Polysaccharides in Fungi. XXXVIII. 1) Anti-diabetic Activity and Structural Feature of a Galactomannan Elaborated by Pestalotiopsis Species

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A fungus of Pestalotiopsis species produced an extracellular, water-soluble polysaccharide (PS-N). PS-N exhibited significant hypoglycemic activity in streptozotocin-induced diabetic mice following intraperitoneal administration and had an effect on oral glucose tolerance in normal mice following oral administration. PS-N (β₁₆, +34.5° in water) was homogeneous on gel chromatography, it is composed of galactose and mannose in a molar ratio of 1:9, and its molecular weight was estimated by gel chromatography to be about 24000. Its structure was investigated by a combination of chemical and spectroscopic methods. The results indicated that PS-N, a highly branched galactomannan, is composed of β-(1→3)-linked D-galactofuranosyl and non-reducing terminal β-D-galactofuranosyl residues, in addition to α-D-mannopyranosyl residues of a yeast mannann type.

Key words: Pestalotiopsis; polysaccharide structure; galactomannan; hypoglycemic activity; anti-diabetes

Our previous studies in this series1) revealed that hypoglycemic polysaccharides have branched structures such as the galactomannan (CS-F30) isolated from the cultured mycelium of Cordyceps sinensis,2) (1→6)-branched (1→3)-β-D-glucan (AG-HN1) from the fruiting bodies of Agrocybe cylindracea3) and glucuronoxylomannans (AC and TAP) from the fruiting bodies of Tremella fuciformis4) and T. aurantia.5)

In the course of studies of hypoglycemic polysaccharides, we attempted to obtain new fungal bioactive polysaccharides. Misaki et al.6) reported the structure of the water-insoluble (1→6)-branched (1→3)-β-D-glucan isolated from the medium of Pestalotia sp. 815 and the antitumor activity of the glucan and its polyol derivative, as well as the presence of a water-soluble glucan containing a small proportion of mannose. However, no other reports of an extracellular, water-soluble polysaccharide elaborated by a fungus of Pestalotia or Pestalotiopsis (synonym) sp. have been published. The present study concerned isolation of a water-soluble polysaccharide (PS-N) from the culture broth of a fungus of Pestalotia sp. KGM96004, 2) the hypoglycemic activity of streptozotocin (STZ)-induced diabetic mice following intraperitoneal i.p. administration and an oral glucose tolerance test, 3) the structural features of PS-N.

MATERIALS AND METHODS

Preparation of Polysaccharides The fungus used in the experiment was isolated from soil in a forest area, Iwate prefecture, Japan. It was identified as Pestalotia sp. or Pestalotia sp. and is kept as Pestalotiopsis sp. KGM96004 in the Research Institute of Kagome Co., Ltd. The fungus was cultivated in a shaker flask in a medium (1 l) containing 50 g glucose, 2.5 g peptone, 2.5 g yeast extract, 1.0 g KH₂PO₄, 0.5 g MgSO₄, and 0.5 g CaCl₂ at 28°C. After 4 d of cultivation, the culture filtrate was heated for 30 min in a boiling water bath, evaporated, and a crude polysaccharide fraction was precipitated by addition of ethanol (yield, 0.4 g/1 medium). The crude polysaccharide fraction was dispersed in water with stirring for 24 h, and the suspension centrifuged to give a water-soluble, crude polysaccharide (PS) as the supernatant. PS was applied to a column of DEAE-Toyopearl 650M (phosphate form) as described previously.2) The column was successively eluted with a 0.1 M phosphate buffer and 0.1 M NaCl, and each eluate was dialyzed; then the soluble fraction of the non-dialyzable fraction was lyophilized to give a neutral polysaccharide fraction (PS-N) and a acidic polysaccharide fraction (PS-A), respectively.

Hypoglycemic Activity Male ddY mice (5 weeks old, Japan SLC) were induced injecting STZ and used as a diabetes model when their plasma glucose was over 400 mg/dl as described previously.9) After i.p. administration of a saline solution of the sample (control: physiological saline), the plasma glucose was measured using a glucose B-test kit (Wako Pure Chemical Ind.) based on the glucose oxidase method.

Oral Glucose Tolerance Test Male ddY mice were fasted for 12 h, and then given oral glucose (1 g/kg) dissolved in water (20 ml/kg) or a mixture of glucose and sample ([1 g + 0.5 g]/kg), control and test group respectively. Plasma glucose was measured at 0, 0.5, 1, 2, and 3 h by the method described above.

Statistical Analysis The data are expressed as means ± S.E. The significance of any difference between the means was evaluated using Student’s t-test.

Gel Chromatography Gel chromatography was performed on a column of Toyopearl HW-55F (Tosoh) in 0.1 M NaCl and the molecular weight was estimated from a calibration curve constructed using standard dextrans, as described previously.7)

Analysis of Component Sugars The sample was hydrolyzed with 2 M trifluoroacetic acid for 8 h at 100°C and the hydrolysate was analyzed by paper chromatography.

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graphy (PPC) and gas chromatography (GC) as alditol acetate derivatives, as described previously.\textsuperscript{5)}

**Methylation Analysis** The polysaccharide was methylated 3 times by the Hakomori method, as previously described.\textsuperscript{7)} The fully methylated product was heated successively with 90\% formic acid for 8 h at 100\(^\circ\)C and 2 M trifluoroacetic acid for 8 h at 100\(^\circ\)C. The partially methylated sugars were converted into alditol acetate derivatives. The resulting partially methylated alditol acetates were analyzed by GC and GC-MS spectrometry. GC was performed on a Shimadzu GC-4CMT gas chromatograph equipped with hydrogen flame-ionization detector, using a glass column (3.0 cm x 2 mm) packed with 3\% ECNSS-M on Gaschrom Q (100—120 mesh) at 170\(^\circ\)C at a flow rate of 60 ml/min (nitrogen) and also on a Shimadzu GC-15A instrument with a fused-silica capillary column (0.25 cm x 30 m, film thickness 0.25 \(\mu\)m) of DB 225 (J&W Scientific) at 180\(^\circ\)C at a helium flow rate of 43 ml/min (splitter vent) with a splitting ratio of 1:53. Retention times and peak areas were measured by a Shimadzu C-R5A Chromatopac. GC-MS was conducted using a Hewlett-Packard Model 5890II combined GC-MS spectrometry system equipped with a DB 225 capillary column.

**Smith Degradation** The polysaccharide (20 mg) was stirred with 25 mm sodium periodate (40 ml) at 4\(^\circ\)C in the dark, and oxidation was complete after 7 d. The reaction mixture was successively treated with ethylene glycol (1 ml) and NaBH\(_4\) (50 mg) for 24 h, then adjusted to pH 5.0 by addition of acetic acid. The solution was dialyzed against water, and the polyalcohol was hydrolyzed with 2 M sulfuric acid for 6 h at 100\(^\circ\)C. The hydrolysate was neutralized with barium carbonate, then analyzed as alditol acetate derivatives by GC with a 3\% ECNSS-M column using a programmed rise in temperature of 4\(^\circ\)C/min from 80\(^\circ\)C to 180\(^\circ\)C.

**Carbon-13 Nuclear Magnetic Resonance (\textsuperscript{13}C-NMR)** The spectrum was recorded on a JEOL FT-NMR EX-400 spectrometer in dimethyl sulfoxide (DMSO)-d\(_6\) (50 mg/0.5 ml) at room temperature.

## RESULTS AND DISCUSSION

A water-soluble, PS was obtained in 22.5\% yield from an extracellular crude polysaccharide fraction (yield, 0.4 g per liter) of the culture broth of *Pestalotiopsis* sp. KGM96004, and further purified by anion-exchange chromatography to give a neutral polysaccharide (PS-N) from the neutral fraction and a polysaccharide (PS-A) from the acidic fraction, in 6.1\% and 3.1\% yield, respectively. We observed the hypoglycemic activity exhibited by PS, PS-N and PS-A in STZ-induced diabetic mice following i.p. administration at a dose of 50 mg/kg (20—100 mg/kg for PS-N), and evaluated the relative plasma glucose with respect to that of saline administration. PS-N lowered the glucose level of the diabetic mice by about 58\% at 6 h, while PS and PS-A had little effect (Table 1). PS-N showed a significant activity until 48 h after administration of a dose of 100 mg/kg and the plasma glucose at 72 h returned to the level at 0 h, while the activity at 3—6 h, after a dose of 100 mg/kg, was weaker than that after a dose of 50 mg/kg. Thus, the administration of a higher dose of PS-N resulted in a longer duration of activity, but the reason for the weaker effect at earlier times (3—6 h) after administration remains obscure. Next, PS-N was subjected to an oral glucose tolerance test in normal mice. A solution of PS-N and glucose (0.5 g + 1 g/l) significantly suppressed the rise in plasma glucose caused by glucose (control, 1 g/l) at 30 min as shown in Fig. 1. The potency of PS-N in the oral glucose tolerance test was lower than the hypoglycemic activity produced by i.p. administration, and this could be due to a difference in the mechanism because a polymer like PS-N should not be absorbed from the intestine.

The anti-diabetic polysaccharide (PS-N) has the appearance of colorless flakes and [\(\alpha\)]\(_D\) = +34.5\(^\circ\) (c=0.4,

![Graph](image)

Table 1. Effect of Water-Soluble Polysaccharide Fractions on Plasma Glucose in STZ-Induced Diabetic Mice by Following Administration

<table>
<thead>
<tr>
<th>Group (n=5)</th>
<th>Dose (mg/kg)</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>24</th>
<th>48 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>101.3 ± 4.2</td>
<td>106.5 ± 6.8</td>
<td>106.7 ± 5.0</td>
<td>104.6 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>50</td>
<td>84.6 ± 9.9</td>
<td>88.7 ± 7.8</td>
<td>90.1 ± 5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS-A</td>
<td>50</td>
<td>85.1 ± 2.9*</td>
<td>90.8 ± 8.8</td>
<td>94.5 ± 2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS-N</td>
<td>20</td>
<td>74.7 ± 9.0*</td>
<td>68.3 ± 12.3*</td>
<td>90.1 ± 4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS-N</td>
<td>50</td>
<td>74.9 ± 1.4***</td>
<td>41.8 ± 5.6***</td>
<td>92.8 ± 6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS-N</td>
<td>100</td>
<td>86.3 ± 3.3*</td>
<td>62.1 ± 10.6**</td>
<td>42.9 ± 9.0**</td>
<td>75.4 ± 4.0**</td>
<td></td>
</tr>
</tbody>
</table>

Plasma glucose at 0 h (500—750 mg/dl) was adjusted to 100. Significantly different from control: *p < 0.05, **p < 0.01, ***p < 0.001.
Table 2. GC and GC-MS of Alditol Acetates Derived from the Methylated Product of PS-N

<table>
<thead>
<tr>
<th>Methylated sugar (as alditol acetates)</th>
<th>Column A</th>
<th>Column B</th>
<th>Primary mass fragments (m/z)</th>
<th>Molar percentage</th>
<th>Mode of linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Me2-Man</td>
<td>1.00</td>
<td>1.00</td>
<td>45, 117, 161, 205</td>
<td>27</td>
<td>[Manp] 1→</td>
</tr>
<tr>
<td>2,3,5,6-Me2-Gal</td>
<td>1.08</td>
<td>1.12</td>
<td>45, 89, 117, 205</td>
<td>5</td>
<td>[Galp] 1→</td>
</tr>
<tr>
<td>3,4,6-Me2-Man</td>
<td>1.77</td>
<td>1.93</td>
<td>45, 161, 189</td>
<td>16</td>
<td>-2 [Manp] 1→</td>
</tr>
<tr>
<td>2,4,6-Me2-Man</td>
<td>1.82</td>
<td>2.12</td>
<td>45, 117, 161, 233</td>
<td>4</td>
<td>-2 [Manp] 1→</td>
</tr>
<tr>
<td>2,3,4-Me2-Man</td>
<td>2.08</td>
<td>2.40</td>
<td>117, 161, 189, 233</td>
<td>16</td>
<td>-6 [Manp] 1→</td>
</tr>
<tr>
<td>2,3,5-Me2-Gal</td>
<td>2.55</td>
<td>3.38</td>
<td>117, 161, 233</td>
<td>3</td>
<td>-6 [Galp] 1→</td>
</tr>
<tr>
<td>4,6-Me2-Man</td>
<td>2.73</td>
<td>3.38</td>
<td>45, 161, 261</td>
<td>2</td>
<td>-2,3 [Manp] 1→</td>
</tr>
<tr>
<td>3,4-Me2-Man</td>
<td>4.08</td>
<td>5.34</td>
<td>189</td>
<td>29</td>
<td>-2,6 [Manp] 1→</td>
</tr>
</tbody>
</table>

a) Relative retention time with respect to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol. Column A: DB 225 (capillary column), column B: 3% ECNSS-M.

Fig. 2. Chromatogram of PS-N on Toyopearl HW-55F
PS-N dissolved in 0.1 M NaCl was applied to a Toyopearl HW-55F column (1.5 x 90 cm), and eluted with 0.1 M NaCl. Each eluted fraction (3 ml) was detected by the phenol-H2SO4 method.

water). It exhibited a symmetrical carbohydrate pattern on Toyopearl HW-55 as shown in Fig. 2 and was completely adsorbed on a column of Con A-Sepharose 4B. The molecular weight of PS-N was estimated by gel chromatography to be about 24000 and PS-N, following PPC and GC of the hydrolysate, was found to be composed of galactose and mannose in a molar ratio of 1:9, but no nitrogen was detected by elementary analysis.

PS-N was fully methylated, and the methylated product was hydrolyzed with acid; this converted the sugars into partially methylated alditol acetates. The results of GC and GC-MS are shown in Table 2. The identification of 2,3,5,6-tetra-O-methyl-D-galactose and 2,3,5-tri-O-methyl galactose indicates that galactose residues are present in the furanose form. The identification of 4,6-di- and 3,4-di-O-methyl mannose derivatives shows the presence of branched mannopyranosyl residues. The results also indicate that PS-N is a highly branched galactomannan. Following sequential borohydride reduction of periodate-oxidized polysaccharide and acid-hydrolysis, PS-N yielded glycerol, threitol, and mannose. Threitol and mannose supported the presence of (1→6)-linked galactofuranosyl residues and (1→3)-linked and (1→2,3)-linked mannopyranosyl residues were suggested by the methylation analysis.

The anomeric configurations were assigned by comparing the 13C-NMR spectrum of PS-N in DMSO-d6 (Fig. 3) with data in the literature.9,10 The lower field signals, at 108.2, 108.0, 106.9 and 106.6 ppm were assigned to the C-1 of the β-D-galactofuranosyl residues. The resonances at 102.3, 102.0 and 101.6 ppm could be attributed to the non-reducing terminal, (1→3)-linked, and (1→2,3)-linked α-D-mannopyranosyl residues, respectively. The signals at 100.7 and 98.1 ppm correspond to the C-1 of (1→6)-linked and (1→2,6)-linked α-D-mannopyranosyl residues.

The foregoing results indicate that the minimal unit of the new water-soluble galactomannan, PS-N, obtained from the culture broth of a fungus of Pestalotiopsis sp. is composed of eight kinds of sugar residues, as shown in Chart 1.

It has been reported that yeast mannans10,11 are composed of (1→6)-linked α-D-mannopyranosyl main chains, some of the residues being substituted at O-2 with (1→2)-linked α-D-mannopyranosyl residues, and contain non-reducing terminal residues with an α-D-(1→3)-linkage. The structural features of the mannose residues in PS-N produced by a fungus Pestalotiopsis sp. resemble that of yeast mannan without galactofuranosyl residues. PS-N contains the non-reducing terminal and (1→6)-linked
\( \beta \)-D-galactofuranosyl residues in addition to the yeast mannan-like mannopyranosyl residues. Since yeast mannan (Nacalai Tesque, Inc.) did not exhibit hypoglycemic activity,\(^{12}\) the galactofuranosyl residues in PS-N would contribute to the hypoglycemic activity. The mechanism for the hypoglycemic activity of polysaccharides is not yet known, and we need further investigation, e.g., studies of the effects on insulin receptors, glucose transporter, and a mediator for the induction.

REFERENCES


