The Structure of the Carbohydrate Moiety of an Acidic Polysaccharide Produced by *Aureobasidium* sp. K-1

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Received August 28, 1981

An acidic polysaccharide was isolated from the culture broth of *Aureobasidium* sp. K-1. The polysaccharide ([α]$_D^{10}$ = -10°) was composed of α-glucose and sulfoacetic acid. The glucan moeity was hydrolyzed by exo-β-1,3-glucanase to give glucose and gentiobiose (molar ratio, ca. 1 : 3). The methylated glucan yielded on acid hydrolysis 2,3,4,6-tetra-O-methyl-, 2,4,6-tri-O-methyl- and 2,4-di-O-methyl-D-glucose (molar ratio, 2.6 : 1.0 : 2.7) together with small amounts of 2,3,6-tri-O-methyl-D-glucose. Smith degradation of the glucan moiety yielded glucose, glyceral and erythritol (molar ratio, 4:3.1:trace). From these results, it was suggested that the glucan consists of a backbone of β-1,3-linked glucose residues containing single branches of glucose residues joined by β-1,6-linkages, roughly three out of every four glucose residues in the backbone.

Microbial polysaccharides have attracted attention because of their unique properties. In attempts to obtain new microbial polysaccharides, we have found that *Aureobasidium* sp. K-1 produces a novel β-1,3-glucan containing a certain acidic component. Although it has already been reported that *Aureobasidium pullulans* produces various α- and β-glucans and an acidic polysaccharide, the polysaccharide produced by our strain differed from those produced by *Aureobasidium pullulans* in the structure of the glucan moiety and in the acidic component. The present paper deals with the structure and the acidic component of the acidic polysaccharide.

MATERIALS AND METHODS

Materials. β-Glucosidase and a Glucostat were purchased from Sigma Chemical Co. and Washington Biochemical Co., respectively. exo-β-1,3-Glucanase was prepared from a commercial enzyme preparation, "Kitalase," which is known as a yeast cell wall lytic enzyme preparation, available from a Kumiai Chemical Co.

Preparation and purification of the polysaccharide. *Aureobasidium* sp. K-1 stocked in our laboratory was cultured at 27°C for 96 hr in a medium containing 3% sucrose, 0.2% NaNO$_3$, 0.1% K$_2$HPO$_4$, 0.05% KCl, 0.5% MgSO$_4$·7H$_2$O, 0.001% FeSO$_4$·7H$_2$O (Czapek medium) and 0.3% vitamin C. After cultivation, cells were removed by centrifugation and a crude polysaccharide solution was obtained. The amount of polysaccharide produced was about 4 mg per ml of broth. The polysaccharide was precipitated by addition of an equal volume of ethanol. The precipitate was dissolved in water and the pH of the solution was adjusted to pH 9 with 1 N NaOH. The polysaccharide was again precipitated by addition of an equal volume of 2% CaCl$_2$. The precipitate was washed with 0.1 N HCl and then dissolved in 1 N NaOH. After removal of insoluble materials by centrifugation, the solution was dialyzed against water. The polysaccharide was precipitated by addition of three volumes of ethanol. The fibrous precipitate thus obtained was dehydrated with acetone and dried over P$_2$O$_5$ in vacuo.

Removal of the acidic component from the polysaccharide. The polysaccharide preparation was dissolved in 0.1 N NaOH and heated at 70°C for 2.5 hr to remove the acidic component. After cooling, the polysaccharide was precipitated by addition of three volumes of ethanol. The precipitate was dissolved in 1 N NaOH and dialyzed against water. The dialyzed solution was subjected to ultracentrifugal analysis to determine the homogeneity. The precipitate was then used for structural analysis.

Analytical methods. Paper chromatography was carried out on Toyo filter paper No. 50, using the following solvents; (a) 1-butanol–pyridine–water (6:4:3 by volume), (b) ethyl acetate–acetic acid–water (3:1:1 by volume) and (c) ether–formic acid–water (5:2:1 by volume). Reducing sugars were detected with alkaline silver nitrate and sugar alcohol with sodium periodate-potassium permanganate.

Gas liquid chromatography of methylated sugars was performed with a Shimadzu gas chromatograph Model GC-4B fitted with a flame ionization detector using a glass column packed with 3% ECNSS-M at 180°C. Methylated sugar components were identified by comparison of the \( T \) values of the corresponding alditol acetates with those of authentic O-methyl sugars, and by their mass spectra.\(^7\) Mass spectra were recorded with a combined GLC-mass spectrometer (Japan Electron Optics Laboratory JMS-07 using a column packed with 1.5% silicon OV-1).

Total carbohydrate and reducing sugar were determined with phenol sulfuric acid\(^8\) and by Somogyi-Nelson's method,\(^9\) respectively. Glucose was assayed with a Glucostat. Determination of sugar alcohols was carried out with chromatropic acid by the procedure of Hanahan and Olley.\(^10\)

Periodate consumption was determined by the usual arsenite method,\(^11\) and formic acid liberation by titration with 0.01 N NaOH.

Sulfoacetic acid was assayed with a Shimadzu liquid chromatograph LC-3A equipped with a UV detector and a column (4 mm × 25 cm) containing Zorbax ODS, using 0.04 N \( \text{KH}_2\text{PO}_4 \) (1 ml/min) as the mobile phase, operated at 50 kg/cm² and 40°C. Detection of sulfoacetic acid was carried out with the absorbancy at 210 nm.

Sulfur was analyzed with sodium salt of the acidic component with an SLFA-200/calculator (Horiba) using fluorescent X-rays and the proportion was converted to that in the free state.

Methylation analysis. The polysaccharide (10 mg) was dissolved in dimethylsulfoxide (0.5 ml) under a nitrogen atmosphere and then methylated by Hakomori's method\(^12\) using methyl iodide (0.5 ml) and methylsulfinyl carbamion (0.5 ml) prepared according to the procedure of Sandford and Conrad.\(^13\) After the reaction was completed, the reaction mixture was dialyzed against water and the methylated product was concentrated and extracted with a small amount of chloroform. Completion of the methylation was judged from the infrared spectrum (OH bond at 3400-3600 cm\(^{-1}\)) of the chloroform extract. The fully methylated polysaccharide was hydrolyzed with concentrated HCOOH (0.2 ml) at 100°C for 5 hr. After removal of formic acid by evaporation, the hydrolysis product was further treated with 0.5 N \( \text{H}_2\text{SO}_4 \) (0.2 ml) at 100°C for 2 hr. The hydrolyzate was treated with Amberlite IR-45 (OH\(^-\)) to remove \( \text{H}_2\text{SO}_4 \) and the mixture of methylated sugars was converted into the corresponding alditol acetates by reduction with sodium borohydride. The methylated sugars thus prepared were analyzed by gas liquid chromatography.

Smith degradation. The polysaccharide (160 mg) was oxidized with 0.05 M sodium metaperiodate (100 ml) at 5°C for 14 days and then the oxidized polysaccharide was reduced with sodium borohydride. The resulting polyalcohol was hydrolyzed with 0.5 N \( \text{H}_2\text{SO}_4 \) at 100°C for 6 hr. The hydrolyzate was neutralized with \( \text{BaCO}_3 \), filtered to remove the precipitate and passed through a column of Amberlite 1R-120 (H\(^+\)). After the solution was evaporated to dryness, methanol was added to the residue, followed by evaporation to remove boric acid and then the residue was dissolved in 1 ml of water. The reducing sugars and sugar alcohols in the hydrolyzate were separated by paper chromatography using solvent (a). Each component was extracted with water and determined by the method of Hanahan and Olley.

RESULTS AND DISCUSSION

1. Properties of the polysaccharide

The polysaccharide was not sufficiently soluble in water but readily soluble in 1 N NaOH giving a highly viscous solution.

The purified polysaccharide was free from nitrogen. The homogeneity of the polysaccharide was examined by ultracentrifugal analysis, which showed \( S_{20} \) (0.4% in 0.5 N NaOH) \( 16.4 \times 10^{-13} \) (Fig. 1). The polysaccharide was found to be composed of glucose and a certain acidic component as revealed by paper chromatography of the hydrolyzate using solvent (a). The specific rotation, \( [\alpha]_D^{20} = -10^\circ \) (0.4% in 0.5 N NaOH), and the characteristic absorption at 890 cm\(^{-1}\) in the infrared spectrum indicated \( \beta\)-D-glucosidic linkages.

![Fig. 1. Ultracentrifugal Pattern of Acidic Polysaccharide Produced by Aureobasidium sp. K-1.](image)
2. Hydrolysis of the polysaccharide by exo-β-1,3-glucanase

In order to determine the structure of the polysaccharide moiety, an aqueous solution of the polysaccharide which was free from the acidic component was prepared by dissolving the polysaccharide in 0.5 M NaOH and removing NaOH from the solution by dialysis against water. The polysaccharide solution thus prepared was treated with an exo-β-1,3-glucanase purified from “Kitalase” (a yeast cell wall lytic enzyme preparation available from Kumiai Chemical Co.). The enzyme used had an activity of 0.1 units per ml which liberated 0.1 μmol of glucose in 1 min from laminarin. After incubation at 40°C for 48 hr, reducing sugars in the reaction mixture were determined by Somogyi-Nelson’s method. The hydrolysis degree was about 24% as glucose. The residual polysaccharide which became insoluble to the enzyme was precipitated by addition of two volumes of ethanol and then dissolved in 0.5 M NaOH. The polysaccharide solution was dialyzed against water to remove NaOH and again treated with the exo-β-1,3-glucanase at 40°C for 24 hr. The polysaccharide was subjected to further enzymic hydrolysis and the final hydrolysis degree reached about 33%. This low efficiency of the enzyme appears to be due to turbidity of the polysaccharide solution as seen in retrogradation of amylose. The products of hydrolysis were examined by paper chromatography using solvent (a). As shown in Fig. 2, glucose and an oligosaccharide were found. The oligosaccharide, which corresponded to gentiobiose on a paper chromatogram, was found to be hydrolyzed by β-glucosidase to give only glucose (Fig. 3). Methylated sugars obtained from the methylated oligosaccharide were 2,3,4,6-tetra-O-methyl glucose (1.1 mol) and 2,3,4-tri-O-methyl glucose (1 mol). The proportions of glucose and gentiobiose produced by the exo-β-1,3-glucanase were determined.


### Table I. Ratio of Gentiobiose to Glucose in the Hydrolyzate of the Polysaccharide during exo-β-1,3-Glucanase Digestion

<table>
<thead>
<tr>
<th>Reaction (min)</th>
<th>Hydrolysis (%)</th>
<th>Gen/Glu (molar ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>4.2</td>
<td>2.3</td>
</tr>
<tr>
<td>30</td>
<td>7.3</td>
<td>3.5</td>
</tr>
<tr>
<td>90</td>
<td>10.6</td>
<td>4.4</td>
</tr>
<tr>
<td>24 (hr)</td>
<td>15.9</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* Gentiobiose/glucose.

from the ratio of glucose and total reducing sugars by the glucose oxidase method (Glucostat) and Somogyi-Nelson's method, respectively. As shown in Table I, the ratio of glucose and gentiobiose in the hydrolyzate was about 1:3. This exo-β-1,3-glucanase can cleave β-1,3-linkages in β-1,3-glucan containing β-1,6-linked branches successively without cessation of the reaction at β-1,6-linked branches. That is, the enzyme acts of scleroglucan (β-1,3-glucan containing single side branches through β-1,6-linkages) to produce glucose and gentiobiose. Therefore, it appeared from the previous results that this polysaccharide consists of a backbone of β-1,3-linked glucose residues with β-linked single glucose units attached, roughly three out of four glucose residues at the O-6 positions in the backbone.

### 3. Position of glucosidic linkages

#### (1) Methylation. The polysaccharide from which the acidic component was removed by alkali-treatment was permethylated by Hakomori's method. The methylated polysaccharide which showed a slight hydroxy absorption in the IR spectrum was hydrolyzed with formic acid and then with sulfuric acid, and the methylated sugars thus formed were analyzed by gas liquid chromatography. The results showed the presence of 2,3,4,6-tetra-O-methyl-(2.6 mol), 2,4,6-tri-O-methyl-(1.0 mol) and 2,4-di-O-methyl-D-glucose (2.7 mol), together with a small amount of 2,3,6-tri-O-methyl-D-glucose (0.1 mol). The insoluble polysaccharide residue after degradation by exo-β-1,3-glucanase was also methylated. Hydrolysis of the methylated polysaccharide yielded 2,3,4,6-tetra-O-methyl-, 2,4,6-tri-O-methyl-, and 2,3,6-tri-O-methyl-D-glucose in the molar ratio of 3.1, 1.0, 2.9 and 0.1, respectively. These results strongly support the structural feature deduced from the enzymic hydrolysis of the native polysaccharide.

#### (2) Smith degradation. The polysaccharide was oxidized with 0.05 M sodium metaperiodate at 5°C as described in methods. The oxidized polysaccharide was reduced with sodium borohydride and the resulting glucan polyalcohol was hydrolyzed with 0.5 N H₂SO₄ at 100°C for 6 hr. The products of hydrolysis were found to be glucose, glycerol and erythritol by means of paper chromatography with solvent (a). The molar ratio of these compounds was 4:3.1:trace. It was assumed that glucose and glycerol arose from 1,3-linked D-glucose units and non-reducing glucose residues, respectively, and the trace erythritol from 1,4-linked D-glucose units. On periodate oxidation of the polysaccharide, the observed consumption of periodate and liberation of formic acid were 0.86 mol/162 g and 0.45 mol/162 g, respectively. The results obtained from enzymic hydrolysis, methylation, Smith degradation and periodate oxidation suggest that the glucan has the structure shown in Fig. 4. The location of a very small proportion of 1,4-linkages in the polysaccharide awaits further investigation. It is known that a similar β-1,3-glucan is produced by Aureobasidium pullulans, but the proportion of the β-1,6-branches in the glucan is less than that in the present polysaccharide. A similar β-1,3-glucan has been also isolated from "Kikurage" by Misaki and his coworkers. The polysaccharide from "Kikurage," however, is insoluble in NaOH, whereas the polysaccharide produced by Aureobasidium sp. K-1 is soluble in it.

### 4. Identification of the acidic component in the polysaccharide

The acidic component liberated from the
Acidic Polysaccharide from *Aureobasidium* sp. K-1

![Possible Structure of the Acidic Glucan Produced by *Aureobasidium* sp. K-1.](image)

Glc, glucose; A, sulfoacetic acid.

![Paper Chromatograms of the Acidic Compound Released from the Polysaccharide by NaOH-Treatment.](image)

S, sulfoacetic acid; A, acidic compound in the polysaccharide.

Solvents: (1) 1-butanol-pyridine-water (6:4:3); (2) ether-formic acid-water (5:2:1); (3) ethyl acetate-acetic acid-water (3:1:1).

In paper chromatography (1), acids were used in the free state and in paper chromatographies (2) and (3), as the sodium salt. Bromophenol blue was used as a detecting reagent.

polysaccharide by NaOH-treatment, was purified according to the following method. After removal of NaOH with Dowex 50W-X8, the acidic compound was adsorbed on DEAE-cellulose and eluted with 4 N formic acid. The eluate was concentrated to dryness and the residue was dissolved in CH$_3$OH, and applied to a column of Sephadex LH-20 equilibrated with CH$_3$OH, the acidic fraction being collected. The acidic compound thus obtained was subjected to infrared analysis. The infrared spectrum showed the characteristic absorption for a CO-group at 1720 cm$^{-1}$ and a SO$_2$-group at 1200 cm$^{-1}$, suggesting that the acidic compound has a carboxyl group and a sulfonyle group. On paper chromatograms, the $R_f$ value of the acidic compound corresponded to that of authentic sulfoacetic acid, as shown in Fig. 5. It appears that this acidic compound is sulfoacetic acid. This may be supported by the results of ultraviolet absorption and elemental analysis. The ultraviolet absorption curve of the acidic compound was identical to that of authentic sulfoacetic acid.

![Ultraviolet Absorption Curve of the Acidic Compound in the Polysaccharide.](image)

S, sulfoacetic acid; A, acidic compound in the polysaccharide.
to that of authentic sulfoacetic acid as shown in Fig. 6. The results of elemental analysis were in fairly good agreement with the calculated values for sulfoacetic acid.

Found: C, 15.7%; H, 2.6%; S, 21.1%
Calcd. for C₂H₄O₅S: C, 17.1%; H, 2.9%; S, 22.9%

That the analysis values for the acidic compound were relatively low compared with those of sulfoacetic acid might be due to some impurities in the sample of the acidic compound.

5. Binding position of the acidic component in the polysaccharide

In order to determine the binding position of the acidic component to the polysaccharide, isolation of the sugar containing acidic component was attempted. The acidic polysaccharide was hydrolyzed with exo-β-1,3-glucanase and then the polysaccharide residue in the digest was precipitated by addition of an equal volume of ethanol. The supernatant was concentrated under reduced pressure and subjected to paper chromatography using solvent (a). A spot of a sugar containing acidic component was detected by staining for sugar and acid. This compound, extracted from filter paper with water, had a degree of polymerization of 2, as estimated by Timell's method.¹⁶ Therefore, the acidic sugar moiety appeared to be gentiobiose binding sulfoacetic acid. From the structure of the polysaccharide moiety and specificity of the enzyme, the sulfoacetic acid residues are probably linked to the glucose residues of side chains in the polysaccharide. The precise binding position of sulfoacetic acid is under investigation.

REFERENCES

13) A. Sandford and H. E. Conrad, Biochemistry, 5, 1508 (1966).