β-Eliminative Cleavage of the Acidic Polysaccharide of *Fusarium* sp. M7-1 by an Enzyme Preparation of *Cellulomonas* sp.

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Received August 8, 1989

By digestion with an enzyme preparation derived from a culture filtrate of *Cellulomonas* sp., an unsaturated disaccharide was produced accompanied by the release of mannose and β1→2mannobiose from the acidic oligomer mixture that was obtained from the acidic polysaccharide of *Fusarium* sp. M7-1 by acetolysis. The unsaturated disaccharide produced was isolated and identified as 4-deoxy-α-L-fuco-β-hex-4-enopyranouronosyl α(1→2)-D-galactose. The same unsaturated sugar linked to the polysaccharide was also produced accompanied by the release of mannose and β1→2mannobiose from the acidic polysaccharide by the enzyme digestion.

Materials and Methods

Materials. The polysaccharide preparation was obtained from the mycelium of *Fusarium* sp. M7-1 by the method described in a previous paper.1 The polysaccharide preparation consisted of about 60% neutral sugar (mannose-galactose-glucose-rhamnose= 10:8:2:1) and about 40% uronic acid. The partially purified enzyme preparation was obtained as described in our previous papers1,3 from the culture filtrate of the *Cellulomonas* sp. The acidic oligomer mixture were obtained from the acidic polysaccharide by mild acetolysis by the method reported previously.4 The oligomers were a mixture of Manβ1→4GlcAα1→2Gal and Manβ1→2Manβ1→4GlcAα1→2Gal in a molar ratio of about 3:1. The reduced oligomer mixture was also used for the preparation of the reduced unsaturated disaccharide. The reducing termini of the oligomers were reduced with NaBH₄ and or with NaBD₄ at room temperature for 24 hr. The reduced oligomer mixture was passed through Dowex 50 (H⁺) with deionized water as an eluant. The eluate was concentrated to dryness under reduced pressure and boric acid was removed by evaporation with methanol. The partial 1H-NMR spectrum of the reduced oligomer mixture is shown in Fig. 1. The details of the isolation methods and the chemical structure of the oligomers will be published in the near future. Chemicals, resins, and other materials were obtained from commercial sources.

General methods. Total carbohydrate was measured by

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1 A part of this study was presented at the Annual Meeting of Kansai Division of the Agricultural Chemical Society of Japan, Takamatsu, October 1988.

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Fig. 1. Partial $^1$H-NMR Spectrum of the Reduced Oligomer Mixture.

the phenol-sulfuric acid method\textsuperscript{5} with D-mannose as a standard. The uronic acid was measured by the meta-hydroxydiphenyl method\textsuperscript{6} with D-glucuronic acid as a standard. Monosaccharides were measured by HPLC by the method described in our previous paper.\textsuperscript{7} The unsaturated sugar was measured by the thiobarbituric acid reaction\textsuperscript{7} or by measuring the absorbance at 230 nm using the isolated unsaturated disaccharide as a standard. 2-Furoic acid was measured by the absorbance at 250 nm using 2-furoic acid as a standard. Lyase activity was measured as follows: the reaction mixture containing 10 mg of the acidic polysaccharide and an appropriate amount of the enzyme solution in a total volume of 1 ml of 0.1 M phosphate buffer, pH 7.0, was incubated for 1 hr at 30°C. The unsaturated sugar produced was measured. One unit of the enzyme was defined as the amount of the enzyme that produces 1 /&micro;mol of the unsaturated sugar per min under these conditions. $^1$H-NMR spectra of the sugar samples were recorded on a JEOL-GX 400 spectrometer at 400 MHz in deuterium oxide at room temperature. $^{13}$C-NMR spectra of the 2-furoic acid were recorded on a JEOL-JNM 90Q spectrometer in CDCl\textsubscript{3} at room temperature. The chemical shifts were measured relative to an external acetone standard.

Results and Discussion

Isolation and identification of the unsaturated sugar

About 50 mg of the acidic oligomer mixture (a mixture of Man$\beta_1\rightarrow4$GlcA$\alpha_1\rightarrow2$Gal and Man$\beta_1\rightarrow2$Man$\beta_1\rightarrow4$GlcA$\alpha_1\rightarrow2$Gal in a molar ratio of about 3 : 1) was incubated with the enzyme preparation (2 units as lyase) for 12 hr at 30°C. After the reaction, the reaction mixture was put on a column of Bio-Gel P-4 (3 cm x 90 cm) equilibrated with 0.1 M NaCl and eluted with the same solvent. As shown in Fig. 2, the oligomers almost disappeared and new peaks appeared during the enzyme digestion. The low molecular weight fractions B and C were identified as $\beta_1\rightarrow2$mannobiose and mannose respectively by the method described in our previous paper.\textsuperscript{7} Fraction A was repeatedly chromatographed on the same column and finally desalted by gel filtration on Bio-Gel P-2 column. The corresponding fractions were combined and concentrated to dryness under reduced pressure below 50°C. Thus, about 20 mg of white powder (compound A) was obtained. The oligomer mixture (about 10 mg), reduced with NaBH\textsubscript{4}, was incubated with the enzyme preparation and purified by
the same method described above. Thus about 2 mg of the reduced unsaturated disaccharide (compound B) was obtained. A reduced unsaturated disaccharide (compound C) was also obtained from the oligomer mixture reduced with NaBD₄ by the same method. The UV spectrum of the isolated compound A had an absorption maximum at 232 nm as shown in Fig. 3. Both compounds B and C also had the same absorption spectrum as that of the compound A. This is characteristic of 4-deoxy-hex-4-enopyranosyl uronic acids⁸,⁹ and is definitive proof of carbon-carbon unsaturation.

These compounds also gave positive thiobarbituric acid test reactions, indicating that a 2-keto-3-deoxy sugar acid is either present or can be formed under the test conditions.¹⁰ The acid hydrolysis of the compounds A, B, and C with 0.1 M HCl at 100°C for 1 hr drastically changed the UV spectra and caused a negative reaction for thiobarbituric acid test. This suggests that the unsaturated sugar acid residue was converted to 2-furoic acid. To analyze the chemical structure of the UV-absorbing substance produced by the acid hydrolysis, 10 mg of the compound A was hydrolyzed with 1 ml of 0.1 M HCl at 100°C for 3 hr in a sealed tube. After the hydrolysis, a UV-absorbing substance was extracted with 10 ml of ethylacetate. The extract was concentrated to dryness under reduced pressure below 30°C. Thus, about 2 mg of fine needle-like crystals was obtained. The crystals melted at 133–134°C, identical with that of the authentic 2-furoic acid. The molecular weight of the crystal was 112 by mass spectrometry. The principal absorption spectra of the crystal and the authentic 2-furoic acid agree well as shown in Fig. 4. The NMR spectral data are as follows;¹¹ ¹H-NMR (90 MHz, CDCl₃): ppm, 10.1 (1H) –COOH, 7.7 (1H) H-5, 7.4 (1H) H-3, and 6.5 (1H) H-4. ¹³C-NMR (22.5 MHz, CDCl₃): ppm, 165 (C-1), 148 (C-2), 144 (C-5), 120 (C-3), and 112 (C-4).

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Fig. 3. UV-Spectrum of the Isolated Compound A. - - - , at pH 2.0; - - - - , at pH 8.0.

Fig. 4. Infrared Spectra of the Isolated Compound (A) and Authentic 2-Furoic Acid (B).
The $R_f$ values on TLC of the isolated compound were also identical with those of authentic sample in several solvent systems. From these results, we concluded that the isolated compound is 2-furoic acid. This indicates the presence of a 4,5-unsaturated sugar residue in compound A. Galactose was found in the acid hydrolyzate of compound A and the amount of galactose was equimolar to that of the 2-furoic acid produced. Compounds B and C gave equimolar amounts of galactitol and 2-furoic acid upon acid hydrolysis. These data indicate that the isolated compound is an unsaturated disaccharide having galactose as a reducing terminus. For further elucidation of the structure of the disaccharide, two-dimensional $^1$H-$^1$H-correlated NMR of the compound B was done at 400 MHz. In the spectrum, characteristic signals at 5.28, 4.35, and 5.80 ppm were observed (Fig. 5) and the characteristic signal of glucuronic acid residue at 4.13 ppm ($J=9.9$ Hz) observed in the substrate (Fig. 1) was not observed. These signals were assigned to H-1, H-3, and H-4 of the unsaturated sugar residue, respectively, from the COSY spectrum. These chemical shifts of the H-1 and H-4 signals of the unsaturated sugar residue agree

Fig. 5. Two-dimensional $^1$H-$^1$H-correlated NMR Spectrum (COSY) of Compound B.
well with those of the unsaturated sugar acid residue of the known oligosaccharide obtained from the acidic heteropolysaccharide of *Rhizobium trifolii* by digestion with bacteriophage-induced acidic heteropolysaccharide lyases.12) In the NOE difference spectrum, peak enhancement of the signal at 4.24 ppm was observed with irradiation at the H-1 signal of the unsaturated sugar residue (Fig. 6). This signal was assigned to H-2 or H-5 of galactitol from the COSY spectrum. To assign this signal, the ¹H-NMR spectrum of the compound C was measured (data were not shown). In the spectrum it was observed that the signal at 4.24 ppm was much simpler than that of the compound B. This indicates that this signal is coupled with C-1 protons of galactitol. Therefore, this signal was assigned to C-2 of galactitol. The low J value (3.9 Hz) of the H-1 signal of the unsaturated sugar residue indicates α-linkage. These data indicate that the unsaturated sugar is linked to C-2 of galactose through an α-linkage. The *threo* configuration was inferred from the fact that the coupling constant between H-3 and H-2 of the unsaturated sugar was 10.0 Hz. Since the C-2 and C-3 of the original glucuronic acid molecule was not involved in the elimination, the configuration of the resulting sugar is expected to be *threo*. From these results, it can be concluded that the isolated compound is 4-deoxy-L-threo-hex-4-enopyranouronosyl α (1→2)-D-galactose. This agrees well with the chemical structure expected from that of the original oligomer mixture.

*Evidence of the production of the unsaturated sugar linked to the polysaccharide*

The acidic polysaccharide (10 mg) was incubated with the enzyme preparation at 30°C for 12 hr and the reaction mixture was analyzed by gel filtration chromatography. As shown in Fig. 7, when the polysaccharide was incubated with the enzyme preparation, low molecular weight sugar (about 30% of the total sugar in the polysaccharide) was produced. This sugar portion was identified as mannose and β1→2mannobiose in a molar ratio of about 3:1 by the method described in our previous paper.1) No low molecular weight unsaturated sugar was observed in the reaction mixture. Therefore, the unsaturated sugar assumed to be linked to the polysaccharide. The enzyme-digested polysaccharide fractions were combined and concentrated to a small volume. About ten volumes of ethanol was added to the concentrate. The precipitate was dissolved

![Figure 6. NOE Difference Spectrum of Compound B.](image)

![Figure 7. Gel Filtration of the Reaction Products on Toyopearl HW-55.](image)
The acidic polysaccharide (10 mg) was incubated with the enzyme preparation (0.2 units as lyase) in a total volume of 1 ml of 0.1 M phosphate buffer (pH 7.0) for 12 hr at 30°C. After the reaction, the reaction mixture was dialyzed against deionized water for 3 days. The UV-spectrum of the dialyzed solution was measured and then the solution was hydrolyzed with 1 M acetic acid for 3 hr at 100°C in a sealed tube. The UV-spectrum of the hydrolyzate was measured. The hydrolyzate was dialyzed against deionized water for 2 days and the UV-spectrum of the dialyzate was measured. The concentration of each sample was about 25 μg/ml as original polysaccharide.

---the acidic polysaccharide; ---the enzyme-digested polysaccharide; ----unsaturated sugar-depleted polysaccharide; ---the acid hydrolyzate of the enzyme-digested polysaccharide; ----authentic 2-furoic acid.

in a small volume of deionized water and dialyzed against deionized water for 2 days.

The dialyzed solution was used for the subsequent experiments. The UV-spectrum of the enzyme-digested polysaccharide had an absorption maximum at 230 nm as shown in Fig. 8. The polysaccharide also gave positive thiobarbituric acid test reaction. As shown in Fig. 9(b), the two characteristic signals, 5.28 and 5.84 ppm, assigned to H-1 and H-4 of the unsaturated sugar, respectively, were observed and a decrease of C-1 protons of glucuronic acid (5.20 ppm), mannose (4.65 ppm) and mannobiose (4.74 and 4.86 ppm) was observed in the NMR spectrum of the enzyme-digested polysaccharide. The connection between the two signals (H-1 and H-4 of the unsaturated sugar) and the presence of the unsaturated linkage was confirmed by cleaving the unsaturated sugar by treatment with 1 M acetic acid for 3 hr at 100°C. The UV-spectral change due to the formation of 2-furoic acid was observed by this treatment (Fig. 8) and the amount of 2-furoic acid was almost equal to that expected from the amount of the unsaturated sugar in the polysaccharide. The thiobarbituric acid test reaction also became negative by this treatment. The acid-treated polysaccharide was chromatographed on Toyopearl HW-55. No low molecular weight sugars were observed on the chromatography. These data indicate that the unsaturated sugar residues in the enzyme-digested polysaccharide were selectively removed as 2-furoic acid by this treatment without release of any low
molecular weight sugars. The effluent containing sugar was dialyzed against deionized water for 2 days and then examined by UV and NMR spectroscopy. Neither the NMR signals assigned to H-4 and H-1 of the unsaturated sugar (Fig. 9(c)) nor the absorption maximum at 230 nm (Fig. 8) was present after this treatment. From these data we concluded that the unsaturated sugar residue was generated in the polysaccharide by the enzyme digestion. This suggests that some lyase(s) are present in the enzyme preparation.

4,5-Unsaturated sugar acids are generated by enzymatic β-eliminative cleavage of natural uronate sugar conjugates such as hyaluronic acid, alginic acid, chondroitin sulfate, dermatan sulfate, heparin, pectin, and pectic acid.7) Recently it was also demonstrated that oligosaccharides with 4,5-unsaturated sugar residues at the non-reducing terminus were produced from the acidic heteropolysaccharide of *Rhizobium trifolii* by digestion with bacteriophage-induced lyases.12) In this study it was found that an enzyme preparation derived from the culture filtrate of *Cellulomonas* sp. releases mannose and β1→2mannobiose from both the acidic polysaccharide and the acidic oligomers with generation of the unsaturated sugar acid. These results indicate that the release of mannose and β1→2mannobiose and the generation of the unsaturated sugar acid from the acidic oligomers and the polysaccharides are due to the function of lyase(s) in the enzyme preparation. It is still unknown that whether the release of mannose and β1→2mannobiose is by the function of a single enzyme or not. This enzyme β-eliminatively cleaves the β1→4 linkage between mannose and glucuronic acid in the polysaccharide and oligosaccharides. Therefore, this enzyme may be useful for the elucidation of mannose and mannobiose units linked to glucuronic acid through β1→4 linkages in the polysaccharide or oligosaccharides if the properties of the enzyme are characterized.

The details of the characteristics of the enzyme are under investigation.

References

4) T. Jikibara, S. Miki, T. Takegawa and S. Iwahara, Abstracts of Papers, the Annual Meeting of Kansai Division of Agricultural Society of Japan, Takamatsu, October, 1988, p. 36.