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Spectral Analysis of Glucan Produced by Wild-Type and Mutant *Saccharomyces cerevisiae*

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ABSTRACT

Carbon-13 NMR was employed to determine the chemical structures of the cell-wall β-glucans isolated from a wild-type and a mutant strain of yeast. The results illustrate distinct differences in frequency and average length of the β(1–6)-glycosidic branching of the glucans. The higher frequency of β(1–6) branching in the mutant strain is consistent with the result of previous enzyme digestion experiments correlating the degree of hydrolysis with the frequency of the β(1–6) linkage.

INTRODUCTION

The glucose homopolymer, β-glucan, is the major component of yeast cell walls and is responsible for the structural rigidity of yeast cells. Generally, yeast glucans have a β-(1–3)-D-linked backbone with periodic branching via β-(1–6)-D-linkages (Manners *et al.*, 1973). In spite of the simplicity of yeast's generally acknowledged structure, the yeast polysaccharides have not yet been thoroughly characterized. To date, structural studies that have been conducted are mainly of traditional chemical techniques (i.e. methylation analysis or Smith degradation) yielding varying results. In contrast, the structures of similar β-glucans from other sources (plants, fungi, bacteria) have been studied with more current techniques, such as ^{13}C-NMR studies on solutions of fractionated glucans (Michell & Scurfield, 1970; Colson *et al.*, 1974; Saito *et al.*, 1977*a*, *b*).

This paper describes a ^{13}C-NMR study for elucidation of structural differences in Yeast glucans. Specifically, a mutant, *Saccharomyces cerevisiae* R4, which synthesizes a modified cell-wall glucan, has been isolated (Jamas *et al.*, 1986). A single gene, *gls*1, which increases the $\beta(1-6)$ branching when transformed into yeast, has been isolated and characterized (Jamas, S. & Sinskey, A. J., unpublished). ^{13}C-NMR spectra have been obtained for glucan preparations from the wild-type strain A364A, the mutant R4, and strains transformed with the *gls*1 allele. The biosynthesis and purification of these samples provided the first opportunity to evaluate structural differences in cell-wall glucans.

BACKGROUND

The glucan particles that make up 80–85% of the total glucan present in yeast cell walls can be described as ghost cells. The ghost cells maintain the original geometry and morphology of the cell. This major glucan component has been shown to retain the structural and mechanical properties of yeast cells. A gross structural characterization of glucan particles from strains A364A and R4 has been made by rheological analysis (Jamas, 1983). The results suggested that the mutant R4 glucans contain a higher degree of $\beta(1-6)$ branching. Subsequent characterization of the hydrodynamic properties of glucan particles in aqueous suspension supported this hypothesis (Jamas *et al.*, 1986). Furthermore, R4 glucans were more resistant to $\beta(1-3)$ hydrolytic enzymes, and this was attributed to the greater extent of $\beta(1-6)$ branching.

EXPERIMENTAL

Strains

The strains of *S. cerevisiae* used in this study are listed in Table 1. All yeast strains were grown in YEPD (1% yeast extract, 2% bacto-peptone, 2% glucose). Agar plates of the above media contained 2% bacto-agar.

TABLE 1
List of *S. cerevisiae* Strains

Strain	*Genotype*
A364A	a, *ade*1, *ade*2, *his*7, *tyr*1, *gal*1
R4	a, *ade*1, *ade*2, *his*7, *tyr*1, *gal*1, *gls*1

Materials

Laminarin ($\beta(1-3)$ glucan, molecular weight = 1600 (Sigma Chemical Co.)) and β-gentiobiose [*O*-β-D-glucopyranosyl-(1–6)-β-D-glucopyranose] were used to obtain standard infrared and ^{13}C-NMR spectra.

Extraction and purification of glucan particles

The extraction procedure is described in detail by Jamas *et al.* (1986).

Infrared spectroscopy

Fourier-transform-infrared spectra were obtained for the cell-wall glucan particles. The samples were prepared in solid potassium bromide discs, containing 2% w/w of the glucan. All the films were made with 100 mg total material compressed at 64 N m torque. An IMB IR/32 FTIR instrument was used to obtain transmission spectra in the 400–4000 cm^{-1} wavelength range.

Sugar composition analysis

Trifluoroacetic acid was used to hydrolyze the glucan particles to their constituent monosaccharide sugars. This procedure is not destructive on mannose and glucose sugars and can therefore be used as a quantitative analysis (Albersheim *et al.*, 1967). The glucan particles (20 mg) from strains A364A and R4 were suspended in 2 ml of 2N trifluoroacetic acid in a sealed tube and held at 121°C for 90 min. Under these conditions, the glucan particles were completely hydrolyzed as indicated by the lack of an insoluble residue. After hydrolysis, the acid was evaporated off at 50°C under a stream of filtered air. The hydrolyzate was dissolved in 2 ml water and evaporated to dryness twice to remove traces of trifluoroacetic acid and was finally dissolved in 2 ml water. An HPLC Waters Model 6000A (Waters Associates, MA), equipped with a lead-loaded cation exchange column (Brownlee Polypore, PB), was used to analyze the hydrolyzates for sugar composition. The column and mobile phase (CH_3CN/H_2O) were kept at 85°C, and a flow rate of 0·5 ml/min was used. The column eluants were monitored by a differential refractometer.

Glucan extraction

A soluble glucan from the alkali insoluble particles was extracted by depolymerizing the minor chitin component as described by Sietsma and

Wessels (1981). Glucan particles (150 mg) were suspended in 15 ml of water in a 50-ml Falcon tube. The nitrous acid was prepared by adding 22·5 ml of 2 M sodium nitrite and 7·5 ml of 2 M hydrochloric acid to the suspension and tightly sealed the tube. The suspension was shaken gently for 1 h at room temperature and then centrifuged at 6000 rpm for 10 min. The pellet was resuspended in 75 ml of 1 M potassium hydroxide and was held at 50°C for 30 min in an Erlenmeyer flask purged with nitrogen. The solution was centrifuged at 8000 rpm for 10 min to remove the insoluble residue. The solubilized glucan was precipitated by the addition of 3 volumes of ethanol, washed twice in ethanol and once in ethyl ether and dried at 37°C to obtain a white powder.

Carbon-13 NMR spectroscopy

The ^{13}C-NMR spectra were recorded with a Bruker 270 at 69·0 MHz, operating in the pulsed Fourier-transform mode with complete proton-decoupling to remove the nuclear Overhauser enhancements. ^{13}C Chemical shifts were expressed in parts per million downfield from external tetramethylsilane and were all measured digitally. Generally, over 2000 scans were necessary to obtain spectra with satisfactory signal/noise ratios. All measurements were made at ambient temperature (22°C). Samples (0·5 ml total volume) were prepared in D_2O (40 mg/ml) or NaOD (80 mg/ml) by heating at 65°C for 10 min and loaded into 5 mm o.d. tubes.

RESULTS AND DISCUSSION

Composition of yeast glucan particles

The procedure for preparing the glucan particles consisted of a series of alkaline extractions that dissolve cytoplasmic material, cell-wall mannans and proteins, and a minor glucan component (approximately 15% of total glucan). These extractions result in an insoluble residue that maintains the morphology of the cell. Carbohydrate and protein assays (Jamas, 1983) on this residue have indicated a $>99\%$ w/w hexose content and a low protein contamination ($<1\%$ w/w). However, chitin, which represents only 1% of the cell-wall mass (or 0·2% of the dry cell weight) is not extracted in this procedure, and the glycogen content may vary depending on the age of the cells.

The homogeneity of the glucan particle preparations was investigated qualitatively by Fourier-transform-infrared spectroscopy (FT-IR). FT-IR

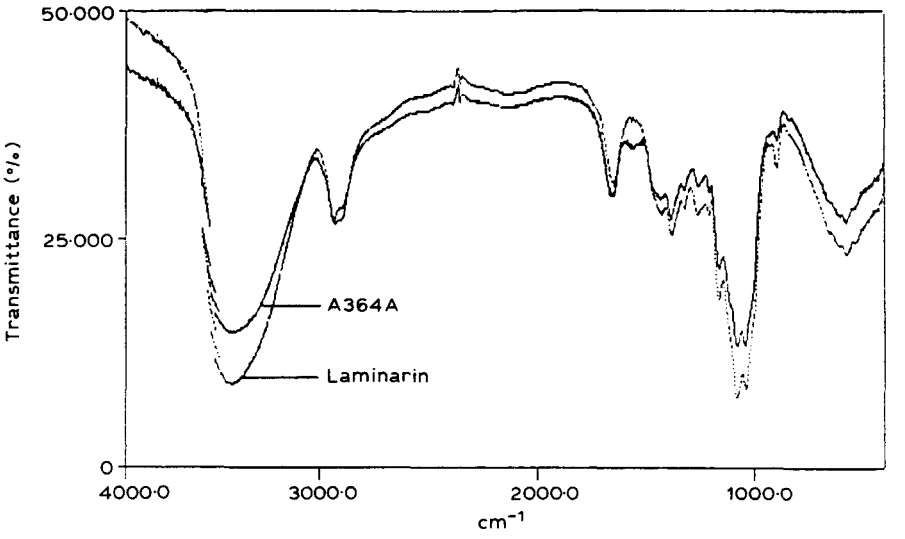

Fig. 1. FT-IR spectra of laminarin and A364A glucan particles. Spectra represent 32 scans and are recorded in percentage transmittance with 4-cm^{-1} resolution.

spectra readily identify the composition of polysaccharides with respect to their predominant monomeric constituents and anomeric structure. FT-IR spectra of the wild-type (Fig. 1) and mutant glucan particles were compared with that of a laminarin of a low molecular weight ($M = 1600$), predominantly $\beta(1-3)$-linked glucan isolated from *Laminaria digitata*.

The FT-IR spectra suggest that the yeast glucan preparations contain a predominantly $\beta(1-3)$-linked polysaccharide. The samples contain the band at 890 cm^{-1}, which is a characteristic feature of polysaccharides having the β-configuration, in addition to the bands that distinguish $\beta(1-3)$ linkages at 2920, 1370, 1250, and 1200 cm^{-1} (Michell & Scurfield, 1970). These spectra could not, of course, indicate any structural differences that exist between the glucans with respect to the relative proportions of $\beta(1-3)$- and $\beta(1-6)$-linkages.

A quantitative sugar analysis of the glucan particles confirmed that the composition derived from the IR spectra. The glucan particles were hydrolyzed with trifluoroacetic acid to their monosaccharide components under optimal conditions for glucose and mannose sugars (Albersheim *et al.*, 1967), and the product was analyzed by high-pressure liquid chromatography. Glucose (retention time = 8·86 min) was the predominant monosaccharide present in both samples.

No differences in the compositions of the parental and mutant glucan structures could be established at that point, which strengthened the

hypothesis that the mutation in strain R4 had targeted the β-glucan structure and not the constituent monomer sugar of the cell-wall polymers.

Carbon-13 NMR structural analysis: determination of side-chains in parental and mutant glucans

The degree of molecular complexity in a polysaccharide molecule can be readily ascertained from the number and relative intensities of ^{13}C signals in a spectrum. A sample of a soluble fraction was prepared to represent the glucan-particle composition accurately and to obtain a well-resolved spectrum for quantitative evaluation of different linkage configurations.

Approximately 50% by weight of the glucan particles were solubilized by treatment with nitrous acid and potassium hydroxide. The water-soluble fractions of this preparation were used to obtain the ^{13}C-NMR spectra. Quantitative ^{13}C-NMR spectra of the soluble glucan samples were obtained in the inverse-gated ^{1}H decoupled mode, in which nuclear Overhauser enhancement was suppressed and the signal-to-noise ratio greatly improved. The decoupler was on during accumulation of data but off during a pulse delay of at least twice the longest relaxation time of the molecule.

The ^{13}C-NMR spectra of laminarin (a low molecular weight $\beta(1-3)$-linked glucan) and soluble yeast glucans in D_2O are shown in Fig. 2. Laminarin, the calibration standard, gave a well-resolved spectrum with shift assignments characteristic of $\beta(1-3)$-D-linked glucose homopolymers (Colson *et al.*, 1974). By contrast, the yeast glucan spectra were characterized by low signal intensities and significant line broadening, resulting in poor resolution. However, the signals of the yeast glucan spectra can be unequivocally assigned to β-D-(1–6)-linked glucose residues by comparison with the ^{13}C chemical shifts of laminarin and gentiobiose (Table 2).

At least two C-6 signals (shown as 1 and 2 in Fig. 2) are recognizable and correspond to a 6-*O*-substituted C-6 (internal β-D-(1–6)-linked unit) that is shifted strongly downfield to 69·2 ppm, and a free (unlinked) C-6 at 61·05 ppm belonging to the non-reducing residue of the side-chain. These results suggested that the side-chain consists of a β-D-(1–6)-linked oligosaccharide. Had the side-chain consisted of a single unit, as has been proposed for the yeast glucan structure (Manners *et al.*, 1973), the molecule would not have had an internal 6-*O*-substituted C-6, and a signal at 69·2 ppm (present in our spectra) would therefore not have been observed. Furthermore, the shoulder at 69·89 ppm (indicated as b

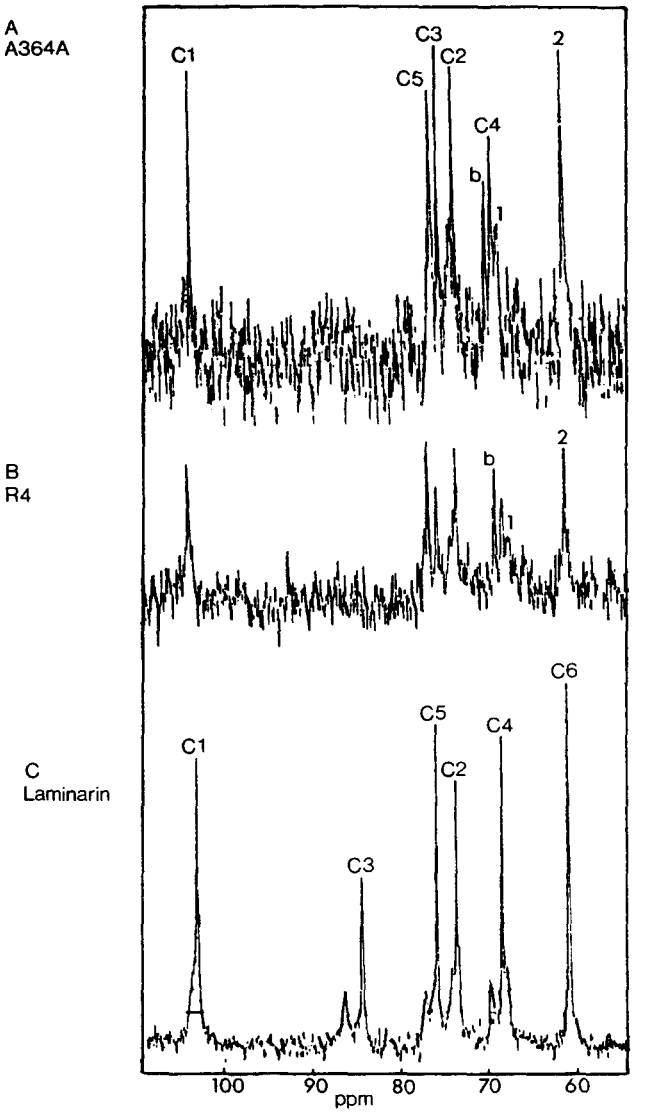

Fig. 2. ^{13}C-NMR spectra of glucans in D_2O. Sample concentration is 40 mg/ml. Carbon assignments in spectra A and B represent the $\beta(1-6)$-linked side-chain residue. Signals in spectrum C represent $\beta(1-3)$-linked glucose units.

in the spectra) might represent the 3,6-Di-*O*-substituted C-6 at the branch point of the main chain. The chemical shift of this carbon atom is expected to be displaced further downfield from the C-6^1 signal (69·2 ppm) owing to the involvement of this residue in the $\beta(1-3)$ backbone linkages.

TABLE 2
^{13}C-NMR Chemical Shifts of Glucans in D_2O

C-atom	*Laminarin β-D-(1–3)*	*Chemical shifts (ppm)*		*R4*
		Gentiobiose β-D-(1–6)	*A364A*	
C-1	102·82	103·30	103·34	103·34
C-2	73·54	73·35	73·37	73·39
C-3	84·34	75·60	75·24	75·20
C-4	68·35	69·70	69·75	69·79
C-5	75·86	76·10	76·01	76·05
C-6	60·92	69·00	69·16	69·17
C-6*		61·10	61·05	61·05

*Unlinked C-6 at non-reducing end.

It is also interesting to note that no signal representing C-3 involved in a β-D-(1–3) glycosidic linkage at 84·3 ppm was detected in spite of the confirmation of the IR spectra that yeast glucans and laminarin predominantly contain β-D-(1–3) linkages. The signals observed for the glucans in D_2O (Fig. 2), probably represent only the β-D-(1–6) linked side-chain portion of the molecules. The ^{13}C signals of the β-D-(1–3)-linked residues of the main chain could be completely suppressed by the immobilization of the main chains in the gel network that was formed by these glucan samples. This has been a general observation in ^{13}C spectral studies of high molecular weight glucans with a β-D-(1–3)-linked backbone (Ogawa *et al.*, 1973; Saito *et al.*, 1977*a*, *b*) and arises from a tendency of these molecules to adopt an ordered conformation in aqueous environments, which limits molecular motion of the carbons in the backbone chain.

In order to resolve the ^{13}C signal of the β-D-(1–3) residues, glucan solutions were prepared in 0·4 M deuterated sodium hydroxide (NaOD). This prevented gel formation, and hence the backbone chains of the glucan molecules would not be immobilized in a conformationally rigid state. The highly resolved spectra of these samples are shown in Fig. 3 in which the chemical shifts characteristic of the β-D-(1–3)-linked backbone are clearly observed. Assignment of the ^{13}C chemical shifts from this spectrum is summarized below in Table 3 (Saito *et al.*, 1977*a*, *b*; Gorin, 1981; Bock *et al.*, 1982).

The C-6^1 and C-6^b signals (displaced approximately 8 ppm downfield from C-6 of the β(1–3) backbone) are detectable in both spectra of Fig. 3. The chemical shift of the unlinked C-6 carbon (C-6^2) on the non-

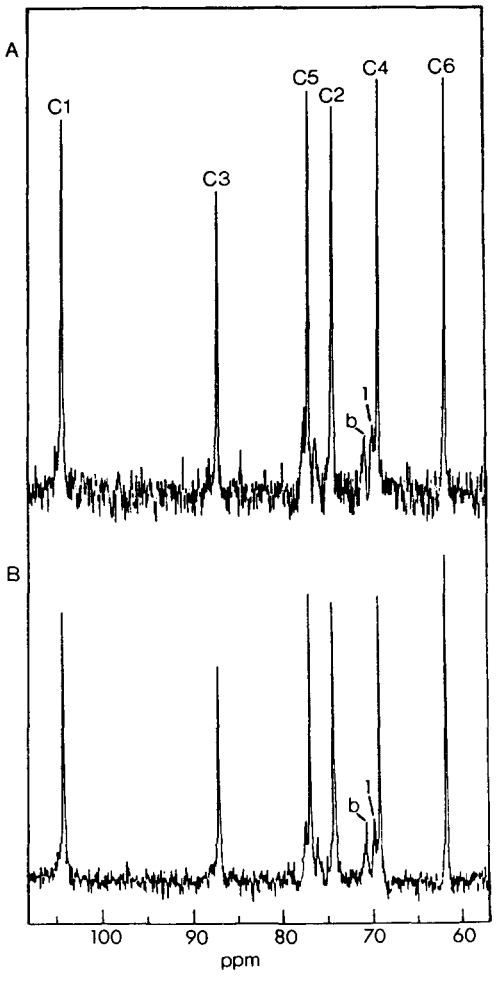

Fig. 3. ^{13}C-NMR spectra of HNO_2/KOH extracted glucans (80 mg/ml) in 0·4 M NaOD: A, A364A glucan; B, R4 glucan. C-1–C-6 assignments represent β(1–3)-linked units.

reducing end of the side-chain at approximately 61·1 ppm is assumed to be hidden under the strong C-6 signal of the backbone residues at 61·05 ppm. In this case, the intensity of the signal would represent the abundance of both nuclei. Signals of the C-2, C-3, and C-5 carbons in the side-chains are very weak but can still be detected, which demonstrates the presence of β-D-(1–6)-linked side-chains.

The sharp resolution of the spectra obtained in NaOD allowed a quantitative analysis to be undertaken of the degree of branching in the parental and mutant glucans solubilized by the HNO_2/KOH procedure.

TABLE 3
^{13}C-NMR Chemical Shifts of Glucans in NaOD

C-atom	*Laminarin*	*Chemical shifts (ppm)* *A364A*	*R4*
C-1	103·66	103·92	104·08
C-2	73·84	74·10	74·24
C-3	86·24	86·84	87·03
C-4	68·60	68·97	69·11
C-5	76·25	76·73	76·84
C-6*	61·15	61·48	61·64
C-6[1]	—	69·56	69·78
C-6[b]	—	70·35	70·60
C-2 (1–6)	—	73·98	74·08
C-3 (1–6)	—	75·87	76·08
C-5 (1–6)	—	77·11	77·10

[1]6–0 Substituted C-6 (linked) in side-chain.
[b]3,6-Di-*O*-substituted at branch point of main chain.
*Includes the free C-6 at non-reducing end of side-chain (C-6[2] in Fig. 4).

The objective of this study was to establish the linkage type of the yeast glucans and the fequency of β-D-(1–6) branching as it was indicated in the earlier study by the resistance of cell walls to β(1–3) glucanase enzymolysis (Jamas *et al.*, 1986). The degree of β(1–6) branching could be calculated by comparing the signal integrals of analogous nuclei in the spectra. In such an analysis, integration rather than peak intensity is a preferable measure of the abundance of nuclei, since line widths may vary in a given spectrum (Rinando & Vincendon, 1982). The peak integrals of the nuclei of interest are summarized in Table 4. These values were used in the quantitative analysis of glucan structure that follows.

The degree of branching was calculated from the abundance ratio of the C-6 nuclei involved in the side-chain (C-6[b]) to the C-6 nuclei of the β(1–3) backbone (C6(1–3)) (see Table 5). Assuming linear side-chains, then the C-6[2] portion of the C-6* signal is equal to the C-6[b] integral, since we can assume that the number of C-6[2] atoms is equal to C-6[b] atoms (Fig. 4). The degree of branching is then equivalent to C-6[b]/(C-6[b] + [C-6* − C-6[2]]) = C-6[b]/C-6*. The reciprocal value of the degree of branching is a measure of the distance (in glucose units) between adjacent side-chains (Table 5). The average length of the side-chain can be determined as the ratio of C-6[1] to C-6[b] plus an additional unit contributed by C-6[2].

TABLE 4
Signal Integration of Glucan ^{13}C-NMR Spectra

C-atom	*A364A*	*R4*
C-1	9·410	14·375
C-2	9·773	12·677
C-3	5·984	10·725
C-4	7·577	10·156
C-5	7·313	13·093
C-6*	8·833	13·371
C-6^{1}	2·248	4·339
C-6^{b}	1·487	3·918

^{1}6–0 Substituted C-6 (linked) in side-chain.
^{b}3,6-Di-*O*-subsituted at branch point of main chain.
*Includes the free C-6 at non-reducing end of side-chain (C-6^{2} in Fig. 4).

TABLE 5
Degree of Branching in HNO_2/KOH-extracted Glucans

	A364A	*R4*
C-6^{b}/C-b* (% β(1–6) branching)	16·8	29·3
C-6*/C-6^{b} (Average distance between branch points)	5·9	3·4
[C-6^{1}/C-6^{b}] + 1 (Average length of side-chain)	2·5	2·1

*Includes the free C-6 at non-reducing end of side-chain (C-6^{2} in Fig. 4).
^{1}6–0 Substituted C-6 (linked) in side-chain.
^{b}3,6-Di-*O*-substituted at branch point of main chain.

These data became the first direct evidence of an increased degree of β(1–6) branching in the mutant R4 glucan; in fact, a greater than 70% increase in the β-D-(1–6) branching is suggested by these results.

On the basis of the extent of branching, the general structure of the repeating unit in the yeast glucan fraction solubilized by treatment with HNO_2 and KOH is now proposed here. For A364A glucan, a side-chain would occur at every sixth glucose unit of the backbone, whereas in R4 glucan the side-chain frequency is increased to about every third or fourth unit. An additional difference between the HNO_2/KOH-soluble parental and mutant glucans is in the average length of the β(1–6) side-chains. In A364A, the ratio C-b^{1}/C-6^{b} is 1·5, suggesting that the side-

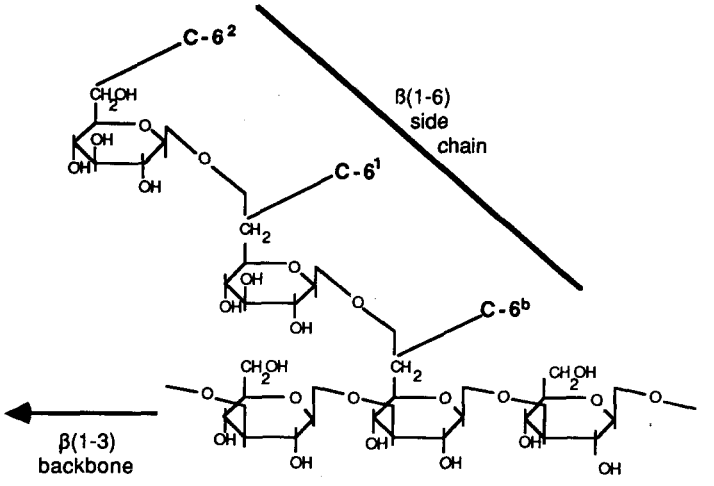

Fig. 4. Side-chain structure of yeast β-glucans: C-6^1, internal $\beta(1-6)$-linked C-6; C-6^2, free (unlinked); C-6^b, branch point on a $\beta(1-3)$-linked residue.

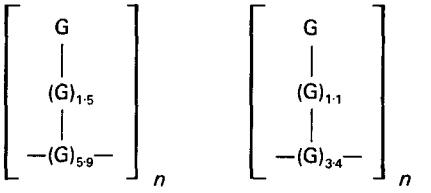

Fig. 5. Proposed structures for the repeating units in wild-type A364A (left) and mutant R4 glucan (right) fractions solubilized by HNO_2/KOH treatment.

chains may have more than two $\beta(1-6)$-linked glucose units. In the mutant glucan, the ratio C-6^1/C-6^b is 1·1, which thus maintains the proposed disaccharide side-chain (see Fig. 4). The general structures for the repeating unit in the parental and mutant glucans fractions are shown in Fig. 5.

These quantitative predictions, however, cannot be taken as absolute representations of glucan structures in yeast cell walls until the various glucan fractions are independently characterized. It is expected that the structure of the glucan will depend on the solubilization technique employed. For example, the signal integrals for the A364A glucan spectrum cannot be taken as absolute values owing to the low signal/noise ratio (the C-6^1 and C-6^b signals for the A364A glucan were not as clearly resolved above the noise at the baseline as those for the R4 spectrum). Furthermore, the lengthy treatment of glucan particles with

KOH and HNO_2 may have resulted in partial hydrolysis of the more acid-labile $\beta(1-6)$ linkages, rendering an absolute determination of $\beta(1-6)$ branching in the cell-wall glucans inaccurate. Thus, until the effects of these variables are quantitatively established, these results should be utilized more as a relative comparison between specific glucan sub-fractions and as an indication of structural differences that can be introduced by mutation of the producing organisms.

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