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Purification of an Unusual α-Glycuronidase from Flavobacteria*

C. Terry Warnick† and Alfred Linker‡

ABSTRACT: An unusual α-glycuronidase was isolated from *Flavobacterium heparinum* and purified. The enzyme acts on degradation products of heparin or heparitin sulfate containing α,β-unsaturated uronic acid. It appears to be specific for α-linked unsaturated uronides derived from glucuronic or iduronic acid. Saturated uronides are not hydrolyzed. Activity is highest with disaccharides and falls off rapidly with increasing molecular weight of oligosaccharides. The glycuronidase in conjunction with eliminases present in the same organism degrades heparin and heparitin sulfate to monosaccharides.

The enzymatic degradation of mucopolysaccharides has been shown to proceed by two different pathways: (1) a hydrolytic pathway characteristic of enzymes found in animal tissues (Mathews, 1966) and (2) an elimination pathway characteristic of enzymes found in bacteria (Linker *et al.*, 1956; Nakada and Wolfe, 1961; Yamagata *et al.*, 1968). Oligosaccharides produced by hydrolysis can be further degraded by glucuronidases which are found in a variety of sources (Levvy and Conchie, 1966).

Oligosaccharides produced by elimination contain a 4,5-unsaturated uronic acid at the nonreducing end (Linker *et al.*, 1956). It has been found that the β-linked oligosaccharides produced from the chondroitin sulfates and hyaluronic acid can be further degraded by β-glucuronidases (Linker *et al.*, 1960) which appear to be specific for the unsaturated uronic acid (Yamagata *et al.*, 1968).

Adapted cultures of *Flavobacterium heparinum* produce an enzyme complex which will degrade the α-linked mucopolysaccharides, heparin and heparitin sulfate (Korn and Payza, 1956; Linker and Hovingh, 1965). These enzymes have been separated into eliminases which degrade heparin and heparitin sulfate to a series of $\Delta^{4,5}$-unsaturated oligosaccharides and an α-glycuronidase which has the unusual property of hydrolyzing the uronic acid from unsaturated α-linked oligosaccharides, the only reported enzyme with this specificity (Linker and Hovingh, 1965, 1968).

As this α-glycuronidase[1] has a specificity which is unique (namely, the hydrolysis of unsaturated α-linked uronic acids), it was thought to be worthwhile to investigate this enzyme and some of its properties further.

Materials and Methods

Paper Chromatography and Electrophoresis. Paper chromatography was carried out on Whatman No. 1 paper in a descending system with butanol–acetic acid–water (50:15:35, v/v) as solvent. Compounds were located by ultraviolet absorption using a short-wavelength ultraviolet light, or by spraying with alkaline silver nitrate to locate reducing sugars (Trevelyan *et al.*, 1950) or *o*-phenylenediamine to locate α-keto acids (Lanning and Cohen, 1951).

Electrophoresis was carried out on cellulose acetate using a Savant flat-plate apparatus, at a potential of 35 V/cm in a buffer system of pyridine–formic acid (pH 3.0) (Mathews, 1961). Reducing sugars were located by spraying with alkaline silver nitrate with the substitution of 95% ethanol for the acetone in the spray.

Chemical Methods. Sugars were reduced with sodium borohydride or hydrogenated as in the previous paper (Linker and Hovingh, 1972). Phenyl α-D-glucopyranoside was oxidized to (phenyl α-D-glucopyranosido)uronic acid by the method of

* From the Departments of Biological Chemistry and Pathology, the University of Utah College of Medicine and the Veterans Administration Hospital, Salt Lake City, Utah 84113. Supported in part by Grant No. AM-13412 from the U. S. Public Health Service.

† Taken from a thesis to the Department of Biological Chemistry, University of Utah, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1970.

‡ To whom to address correspondence.

[1] The enzyme has been called a glucuronidase previously. As it acts on 4,5-unsaturated uronides this is not strictly correct as it would also act on unsaturated uronides derived from L-iduronic acid. We are, therefore, using the term α-glycuronidase in this paper.

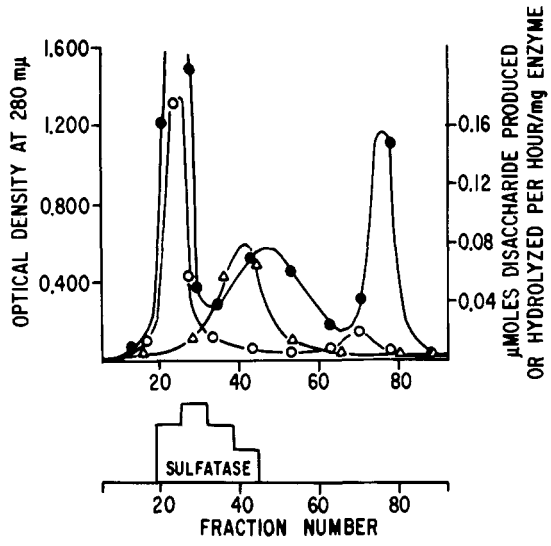

FIGURE 1: Elution pattern of Flavobacterium enzymes from a Sephadex G-200 column. (●) Optical density at 280 mμ; (○) eliminase activity; (△) α-glycuronidase activity, pH 7.0.

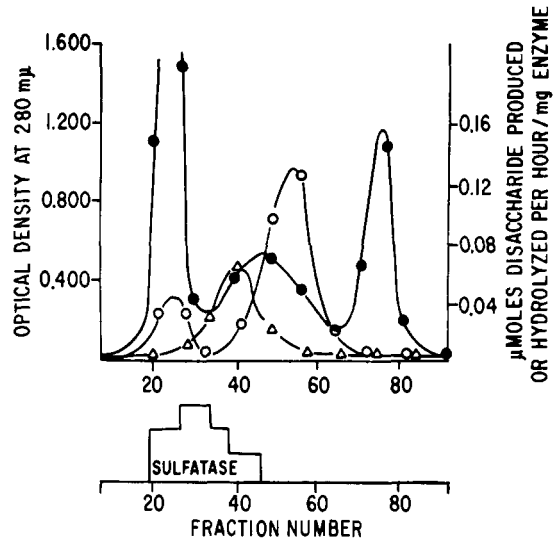

FIGURE 2: Elution pattern of Flavobacterium enzymes from a Sephadex G-200 column at pH 5.5–6.0. (●) Optical density at 280 mμ; (○) eliminase activity; (△) α-glycuronidase activity.

Mehltretter (1963) using a platinum catalyst prepared by bubbling hydrogen gas through a solution of platinum oxide in glacial acetic acid. The glucuronide had a melting point of 146–147° (lit. mp 147°, Marsh and Levvy, 1958).

Purification of Substrates and Assay of the Enzymes. The substrates for the α-glycuronidase are the unsaturated α-linked oligosaccharides from heparitin sulfate (Linker and Hovingh, 1968) and from heparin (Linker and Hovingh, 1972). In addition a disaccharide was prepared from chondroitin 6-sulfate by digesting 500 mg of chondroitin 6-sulfate with 10 mg of a chondroitinase from *Flavobacterium heparinum* which had been adapted for growth on chondroitin 6-sulfate (Hoffman *et al.*, 1960). The pH of the digest was adjusted to 6.0 with HCl and applied to a column (2.1 × 30 cm) of Dowex 1-X4 (chloride form, 200–400 mesh) and eluted with a linear gradient of 0.0–2.0 M LiCl in 0.01 M HCl. The column was monitored by measuring the ultraviolet absorbance of the fractions at 232 mμ. The fractions corresponding to the disaccharide, identified by paper chromatography (Yamagata *et al.*, 1968) were combined, neutralized with KOH, concentrated on a flash evaporator, desalted on a Sephadex G-10 column, and lyophilized.

The N,O-disulfated glucosamine which was used as the substrate when assaying for sulfatase was prepared by digesting heparin with a crude enzyme mixture from *Flavobacterium heparinum* (see preparation of the enzyme). The digest was mixed with a small amount of dry cellulose powder and lyophilized. The mixture was then applied to a cellulose column (2.2 × 30 cm) and eluted with butanol–acetic acid–water (50:12:20, v/v). The column was monitored by measuring the reducing sugar (Rapport *et al.*, 1950) of each sample and fractions containing the N,O-disulfated glucosamine, identified by paper chromatography (Linker and Hovingh, 1965), were combined and taken to dryness. The N,O-disulfated glucosamine was then dissolved in a small amount of water, chromatographed on Sephadex G-10 to remove extraneous material and lyophilized (see also preceding paper (Linker and Hovingh, 1972)).

The α-glycuronidase was assayed by measuring the decrease in ultraviolet absorbance at 232 mμ of the unsaturated α-linked disaccharide which served as substrate. As the unsaturation in the disaccharide or the oligosaccharides is α,β to the carbonyl moiety of the uronic acid, hydrolysis of the uronic acid from the oligosaccharide by the enzyme leads to loss of absorbance (see Discussion). The reaction mixture contained substrate (10 mg/ml) and enzyme (1 mg/ml) in 0.1 M phosphate buffer (pH 7.0) and the reaction was run at 25° in a constant-temperature water bath. The reaction was linear up to 2 hr. The specific activity of the enzyme is defined as the micromoles of unsaturated disaccharide (H-I) (mol wt 560) hydrolyzed per hour per milligram of enzyme.

The eliminase was assayed as reported earlier (Linker and Hovingh, 1965). The presence of sulfatase was determined by incubating the enzyme (2 mg/ml) with N,O-disulfated glucosamine (10 mg/ml) in 0.1 N sodium acetate (pH 7.0); after incubation for 18 hr at 25° (at 45° the sulfatase assay was erratic), the mixture was chromatographed on paper in butanol–acetic acid–water (50:15:35, v/v). The compounds were located by spraying with alkaline silver nitrate and the presence of sulfatase noted by the formation of N-sulfated glucosamine from N,O-disulfated glucosamine.

Results

Preparation of the Enzyme. The procedure followed for growth of the *Flavobacterium heparinum* was patterned after Payza and Korn (Payza and Korn, 1956). After growth, the cells were collected by centrifugation and washed with 0.025 M phosphate buffer. The following procedures were carried out at 4°. The cells were suspended in 0.69 M ammonium carbonate (pH 7.0) and sonicated for 25 min in an ice bath at full power on a Bronwill Biosonik Model BP1 sonifier. The suspension was then centrifuged at 25,000*g* for 1.5 hr. The supernatant fluid from this centrifugation was recentrifuged at 25,000*g* for an additional 1.5 hr and the supernatant dialyzed overnight against distilled water and lyophilized. The enzyme preparation at this point contained all the enzymes responsible for the degradation of heparin to monosaccharides (eliminase, α-glycuronidase, and sulfatase) and is designated as the "crude enzyme."

The α-glycuronidase, eliminase, and sulfatase were fractionated by chromatography on a Sephadex G-200 column

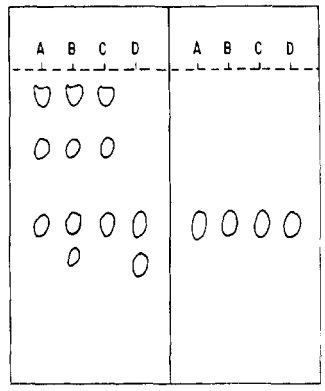

FIGURE 3: Tracing of paper chromatograms of the products of α-glycuronidase digestion. (A) Crude heparinase digest of heparin; (B) crude heparinase digest of heparitin sulfate; (C) α-glycuronidase digest of unsaturated disulfated disaccharide (H-I) from heparin; (D) α-glycuronidase digest of unsaturated N-acetylated disaccharide (nonsulfated) from heparitin sulfate. Left panel stained with alkaline silver nitrate, right panel with *o*-phenylenediamine.

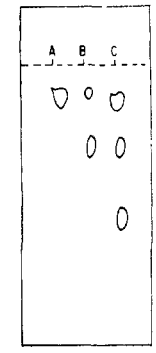

FIGURE 4: Tracing of paper chromatogram showing action of Flavobacterium sulfatase. (A) N,O-Disulfated glucosamine; (B) N,O-disulfated glucosamine after incubation with crude heparinase for 16 hr at 25°; (C) crude heparinase digest of heparin.

prepared as reported earlier (Linker and Hovingh, 1965). A typical elution pattern is shown in Figure 1. It was found that the pH of the enzyme solution as it is applied to the Sephadex column is very important in achieving a separation of the eliminase and α-glycuronidase. The pH of the crude enzyme when dissolved in 0.1 M sodium acetate is usually 5.5–6.0 and must be adjusted to neutrality for best results on the Sephadex column. A low pH can change the elution pattern markedly as shown in Figure 2.

The α-glycuronidase fractions from the Sephadex G-200 columns were pooled and lyophilized. The lyophilized enzyme (20 mg) was dissolved in 2 ml of 0.1 M potassium phosphate buffer (pH 7.0) and further fractionated with ammonium sulfate. The material which precipitated between 65 and 75% ammonium sulfate saturation was found to contain the α-glycuronidase activity and was recovered by centrifugation. It was then dissolved in 0.1 N potassium phosphate buffer and dialyzed against ten volumes of the same buffer. The purification steps and the amount of purification achieved in each step are shown in Table I. An overall purification of 13-fold and a yield of 13% were obtained with this procedure.

TABLE I: Purification of the α-Glycuronidase from *Flavobacterium heparinum*.

Step	Total Protein (mg)	Sp Act.[a]	Total Act.[b]	Purifcn	Yield (%)
Crude enzyme	150	0.058	8.7	1.0	100
Sephadex G-200	20	0.085	1.7	1.5	20
Ammonium sulfate	1.4	0.780	1.1	13.4	13

[a] The specific activity is expressed in terms of micromoles of unsaturated disaccharide hydrolyzed per hour per milligram of enzyme. [b] The total activity is expressed in terms of the total micromoles of unsaturated disaccharide hydrolyzed per hour.

The products of the action of the α-glycuronidase on the unsaturated disulfated disaccharide from heparin and the unsaturated, N-acetylated nonsulfated disaccharide from heparitin sulfate (Linker and Hovingh, 1968) were identified as follows. After digestion for 24 hr, the reaction mixtures were chromatographed on paper in a descending system of butanol–acetic acid–water (50:15:35, v/v) and the products located by staining with alkaline silver nitrate or *o*-phenylenediamine. The results are shown in Figure 3. Digests of heparin and heparitin sulfate by the crude enzyme mixture from *F. heparinum* were chromatographed for comparison; the products in these crude enzyme digests have been previously isolated and identified (Linker *et al.*, 1960; Linker and Sampson, 1960). They are in order of increasing rate of migration, N,O-disulfated glucosamine, N-sulfated glucosamine, α-keto acid (4-deoxy-L-*threo*-5-hexulosuronic acid), and *N*-acetylglucosamine. As seen in Figure 3, the spots in the α-glycuronidase digests of the disaccharides migrate exactly like those in the crude enzyme digest. The spots corresponding to the α-keto acid also stain with *o*-phenylenediamine, a reagent specific for α-keto acids (Lanning and Cohen, 1951).

The N-sulfated glucosamine seen in each of the α-glycuronidase digests is a result of contaminating sulfatase in the preparation. This can be seen in Figure 4, which shows a paper chromatogram of N,O-disulfated glucosamine before and after incubation with a crude enzyme preparation from *F. heparinum* for 17 hr at 25°. The N,O-disulfated glucosamine has been almost entirely converted to N-sulfated glucosamine under these conditions. Although an *N*-sulfatase is also present (Dietrich, 1969a) its activity does not appear significant under these conditions.

To check the identification of the products, the α-glycuronidase digest along with the standard heparin and heparitin digests (containing known components) were subjected to electrophoresis on cellulose acetate in pyridine–formate buffer (pH 3.0). The results are shown in Figure 5. The electrophoresis reverses the paper chromatography pattern but the compounds from the α-glycuronidase digest of the disaccharides still correspond to the known compounds in the crude enzyme digests of heparin and heparitin sulfate.

Some of the properties of the α-glycuronidase were then investigated. The pH optimum of the enzyme was determined in potassium phosphate buffer using the unsaturated disulfated disaccharide from heparin as substrate. The enzyme has a pH optimum at about 6.5–7.0 under these conditions. The temperature optimum was at 45°, again using the unsaturated disulfated disaccharide from heparin as the substrate.

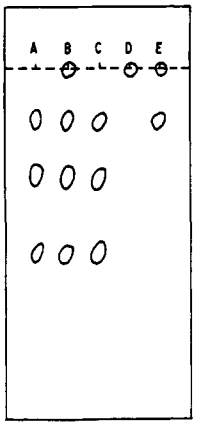

FIGURE 5: Tracing of electrophoresis pattern of α-glycuronidase digests of unsaturated disaccharides. Electrophoresis was carried out at 1000 V for 1 hr. (A) Crude heparinase digest of heparin, (B) crude heparinase digest of heparitin sulfate, (C) α-glycuronidase digest of unsaturated disulfated dissaccharide (H-I) from heparin, (D) *N*-acetylglucosamine, and (E) α-glycuronidase digest of unsaturated N-acetylated disaccharide (nonsulfated) from heparitin sulfate.

The activity of the enzyme on various unsaturated disaccharides is shown in Figure 6. The enzyme activity is expressed as the per cent loss of ultraviolet absorbance at 232 mμ which is the equivalent of putting all the substrates on a molar ratio basis, as a 50% loss of ultraviolet absorbance means that 50% of the disaccharide have been hydrolyzed. It was found that the enzyme hydrolyzes the nonsulfated disaccharide from heparitin sulfate best, hydrolyzes the unsaturated disulfated disaccharide from heparin less well and has essentially no activity on the β-linked unsaturated disaccharide from chondroitin 6-sulfate. Activity of the glycuronidase on unsaturated (nonreducing end only) oligosaccharides is compared with activity on disaccharide in Figure 7. It is very significant that the rate falls off rapidly with increase in size, hexasaccharide is hardly acted upon.

The substrate specificity of the α-glycuronidase was then investigated using chemically modified substrates. Tested were an unsaturated disulfated disaccharide from heparin which had been reduced with sodium borohydride (Linker and Hovingh, 1972), a disulfated disaccharide from heparin which had been hydrogenated to saturate the double bond (Linker and Hovingh, 1972) and α-phenyl glucuronide prepared by oxidizing phenyl α-glucoside. The results are shown in Table II. The unsaturated compounds were assayed as before, but for compounds with no α,β-unsaturation, enzyme activity was determined by measuring the increase in reducing sugar. The borohydride reduced compound was found to be as good a substrate as the original disaccharide, but any loss of the unsaturation caused a complete loss of enzyme activity toward that molecule. Neither the catalytically hydrogenated disaccharide nor the α-phenyl glucuronide were hydrolyzed by this α-glycuronidase.

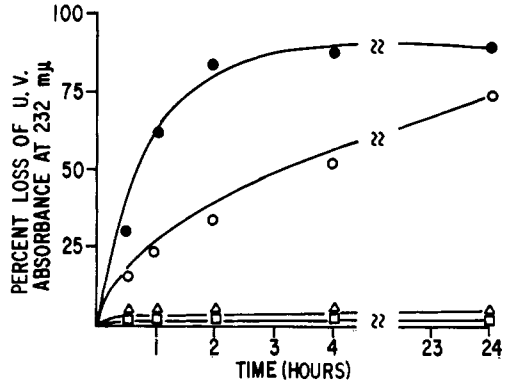

FIGURE 6: Activity of α-glycuronidase on various unsaturated disaccharides (●), N-acetylated disaccharide (nonsulfated) from heparitin sulfate; (○) disaccharide H-I from heparin; (△) disaccharide from chondroitin 6-sulfate; (□) boiled enzyme control.

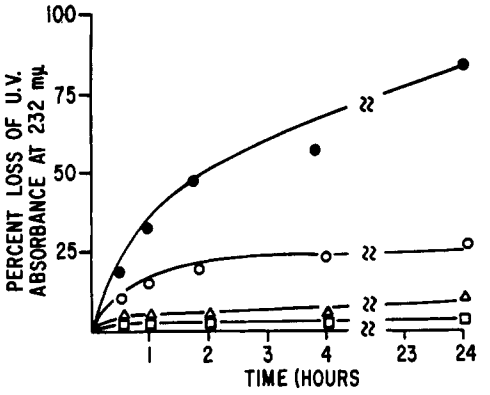

FIGURE 7: Activity of the α-glycuronidase on unsaturated oligosaccharides isolated from a heparin digest. (●) Disulfated disaccharide (H-I); (○) fully sulfated tetrasaccharide (H-II); (△) sulfated hexasaccharide (H-III); (□) boiled enzyme control.

Discussion

Extracts of Flavobacteria degrade hyaluronic acid and the chondroitin sulfates by an elimination mechanism forming α,β-unsaturated oligosaccharides (Yamagata *et al.*, 1968; Linker *et al.*, 1960). In order to produce significant amounts of enzymes which further degrade these unsaturated oligosaccharides, the organism must be adapted for growth on these mucopolysaccharides. Such extracts show no significant activity on heparin or heparitin sulfate which is not surprising in view of the α-glycosidic linkages in these poly-

TABLE II: Activity of the α-Glycuronidase on Chemically Modified Substrates.

Substrate[a]	μmole of Disaccharide Hydrolyzed/ hr per mg	Act. Rel to Unsatd Disaccharide from Heparin
Unsaturated disaccharide	0.750	1.00
Chemically reduced disaccharide ($NaBH_4$)	0.710	0.95
Catalytically reduced disaccharide (H_2)	0.000	0.00
α-Phenyl glucuronide	0.000	0.00

[a] The substrates were an unsaturated disulfated disaccharide from heparin and the same compound after chemical reduction with sodium borohydride and catalytic reduction with hydrogen and palladium.

saccharides. However, when Flavobacteria are grown on either heparin or heparitin sulfate, enzymes are produced that degrade both polysaccharides to monosaccharides (Linker and Hovingh, 1965). This degradation was shown to proceed also by an elimination mechanism catalyzed by enzymes which produce α-linked unsaturated oligosaccharides and a glycuronidase which hydrolyzes the α-linked unsaturated oligosaccharides further.

In this report the purification of the α-glycuronidase is described. It should be emphasized that though the purification as such is not very extensive (about 13-fold), this is the first time that the enzyme has been obtained free of eliminases. This made it possible to characterize its activity and particularly its specificity on oligosaccharide substrates.

The products of the hydrolysis of the unsaturated disaccharides from heparin and heparitin sulfate by the α-glycuronidase were shown to be an α-keto acid and an amino sugar, either *N*-acetylglucosamine if the disaccharide came from heparitin sulfate or N,O-disulfated glucosamine if the disaccharide came from heparin. These products are the same type as Linker *et al.* (1960) found in the hydrolysis of unsaturated chondroitin sulfate disaccharides. This leads us to postulate that the same type of pathway proposed for the β-glycuronidase (Linker *et al.*, 1960) also holds for the α-glycuronidase. Only glycosidic cleavage is necessary to obtain the products. The α-glycuronidase releases the unsaturated uronic acid, which is stabilized by the glycosidic linkage, from the disaccharide. The hydrolyzed uronic acid now equilibrates with the open chain form, thus releasing a hydroxyl group that is in position for a keto–enol isomerization. The keto form is evidently more stable and gives the α-keto acid. The amino sugar of the disaccharide released from the uronic acid, is desulfated to some extent by sulfatases in the preparation. These reactions account for all the products found in the α-glycuronidase digests.

While this pathway is similar to that proposed for the β-glycuronidase, the α-glycuronidase of this report seems to be a different enzyme. The α-glycuronidase has been shown here to have very little activity on an unsaturated disaccharide from chondroitin 6-sulfate. The β-glycuronidase, on the other hand, hydrolyzes this compound rapidly while it has no activity on α-linked compounds.

It is interesting to note that both enzymes appear to be specific for unsaturated oligosaccharides. The α-glycuronidase lost all activity toward the disaccharide from heparin when its unsaturation was removed by hydrogenation and it had been found that the β-glycuronidase also has no activity towards several saturated compounds (Yamagata *et al.*, 1968). This is in contrast to a report by Dietrich that *F. heparinum*, induced for growth on heparin, produces enzymes which degrade heparin by hydrolysis instead of by elimination (Dietrich, 1968). Dietrich finds only saturated oligosaccharides after digestion of heparin with enzymes from Flavobacteria, and reports finding an α-glucuronidase which hydrolyzes glucuronic acid from these oligosaccharides, although, curiously enough, free glucuronic acid as such could not be demonstrated (Dietrich, 1969b). Since the enzyme reported here appears to have no activity on saturated uronides, these enzymes must be different. The growth of the bacteria and the isolation of the enzymes was similar in both cases, however, we used an enzyme to substrate ratio of 1:10 (on a weight basis) while Dietrich used a ratio of 2:1 (also on a weight basis). The discrepancy in the mechanism is, therefore, hard to understand. It is unlikely that two completely different pathways exist in one organism for the metabolism of a single substrate.

It is important to note that the α-glycuronidase has some activity on unsaturated oligosaccharides larger than disaccharide. The activity decreases as the size of the oligosaccharide increases which is similar to a report by Nagel and Hasegawa (1968) who found an α-1,4-($\Delta^{4,5}$-dehydrogalacturonosyl)galacturonate hydrolase, which attacks only the α-1,4-glycosidic linkage adjacent to the terminal 4,5-dehydrogalacturonate of unsaturated oligogalacturonides. They found digalactosyluronic acids to be hydrolyzed best with decreasing activity on tri-, tetra-, and pentagalactosyluronic acids.

In general the enzyme complex of Flavobacteria has been shown to be able to degrade heparin and heparitin sulfate to nonsulfated monosaccharides by the action of eliminase, α-glycuronidase and sulfatases. Some evidence had been presented for analogous situations (Preiss and Ashwell, 1962; Karapally and Dietrich, 1970) that further degradation of the α-keto acids to metabolically useful products could be carried out by the organisms.

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