Biochimica et Biophysica Acta, 657 (1981) 344—355

BBA 69190

PURIFICATION AND PROPERTIES OF HUMAN *N*-ACETYLGALACTOSAMINE-6-SULFATE SULFATASE

CHANG T. LIM and ALLEN L. HORWITZ *

Department of Pediatrics and The Committee on Genetics Joseph P. Kennedy, Jr. Mental Retardation Research Center, Pritzker School of Medicine, University of Chicago, Chicago, IL 60637 (U.S.A.)

(Received August 25th, 1980)

Key words: N-Acetylgalactosamine-6-sulfate sulfatase; Glycosaminoglycan; Chondroitin sulfate; (Man)

Summary

1. Human *N*-acetylgalactosamine-6-sulfate sulfatase (EC 3.1.6.-) from human placenta has been purified more than 3000-fold by gel filtration, ion-exchange and substrate affinity chromatography. The enzyme has a molecular weight of 90 000 by gel filtration chromatography and 85 000 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Enzyme purified from cultured human skin fibroblasts has similar properties.

2. The tritium-labeled chondroitin 6-sulfate trisaccharide *N*-acetylgalactosamine 6-sulfate-(β,1—4)-glucuronic acid-(β,1—3)-*N*-acetyl[1-^{3}H]galactosaminitol 6-sulfate as substrate demonstrated a K_m of 0.12 mM at pH 4.5. Sulfate was hydrolyzed only from the non-reducing terminal of this disulfated trisaccharide. Hyaluronic acid, dermatan sulfate, chondroitin 4-sulfate, heparin and chondroitin 6-sulfate tetrasaccharide were slightly inhibitory, whereas 6-sulfated pentasaccharides and heptasaccharides were strongly inhibitory. The enzyme does not hydrolyze sulfate from *N*-acetylglucosamine 6-sulfate.

Introduction

The degradation of glycosaminoglycans requires the sequential action of both glycosidases and sulfatases for complete hydrolysis. The absence of any of these enzymes leads to the accumulation of one or more glycosaminoglycans in cells and tissues, resulting in the clinical conditions known as mucopolysac-

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

* To whom reprint requests should be addressed.

charidoses [1,2]. Morquio disease is due to a deficiency of *N*-acetylgalactosamine-6-sulfate sulfatase [3—6]. This enzyme is required for the degradation of chondroitin 6-sulfate. Cells of patients with Morquio disease presumably lack galactose-6-sulfate sulfatase activity, which is necessary for keratan sulfate degradation. A rapid, sensitive radiochemical assay for *N*-acetylgalactosamine-6-sulfate sulfatase was developed, in which a tritiated trisaccharide with *N*-acetylgalactosamine 6-sulfate at the non-reducing terminus is used as a substrate. This assay is intended for use in studies on the purification and properties of this enzyme. While this manuscript was in preparation, a report on the purification of human placental *N*-acetylgalactosamine-6-sulfate sulfatase appeared [7]. Many of the properties of the enzyme described by Glössl et al. are verified in this study. We report on additional characteristics, especially with regard to substrate specificities and inhibitors. Cultured skin fibroblasts are described as an additional source of *N*-acetylgalactosamine-6-sulfate sulfatase.

Materials and Methods

Materials

Chondroitin 6-sulfate, chondroitin 4-sulfate, hyaluronic acid, dermatan sulfate and chondroitinase ABC were purchased from Miles Laboratories, Inc. Heparin with a sulfate-uronic acid ratio of 1.8 was a gift from Dr. J.A. Cifonelli. Galactose 6-sulfate, a product of Sekiagaku Koyo Co., Tokyo, was gift from Dr. Elizabeth Neufeld; β-glucuronidase from bovine liver (Type B-10) was purchased from Sigma Chemical Co., DEAE-cellulose from Whatman, Inc., and tritiated sodium borohydride was from New England Nuclear.

Substrates

For preparation of chondroitin sulfate oligosaccharides, 5 g chondroitin 6-sulfate were dissolved in 200 ml 0.1 M sodium acetate, pH 5.0/0.15 M NaCl and digested with 100 000 units of testicular hyaluronidase (A.B. Leo, Helsingborg, Sweden) at 37°C for 40 h. The digestion mixture was applied to a 4 × 30 cm column of Dowex 1 × 2 200--400 mesh) and eluted with a 4 l linear gradient from 0.2 to 2 M NaCl. On the basis of uronic acid analyses, fraction peaks corresponding to tetra-, hexa- and octasaccharides were pooled, concentrated and desalted on a column of Sephadex G-25. The hepta- and pentasaccharides were prepared by digestion of the octa- and hexasaccharides, respectively, with 200 U of β-glucuronidase/mg oligosaccharide in 0.05 M sodium acetate, pH 5.5. The pentasaccharide and heptasaccharide were purified by chromatography on a column of Sephadex G-25, 1 × 180 cm eluted with 0.1 M pyridium acetate, pH 5.0. The resultant oligosaccharides had the uronic acid, hexosamine and sulfate analyses as well as the relative paper-chromatographic mobilities expected for the various sizes of oligosacchides [8]. Oligosaccharides of chondroitin 4-sulfate were prepared in a similar manner.

The chondroitin 6-sulfate tetrasaccharide was digested with chondroitinase ABC and subjected to chromatography on Whatman 3-MM paper in 1-butanol/acetic acid/1 N NH_4OH (3 : 1 : 1) for 48 h. Upon staining with alkaline silver reagent [9], the only products detectable were saturated 6-sulfated disac-

charide, unsaturated 6-sulfated disaccharide and a small amount (less than 5% of the total) of unsaturated 4-sulfated disaccharide. Within the sensitivity of the stain, the tetrasaccharide had less than 1% 4-sulfate on the non-reducing penultimate *N*-acetylgalactosamine.

For preparation of a radioactive substrate for the assay of *N*-acetylgalactosamine-6-sulfate sulfatase, 20 mg chondroitin 6-sulfate tetrasaccharide were dissolved in 2 ml 0.2 M $NaHCO_3$ and reduced by addition of 5 mCi (0.2 mmol) sodium borotritide. Following repeated evaporations in the presence of methanol, the resulting material was digested with 8000 units of β-glucuronidase in 10 ml 0.1 M sodium acetate, pH 5.5, followed by isolation of the disulfated trisaccharide, *N*-acetylgalactosamine 6-sulfate-(β,1—4)-glucuronic acid-(β,1—3)-*N*-acetyl[1-^{3}H]galactosaminitol 6-sulfate by elution from a 1 × 6 cm column of Dowex 1-X8, with a 300 ml gradient of NaCl from 0.2 to 1.5 M. Based on glucuronic acid content, the substrate had a specific activity of 630 dpm/pmol.

For the assay of *N*-acetylglucosamine-6-sulfate sulfatase, cartilage keratan sulfate (a gift from Dr. J.A. Cifonelli) with a sulfate/hexosamine ratio of 0.9 was digested by an endo-β-galactosidase from *Escherichia freundii* (a gift from Dr. E. Kiatamikado, Tokyo, Japan), which digests keratan sulfate into oligosaccharides including the disaccharide *N*-acetylglucosamine 6-sulfate-(β,1—4)-galactose [10,11]. This disaccharide, isolated by chromatography on a 1 × 180 cm column of Sephadex G-25, had a molar ratio of glucosamine/galactose/sulfate of 1.0 : 1.1 : 0.96 and contained less than 0.2% of the hexosamine as galactosamine. The product had chromatographic mobilities as previously reported [5,11]. The disaccharide was reduced with borotritide as described above to yield *N*-acetylglucosamine 6-sulfate-(β,1—4)-[1-^{3}H]galactitol. For the preparation of [^{3}H]galactitol 6-sulfate, 10 mg galactose 6-sulfate in 2 ml 0.1 M $NaHCO_3$ were reduced with 10 mCi (1 mol) sodium borotritide for 30 min, followed by addition of 2 mg sodium borohydride. 30 min later, 1 g Dowex 50W-X8 (H^+ form) was added and the mixture was filtered. After neutralization with NH_4OH, the filtrate was applied to a 1.2 × 5 cm column of Dowex 1-X8, in the formate form. The column was eluted with 0.1 M ammonium formate. After lyophilization, the [^{3}H]galactitol 6-sulfate was further purified by electrophoresis on Whatman 3-MM paper in 0.07 M pyridine acetate, pH 5.3, at 2500 V for 1 h. The [^{3}H]galactitol 6-sulfate had a spec. act. of 350 dpm/pmol based on sulfate content.

Enzyme assays

Assay conditions for *N*-acetylgalactosamine-6-sulfate sulfatase were those previously described [5], except that the ^{3}H-labeled 6-sulfated trisaccharide was used. Routine incubation mixtures contained 20—50 μg enzyme preparation, 7.5 nmol sodium acetate, pH 4.5/14 μg bovine serum albumin/13 nmol trisaccharide in a total volume of 50 μl. Following incubation at 37° C for times ranging from 4 to 16 h, 1 ml water was added, and the mixture was applied to a 0.7 × 2 cm column of ECTEOLA-cellulose, formate form, prepared as described by Hall et al. [12]. The column was washed with 1 ml water and the reaction product was eluted with 4 ml 0.07 M sodium formate, pH 7. Upon addition of 12 ml Aquasol scintillation fluid (New England Nuclear), the material was counted in a Packard Tri-Carb scintillation counter.

Purifications of N-acetylgalactosamine-6-sulfate sulfatase

All purification steps were carried out at 4°C. Human placentas were refrigerated immediately after delivery and washed with 0.15 M NaCl in 0.05 M sodium phosphate, pH 7.0. 900 g placenta were cut into fragments, suspended in 4 vol. 0.02 M Hepes/0.02 M NaCl, pH 7.0, and homogenized by a Polytron homogenizer (Brinkman Instruments) operated at 70% of full speed for 2 min. After centrifugation at 9000 × *g* for 15 min, $(NH_4)_2SO_4$ was added to the supernatant solution to 30% saturation at 4°C. The precipitate, which formed after 18 h, was collected by centrifugation at 9000 × *g* for 15 min, dialyzed against 0.05 M sodium acetate, pH 5.0 and saved for assay. $(NH_4)_2SO_4$ was added to the supernatant to give 55% saturation. After standing for 18 h at 4°C, the precipitate was collected by centrifugation at 9000 × *g* for 15 min. The precipitate was dissolved in 20 vol. 0.02 M Hepes/0.02 M NaCl, pH 7.0, and the solution was chromatographed on a column of Sephadex G-200 (2.5 × 150 cm) with 0.02 M Hepes/0.02 M NaCl, pH 7.0. Fractions containing *N*-acetylgalactosamine-6-sulfate sulfatase activity were pooled and dialyzed against 0.02 M Tris-HCl, pH 7.0. The preparation was applied to a column (2.5 × 20 cm) of DEAE-cellulose and eluted with a 1 l linear gradient from 0 to 0.35 M NaCl in 0.02 M Tris, pH 7.0. Fractions containing enzyme activity (Fig. 1) were pooled, dialyzed against 0.02 M Tris-HCl, pH 7.0, applied to a second DEAE-cellulose column of the same size, and eluted with a 1 l linear gradient from 0 to 0.3 M NaCl in 0.02 M Tris, pH 7.0. The NaCl concentration was diluted to less than 5 mM during the assay. This concentration of NaCl is not significantly inhibitory. The fractions corresponding to the peak of enzyme activity were pooled, dialyzed against 1 mM sodium acetate, pH 6.0, and lyophilized.

Affinity chromatography medium was prepared by addition of 8 mg chondroitin 6-sulfate pentasaccharide to 20 ml hydrazido-Sepharose 4B, followed by reduction with cyanoborohydride as described by Christner et al. [13]. Hydrolysis by 4 N HCl at 100°C for 14 h, followed by determination of hexosamine by the method of Boas [14], indicated that 62 μg pentasaccharide were bound per ml gel. Columns, 0.7 × 10 cm, were equilibrated with 0.05 M sodium acetate, pH 4.5. The DEAE-cellulose purified enzyme was applied and the column was washed with 10 ml sodium acetate buffer. The enzyme was then eluted with 20 ml chondroitin 6-sulfate pentasaccharide, 600 μg/ml, in acetate buffer. The fractions were dialyzed in 10 mM sodium acetate, pH 5.5, prior to assay.

Human skin fibroblasts were grown for 2 weeks on plastic dishes in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum. The plates were rinsed with 0.9% NaCl in 0.05 M sodium acetate, pH 5.5 and cells were removed by scraping with a rubber policeman. Disruption of the cells was by 6 cycles of freeze-thawing. The material was centrifuged at 10 000 × *g* for 10 min and *N*-acetylgalactosamine-6-sulfate sulfatase was purified from the supernatant as described above for the placental enzyme.

Analytical methods

Uronic acid analyses were performed by the method of Dische [15]. Total hexosamine was determined after hydrolysis in 4 N HCl for 14 h by the method of Boas [14]. Glucosamine and galactosamine were distinguished by separation on a Technicon amino acid analyzer. Sulfate analyses were performed accord-

ing to the method of Dodgson and Price [16]. Protein determination was carried out by the method of Lowry et al. [17], with bovine serum albumin used as a standard.

Polyacrylamide-gel electrophoresis in buffer containing 0.1% SDS was carried out by the method of Weber and Osborn [18], with 10% polyacrylamide.

Results

Purification of N-acetylgalactosamine-6-sulfate sulfatase

The assay of the enzyme was based on the hydrolysis of sulfate from the non-reducing terminal *N*-acetylgalactosamine of the disulfated trisaccharide to form a monosulfated trisaccharide as described previously [5]. This product eluted from ECTEOLA-cellulose with 0.07 M sodium formate and had the electrophoretic mobility of a monosulfated trisaccharide. The hydrolysis varied linearly with the amount of enzyme and with the time of incubation at 37°C up to 16 h.

The results of each step of purification of placental enzyme are shown in Table I. Insignificant amounts of *N*-acetylgalactosamine-6-sulfate sulfatase were found in the 0—30°C saturated $(NH_4)_2SO_4$ precipitate and in the 55% saturated $(NH_4)_2SO_4$ supernatant solution. Gel filtration chromatography was carried out in a column previously calibrated for molecular weight estimation [19]. The activity eluted as a single peak, with a molecular size equivalent to that of standard globular proteins of 90 000. The patterns of elution from DEAE-cellulose are shown in Fig. 1. Apparent charge heterogeneity, resulting in multiple peaks of activity on elution from DEAE-cellulose, was noted by Glössl et al. [7]. The broad activity peak shown in Fig. 1 (top) may represent such heterogeneity. The relatively high purification resulted from the use of a more shallow gradient and a more selective pool of fractions in our study than in those reported by Glössl et al. [7].

In affinity chromatography of *N*-acetylgalactosamine-6-sulfate sulfatase, we used a chondroitin 6-sulfate pentasaccharide bound to a support medium in a manner which is expected to leave the non-reducing terminal *N*-acetylgalactosamine 6-sulfate exposed. More than 80% of the enzyme activity was bound when the DEAE-cellulose-purified enzyme was applied in 0.05 M sodium acetate, pH 4.5. Less than 10% of the enzyme was bound in 0.05 M sodium acetate, pH 7.0. For determination of the specificity of the affinity column method of purification, chondroitin 6-sulfate hexasaccharide was bound to Sepharose 4B in the same manner as the pentasaccharide. This material contained slightly more oligosaccharide (79 μg hexasaccharide compared to 62 μg pentasaccharide/ml of Sepharose). *N*-Acetylgalactosamine-6-sulfate sulfatase was not bound to the hexasaccharide-Sepharose 4B under the conditions used for binding to the pentasaccharide derivative. This may reflect specificity of the enzyme affinity for a free *N*-acetylgalactosamine 6-sulfate group. The enzyme was eluted from the pentasaccharide-Sepharose 4B column by 0.46 mM chondroitin 6-sulfate pentasaccharide, but could not be eluted with chondroitin 6-sulfate trisaccharide at a concentration of 1.2 mM.

As noted in Table I, affinity chromatography of placental enzyme resulted in an additional 2-fold purification. It is evident from the results of polyacryl-

TABLE I

PURIFICATION OF *N*-ACETYLGALACTOSAMINE-6-SULFATE SULFATASE

Enzyme was purified from 900 g fresh human placenta and 50 ml (packed cell volume) cultured human skin fibroblasts. Enzyme activities were determined on each fraction with the [^{3}H]-labeled trisaccharide following dialysis against 0.002 M sodium acetate, pH 5.5, for removal of inhibitors. Protein was determined by the method of Lowry et al. [17].

Purification step	Placenta			Fibroblasts		
	Total activity (nmol/h)	Specific activity (nmol/h/mg)	Purification (-fold)	Total activity (n/mol/h)	Specific activity (nmole/h/mg)	Purification (-fold)
10 000 × *g* supernatant	925	0.014	—	211	0.36	—
30—55% $(NH_4)_2SO_4$	671	0.056	4	193	1.05	3
Sephadex G-200	578	0.33	23	152	1.68	5
DEAE-cellulose I	307	2.36	168	117	17.6	49
DEAE-cellulose II	220	22	1570	90	39.2	109
Affinity chromatography pentasaccharide elution	57	44	3140			

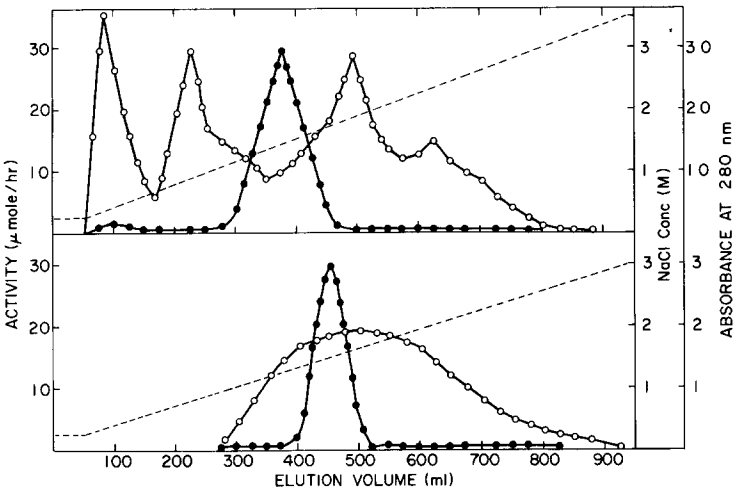


Fig. 1. Chromatography of *N*-acetylgalactosamine-6-sulfate sulfatase on DEAE-cellulose. The top figure demonstrates the first chromatography with a linear NaCl gradient from 0 to 0.35 M in 0.02 M Tris, pH 7.0. The material eluting between 240 and 420 ml as displayed in the upper figure was applied to a second DEAE-cellulose column (bottom figure) and eluted with a linear NaCl gradient from 0 to 0.3 M in 0.02 M Tris, pH 7.0. Absorbance at 280 nm (○———○), concentration of NaCl (- - - - - -), and *N*-acetylgalactosamine-6-sulfate sulfatase activity (●———●) are plotted.

amide gel electrophoresis (Fig. 2) that a considerable quantity of contaminating proteins were removed. No additional activity could be eluted from the affinity column with 1 M NaCl in 0.05 M sodium acetate, pH 5.0. Upon standing at 4°C for 36 h, nearly all of the activity of affinity chromatography-purified material was lost. When 100 μg/ml bovine serum albumin was included in the pentasaccharide elution, more than 90% of the activity was recovered and remained stable at 4°C. Enzyme eluted in the presence of albumin had a specific activity of 154 nmol/h per mg, if the protein contribution of the added albumin is subtracted. This specific activity then represents an $11 \cdot 10^3$-fold purification. Thus, the degree of purification by affinity chromatography is much greater than indicated in Table I. As shown in Fig. 2, SDS-polyacrylamide gel electrophoresis of enzyme purified by affinity chromatography showed only one band of protein. Compared with protein standards run in parallel gels, this location corresponds to a molecular weight of 85 000. Table II indicates that *N*-acetylgalactosamine-6-sulfate sulfatase can also be purified from cultured human skin fibroblasts. The fibroblast homogenate has a much higher initial specific activity than does the initial extract of placenta and results in a higher degree of purification for the same number of purification steps. The molecular size of the fibroblast enzyme was the same as that of enzyme from placenta.

Properties of N-acetylgalactosamine-6-sulfate sulfatase

The K_m for hydrolysis of the ^{3}H-labeled trisaccharide determined from the Lineweaver-Burk plot shown in Fig. 3 was 0.12 mM. We attempted the digestion of [^{3}H]galactitol 6-sulfate by using 20 μg purified enzyme with an activity

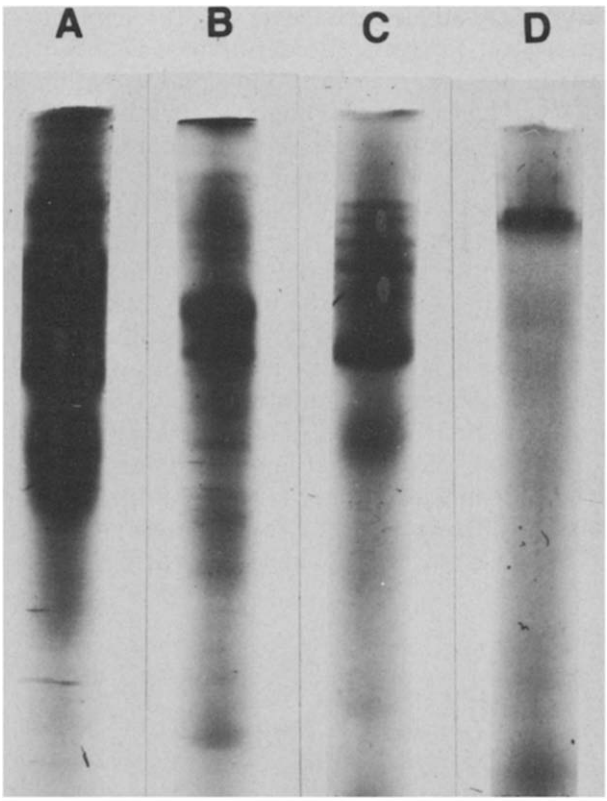


Fig. 2. Polyacrylamide gel electrophoresis in the presence of SDS. The gels were formed, run and stained with Coomassie blue as described by Weber and Osborn [18]. 20 μg protein were added to each gel. (A) Concentrated pooled fractions from Sephadex G-200 chromatography of placental enzyme; (B) enzyme from first DEAE-cellulose chromatography; (C) enzyme after second DEAE-cellulose chromatography; (D) placental enzyme purified by affinity chromatography.

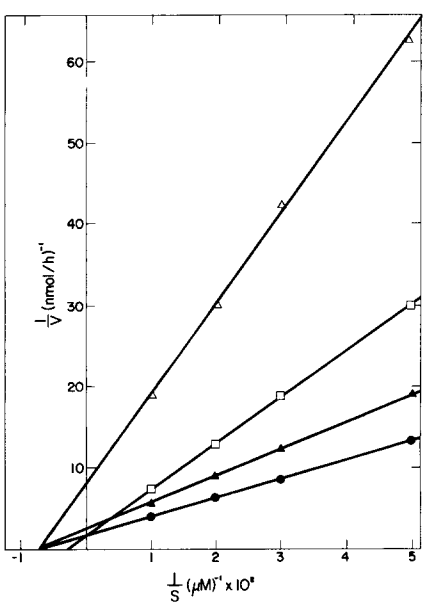
TABLE II

EFFECTS OF POLYSACCHARIDES AND IONS ON *N*-ACETYLGALACTOSAMINE-6-SULFATE SULFATASE ACTIVITY

Incubation mixtures contained [^{3}H]chondroitin 6-sulfate trisaccharide as substrate. The compounds were added at pH 4.5. The activity of the enzyme in the absence of inhibitor was 100.4 pmol/h.

Addition	Concentration	Activity remaining (percent)
None		100.0
Hyaluronic acid	0.1 mg/ml	62.5
Dermatan sulfate	0.1 mg/ml	63.0
Chondroitin 4-sulfate	0.1 mg/ml	54.8
Chondroitin 6-sulfate	0.1 mg/ml	48.0
Heparin	0.1 mg/ml	33.5
Dextran sulfate	0.1 mg/ml	8.4
$Na_2P_2O_7$	1.0 mM	6.7
Na_2HPO_4	1.0 mM	5.7
$MgCl_2$	20 mM	60
NaCl	20 mM	56
Na_2SO_4	10 mM	1

of 0.5 nmol/h as measured with the ^{3}H-labeled trisaccharide. The incubation mixture contained, in addition to enzyme, [^{3}H]galactitol 6-sulfate at a concentration of 170 mM and 50 mM sodium acetate, pH 4.5. Following incubation at 37° C for 24 h, 0.04 nmol presumed [^{3}H]galactitol was liberated, as measured by the absence of binding of reaction products to Dowex 1 resin according to the method of Di Ferrante et al. [6]. However, when the same reaction product was applied to Whatman 3-MM paper and subjected to electrophoresis in 0.02 M pyridine acetate at pH 5.3, there was no evidence of [^{3}H]galactitol at the origin. Variation in the [^{3}H]galactitol 6-sulfate concentration between 20 and 400 mM, and in the pH of the incubation mixture between 4 and 5.5, did not result in hydrolysis of [^{3}H]galactitol 6-sulfate. Addition of 20 mM galactose 6-sulfate to the *N*-acetylgalactosamine-6-sulfate sulfatase reaction with [^{3}H]chondroitin 6-sulfate trisaccharide resulted in mild non-competitive inhibition (Fig. 3). The ^{3}H-labeled keratan sulfate-derived disaccharide having *N*-acetylglucosamine 6-sulfate at the non-reducing terminus was not affected by the purified enzyme. The enzyme also had no activity toward the heparin-derived disulfated disaccharide used in the assay of iduronosulfate sulfatase [12], or toward methylumbelliferone sulfate at pH 5 or 6.5. There was no detectable activity of β-*N*-acetyl-



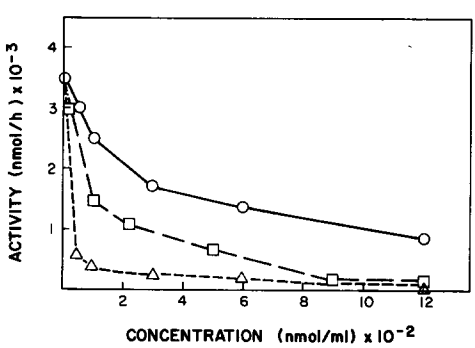


Fig. 3. Activity of *N*-acetylgalactosamine-6-sulfate sulfatase as a function of substrate concentration. The reaction was carried out with disulfated ^{3}H-labeled trisaccharide substrate added at various concentrations as indicated. Indicated in the Lineweaver-Burk plot are data for ^{3}H-labeled trisaccharide alone (●———●) and for the same concentrations of ^{3}H-labeled trisaccharide assayed in the presence of 20 mM galactose 6-sulfate (▲———▲), 0.3 mM chondroitin 6-sulfate hexasaccharide (△———△), and 0.12 mM chondroitin 6-sulfate pentasaccharide (□———□).

Fig. 4. Inhibition of *N*-acetylgalactosamine-6-sulfate sulfatase by chondroitin sulfate oligosaccharides. Oligosaccharides were added at the concentrations indicated. The results shown were obtained with addition of chondroitin 6-sulfate heptasaccharide (△———△), chondroitin 6-sulfate pentasaccharide (□———□), and chondroitin 6-sulfate hexasaccharide (○- - - - -○).

glucosaminidase, β-glucuronidase, or β-galactosidase when the 4-methylumbelliferone substrates were used.

The purified enzyme was inhibited approx. 50% by 100 μg/ml hyaluronic acid, dermatan sulfate, chondroitin 4-sulfate and chondroitin 6-sulfate. The same concentration of heparin gave 70% inhibition, while dextran sulfate caused a 92% decrease in activity. Sulfate, phosphate and pyrophosphate ions were strongly inhibitory (over 95% at 1 mM), while chloride was mildly inhibitory as previously demonstrated [4,7].

Addition of various chondroitin 6-sulfate oligosaccharides resulted in inhibition of the hydrolysis of sulfate from the ^{3}H-labeled trisaccharide substrate. Fig. 4 indicates that chondroitin 6-sulfate heptasaccharide and pentasaccharide were much more inhibitory at low concentration that the hexasaccharide, which has a non-reducing terminal glucuronic acid. Fig. 3 indicates that chondroitin 6-sulfate pentasaccharide acts as a competitive inhibitor of *N*-acetylgalactosamine-6-sulfate sulfatase, whereas chondroitin 6-sulfate hexasaccharide causes non-competitive inhibition at higher concentrations. The inhibitory effect of hexasaccharide at high concentration may be due to its action as a sulfated polyanion as was seen for dextran sulfate.

Discussion

The production of a radioactive oligosaccharide with *N*-acetylgalactosamine 6-sulfate at the non-reducing terminus provides a substrate for a sensitive assay for *N*-acetylgalactosamine-6-sulfate sulfatase. The purification scheme summarized in Table I resulted in more than 3000-fold purification of a single enzyme protein, as indicated by polyacrylamide gel electrophoresis. The specific activity which we report after substrate affinity column chromatography is less than that reported by Glössl et al. [7], but if loss of activity due to instability of the enzyme in our procedure is considered, the degree of purification is of the same order of magnitude with both methods. The substrate affinity column can be used at earlier stages of purification and should be capable of handling more material than is the case with preparative polyacrylamide gel electrophoresis. If bovine serum albumin is included in the final purification steps, the stability and yield of the enzyme are greatly improved. Purified *N*-acetylgalactosamine-6-sulfate sulfatase from extracts of cultured human skin fibroblasts had kinetic properties and substrate specificities which were the same as those for enzyme from placenta.

The properties of *N*-acetylgalactosamine-6-sulfate sulfatase resemble those of many lysosomal hydrolases that have an acid pH optimum and are glycoproteins. Inhibition by SO_4^{2-} and PO_4^{3-} has been found for a number of other sulfatases [6,7,20]. The substrate specificities are consistent with a requirement for sequential degradation of polysaccharides from the non-reducing terminus, as has been suggested for other hydrolases involved in the metabolism of sulfated glycosaminoglycans [1—3].

Our results with polysaccharides and oligosaccharides as inhibitors of *N*-acetylgalactosamine-6-sulfate sulfatase confirm that competitive inhibition is strongest when 6-sulfate is present on the non-reducing terminal *N*-acetylgalactosamine in the polysaccharide, but that anion inhibitory effects are also con-

siderable. Molecular-size determinations by gel filtration and SDS-polyacrylamide gel electrophoresis resulted in molecular weights of 90 000 and 85 000, respectively. These results suggest that this enzyme has no subunit structure.

There was no evidence of hydrolysis of galactitol 6-sulfate by placental *N*-acetylgalactosamine-6-sulfate sulfatase in these or other studies [7]. We found that enzyme prepared from human fibroblasts, spleen and urine, likewise, did not act upon galactitol 6-sulfate. The absence of hydrolysis of galactitol 6-sulfate does not preclude the possibility that the enzyme under study serves in the stepwise degradation of keratan sulfate by removing sulfate from galactose 6-sulfate. Genetic evidence from patients with Morquio disease [3—6] strongly suggests that the accumulation of keratan sulfate in this disease results from a lack of galactose-6-sulfate sulfatase activity. Nakazawa and Kagabe [21] have described a bacterial enzyme which has sulfatase activity when either galactose 6-sulfate or *N*-acetylgalactosamine 6-sulfate is used as substrate. The latter substrate gave a rate of sulfate hydrolysis 2.4-times that for galactose 6-sulfate. A recent report by Habuchi et al. [22] indicates that a *N*-acetylgalactosamine-6-sulfate sulfatase partially purified from rat skin has specificities toward chondroitin 6-[^{35}S]sulfate oligosaccharides similar to those which we have described for human placental *N*-acetylgalactosamine-6-sulfate sulfatase. The rat skin *N*-acetylgalactosamine-6-sulfate sulfatase was noted to release sulfate from [^{35}S]keratan sulfate in the absence of *N*-acetylglucosamine-6-sulfate sulfatase activity, thus further suggesting that *N*-acetylgalactosamine-6-sulfate sulfatase can release sulfate from galactose 6-sulfate residues. It is likely that a fragment of keratan sulfate with non-reducing terminal galactose 6-sulfate would be much more active than galactitol 6-sulfate as a substrate for this enzyme. Such a keratan sulfate-derived substrate would supply direct evidence for galactose-6-sulfate sulfatase activity.

The enzyme described here appears to be similar to an *N*-acetylgalactosamine-6-sulfate sulfatase from quail oviduct [23] that hydrolyzes sulfate from UDP-*N*-acetylgalactosamine 6-sulfate as well as from *N*-acetylgalactosamine 6-sulfate of a chondroitin 4/6-[^{35}S]sulfate trisaccharide.

Further study is necessary to determine whether heterogeneity of *N*-acetylgalactosamine-6-sulfate sulfatase exists in a variety of tissues. It has been suggested that the forms of lysosomal enzymes obtained from various sources differ with regard to receptor-mediated uptake of enzyme into cells [24—27], based at least partially on carbohydrate and/or phosphohexose content. Preliminary studies have shown that *N*-acetylgalactosamine-6-sulfate sulfatase can be taken up by cultured fibroblasts and chondrocytes derived from patients with Morquio disease [28]. This uptake is inhibited by mannose 6-phosphate. Thus, *N*-acetylgalactosamine-6-sulfate sulfatase resembles a number of other lysosomal hydrolases in its specific uptake.

Acknowledgements

This work was supported by NIH Grants AM-05996, HD-09402, and HD-04583. The authors wish to thank Dr. Albert Dorfman for his evaluation of this study and Mrs. E. Lanzl for reading the manuscript.

References

1 Dorfman, A. and Matalon, R. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 630—637
2 McKusick, V.A., Neufeld, E.F. and Kelly, T.E. (1978) in The Metabolic Basis of Inherited Disease (Stanbury, J.B., Wyngaarden, J.B. and Fredrickson, D.S., eds.), 4th, edn., pp. 1282—1307, McGraw-Hill, New York
3 Matalon, R., Arbogast, B., Justice, P., Brandt, I.K. and Dorfman, A. (1974) Biochem. Biochem. Res. Commun. 61, 759—765
4 Singh, J., Di Ferrante, N., Niebes, P. and Tavella, D. (1976) J. Clin. Invest. 57, 1036—1040
5 Horwitz, A.L. and Dorfman, A. (1978) Biochem. Biophys. Res. Commun. 80, 819—825
6 Di Ferrante, N., Ginsberg, L.C., Donelli, P.V., Di Ferrante, D.F. and Caskey, C.T. (1978) Science 199, 79—81
7 Glössl, J., Truppe, W. and Kresse, H. (1979) Biochem. J. 181, 37—46
8 Telser, A., Robinson, H.C. and Dorfman, A. (1966) Arch. Biochem. 116, 458—465
9 Partridge, S.M. and Westall, R.G. (1948) Biochem. J. 42, 238—248
10 Kitamikado, M. and Ueno, R. (1970) Bull. Jap. Soc. Sci. Fish. 36, 1175—1180
11 Reference deleted
12 Hall, C.W., Liebaers, I., Di Natale, P. and Neufeld, E.F. (1978) Methods Enzymol. 50, 439—456
13 Christner, J.E., Brown, M.L. and Dziewiatkowski, D.D. (1978) Anal. Biochem. 90, 22—32
14 Boas, N.F. (1953) J. Biol. Chem. 204, 553—563
15 Dische, Z. (1947) J. Biol. Chem. 167, 189—198
16 Dodgson, K.S. and Price, R.G. (1962) Biochem. J. 84, 106—110
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
19 Andrews, P. (1964) Biochem. J. 91, 222—233
20 Bashner, R., Kresse, H. and von Figura, K. (1979) J. Biol. Chem. 254, 1151—1158
21 Nakazawa, K. and Kagabe, K. (1978) Biochim. Biophys. Acta 527, 391—402
22 Habuchi, H., Tsuji, M., Nakanishi, Y. and Suzuki, S. (1979) J. Biol. Chem. 254, 7570—7578
23 Nakanishi, Y., Tsuji, M., Habuchi, H. and Suzuki, S. (1979) Biochem. Biophys. Res. Commun. 89, 863—870
24 Hieber, V., Disler, J., Myerowitz, R., Schmickel, R.D. and Jourdian, G.W. (1976) Biochem. Biophys. Res. Commun. 73, 710—717
25 Kaplan, A., Rischer, D., Achord, D. and Sly, W. (1977) J. Clin. Invest. 60, 1088—1093
26 Sando, G.N. and Neufeld, E.F. (1977) Cell 12, 619—627
27 Von Figura, K. (1977) Eur. J. Biochem. 80, 535-542
28 Horwitz, A.L. and Lim, C. (1979) Pediatr. Res. 13, 566