). In vertebrates, a chondrosulphatase that attacks only polymer chondroitin 4-sulphate has been separated from bovine aorta (Held & Buddecke, 1967), and an enzyme that desulphates octasaccharide sulphate has been found in rat liver lysosomes (Tudball & Davidson, 1969).

It has previously been reported from this laboratory that liver extracts of the squid (*Ommastrephes sloani pacificus*) contain hyaluronidase (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) (Kawai *et al.*, 1969; Kawai & Anno, 1971). The present paper describes the purification and characterization of the chondrosulphatase of squid liver.

Experimental

Materials. Potassium chondroitin 4-sulphate was prepared from whale cartilage and sodium chondroitin 6-sulphate was from shark cartilage. Analyses of these compounds by chondroitinase ABC indicated 4-sulphate:6-sulphate molar ratios of 80:20 and 5:95 respectively. Sodium chondroitin 4,6-disulphate (4-sulphate:6-sulphate:4,6-disulphate molar proportions 33:8:59) was prepared from squid cartilage (Kawai *et al.*, 1966). Sodium keratan sulphate was a preparation from whale cartilage (Toda & Seno, 1970), and sodium hyaluronate was from umbilical cord. Sodium dermatan sulphate, 2-acetamido-2-deoxy-3-O-(β -D-glucosyl)-4-enepyransyluronic acid)-D-galactose, 2-acetamido-2-deoxy-3-O-(β -D-glucosyl)-4-enepyransyluronic acid)-4-O-sulpho-D-galactose and 2-acetamido-2-deoxy-3-O-(β -D-glucosyl)-4-enepyransyluronic acid)-6-O-sulpho-D-galactose were purchased from Seikagaku Kogyo Ltd. (Tokyo, Japan). 2-Acetamido-2-deoxy-3-O-(β -D-glucosyl)-4-enepyransyluronic acid)-4,6-O-disulpho-D-galactose was isolated from the digestion mixture of chondroitin 4,6-disulphate with chondroitinase ABC.

Bovine testicular hyaluronidase (280 National Formulary units/mg) was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and chondroitinase ABC was obtained from Seikagaku Kogyo Ltd.

Chondrosulphatase assay. (a) The reaction mixture consisted of 1.0 ml of 0.1 M-sodium acetate buffer, pH 5.0, containing substrate (2.0 mg) and enzyme (1.2 mg of protein). After incubation for the indicated time at 37°C, the released inorganic sulphate was measured by the cetylpyridinium chloride turbidimetric method (Kawai *et al.*, 1969).

(b) The reaction mixture consisted of 50 μ l of 0.1 M-sodium acetate buffer, pH 5.0, containing chondroitin [35 S]sulphate (approx. 2.5×10^3 c.p.m.; prepared from chicken tibiofemoral epiphyseal cartilage) and enzyme (58 μ g of protein). After incubation at 37°C for 16 h, inorganic [35 S]sulphate was separated by high-voltage electrophoresis (Yamagata *et al.*, 1968), and its radioactivity was counted in a gas-flow counter.

Hyaluronidase assay. Hyaluronate (16.5 μ g) was incubated with enzyme solution at 37°C for 16 h in a final volume of 0.1 ml (0.1 M-sodium acetate buffer, pH 4.5); 0.5 ml of water was added to the incubation mixture and then the increase of reducing power was measured as glucose by the method of Park & Johnson (1949).

Electrophoresis. Cellulose acetate electrophoresis was carried out in 1 M-acetic acid-pyridine buffer, pH 3.5, at 0.5 mA/cm for 20 min (a) and in 0.3 M-calcium acetate, pH 7.6, at 1 mA/cm for 4 h (b), and staining was performed with 0.5% Alcian Blue (Seno *et al.*, 1970).

Chondroitinase ABC digestion. Chondroitin sulphate (300 μ g) was incubated with the squid chondrosulphatase (1.5 mg of protein) at 37°C for 16 h in a final volume of 0.25 ml. The mixture was heated at 100°C for 1 min and centrifuged. The supernatant was dried *in vacuo*, 0.25 M-tris-HCl buffer, pH 8.0 (30 μ l), and chondroitinase ABC (0.2 unit, 20 μ l)

were added to it, and the mixture was incubated at 37°C for 16h. The unsaturated disaccharides produced were determined by paper chromatography (Saito *et al.*, 1968).

Results and discussion

The acetone-dried powder (10g) of squid liver was homogenized with 10vol. of 0.1M-sodium acetate buffer, pH7.0, in a Potter-Elvehjem homogenizer in an ice bath. All procedures were performed at 0–4°C. The extract obtained by centrifuging at 9500g for 30min was adjusted to pH4.5. The mixture was allowed to stand in a refrigerator overnight, and the supernatant obtained by centrifuging at 9500g for 30min was adjusted to pH5.0. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the crude enzyme, and the precipitate obtained at 30–70% saturation was dissolved in 30ml of 0.1M-sodium acetate buffer, pH5.0. Portions (10ml) of this solution were applied to a column (2.5cm×95cm) of Sephadex G-100 and eluted with 0.1M-NaCl. Chondrosulphatase was eluted between 180 and 300ml, and hyaluronidase between 280 and 350ml. The fractions (from 180 to 250ml) containing the chondrosulphatase activity were collected, concentrated in a Visking tube to 10ml *in vacuo* and dialysed against 0.1M-sodium acetate buffer, pH5.0. This enzyme fraction was almost free from hyaluronidase and was purified 15-fold from the crude enzyme, its specific activity being 0.31 (expressed as μmol of sulphate released/h per mg of protein). The partially purified chondrosulphatase was stored at –20°C, without appreciable loss of activity for a month.

The optimum pH of this enzyme was found to be about 5.0.

The chondrosulphatase activity was extremely inhibited by the presence of CuSO_4 , sodium phosphate, NaF, borax (final concn. 10mM in each case) and heparin (final concn. 1 mg/ml). The hyaluronidase activity of squid liver was inhibited by about 50% by zinc acetate (final concn. 10mM), whereas the chondrosulphatase was activated. The chondrosulphatase was also activated by NaCl, CaCl_2 , EDTA and cysteine hydrochloride (final concn. 10mM in each case). In these experiments the chondrosulphatase activity was assayed by using chondroitin [^{35}S]-sulphate and incubating at 37°C for 16h.

The apparent K_m of this chondrosulphatase was estimated by the Hofstee (1952) plot to be 0.49mM (expressed as mol of disaccharide dipotassium salt) when the reaction was carried out at pH5.0 and 37°C for 3h, with chondroitin 4-sulphate as the substrate (final concns. 0.54–6.34mM).

The substrate specificity of this enzyme was studied on the various mucopolysaccharides by quantitative determination of the liberated inorganic sulphate, by using the cetylpyridinium chloride turbidimetric method. The results are shown in

Fig. 1. Substrate specificity of chondrosulphatase from squid liver

The reaction mixture contained substrate (2.0mg) and enzyme (1.2mg of protein) in 1.0ml of 0.1M-sodium acetate buffer, pH5.0. After incubation for the indicated time at 37°C, liberated sulphate was measured by the cetylpyridinium chloride turbidimetric method. ■, Chondroitin 4-sulphate; ●, chondroitin 6-sulphate; ○, chondroitin 4,6-disulphate; □, keratan sulphate; ▲, dermatan sulphate; △, heparin.

Fig. 1. The squid chondrosulphatase attacked chondroitin 4-sulphate at greater rate than it did chondroitin 4,6-disulphate. Chondroitin 6-sulphate was also desulphated to a slight extent, but keratan sulphate, dermatan sulphate and heparin were not desulphated with this enzyme. A sulphated tetrasaccharide (reducing value 26.1%) and a mixture of sulphated oligosaccharides (reducing value 14.7%), both prepared from chondroitin 4-sulphate by testicular hyaluronidase digestion, were respectively desulphated to the extent of about 32% and 56% of the sulphate released from chondroitin 4-sulphate polymer. Desulphation from the sulphated disaccharide 2-acetamido-2-deoxy-3-O-(β -D-glucopyranosyluronic acid)-4-O-sulpho-D-galactose was negligible.

The products obtained from chondroitin sulphates by exhaustive treatment with the chondrosulphatase were digested with chondroitinase ABC, and the unsaturated disaccharides yielded were separated by paper chromatography by the method of Saito *et al.* (1968). After chondrosulphatase treatment of chon-

Fig. 2. Cellulose acetate electrophoresis of the reaction products of chondroitin sulphates with squid chondrosulphatase

Electrophoresis was carried out in 1M-acetic acid-pyridine buffer, pH 3.5, at 0.5mA/cm for 20min (a) and in 0.3M-calcium acetate, pH 7.6, at 1mA/cm for 4h (b). 1, Products from chondroitin 4-sulphate with chondrosulphatase; 2, standards (order of migration rates: chondroitin 6-sulphate>chondroitin 4-sulphate>dermatan sulphate>chondroitin); 3, products from chondroitin 6-sulphate with chondrosulphatase.

droitin 4-sulphate almost all of the spot corresponding to 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enopyranosyluronic acid)-4-*O*-sulpho-D-galactose disappeared and the corresponding desulphated disaccharide was produced. With regard to chondroitin 4,6-disulphate, 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enopyranosyluronic acid)-4,6-*O*-disulpho-D-galactose disappeared and 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enopyranosyluronic acid)-4-*O*-sulpho-D-galactose also decreased, and 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enopyranosyluronic acid)-6-*O*-sulpho-D-galactose increased and the desulphated disaccharide appeared. With regard to chondroitin 6-sulphate, 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enopyranosyluronic acid)-6-*O*-sulpho-D-galactose decreased slightly and a small amount of the desulphated disaccharide was produced. These findings suggest that the chondrosulphatase preferentially attacks 4-sulphate in the chondroitin sulphates, although it also affects 6-sulphate to some extent.

The same results were also demonstrated by cellulose acetate electrophoresis before and after chondrosulphatase action. Chondroitin 4-sulphate and chondroitin 6-sulphate (300 μ g of each) were incubated with the chondrosulphatase (0.1ml containing 0.35mg of protein) at 37°C for 16h, and the reaction mixture was heated at 100°C for 1min and then centrifuged. The supernatant, after concentration, was subjected to electrophoresis. The results are shown in Fig. 2. In 1M-acetic acid-pyridine buffer, pH 3.5, the mobility of the product from chondroitin 4-sulphate with the chondrosulphatase decreased

considerably, whereas that of chondroitin 6-sulphate decreased only slightly. Electrophoresis in 0.3M-calcium acetate, pH 7.6, gave similar results.

From these studies it is evident that, unlike the chondrosulphatases of *P. vulgaris*, which attack only disaccharide sulphates, the squid chondrosulphatase is capable of desulphating mainly 4-sulphate in chondroitin sulphates without prior depolymerization.

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