Sequential Degradation of Keratan Sulfate by Bacterial Enzymes and Purification of a Sulfatase in the Enzymatic System*

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SUMMARY

Pseudomonas sp. IFO-13309 and Actinobacillus sp. IFO-13310, bacteria which exhibit a symbiotic growth in a medium containing keratan sulfate as a sole carbon source, were isolated from soil.

Extracts of these organisms were shown to contain an endoglycosidase, a sulfatase, an exo-β-D-galactosidase, and an exo-β-D-N-acetylglucosaminidase which, together, catalyze an extensive cleavage of corneal keratan sulfate. The Pseudomonas extract was particularly rich in the endoglycosidase activity and poor in the exoglycosidase activities. The Actinobacillus extract, in sharp contrast, contained principally the exoglycosidases.

The sulfatase activity did not show this marked difference in distribution. A sulfatase was purified from the crude extract of Actinobacillus. The purified sulfatase reacted little or not at all with keratan sulfate, but acted on 2-acetamido-2-deoxy-6-O-sulfo-D-glucose, 2-acetamido-2-deoxy-6-O-sulfo-β-D-glucosyl(1→3)-D-galactose, and a tetrasaccharide trisulfate having 2-acetamido-2-deoxy-6-O-sulfo-D-glucose at the nonreducing end (prepared from keratan sulfate with an endogalactosidase). The enzyme removed one sulfite group from the tetrasaccharide trisulfate, producing an oligosaccharide which, unlike the parent oligosaccharide, was susceptible to hydrolysis with exo-β-D-N-acetylglucosaminidase. The data suggest that the nonreducing end is the only site at which enzymatic desulfation is carried out.

Previous studies in this laboratory centered around the purification of depolymerases and sulfatas which preferentially attack chondroitin sulfate, dermatan sulfate, and related substances (e.g. chondroitinase-ABC, chondroitinase-AC, chondro-4-sulfatase, chondro-6-sulfatase, and Δ-glucuronidase hydrdrolase).

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(1). These enzymes of defined specificity have already been proven useful as reagents for proteoglycan research (for a review see Ref 7). None of these preparations, however, could catalyze the degradation of keratan sulfate, a mucopolysaccharide which is frequently present in proteochondroitin sulfate preparations (for a review of this type of proteoglycans see Ref 3). The main structure of keratan sulfate is shown by chemical methods to contain repeating units of N-acetylgalactosamine polymerized via β-1,3 linkages and sulfated in position 6 of the galactosamine and in part of the galactose (cf. Refs 4 and 5), but available information indicates that keratan sulfate is much more variable than is chondroitin sulfate in chain length, in the degree of sulfation, and in the proportion of minor sugar components (6-11). It would thus appear that the development of enzymatic methods for structure analysis is necessary to further the study of keratan sulfate and related proteoglycans.

This paper describes the isolation of soil bacteria which are able to use keratan sulfate as a sole source of carbon and the characteristics of the degradation of keratan sulfate by cell-free extracts of the bacteria. The ultimate goal of this investigation is to obtain purified enzymes which are involved in the complete stepwise degradation of keratan sulfate. So far, two such enzymes, an endogalactosidase producing oligosaccharides from keratan sulfate and a sulfatase releasing SO₄²⁻ from the oligosaccharides, have been purified from the extracts. This paper describes, as a second subject, how the sulfatase may be purified and characterized. The accompanying paper (12) will deal with the isolation of the endogalactosidase and its action on keratan sulfate.

Several authors have already described the occurrence of keratan sulfate-degrading enzymes in bacteria (13, 14), marine gastropod (15), and rabbit liver and kidney (16). It is not clear at present whether the preparations of these authors involve the same enzymes as described here.

EXPERIMENTAL PROCEDURES

Materials

We are grateful to the following individuals for gifts of the indicated substances: β-galactose 3-sulfate and β-galactose 6-sulfate from Dr. H. Jatkovitz, Max Planck Institut, Munich; dermatan sulfate (hog intestinal mucosa) and heparan sulfate (by-product of heparin preparation) from Dr. M. B. Mathews and Dr. J. A. Cifonelli, University of Chicago; chondroitin 4-sulfate (whale cartilage), chondroitin 6-sulfate (shark cartilage), and heparin from Dr. M. Nomoto, Seikagaku Kogyo Company, Tokyo; phenyl...
β-D-galactoside and phenyl 2-acetamido-2-deoxy-β-D-glucoside from Dr. H. Kushida, Kyoto General Medical Chemical Laboratory, Kyoto; 2-acetamido-2-deoxy-6-0-sulfo-D-galactose (17), and 2-acetamido-2-deoxy-6-O-sulfo-D-galactose from Dr. Y. Nakanishi in this laboratory. Keratan sulfates of bovine cornea and shark cartilage were prepared according to Meyer et al. (18) and Furuhashi (19), respectively, with the exception that, in each case, further digestion with chondroitinase-ABC (1) was carried out after the ethonal fractionation. The resulting digests were deproteinized with 5% trichloroacetic acid, dialyzed against running tap water, and mixed with 2 volumes of ethanol containing 1% potassium acetate. The precipitates were washed successively with ethanol and ether and dried over P2O5 in a vacuum. The yields were 1.5 g from 100 g (dry weight) of cornea and 700 g from 20 kg (dry weight) of cartilage.1 The following analyses were obtained and calculated on a dry weight basis. Cornea sample: hexose (as galactose), 30.4%; hexosamine (as glucosamine), 32.3%; sulfate, 17.4%; β-deoxyhexose (as fucose), 0.9%; sialic acid (as N-acetylgalactosaminic acid), 1.2%; uronic acid (as glucuronic acid), 2.2%; and protein (as Lowry protein), 2.5%. Cartilage sample: hexose (as galactose), 26.5%; hexosamine (as glucosamine), 25.7%; sulfate, 23.0%; β-deoxyhexose (as fucose), 0.9%; sialic acid (as N-acetylgalactosaminic acid), 1.8%; uronic acid (as glucuronic acid), 2.5%; and protein (as Lowry protein), 4.0%.

Keratan sulfate oligosaccharide (substrate for the assay of sulfatase, see below) was prepared from 30 mg of corneal keratan sulfate with 30 units of a purified endogalactosidase preparation as described in the accompanying paper (12). Available evidence has indicated that the disaccharide monosulfate is 2-acetamido-2-deoxy-6-O-sulfo-β-D-glucosyl-1-3)-6-sulfo-D-galactose (conveniently designated in the formulation 6-sulfo-GlcNAc(1-3)-6-sulfo-Gal) and the tetrasaccharide trisulfate is 2-acetamido-2-deoxy-6-O-sulfo-β-D-glucosyl-1-3)-6-sulfo-D-galactosyl(1-4)-2-acetamido-2-deoxy-6-O-sulfo-β-D-glucosyl(1-3)-6-sulfo-D-galactosyl (designated 6-sulfo-GlcNAc(1-3)-6-sulfo-Gal(1-4)-6-sulfo-GlcNAc(1-3)-6-sulfo-Gal).

The following commercial materials were used: CM-cellulose from the Brown Co., Keene, N. H.; Sephadex G-200 from Pharmacia, Uppsala; potassium p-nitrophenyl sulfate from Sigma, St. Louis, Mo.

Methods

Analytical Procedures

The following compounds were determined by the indicated methods: phenol released enzymatically from phenyl glucosides by the method of Hockenhull et al. (20); p-nitrophenol released enzymatically from p-nitrophenyl sulfate by the method of Dodgson and Spencer (21); sulfate by the method of Dodgson (22) as modified by Kawai et al. (23); reducing activity of sugar by the method of Park and Johnson (24); uronic acid by the method of Bitter and Muir (25); protein by the method of Lowry et al. (26); hexosamine by the method of Trevelyan and Harrison (27); hexosamine by the method of Rosenberg et al. (28); 6-deoxy-3-α-galactose by the method of Dieche and Shettles (29); and sialic acid by the method of Werner and Odin (30). For the determination of exter sulfate and hexosamine in keratan sulfate, samples were hydrolyzed with 4 N HCl at 100°C for 7 hours.

1 We wish to thank Dr. T. Fujiiwara, Seikagaku Kogyo Company, for assistance in preparing the keratan sulfates.

2 The abbreviations used are: 6-sulfo-Gal, D-galactose 6-sulfate; 6-sulfo-GlcNAc, 2-acetamido-2-deoxy-6-0-sulfo-D-glucose; GlcUA(1-3)-6-sulfo-GlcNAc and Δ4-GlcUA(1-3)-6-sulfo-GlcNAc, 2-acetamido-2-deoxy-3-O-(β-D-glucuronosyl-4-epenepropanoyl) acid; 4-O-sulfo-D-galactose and 2-acetamido-2-deoxy-3-O-(β-D-glucuronosyl-4-epenepropanoic acid) residue; and 4-O-sulfo-D-galactose, respectively (i.e., unsaturated disaccharides derived from chondroitin sulfates by lyase reaction).

Results

Isolation of Bacteria

In an attempt to find a source of enzymes which would decompose keratan sulfate, a number of soil bacteria were examined and it was found that Pseudomonas sp. IFO-13309 and Actinobacillus sp. IFO-13310 could grow interdependently in a liquid medium containing shark keratan sulfate as a sole source of carbon (Medium A, see “Methods”). Thus, when the organisms were grown together in a single bottle they reached a density of about 1.5 (expressed as ΔOD) within 14 hours. However, when they were grown separately, it was obvious that the growth rates of Pseudomonas and Actinobacillus were at best 10% and 50%, respectively, of that attained by the mixed culture. This phenomenon could be interpreted as suggesting that some enzymes required for keratan sulfate utilization are distributed between the two organisms in a complementary manner (see below).

Culture Conditions

The organisms were grown at 30°C with vigorous aeration in the following media: Medium A (for screening and stock culture); 1.0% shark cartilage keratan sulfate, 0.2% NaH2PO4, 0.1% K2HPO4, 0.1% MgCl2·6H2O, 0.01% FeCl3·6H2O (adjusted to pH 7.0); Medium B (for adaptive growth of Pseudomonas); 1.0% shark cartilage keratan sulfate, 1.5% peptone, 0.45% meat extract, and 0.15% NaCl (adjusted to pH 7.0); and Medium C (for adaptive growth of Actinobacillus): 1.0% shark cartilage keratan sulfate, 0.1% glucose, 0.1% Casamino acid, 0.01% yeast extract, 0.2% NaH2PO4, 0.1% K2HPO4, 0.05% KCl, 0.05% MgCl2·6H2O, and 0.001% FeCl3·6H2O (adjusted to pH 7.0).

Enzyme Assay

Assay I—This assay measured the liberation of reducing substances from corneal keratan sulfate. Routine incubation mixture (100 μl) contained 5 μmol of Tris-HCl, pH 7.2, 0.1 μmol (as galactose) of keratan sulfate, and enzyme. Controls contained heat-inactivated enzyme. The reaction mixture was incubated at 37°C for 30 to 180 min, depending on the enzyme activity present, and then heated in a boiling water bath for 2 min. The resulting precipitate was removed by centrifugation and aliquots of the supernatant solution were assayed for reducing activity. This assay obviously does not indicate whether the reducing material is monosaccharide or oligosaccharide. If, however, the assay is made with an enzyme preparation which contains little exoglycosidase activity, e.g., the extract of Pseudomonas, the value may actually represent an “endoglycosidase” activity. One unit of enzyme was defined as the quantity that catalyzed the release of 1 μmol (as galactose) of product per hour.

Assay II—This assay measured the liberation of SO4− from keratan sulfate oligosaccharides (for the preparation of the oligosaccharides see above). Routine incubation mixture (200 μl) contained 10 μmol of Tris-HCl, pH 7.2, 0.65 μmol (as galactose) of oligosaccharides, and enzyme. Controls contained heat-inactivated enzyme. The reaction mixture was incubated at 37°C for 1 to 2 hours, depending on the enzyme activity present. At the end of the incubation, 200 μl of a solution containing 1% cetylpyridinium chloride in 0.3 N HCl were added to the mixture. The resulting precipitate was removed by centrifugation and aliquots of the supernatant solution were assayed for SO4−. One unit of enzyme was defined as the quantity that catalyzed the release of 1 μmol of SO4− per hour.

Assay III—This assay measured the liberation of phenol from phenyl 2-acetamido-2-deoxy-β-D-glucoside or phenyl β-D-galactoside. Routine incubation mixture (300 μl) contained 5 μmol of phenyl 2-acetamido-2-deoxy-β-D-glucoside or phenyl 2-acetamido-2-deoxy-β-D-galactose, 0.1% glucose, 0.1% NaH2PO4, 0.1% K2HPO4, 0.05% KCl, 0.05% MgCl2·6H2O, and 0.001% FeCl3·6H2O (adjusted to pH 7.0).
TABLE I
Characteristics of bacteria

<table>
<thead>
<tr>
<th>Test</th>
<th>Pseudomonas sp. IFO-13309</th>
<th>Actinobacillus sp. IFO-13310</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>Shape</td>
<td>Rods, 0.2 to 0.3 by 1.2 to 1.5 micrometers, occurring singly</td>
<td>Rods, 0.6 by 2 to 3 micrometers, occurring singly in pairs, in groups, and sometimes as filaments</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile by means of a polar flagellum</td>
<td>Non-motile</td>
</tr>
<tr>
<td>O₂-requirement</td>
<td>Aerobic, slightly oxidative</td>
<td>Aerobic, fermentative</td>
</tr>
<tr>
<td>Acid formation from glucose</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Catalase</td>
<td>positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Oxidase</td>
<td>weakly positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Gelatin</td>
<td>yellow, circular, viscid colonies; slow liquefaction</td>
<td>yellow colonies</td>
</tr>
<tr>
<td>Agar</td>
<td>yellow colonies</td>
<td>grayish white colonies (later become yellowish brown)</td>
</tr>
<tr>
<td>Broth</td>
<td>turbid with pellicle and yellow sediment</td>
<td>turbid with pellicle and gray (viscid) sediment</td>
</tr>
<tr>
<td>Litmus milk</td>
<td>coagulation occurs; casein is hydrolyzed slowly; litmus is reduced</td>
<td>coagulation occurs; casein is not hydrolyzed; litmus is reduced</td>
</tr>
<tr>
<td>Potato</td>
<td>scant, yellow growth</td>
<td>spreading slimy growth; luxuriant yellow colonies; potato is darkened and decomposed</td>
</tr>
<tr>
<td>Glucose mineral salts</td>
<td>no growth</td>
<td>no growth</td>
</tr>
<tr>
<td>7% NaCl broth</td>
<td>no growth</td>
<td>no growth</td>
</tr>
<tr>
<td>Indole production</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>Methyl red reduction</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>30~37°C</td>
<td>30~37°C</td>
</tr>
</tbody>
</table>

The classification of the organisms was based on their morphological, cultural, and physiological characteristics which are summarized in Table I.¹

Reactions Involved in Keratan Sulfate Degradation

Pseudomonas and Actinobacillus were grown in Mediums B and C, respectively, at 30°C with aeration. The cells were harvested by centrifugation at 3°C at the end of the logarithmic phase (usually between 9 and 13 hours), and were washed twice with cold water. Approximately 13 g (wet weight) of Pseudomonas cells or 10 g (wet weight) of Actinobacillus cells were obtained from 1-liter cultivation. The cells were suspended in 3 volumes of 0.05 M Tris-HCl, pH 7.2, and treated at 0°C in a 10-kHz sonic oscillator for 9 min. The supernatant solutions, obtained after centrifugation at 17,000 X g for 30 min, were tested for their capacity to decompose keratan sulfate. Fig. 1 shows the time courses of the degradation of keratan sulfate by the extracts. Samples were withdrawn at suitable time intervals and assayed for release of reducing substance. It can be seen that the release of reducing substance proceeded more rapidly with the Pseudomonas extract than with the equivalent amount of the Actinobacillus extract.

Aliquots of the 5-hour digests were chromatographed on paper with butyric acid-0.5 N ammonium (3:3). In all cases control experiments were made in which enzyme preparation and substrate were incubated separately and chromatographed under the same condition. The results are shown in Fig. 2. The profiles of the two systems differ markedly from each other. Thus, it will be noted that the chromatogram of the Actinobacillus digest shows the presence of two major components corresponding to galactose

¹The authors thank Dr. K. Komagata, Institute of Applied Microbiology, University of Tokyo, and Dr Y. Iwaike, Department of General Education, Nagoya University, for assistance with the identification of the organisms. The strains are deposited in the Institute for Fermentation, 4-54, Juso-Nishinocho, Higashiyodogawa-ku, Osaka 532, Japan. Requests for the strains should be addressed to this institute.
Pseudomonas extract (Curve A) than with the Actinobacillus extract. The release of SO$_4^{2-}$ proceeded more rapidly with the Pseudomonas digest (Curve A) of the Pseudomonas extract (Fig. 1), there was a distinct lag in the appearance of SO$_4^{2-}$ in each material possessing reducing activity began immediately (see "Methods Assay 1.").

Fig. 1 (left). Time-activity curves for the release of reducing activity from corneal keratan sulfate by the extracts of Pseudomonas and Actinobacillus. Each tube contained 5 pmol of Tris-HCl, pH 7.2; 0.1 pmol (as galactose) of keratan sulfate, and 30 µg (as protein) of each crude extract in a total volume of 100 µl. At the indicated times, one tube was removed and assayed for reducing substance as described under "Methods Assay 1."

Fig. 2 (center). A chromatogram showing the products of the enzymatic degradation of corneal keratan sulfate. Descending paper chromatography was carried out on Toyo No. 51A filter paper with butyric acid-0.5 N ammonia (5:3) at 20° for 24 hours. Reducing sugars were examined with the silver nitrate reagent (31). A, 5-hour digest with the Pseudomonas extract; B, 5-hour digest with the Actinobacillus extract. The latter extract, when incubated without keratan sulfate, gave a faint spot between the galactose and 2-acetamido-2-deoxy-d-glucose, respectively, whereas the Pseudomonas digest shows five or more spots with relatively slow mobilities. Evidence will be presented in the accompanying paper (12) to indicate that the slower moving components of the Pseudomonas digest represent sulfated oligosaccharides with varying molecular size. Therefore, the results of paper chromatography may reflect a difference in relative composition of endoglycosidase and exoglycosidase in the two organisms; i.e., it is supposed that the ratio of endoglycosidase to exoglycosidase activity is much higher in the Pseudomonas extract than in the Actinobacillus extract. This difference in enzyme composition would account for the symbiotic growth of the two organisms in Medium A (see above).

The appearance of galactose and 2-acetamido-2-deoxy-d-glucose in the chromatogram (Fig. 2) suggests the participation of a sulfatase system in the degradation of keratan sulfate. In order to obtain more information on the type and nature of such a sulfatase or sulfatases, the time-activity curves of the crude extracts were examined (Fig. 3). Enzyme preparations were used at 100 µg (as protein)/200 µl, and the increase in SO$_4^{2-}$ with time was measured. It can be seen from Fig. 3 that, whereas liberation of material possessing reducing activity began immediately (see Fig. 1), there was a distinct lag in the appearance of SO$_4^{2-}$ in each reaction. The release of SO$_4^{2-}$ proceeded more rapidly with the Pseudomonas extract (Curve A) than with the Actinobacillus extract (Curve B), but the sulfatase action of the latter was greatly facilitated by addition of a small amount (about 30% of that used for the experiment of Curve A) of the Pseudomonas extract (Curve C). These findings suggest that sulfatase activity is dependent on preliminary endoglycosidase attack. Indeed, it seems highly probable that the enzyme is without action on polymerized keratan sulfate and that the low rate which was observed with the Actinobacillus extract merely reflects the deficiency of endoglycosidase to degrade keratan sulfate.

Further support for this view comes from experiments in which the glycosidase and sulfatase activities were assayed separately by using polymerized keratan sulfate, phenyl 2-acetamido-2-deoxy-β-D-glucoside, phenyl β-D-galactoside, and keratan sulfate oligosaccharides as substrates. It can be seen in Table II that the Actinobacillus extract is rich in β-D-N-acetylgalactosaminidase and relatively poor in an endoglycosidase activity. The Pseudomonas extract, in sharp contrast, has a low β-D-N-acetylgalactosaminidase activity and a distinctly high endoglycosidase activity. β-D-Galactosidase activity is very low in both extracts, but it shows the same trend of distribution as that shown by β-D-N-acetylgalactosaminidase. In regard to sulfatase activity, it appears that the Actinobacillus extract is about as active on a protein basis as the Pseudomonas extract, provided the activities were measured using the oligosaccharide mixture as a substrate.

**Enzyme Induction**

When crude extracts from the cells grown in the presence of 1% keratan sulfate and from control cells grown in the absence of keratan sulfate were tested for endoglycosidase, sulfatase, and exoglycosidase (measured by Assays 1, 2, and 3, respectively), it was noticed that the endoglycosidase and sulfatase activities, but not the exoglycosidase activities, were greatly enhanced by the adaptive growth. Thus, the extract of unadapted Pseudomonas cells was shown to contain only one-thirtieth the endoglycosidase activity of the adapted cell extract on a protein basis. Moreover, neither this extract of unadapted Pseudomonas cells nor the extract of unadapted Actinobacillus cells could catalyze any significant release of SO$_4^{2-}$ from the oligosaccharide mixture under the standard assay conditions.
Enzyme activities involved in degradation of keratan sulfate

The standard assay systems described under "Methods" were used.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pseudomonas</td>
</tr>
<tr>
<td>Endoglycosidase</td>
<td>1.82a</td>
</tr>
<tr>
<td>Sulfatase</td>
<td>0.22</td>
</tr>
<tr>
<td>β-D-N-Acetylglucosaminidase</td>
<td>0.39</td>
</tr>
<tr>
<td>β-D-Galactosidase</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>

* These values are based on the reducing activities of products released from keratan sulfate (Assay 1, see "Methods"). Essentially all of the reducing substances produced by the Pseudomonas extract are in oligosaccharide forms (see Fig. 2), indicating that the reducing value may actually represent the amount of oligosaccharides released by an endoglycosidase action. However, it should be noted that most of the products of the Actinobacillus system are in monosaccharide forms (see Fig. 2) which may be formed by a combined action of endo- and exoglycosidases.

Purification of Sulfatase from Actinobacillus

The results described above suggested the existence of an inducible sulfatase or sulfatases which preferentially attacks the oligosaccharides from keratan sulfate. In order to characterize this sulfatase activity, purification experiments were carried out with Actinobacillus cells grown in Medium C.

All operations were conducted between 0° and 4°. All centrifugations were at 17,000 X g for 30 min.

Step 1: Preparation of Extracts—Approximately 120 g (wet weight) of cells were suspended in 4 volumes of 0.05 M Tris-HCl, pH 7.2, and treated at 0° in a 10-kHz sonic oscillator for 9 min. The suspension resulting from this treatment was centrifuged. The supernatant fluid thus obtained could be stored in an ice bath for at least 1 month without significant loss of activity.

Step 2: Streptomycin and Ammonium Sulfate Precipitation—

To 447 ml of the crude extract were added, with stirring, 112 ml of a 5% streptomycin sulfate solution in 0.05 M Tris-HCl, pH 7.2. The resultant precipitate was removed by centrifugation. Solid ammonium sulfate was added to the supernatant fraction to attain 50% saturation and the precipitate formed was removed by centrifugation. The concentration of ammonium sulfate was then raised to 90% saturation and the precipitate formed was collected by centrifugation. The precipitate was dissolved in 20 ml of 0.05 M Tris-HCl, pH 7.2, and dialyzed for 24 hours against four 2-liter changes of the same buffer.

Step 3: CM-cellulose Chromatography—A column (1.8 X 59 cm) of CM-cellulose was prepared and equilibrated with 0.05 M Tris-HCl, pH 7.2. The dialyzed ammonium sulfate fraction was applied to the column and washed with 250 ml of 0.05 M Tris-HCl, pH 7.2. The column was developed by linear gradient elution with 600 ml of 0.05 M Tris-HCl, pH 7.2, in the mixing flask and 600 ml of 0.5 M NaCl in the same buffer in the reservoir. The flow rate was 13 ml per hour and fractions of 4 ml were collected. Although a small portion of the sulfatase activity appeared in the wash (for which there is no explanation at this moment), most of the sulfatase activity was found in the fractions from tubes 120 to 160 (Fig. 4). The sulfatase activity was well separated from β-D-galactosidase, but it was accompanied by some β-D-N-acetylglucosaminidase and keratan sulfate-depolymerizing activity. Fractions 124 to 160 were pooled and concentrated to 7 ml on a Diaflo PM-10 (membrane filter).

Step 4: Sephadex G-200 Chromatography—The CM-cellulose fraction was applied to a Sephadex G-200 column (1.7 X 114 cm) previously equilibrated with 0.1 M NaCl in 0.05 M Tris-HCl, pH 7.2. The enzyme was eluted with the same salt solution. The flow rate was 5 ml per hour and fractions of 4 ml were collected. A single peak was observed (Fig. 5). The fractions (tubes 46 to 52) which were devoid of the glycosidase activities were pooled, concentrated to 8 ml on the membrane filter, and dialyzed for 4 hours against two 2-liter changes of 0.05 M Tris-HCl, pH 7.2.

A summary of the purification procedure is shown in Table III. Although a large loss in activity has been observed during the Sephadex G-200 step, this procedure appears to be essential to remove the glycosidase present in the CM-cellulose fraction. The absence of reducing material after extensive treatment (8 hours) of keratan sulfate (0.1 μmol as sulfate) by the Sephadex G-200 fraction (2.0 μg as protein) indicated that this enzyme preparation is free from endo- and exoglycosidase activities. It also showed no activity on phenyl 2-acetamido-2-deoxy-β-D-glucoside and phenyl β-n-galactoside.

FIG. 4. Purification of sulfatase by CM-cellulose chromatography. Fractions were obtained as described in the text and checked for sulfatase activity (O——O) (by Assay 2), glycosidase activity (●——●) (by Assay 1), and ultraviolet (280 nm)-absorbing material (······). Solid bars above curve indicate fractions containing the indicated exoglycosidase activities measured by Assay 3. Dashed line refers to the gradient of NaCl.

FIG. 5. Step 4 of the purification procedure. Sephadex G-200 chromatography of the sulfatase peak from CM-cellulose (see Fig. 4) is shown. Fractions were obtained as described in the text and checked for sulfatase activity (O——O) (by Assay 2), glycosidase activity (●——●), and β-D-N-acetylglucosaminidase activity (△——△).
Activities are catalyzed by the same enzyme.

It seems likely therefore that these activities were eluted in Fractions 38 to 51 by the gel chromatography. Thus, both activities were quantitatively cleaved by the enzyme to give rise to SO$_4^{2-}$ in the case of the monosaccharide and disaccharide.

The enzyme was tested for its ability to remove the sulfates of various synthetic and naturally occurring sulfate esters; i.e. N-p-nitrophenyl sulfate, galactose 3-sulfate, galactose 6-sulfate, 2-acetamido-2-deoxy-6-O-sulfo-n-glucose, 2-acetamido-2-deoxy-4-O-sulfo-d-galactose, 2-acetamido-2-deoxy-6-O-sulfo-d-galactose, and 2-acetamido-2-deoxy-6-O-sulfo-d-galactose, the products of the sulfatase would then become the substrates for the third enzyme, exo-$eta$-N-acetylglucosaminidase, forming free 2-acetamido-2-deoxyglucose and either galactose (from the disaccharide) or oligosaccharides having galactose 6-sulfate at the nonreducing end (from the tetra- or higher oligosaccharides). For further degradation of these oligosaccharides, one would expect the presence in the enzymatic system of a second sulfatase which can remove the sulfate group from the galactose 6-sulfate residue. It should be noted in this regard that our preliminary survey indicated the appearance of SO$_4^{2-}$ after incubation of the crude extract of *Actinobacillus* in the presence of galactose 6-sulfate. Upon chromatography on CM-cellulose (cf. Fig. 4), this activity, unlike the activity on 2-acetamido-2-deoxy-6-O-sulfo-d-glucose, was recovered almost exclusively in Fractions 15 to 40 with an average specific activity of 0.1 pmol of SO$_4^{2-}$ released per hour per mg of protein. Although it is not possible at this stage to define the function of this second sulfatase, it is logical to assume that the enzyme may attack the nonreducing galactose 6-sulfate terminus, thereby exposing the terminal group for further hydrolysis by the exo-$eta$-p-galactosidase present in the *Actinobacillus* extract.

Nishida-Fukuda and Egami (15) have reported the occurrence in the marine gastropod liver of a multienzyme system capable of degrading keratan sulfate to yield galactose, 2-acetamido-2-deoxyglucosamine, and sulfate. They have purified an exo-$eta$-p-galactosidase, an exo-$eta$-N-acetylglucosaminidase, and two sulfatase fractions catalyzing a desulfation of polymerized keratan sulfate. No endoglycosidase activity has been encountered in this tissue. Thus, the liver system appears to differ from the bacterial system in that the desulfation occurs at the polysaccharide level prior to the action of the glycosidases. In any event, our bacterial system which has the capacity of degrading keratan sulfate to a great extent should be an excellent source from which sulfatases and glycosidases of defined specificity may be obtained.

The term “glycosulfatase” (sugar-sulfate sulfohydrolase, EC 3.1.6.3) has long been used to describe a group of enzymes capable of hydrolyzing ester-sulfate linkages in a variety of sugars. Although earlier studies demonstrated a number of enzymes of this type in many organisms, the results of much of the earlier studies should be accepted with reserve in view of the scarcity of reliable information on the specificities. In more recent years, it has become increasingly evident that there are many different types of glycosulfatases, each being characterized by a remarkably high specificity toward the sugar part of the substrate molecule. Some examples of such enzymes related to hexoseamine or mucopolysaccharide metabolism are chondro-4-sulfatase (EC 3.1.6.9) (1), chondro-6-sulfatase (EC 3.1.6.10) (1), disulfoglucosamine 6-sulfatase (EC 3.1.6.11) (22), disulfoglucosamine N-sulfatase (32), UDP-N-acetylglucosamine 4-
sulfatase (33), sulfiduronate sulfatase (34), a sulfatase active
toward an oligosaccharide derived from chondroitin sulfate (35),
and a sulfatase catalyzing the desulfation of polymerized chon-
droitin 4-sulfate (36). The present demonstration of the sul-
fatase active toward the nonreducing terminal 2-acetamido-2-
deoxy-6-O-sulfoglucose will add a further interest to the problems
concerning the role occupied in mucopolysaccharide metabolism
of the sulfatases with such high, bewildering specificities.

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REFERENCES
J. Biol. Chem. 243, 1523-1535
Mol. Cell. Biochem. 1, 211-228
3. Muir, H. (1973) in Adult Articular Cartilage (Freeman,
242, 4352-4359
243, 1052-1059
J. Biol. Chem. 240, 1005-1010
240, 4140-4145
10. Antonopoulos, C. A., Fransson, L. Å., Gardell, S., and
227-235
912-917
Commun. 44, 1371-1375
Fish. 38, 497-502
39-47
Hoppe-Seyler’s Z. Physiol. Chem. 350, 669
Chem. 245, 6046-6051
(1953) J. Biol. Chem. 205, 611-616
20. Hockenhuill, D. J. D., Ashton, G. C., Fantes, K. H., and
Anal. 4, 246-247
32, 314-321
24. Park, J. T., and Johnson, M. J. (1949) J. Biol. Chem. 181,
149-151
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Ran-
dall, R. J. (1951) J. Biol. Chem. 193, 265-275
27. Trevelyan, W. E., and Harrison, J. S. (1952) Biochem. J. 50,
298-303
J. Biol. Chem. 234, 3263-3268
29. Dische, Z., and Shetlles, L. B. (1951) J. Biol. Chem. 192,
570-582
230-241
31. Trevelyan, W. E., Proctor, D. P., and Harrison, J. S.
J. Biol. Chem. 248, 6498-6410
33. Tsuji, M., Hamano, M., Nakanishi, Y., Ishihara, K., and
34. Bach, G., Eisenberg, F., Jr., Cantz, M., and Neufeld,
Acta 111, 113-120
Chem. 348, 1047-1060
Sequential degradation of keratan sulfate by bacterial enzymes and purification of a sulfatase in the enzymatic system.
K Nakazawa, N Suzuki and S Suzuki


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