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GALACTOSE-6-SULFATASE FROM *ACTINOBACILLUS* SP. IFO-13310 AND ITS ACTION ON SULFATED OLIGOSACCHARIDES FROM KERATAN SULFATE

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Summary

A 6-sulfatase specific for sugars of the galactose configuration was purified 81-fold from the crude extract of *Actinobacillus* sp. IFO-13310. This preparation contained activitiy towards both *N*-acetylgalactosamine 6-sulfate and galactose 6-sulfate (relative activity, 2.4 : 1). The enzyme also released inorganic sulfate from the non-reducing galactose 6-sulfate end group of a trisaccharide disulfate prepared from keratan sulfate by sequential degradation with endo-β-galactosidase, *N*-acetylglucosamine-6-sulfatase and exo-β-*N*-acetylglucosaminidase. In addition, a tetrasaccharide trisulfate bearing the non-reducing *N*-acetylglucosamine 6-sulfate end group, also enzymatically prepared from keratan sulfate, was degraded to give rise to inorganic sulfate, *N*-acetylglucosamine and galactose by the sequential action of this enzyme, *N*-acetylglucosamine-6-sulfatase, exo-β-*N*-acetylglucosaminidase and exo-β-galactosidase (*Charonia lampas*).

Introduction

Keratan sulfate has been found in various connective tissues such as cornea, nasal cartilage, costal cartilage and nucleus pulposus and it is known to be a requisite constitutent of proteoglycans in tissues [1—4]. However, our knowledge of the structure and metabolism of keratan sulfate is still limited. The structure of keratan sulfates seems to be considerably complex in regard to their chain lengths, degrees of sulfation, and proportions of minor components such as sialic acid and fucose [5—12]. These variations have been observed among different tissues, different species, or with advancing age. But it is yet

The abbreviations used are: Gal(6S), galactose 6-sulfate; GlcNAc(6S), *N*-acetylglucosamine 6-sulfate; Δ^4GlcUA-GalNAc(6S), O-β-Δ^4-glucuronyl-(1 → 3)-*N*-acetylgalactosamine 6-sulfate.

unknown how these variations are concerned with a role of keratan sulfate on the architecture of connective tissues. To clarify this, it seems necessary to develop a method permitting more precise examination of the structure of keratan sulfates in different species and tissues.

In an attempt to develop such a method, we have purified and characterized bacterial enzymes from *Pseudomonas* sp. IFO-13309 and *Actinobacillus* sp. IFO-13310, capable of degrading keratan sulfate. In our earlier papers [13,14], we examined three different enzymes purified from the cell-free extracts of these bacteria: (1) endo-β-galactosidase from *Pseudomonas* capable of degrading keratan sulfate to oligosaccharides having *N*-acetylglucosamine 6-sulfate at the nonreducing end; (2) *N*-acetylglucosamine-6-sulfatase and (3) exo-β-*N*-acetylglucosaminidase from *Actinobacillus* capable of releasing inorganic sulfate and *N*-acetylglucosamine, respectively, from the nonreducing *N*-acetylglucosamine 6-sulfate terminus of the oligosaccharides. Furthermore, it was shown that a new sulfatase ("galactose-6-sulfatase") was present in the extract of *Actinobacillus* which would act on a non-reducing galactose 6-sulfate terminus of oligosaccharides produced by degradation of keratan sulfate with the preceding three enzymes.

This paper presents the purification and characterization of the galactose-6-sulfatase. Sequential degradation of keratan sulfate tetrasaccharide [14], GlcNAc(6S)-Gal(6S)-GlcNAc(6S)-Gal[1], by the action of this sulfatase coupled with *N*-acetylglucosamine-6-sulfatase, exo-β-*N*-acetylglucosaminidase and exo-β-galactosidase is also described.

Materials

Disaccharide monosulfate, GlcNAc(6S)-Gal, and tetrasaccharide trisulfate, GlcNAc(6S)-Gal(6S)-GlcNAc(6S)-Gal, from the endo-β-galactosidase-digest of bovine corneal keratan sulfate were prepared as described previously [14].

We are grateful to the following individuals for gifts of the indicated substances: galactose 6-sulfate, glucose 6-sulfate, and Δ^4GlcUA-GalNAc(6S) from Dr. S. Suzuki, Nagoya University, Nagoya; *N*-acetylgalactosamine 6-sulfate, *N*-acetylglucosamine 6-sulfate, *N*-acetylgalactosamine 4-sulfate, *N*-acetylgalactosamine 4,6-bissulfate, and *N*-acetylgalactosamine 3,6-bissulfate [15] from Dr. Y. Nakanishi, Nagoya University, Nagoya; *N,N'*-diacetylchitobiose from Dr. T. Osawa, Tokyo University, Tokyo; bovine cornea and keratan sulfate (whale cartilage) from Dr. T. Harada, Seikagaku Kogyo Co., Tokyo.

The following commercial materials were used: *p*-nitrophenyl β-galactoside, *p*-nitrophenyl *N*-acetyl β-glucosaminide, and exo-β-galactosidase (*Saccharomyces fragilis*) from BDH Chemicals; *p*-nitrophenylsulfate from Sigma Chemical; chondroitin 6-sulfate (whale cartilage) and exo-β-galactosidase (*Charonia lampas*) from Seikagaku Kogyo Co.; Galactose-UV-Test and exo-β-galactosidase (*Escherichia coli*) from Boehringer Mannheim; Sephadex G-200, DEAE-Sephadex A-25, and Sephacryl S-200 (superfine) from Pharmacia; CM-cellulose and DEAE-cellulose from Whatman.

Methods

Enzyme assay

Endo-β-glycosidase activity was assayed as described previously [13]. Exo-β-

galactosidase and exo-β-*N*-acetylglucosaminidase activities were assayed as follows: the incubation mixture (50 μl) contained 0.1 μmol *p*-nitrophenyl β-glycoside, 1.5 μmol potassium phosphate buffer (pH 6.0) and enzyme solution. The reaction mixture was incubated at 37°C for 10—120 min, depending on the activity of the enzyme. The reaction was terminated by the addition of 1 ml 0.25 M Na_2CO_3 and the *p*-nitrophenol released was measured by its absorbance at 400 nm ($\epsilon = 1.8 \cdot 10^4$) [16]. For assay of exo-β-*N*-acetylglucosaminidase activity with *N,N'*-diacetylchitobiose as substrate, the incubation mixture (100 μl) contained 0.1 μmol substrate, 5 μmol potassium phosphate buffer (pH 6.0) and enzyme. After incubation at 37°C for 15—180 min, the reaction mixture was heated in a boiling water bath for 1 min. The resulting precipitate was removed by centrifugation and free *N*-acetylglucosamine in the supernatant fraction was measured by the method of Reissig et al. [17].

Sulfatase activity of crude enzyme preparations was assayed as described previously [13] except that the incubation mixture contained 0.3 μmol galactose 6-sulfate or *N*-acetylglucosamine 6-sulfate as substrate in place of keratan sulfate-oligosaccharides. When sulfatase activity of the most purified enzyme preparation was assayed, the incubation mixture (100 μl) contained 5 μmol Tris-HCl (pH 7.2), 0.2 μmol sugar sulfate and enzyme. After incubation at 37°C for 1—4 h, the reaction mixture was heated in a boiling water bath for 1 min. The resulting precipitate was removed by centrifugation and inorganic sulfate in the supernatant fraction was measured by the method of Dodgson [18]. For all the enzyme assays, one unit was defined as the quantity that catalyzed the release of 1 μmol product per min.

Culture conditions

Actinobacillus sp. was grown as described previously [13].

Analytical procedures

Unless otherwise indicated, the methods used were the same as those employed in the preceding papers [13,14]. *p*-Nitrophenol released enzymatically from *p*-nitrophenyl-β-glycoside or *p*-nitrophenylsulfate was determined by the method of McGuire et al. [16]; free *N*-acetylglucosamine by the method of Reissig et al. [17]; free galactose specifically by the method of Rommel et al. [19] using Galactose-UV-Test from Boehringer Mannheim.

Paper chromatography and paper electrophoresis

Descending paper chromatography was carried out at room temperature on 50-cm long strips of Toyo No. 51A filter paper in 1-butanol/ethanol/water (13 : 8 : 4, v/v).

Paper electrophoresis was carried out on 60-cm long strips of Toyo No. 51A filter paper at a potential gradient of 35 v/cm for 60 min. The buffer used was 0.05 M ammonium acetate/acetic acid (pH 5.0).

On a preparative scale, the sample was applied as a thin zone on filter paper and then subjected to chromatography or electrophoresis. Saccharide on filter paper was located with alkaline/$AgNO_3$ [20].

TABLE I

PURIFICATION OF GALACTOSE-6-SULFATASE

Step		Total protein (mg)	Total activity (units)	Specific activity (unit/mg protein)	Yield (%)
1	Crude extract	7840	13.3	0.0017	100
2	Streptomycin and $(NH_4)_2SO_4$	675	11.6	0.017	87.1
3	CM-cellulose	386	6.67	0.017	50.0
4	DEAE-Sephadex A-25	47.6	3.43	0.072	25.7
5	Sephadex G-200	19.3	1.88	0.098	14.1
6	Sephacryl S-200 *	6.6	0.91	0.138	6.8

* Values on this step were calculated from the data obtained by Sephacryl S-200 chromatography on a small scale (see text).

Results

*Purification of galactose-6-sulfatase * from Actinobacillus*

Table I shows a summary of the purification of galactose-6-sulfatase. Unless otherwise indicated, all operations were carried out at 0—4°C and all centrifugations were at 17 500 X*g* for 30 min.

Steps 1 and 2: Preparation of extracts and streptomycin and $(NH_4)_2SO_4$ precipitation. Crude extract (600 ml) was prepared from approximately 150 g (wet wt.) of cells, and streptomycin and $(NH_4)_2SO_4$ treatment were performed as described previously for the purification of *N*-acetylglucosamine-6-sulfatase [13]. A large proportion of galactose-6-sulfatase activity was recovered in the 50—90% $(NH_4)_2SO_4$ fraction in which most of the *N*-acetylglucosamine-6-sulfatase activity was also found [13].

Step 3: CM-cellulose chromatography. A column (1.6 X 60 cm) of CM-cellulose was prepared and equilibrated with 0.05 M Tris-HCl (pH 7.2). The dialyzed $(NH_4)_2SO_4$ fraction was applied to a column of CM-cellulose (1.6 X 60 cm) and eluted with 0.05 M Tris-HCl (pH 7.2) (flow rate 80 ml/h, 5-ml fractions). The column was then eluted with a 1200 ml gradient of 0—0.5 M NaCl in 0.05 M Tris-HCl (pH 7.2) (flow rate 50 ml/h, 5-ml fractions; Fig. 1).

Galactose-6-sulfatase activity was found in fractions 14—30 (fraction I) and the *N*-acetylglucosamine-6-sulfatase activity in fractions 140—166 (fraction IV). Keratan sulfate-endo-β-glycosidase activities were found in two separate fractions (15—30 (fraction I) and 142—160 (fraction IV) [13].

These fractions were separately pooled and fraction I was concentrated by ultrafiltration in an Amicon Diaflo apparatus using a PM-10 membrane for further purification of galactose-6-sulfatase.

Step 4: DEAE-Sephadex A-25 chromatography. The concentrated CM-cellulose fraction was applied to a DEAE-Sephadex A-25 column (3.5 X 44 cm) equilibrated with 0.05 M Tris-HCl (pH 7.2). The adsorbent was washed with

* Although this enzyme has been shown to be a sulfatase specific for sugars of galactose configuration (see below for the specificity), it will be referred to as galactose-6-sulfatase.

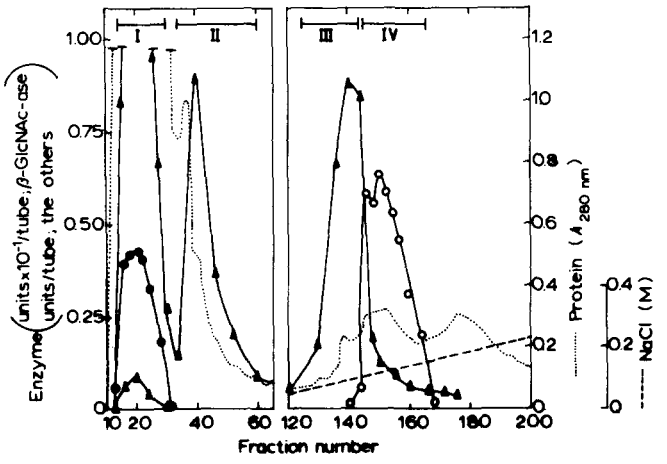


Fig. 1. Purification of sulfatases by CM-cellulose chromatography. 2- to 20-μl aliquots of the fractions were checked for galactose-6-sulfatase (●———●), *N*-acetylglucosamine-6-sulfatase (○———○), exo-β-*N*-acetylglucosaminidase (▲———▲), exo-β-galactosidase (△———△), and (280 nm) ultraviolet-absorbing material (· · · · · ·). (— — —) gradient of NaCl. Exo-β-*N*-acetylglucosaminidase (β-GlcNAc-ase) activity was assayed by *p*-nitrophenyl-*N*-acetyl-β-glucosaminide as substrate.

the same buffer (flow rate, 40 ml/h; 5-ml fractions). Approx. 51% of the galactose-6-sulfatase activity was recovered in the washing, whereas approx. 88% of protein was retarded by the column.

Step 5: Sephadex G-200 chromatography. The enzyme fraction was concentrated to 7 ml with a Diaflo PM-10 membrane filter and the concentrated solution was applied to a Sephadex G-200 column (1.6 X 110 cm) equilibrated with 0.1 M NaCl in 0.05 M Tris-HCl (pH 7.2). The enzymes were eluted with the same salt solution (flow rate 12 ml/h, 3-ml fractions). Galactose-6-sulfatase activity was found in fractions 46—64 (Fig. 2). Fractions 51—60 were pooled

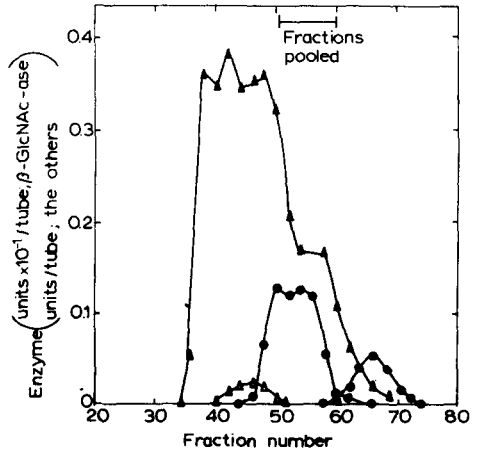


Fig. 2. Sephadex G-200 chromatography of the galactose-6-sulfatase fraction from the DEAE-Sephadex A-25 column (Step 5 of the purification procedure). 5- to 10-μl aliquots of the fractions were checked for galactose-6-sulfatase (●———●), exo-β-*N*-acetylglucosaminidase (β-GlcNAc-ase, ▲———▲), exo-β-galactosidase (△———△), and endo-β-glycosidase (○———○).

and concentrated to 9 ml with a Diaflo PM-10 membrane filter. By this step, the activities of exo-β-galactosidase and endo-β-glycosidase were successfully separated from the galactose-6-sulfatase activity, but exo-β-*N*-acetylglucosaminidase activity was not. This preparation hydrolyzed, besides galactose 6-sulfate, the following three sugar sulfates, to give rise to inorganic sulfate and the corresponding sugars: *N*-acetylgalactosamine 6-sulfate, *N*-acetylglucosamine 6-sulfate and glucose 6-sulfate; the rate of hydrolysis of these substrates were 240%, 5%, and 40%, respectively, of that of galactose 6-sulfate (the fractions obtained by Steps 1—5 procedures were not monitored with these substrates).

Step 6: Sephacryl S-200 chromatography. 1.4 ml (0.3 unit galactose-6-sulfatase) of the Sephadex G-200 fraction was applied to a Sephacryl S-200 column (1.5 × 59 cm) equilibrated with 0.1 M NaCl in 0.05 M Tris-HCl (pH 7.2) (Fig. 3). The enzymes were eluted with the same salt solution (flow rate, 15 ml/h; 1.3-ml fractions). Fig. 3 suggests that at least two different sulfatases were present in the Sephadex G-200 fraction; a faster-eluting fraction which may have activities for galactose 6-sulfate and *N*-acetylgalactosamine 6-sulfate (a substrate with the galactose configuration) and a slower-eluting fraction with activities for glucose 6-sulfate and *N*-acetylglucosamine 6-sulfate (a substrate with the glucose configuration). Fractions 45—48 were pooled and concentrated with a Diaflo PM-10 membrane filter. The galactose-6-sulfatase was purified 81-fold from the crude extract (Table I) and contained no detectable glucose-6-sulfatase and *N*-acetylglucosamine-6-sulfatase activities. The galactose-6-sulfatase activity, however, had been lost considerably during Sephacryl S-200 column chromatography (Table I). Thus, in some of the experiments described below (e.g. sequential degradation of keratan sulfate tetrasaccharide trisulfate) the Sephadex G-200 fraction was used without further purification.

On the other hand, the exo-β-*N*-acetylglucosaminidase activity presented in the Sephadex G-200 fraction was found in the fractions 40—52 on the Sephacryl S-200 chromatography (not indicated in the figure) and could not be separated from the galactose-6-sulfatase activity.

The *N*-acetylglucosamine-6-sulfatase was purified from CM-cellulose Fraction IV (145—166 in Fig. 1) as described previously [13].

Properties of purified galactose-6-sulfatase

The most purified preparation of galactose-6-sulfatase (Sephacryl S-200 fraction) was tested for its ability to remove the sulfate of various sulfate esters as well as galactose 6-sulfate. The following compounds were used: *p*-nitrophenylsulfate, *N*-acetylgalactosamine 6-sulfate, *N*-acetylgalactosamine 4-sulfate, *N*-acetylglucosamine 6-sulfate, *N*-acetylgalactosamine 4,6-bissulfate, *N*-acetylgalactosamine 3,6-bissulfate, glucose 6-sulfate, Δ^4GlcUA-GalNAc(6S), keratan sulfate (bovine cornea) and chondroitin 6-sulfate (whale cartilage). In these tests, $1.3 \cdot 10^{-3}$ unit enzyme was incubated with 0.2 μmol of each sulfate ester in 100 μl 0.05 M Tris-HCl (pH 7.2) at 37°C for 15 h. Only *N*-acetylgalactosamine 6-sulfate was hydrolyzed, to give rise to inorganic sulfate and *N*-acetylgalactosamine. The rate of desulfation of *N*-acetylgalactosamine 6-sulfate was approx. 2.4 times that of galactose 6-sulfate. Fig. 4 shows the effect of pH on the enzymatic release of SO_4^{2-} from galactose 6-sulfate and *N*-acetylgalactosa-

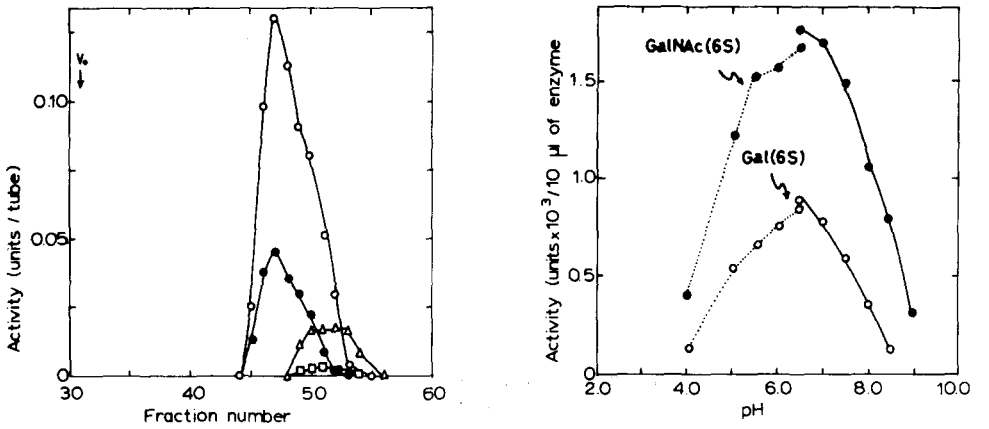


Fig. 3. Sephacryl S-200 column chromatography of the Sephadex G-200 fraction (Step 6 of the purification procedure). The Sephadex G-200 fraction (1.4 ml, 0.3 unit as galactose-6-sulfatase) was applied to the column. The sulfatase activities in 20-µl aliquots from each tube were measured. Galactose-6-sulfatase (●———●), *N*-acetylgalactosamine-6-sulfatase (○———○), glucose-6-sulfatase (△———△), and *N*-acetylglucosamine-6-sulfatase (□———□).

Fig. 4. Effects of pH on the enzymatic release of SO_4^{2-} from galactose 6-sulfate and *N*-acetylgalactosamine 6-sulfate by the galactose-6-sulfatase preparation (the Sephacryl S-200 fraction). The buffers used were 5 µmol Tris-HCl (———) or sodium acetate/acetic acid (· · · · ·) and the substrate used 0.2 µmol *N*-acetyl-galactosamine 6-sulfate [GalNAc(6S), ●] or galactose 6-sulfate [Gal(6S), ○].

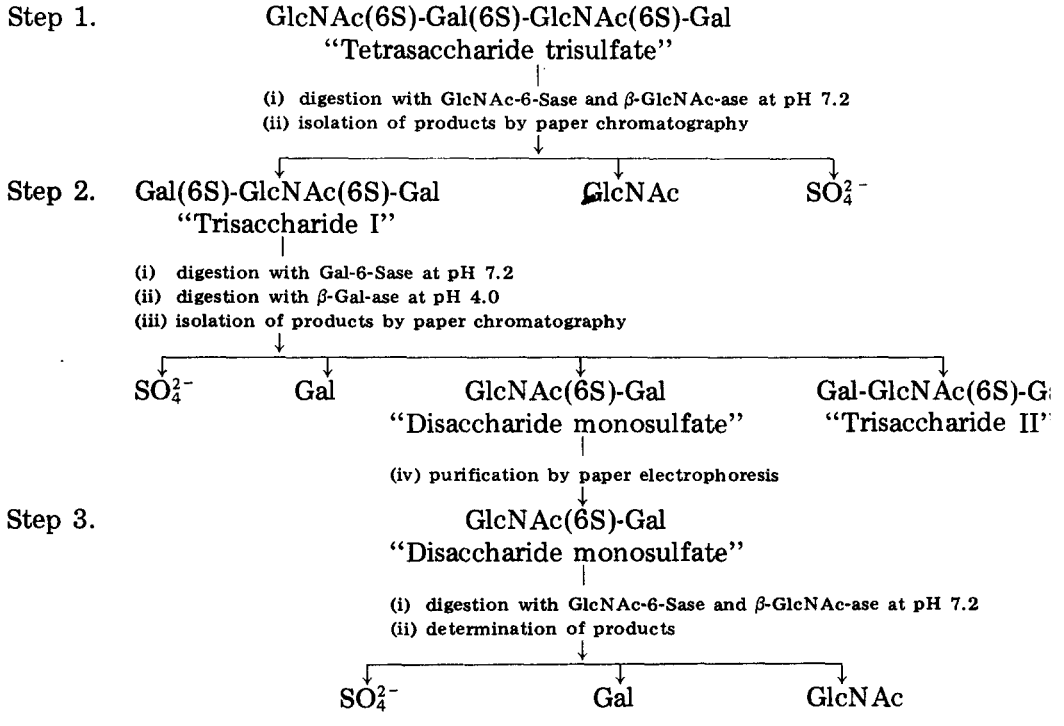
mine 6-sulfate. The two activities exhibited the same pH optimum of 6.5. On the other hand, the glucose 6-sulfatase preparation (49—56 in Fig. 3) exhibited a broad pH optimum of 7.5—8.5, measured by the release of SO_4^{2-} from glucose 6-sulfate. These results suggest that galactose-6-sulfatase is specific for the 6-sulfate linked to hexose or *N*-acetylhexosamine with the galactose configuration.

Sequential degradation of keratan sulfate tetrasaccharide trisulfate

On CM-cellulose chromatography, three peaks of exo-β-*N*-acetylglucosaminidase activities were detected (Fig. 1). To determine whether the exo-β-*N*-acetylglucosaminidases in three fractions (I, II, III in Fig. 1) were distinct from one another in their substrate specificities, the activities were measured with two substrates, *p*-nitrophenyl *N*-acetyl β-glucosaminide and *N,N'*-diacetylchitobiose. The ratio of activity on *N,N'*-diacetylchitobiose to that on *p*-nitrophenyl *N*-acetyl β-glucosaminide was 12.8 for fraction I, 18.5 for fraction II and 109 for fraction III. Apparently fraction III is different from the other two fractions (I, II) since it showed a markedly higher activity toward *N,N'*-diacetylchitobiose. Based on these results, fraction III was used as exo-β-*N*-acetylglucosaminidase in the experiments described below. The exo-β-*N*-acetylglucosaminidase in fraction III exhibited a pH optimum of 6.0 (in 0.03 M potassium phosphate buffer and with *N,N'*-diacetylchitobiose as substrate).

The exo-β-galactosidase in fractions 40—50 from Sephadex G-200 (Fig. 2) could release galactose from lactose, but not from a trisaccharide monosulfate, Gal-GlcNAc(6S)-Gal (obtained enzymatically from a keratan sulfate tetrasaccharide trisulfate). It is likely that this exo-β-galactosidase may not attack

a galactoside bond having sulfate residue on the aglycone. This property contrasts with the exo-β-galactosidase from *Charonia lampas* which could release galactose from the trisaccharide monosulfate. Exo-β-galactosidase preparations from *Escherichia coli* and *Saccharomyces fragilis* were inactive in regard to this reaction. Thus, *N*-acetylglucosamine-6-sulfatase, galactose-6-sulfatase, and exo-β-*N*-acetylglucosaminidase (fraction III) from *Actinobacillus*, coupled with exo-β-galactosidase from *Charonia lampas*, should be useful as reagents for studying the structure of keratan sulfate.



Scheme 1. Procedure for sequential degradation of Tetrasaccharide trisulfate. Details are described in the text. GlcNAc-6-Sase, *N*-acetylglucosamine-6-sulfatase; Gal-6-Sase, galactose-6-sulfatase; β-GlcNAc-ase, exo-β-*N*-acetylglucosaminidase; β-Gal-ase, exo-β-galactosidase (*Charonia lampas*).

Scheme 1 shows a summary of such experiments in which tetrasaccharide trisulfate *, GlcNAc(6S)-Gal(6S)-GlcNAc(6S)-Gal, are sequentially degraded by stepwise action of these enzymes. All enzyme digestions were performed at 37°C.

Step 1: Removal of nonreducing N-acetylglucosamine 6-sulfate terminus. The tetrasaccharide trisulfate (3.50 μmol as glucosamine) was digested with 0.06 unit *N*-acetylglucosamine-6-sulfatase and 0.6 unit (assayed with *N,N'*-

* Apparently, this oligosaccharide originates from the repeating tetrasaccharide unit of corneal keratan sulfate, the structure of which has been shown to be 2-acetamido-2-deoxy-6-*O*-sulfo-β-glucosyl-(1 → 3)-6-*O*-sulfo-β-galactosyl-(1 → 4)-2-acetamido-2-deoxy-6-*O*-sulfo-β-glucosyl-(1 → 3)-galactose [21].

TABLE II

ANALYSES OF PRODUCT FORMED BY SEQUENTIAL DEGRADATION OF TETRASACCHARIDE TRISULFATE

Galactose in the oligosaccharides was measured by the method of Trevelyan and Harrison (22). Sulfate and glucosamine in the oligosaccharides were measured by the method of Dodgson (18) and of Strominger et al. (23), respectively, after hydrolysis with 4 M HCl at 100° C for 7 h.

Step	Products	Yield (nmol)	Chemical composition * of product (Molar ratios)		
			GlcN	Gal	Sulfate
1.	"Trisaccharide I"	1610 **	1.00	2.21	2.19
	GlcNAc	1450	—	—	—
2.	"Trisaccharide II"	105 **	1.00	1.83	1.31
	"Disaccharide monosulfate"	858 **	1.00	1.03	1.05
	Gal	1060	—	—	—

* The figures represent the mean of values from three independent experiments.
** Expressed as nmol glucosamine (GlcN).

diacetylchitobiose as substrate) exo-β-*N*-acetylglucosaminidase in 0.05 M Tris-HCl (pH 7.2) for 24 h. During the incubation (10 h after the initiation), the mixture was supplemented with *N*-acetylglucosamine-6-sulfatase (0.06 unit) and exo-β-*N*-acetylglucosaminidae (0.6 unit). At the end of the incubation, aliquots of the digest were assayed for the release of inorganic sulfate and *N*-acetylglucosamine. The amounts of *N*-acetylglucosamine and inorganic sulfate released were 1.72 and 1.83 μmol, respectively, indicating that the non-reducing *N*-acetylglucosamine-6-sulfate terminus of the tetrasaccharide trisulfate was quantitatively hydrolyzed by this procedure. The remainder of the reaction mixture (95% of total volume) was heated in a boiling water bath for 2 min and the precipitate removed by centrifugation. The resulting supernatant fraction was subjected to paper chromatography in 1-butanol/ethanol/water (13 : 8 : 4, v/v). Two compounds were located on the chromatogram by staining guide strips with $AgNO_3$. One (designated as Trisaccharide I, R_{Gal} = 0.12) migrated faster than the tetrasaccharide trisulfate (R_{Gal} = 0.06) and the other co-migrated with the standard *N*-acetylglucosamine (R_{Gal} = 1.65). They were eluted from the filter paper with distilled water and lyophilized. The yields and chemical compositions of these compounds are shown in Table II. The results with Trisaccharide I show that it is a trisaccharide disulfate, Gal(6S)-GlcNAc(6S)-Gal.

Step 2: Removal of nonreducing galactose 6-sulfate terminus of the Step 1 product, "Trisaccharide I". Trisaccharide I (1.40 μmol as glucosamine) obtained above was digested with 0.02 unit galactose-6-sulfatase (the Sephadex G-200 fraction) in 0.05 M Tris-HCl (pH 7.2) for 20 h. During the incubation (8 h after the initiation), an additional aliquot of enzyme (0.02 unit) was added. Aliquots of the digest were assayed for inorganic sulfate. Since the amount of inorganic sulfate released was 1.50 μmol, the remainder of the reaction mixture (95% of total volume) was adjusted to pH 4.0 with a small volume

of 0.05 M HCl and further digested with 0.1 unit exo-β-galactosidase *. After 10 h, an additional aliquot of enzyme (0.1 unit) was added and reaction was continued for another 10 h. Aliquots of the mixture were assayed for galactose by the Galactose-UV-Test. The amount of galactose released was 1.19 μmol, indicating that the liberation of galactose was still incomplete. Thus, fresh enzyme (0.05 unit) was added to the mixture and further digestion was carried out for 10 h. But no appreciable increase in the amount of galactose was observed. The reason for this incomplete hydrolysis is unknown. The final reaction mixture (containing 90% of the amount of Trisaccharide I originally present) was heated in a boiling water bath for 2 min and centrifuged. The resulting supernatant fraction was subjected to paper chromatography as described under Step 1. Three spots were found on the chromatogram by staining guide strips with $AgNO_3$. One (Trisaccharide II) exhibited a mobility of R_{Gal} = 0.20; it was a relatively faint spot. The other two exhibited the same mobilities as standard disaccharide monosulfate, GlcNAc(6S)-Gal (R_{Gal} = 0.38) and galactose, respectively. The three compounds were eluted from the filter paper with distilled water and lyophilized. The fraction, corresponding in position to standard disaccharide monosulfate, was further purified by paper electrophoresis to remove contaminants from the enzyme preparations. The yields and compositions of the compounds are shown in Table II. The yield of "disaccharide monosulfate" is rather low compared with that of galactose. This may be due to a relatively low recovery of the compound from paper chromatogram and paper electrophoretogram. The data in Table II are consistent with the formulation of disaccharide monosulfate and Trisaccharide II as GlcNAc(6S)-Gal and Gal-GlcNAc(6S)-Gal, respectively, except for slight discrepancies among the GlcN, Gal and sulfate contents. These discrepancies could be due to contamination with other oligosaccharides. The Trisaccharide II compound could have arisen from an incomplete removal of nonreducing galactose end group by exo-β-galactosidase.

Step 3: degradation of disaccharide monosulfate. Disaccharide monosulfate (130 nmol) was digested with 0.003 unit *N*-acetylglucosamine-6-sulfatase and 0.03 unit exo-β-*N*-acetylglucosaminidase in 0.05 M Tris-HCl (pH 7.2). After 10 h incubation, 129 nmol *N*-acetylglucosamine, 126 nmol inorganic sulfate and 120 nmol galactose were detected in the reaction mixture, indicating that "Disaccharide monosulfate" was degraded quantitatively.

Discussion

Sephacryl S-200 chromatography of the Sephadex G-200 fraction of galactose-6-sulfatase (Fig. 3) suggested that at least three glycosulfatases with distinct substrate specificity were present in *Actinobacillus*. Among these, two sulfatases passed through a CM-cellulose column and were separated from each other on Sephacryl S-200 chromatography. The first sulfatase has been characterized as galactose-6-sulfatase in this paper, which can hydrolyze *N*-acetyl-

* The enzyme was dissolved in 0.05 M ammonium acetate/acetic acid (pH 4.0). One unit was defined as the quantity that catalyzed the release of 1 μmol of *p*-nitrophenol per min from *p*-nitrophenyl β-galactoside in the same buffer.

galactosamine-6-sulfate as well. The second sulfatase is a glucose-6-sulfatase with a low activity toward *N*-acetylglucosamine 6-sulfate (Fig. 3). The third sulfatase was retarded by a CM-cellulose column (fraction IV in Fig. 1); this enzyme has been characterized as *N*-acetylglucosamine-6-sulfatase in the preceding paper [13]. Although the last sulfatase can also cleave glucose 6-sulfate, its activity toward this substrate is very low (approx. 4% of activity toward *N*-acetylglucosamine-6-sulfate; Nakazawa, K., unpublished data) unlike the second sulfatase. In no case is it clear that two different activities are due to a single enzyme. Recently, Di Ferrante et al. [24] found that in fibroblasts of the Morquio disease (mucopolysaccharidosis IV), two activities of galactose-6-sulfatase and *N*-acetylgalactosamine-6-sulfatase were defective in parallel, whereas an *N*-acetylglucosamine-6-sulfatase was present at a normal level, suggesting the existence of two different sulfatases, specific for the galactose and glucose configuration, respectively. The data reported here are consistent with these observations with human fibroblasts.

We also purified an exo-β-galactosidase (fractions 40—50, Fig. 2) from the extract of *Actinobacillus*, which might be useful for analyzing the keratan sulfate oligosaccharides. This enzyme, however, could not hydrolyze the nonreducing galactose end of Gal-GlcNAc(6S)-Gal. Since *Actinobacillus* can grow in a medium containing shark keratan sulfate as a sole source of carbon [13], it is possible that this organism contains an exo-β-galactosidase capable of hydrolyzing the nonreducing end of Gal-GlcNAc(6S)-Gal, an enzyme required for the utilization of medium keratan sulfate. In the process of purifying galactose-6-sulfatase, some exo-β-galactosidase activity was in fact found in the 50% $(NH_4)_2SO_4$ fraction.

The result presented here showed that *Actinobacillus* galactose-6-sulfatase and *N*-acetylglucosamine-6-sulfatase could hydrolyze sulfate groups on the nonreducing end of oligosaccharides (tetrasaccharide trisulfate and trisaccharide disulfate), a finding which makes it possible to determine the sugar sequence of keratan sulfate oligosaccharides. Although it remains to be determined whether the galactose-6-sulfatase and *N*-acetylglucosamine-6-sulfatase can attack the nonreducing end of macromolecular keratan sulfates, the results presented in this paper suggest that these enzymes, together with endo-β-galactosidase [14], afford a new method in studying the structure of keratan sulfates and proteoglycans.

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