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Structural Characterization of Pichilan, a β-D-Glucan Immunostimulant from *Pichia fermentans*

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An insoluble polysaccharide with immunostimulating activity, which we have designated as pichilan, was isolated from the cell wall of *Pichia fermentans* (CBS 187) by repeated treatment with hot alkali and hot acetic acid. Partial hydrolysis led to the liberation of laminariosides and gentiobiose. A study of the products obtained by methylation, Smith degradation and endo-glucanase hydrolysis showed that the polysaccharide is a moderately branched, (1→3)-β-D-glucan with a high molecular weight (approximately 200000) containing 10% β-(1→6)-linked gluco-pyranosyl residues and two different types of branches, a single glucosyl group and a long glucosyl chain.

Keywords——*Pichia fermentans*; pichilan; β-D-glucan; immunostimulating activity; methylation analysis; polysaccharide structure

Introduction

The immunopotentiating activity of polysaccharides extracted from various sources is well documented.[1] Among the polysaccharides of microbial origin, the β-D-glucans have been found to be the most active.[2] We have described the immunostimulating activity of several glucans from the cell wall of *Pichia fermentans*.[3,4] The alkali- and acid-insoluble glucan, which we have designated as pichilan, has immunostimulating activity[5] which appears to be based on a different mechanism from that described for other glucans.[2] For this reason, we decided to determine the structure of pichilan. In the present report we describe the structural characteristics deduced from analyses of the products obtained by methylation, Smith degradation and enzymatic hydrolysis. The results show that pichilan has an unusual structure; it is a (1→3)-β-D-glucan containing 10% (1→6) linkages and having two different branches, a single, glycopyranosyl group and a long glycopyranosyl chain.

Materials and Methods

General Experimental Conditions——The solutions were concentrated under reduced pressure at bath temperatures not exceeding 40 °C. Infrared (IR) spectra were taken on a Perkin Elmer spectrometer as KBr pellets. Ascending paper chromatography (PC) was carried out on Whatman No. 1 paper using the following solvent system (v/v): (A) 6:1:3, 1-propanol–ethyl acetate–water. Thin-layer chromatography (TLC) was performed on Merck plates. The following solvent systems (v/v) were used: (B) 14:2:7, 1-propanol–ethyl acetate–water on a cellulose support and (C) 9:6:3:1, 1-butanol–acetic acid–ether–water on a silica gel support. The chromatograms on cellulose (paper and thin-layer) were developed with alkaline silver nitrate[6] and those on silica gel with resorcinol-sulfuric acid. The partially methylated monosaccharides were analyzed as alditol acetates by gas-liquid chromatography (GLC) using a Girdel 3000 instrument with flame detection. A stainless-steel column (140 × 0.2 cm) of 3% OV225 on Chromosorb WHP (100—120 mesh) at 200 °C was used. The mass spectra of the partially methylated alditol acetates were recorded

on a Kratos MS-50 mass spectrometer using a 70-eV beam.

Isolation of the β-D-Glucan——A method similar to that described by Bell and Northcote[7] and by Manners *et al.*[8] was used. *Pichia fermentans* CBS 187 was cultured[3] in a complex medium for 48 h at 27 °C.

1) Separation of the Yeast Cell Wall: The yeast (30 g, dry weight) was treated successsively with 300 ml of each of the following and then centrifuged: 6% NaOH for 90 min at 80 °C, 3% NaOH for 18 h at room temperature, water, 3% NaOH for 2 h at 80 °C.

2) Isolation of the Glucan: Cell-wall material obtained above was treated with 0.5 M acetic acid for 1 h at 80 °C and then with water; the two treatments were repeated once. The insoluble fraction was recovered by centrifugation after each extraction. The final insoluble fraction was suspended in 0.02 M sodium acetate (300 ml) and autoclaved at 120 °C for 90 min. The product was washed several times with water and spray-dried.

Strong Acid Hydrolysis——The glucan was hydrolyzed with strong acid by a method similar to that described by Peat *et al.*[9] The neutral sugars in the hydrolyzate were assayed by the anthrone method described by Fairbain.[10] The glucan (75 mg) in 99% formic acid (5 ml) was sonicated for 1 h and then the suspension was heated for 4 h at 100 °C with stirring. The mixture was evaporated to dryness by repeated addition of water. The residue was dissolved in 3 N sulfuric acid (20 ml) and was heated for 6 h at 100 °C with stirring. The hydrolyzate was neutralized and adjusted to 100 ml; the neutral sugars were assayed using glucose as a standard.

Partial Acid Hydrolysis——The glucan was subjected to partial acid hydrolysis by a slight modification of the technique described by Manners *et al.*[11] The glucan (20 mg) in 99% formic acid (20 ml) was sonicated for 1 h and then heated for 1 h at 100 °C with stirring. Formic acid was removed by repeated addition and evaporation (five times) of water (30 ml). The residue was dissolved in 0.2 M sulfuric acid (20 ml) and heated for 1 h at 100 °C with stirring. The hydrolyzate was neutralized with barium carbonate and then deionized by contact with a mixed anion–cation exchange resin. After filtration, the solution was concentrated for analysis by TLC on cellulose and on silica gel using solvent systems B and C. On cellulose, the *Rf* values were 0.36, 0.31, 0.21, 0.14 and 0.09 for the products from pichilan, and 0.36 (glucose), 0.33 (galactose), 0.31 (laminaribiose) and 0.21 (gentiobiose) for the reference substances. On silica gel, the *Rf* values were 0.29, 0.17, 0.12, 0.07 and 0.03 for the products from pichilan, and 0.31 (mannose), 0.29 (glucose), 0.17 (laminaribiose) and 0.12 (gentiobiose) for the reference substances.

Enzymatic Degradation——The glucan was hydrolyzed according to the method of Ukai *et al.*[12] and Fleet and Manners.[13] Laminarinase from *Penicillium* (10 units, Sigma) was added to a suspension of glucan (150 mg) in pH 5 acetate buffer (12 ml). Several suspensions were prepared. They were incubated for 1 to 96 h with gentle mixing. At intervals, a suspension was heated for 15 min at 100 °C and deionized. After filtration, the filtrate was adjusted to 100 ml and assayed for glucose enzymatically using hexokinase.[14,15] The hydrolyzate was concentrated for analysis by TLC on both supports using solvent systems B and C. On cellulose, the *Rf* values were 0.36, 0.21, 0.05 (trace amount) for the products from pichilan, and 0.37 (glucose), 0.33 (galactose), 0.31 (laminaribiose) and 0.22 (gentiobiose) for the reference compounds. On silica gel, the *Rf* values were 0.29, 0.13 and 0.06 (trace amount) for the products from pichilan, and 0.31 (glucose), 0.18 (laminaribiose) and 0.12 (gentiobiose) for the reference substances.

Acetolysis——Acetolysis of the glucan was performed according to the method of Ukai *et al.*[12] and Nanba and Kuroda.[16] A suspension of glucan (50 mg) in 98% acetic anhydride (5 ml) was sonicated for 1 h and then acetic acid (3 ml) and sulfuric acid (0.5 ml) were added. After 3 d at room temperature with intermittent stirring, the mixture was heated for 30 min at 80 °C and then poured into ice-cold water (50 ml). The solution was neutralized with sodium bicarbonate and extracted with chloroform (5 × 30 ml). The combined extracts were washed with water (50 ml), dried over anhydrous sodium sulfate for 12 h at 4 °C amd filtered. The filtrate was evaporated to dryness and the residue was dissolved in a solution (15 ml) of 0.05 M sodium methanolate in methanol. After 16 h at 4 °C, water (5 ml) was added and the solution wad deionized and filtered. The filtrate was concentrated for analysis by PC using solvent system A. The degree of polymerization (DP) of the oligosaccharides was determined by the method of French and Wild,[17] who showed that there is a linear relationship between the DP and the $\log(Rf/1-Rf)$.

Periodate Oxidation——The glucan was oxidized according to the procedure of Hay *et al.*[18] A sample (75 mg) of glucan was suspended in 0.1 M sodium metaperiodate (25 ml). Several suspensions were prepared and kept in the dark for 7 to 135 h at 4 °C with stirring. At various intervals, excess metaperiodate was destroyed by addition of 50% ethylene glycol (5 ml). After stirring for 10 min, the suspension was filtered. Formic acid in the filtrate was assayed enzymatically using formaldehyde dehydrogenase.[19] To correct for overoxidation, the amount of formic acid produced was plotted against the time of oxidation and extrapolated to zero time.

Methylation——The glucan was methylated by the Hakomori method.[20] The glucan was left in contact with the dimethylsulfoxide (DMSO) for 16 h with stirring and then sonicated for 1 h. The methylsulfinyl carbanion was prepared just before use by the method of Sjöberg.[21] The sodium hydride (55—60% suspension in oil) was extracted (6 times) with hexane before mixing with methyl sulfoxide. The absence of hydroxyl groups in the methylated glucan was verified by infrared spectroscopy. The methylated glucan (10 mg) was hydrolyzed according to the method described by Lindberg.[22] For GLC, the hydrolyzate of the methylated glucan was reduced with sodium borohydride and then acetylated (pyridine–acetic anhydride, 1 : 1; 60 min, 100 °C). After cooling, portions (1—5 μl) were injected onto the OV 225 column. Retention times of the alditol acetates of the methylated sugars were determined relative to that of the alditol acetate of 2,3,4,6-tetra-*O*-methyl D-glucopyranose (mixture of α and β anomers).

Smith Degradation——The procedure used was adapted from that described by Goldstein *et al.*[23] The glucan (370 mg) was suspended in 0.04 M sodium metaperiodate (250 ml) and kept in the dark for 135 h at 4 °C. Ethylene glycol was added, the mixture was stirred and dialyzed. The non-dialyzable fraction was reduced with sodium borohydride (2%, final concentration). After 24 h at room temperature, the base was neutralized with HCl added dropwise and the suspension was concentrated to 75 ml. Hydrolysis was performed with 1 N HCl (75 ml) for 5 h at room temperature and the mixture was centrifuged. The residue was washed with water (3 × 20 ml) and the washings were added to the supernatant. The insoluble fraction was washed with methanol (3 × 20 ml) and then with ether (2 × 10 ml); the solvents were removed by evaporation. The insoluble fraction was dried over phosphorus pentoxide and weighed. The supernatant was deionized and the glycerol content was determined enzymatically using glycerokinase.[24] The supernatant was concentrated for analysis by TLC on silica gel. The *Rf* values were 0.53, 0.32, 0.26 (trace amount) and 0.10 (trace amount) for the products from pichilan, and 0.53 (glycerol), 0.48 (erythritol), 0.34 (mannitol), 0.31 (glucitol), 0.29 (*N*-acetylglucosamine) and 0.12 (glucosamine) for the reference products.

Modified Smith Degradation——The glucan (370 mg) was oxidized and reduced as described above. The excess sodium borohydride was neutralized with concentrated acetic acid, and the mixture was evaporated and freed from boric acid by repeated evaporation with methanol (3 × 20 ml) and then ether (2 × 50 ml). The solvents were removed by evaporation. The residue (about 250 mg) was treated as described by Nánási and Lipták.[25] The concentrated hydrolysate was converted into the corresponding alditol acetate and analyzed by GLC as described above.

DP——Sodium borohydride (5 mg) was added to the glucan (25 mg) in water (5 ml). The mixture was sonicated for 1 h. After 24 h at 37 °C, the mixture was cooled and acidified to pH 4 with HCl and then evaporated to dryness. The resulting boric acid in the residue was removed by repeated evaporation (six times) with methanol (10 ml). The residue was hydrolyzed with 99% formic acid (5 ml) and heated for 2 h at 100 °C with stirring. The mixture was evaporated repeatedly by addition of water (6 × 10 ml). The residue was treated with 2 N HCl (2 ml) and stirred for 4 h at 100 °C. The pH of the hydrolyzate was adjusted to 9.5 with sodium hydroxide and the volume to 25 ml. The sorbitol content of the hydrolyzate was determined enzymatically using sorbitol dehydrogenase.[26] The glucose content was determined enzymatically using hexokinase after strong acid hydrolysis of the glucan. The DP was calculated according to Sturgeon[27] and Manners *et al.*[28]:

$$\mathrm{DP}=\frac{\mu\text{mol D-glucose}}{\mu\text{mol D-sorbitol}}+1$$

The method was verified by treating laminarin under the same conditions.

Results and Discussion

The yield of β-D-glucan obtained from the cell wall of *Pichia fermentans* was about 9% of the dry weight of the yeast starting material. This polysaccharide was found to be insoluble in 4 N sodium hydroxide, 1 N hydrochloric acid and 1 N acetic acid and slightly soluble in 1.5 N sodium hydroxide (90 min, 80 °C) and DMSO. Strong acid hydrolysis followed by assay of the neutral sugars liberated showed that the glucan contained approximately 90% carbohydrate (composed of 98.5% glucose), 1.5—2.0% glucosamine or *N*-acetylglucosamine, <1% mannose, 2% lipids, 1.6—1.7% protein, 1% mineral substances and traces of heavy metals. The amount of total nitrogen determined by the Kjeldahl method was 0.3%. The IR spectrum of pichilan revealed an absorption band at 890 cm^{-1} characteristic of β-glycosidic linkages.[29]

Partial acid hydrolysis liberated glucose, gentiobiose, laminaribiose and other laminariosides indicative of β-(1→3)- and β-(1→6)-linked glucopyranosyl residues. No other sugar such as galactose or xylose could be detected by TLC. These results indicate that pichilan is mainly composed of glucose.

Treatment of the glucan with laminarinase gave glucose and gentiobiose. This hydrolysis was relatively rapid; 20% of the glucose was liberated within 1 h, 30% after 3 h, 50% after 24 h and 62% after 48 h. The percentage of glucose liberated was not increased by further incubation. A small part (8—9%) of the glucan was resistant to enzyme hydrolysis; however, after methylation, more than 87% of this resistant fragment was still composed of β-(1→3)-linked glucopyranosyl residues. 2,4-Di-*O*-methyl D-glucopyranose (6%) was one of the products of methylation of the residue. This indicates that the residual glucan had a branched structure. There was also 5% 2,3,4-tri-*O*-methyl D-glucopyranose. Resistance of the glucan to hydrolysis by the endo-glucanase may be explained by the very complex physical state of the

TABLE I. GLC and GLC-MS Data for the Alditol Acetates Derived from the Methylated Glucan

Methylated sugar (as alditol acetate)	t_R [a]	Prominent peaks (m/z)	Mode of linkage	Mol proportion (%)
2,3,4,6-Me_4-D-Glc	1	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	Glc_p-(1→	4
2,4,6-Me_3-D-Glc	1.66	43, 45, 71, 87, 101, 117, 129, 161, 233	→3)-Glc_p-(1→	81
2,3,4-Me_3-D-Glc	1.95	43, 71, 87, 99, 101, 117, 129, 161, 173, 189	→6)-Glc_p-(1→	10
4,6-Me_2-D-Glc	2.79	43, 45, 85, 101, 129, 161, 261	→2) →3)-Glc_p-(1→	Trace
2,4-Me_2-D-Glc	3.30	43, 87, 117, 129, 189	→3) →6)-Glc_p-(1→	4

a) Relative retention time. Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl D-glucitol. Glc_p: glycopyranose.

molecule, making it difficult for the enzyme to reach the core of the glucan.

It is known that (1→6) linkages in polysaccharides are preferentially cleaved during acetolysis.[30] Acetolysis of pichilan gave glucose, laminaribiose, laminaritriose, laminaritetrose and a high-molecular-weight polysaccharide representing the acetolysis-resistant polymer.

The nature of the linkages in the β-D-glucan was studied in several ways. The results of the methylation experiments are shown in Table I. The partially methylated sugars were analyzed as the alditol acetate derivatives by GLC and GLC-mass spectrometry (GLC-MS) and identified by reference to the retention times of authentic standards and MS analyses. The glucan was found to contain 81% β-(1→3)- and 10% β-(1→6)-linked glycopyranosyl residues. The presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylglucitol is proof that the polysaccharide has a branched structure; the analysis showed one branched (1→3,6)-linked glucose unit for every 24 glucosyl residues. The presence of 4,6-di-*O*-methylglucitol, which increased with repeated methylation, can probably be attributed to incomplete methylation. 4-*O*-Methyl- or 6-*O*-methylglucitol (very long GLC retention times) might have been produced by incomplete methylation and then methylated to 4,6-di-*O*-methylglucitol with repeated methylation.

Periodate oxidation produced 0.145 mol of formic acid per mol of glucosyl residue; in other words, one metaperiodate-sensitive glucosyl residue for every 6.9 glycosyl residues. These formic acid units were derived from glucosyl residues nonsubstituted on positions 2, 3 and 4 and from nonreducing terminal residues. The results of the oxidation experiment corroborate the results of the methylation experiment, where we found 4% nonreducing terminal residues and 10% (1→6) linkages, or in other words, one periodate-sensitive residue for every 7.1 glucose units.

Analysis of the products obtained by Smith degradation indicated that the major part of pichilan was not attacked. Laminarinase hydrolysis of the fraction from this degradation liberated approximately 86% glucose after 48 h, and partial acid hydrolysis of the fraction liberated glucose, gentiobiose (trace amount), laminaribiose and high-molecular-weight polysaccharides. These results confirm that this fraction was mainly composed of β-(1→3)-linked glucopyranosyl residues.

Analysis of the supernatant from the Smith degradation revealed the presence of glycerol, which corresponded to the periodate oxidation of 14% of the glucose molecules in pichilan; the glycerol was derived from glucose units involved in β-(1→6) linkages and from nonreducing terminal units. No erythritol was detected, ruling out the presence of β-(1→4)-linked glucosyl residues in the glucan.

When pichilan was subjected to modified Smith degradation, namely, methylation of the oxidized and reduced glucan and hydrolysis of the fully methylated polysaccharides, the results in Table II were obtained. Compared to the results of the methylation of the native

glucan (Table I), the modified Smith degradation led to an increase in the number of nonreducing terminal residues and a decrease in the number of glucosyl residues linked through positions 1, 3 and 6. The increase in the number of terminal units can be explained by the presence of intra-chain β-(1→6)-linked units. In this case, periodate oxidation of the glucan would produce chain fragments each bearing a nonreducing terminal unit after hydrolysis. The decrease in the number of (1→3,6)-linked glucosyl residues is probably due to the single glucosyl branches in the initial polysaccharide.

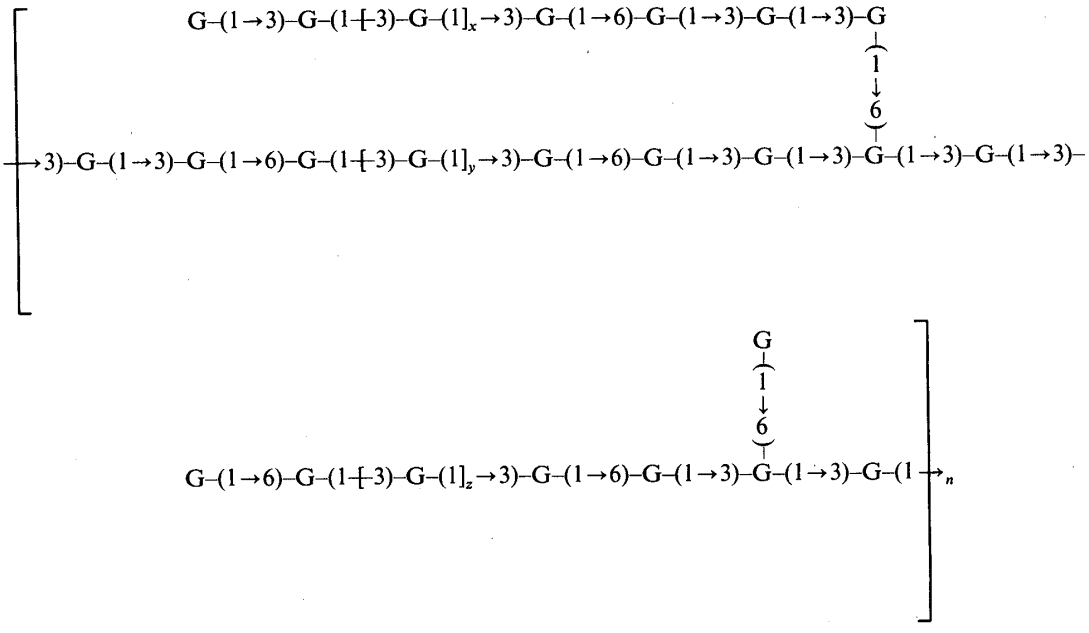
Determination of the molecular size of the β-D-glucan was rendered difficult because of its relative insolubility. Since the glucan is not highly branched, however, it was possible to determine the DP by the technique of Manners *et al.*[28] which is based on the measurement of sorbitol produced by reduction and hydrolysis of the polysaccharide. A DP value of 1200 ± 200 was found; this corresponds to an average molecular weight of approximately 200000.

Taken together, the results suggest that pichilan, the insoluble polysaccharide we obtained from the cell wall of *Pichia fermentans*, is a high-molecular-weight glucan whose repeating unit contains about 50 glucosyl residues composed of 81% β-(1→3) linkages and

TABLE II. GLC and GLC-MS Data for the Alditol Acetates Derived from the Methylated Glucan after Modified Smith Degradation

Methylated sugar (as alditol acetate)	t_R[a]	Prominent peaks (m/z)	Mode of linkage	Mol proportion (%)[b]
2,3,4,6-Me_4-D-Glc	1	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	Glc_p-(1→	14
2,4,6-Me_3-D-Glc	1.66	43, 45, 71, 87, 101, 117, 129, 161, 233	→3)-Glc_p-(1→	70
2,3,4-Me_3-D-Glc	1.93	43, 71, 87, 99, 101, 117, 129, 161, 173, 189	→6)-Glc_p-(1→	<1
2,4-Me_2-D-Glc	3.29	43, 87, 117, 129, 189	→3) →6)-Glc_p-(1→	2

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. *b*) The remaining 14% corresponds to glucose molecules transformed into glycerol.



G = β-D-Glc_p; $x+y+z \simeq 28$, $n \simeq 24$

Fig. 1. Possible Structure of the Average Repeating Unit of Pichilan

10% intrachain β-(1→6) linkages. In addition, the average repeating unit has 4% (1→3,6)-linked glucosyl residues, that is, only two branches; one of the glucosyl residues is linked to a single glucosyl group and the other to a long glucosyl chain. A possible structure for this repeating unit is shown in Fig. 1. This rather unusual structure might explain the differences between the mode of action proposed for pichilan[5] and that of other insoluble glucans[2] which have similar biological activity.

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