Purification of Keratan Sulfate-endo-galactosidase and Its Action on Keratan Sulfates of Different Origin*

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SUMMARY

A glycosidase which attacks corneal keratan sulfate was purified from extracts of Pseudomonas sp. IFO-13309. When corneal keratan sulfate was degraded by the purified enzyme, Sephadex G-50 chromatography indicated the presence of a number of oligosaccharides differing in size and sulfate content. The characterization of two major fractions of the oligosaccharides indicated that the point of enzyme attack is limited to the endo-β-D-galactoside bonds in which nonsulfated D-galactose residues participate. The enzyme, unlike ordinary exo-β-D-galactosidases, did not catalyze the hydrolysis of phenyl β-D-galactoside. Moreover, β-D-galactosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucosyl-(1→3)-β-D-galactosyl-(1→4)-D-glucose ("lacto-N-tetraose") was completely refractory to the action of this enzyme, suggesting that a structure of the type, X-(1→3)-β-D-galactosyl-(1→4)-Y, is not the only specificity-determining factor, i.e. neighboring sugars, X and Y, or even larger portions of substrate molecule must have an important effect. Compared with corneal keratan sulfate, keratan sulfates from human nucleus pulposus and shark cartilage were attacked at lower rates with a resultant production of oligosaccharides of relatively large size. The result is in agreement with the view that considerable variations exist in the structure of keratan sulfates of different origin, and further suggests that the enzyme may serve as a useful reagent in studying these variations.

In the preceding paper (1) we have described the isolation of Pseudomonas sp. IFO-13309 and Actinobacillus sp. IFO-13310 and experiments on the degradation of corneal keratan sulfate by cell-free extracts of the bacteria. The isolation of these organisms was originally undertaken with the purpose of obtaining purified enzymes which could be used as analytical tools for the structure of keratan sulfate. The present report deals with one such enzyme from Pseudomonas which preferentially attacks corneal keratan sulfate, yielding a mixture of oligosaccharides with varying size. This enzyme produces scissions at galactose bonds along the keratan sulfate chain and hence can be classed as an endo-β-D-galactosidase.

Because of its potential utility in clarifying the structure and function of keratan sulfates present in various tissues, the susceptibility of keratan sulfates of different origin to the action of this enzyme was determined, and the released fragments on hydrolysis were examined by gel chromatography. The results indicate that marked variations exist in the structure of keratan sulfates from bovine cornea, human nucleus pulposus, and shark cartilage.

MATERIALS AND METHODS

Unless otherwise indicated, the materials and general methods used are the same as those employed in the preceding paper (1). Lacto-N-tetraose, i.e. β-D-galactosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucosyl-(1→3)-β-D-galactosyl-(1→4)-D-glucose, was kindly given by Dr. A. Kobata, Kobe University, Department of Medicine, Kobe.

Sephadex G-50, Sephadex G-200, and DEAE-Sephadex A-25 were purchased from Pharmacia, Uppsala, and DEAE-cellulose from Brown Co., Keene, N. H.

Human nucleus pulposi were collected from normal subjects aged 60 to 75 years in the autopsy room of the Medical School of this university. The tissues were dissected free from annulus fibrosus, dried in acetone, and ground in a mill. The keratan sulfate was prepared from this material by the procedure that was used in the preceding paper (1) to prepare corneal keratan sulfate. The yield was 1.0 g from 34 g of dry tissue. The following analyses were obtained and calculated on a dry weight basis: hexose (as galactose), 36.4%; hexosamine (as glucosamine), 21.4%; sulfate, 13.6%; 6-deoxyhexose (as fucose), 1.7%; sialic acid (as N-acetylmuramuramic acid), 3.8%; uronic acid (as glucuronic acid), 2.8%; and protein (as Lowry protein), 6.5%.

Chemical desulfation of shark cartilage keratan sulfate was carried out according to the method of Bhavanandan and Meyer (3). As was the case of the rib cartilage preparation described by these authors, part of the desulfated polysaccharide from shark keratan sulfate was insoluble in water. In the present experiment, the insoluble product was removed by centrifugation and the resulting supernatant solution was lyophilized to yield the water-soluble fraction. The yield was 100 mg from 500 mg of keratan sulfate of shark cartilage. Its residual sulfate content was 9.5% (about two-fifths the sulfate content of the parent polysaccharide).

Periodate oxidation of oligosaccharide (Component C-7aa, see below) was carried out as follows. The dried sample, 4 μmol as galactose, was dissolved into 100 μl of 2% NaB₃H₄ solution (adjusted to pH 8.0), and the mixture was kept at room temperature for 2 hours. After cooling, about 50 μl of 2% NaBH₄ solution were added and the mixture was kept at 0° for 15 hours. The mixture...
was then brought to pH 4.0 with acetic acid and subjected to paper electrophoresis (see below). The reduced compound (mobility = 15 cm per hour) was located by staining guide strips with periodic acid-Schiff reagent, and eluted from the remainder of the electrophoretic strip with water. The material was chromatographed on paper with Solvent D (for desalting) in which the reduced oligosaccharide had little mobility. The compound was eluted from the chromatographic strip with water and dried in a vacuum. No trace of the reducing power of the parent oligosaccharide was retained with this preparation. Finally, a 0.7-mmol (as hexosamine) portion of the reduced tetrasaccharide was treated with 8 mmol of NaIO4 in 200 μl of 0.01 M acetate buffer, pH 4.5, at 4°C for 24 hours in the dark.

Descending chromatography was carried out at room temperature on 60-cm long strips of Toyo No. 54A filter paper in Solvent A, 1-butanol-acetic acid-water (10:3:7, by volume), Solvent B, butyric acid-O.5 N ammonia (5:3, by volume), Solvent C, ethylacetate-pyridine-water-acetic acid (5:5:3:1, by volume), and Solvent D (for desalting) in which the atmosphere of the chamber saturated with ethylacetate-pyridine-water (11:40:6, by volume), or Solvent I, 1-butanol-ethanol-water (13:8:4, by volume).

Paper electrophoresis was carried out on 60-cm long strips of Toyo No. 54A filter paper in the apparatus described by Markham and Smith (5) at a potential gradient of 30 volts per 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. Afterward, guide strips were cut and stained with AgNO3 (6) (for reducing sugars) or with periodate-benzidine (4) (for glycitols). The compounds were then eluted from the remainder of the chromatogram or the electrophoretic strip with water.

RESULTS

Purification of Keratan Sulfate-Endogalactosidase

Table I shows a summary of the purification of an endogalactosidase which, as will become evident, preferentially attacks keratan sulfates, yielding oligosaccharide fragments with a reducing galactose end (for this reason, the enzyme will be called "keratan sulfate-endogalactosidase"). Details of the purification procedure are described below. Unless otherwise indicated, all operations were carried out at 0-4°C. All centrifugations were at 17,500 x g for 30 min.

Step 1: Preparation of Extracts—Pseudomonas IFO-13309 was grown in Medium B as described previously (1). The cells (170 g, wet weight) were suspended in 600 ml of 0.05 M Tris-HCl, pH 7.2, disrupted with a French press, and centrifuged. The resulting supernatant solution was collected.

Step 2: Streptomycin and Ammonium Sulfate Precipitation—To 640 ml of the extract were added, with stirring, 160 ml of a 5% streptomycin sulfate solution. After standing for 30 min, the suspension was centrifuged, and the supernatant solution was collected. Solid ammonium sulfate was added to the supernatant fraction to attain 25% saturation. After standing for 30 min, the mixture was centrifuged, and the supernatant solution was collected. The concentration of ammonium sulfate was then raised to 50% saturation. After 30 min, the mixture was centrifuged, and the supernatant solution was discarded. The precipitate was dissolved in 25 ml of 0.05 M Tris-HCl, pH 7.2, and dialyzed for 20 hours against three 3-liter changes of the same buffer.

Step 3: DEAE-cellulose Chromatography—A column of DEAE-cellulose (4.5 x 39 cm) was prepared and equilibrated with 0.2 M NaCl in 0.05 M Tris-HCl, pH 7.2. The dialyzed ammonium sulfate fraction (80 ml) was applied to the column. The column was washed with 1.4 liters of 0.2 M NaCl in 0.05 M Tris-HCl, pH 7.2, and then eluted with a gradient formed by addition of 1 liter of 0.7 M NaCl in 0.05 M Tris-HCl, pH 7.2, to a mixing vessel containing 1 liter of 0.2 M NaCl in the same buffer. Fractions of 10 ml were collected and checked for the enzyme activities indicated in Fig. 1. As can be seen, the endogalactosidase activity showed a large peak (tubes 201 to 290), plus two minor peaks (tubes 48 to 50 and tubes 90 to 200). At the present time, there is no explanation for the minor peaks. Fractions in the major peak which was eluted at almost the same region as the peaks of β-D-α-N-acetylgalcosaminidase, β-N-galactosidase, and sulfatase were combined and precipitated with 472 g of solid ammonium sulfate. The precipitate was dissolved in 30 ml of 0.05 M Tris-HCl, pH 7.2, and concentrated to about 10 ml by pressure dialysis against the same buffer.

Step 4: Sephadex G-200 Chromatography—The concentrated fraction (the major peak fraction from DEAE-cellulose) was applied to the top of a Sephadex G-200 column (3 x 94 cm) which had been equilibrated with 0.1 M NaCl in 0.05 M Tris-HCl, pH 7.2. The column was then eluted in 4-ml fractions.
with the same salt solution. The elution pattern is shown in Fig. 2. The endogalactosidase activity showed a single peak, plus a minor peak which is presumably due to a contamination by the early peak fraction of DEAE-cellulose column. Essentially all of the $\beta$-D-N-acetylgalactosaminidase and sulfatase activities present in the DEAE-cellulose fraction were removed by this procedure. Fractions in the major peak (tubes 53 to 72) were combined and concentrated to about 7 ml by pressure dialysis against 0.05 M Tris-HCl, pH 7.2.

**Step 5: DEAE-Sephadex A-25 Chromatography**—The concentrated Sephadex G-200 fraction was applied to the top of a DEAE-Sephadex A-25 column (2.7 x 19 cm) which had been equilibrated with 0.2 M NaCl in 0.05 M Tris-HCl, pH 7.2. The column was washed with 300 ml of the same salt solution and then eluted with a gradient formed by addition of 500 ml of 0.7 M NaCl in 0.05 M Tris-HCl, pH 7.2, to a mixing vessel containing 500 ml of 0.2 M NaCl in the same buffer. Fractions of 4 ml were collected and checked for the enzyme activities indicated in the inset of Fig. 2. As can be seen, a large portion of endogalactosidase activity was eluted in a peak from tubes 100 to 167. $\beta$-D-Galactosidase activity was well separated from the endogalactosidase activity. Fractions in the endogalactosidase peak were combined and concentrated to about 6 ml by pressure dialysis against 0.05 M Tris-HCl, pH 7.2.

**Properties of Keratan Sulfate-Endogalactosidase**

**Stability**—The purified enzyme has been stored in an ice bath for as long as 1 year with no loss in activity.

**pH Optimum**—Fig. 3 shows the effect of pH on the rates of degradation of corneal keratan sulfate by keratan sulfate-endogalactosidase. As can be seen, the optimum lies between pH 7.2 and 7.4.

**Temperature Optimum**—The optimal temperature for keratan sulfate-endogalactosidase was approximately 37°C. At 30° and 45°C, the increases of reducing activity per hour were about 80% and 50%, respectively, of that observed at 37°C.

**Effects of Cations and Anions**—Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ had little effect on the enzyme when they were tested as chloride salts at 1 mM. Acetate and maleate, on the other hand, elicited a slight activation (about 15%) of the activity when they were tested as sodium salt at 0.05 M.

**Specificity**—As seen in Fig. 4, this enzyme showed fairly good activity with corneal keratan sulfate as a substrate. After prolonged incubation for 24 hours, during which time further additions of enzyme were made, the level of reducing activity in the digest rose to about 50% of the total galactose in the polysaccharide. The keratan sulfates from nucleus pulposus and shark cartilage, in contrast, were attacked much more slowly. The extent of degradation of the keratan sulfates from nucleus pulposus and shark cartilage were virtually 40% and 10%, respectively, of that of corneal preparation. The differences in observed rates and extents of degradation can be interpreted as reflecting certain inherent, but as yet obscure, features of their structure which in some way alter the ability of the enzyme to attack a specific glycoside linkage within the polymer.

Treatment of chondroitin 4-sulfate, chondroitin 6-sulfate, hyaluronic acid, dermatan sulfate, and heparin with the enzyme for a period of 24 hours yielded no significant increase in reducing activity (Fig. 4). Similarly, when laco-N-tetraose (for structure see “Materials and Methods”) was treated by this enzyme, no hydrolysis of this oligosaccharide could be detected on subsequent chromatography in Solvent A. As will become evident, the bond susceptible to hydrolysis with this enzyme is associated with $\beta$-D-galactosyl-(1 → 4)-2-acetamido-2-deoxy-6-O-sulfoglucose in keratan sulfate. The failure of the enzyme to attack laco-N-tetraose, therefore, suggests that a structure of the type, X-(1 → 3)-$\beta$-D-galactosyl-(1 → 4)-Y, is not the only specificity-determining factor, i.e. neighboring sugars, X and Y, or even larger portions of substrate molecule must have an important effect. Also to be noted in this respect is the fact that this enzyme did not act on phenyl $\beta$-D-galactoside, a typical substrate for ordinary exo-$\beta$-D-galactosidases.

**Product Identification**—To obtain a larger amount of the enzymatic products, 120 $\mu$mol (as galactose) of corneal keratan sulfate were digested with 15 units of the purified endogalactosidase for 60 hours. During that time, additional enzyme (10 units) was added thrice, together with a small amount of toluene as a preservative. The digest was then submitted to chromatography on Sephadex G-50 (Fig. 5). The elution profile indicates that the end products of enzyme action comprised a number of fragments with various sizes. About 54% of the total galactose was recovered in the last two fractions, C-7 and C-8.

Table II shows the results of analysis of Fractions C-1 to C-8. The molar ratio of galactose to glucosamine varied slightly from about 0.9 to 1.1 except for C-1 which had a considerably low value. This low value is probably due to a contamination by another polysaccharide (e.g. chondroitin sulfate) since the keratan sulfate preparation contained 2.2% uronic acid (I), and since the bulk of the uronic acid was recovered in Fraction C-1. The ratio of sulfate to glucosamine varied from about 0.9 to 1.36.

To isolate the materials in Fractions C-7 and C-8 for further characterization, these fractions were subjected to paper chromatography in Solvent B (Fig. 6, left). Fraction C-7 gave one major component (C-7a) plus one minor component, and Fraction C-8 one major component (C-8b) plus three minor components. The main component of each fraction was eluted from the chromatographic strip with water and submitted to paper electrophoresis (Fig. 6, right). Component C-7a gave only one fraction (C-7aa), but component C-8b yielded one major fraction (C-8bb) plus two minor fractions. The main fractions were eluted from the electrophoretic strips and chroma-
Fig. 3 (left). Effect of pH on the activity of Pseudomonas endo-
galactosidase. The conditions of the experiment were those of
Assay 1 described in Ref. 1, except for the pH of the buffers. The
buffers used were 5 μmol of Tris-HCl (pH 6.5 to 10.2), Tris-maleate
(pH 4.0 to 8.2), or sodium acetate-acetic acid (pH 3.5 to 6.1) per
incubation mixture.

Fig. 4 (center). Rate and extent of degradation of keratan sul-
fates and related polysaccharides with Pseudomonas endo-
galactosidase. The purified enzyme from Step 5 (see the text) was used.
The conditions of the experiment were those in Assay 1 (1) with
0.05 unit of enzyme and 0.1 pmol (as galactose or uronic acid) of
the following substrates: O—O, corneal keratan sulfate; •—•, nucleus pulposus keratan sulfate; △—△, shark cartilage keratan sulfate;
▲—▲, hyaluronic acid, chondroitin 6-sulfate, dermatan sulfate, or heparin.
At the 4th and 8th hour, 0.5 unit each of enzyme was added to the
incubation mixture. The reducing activity equivalent to 50% of the
total galactose in keratan sulfate is indicated by the arrow.

Fig. 5 (right). Sephadex G-50 chromatography of endogalac-
tosidase digest of keratan sulfate. The reaction mixture (1.0 ml)
contained 50 μmol of Tris-HCl, pH 7.2, 120 μmol (as galactose) of
keratan sulfate, 15 units of enzyme, and a small amount of tolune
(preservative). Incubation was carried out at 37° for 60 hours;
during that time, additional enzyme (10 units) was added at the
15th, 24th, and 42nd hour. Solid NaCl was added to the mixture to
give a final concentration of 0.5 M. The solution was then
transferred to a column (2 X 102 cm) of Sephadex G-50. Elution
was carried out with 0.5 M NaCl at a rate of 14 ml per hour. Two-
milliliter fractions were collected and analyzed for hexose by the
anthrone reaction. a, the elution profile of digest of corneal kera-
tan sulfate; b, the elution profiles of digests of nucleus pulposus keratan sulfate (---) and shark cartilage keratan sulfate (••••).
Note the difference in the scale of the ordinate in a and in b.

Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Composition</th>
<th>Molar ratios</th>
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<tr>
<td></td>
<td>Glucosamine</td>
<td>Galactose</td>
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<tr>
<td></td>
<td>μmol</td>
<td></td>
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<tr>
<td>C-1</td>
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<tr>
<td>C-2</td>
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<td>10.6</td>
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<td>C-7</td>
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</tr>
<tr>
<td>C-8</td>
<td>21.9</td>
<td>28.0</td>
</tr>
</tbody>
</table>

*Expressed as the total amount in each pooled fraction (see Fig. 5).

tographed on paper for desalting (with Solvent D in which the
compounds had little mobility). The compounds were eluted
from the strips with water and lyophilised.

Analyses of C-7aa and C-8bb gave the results shown in Table
III. The data clearly indicate that C-7aa and C-8bb are a
tetrasaccharide trisulfate and a disaccharide monosulfate,
respectively, with galactose at the reducing end. When C-8bb
was hydrolyzed with 0.1 N HCl at 100° for 2 hours, paper elec-
rophoresis indicated the presence of a negatively charged sugar
with the same mobility as authentic 2-acetamido-2-deoxy-0-
O-sulfo-N-glucose (17.4 cm per hour). Products corresponding
to neutral sugar were also present near the origin and identified as
2-acetamido-2-deoxyglucose and galactose by paper chromatog-
raphy in Solvent B. Using the same procedure, C-7aa was
shown to yield, in addition to the products indistinguishable from
those derived from C-8bb, a distinct substance with the mobility
of galactose 6-sulfate (19.0 cm per hour), a clue which led to the
subsequent finding that one of the sulfate groups of C-7aa is
present in the internal galactose moiety (see below).

Upon digestion with the sulfatase purified from Actinobacillus
(1), the sulfate of C-8bb was quantitatively released. Subse-
quent treatment of the sulfatase digest with the β-D-N-acetyl-
glucoaminidase preparation from Actinobacillus (1) resulted in
formation of galactose and 2-acetamido-2-deoxyglucose, as
d judged by paper chromatography (Solvent C) of the digest
which had been passed through Dowex 50 (H+) and concen-
trated to dryness in a vacuum (for desalting). This result indi-
cates that the sulfate is located on the nonreducing 2-acetamido-
2-deoxyglucose unit and further suggests that the compound is a
disaccharide derived from the main structure unit of keratan
sulfate, namely, 2-acetamido-2-deoxy-6-O-sulfo-β-D-glucosyl
(1→3)-β-D-galactose.

The tetrasaccharide trisulfate, C-7aa, was subjected to the
same procedure. Only one-third of the total sulfate was re-
moved by the action of Actinobacillus sulfatase. The sulfatase-
treated compound was shown to be susceptible to the digestion
with Actinobacillus β-D-N-acetylglucosaminidase, liberating 1
eq mol of 2-acetamido-2-deoxyglucose, assessed by the Morgan-
Elson reaction (7). Therefore, the nonreducing terminal unit of C-7aa must be a 2-acetamido-2-deoxyglucose bearing a
sulfate group presumably at position 6.
sulfated sugar units can be considered as originating from the internal disaccharide moiety of the tetrasaccharide, since the sugars at both reducing and nonreducing end should be destroyed by the periodate oxidation. It thus appears that C-7aa is a fragment derived from the known tetrasaccharide units of corneal keratan sulfate (8), namely 2-acetamido-2-deoxy-6-O-sulfo-β-D-glucosyl-(1→3)-6-O-sulfo-β-D-galactosyl-(1→4)-2-acetamido-2-deoxy-6-O-sulfo-β-D-galactosyl-(1→3)-D-galactose, although the exact type of the linkages cannot be evaluated from the present studies.

These results of product identification confirm the ability of the enzyme to hydrolyze keratan sulfate at endogalactoside bonds and further suggest that the enzyme has little or no action on a galactoside bond in which galactose 6-sulfate participates.

**Use of Endogalactosidase for Studies on Structural Variability of Keratan Sulphates of Different Origin**—The results presented thus far indicate that the endogalactosidase can be used to analyze the structures of keratan sulfates. For example, the proportion and distribution of endogalactosidase-susceptible bonds and endogalactosidase-resistant sections can be assessed by the Sephadex G-50 elution profiles of endogalactosidase digests (cf. Fig. 5).

This approach was used to study the properties of keratan sulfates from human nucleus pulposus and shark cartilage. As has already been shown in Fig. 4, the endogalactosidase showed a relatively slow activity with nucleus pulposus keratan sulfate that proceeded to about 40% of its limit with corneal keratan sulfate. Sephadex G-50 chromatography of the 60-hour digest of nucleus pulposus keratan sulfate (Fig. 5b) showed a profile with multiple peaks, which differed from the profile of corneal keratan sulfate with respect to the molecular size distribution of the fragments. Apparently, the nucleus pulposus sample gave major peaks in the region expected for higher oligosaccharides. The fractions designated as N-7 and N-8 (Fig. 5b) were eluted in the positions coinciding with Fractions C-7 and C-8, respectively, of the corneal sample (cf. Fig. 5a). The molar ratios, hexosamine to hexose to sulfate were 1.00:1.06:1.57 for N-7 and 1.00:1.00:1.07 for N-8. Since the correspondence between N-7 and C-7 and that between N-8 and C-8 were also excellent upon paper chromatography in Solvent B and paper electrophoresis, peaks N-7 and N-8 may actually represent the same tetrasaccharide trisulfate and disaccharide monosulfate, respectively, released from the corneal sample.

When shark cartilage keratan sulfate was subjected to digestion with the endogalactosidase, the rate and extent of degradation were much lower than those observed with nucleus pulposus sample (Fig. 4). The gel chromatography profile of the 60-hour digest (Fig. 5b) indicated that most of the materials in the digest were eluted in or near the void volume. Since the shark keratan sulfate is characterized by a comparatively high sulfate content (23.97%, see Ref. 1) the data of Fig. 4 and Fig. 5b may be accounted for, at least in part, by a high degree of sulfation of the galactose residues in the polymer chain. Compatible with this postulate was the observation that the chemically desulfated keratan sulfate (shark) with a low sulfate content (9.5%) could be hydrolyzed to a greater extent (corresponding to about 20% of the total galactose) by the endogalactosidase.

**DISCUSSION**

From the results presented in this paper, it would appear that the bond susceptible to hydrolysis with *Pseudomonas* endogalactosidase is associated with β-D-galactosyl-(1→4)-2-acetamido-2-deoxy-6-O-sulfo-D-glucose, since the major oligosacchari-
ride products in the digest are terminated by galactose and 2-acetamido-2-deoxy-6-O-sulfoglucose at the reducing and non-reducing end, respectively. Substitution of the galactose moiety at position 6 with a sulfate ester group may impair the hydrolysis, for 2-acetamido-2-deoxy-6-O-sulfo-β-D-glucosyl-(1→3)-6-O-sulfo-β-D-galactosyl-(1→4)-2-acetamido-2-deoxy-6-O-sulfo-β-D-glucosyl-(1→3)-β-D-galactose remains unchanged even when exhaustively treated with the endogalactosidase. It has been shown previously (8) that about 40% of the galactosyl groups in corneal keratan sulfate are substituted in position 6, at least in the major portion by sulfate ester groups. If these sulfated galactosyl groups are distributed unevenly along the polysaccharide chain, treatment with the endogalactosidase would result in a formation of fragments of rather varying size. This would be observed as a dispersed gel chromatography profile. The profile of Fig. 5a indicates that this is indeed the case. Although the fragments apparently vary in sulfate content (Table II), some of them are not so high in sulfate content as would be anticipated from the presence of "extra" sulfate in the internal galactosyl groups. An adequate explanation for the resistance of such fragments to hydrolysis is not immediately available. These fragments would contain nonsulfated 2-acetamido-2-deoxyglucose groups so that their sulfate contents per disaccharide unit may not exceed unity to any large extent. Another possibility is the presence of sugar branches (e.g. galactosyl-, fucosyl-, or sialyl-branches; cf. Ref. 8) which could possibly restrict the enzyme's access to the susceptible galactosidic bonds. These problems of specificity will be solved as more precise information on the structure of the released fragments becomes available.

Comparison of the chemical properties of keratan sulfates from different origin (9, 10) suggested division of these preparations into two classes derived, respectively, from cornea (KSI) and from skeletal tissues (KSII). The basic structure appears to be similar for both polysaccharides, but differences were noticed mainly in the average length of the chains and in the occurrence of a greater degree of branching in KSII (3, 8). It is difficult, however, to assess the actual number of the branches or the points of branching. The difficulty appears to come from the scarcity of methods for fragmentation in which various enzymes of known specificity may be employed to degrade the polysaccharides. From the data obtained in the present study (e.g. the data in Figs. 4 and 5), it can be expected that the endogalactosidase will be of value as a reagent for fragmentation in studying the fine structure of polymorphous keratan sulfates.

The existence of an endoglycosidase in a Coccobacillus has been reported by Hirano and Meyer (11), who observed the appearance of sulfated oligosaccharide products after degradation of keratan sulfates by this enzyme. A similar enzyme has also been found in an enteric bacterium by Kitamikado and Ueno (12). Strict comparison among the results obtained with different enzyme sources is difficult at the present time in view of inherent differences in the principles of the methods used, but the available information suggests that the enzymes from the three different bacteria have a similar, if not identical, specificity.

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