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The Structure of a β-(1→6)-D-Glucan from Yeast Cell Walls

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By selective enzymolysis, or chemical fractionation, a minor polysaccharide component has been isolated from yeast (*Saccharomyces cerevisiae*) glucan. This minor component has a degree of polymerization of about 130–140, a highly branched structure, and a high proportion of β-(1→6)-glucosidic linkages. The molecules also contain a smaller proportion of β-(1→3)-glucosidic linkages that serve mainly as interchain linkages, but some may also be inter-residue linkages.

Previous studies by Bacon and his co-workers (Bacon & Farmer, 1968; Bacon *et al.*, 1969) and by ourselves (Manners & Masson, 1969; Manners *et al.*, 1973) have shown that yeast glucan, as normally prepared, is heterogeneous, and is in fact a mixture of two structurally related β-glucans. The major component (about 85%) is a branched β-(1→3)-glucan of degree of polymerization about 1500 containing 3% of β-(1→6)-glucosidic interchain linkages. The minor component, as shown by i.r. spectroscopy and partial hydrolysis by acid or enzymes, is a β-(1→6)-glucan. We now describe the results of a detailed structural analysis of this glucan. A preliminary account of some of the results has been given elsewhere (Manners & Masson, 1969).

In our earlier studies on yeast glucan (Manners & Patterson, 1966), the insoluble polysaccharide preparation was incubated with a bacterial laminarinase giving glucose, laminaribiose, laminaritriose and about 10% of an enzymically resistant water-soluble glucan. At this time, the heterogeneity of yeast glucan was not known, and the residual glucan was considered to be a 'limit dextrin', by analogy with the well-known action of β-amylase or phosphorylase on starch-type polysaccharides. This view is now known to be incorrect, and it is apparent that the bacterial enzyme preparation had selectively hydrolysed one of the two β-glucan components. Hence, this enzymic degradation provides a method for the preparation of the minor component.

During the studies on the major β-(1→3)-glucan component (Manners *et al.*, 1973), a scheme for the fractionation of yeast cell walls was also developed, which gave substantial amounts of the purified major and minor components. The latter has now been examined, and shown to be very similar to the polysaccharide obtained by enzymic degradation.

Materials and Methods

Analytical methods

The methods used for the partial or total acid hydrolysis of oligosaccharides, glucans and methylated glucans, and for the determination of normal and methylated sugars are described by Manners *et al.* (1973). The following solvents were used for qualitative paper chromatography: *A*, ethyl acetate–pyridine–water (10:4:3, by vol.); *B*, methyl ethyl ketone–water–NH_3 (sp.gr. 0.88) (200:17:1, by vol.); *C*, butan-1-ol–ethanol–water (4:1:5, by vol.); *D*, ethyl acetate–acetic acid–90% formic acid–water (18:3:1:4, by vol.); *E*, propan-1-ol–ethanol–water (14:2:7, by vol.); *F*, butan-1-ol–pyridine–water (6:4:3, by vol.).

Preparative methods

Glucans were converted into the corresponding alcohols (glucanitols) by reduction with KBH_4. In a typical experiment, the enzymically resistant glucan (about 300mg) was dissolved in water (12.5ml), an equal weight of borohydride added, and the mixture kept at 20°C for 3 days. The solution was then adjusted to pH 6.7 with acetic acid, freed from inorganic material by dialysis and the glucanitol isolated by freeze-drying. In the various experiments, the yield varied between 90–98%.

Polysaccharide samples

Yeast (*Saccharomyces cerevisiae*) glucan A2, pustulan, luteose and laminarin were the samples described previously (Manners *et al.*, 1973). Repeated extraction of the alkali-insoluble residue of glucan A2 with 0.5M-acetic acid at 90°C gave a mixture of glucan

and glycogen. The latter was removed by precipitation with iodine, followed by treatment with α-amylase, to give the purified minor component.

Enzyme preparations

Endo-β-(1→3)-glucanase from *Rhizopus arrhizus* was kindly provided by Dr. E. T. Reese (see Reese & Mandels, 1959). The bacterial laminarinase and endo-β-(1→6)-glucanase from *Penicillium brefeldianum* were described by Manners *et al.* (1973). Almond β-glucosidase was prepared by Dr. W. L. Cunningham (see Cunningham *et al.*, 1962).

Preparation of the enzymically resistant glucan

Glucan A2 (7.19g) was incubated at 39°C with the bacterial laminarinase preparation (887mg) dissolved in 25mM-sodium citrate buffer, pH5.0 (750ml). The digest was slowly stirred throughout the incubation; toluene was present, to minimize microbial contamination. Samples (1ml) were withdrawn at intervals, diluted to 50ml with water, and the reducing power measured. After 125h a further 206mg of enzyme preparation was added. Paper-chromatographic analysis showed the presence of large amounts of glucose, laminari-biose, and -triose, and much smaller amounts of laminari-tetraose and -pentaose, showing that extensive degradation had taken place. After 9, 24, 95, 121, 167 and 191h, the apparent percentage conversion into glucose was 17, 29, 34, 34, 35 and 34 respectively. After 195h, the digest was heated to 100°C for 30min, and the solution dialysed against distilled water (six successive changes, each of 3 litres). The non-diffusible fraction was centrifuged to remove an insoluble grey precipitate (fraction A). The supernatant solution was concentrated and again dialysed against distilled water. Ethanol (2.5vol.) was then added at 0°C, and the white precipitate (fraction B) that formed (resistant glucan) was washed with ethanol and ether and then air-dried (yield, 0.86g). The ethanol washings and aq. ethanol supernatant solution were evaporated to dryness on a rotary evaporator at 40°C to give 0.15g of a brown syrup (fraction C).

Qualitative analysis (see Table 1) showed that the enzyme preparation contained substantial amounts of carbohydrate including polymers of galactose and mannose, but that fraction B was almost free of this contaminating material. Fraction C resembled fraction B in overall composition, but had a higher solubility in ethanol and hence presumably a lower molecular weight, and contained rather more contaminating carbohydrate from the enzyme preparation. Fraction A was not considered further. The overall yield of enzymically resistant glucan was about 13% of the original yeast glucan.

Fraction B (10mg) was incubated with the enzyme

Table 1. *Composition of products of enzymic degradation of yeast glucan*

Hydrolysates obtained by the total acid hydrolysis of fraction A and the enzyme preparation, and by the partial acid hydrolysis of fractions B and C, were examined qualitatively by paper chromatography (solvents *A* and *B*). The intensity of spots is indicated by: ++++, heavy; +++, strong; ++, medium; +, light; ±, trace; −, nil.

Hydrolysate	Products						
	Glucose	Galactose	Mannose	Gentiobiose	Gentiosaccharides	Laminaribiose	Laminarisaccharides
A	+++	+	+	+	−	−	−
B	++++	++	++	+++	++	++	−
C	++++	+	+	+++	++	+	−
Enzyme preparation	++	+++	+++	−	−	−	−

preparation (1mg) in 1ml of buffer at 37°C for 3 days. By paper chromatography, only trace amounts of glucose, laminaribiose and laminaritriose could be detected with difficulty, so that degradation of the original yeast glucan was therefore essentially complete. Fraction B was subsequently used for structural analysis.

Results

Properties of the enzymically resistant glucan

As shown in Table 1, partial acid hydrolysis gave a mixture of glucose, gentiobiose and higher gentiosaccharides and a trace of laminaribiose, but higher laminarisaccharides were not produced. On incubation with an endo-β-(1→6)-glucanase from *P. brefeldianum*, glucose and gentiobiose were produced initially; glucose was the final product.

Fraction B was converted into the corresponding alcohol (glucanitol) by treatment with KBH_4. On total acid hydrolysis it gave glucose and a trace of D-glucitol (sorbitol) as the only products of hydrolysis (paper chromatography in solvents *A*, *D* and *F*). By visual inspection, the proportion of sorbitol was less than the proportion of mannitol in a similar hydrolysate of laminarin (which contains 2–3% of mannitol; Annan *et al.*, 1965), indicating that the glucanitol must have a higher molecular weight than laminarin. The degree of polymerization of fraction B, as determined by the sorbitol dehydrogenase method (Manners *et al.*, 1971), was 127±10.

The glucanitol (147mg) was methylated by the procedure of Haworth (1915), and then by treatment with dimethyl sulphate and solid NaOH with tetrahydrofuran as solvent. The methylated glucanitol (yield, 113mg or 75%; OMe, 42.5%) was methanolysed and examined by g.l.c. on a column of polyethylene glycol adipate at 175°C. The following methyl glucosides were identified: 2,3,4,6-tetra-, 2,3,4-tri-, 2,4,6-tri-, 2,4-di- and 2,3-di-*O*-methylglucose. The relative peak areas of the two tri-*O*-methyl isomers was 4.4 to 1, and of the two di-*O*-methyl isomers was 12:1.

An acid hydrolysate of the methylated glucanitol, on examination by paper chromatography (solvent *B*), contained the same mixture of methylated sugars. The 2,3-di-*O*-methyl isomer gave a brown colour with aniline oxalate, and was differentiated from all the others, which gave a red stain.

Methylated glucanitol (48mg) was hydrolysed successively with 90% formic acid followed by 1M-H_2SO_4. The neutralized hydrolysate was analysed by quantitative paper chromatography (solvent *B*) by using hypoiodite oxidation. The relative amounts of tetra-, tri-, di- and mono-*O*-methyl sugars were 12, 59, 26 and 3% respectively. With the information from the g.l.c., the full composition of the hydrolysate is therefore: 2,3,4,6-tetra-, 12%; 2,3,4-tri-, 48%; 2,4,6-tri-, 11%; 2,4-di-, 24%; 2,3-di, 2%; mono-*O*-methylglucose, 3%. The latter two sugars are considered to be the result of undermethylation, and not to be structurally significant. It is concluded that in the glucan, (a) about one-half of the glucose residues are linked only by C-1 and C-6, (b) there are about 12% of non-reducing terminal glucopyranose residues, (c) there are about 11% of glucose residues linked only by C-1 and C-3 and (d) about one-quarter of the glucose residues are triply linked at C-1, C-3 and C-6.

When the glucanitol was oxidized by sodium metaperiodate, the initial reduction of periodate corresponded to 1.62mol per anhydroglucose residue. Under similar conditions, the alcohols prepared by the borohydride reduction of luteose and pustulan reduced 1.95 and 1.82mol. prop. respectively.

The glucanitol (100mg) was subjected to the Smith-degradation procedure (see Goldstein *et al.*, 1965), the resulting polyalcohol being partially hydrolysed by 0.05M-H_2SO_4 at 20°C for 15h. The hydrolysate was neutralized ($BaCO_3$), the solution centrifuged to remove $BaSO_4$, and then concentrated to a syrup. This was then chromatographed on Whatman 3MM paper with solvent *D*, and the components eluted, concentrated and identified. Fraction 1 was non-reducing, and had the same R_{Glc} value as glycerol in solvents *A*, *D* and *E*. Fraction 2 was reducing, and had the same R_{Glc} value as glycollic aldehyde in the same three solvents. Fraction 3 had R_{Glc} 1.00 in solvent *D*, but was non-reducing. After total acid hydrolysis, it gave equal amounts of glucose and glycerol (visual estimation of paper chromatograms) and is tentatively identified as a glucosylglycerol.

Properties of the glucan obtained by the fractionation of yeast cell walls

The glucan had $[\alpha]_D^{20}$ −32° (*c* 1 in water), and gave an i.r. spectrum which was very similar to that of the β-(1→6)-glucans pustulan and luteose, and differed from that of laminarin. On partial acid hydrolysis, it gave sugars having the R_{Glc} values (in solvent *A*) of glucose, gentiobiose, gentiotriose, gentiotetraose and gentiopentaose with a trace of laminaribiose.

The glucan (5mg) was methylated by treatment with methylsulphinyl sodium and methyl iodide in methyl sulphoxide (Hakomori, 1964). The methylated polysaccharide was hydrolysed successively with 90% formic acid and 0.25M-H_2SO_4 and neutralized ($BaCO_3$), and the sugars were converted into alditol acetates and analysed by quantitative g.l.c. and mass spectrometry (Björndal *et al.*, 1967*a*,*b*). The results are given in Table 2. There was evidence for the presence of a trace of the 2,3-di-*O*-methyl isomer (retention time 5.39) near the peak due to the 2,4-di-*O*-methyl sugar, which may indicate a slight degree

Table 2. *Composition of acid hydrolysate of methylated β-(1→6)-glucan*

The retention time of the derived alditol acetate was obtained by using an ECNSS-M column at 170°C and is given relative to the alditol acetate of 2,3,4,6-tetra-*O*-methylglucose. (ECNSS-M is a co-polymer of ethylene glycol succinate and cyanoethylsilicone.)

Sugar	Retention time	Amount (mol/100mol)
2,3,4,6-Tetra-*O*-methylglucose	1.00	16
2,4,6-Tri-*O*-methylglucose	1.95	5
2,3,4-Tri-*O*-methylglucose	2.50	65
2,4-Di-*O*-methylglucose	5.13	14

of undermethylation. These results show that (a) about two-thirds of the glucose residues are linked only by C-1 and C-6, (b) there are about 16% of non-reducing terminal glucopyranose residues, (c) there are about 5% of glucose residues linked only by C-1 and C-3 and (d) about 14% of the residues are triply linked at C-1, C-3 and C-6.

On oxidation with sodium metaperiodate, the initial reduction of periodate amounted to 2.06mol. prop., thus confirming the presence of a high proportion of triol groups. Application of the Smith-degradation procedure again gave a mixture of glycerol, glycollic aldehyde and glucosylglycerol.

The borohydride-reduced glucan was subjected to acid hydrolysis, and the neutralized ($BaCO_3$), concentrated hydrolysate chromatographed on paper by using solvent *A*, together with synthetic mixtures containing (a) 1% of sorbitol and 99% of glucose and (b) 10% of sorbitol and 90% of glucose. The hydrolysate contained only a trace of sorbitol which was similar to the 1% standard, suggesting a degree of polymerization of the order of 100. This value was confirmed quantitatively by using the sorbitol dehydrogenase method, which indicated a degree of polymerization of 141 ± 10.

The enzymic degradation of the glucan was examined. In qualitative experiments, the polysaccharide was not attacked by the bacterial laminarinase preparation, by almond β-glucosidase or by an endo-β-(1→3)-glucanase from *R. arrhizus*. However, an endo-β-(1→6)-glucanase from *P. brefeldianum* caused random hydrolysis of the glucan, and also of pustulan and luteose, giving a mixture of glucose and gentiosaccharides, having a degree of polymerization 2–5. In addition, the yeast β-(1→6)-glucan gave an oligosaccharide (R_{Glc} 0.31 in solvent *A*), which reached a maximum concentration after 24h and then gradually disappeared.

This oligosaccharide was isolated from a digest of 20mg of β-(1→6)-glucan by preparative paper chromatography in solvent *A*. It had R_{Glc} 0.32 and differed from an authentic sample of 6^2-β-glucosyllaminaribiose, which had R_{Glc} 0.39. A partial acid hydrolysate contained glucose, gentiobiose and laminaribiose. After borohydride reduction and partial acid hydrolysis, a mixture of glucose and laminaribiose as reducing sugars, and sorbitol and gentiobiitol as non-reducing carbohydrates was produced. On incubation with almond β-glucosidase, a mixture of glucose and gentiobiose was liberated. Collectively, these results characterize the oligosaccharide as 3^2-β-glucosylgentiobiose.

Discussion

The polysaccharides isolated from yeast glucan either by selective enzymolysis of the major β-(1→3)-glucan component, or by fractionation involving prolonged extraction with hot acetic acid, appear to be very similar to each other. Both are branched β-(1→6)-glucans, having a degree of polymerization of about 130–140 and containing a small proportion of β-(1→3)-glucosidic linkages, which may serve as both inter-residue and interchain linkages. Hence one may conclude that this represents the general structure of the minor component of yeast glucan. The structural analysis of both polysaccharide preparations was necessary, since it was possible that the minor component could have been partly degraded by the action of the bacterial laminarinase or during the fractionation. The general similarity of the results would appear to rule out both of these possibilities.

Although β-(1→3)-glucans are widely distributed in Nature, there are surprisingly few reports of β-(1→6)-glucans. Luteose produced by *Penicillium* moulds, pustulan from the lichen *Umbilicaria pustulata* and a glucan from the yeast *Candida albicans* all contain a high proportion of β-(1→6)-glucosidic linkages (Anderson *et al.*, 1939; Lindberg & McPherson, 1954; Bishop *et al.*, 1960), and have $[\alpha]_D$ −33°, −46° and −30° (in water) respectively. These values may be compared with $[\alpha]_D$ −32° for the yeast β-(1→6)-glucan, −9° for laminarin and −10° for yeast glucan (Anderson *et al.*, 1958; Misaki *et al.*, 1968).

The presence of β-(1→6)-linkages in the minor component was indicated by the results of partial

acid and enzymic hydrolysis and the i.r. spectrum; the identity of the (1→6)-linkages was also indicated by the periodate oxidation results, and was established unequivocally by methylation. The two methylation analyses are in qualitative agreement with respect to the types of inter-residue and interchain linkages, and the proportion of non-reducing end-groups. The results from the g.l.c.–mass spectrometry study have the greatest quantitative significance. They clearly show the branched nature of the polysaccharide, since the proportion of end-groups (about one per six residues) is so much less than the degree of polymerization (about 140), and in fact represents a relatively high degree of branching. This latter feature may be responsible for the relative solubility of the polysaccharide, since the possible alignment of adjacent molecules to form insoluble aggregates will be restricted.

The resistance of the polysaccharide to hydrolysis by endo-β-(1→3)-glucanases shows that sequences of adjacent β-(1→3)-glucosidic linkages were absent, a conclusion supported by the absence of laminaritriose and higher laminarisaccharides from partial acid hydrolysates. These facts also make it improbable that the small amount of 2,4,6-tri-*O*-methylglucose arose from a contaminating soluble β-(1→3)-glucan. The location of all the β-(1→3)-glucosidic linkages has not been established. About 75% of them are present as interchain linkages at triply linked glucose residues, and the remainder as inter-residue linkages. The latter may be the source of the trisaccharide 3^2-β-glucosylgentiobiose which was obtained by enzymic degradation of certain β-(1→6)-glucosidic linkages. However, it is not yet possible to describe the glucan in terms of singly branched, side-chain branched, or multiply branched structures.

The characterization of yeast glucan as a mixture of a high-molecular-weight β-(1→3)-glucan containing about 3% of β-(1→6)-glucosidic linkages, and a lower-molecular-weight β-(1→6)-glucan containing about 19% of β-(1→3)-glucosidic linkages is in accord with the partial acid hydrolysis studies on yeast glucan (Peat *et al.*, 1958). These workers isolated eight sugars including laminari-biose and -triose, gentio-biose, -triose and -tetraose, and the isomeric trisaccharides 6^2-β-glucosyl-laminaribiose and 3^2-β-glucosylgentiobiose. However, this work indicated a linear structure, and the present results emphasize the need for the determination of both the proportion of non-reducing end-groups and the degree of polymerization to enable the possibility of a branched structure to be decided.

The proposed structure for the minor component is generally similar to that of the glucan synthesized by *C. albicans*, which on methylation analysis gave the same di- and higher-methylated sugars as shown in Table 2, but in rather different proportions (Bishop *et al.*, 1960). The other known β-(1→6)-glucans (luteose and pustulan) appear to have essentially linear structures (Anderson *et al.*, 1939; Hellerqvist *et al.*, 1968).

The presence of two distinct, but chemically related, glucans in the cell walls of baker's yeast poses questions, as yet unanswered, concerning their respective functions and their modes of biosynthesis, including the biological control of the relative rates of synthesis. It may well be that some of the variations in the composition and structure of yeast cell walls when grown under different conditions, e.g. under NH_4^+ limitation, are caused, in part, by alterations in the relative amounts of the two glucan components (see for example, McMurrough & Rose, 1967).

Since the above work was carried out on baker's yeast (*S. cerevisiae*), it was of interest to examine cell-wall preparations from other yeasts. Preliminary studies have shown that preparations from *Kloeckera apiculata*, *Schizosaccharomyces pombe*, *Saccharomyces fragilis* and *Saccharomyces fermentati* were also heterogeneous, and periodate-oxidation analysis suggests that the content of β-(1→6)-glucan is rather greater than in baker's yeast (D. J. Manners, A. J. Masson & J. C. Patterson, unpublished work).

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