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PURIFICATION OF AN ANTITUMOR-ACTIVE, BRANCHED (1→3)-β-D-GLUCAN FROM *Volvariella volvacea*, AND ELUCIDATION OF ITS FINE STRUCTURE*

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ABSTRACT

A (1→3)-β-D-glucan branched by O-6 substitution (FCAP), obtained from the cold-alkali extract of the fruiting body of *V. volvacea*, exhibited potent growth-inhibitory activity against implanted tumors in mice. It contained protein and appeared to be heterogeneous. Fractionation by DEAE-Toyopearl column chromatography yielded an unbound, protein-free glucan fraction ($[\alpha]_D$ −30° in M NaOH, mol. wt. 1.5–2 × 10^6), which showed the highest antitumor activity. The polysaccharide had a moderately branched structure, consisting of a backbone chain of β-(1→3)-linked-D-glucose residues, one out of five or six being substituted at O-6 with single glucosyl or β-(1→6)-linked diglucosyl groups. Digestion of the glucan with exo-(1→3)-β-D-glucanase yielded glucose and gentiobiose (molar ratio, 8.2:1.0), and a highly branched (d.b. 1/3), degraded glucan. Digestion with endo-(1→3)-β-D-glucanase gave D-glucose, laminarabiose, a trisaccharide β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→3)-D-Glc, a tetrasaccharide β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-[β-D-Glc*p*-(1→6)]-D-Glc, and a highly branched (d.b. 1/2), enzyme-resistant glucan. The results suggest that the *Volvariella* glucan is structurally heterogeneous with regard to the distribution of branches, having less branched, moderately branched, and highly branched segments.

INTRODUCTION

In our investigations on the interrelations of structure and the immunomodulating action of fungal polysaccharides, we have isolated from edible mushrooms[1–3], and also from growing cultures of fungi[4], various branched (1→3)-β-D-glucans having host-mediated antitumor action on transplanted tumors in mice.

As reported in a previous paper[3], fractionation of the polysaccharide compo-

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nents of the fruiting body of *Volvariella volvacea** gave, in addition to glycogen and a D-manno-D-galactan, three kinds of antitumor-active, branched (1→3)-β-D-glucans. Among these the cold alkali-extracted glucan (FCAP, d.b.† 1/5) showed the highest activity (97% inhibition at 5 mg/kg) against Sarcoma 180 in ICR-JCL mice, whereas the more highly branched, hot alkali-extracted and alkali-insoluble glucans showed low or moderate activities (inhibition 34 and 48%, respectively). The fact that FCAP contained ~10% of protein and appeared to be heterogeneous in molecular composition prompted us to purify it by careful fractionation and elucidate its structure.

This paper reports on the fractionation of FCAP by anion-exchange column chromatography, and the determination of its fine-structural features, particularly the distribution and localization of branches along the (1→3)-β-D-glucan backbone.

RESULTS AND DISCUSSION

Fractionation of the antitumor glucan, FCAP. — The cold alkali-extracted glucan ($[\alpha]_D$ −20°, *c* 0.5, M NaOH), designated FCAP, analyzed for 75% glucose, and appeared to contain a small amount of protein. Purification was attempted by ion-exchange chromatography, which has been used for the fractionation of neutral polysaccharides under slightly basic conditions[5]. A preliminary study indicated that highly branched (1→3)-β-D-glucans are retained strongly on a DEAE-Toyopearl column at pH 7.8. Therefore, FCAP was applied to the column in 0.01M phosphate buffer, pH 7.8. After elution of unbound components with this buffer, the retained components were fractionated by a salt gradient, and finally the column was washed with dilute alkali. Fig. 1 shows the elution profile of the chromatography, which afforded four fractions. The unbound material (fraction I, recovery 14.3% of the dry weight of the sample) was composed solely of glucose. The next two fractions (II, 4.9%, and III, 7.5%), eluted as the salt concentration increased to 0.6M, analyzed for 78.8 and 45.0% of carbohydrate (as glucose), respectively, and appeared to be associated with protein, as they showed absorption at 280 nm. Since the recovery of total carbohydrate (fractions I, II and III) from the column was low, it was further eluted with 0.1M NaOH, to give an additional polysaccharide fraction (IV, recovery 13.5%). This fraction contained 92.6% of glucose. The relatively low recovery of total carbohydrate from the column may be due to the low solubility of the slightly branched β-(1→3)-linked D-glucan components.

Among these four fractions, fraction III showed peak carbohydrate content coinciding with a peak in 280-nm absorbance, suggesting that it may be a polysaccharide–protein conjugate, such as an antitumor polysaccharide from *Corilus versicolor*[6], or *Flammulina velutines*[7]. Fraction III contained aspartic acid, glutamic

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*Called "tsao ru" in Chinese, "fukurotake" in Japanese; cultivated in Taiwan.

†Degree of branching, expressed as the ratio of O-6-substituted D-glucose residues to the total (1→3)-linked D-glucose residues of the backbone chain.

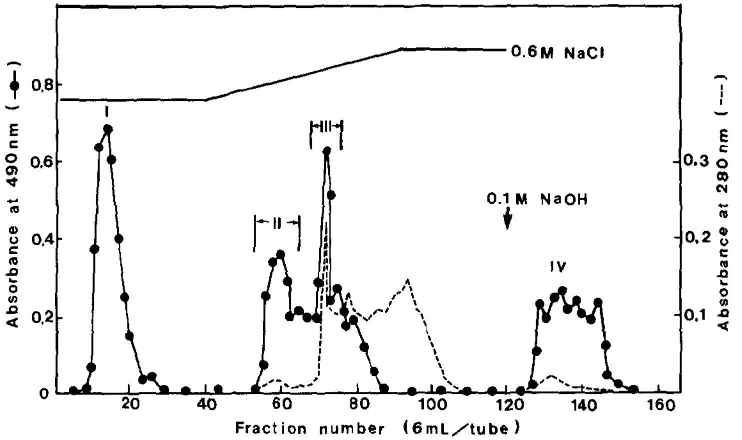


Fig. 1. Fractionation of FCAP by column chromatography. A solution of FCAP was applied to a DEAE-Toyopearl column (2.2 × 35 cm). The column was washed with 0.01M phosphate buffer (pH 7.8) to elute the unbound glucan (fraction I), and the retained components were eluted with a salt gradient (0 to 0.6M NaCl in the same buffer) followed by 0.1M NaOH.

TABLE I

METHYLATION ANALYSIS OF β-D-GLUCAN FRACTIONS FROM DEAE-TOYOPEARL COLUMN CHROMATOGRAPHY [a]

O-Methyl-D-glucose	*Linkage indicated*	*Molar ratios*			
		Fr. I	*Fr. II*	*Fr. III*	*Fr. IV*
2,3,4,6-Tetra-	Glc(1→	1.0	1.0	1.0	1.0
2,4,6-Tri-	→3)Glc(1→	4.6	2.3	1.9	2.2
2,3,4-Tri-	→6)Glc(1→	0.5	0.4	0.3	
2,4-Di-	→3)Glc(1→ 6 ↑	1.0	1.0	0.9	1.0

[a] See Fig. 1.

acid, glycine, and alanine, so is similar in amino acid composition to the *F. velutipes* polysaccharide. However, fraction III gave, by gel-chromatography on Sephadex G-150, one sharp carbohydrate peak at the void volume, and another broad carbohydrate peak, corresponding to a molecular weight of $\sim 3 \times 10^4$, which differed slightly from the protein peak. This result indicates that fraction III may not be a glycoconjugate, but rather a polysaccharide firmly associated with protein.

The pattern of glucosidic linkages in the four fractions, revealed by methylation analysis, is shown in Table I. All the fractions have similar branched structures, based on a (1→3)-D-glucan backbone, but fraction I was less highly branched (d.b. 1/5-6) than the other fractions, which had d.b. 1/3. All the fractions except IV contained a small proportion (7%) of (1→6)-linked interior residues.

TABLE II

ACTIVITIES AGAINST SARCOMA 180 OF β-D-GLUCAN FRACTIONS FROM DEAE-TOYOPEARL COLUMN CHROMATOGRAPHY

Fraction	*Average tumor weight in g*		*Tumor growth inhibition (%)*	*Complete regression (number/number in group)*
	Treated	*Control*		
I	0	10.5	100	4/4
II	0	6.1	100	4/4
III	3.1	6.1	49	1/5
IV	0	6.1	100	6/6

The antitumor activities of the four polysaccharide fractions against Sarcoma 180 solid tumor were compared. As listed in Table II, fractions I, II, and IV exhibited very high immunomodulating activities, with complete growth-inhibition of the tumor in mice at dosages of 5 mg/kg for 10 days, whereas fraction III, which is a glucan–protein mixture, showed lower activity (inhibition, 49%, rate of complete regression, 1/5) under the same conditions. This result supported the proposal that the antitumor-active substance is most likely the (1→3)-β-D-glucan and not the protein moiety.

Elucidation of the fine structural features of the purified glucan. — Fraction I, which was not bound to DEAE-Toyopearl, showed the highest antitumor activity, and thus should be regarded as the active principle of FCAP. It gave a broad but single, symmetrical peak on Sepharose CL-2B when eluted with 0.1M sodium hydroxide. Its molecular weight was in the range $1.5–2.0 \times 10^6$ as estimated by liquid chromatography at 3 MPa on a column of TSK G-5000 PW eluted with 0.1M Tris–HCl buffer[4], pH 8.0. Fraction I was composed solely of D-glucose not appreciably contaminated by protein. Its optical rotation ($[\alpha]_D$ −30.0° at *c* 0.2, M NaOH), and the characteristic absorption band at 890 cm^{-1} in the i.r. spectrum supported a β-anomeric linkage for the D-glucose residues. ^{13}C-N.m.r. of the glucan gave essentially the same spectrum as recorded for FCAP[3], including signals for C-1 at 103.56 p.p.m., *O*-substituted C-3 at 86.76 p.p.m., and *O*-substituted C-6 at 70.55 p.p.m.

Since the antitumor activity of the purified glucan has been shown to be closely related to the triple-stranded conformation of its backbone chain[8], and also the arrangement and molecular shape of the side chains[3], determination of its fine structure was attempted by a combination of chemical analysis and enzymic fragmentation.

The methylated glucan gave, on acidic hydrolysis, 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, and 2,4-di-*O*-methyl-D-glucose in a molar ratio of 1.0:4.6:0.5:1.0 (Table I). This result was supported by analysis of the products of complete hydrolysis of the glucan polyalcohol, derived by periodate oxidation and borohydride reduction. G.l.c. of the mixture showed the presence of glycerol and D-glucose in a molar

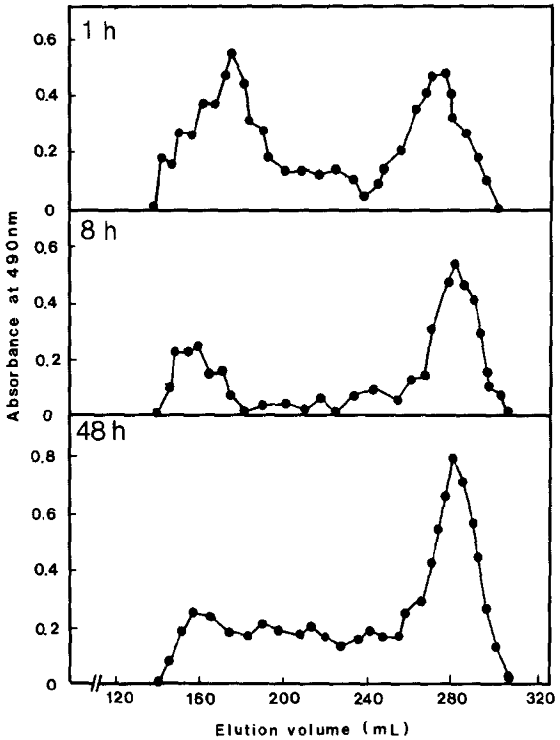


Fig. 2. Gel chromatography profiles of the glucan in the course of hydrolysis by exo-(1→3)-β-D-glucanase.

ratio of 1.0:4.0, in fairly good agreement with the value 1.0:3.7 calculated from the methylation data. No erythritol was detected, confirming the absence of (1→4)-linked D-glucose units.

When the glucan polyalcohol was hydrolyzed by heating with 0.2M TFA, at 90° for 1 h (Smith degradation)[1], the periodate-resistant, degraded glucan was obtained as an insoluble product (yield 74%), which on methylation gave 2,4,6-tri-*O*-methyl-D-glucose together with only a very small amount of the 2,3,4,6-tetra-*O*-methyl-derivative. This result suggested that the backbone chain of the glucan consists essentially of consecutive (1→3)-linked D-glucose residues. Detection of only D-glycerol in the soluble fraction from the Smith degradation indicates the absence of (1→4)-linked interior units in the side chains. Thus, it is apparent from the methylation data and the results of Smith degradation that one out of five or six D-glucose residues in the main chain are substituted at O-6 with single D-glucosyl groups, or possibly with short chains of (1→6)-linked glucosyl units.

For further characterization of the distribution of side chains, the glucan was incubated with the exo-(1→3)-β-D-glucanase [EC 3.2.1.6] of basidiomycete QM 806. The release of reducing sugars was monitored by gel chromatography on Toyopearl HW 65S (Fig. 2). The degree of hydrolysis reached 53% (as glucose) after 48 h, and no further increase was observed. After heat inactivation of the enzyme the reaction mixture was centrifuged to give soluble and insoluble products. Analysis of the soluble products (supernatant solution) by gel chromatography on Bio-Gel P-2 revealed glucose and gentiobiose in a molar ratio of 8.2:1.0. In another analysis, glucose and gentiobiose were separated by preparative paper chromatography, and the molar ratio was estimated colorimetrically as 7.1:1.0. These values are somewhat higher than the value 5.6:1.0 calculated from the methylation data. Considering the specificity of this exoenzyme, which can hydrolyze a (1→3)-β-D-glucan from the non-reducing end to produce glucose, and also gentiobiose, if the glucan is branched at O-6 with a single glucosyl group, the difference in the ratio between enzymic and methylation data might be due to a molecular heterogeneity of the glucan with regard to the distribution of branch points. That is, part of the molecule, corresponding to 59% of total sugar residues, would be less branched and more susceptible to the exoenzyme action, so that hydrolysis would proceed from the nonreducing end, until an anomalous linkage interfered with further action.

The insoluble, degraded glucan, corresponding to 42% of the total glucose residues, was methylated and hydrolyzed with acid to yield 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, and 2,4-di-*O*-methyl-D-glucose, in a molar ratio of 1.0:2.1:0.5:1.1. This result indicates that the enzyme-resistant glucan portion contains more branches, *i.e.*, two branch points per six (1→3)-linked D-glucose residues, approximately twice the branching of the original glucan. The nonsusceptibility of this segment to enzymic hydrolysis may be attributable to the presence of densely localized branch points, or side chains of short β-(1→6)-linked D-glucosyl units.

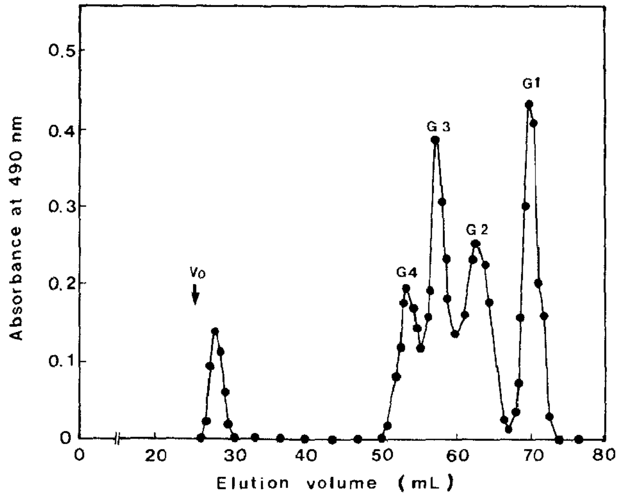


Fig. 3. Gel chromatography of the endo-(1→3)-β-D-glucanase-digested glucan on a Bio-Gel P-2 column (0.8 × 190 cm), G1, D-glucose; G2, disaccharide; G3, trisaccharide; G4, tetrasaccharide.

TABLE III

METHYLATION ANALYSIS OF THE PRODUCTS OF THE ENDO-(1→3)-β-D-GLUCANASE TREATMENT OF THE PURIFIED GLUCAN FRACTION I

*O-Methyl-*D*-glucose*	*Linkage indicated*	*Molar ratios*[a]			
		Degraded glucan, insoluble fraction	*Oligosaccharides*		
			Tetra-	*Tri-*	*Di-*
2,3,4,6-Tetra-	Glc(1→	1.0	2.0 (2.0)	1.0 (1.0)	1.0
2,4,6-Tri-	→3)Glc(1→	0.6	1.0 (0.9)	1.1 (0)	1.0
2,3,4-Tri-	→6)Glc(1→			1.0 (1.0)	
2,4-Di-	→3)Glc(1→ 6 ↑	0.9	1.1 (0)		

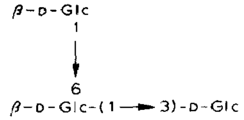
[a] Values in parentheses are those found by methylation of the borohydride-reduced oligosaccharides.

More structural information was provided by enzymic fragmentation, using Zymolyase 60 000, a partially purified endo-(1→3)-β-D-glucanase [EC 3.2.1.39] of *Arthrobacter luteus*. After 48 h (degree of hydrolysis 43%, as glucose), the enzyme-resistant, degraded glucan (~34%), which became insoluble during incubation, was removed by centrifugation. The mixture of saccharides in the supernatant solution was applied to a column of Bio-Gel P-2 (Fig. 3). This procedure afforded four fractions, namely glucose (24.5% by weight), di- (26.6%), tri- (27.2%), and tetra-glucosaccharide (13.7%), corresponding to a molar ratio of 7.3:3.4:2.6:1.0. In addition, a small amount of a high-molecular-weight saccharide (8.0%) eluted at the void volume. The oligosaccharides were purified by preparative paper chromatography, and characterized, mainly by methylation analysis. The data are presented in Table III.

The disaccharide ($[\alpha]_D$ +19.0° at *c* 0.5, water) had R_{Glc} 0.85. Methylation analysis revealed that the D-glucose residues were joined by a β-(1→3) linkage. Thus, the disaccharide was laminarabiose.

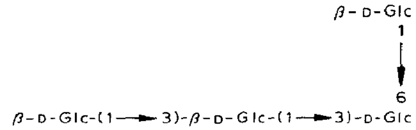
The trisaccharide ($[\alpha]_D$ +1.8° at *c* 0.2, water) had R_{Glc} 0.45. It was comprised of one mole each of nonreducing-terminal, (1→3)-linked, and (1→6)-linked D-glucose residues. Reduction of the reducing-terminal group resulted in the elimination of 2,4,6-tri-*O*-methyl-D-glucose, indicating the trisaccharide was *O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→3)-D-glucose (**1**).

The tetrasaccharide ($[\alpha]_D$ −4.0° at *c* 0.3, water) had R_{Glc} 0.25. It was comprised of one each of 3-*O*-substituted and 3,6-di-*O*-substituted D-glucose residues, and two nonreducing terminal groups. Reduction of the reducing group resulted in the elimination of 2,4-di-*O*-methyl-D-glucose. Thus, the tetrasaccharide had the



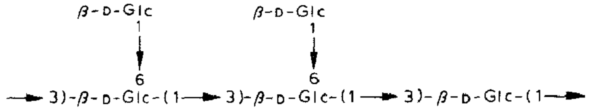
$$\begin{array}{l} \beta\text{-D-Glc} \\ \quad 1 \\ \quad\downarrow \\ \quad 6 \\ \beta\text{-D-Glc-}(1\rightarrow 3)\text{-D-Glc} \end{array}$$

1



$$\begin{array}{r} \beta\text{-D-Glc} \\ 1 \\ \downarrow \\ 6 \\ \beta\text{-D-Glc-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc-}(1\rightarrow 3)\text{-D-Glc} \end{array}$$

2



$$\begin{array}{lll} \quad\beta\text{-D-Glc} & \quad\beta\text{-D-Glc} & \\ \qquad 1 & \qquad 1 & \\ \qquad\downarrow & \qquad\downarrow & \\ \qquad 6 & \qquad 6 & \\ \rightarrow 3)\text{-}\beta\text{-D-Glc-}(1 & \rightarrow 3)\text{-}\beta\text{-D-Glc-}(1 & \rightarrow 3)\text{-}\beta\text{-D-Glc-}(1\rightarrow \end{array}$$

3

branched structure *O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→3)-*O*-[β-D-glucopyranosyl-(1→6)]-D-glucose (**2**).

Since the *Arthrobacter* enzyme is known to act on unbranched segments of β-(1→3)-linked D-glucose residues, the production of laminarabiose and **2** indicates the presence of three or more consecutive β-(1→3)-linked D-glucose units not having branches. Tetrasaccharide **2** presumably arose from branched segments having single D-glucosyl groups as side chains. Trisaccharide **1** may have been formed in two ways: either from branch-point units substituted with β-D-glucosyl groups at O-6, or from the sequence →3)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→, interposed in the main chain. However, the isolation of mainly water-insoluble, (1→3)-linked linear glucan from the Smith degradation of the polysaccharide supports the first possibility. That is, the units of the backbone are linked by (1→3)-β-D-glucosidic bonds, and there are probably no (1→6)-linked residues in the main chain.

Concerning the presence of glucose and laminarabiose in the Zymolyase digest of the glucan: although this enzyme would be expected to give laminarapentaose as the smallest oligosaccharide product from a linear (1→3)-β-D-glucan (*cf.* the specifications for Zymolyase 60 000, Kirin Brewing Co.), we found that the preparation used in this study hydrolyzed laminarapentaose to glucose and laminarabiose. However, it failed to hydrolyze gentiobiose or trisaccharide **1**. Hiura and coworkers also obtained glucose, laminarabiose, and **1** when the endo-(1→3)-β-D-glucanase of *B. circulans* acted on a branched glucan of the *Neurospora crassa* cell wall[9].

The Zymolyase-resistant, high-molecular-weight saccharide fraction, which emerged from the Bio-Gel P-2 column in the void volume, contained (1→6)-D-glucosidic linkages. The methylated product gave, on acid hydrolysis, 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, and 2,4-di-*O*-methyl-D-glucose, in a molar ratio of 1.0:1.1:0.8:1.1, indicating that this product (mol. wt. 2.5–4 × 10^4) represents highly branched segments, containing a high proportion of (1→6)-D-glucosidic linkages, most probably located in side chains.

The degraded glucan, obtained as a water-insoluble residue after the endo-(1→3)-β-D-glucanase action, had essentially no antitumor activity on Sarcoma 180 (data not shown). Methylation analysis indicated that this product (mol. wt. 5×10^4) had a very highly branched structure in which, and on average, two out of three (1→3)-linked glucose residues carry single D-glucosyl groups as side chains (see **3** and Table III). Although the degraded glucan was devoid of (1→6)-linked side chains containing more than one glucosyl unit, it was not susceptible to exo-(1→3)-β-D-glucanase, probably because of its highly branched structure.

From the structures of the digestion products of the exo- and endo-(1→3)-β-D-glucanases, it is apparent that in the glucan purified from FCAP there is some structural heterogeneity with regard to the distribution of branches. The glucan molecule may consist of structurally[24] different types of segments, namely less branched (d.b. 1/7–8), moderately branched (d.b. 1/3) by substitution with both single and β-(1→6)-linked diglycosyl groups, and highly branched (d.b. 2/3) but having only single-unit side chains. The less branched segment, which contains single D-glucosyl branches and makes up 60% of the whole molecule, beginning at the nonreducing terminal, is hydrolyzed stepwise by the exoenzyme. Most parts of this segment would also be degraded by Zymolyase to produce glucose, laminarabiose, and trisaccharide **1**. The moiety resistant to exoenzyme may comprise the relatively highly branched segments, whereas the endoglucanase-resistant portion may be only the very highly branched segment.

Heterogeneity with regard to degree of branching has been reported for the antitumor (1→3)-β-D-glucans from the fruiting bodies of *Basidiomycetes* and the cell walls of fungi, such as *Lentinus edodes*[10], *Ganoderma lucidum*[2], and *Audiobasidum pullulans*[11]. These glucans might be resolved by careful fractionation involving sequential solvent extraction and column chromatography. However, like the antitumor glucan of *V. volvacea* described in this paper, some fungal cell-wall glucans may have heterogeneity in their molecular structures, particularly with regard to distribution and length of branches. Recently Hara *et al.* reported that the (1→6)-branched (1→3)-β-D-glucan from *Dictyophora indusiata*[12] is heterogeneous with respect to the distribution of D-glucosyl side chains, and showed that these are mainly located near the nonreducing end of the main chain. The presence of exoglucanase-resistant D-glucosidic linkages, resulting from the mode of branching, has also been reported for the antitumor (1→3)-β-D-glucan of *Grifola frondosa*[13].

The present studies provide evidence that the structural features required for the antitumor activity of the polysaccharides from *Volvariella volvacea* are a 6-*O*-substituted, (1→3)-linked β-D-glucan chain having an appropriate distribution of side chains [ranging one per three to one per five (1→3)-linked D-glucose residues], and adequate molecular size. The latter parameter and the tendency to triple-helix formation are probably related. The mechanism of immunomodulation exhibited by such a (1→6)-branched (1→3)-β-D-glucan is a subject for further research.

EXPERIMENTAL

Materials. — Air-dried fruiting bodies of *Volvariella volvacea* (fukurotake) were a commercial product from T'aichung, Taiwan. Exo-(1→3)-β-D-glucanase of basidiomycete QM 806 was a gift from the research laboratories of the Taito Co., Ltd., Kobe. Zymolyase 60 000 was purchased from the Kirin Beer Co., Ltd., Tokyo. DEAE-Toyopearl 650, Toyopearl HW 65S, and TSK G-5000 PW were obtained from Toyo Soda Manufacturing Co., Ltd., Tokyo, and Bio-Gel P-2 from Bio-Rad Laboratories, Richmond, CA, U.S.A. Other chemicals were standard commercial products.

General analytical methods. — All evaporations were done under reduced pressure at temperatures below 40°, unless otherwise stated.

Total carbohydrate was determined by the phenol–sulfuric acid method[14] and reducing sugar by the Somogyi–Nelson method[15].

Paper chromatography was performed on Toyo No. 50 filter paper, irrigated with 6:4:3 (v/v) 1-butanol–pyridine–water. Sugars on paper chromatograms were detected with alkaline silver nitrate.

For gas-liquid chromatography, neutral sugars and their methylated derivatives were converted into their corresponding alditol acetates. Separations were accomplished on a Hitachi gas chromatograph, model 163, equipped with a flame-ionization detector and a glass column (0.4 × 200 cm) packed with 3% ECNSS-M on Gas Chrom Q, or a glass capillary column (0.24 mm × 25 m) coated with CP-Sil 88, and operated at 190° (free sugars) or 180° (methylated sugars). Nitrogen was used as carrier gas at a flow rate of 40 mL/min. G.c.–m.s. of the alditol acetates of partially methylated sugars was performed with a Shimadzu LKB-9000B GC-MS, using a column of 3% ECNSS-M on Gas Chrom Q.

Polysaccharide samples (1–5 mg) were usually completely hydrolyzed by heating in 90% formic acid (0.4 mL) for 12 h at 100°, followed by evaporation and heating of the residue in 2M trifluoroacetic acid (TFA, 0.8 mL) for 7 h at 100°. After evaporation of the acid the carbohydrate components were analyzed by g.l.c.

Optical rotations were measured at 20° in water or dilute alkali solution with an automatic polarimeter, model PM 101, from Union Giken, Osaka, Japan.

Fractionation of cold alkali-extracted polysaccharide. — The cold alkali-extracted polysaccharide (FCAP) was prepared by the method described in the previous paper[3]. After successive extraction with phosphate-buffered saline (PBS) and hot water, the extraction residue was treated twice with M NaOH for 2 h at 25° to give FCAP. FCAP (50 mg) was dissolved in 0.1M NaOH (5 mL), and the solution dialyzed against 0.01M phosphate buffer, pH 7.8. As the solution after dialysis was very viscous, it was diluted with 20 mL of the same buffer and applied to a DEAE-Toyopearl column (see Fig. 1). Each polysaccharide fraction was lyophilized.

Determination of molecular weight. — Average molecular weights of glucan fractions were estimated by 3-MPa l.c., using a column (0.75 × 60 cm) of TSK

G-5000 PW with 0.1M Tris·HCl buffer, pH 8.0, as the carrier, at a flow rate 0.8 mL/min[4]. Pullulan fractions having defined molecular weights (2×10^4–8×10^5, Hayashibara Biochemical Lab. Inc., Okayama) and also purified dextran fractions (mol. wt. 2×10^4–2×10^6, provided by Meito Sangyo Co. Ltd.) were used as standards.

Methylation analyses. — A sample (1–5 mg) of polysaccharide or oligosaccharide, dissolved in dimethyl sulfoxide (2 mL), was methylated by the method of Hakomori[16]. Some samples of oligosaccharide (3 mg), as noted, were reduced with $NaBH_4$ (1–1.5 mg) before methylation. After methylation of a polysaccharide sample, the reaction mixture was dialyzed, the nondialyzable fraction was concentrated to dryness, and the methylated polysaccharide extracted with chloroform.

For purification of methylated oligosaccharides, reaction mixtures were sparged with a stream of nitrogen to remove the excess methyl iodide, then passed through a Sep Pak C18 cartridge (Waters Association, Milford, MA, U.S.A.), that had been prewashed with methanol (10 mL) and water (10 mL). The cartridge was eluted successively with water (5 mL × 2), 1:1 methanol–water (2 mL × 3), and methanol (2 mL × 3). On examination of the eluates by t.l.c., the methylated oligosaccharides were usually found in the methanol or the methanol–water fractions. The tubes containing methylated oligosaccharide were combined and concentrated to dryness.

The fully methylated polysaccharide or oligosaccharide (1–5 mg) was hydrolyzed by heating in 90% formic acid (0.4 mL) at 100° for 12 h, and then (after removal of the formic acid) in 2M TFA (0.8 mL) for 7 h. Partially methylated sugars in the hydrolysate were successively reduced with $NaBH_4$ and acetylated.

Smith degradation. — A glucan sample (10 mg) was stirred with 0.05M sodium periodate (10 mL) at 5° in the dark. After complete oxidation, the polyaldehyde was treated with $NaBH_4$ at room temperature. After 24 h, $NaBH_4$ was decomposed by the addition of acetic acid, and the solution dialyzed against water. The glucan polyol was recovered by concentration and lyophilization of the dialysand (yield, usually 7 mg). The glucan polyalcohol was hydrolysed with 0.2M TFA for 1 h at 90°.

Enzymic hydrolysis of the purified glucan by exo-(1→3)-β-D-glucanase. — The purified glucan (fraction I, 20 mg) was dissolved in 0.1M NaOH (2 mL) and the solution neutralized with acetic acid. After dilution with 0.1M acetate buffer, pH 5.0 (3 mL), exo-(1→3)-β-D-glucanase from Basidiomycete QM 806 (1.0 unit) was added, and the solution was incubated for 48 h at 37° in the presence of a few drops of toluene. The degree of hydrolysis of the glucan was estimated by measurement of the release of the reducing sugar (expressed as glucose). Aliquots (1.5 mL) were taken at 1, 8, and 48 h. Each was heated to inactivate the enzyme, and centrifuged to separate insoluble residue from the reaction mixture. The insoluble, degraded glucan was washed with distilled water and lyophilized. It was then dissolved in 0.1M NaOH (1 mL) and applied to a Toyopearl HW 65S column (2.2 × 100 cm). When there was no further increase in reducing sugar (48 h), the incubation mixture was heated and centrifuged, and the supernatant was concentrated for examination of the hydrolysis products.

*Endo-(1→3)-β-*D*-glucanase treatment of the glucan.* — The glucan (15.4 mg) was dissolved in 0.1M NaOH (1.0 mL). The alkaline solution was neutralized with acetic acid, diluted with 0.067M phosphate buffer, pH 7.5 (3.5 mL), and incubated with Zymolyase 60 000 (3.5 mg, 210 units) for 48 h at 37°. After heat-inactivation of the enzyme, the reaction mixture was centrifuged to separate insolubilized, degraded glucan, which was washed with water and after lyophilization examined by methylation analysis. The supernatant was concentrated to a small volume for examination of the hydrolysis products.

Assay of antitumor activities. — The growth-inhibitory activities of the glucan and related polysaccharide fractions were assayed by a method similar to that described in a previous paper[3]. Sarcoma 180 ascites cells (0.1 mL, 6×10^6 cells) were transplanted subcutaneously into the right groin of ICR-JCL mice (female, average weight 25 g). The test samples, dissolved or suspended in phosphate-buffered saline (0.01M, pH 7.2), were injected intraperitoneally daily for 10 days, starting 24 h after tumor implantation. The growth of tumors was observed for 35 days, then the mice were killed and the tumors excised. The inhibition ratio was calculated from the following equation:

$$\text{Inhibition ratio } (\%) = 100(A - B)/A$$

where A is the average tumor weight of the control group and B is that of the polysaccharide-treated group.

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