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# ANTITUMOR POLYSACCHARIDES FROM *P. ostreatus* (FR.) QUÉL.: ISOLATION AND STRUCTURE OF A β-GLUCAN

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ABSTRACT

We isolated an antitumor glucan (HA β-glucan) from the neutral polysaccharide fraction ($A_3$) of a hot-water extract of the edible mushroom *P. ostreatus* (Fr.) Quél. Purification was accomplished by extractions with 20% sodium chloride solution saturated with thymol and by precipitations with ethanol from dimethyl sulfoxide solution. The glucan showed marked antitumor activity at a dose of 0.1 mg/kg. It is a highly branched (1→3)-β-glucan having an average structure represented by a pentasaccharide segment consisting of one nonreducing terminal, one 3,6-di-*O*-substituted, and three 3-mono-*O*-substituted β-D-glucopyranosyl residues. This structure was confirmed by examining $^{13}$C-n.m.r. spectra taken at 75.46 MHz.

## INTRODUCTION

It has been shown that the antitumor activity of (1→3)-β-D-glucans from various fungi, such as lentinan[1], schizophyllan[2], scleroglucan[3], and others[4,5] is exhibited at doses of 10–0.2 mg/kg. By contrast, the effective doses of α-glucans are above 20 mg/kg[4,6–8]. However, we showed that a neutral glucan (fraction $A_3$) from *Pleurotus ostreatus* (Fr.) Quél. (Basidiomycetes) was active at a dose of 5 mg/kg in spite of a high positive specific rotation (+111°)[9,10].

The major structural features of this glucan as revealed by $^{13}$C-n.m.r. spectra in dimethyl sulfoxide-$d_6$ solution were (1→3)-linked β-glucosyl residues (over 50%), together with (1→4)-linked α-glucosyl residues[11,12]. However, the $^{13}$C-n.m.r. signals of the β-residues were almost completely suppressed in neutral aqueous or acidic media, whereas the $^{13}$C-n.m.r. peaks of the α-residues were not[11]. Thus there remained a possibility that fraction $A_3$ consisted of two components (β-glucan and α-glucan). For this reason, we examined the fraction to see whether or not the two components were separable.

Two kinds of fractionation procedures were found to be useful for the isolation of the β-glucan (HA β-glucan). In addition, the other component, an α-glucan, was purified by the use of concanavalin A. Interestingly, the antitumor activity of

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the HA β-glucan exhibited marked enhancement (effective dose 0.1 mg/kg) as compared with that of the original $A_3$ fraction (1 mg/kg).

## EXPERIMENTAL

*General methods.* — Gas–liquid chromatography was performed at 210° on a glass column (3 m × 4 mm) packed with 3% OV-225 on Gas Chrom Q. The g.c.-mass spectra were recorded with a JEOL-01SD-2 mass spectrometer combined with a JGC-20KFP gas chromatograph; an ionizing potential of 23 eV, an ionizing current of 200 μA, and an ion source temperature of 260° were employed. The latter instrument was equipped with a glass column (2 m × 3 mm) containing 3% OV-225 on Gas Chrom Q, operated at 200°. Helium at a flow rate of 30 mL/min was used as the carrier gas.

*Isolation of the β-glucan.* — (*a*) *Fractionation by 20% NaCl solution saturated with thymol.* A suspension of fraction $A_3$ (ref. 9) in a solution of thymol (10 mg/mL) in 20% aqueous NaCl was vigorously stirred overnight at room temperature, then centrifuged at 12,000 × *g* for 60 min. The precipitated gel was washed with the solvent, dialyzed against water, collected, washed with ethanol (3 vol.) to remove thymol, and recovered by lyophilization after removing the ethanol. This procedure was repeated to furnish the insoluble fraction (Tpp) in 47% yield based on fraction $A_3$, $[\alpha]_D$ −12° (*c* 1, $Me_2SO$); i.r.: 890 $cm^{-1}$.

(*b*) *Precipitation by ethanol from dimethyl sulfoxide solution.* — Fraction $A_3$ was dissolved in $Me_2SO$ to a concentration of 10 g/L by stirring for 24 h at room temperature, and the solution was freed from a trace of insoluble material. To the $Me_2SO$ solution was added 1 vol. of ethanol under vigorous stirring, and the mixture was kept overnight at 4°. A precipitate, consisting mainly of α-glucan, was removed by centrifugation at 12,000 × *g* for 30 min. To the supernatant was added ethanol up to a concentration of 63% (v/v), and the mixture was centrifuged. To the final supernatant was further added ethanol up to final concentration of 80% (v/v), and the mixture was kept overnight at 4°, then centrifuged. The precipitate was dialyzed against water until free from $Me_2SO$, and glucan (fraction $B_4$) was recovered by lyophilization in about the same yield as Tpp; $[\alpha]_D$ −12° (*c* 1, $Me_2SO$); i.r.: 890 $cm^{-1}$.

(*c*) *Repeated fractionation.* Finally, the highly purified β-glucan (HA β-glucan) was isolated by the alternating application of the above two fractionations until the specific rotation achieved a constant value, $[\alpha]_D$ of −18° (*c* 1, $Me_2SO$). The i.r. absorption bands corresponded to those of laminaran.

*Isolation of α-glucan by precipitation with concanavalin A (Con A).* — The fraction soluble in 20% NaCl–thymol solution was dialyzed, precipitated with ethanol, washed, and recovered by lyophilization after removal of the ethanol. A fraction (Tss) was obtained after repetition of this procedure in 20% yield based on fraction $A_3$; $[\alpha]_D$ +141°; i.r.: 930, 840 and 760 $cm^{-1}$; color test with iodine: reddish brown.

A Con A solution (25 mg/mL in 4M NaCl) was added to the fraction Tss (2 mg/mL) dissolved in 1M NaCl buffered at pH 6.8 with 0.018M phosphate buffer. The precipitate was recovered by centrifugation at 5,500 × *g* for 20 min, and washed with the buffer[13]. The component complexed with Con A was extracted with 10% trichloroacetic acid, precipitated with excess ethanol after neutralization of the solution, and dissolved in water. Dialysis of the solution, after removal of a trace of insoluble material, furnished a pure α-glucan (Ap), which was obtained from the dialyzate by lyophilization (56% yield based on fraction Tss); $[\alpha]_D$ +174° (*c* 0.9, $Me_2SO$), and +186° (*c* 0.9, $H_2O$); i.r.: 845 $cm^{-1}$.

The other component of the mixture was recovered from the supernatant after no further precipitate was formed by Con A. This supernatant was deproteinized by heating at 100° and centrifuging. The glucan was precipitated with ethanol, again deproteinized by adding trichloroacetic acid to a concentration of 10%, reprecipitated with ethanol, and recovered by dialysis and lyophilization. The preparation of mainly β-glucan (As) was obtained in the yield of ~10% based on fraction Tss; $[\alpha]_D$ −3° (*c* 0.9, $Me_2SO$); i.r.: 890 $cm^{-1}$.

*Methylation analysis of HA β-glucan.* — Methylation was performed by the method of Hakomori[14] using methylsulfinyl carbanion[15]. The procedure was repeated four times, when no i.r. absorption due to free hydroxy groups could be detected. The methylated glucan was dissolved in 90% formic acid, and hydrolyzed with 0.25M sulfuric acid for 12 h at 100° (ref. 16). The hydrolyzate was reduced with sodium borodeuteride, acetylated[17], and then injected onto a column of 3% OV-225 on Gas Chrom Q. The identification of the peaks was confirmed by g.c.-mass spectrometry[18].

*Periodate oxidation of the HA β-glucan.* — The HA β-glucan was oxidized by treatment with 0.02M sodium metaperiodate at 4° in the dark, with stirring. The periodate uptake was determined by the spectrophotometric method[19]. The released formic acid was determined potentiometrically by titration with 10mM sodium hydroxide[20], using a Metrohm AG Herisau Potentiograph E 336 equipped with a glass electrode, after degradation of the oxidant by ethylene glycol.

*$^{13}C$-N.m.r. spectrometry.* — $^{13}C$-N.m.r. spectra were recorded at 75.46 MHz on a Bruker CXP-300 spectrometer, for samples dissolved in $Me_2SO$-$d_6$. To achieve well resolved $^{13}C$ spectra, the glucan was dispersed by placing the 10 mm o.d. sample tubes in a bath-type sonicator, cooled with ice, for 30 min or more. Spin-lattice relaxation times were measured in the usual manner, using 180°–t–90° pulse sequences. The estimated error of the $T_1$ values is less than ±5%.

*Bioassay of antitumor activity.* — Ascites of sarcoma 180 (~5 × $10^6$ cells) were transplanted subcutaneously into female mice of the ICR-JCL strain. After the elapse of 24 h, a solution of the sample in saline was injected intraperitoneally, and injections were continued daily for 10 days. The tumor was weighed at the end of 5 weeks. The inhibition ratio, expressed in per cent, was calculated by comparing the average weight of the tumors of treated mice with that of untreated controls.

## RESULTS AND DISCUSSION

*Fractionation and bioassay.* — We found that an insoluble component was separated from fraction $A_3$ by stirring in 20% NaCl–thymol solution. The insoluble fraction, Tpp, showed the physical properties characteristic of a β-glucan, whereas the properties of the soluble fraction, Tss, were reminiscent of a (1→4)-linked α-glucan. Fraction Tpp at a dose of 0.2 mg/kg/day strongly inhibited the growth of the tumor, with activity of 93 ±7% (mean ±S.E.), in comparison with an activity for the original fraction $A_3$ of 82 ±5% at a dose of 1 mg/kg. Fraction Tss at a dose of 0.2 mg/kg showed decreased activity (52% ±31), although at a dose of 1.0 mg/kg it had strong activity (95 ±5%).

Previously, it had been suggested that the original fraction $A_3$ contained a glycogen-like α-glucan, since α-(1→6)-linked diglucose units with nonreducing terminal D-glucose were found by serological tests against antipneumococcal horse sera types I–XXIX and antiparatyphi A serum[21]. The α-glucan was successfully purified from fraction Tss by using Con A. The pure α-glucan (Ap), isolated from the complex with Con A, showed physical properties similar to those of glycogen (including a positive color test with iodine), but the preparation As, recovered from the supernatant, was mainly β-glucan. The activity of Ap decreased to 55% at a dose of 1.0 mg/kg as compared with 95% for Tss, while that of preparation As increased to 100%, even at the low dose of 0.2 mg/kg. Thus, the active component was contained in preparation As, but scarcely in Ap. Therefore, the antitumor activity arose from the β-glucan, but not from the α-glucan. Clearly, the minimum effective dose of the active component is less than 0.2 mg/kg.

The fractionation by 20% sodium chloride–thymol solution was useful for exclusion of the α-glucan from fraction $A_3$, but insoluble contaminants were introduced into the β-glucan. We found that the β-glucan in fraction $A_3$ was soluble in dimethyl sulfoxide even in the presence of ethanol (63%), but the α-glucan was precipitated by the presence of 30–63% ethanol in the dimethyl sulfoxide solution. The β-glucan could be recovered by the further addition of ethanol up to 80%. This β-glucan preparation ($B_4$) showed an activity of 77 ±13% at a dose of 0.2 mg/kg.

The pure β-glucan finally obtained (HA β-glucan) exhibited the strong activity of 95 ±3% at a dose of 0.2 mg/kg. Furthermore, the inhibition ratio was 74 ±10% even at a dose of 0.1 mg/kg. Thus, it is likely that even α-glucan containing 10% of such a potent antitumor component could produce an antitumor response at a dose of 1.0 mg/kg, as in the case of fraction Tss. The HA β-glucan showed the strongest antitumor activity of all the (1→3)-β-glucans in the series so far assayed in the S-180 system.

*Chemical analyses of HA* β-glucan. — The g.l.c. of the alditols from fully methylated HA β-glucan showed three peaks corresponding to tetra-*O*-methyl, tri-*O*-methyl, and di-*O*-methyl derivatives. These peaks were identified as the alditols from 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methylglucose (molar proportions 1:3:1 based on peak areas), as confirmed by g.c.-mass spectrometry of their 1-deu-

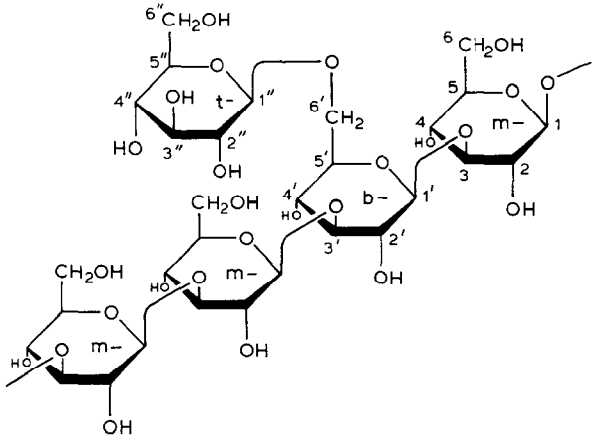

Fig. 1. Structure showing the kinds of glucose residues contained in HA β-glucan, and the numbering of the carbon atoms in these residues.

terated derivatives. There were no peaks resulting from sugars other than glucose in the g.l.c. Accordingly, the HA β-glucan is a (1→3)-linked glucan highly branched at O-6, and having no (1→6) linkages except at the branching point, as illustrated in Fig. 1.

This structure of HA β-glucan was further confirmed by periodate oxidation. The reaction mixture consumed 0.38 ±0.02 (S.D.) mol of periodate per mol Glc, and 0.18 ±0.01 mol of formic acid per mol Glc was liberated, corresponding to an average subunit of 5.2–5.5 glucose residues. These results are consistent with the data obtained by the methylation analysis.

*$^{13}$C-N.m.r. spectrometry of HA β-glucan.* — The 75.46 MHz $^{13}$C-n.m.r. spectrum of HA β-glucan in $Me_2SO$-$d_6$ solution exhibited six intense signals and seven weak signals, as shown in (a) of Fig. 2.

The spectral features of HA β-glucan are very similar to those of scleroglucan, reported by Rinaudo and Vincendon[22], although there are significant differences in the relative intensities of peaks, reflecting the difference in the extent of branching. Thus, it is possible to confirm the proposed assignment in view of the relative peak intensities together with the $T_1$ values[23]. Our present data for peak intensities and $T_1$'s are consistent with the assignments of Rinaudo and Vincendon except for the C-5′ peak, as summarized in Table I.

Peak G (75.4 p.p.m.) was assigned to the C-5′ carbon of the 3,6-di-*O*-substituted β-glucopyranosyl residue (b-residue)[12,24,25] of a variety of branched (1→3)-β-glucans in previous papers. This assignment was confirmed by the very short $T_1$ value (185 ms) of signal G, reflecting its location in the rigid portion of the molecule. Previously, this signal at 75.7 p.p.m. had been assigned to the C-5 carbon in the 3-monosubstituted β-glucosyl residue (m-residue) by Rinaudo and Vincendon[22]. However, the C-5′ signal in the b-residue should be at higher field than the C-5 signal in the m-residue, because glycosylation at the C-6′ carbon induces upfield

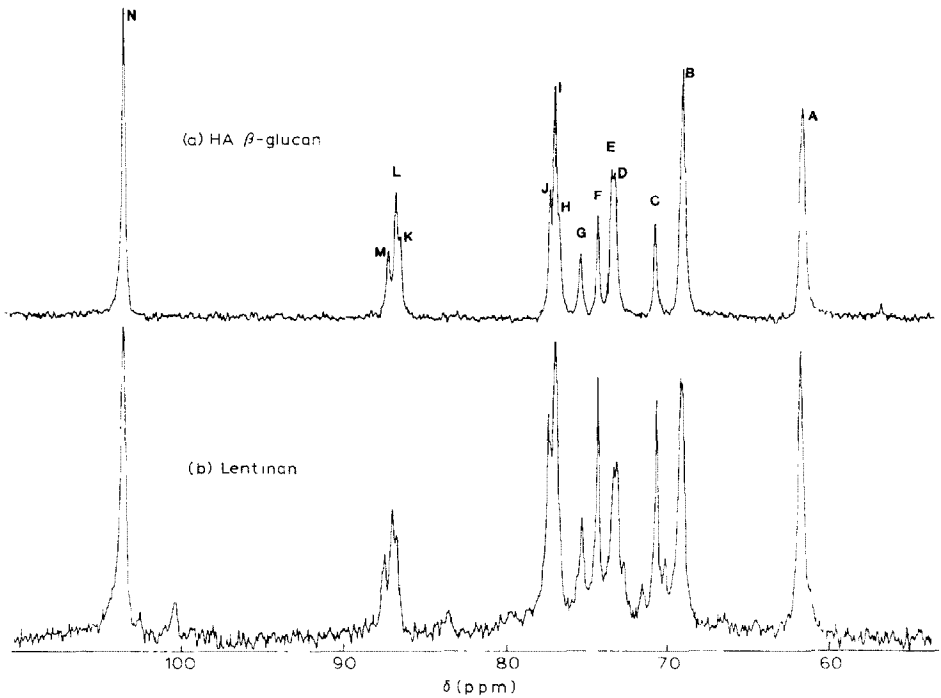


Fig 2. $^{13}$C-N.m.r spectra at 75.46 MHz of (a) HA β-glucan and (b) lentinan in $Me_2SO$-$d_6$.

and downfield shifts of the nearby β- and α-carbons, respectively[26]. Further, signal B at 69.3 p.p.m. was assigned to the C-6′ carbon by taking into account the difference (+7.6 p.p.m.) between the corresponding carbons of curdlan [linear (1→3)-β-glucan][25,27] and GE-3 [linear (1→6)-β-glucan][12,28], as presented in Table I.

Signal K, with the shortest $T_1$ value among K, L, and M, was assigned to the C-3′ carbon of the b-residue located at the most rigid part of the backbone (Table I). Peaks L and M are ascribed to the C-3 carbons of the m-residues, the latter peak being displaced 0.3 p.p.m. downfield with respect to the former, reflecting a neighboring effect of the b-residue. In addition, the intensity ratio of 1:2:1 for peaks K, L, and M is in good agreement with the value predicted from the chemical analyses.

Therefore, the characteristic structural feature of the HA β-glucan consists of a pentasaccharide segment having a nonreducing terminal β-glucosyl residue and four internal β-glucosyl residues, of which one is 3,6-disubstituted and three are 3-monosubstituted.

In conclusion, the HA β-glucan, isolated from fraction $A_3$, possesses strong antitumor activity at a dose of 0.1 mg/kg. This glucan is a highly branched (through O-6) (1→3)-β-glucan. Its fundamental structure is found to be analogous to that of other (1→3)-β-glucans having antitumor activity in doses of 1.0–0.2 mg/kg, such as lentinan, shizophyllan, scleroglucan, etc. In structural details it seems to differ (a

TABLE I

THE $^{13}$C-N M R SPECTRAL LINES OF HA $\beta$-GLUCAN AND THEIR ASSIGNMENTS

| *Peak* | *Observed value* | | | *Peak assignment* | | | *Standard* | |
|---|---|---|---|---|---|---|---|---|
| | *δ (p.p.m.)* | *Intensity ratio*[a] | *$NT_1$ (ms)* | *Residue* | | | *Curdlan*[b] *(→3Glc→) δ (p.p.m.)* | *GE-3*[c] *(→6Glc→) δ (p.p.m.)* |
| | | | | *m-* | *b-* | *t-* | | |
| A | 61.8 | 9.1 | 143, 181 | C-6 | | C-6″ | 62.4 | |
| B | 69.3 | 9.5 | 172 | C-4 (−1.6)[d] | C-4′, C-6′ (+7.5) | | 69.9 (−1.5) | 70.0 (+7.6) |
| C | 70.9 | 2.4 | 286 | | | C-4″ | | 71.4 |
| D | 73.6 | D, E 7.4 | 210 | C-2 | C-2′ | | | |
| E | 73.8 | | 200 | C-2 (−0.7) | | | 74.3 (−0.7) | |
| F | 74.5 | 2.7 | 262 | | | C-2″ | | 75.0 |
| G | 75.4 | 2.0 | 185 | | C-5′ (−1.6) | | | 77.1 (−0.6) |
| H | 76.7 | H | 224 | C-5 | | C-5″ | | |
| I | 77.0 | I 10.4 | 217 | C-5 | | | 77.7 | |
| J | 77.4 | J | 265 | | | C-3″ | | 78.1 |
| K | 86.4 | K | 182 | | C-3′ | | | |
| L | 87.0 | L 7.1 | 199 | C-3 (+9.6) | | | 87.5 (+9.4) | |
| M | 87.7 | M | 200 | C-3 | | | | |
| N | 103.7 | 9.5 | 224 | C-1 | C-1′ | C-1″ | 104.5 | 104.9 |

[a]Integrated peak-areas normalized to 6 × 10. [b]Ref. 25. [c]Ref. 12. [d]The shift increments representing the $\alpha$- and $\beta$-effects of glucosylation at the C-6 and C-3 positions are given in parentheses.

different degree of branching and/or a branch free from 6-monosubstituted residues) from the others[29], as suggested by the $^{13}$C-n.m.r. spectrum of lentinan, displayed in (b) of Fig. 2.

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