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Demonstration of chondroitin sulfates degrading endo-β-glucuronidase activity in rabbit liver

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Reduced chondroitin sulfate was incubated with rabbit liver extracts followed by reduction once more with sodium [^{3}H]borohydride, and then passed through a Sephadex G-100 column. Chondroitin sulfate obtained from the incubation medium at pH 4 was only slightly depolymerized and was highly radioactive. Paper chromatographic analyses showed that glucuronic acid residues became exposed at the reducing terminal of chondroitin sulfate after incubation with the liver extracts. These results suggest that endo-β-glucuronidase activity which degrades chondroitin sulfate is present in the rabbit liver.

Chondroitin sulfate *Endo-β-glucuronidase* *Rabbit liver*

1. INTRODUCTION

Proteoglycans in tissue are thought to be catabolized by a concerted action of proteolytic enzymes, glycosidases and sulfatases. Glycosaminoglycans, the carbohydrate chains of proteoglycans, are acted on by a number of exo-glycosidases [1]. As a result, monosaccharide residues are removed stepwise from non-reducing termini. In addition, endo-glycosidases, as exemplified by hyaluronidase, cleave non-terminal sites of glycosaminoglycan chains and increase the number of target sites for exo-glycosidases.

Endo-glucuronidases which degrade heparin and heparan sulfate have been found recently [2–7]. Endo et al. [8] and Majima et al. [9,10] showed that some molecules of urinary chondroitin sulfate and chondroitin bear either glucuronic acid or iduronic acid residues at the reducing termini. These observations demonstrated that the glucuronide and iduronide linkages at non-terminal sites of carbohydrate chains were cleaved in tissues. It was thus suggested that endo-uronidases (endo-β-glucuronidase and endo-α-iduronidase), which act on chondroitin sulfate or dermatan sulfate, are present in tissues.

The present communication describes the presence in the rabbit liver of a chondroitin sulfate degrading endo-β-glucuronidase. Apparently this enzyme cleaved glucuronide linkage at non-terminal sites of the carbohydrate chain of chondroitin 6-sulfate and 4-sulfate. The resultant products had glucuronic acid residues at the reducing termini of the depolymerized chondroitin sulfate chains.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Glucuronic acid, gulonic acid, idonic acid, gulonolactone and idonolactone were the same as in [9]. Chondroitin 6-sulfate (super special grade from shark cartilage), and chondroitin 4-sulfate (super special grade from whale cartilage) were purchased from Seikagaku Kogyo (Tokyo). Saccharo-1,4-lactone and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St.Louis, MO). Sephadex G-100, and AG 1-X2

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(200–400 mesh) and AG 50W-X8 (200–400 mesh) were purchased from Pharmacia (Uppsala) and Bio-Rad (Richmond, VA), respectively. Sodium [^{3}H]borohydride (spec. act. 347.8 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Other reagents and chemicals were obtained from commercial sources.

2.2. *Analytical methods*

Uronic acid was determined by the method of Bitter and Muir [11]. Paper chromatography on Whatman no.1 paper was carried out by the descending technique in the following solvents (v/v): solvent 1, *n*-butanol–acetic acid–water (50:12:15); solvent 2, *n*-butanol–pyridine–water (6:4:3). The sugars on paper were located by staining with the alkaline silver nitrate reagent [12]. Radioactive components on paper were determined by scintillation spectrometry, after the paper was cut into 1-cm strips. They were transferred into scintillation vials containing scintillation medium and liquid scintillation counting was performed in an Aloka model LSC-683 liquid scintillation counter.

2.3. *Preparation of substrate (reduced chondroitin sulfates)*

Chondroitin 6-sulfate or 4-sulfate was reduced with sodium borohydride according to the method of Perry and Hulyalkar [13], dialyzed, lyophilized and used as a substrate.

2.4. *Preparation of crude enzyme*

A 10% homogenate of fresh rabbit liver (12-month-old male, Japanese white rabbit, JW, supplied by the Institute for Animal Experiment, Hirosaki University School of Medicine) was prepared in 0.01 M phosphate buffer (pH 7.3) containing 0.1 mM PMSF, and was centrifuged at 24000 × *g* for 30 min. Protamine sulfate was added to the resultant supernatant to a final concentration of 0.2%. After centrifugation, the fraction emerging within the range from 30 to 50% saturation of ammonium sulfate was collected from the supernatant, dialyzed against the buffer as mentioned above, and used as a crude enzyme fraction.

2.5. *Incubation with crude enzyme*

A 1.0-ml standard incubation mixture which contained reduced chondroitin 6-sulfate (4 mg), enzyme protein, 50 mM acetate buffer (pH 4.0) and 0.1 mM PMSF was sealed tightly after receiving an addition of a drop of toluene as preservative. The reaction mixture incubated at 37°C for 12 h was inactivated by heating at 100°C for 3 min and was centrifuged to remove insoluble materials. To the supernatants were added 4 vols ethanol saturated with NaCl and the resultant precipitates were collected by centrifugation.

2.6. *Analytical method for hexuronic acid residues at the reducing termini*

The analytical procedure was carried out as in [9]. Chondroitin 6-sulfate was reduced by the method of Perry and Hulyalkar [13], incubated as above and reduced once more with sodium [^{3}H]borohydride. The resultant products were hydrolyzed with trifluoroacetic acid and nitrous acid, and passed through columns of AG 50W-X8 and AG 1-X2 in the order described. The aldonic acid was lactonized. The lactone, thus prepared, was developed by paper chromatography, and radioactivities on paper were determined.

2.7. *Gel filtration*

Gel filtration was carried out on a column (1.8 × 95 cm) of Sephadex G-100, using 0.1 M acetic acid.

3. RESULTS

Reduced chondroitin 6-sulfate was incubated with the enzyme fraction from the rabbit liver at various pH values for 12 h. The chondroitin 6-sulfates recovered after incubation were reduced once more with sodium [^{3}H]borohydride, and then passed through a Sephadex G-100 column (fig.1).

No marked depolymerization of chondroitin 6-sulfate was found, as shown by the elution profiles of gel filtration which were depicted with carbazole reaction. No remarkable difference was found among elution profiles at various pH values except for a very slight degree of depolymerization at pH 4, whereas the product of tritiation with sodium [^{3}H]borohydride of the reducing termini of the chondroitin 6-sulfate recovered after incubation was most abundant at pH 4. The radioactivities in the area of low molecular size as shown by gel filtration were higher than those of high molecular size. The reaction rate, as evidenced by

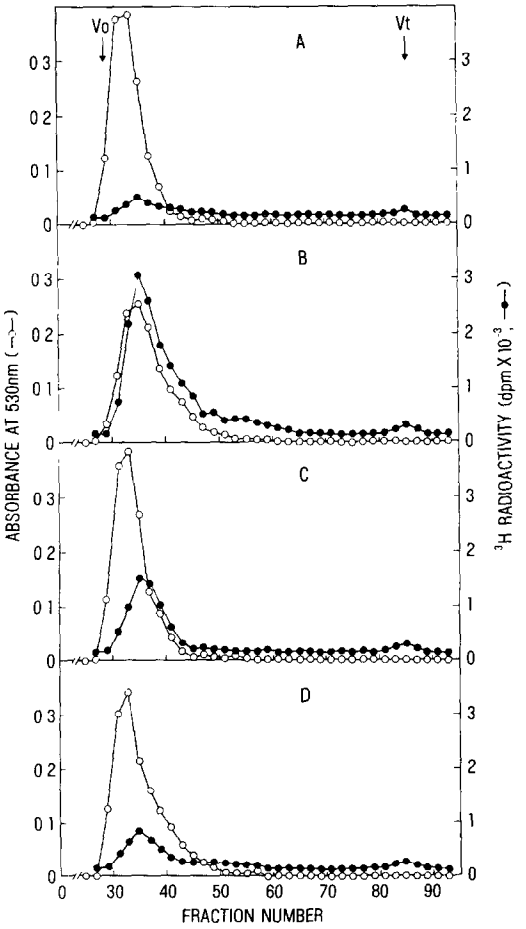

Fig.1. Gel filtration chromatography on Sephadex G-100 of chondroitin 6-sulfate. Reduced chondroitin sulfate was incubated with the rabbit liver extracts followed by reduction once more with sodium [^{3}H]borohydride, and then passed through the column. The buffers for incubation were as follows: (A) 50 mM glycine buffer (pH 3.0); (B) 50 mM acetate buffer (pH 4.0); (C) 50 mM acetate buffer (pH 5.0); (D) 50 mM phosphate buffer (pH 6.0). (○—○) Carbazole reaction for uronic acid. (●—●) ^{3}H radioactivity.

the increase with time of radioactivity, was linear with time during incubation and plateaued off at 40 mg liver extracts.

The tritiated derivatives prepared as above were alditols, which indicated that the new reducing termini became exposed during incubation. Theoretically, treatment with sodium [^{3}H]borohydride of glucuronic acid reduces glucuronic acid to L-[^{3}H]gulonic acid (an aldonic acid). To identify the aldonic acid at the reducing termini, [^{3}H]chondroitin 6-sulfate recovered from the incubation medium at pH 4 was collected from the effluents of gel filtration, hydrolyzed to monosaccharides, and passed through AG 50W-X8. The radioactivity of the non-adsorbed fraction (containing aldonic acid) was 24% of total radioactivities.

The non-adsorbed fraction after alkaline treatment (0.25 M ammonium hydroxide) was applied to AG 1-X2. The radioactivity of the effluents with 2 M acetic acid (containing aldonic acid) was 11% of total radioactivity. The above effluent was lactonized and resolved by paper chromatography in solvent 1 (fig.2). The radioactive substances from the area corresponding to the standard gulonolactone on chromatograms was rechromatographed on paper in solvent 2 (fig.3). On rechromatography, the radioactive spot (4% of the total activities) was found only at the area of gulonolactone.

On the other hand, the tritiated chondroitin 6-sulfate was directly hydrolyzed with 4 N HCl at 100°C for 8 h, and applied to AG 50W-X8. The radioactivity of the effluent with 1 N HCl from AG 50W-X8 was found at the area of galactosaminitol by paper chromatography in solvents 1 and 2 (not shown). When reduced chondroitin

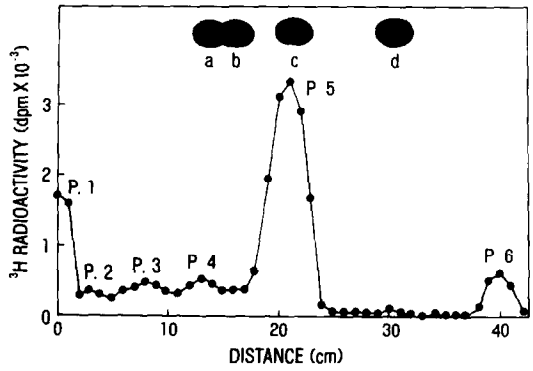

Fig.2. Paper chromatography of aldonolactone derived from reducing termini of chondroitin 6-sulfate. Chondroitin 6-sulfate reduced with sodium [^{3}H]borohydride after incubation with the enzyme fraction was passed through Sephadex G-100 (fig.1). Gulonic acid (a), idonic acid (b), gulonolactone (c) and idonolactone (d) were used as a reference. Peak 1 (P.1) corresponds to 11%, peak 2 (P.2) to 3%, peak 3 (P.3) to 9%, peak 4 (P.4) to 10%, peak 5 (P.5) to 60% and peak 6 (P.6) to 7% of radioactivity recovered.

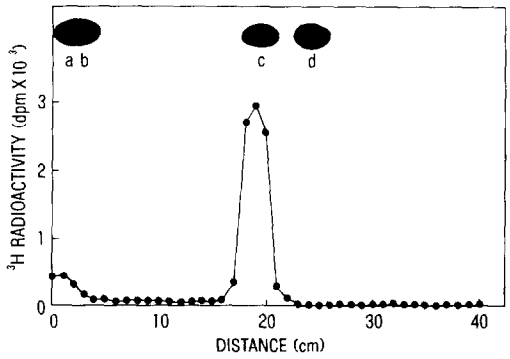

Fig.3. Paper chromatography of aldonolactone recovered from the first paper chromatography (fig.2). Sample was eluted from the area corresponding to the standard gulonolactone on paper, and then developed in solvent 2, *n*-butanol–pyridine–water (6:4:3, v/v). The method for radioactivity measurements and the standard samples are the same as in fig.2.

4-sulfate was used as a substrate instead of reduced chondroitin 6-sulfate, gulonic acid was detected at reducing termini just as in the experiment with chondroitin 6-sulfate. Saccharo 1,4-lactone (0.1 mM), an inhibitor of exo-β-glucuronidase, when added to the incubation medium, caused no hindrance to degradation by endo-β-glucuronidase of chondroitin 6-sulfate.

4. DISCUSSION

Endo-β-glucuronidases, which act on heparin and heparan sulfate have been found in several tissues and cells [2–7]. However, the presence of an endo-β-glucuronidase, which is active towards chondroitin sulfates has not hitherto been reported. The presence of hexuronic acid residues at reducing termini of low M_r chondroitin sulfate and low-sulfated chondroitin sulfate in urine has already been reported [8–10]. These findings might be explained by the presence of endo-uronidases, which act on non-terminal sites of chondroitin sulfate chains in tissue.

This paper shows that an endo-β-glucuronidase, which acts on chondroitin sulfates, is present in the rabbit liver. This enzyme exposes glucuronic acid residues at the reducing termini of chondroitin sulfates.

It was also indicated that *N*-acetylgalactosamine as well as glucuronic acid was exposed at the reducing termini of chondroitin 6-sulfate chains. Therefore, it is likely that at least two different types of endo-glycosidase participate in the degradation of chondroitin sulfates in tissue. Since the radioactivity of the effluent with 1 N HCl from the column of AG 50W-X8 was found at the area of galactosaminitol, it is likely that the rabbit liver extracts contained an endo-glycosidase, which exposed *N*-acetylgalactosamine at reducing termini. Probably this enzyme is an endo-β-*N*-acetylgalactosaminidase which is similar to hyaluronidase.

The catabolism of chondroitin sulfates is not yet fully understood. However, our results suggested that endo-β-glucuronidases participate in the degradation of chondroitin sulfates in tissue.

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