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Characterization of Two Glucans Activating an Alternative Complement Pathway from the Fruiting Bodies of Mushroom *Pleurotus ostreatus*

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Abstract Two glucans (PONGa and PONGb) differing in their anomeric and glycosidic linkage structures were isolated from the water-insoluble materials (PON) of *Pleurotus ostreatus* basidiocarps, which activated the complement system and were almost soley composed of D-glucose. The isolation was achieved by repeated precipitations with ethanol and adsorption on concanavalin A (Con A) of PON suspension in thymol/NaCl. Based on methylation analysis, IR, GLC-MS, ^{1}H, and ^{13}C-NMR spectroscopies, PONGa was found to be a branched α-glucan composed of α-1,3-linked D-glucopyranose residues and α-1,3-linked units with 6-branching points, whereas PONGb was a linear β-glucan composed mainly of β-1,3-linked D-glucopyranose residues. The PONGb particles reacted more potently than the PONGa particles as C3 activator in alternative complement hemolysis and crossed-immunoelectrophoresis using anti-human C3, thereby suggesting that the complement activating components of PON were β-(13)-glucans rather than α-glucan components.

Key words: β-glucans, alternative complement pathway, *Pleurotus ostreatus*

It has been reported that polysaccharides isolated from certain higher fungi, for example, lentinan from *Lentinus edodes* and PS-K from *Coriolus versicolor*, have powerful anti-tumor activities against sarcoma-180 and other tumors [4]. Although the mechanism of the anti-tumor activity of these polysaccharides is not well understood, it has been suggested that the activity is due to host-mediated immunity rather than a direct cytotoxic effect on the tumor cells [9]. Egwang and Befus [3] described the importance of an activated complement component in the macrophage activation which releases lysosomal enzymes to inhibit the growth of tumor cells. In particular, the anti-tumor β-glucans, such as lentinan and griforan from *Grifora frondosa*, are known to activate an alternative complement pathway. Recently, potent anti-complementary activity was found in the polysaccharides fractions obtained from an alkaline extraction of fruit bodies of an edible mushroom *Pleurotus ostreatus*. Previously, the authors have purified and characterized two water-soluble hetero-polysaccharides (PO-IIIa-1 and PO-IIIa-2) which activated both classical and alternative complement pathways [7]. Furthermore, it was found that the water-insoluble material (fraction PON) remained inside the dialysis bag during the purification of the PO-IIIa-1 and PO-IIIa-2. It also activated the complement system and consisted of only D-glucose in addition to a few *O*-acetyl groups. Accordingly, in this study, two classes of water-insoluble glucans (PONGa and PONGb) were isolated from the fraction PON, and structural features and alternative anti-complementary activities of these two glucans are deseribed.

A suspension of the fraction PON in thymol (10 mg/ml) solution containing 20% NaCl was vigorously stirred overnight at room temperature and then centrifugated at 12,000 ×g for 60 min. The supernatant was dialyzed and precipitated with ethanol. The precipitates were then washed with ethanal and lyophilized to remove any ethanol. A Con A solution (25 mg/ml in 4 M NaCl) was then added to the lyophilized precipitates and the suspension dissolved in 0.018 M phosphate buffer (pH 6.8) containing 1 M NaCl. The component adsorbed on Con A was extracted with 10% trichloroacetic acid (TCA), precipitated with an excess ethanol after neutralization of the solution, and dissolved in 5% aqueous dimethylsulfoxide (DMSO). Dialysis and lyophilization of the solution yielded the PONGa glucan (18% yield based on PON). The other component of the Con A mixture was recovered from the supernatant without further precipitation. This supernatant was deproteinized by heating at 100°C followed by centrifugation. Thereafter, the supernatant obtained was precipitated with ethanol. After repeating the precipitation with ethanol and redeproteinization with trichloroacetic

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acid, the water-insoluble glucan PONGb (53% yield based on PON) was obtained.

The anti-complementary activity was measured by the complement fixation test, based on the complement consumption and degree of red blood cell lysis by the residual complement protein [13]. Briefly, each sample was diluted in 5% aqueous dimethylsulfoxide (DMSO) at various ratios and incubated with normal human serum (NHS) and gelatin-veronal-buffered saline (pH 7.4) containing 500 μM Mg^{2+} and 150 μM Ca^{2+} (GVB^{2+}). The residual total hemolytic complement (TCH_{50}) was determined using immunoglobulin M-sensitized sheep erythrocytes (Nippon Biotest Lab., Tokyo, Japan). As a control, NHS was incubated with 5% aqueous DMSO and GVB^{2+}. The activity of each sample was expressed as % of $ITCH_{50}$, a percentage of 50% inhibition against the total complement hemolysis of the control. For an assay of the alternative anti-complementary activity, the samples were dissolved in GVB^{2+} containing 10 mM ethylenediamine tetraacetate (EDTA-GVB^{2-}) or GVB^{2-} containing 10 mM ethylene glycol bis-amino tetraacetic acid and 2 mM $MgCl_2$ (Mg^{2+}-EGTA-GVB^{2-}). The alternative activation of the C3 component was examined using the following crossed immuno-electrophoresis (IEP) method. The purified glucan was incubated with NHS in Mg^{2+}-EGTA-GVB^{2-} buffer at 37°C for 30 min. The reacted sample was subjected to electrophoresis on 1% agarose gel plates (4.5×4.5 cm, 1.5 mm) using 25 mM barbital buffer (pH 8.6) at 5 mA/cm and 4°C for 90 min. After cutting along the upper part of the agarose gel, a second electrophoresis was conducted with the remaining agarose gel containing 0.5% goat anti-human C3 (Sigma Co., St. Louis, U.S.A.) at 1 mA/cm at 4°C for 12 h. The C3 precipitin line was visualized by staining the gels with Ponceau 3R.

The *O*-acetyl content was measured by the method described by Tomoda *et al.* [16]. Exhaustive methylation of the polysaccharide was performed with methylsulfinyl carbanion and methyl iodide in dimethylsulfoxide using the method of Hakomori [5]. The completeness of methylation of the polysaccharide was examined using triphenylmethane, and the methylated polysaccharides were recovered by a Sep-Pak C_{18} cartridge (Waters, Miliford, MA., U.S.A.) The methylated polysaccharides were then hydrolyzed with 2 M trifluoroacetic acid, followed by reduction and acetylation, as previously reported [7]. The partially methylated alditol acetates obtained were analyzed by a GC/MS (HP5973 MSD, Hewlett Packard, Honover, U.S.A.) on a fused silica capillary column (0.32 mm× 30 m) of SP-2380 (Supelco Co., Bellefonte, U.S.A.) with a programmed temperature; one minute at 60°C, a linear gradient to 150°C at a rate of 18°C/min (held at 150°C for 5 min), and a subsequent linear gradient to 210°C at a rate of 2°C/min (held at 210°C for 6 min). The compound at each peak was characterized by their characteristic mass spectra and retention times relative to the standard sugar derivatives and by comparison with library spectra. A calibration of the molar ratio for each sugar derivative was performed using the peak areas and response factors.

All NMR experiments were performed using a Bruker AMX-500 spectrometer (Rheinstetten, Germany) at 500 MHz for ^1H and 125 MHz for ^{13}C. The spectra were recorded using a 1% (w/v) solution of each glucan in 0.5 ml of dimethylsulfoxide-d_6 (Me_2SO-d_6) at 25°C. The chemical shifts were expressed in ppm downfield, using 3-trimethylsilyl-propane-1-sulphonate-d_4 (δ_H 0.00 and δ_C 0.00) and Me_2SO-d_6 (δ_H 2.45 and δ_C 39.5) as the external and internal references, respectively.

Structural features of PONGa and PONGb

The two glucans isolated from the water-insoluble fraction (PON) of *P. ostreatus* consisted almost completely of D-glucose with only a small amount of *O*-acetyl substituents (PONGa 3.2%, PONGb 4.5%; data not shown). The conventional methylation analysis of the partially methylated alditol acetates using a GC-MS determination revealed the composition of the glycosidic linkage. As shown in Table 1, a high proportion of the 3-linked glucopyranosyl residues (2,4,6-tri-*O*-methyl-D-glucose) was detected in PONGb, whereas PONGa was composed of 3-linked glucopyranose (2,4,6-tri-*O*-methyl-D-glucose) and 3,6-linked glucopyranose (2,4-di-*O*-methyl-D-glucose) in a molar ratio of 2.5:1. PONGa was identified as a kind of α-glucan due to the characteristic IR absorption peaks (Table 2) of 930, 845, and 760 cm^{-1}, and ^1H signals (5.03, 5.36 ppm) of anomeric α-protons [10]. The ^{13}C-NMR spectrum (not shown) showed a triplet signal, 85.1, 84.5, and 83.9 ppm (Table 2) due to the C-3 of the (1→3) and (1→3, 1→6)-linked glucopyranosyl units and was similar to the spectra of the other (16)-branched (1→3)-glucans [5]. Regardless of the differences in the types of side chains attached to the backbone of the (1→3)-glucans, the C-6'-chemical shift (71.5 ppm) of PONGa showed a typical downfield effect

Table 1. Glycosidic linkage analysis of glucans, PONGa and PONGb.

	Methylated sugars (as alditol acetates)	Relative retention times[a]	Molar ratio	Mode of linkage
PONGa	2,3,4,6-Me_4-D-glucose	1.00	4	Glc1→
	2,4,6-Me_3-D-glucose	1.26	45	→3Glc1→
	2,3,4-Me_3-D-glucose	1.53	5	→6Glc1→
	2,4-Me_2-D-glucose	1.89	18	→3,6Glc1→
PONGb	2,3,4,6-Me_4-D-glucose	1.00	6	Glc1→
	2,4,6-Me_3-D-glucose	1.27	72	→3Glc1→
	2,3,4-Me_3-D-glucose	1.52	1	→6Glc1→

[a]Relative to 1,5-di-*O*-actetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
Abbreviations Me, methyl; Glc, D-glucopyranose.

Table 2. Spectroscopic data of glucans, PONGa and PONGb, isolated from *P. ostreatus*.

	IR (cm^{-1})	NMR Chemical shifts δ (ppm)[a]		
		^1H	^{13}C	
PONGa	3,440	5.36[b]	C-1 (100.8[b], 103.4[d])	21.4[c]
	2,850	5.03[b]	C-2 (75.8)	74.5[e]
	1,420	2.45[c]	C-3 (85.1, 84.5, 83.9)	76.0[e]
	1,060		C-4 (67.7)	72.5[e]
	930[b]		C-5 (73.9)	74.1[e]
	845[b]		C-6 (62.4)	63.5[e]
	760[b]		C'-6 (69.5)[d]	
PONGb	3,440	4.85[b]	C-1 (105.6)	21.6[c]
	2,854	2.51[c]	C-2 (74.9)	73.5[e]
	1,413		C-3 (84.4)	78.5[e]
	1,041		C-4 (68.9)	71.0[e]
	891[b]		C-5 (77.7)	75.8[e]
			C-6 (61.7)	60.8[e]

[a]Relative to 3-trimethylsilyl-propane-1-sulfonate-d_4 (δ_H 0.00 and δ_C 0.00) and Me_2SO-d_6 (δ_H 2.45 and δ_C 39.5).
[b]Anomeric region of glucans.
[c]*O*-acetyl substitutions of glucosyl residues.
[d]Branch point of α-D-(1→3)-glucan main chain.
[e]Minor signals of nonreducing terminal glucosyl units

(+1.5 ppm) [5]. In addition, the intensity ratio of the C-6 and C-6' peaks (spectra not shown) was in good agreement with the results (Table 1) obtained from the glycosidic linkage analysis. Accordingly, the results confirmed that PONGa was a branched α-glucan composed of α-1,3-linked D-glucopyranose residues and α-1,3-linked units with 6-branching points.

PONGb showed a characteristic absorbance at 890 cm^{-1} in the IR and an intense signal at 4.85 ppm (Table 2) in the ^1H-NMR spectrum (spectrum not shown), indicating the presence of β-D-glycosidic linkages. The ^{13}C-NMR data (Table 2) showed a downfield signal (84.4 ppm) due to the C-3 of the (1→3)-linked glucopyranosyl units, which was similar to the spectra of other linear β-D-(1→3) glucans [6]. The signals at 103.8, 74.9, 68.9, 77.7, and 61.7 ppm were assigned to the C-1, C-2, C-4, C-5, and C-6 of the linear β-D-(1→3) glucan, respectively. The other minor signals at 78.5, 75.8, 73.5, 71.0, and 60.8 ppm were attributable to C-3, C-5, C-2, C-4, and C-6, respectively of the nonreducing terminal residues. The ^1H signal at δ 2.5 and ^{13}C signal at δ 21.6 reflected the presence of *O*-acetyl groups in the glucosyl residues [12], and the content was determined as 4.5% (data not shown). These NMR and IR spectroscopic data together with those from the methylation analysis indicated that PONGb was a linear β-D-(1→3) glucan containing a small amount of *O*-acetyl groups.

Anti-Complementary Properties of PONGa and PONGb

When the NHS was incubated with some complement regulators and the remaining complement titer was measured with sensitized sheep erythrocytes as an antigen-antibody complex, the inhibition of hemolysis by the complement was considered as anti-complementary activity. It is generally accepted that the hemolysis of sensitized SRBC by a human complement is mediated *via* the classical and the alternative complement pathway in the presence of both Mg^{2+} and Ca^{2+} [14]. Thomas and Ishizaka [13] reported the use of EGTA buffers to distinguish the complement pathway that requires only Mg^{2+}, and it has been well accepted that complement fixations occurring in the presence of EGTA are *via* the alternative pathway. Since EGTA binds Ca^{2+} as strongly as EDTA, yet only binds Mg^{2+} rather weakly, it is assumed that an EGTA buffer containing a sufficient amount of Mg^{2+} could be used to measure the alternative complement pathway activity while still blocking the complement component C1. Table 3 shows the anti-complementary activities and activation pathways of PON, PONGa, and PONGb. The $ITCH_{50}$ value of the polysaccharide was measured by incubating with NHS in the presence of Mg^{2+} and Ca^{2+} (GVB^{2-} buffer), or Ca^{2+}-free gelatin veronal buffered saline (Mg^{2+}-EGTA-GVB^{2-}), or buffer free of both metal ions (EDTA-GVB^{2-}). In the Ca^{2+}-free condition, the anti-complementary activity of the fraction PON (1 mg/ml) was 45.3%, which was significantly lower than that in the GVB^{2+} buffer (70%), and was abrogated completely in the absence of both metal ions. This indicated that the complement activation by PON was generated *via* both the classical and the alternative complement pathways. PONGa showed considerably lower anti-complementary activities than the fraction PON; however, the mode of the complement pathway was also similar to that of the fraction PON. Among the three polysaccharides, PONGb (1 mg/ml) showed the highest anti-complementary activity in the GVB^{2+} buffer (75.3%). Moreover, the alternative pathway activity, which was measured at Mg^{2+}-EGTA-GVB^{2-}, was hardly reduced, suggesting that PONGb was a potent alternative complement activator.

Table 3. Effect of Ca^{2+} and Mg^{2+} ions on anti-complementary activity of the glucans from *P. ostreatus*.

Buffers	Inhibiton of TCH_{50} (%)		
	PON	PONGa	PONGb
GVB^{2+} (Both Ca^{2+} and Mg^{2+} existed)	70±3.0	53.2±1.5	75.3±2.3
Mg^{2+}-EGTA-GVB^{2-} (Only Mg^{2+} existed)	45.3±0.7	23.6±0.9	68.2±1.7
EDTA-GVB^{2-} (Neither existed)	3.1±0.1	1.6±0.5	2.8±0.2

It is known that the cleavage of the C3 complement component (C3) through the alternative pathway is promoted by a variety of substances which often comprise of yeast and fungal carbohydrates containing repeating units [2].

Fig. 1. Crossed immuno-electrophoresis of C3 component treated by PONGa and PONGb.
The polysaccharides were dissolved in a Mg^{2+}-EGTA-GVB^{2-} buffer and incubated with an equal volume of NHS at 37°C for 30 min. The sera were subjected to crossed immuno-electrophoresis using anti-human C3 to locate the cleaved C3 product. The precipitin line was visualized by staining the gels with Ponceau 3R. The anode is to the right. A: NHS only; B: NHS treated by PONGa; C: NHS treated by PONGb. Arrows indicate C3a and C3b components.

C3 is present in plasma in its largest quantities (800–1800 mg/ml) and its cleavage to C3a and C3b is the major reaction in the alternative complement activation [11]. To examine the C3 cleavage using PONGa and PONGb, an immuno-electrophoresis using anti-human C3 and NHS treated with the glucans was carried out. As expected, the PONGb β-glucan exhibited a significant second precipitin line by C3 cleavage components in the Ca^{2+}-free condition (Fig. 1C), whereas the control group failed to cleave C3 (Fig. 1A). In the case of PONGa, the second peak was much lower than that of PONGb (Fig. 1B). The data shown in Table 3 and Fig. 1 suggested that the β-anomeric configuration of the glucose was more optimal for the alternative pathway activation than the α-anomeric one. This result was in good agreement with other investigations [4] wherein it was concluded that the cleavage of C3 through the alternative complement pathway is promoted by β-glucans with 1,3 and/or 1,6 linkages, such as pachyman, nigeran, and zymosan particles. Thus, it also appears that the capacity of glucans to activate the alternative complement pathway is dependent on their chemical structure.

Based on the determination of its structure and anti-complementary properties, PONGb can be considered as an immunomodulator. It is hoped that the present study will encourge further investigation on the anti-tumor activity of glucans, to provide more information and to clarify the relationship between glucan structures and biological activities.

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