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THE MOLECULAR STRUCTURES OF SOME GLUCANS FROM THE CELL WALLS OF *Schizosaccharomyces pombe**

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ABSTRACT

Extraction of the cell walls of *Schizosaccharomyces pombe* with dilute alkali at 4° yields a mixture of polysaccharides including galactomannan, (1→3)-α-D-glucan, and a branched (1→3)-β-D-glucan. The alkali-insoluble residue contains a lightly branched (1→3)-β-D-glucan, together with smaller amounts of an extremely highly branched (1→6)-β-D-glucan. The properties of the three distinct β-D-glucans are compared with those isolated from other yeasts.

INTRODUCTION

The carbohydrate components of the walls of *Schizosaccharomyces* yeast, unlike baker's yeast (*Saccharomyces cerevisiae*), do not contain mannan or chitin, but consist of glucans and galactomannan[1]. The heterogeneous nature of the cell-wall glucans of baker's yeast has been established; an alkali-insoluble acetic acid-insoluble (1→3)-β-D-glucan[2], an alkali-soluble (1→3)-β-D-glucan[3], and an alkali-insoluble acetic acid-soluble (1→6)-β-D-glucan[4] have been isolated and characterized. Preliminary results[5,6] have suggested that the cell-wall glucans in other yeast genera may also be a complex mixture.

Extraction of cell walls of *Schizosaccharomyces pombe* with hot alkali by Deshusses and co-workers[7] resulted in solubilization of ~84% of the wall polysaccharide. The soluble portion was resolved into a glucan component that precipitated upon neutralization of the extract, and a supernatant fraction that contained a mixture of glucan, mannan, and galactan. The insoluble residue likewise consisted of glucan.

The existence of a (1→3)-linked α-D-glucan that was solubilized by alkali and precipitated upon neutralization of the extract has been known for some time[8]. This particular glucan has been investigated by Bush and co-workers[9], after solubilization of the β-D-glucan from the cell walls by an exo-(1→3)-β-D-glucanase. The insoluble residue from this digest, containing 28% of the wall carbohydrate, was characterized as, most probably, a linear (1→3)-α-D-glucan containing some 7% of (1→4)-linkages.

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*Dedicated to the memory of Sir Edmund Hirst, C.B.E., F.R.S.

Fractionation of the whole yeast cell-walls by hot alkali, hot acetic acid, and hot water has indicated[6] that the glucans in *Schiz. pombe* are also a mixture, as in *S. cerevisiae*[4]. The content of a minor glucan component, which was alkali-insoluble, but acetic acid-soluble, and probably (1→6)-β-linked, appeared to be rather greater than in *S. cerevisiae*. We now describe the results of an investigation of the alkali-soluble and alkali-insoluble acetic acid-soluble β-D-glucans isolated from the cell walls of *Schiz. pombe*.

MATERIALS AND METHODS

Organism and preparation of cell walls. — Cell walls of *Schizosaccharomyces pombe* C-277 were kindly provided by Professor H. J. Phaff (Department of Food Science and Technology, University of California, Davis, U.S.A.). The yeast was maintained and grown, and the cell walls were prepared as previously described[10]. The method of isolation and purification of the cell walls carefully avoided any endogeneous glucanase degradation.

Analytical procedures. — Procedures for partial and total acid hydrolysis, for the determination of reducing sugar, D-glucose, total hexose, and nitrogen, and for methylation analysis have been previously described[3]. Periodate oxidation and Smith degradations were also performed as described, except that the temperature of the oxidation was 2.5°. Additionally, for the Smith degradations, the oxidation was halted after 120 h. The d.p. of glucan preparations was determined from the D-glucitol content of an acid hydrolysate of the borohydride-reduced glucan[11]. Protein was determined by the method of Lowry and co-workers[12], with bovine serum albumin as standard protein. The quantitation of monosaccharide composition was performed by conversion of total acid hydrolysates into the corresponding alditol acetates[13]. These were then analysed by using the same g.l.c. column and conditions as for the analysis of methylated sugars, with the exception that the nitrogen flow rate was 60 ml/min.

Glycerol was analysed according to the procedure of Noble and Sturgeon[14]. All samples were centrifuged prior to assay, and a control sample (water) was included.

Qualitative descending paper chromatography was performed with ethyl acetate–pyridine–water (10:4:3), and alkaline silver nitrate was used to detect the sugars.

Materials. — Laminarin, pustulan[15], and cellobiose were obtained from the Departmental collection. Purified (1→3)-α-D-glucan from *Aspergillus niger*[16] was kindly provided by Professor H. J. Phaff. Dextran T-110 was purchased from Pharmacia.

Enzyme preparations. — Exo-(1→3)-β-D-glucanase (fungal "Glucanase GV", supplied by A/S Grindstedvaerket, Braband, Denmark) was purified as described previously[17]. The partly purified enzyme preparation showed some β-D-glucosidase activity, which was subsequently eliminated by adsorption of the glucanase on

CM-cellulose ion-exchanger (equilibrated with 9mM sodium acetate buffer pH 4.0) and elution with a linear salt gradient. The enzyme was eluted by 75mM sodium chloride. At this stage of purification, a trace of (1→3)-α-D-glucanase activity noted in the original preparation was no longer detectable. Salivary alpha-amylase was prepared and used as described[2].

Endo-(1→3)-α-D-glucanase[18], endo-(1→3)-β-D-glucanase[19], and endo-(1→6)-β-D-glucanase[19] were purified preparations from *Bacillus circulans* WL-12. Control experiments showed that the α-D-glucanase had no action on (1→3)-β-D-glucans, and similarly, that the endo-(1→3)-β-D-glucanase had no action on a (1→3)-α-D-glucan.

EXPERIMENTAL

Preparation of alkali-soluble glucan. — *Schizosaccharomyces pombe* cell-walls (9.96 g) were extracted with 3% sodium hydroxide (1.2 litres), under nitrogen, for 6 days at 4° (see Fig. 1). The material was stirred continuously, and re-flushed with nitrogen every 2 days. The extract was subsequently centrifuged at 24,000 *g* for 30 min at 0°, and the insoluble pellet was washed with a small portion of fresh alkali solution and re-centrifuged. The wash supernatant was combined with the main extract. The insoluble material was extracted a second time, as described above, with 500 ml of alkali; it was then neutralized with acetic acid, washed extensively with alcohol, and ethyl ether, and dried at 43° in a vacuum oven for 48 h. The alkali-insoluble material (R, 1.95 g) was used for the acetic acid extraction to be described later.

The supernatant solution from each alkali extraction, immediately after separation from the insoluble material, was filtered through glass paper to ensure that no particulate material was present. The clear solution was neutralized with dilute (~6M) acetic acid to pH 6.5 immediately after filtration. The precipitate that formed immediately was kept for 48 h at 4°, after which it was centrifuged (17,500 *g* for 30 min, 0°). The precipitate was washed twelve times with distilled water (at ~55°); this was facilitated by Potter homogenization to ensure efficient dispersion. The supernatant solutions from each wash, after centrifugation (17,500 *g*, 30 min and 0°), were combined with the original supernatant solution associated with this precipitate. The well-washed precipitate was finally washed with methanol and ethyl ether, and dried (vacuum oven at 43° for 48 h) to give fraction G-1 (3.87 g, as carbohydrate). Similar treatment of the second (500 ml) alkaline extract gave fraction G-2 (0.087 g, as carbohydrate).

The slightly hazy, supernatant solution, derived from G-1 and the washing steps, was concentrated by vacuum evaporation (40°) to 400 ml, and methanol (4 vol.) was added. After 48 h at 4°, the solution was centrifuged (17,500 *g*, 30 min, 0°) to give an alcohol-insoluble fraction and a supernatant (S-3) fraction. The insoluble fraction was resuspended in a small volume of distilled water (90°), Potter-homogenized, cooled, and reprecipitated by 4 vol. of methanol. After 48 h at 4°, the solution was centrifuged as before (the supernatant solution being discarded), washed with ethyl ether, and dried. The yield of alcohol-insoluble fraction (S-1) from the first alkali extraction was 2.11 g; S-2, the comparable fraction from the second alkali extraction, amounted to 0.15 g, but was not examined further. The methanol supernatant solution, S-4, obtained during this second alkali extraction was combined with the similar S-3 supernatant solution; S-3 and S-4 contained 0.1 g of unidentified carbohydrate and 0.4 g of solubilized protein.

The alcohol-insoluble material S-1 was further fractionated by treatment with Fehling's solution[20] at 2° under nitrogen. Immediately upon addition of the reagent (300 ml) to S-1, dissolved in 20 ml of distilled water, a precipitate formed, which necessitated a brief homogenization to obtain a uniform suspension. The mixture was kept at 2° for 24 h before centrifugation (20,000 *g*, 20 min, 0°). The pellet was added to 100 ml of fresh Fehling's solution, and treated as described previously, while the supernatant solution was kept for a further 24 h at 2° and re-flushed with nitrogen. Both solutions were centrifuged as previously described; the two pellets and the two supernatant solutions were each combined. The insoluble material was neutralized with 6M acetic acid and dialysed against frequently changed distilled water containing a trace of EDTA. Dialysis was continued for 14 days at 2°; in the last 24 h, only distilled water was used. The deionized solution was concentrated by vacuum evaporation (40°), after which methanol (4 vol.) was added (with a trace of ammonium acetate) to give a precipitate (C-1) that was washed with methanol and ethyl ether, and finally dried (vacuum oven) at 43° for 48 h (yield 0.54 g, as carbohydrate).

The combined supernatant (Fehling's) solutions were adjusted to pH 10.5 with 6M acetic acid, sparged with nitrogen for ~5 min, and kept at 2° for 24 h. During this time, another precipitate (C-2) formed that was separated by centrifugation (20,000 *g*, 20 min, 0°), neutralized, dialysed, concentrated, and dried (as described above for C-1), yielding 0.72 g as carbohydrate. The supernatant solution from C-2 was neutralized and dialysed as described above; again, precipitation occurred, but slowly over the period (14 days) of dialysis. Centrifugation (as for C-2) and drying (as for C-1) yielded C-3 (0.19 g, as carbohydrate). The supernatant solution from this last fraction was precipitated by 4 vol. of methanol, as for C-1, and gave a final fraction, C-4 (0.28 g, as carbohydrate).

Enzymic fractionation of G-1 to give separate α- and β-D-glucan fractions. — Aliquots of fraction G-1 were treated with either an exo-(1→3)-β-D-glucanase or an endo-(1→3)-α-D-glucanase; in both cases, the enzyme was still active at the end of a prolonged digestion period. A portion of G-1 (0.29 g, as carbohydrate) was uniformly suspended in distilled water by Potter homogenizations, and centrifuged (10,000 *g*), and the pellet was added to a solution (10 ml) of purified exo-(1→3)-β-D-glucanase (0.05 mg of protein/ml). The mixture was incubated in a dialysis tube, with dialysis against 0.1M sodium acetate buffer (pH 5.0, 500 ml) and 0.01% of sodium azide (as a bacteriostat). The temperature was maintained at 37° in a shaking water-bath. The ambient buffer solution was monitored for increases in reducing power, and was replaced with fresh buffer every 1–2 days until no further increases in reducing sugar were noted. After incubation for 27 days, the undigested material was collected by centrifugation (10,000 *g*, 20 min, 0°), washed with distilled water with Potter homogenization (repeated five times), washed with methanol (five times) and finally with ethyl ether, and dried (vacuum oven, 48 h at 43°). The yield of undigested residues (α-D-glucan) was 0.11 g (as carbohydrate). Another portion of G-1 (0.59 g, as carbohydrate) was suspended in a solution (35 ml) of endo-(1→3)-α-D-glucanase (0.09 mg of protein/ml). This mixture was placed in a dialysis tube and dialysed against distilled water (900 ml) containing mM EDTA and 0.01% of sodium azide. The incubation temperature was maintained at 30°, again in a shaking water-bath, and the ambient solution was changed every second day until no further increases in reducing power were noted. After 22 days, the insoluble material was collected by centrifugation, washed, and dried, as described for the exo-(1→3)-β-D-glucanase digest. The yield of undigested residue (β-D-glucan) was 0.36 g (as carbohydrate).

Enzymic fractionation of C-2. — A sample of C-2 (0.67 g) was digested with the endo-(1→3)-α-D-glucanase for 32 days, under conditions similar to those used for G-1. The yield of undigested carbohydrate (β-D-glucan) was 0.42 g (as carbohydrate) or 63% of the original C-2 sample. The reducing sugars which had been produced were analysed by paper chromatography after concentration of the solution.

Acetic acid extraction of alkali-insoluble residue. — The alkali-insoluble material (R, 1.95 g) was exhaustively extracted with 0.5M acetic acid (100 ml) at 90° for 3 h, as described previously[2]. After cooling to 4°, the solution was centrifuged (23,000 *g*, 30 min, 0°), and the pellet washed with fresh acid of the same concentration. After re-centrifugation, the wash supernatant solution was combined with the clear supernatant solution of the main extract. This combined extract was filtered through glass-fibre filter paper, neutralized, and dialysed against distilled water (4 l) at 4° for 48 h (the water being changed three times). The dialysed extract was then concentrated to 5 ml by vacuum evaporation (40°), methanol (4 vol.) was added, and the precipitate was washed with methanol (repeated five times) and ethyl ether, and dried (vacuum oven at 43°, 48 h). This acid extraction of R was repeated a total of seven times, giving yields of 0.106, 0.086, 0.065, 0.057, 0.050, 0.042, and 0.032 g, respectively, in order of extraction. The amount of residual, acid-insoluble, alkali-insoluble material (R-1) after these extractions was 1.505 g (as carbohydrate).

The first three fractions (A-1, A-2, and A-3; total, 0.258 g) were combined and then treated with salivary alpha-amylase (30 ml) to eliminate any possible contamination with glycogen[2]. Digestion (at 30° with shaking) in a dialysis tube, against 0.01M sodium chloride (75 ml) changed every 24 h, was continued for 10 days, at which time there was no further increase in reducing power in the salt solution. The residual carbohydrate solution was adjusted to 0.5M acetic acid, heated to 85° for 3 h, centrifuged (10,000 *g*, 30 min), and then dialysed against distilled water for 3 days at 2°. A precipitate formed (non-carbohydrate) which was discarded after re-centrifugation. Additional non-carbohydrate material was removed by treatment with trichloroacetic acid (10%) at 0° for 4 h, followed by centrifugation. After dialysis against distilled water for 2 days, the solution was concentrated by vacuum evaporation, and the carbohydrate precipitated with ethanol (2 vol.) containing a trace of ammonium acetate. The precipitate was collected by centrifugation, re-dissolved in a small volume of water, and re-precipitated with ethanol solution 9 additional times. The supernatant solutions were free of carbohydrate (by the phenol–sulphuric assay), but gave a positive reaction for protein. The material was washed with methanol and ethyl ether, and dried (vacuum oven, 43° for 24 h), giving a yield of 0.165 g (100%, as carbohydrate).

RESULTS

Wall extraction by alkali, followed by enzymic fractionation. — The extraction and fractionation of cell walls of *Schiz. pombe* were performed essentially as described earlier[3] (see Fig. 1); 80% of the initial weight was solubilized. The carbohydrate contents of the derived fractions, including the insoluble residue, accounted for 79% of the weight of the starting material. Considerable protein (N, 2.7%) and some lipid material (~2%) were also present. The initial extraction solubilized the majority of the material, a second extraction having only a minor effect (Table I). Part of this

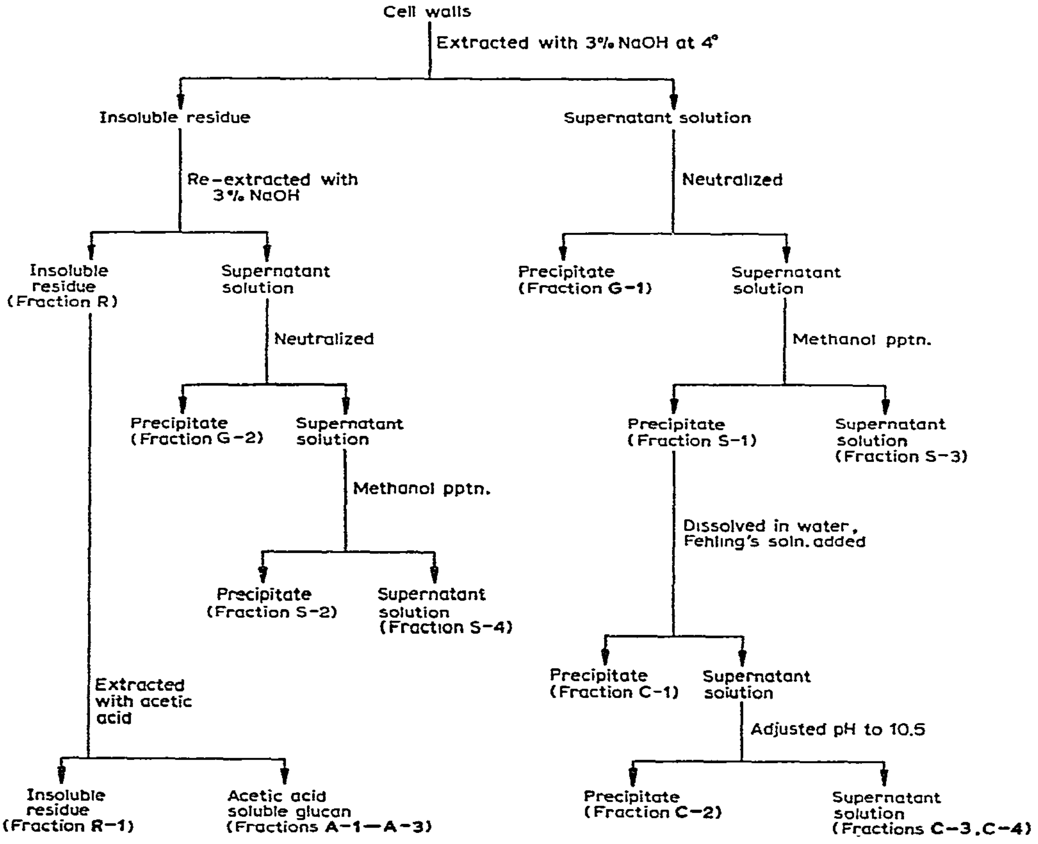

Fig. 1. Fractionation scheme for the *Schiz. pombe* cell-walls.

investigation is concerned with the fraction (G-1) that precipitated upon neutralization of the alkaline extract, and with that fraction (C-2) which was subsequently isolated from the supernatant solution after G-1 had been removed.

The main alkali-soluble fraction (G-1) represented 39% of the cell walls, and contained minor amounts of mannose (0.7%) and galactose (0.3%), presumably as galactomannan, in addition to glucose (98%). Paper chromatography of a partial, acid hydrolysate indicated that both α and β linkages were present, as the laminari- and nigero-series of oligosaccharides were produced. Partial solubilization of G-1 by an exo-(1→3)-β-D-glucanase was possible, as was a converse, partial solubilization by an endo-(1→3)-α-D-glucanase. Exhaustive digestion of separate aliquots of G-1 by each glucanase yielded insoluble residues that accumulatively accounted for virtually 100% of the initial carbohydrate (38% and 61% residues from the treatments with β- and α-D-glucanases, respectively). Paper chromatography of partial, acid hydrolysates of each residue indicated only the oligosaccharides expected of a homogeneous α- or β-linked D-glucan. After this enzymic digestion, each residue was free of the minor galactomannan contaminant previously noted in G-1; however, the protein content in the β-D-glucan had increased slightly. The additional protein could not be removed by exhaustive homogenization and washing. In G-1, the original cell-wall material contained ~24% of an alkali-soluble β-linked D-glucan {$[\alpha]_D^{23}$ +20° (*c* 0.8, M NaOH)} and 15% of an α-linked D-glucan, both of which co-precipitated upon neutralization of the alkaline extract.

Fraction S-1, when further fractionated by Fehling's solution[20], yielded an immediate precipitate (C-1) upon addition of the reagent. C-1 represented 5.4% of the initial cell-wall weight, and consisted of mannose, galactose, and glucose in the molar ratios of 7.4:2.0:0.6. Subsequent to the removal of C-1 and lowering of the pH of the solution to 10.5, a second precipitate formed overnight (C-2). C-2 (7.3% of the initial walls), which was composed only of glucose, contained α- and β-D-glucans in proportions similar to those in G-1. The endo-(1→3)-α-D-glucanase-resistant material {$[\alpha]_D^{23}$ +19° (*c* 0.66, M NaOH)} amounted to ~64% of C-2 or 4.7% of the initial cell-wall weight. By difference, the yield of α-linked polymer in C-2 corresponds to 2.6% of the weight of the cell walls. As the β-linked residues of G-1 and C-2 were resistant to further endo-(1→3)-α-D-glucanase hydrolysis, and were evidently homogeneous as shown by paper-chromatographic analysis of their partial, acid hydrolysates, they were then subjected to structural analysis.

Additional fractions derived from the treatment of S-1 with Fehling's solution were C-3 and C-4. The former contained mannose and glucose, in the molar ratio of 2.5:97.5, and precipitated upon neutralization and dialysis of the supernatant solution after the separation of C-2. Fraction C-4, which contained mannose, galactose, and glucose in the molar ratios of 3.9:2.6:3.5, was obtained by alcohol precipitation of the residual supernatant solution. Fractions C-1, C-3, and C-4, in addition to G-2 and S-2 from the second alkali extraction of the cell walls, were not investigated further. Fractions C-3 and C-4 represented ~2.0 and ~2.8%, respectively, of the cell walls. G-2 and S-2 accounted for less than 2.5% of the initial weight of the cell walls (see

Table I). The α-linked polymers present in G-1 and C-2 were also studied, mainly with respect to some properties not previously determined.

TABLE I

PRODUCTS OF THE FRACTIONATION OF THE CELL WALLS OF *Schiz. pombe*

Fraction	*Nature*	*Yield*[a] (%)
First extraction with alkali		
G-1	Mixture of α- and β-D-glucans, obtained on neutralization	38.8
S-1	Mixture of α- and β-D-glucans, obtained by precipitation with methanol	21.2
R	Residue, including (1→3)- and (1→6)-linked β-D-glucans	19.5
Subfractionation of S-1 with Fehling's solution		
C-1	Mainly galactomannan, with some glucan	5.4
C-2	Mixture of α- and β-D-glucans	7.3
C-3	Mixture of glucan and mannan	2.0
C-4	Mixture of galactomannan and glucan	2.8
Second extraction with alkali		
G-2	Polysaccharide obtained on neutralization	0.9
S-2	Polysaccharide obtained by precipitation with methanol	1.5

[a] From 9.96 g of cell walls, and based on carbohydrate contents determined by the phenol–sulphuric acid method.

Acetic acid extraction. — The alkali-insoluble residue (R) was extracted seven times with 0.5M acetic acid at 90° for 3 h, but the extraction was then still incomplete. Extrapolation of the decreasing, linear rate of solubilization indicated that the seven extractions had solubilized ~80% of the potentially acid-soluble material. The first three fractions extracted, comprising the bulk of the acetic acid-soluble material, were combined and then exhaustively digested with salivary alpha-amylase. The final yield (0.165 g) of residual material {$[\alpha]_D^{23}$ −27° (*c* 0.45, water)} corresponded to ~1.7% of the original cell-walls. Partial, acid hydrolysis and paper chromatography (see below) failed to indicate any maltose, and hence residual (1→4)-α-D-linkages; however, there were traces of galactose (1.3%) and mannose (2.5%) that could not be eliminated by treatment with Fehling's solution.

Partial hydrolysis with acid. — Partial, acid hydrolysates of the β-linked residue (10 mg) from G-1 and C-2 yielded similar products, as indicated by paper-chromatographic analysis. In addition to glucose, the laminarisaccharides from d.p. 2–5 were identified, as well as gentiobiose, and some unidentified products of lower mobility (R_{GLC} 0.33, 0.16, and 0.11). The absence of the nigerosaccharides was confirmed by using as reference a partial acid hydrolysate of the (1→3)-α-D-glucan from *A. niger*.

Paper-chromatographic analysis of the acetic acid-soluble glucan, after partial hydrolysis with acid, showed glucose, laminaribiose, gentiobiose, gentiotriose, gentiotetraose, and two unidentified spots of lower mobility (R_{GLC} 0.28 and 0.12).

The absence of a product corresponding to maltose indicated that the glycogen contaminant had been effectively eliminated.

These results indicate that the two alkali-soluble β-D-glucans were generally similar, being predominantly (1→3)-linked with some (1→6)-linkages also present. The acetic acid-soluble β-D-glucan was quite different, the major repeating-linkage being (1→6) with a very minor occurrence of (1→3)-linkages.

Hydrolysis by exo-(1→3)-β-D-glucanase. — Whereas the two alkali-soluble β-D-glucans (from G-1 and C-2) were susceptible to hydrolysis by the exo-(1→3)-β-D-glucanase, this was not the case with the acetic acid-soluble glucan. Only 5% of the D-glucose residues in the latter glucan were released as the monosaccharide after 24 h at 37°, with the majority of the hydrolysis (81%) occurring within the first 3 h. The agreement of the results obtained in D-glucose oxidase and reducing-equivalent assays, and also paper chromatography of the enzymic hydrolysate, confirmed that D-glucose was the sole product. In a control experiment, the rate of hydrolysis of pustulan [a linear (1→6)-β-D-glucan] was only 2.8% of that of laminarin, showing that the enzyme had a high, but not absolute, specificity for the (1→3)-β-D-glucosidic linkage.

When the digest of acetic acid-soluble glucan and exo-β-D-glucanase was heated to inactivate the enzyme and then cooled, and endo-(1→6)-β-D-glucanase was added, there was a barely significant increase in reducing power. In 24 h, this amounted to only 2% apparent conversion into glucose. Paper-chromatographic analysis showed the presence of some gentiobiose and another oligosaccharide (R_{GLC} 0.29), in addition to the glucose from the original digestion with exo-β-D-glucanase.

Average degree of polymerisation. — As both alkali-soluble β-D-glucans were insoluble in water (although C-2 was originally soluble at an intermediate stage of purification), the glucans were maintained in a soluble state at pH 9.5, as described

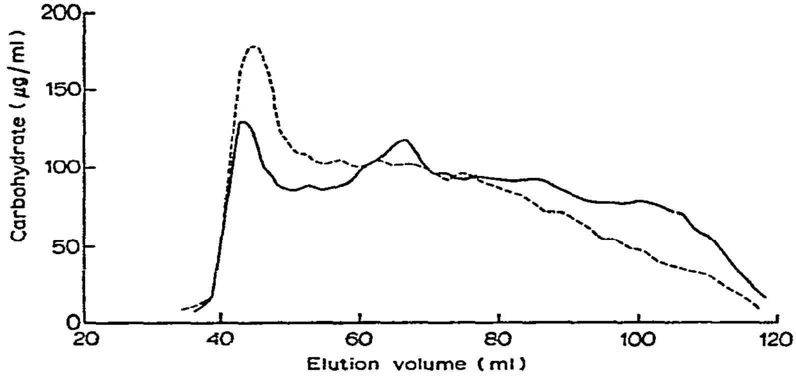

Fig. 2. Elution patterns of fractions G-1 (- - - -) and C-2 (——) from Sepharose 2B. The column (84 × 1.5 cm) was equilibrated and eluted with 0.05M sodium tetraborate buffer (pH 9.5). The flow rate was 6 ml/h; 2-ml fractions were collected, and 0.2-ml samples analysed by the phenol–sulphuric acid method.

previously[3], for chromatography on Sepharose 2B. Both preparations gave fairly similar elution-profiles (Fig. 2), except that the C-2 preparation contained more material of intermediate size than that from G-1. The number-average degree of polymerisation (d.p.), as determined by the D-glucitol dehydrogenase method, was 809 ±17 for the G-1 glucan, and 544 ±20 for the glucan from C-2.

The acetic acid-soluble glucan could not be chromatographed by gel filtration, as insufficient sample was available. However, the d.p. was 186 ±4, when determined by the stated assay system. In a control experiment to confirm the validity of the assay system, dextran T-110 gave a d.p. of 391, which compares well with the M_n value of 62,000 assigned by Pharmacia.

Methylation analysis. — The three glucan samples (G-1, C-2, and acetic acid-soluble) were methylated four times by the Hakomori method[21]. The glucans were soluble in methyl sulphoxide at room temperature, but were reluctantly soluble after the first methylation. After three methylations, a slight undermethylation was still evident, as shown by the presence of small amounts of the 2,6-di-*O*-methyl-D-glucose derivative, possibly due to some incomplete solubilisation. However, four treatments resulted in complete methylation; the molecular percentage of the tetra- and tri-*O*-methyl derivatives was not changed significantly by the additional methylation step.

The results (Table II) indicate that the two alkali-soluble glucans have similar structures as expected, containing mainly (1→3)-linked residues with some degree of branching at C-6, corresponding to an average chain-length of ~7 D-glucose residues. A very minor proportion (~4%) of (1→6)-linked residues is also evident.

TABLE II

A COMPARISON OF THE PROPERTIES OF SOME ALKALI-SOLUBLE (1→3)-β-D-GLUCANS

	Schiz. pombe		S. cerevisiae[3]	
	Fraction G-1	*Fraction C-2*	*NCYC 1109*	*Baker's yeast*
Approx. yield (%)	24.0	4.7	22	13.3
$[\alpha]_D$ (M NaOH) (degrees)	+20	+19	n.d.[a]	n.d.[a]
D.p.	809	544	1330	1810
Methylation analysis				
Mol. per cent of alditol acetates of				
(*a*) Tetra-*O*-methyl-D-glucose	15.0[b]	12.7[b]	3.7	4.7
(*b*) 2,4,6-Tri-*O*-methyl-D-glucose	66.3	71.2	84.7	79.3
(*c*) 2,3,4-Tri-*O*-methyl-D-glucose	4.2	3.7	8.3	12.0
(*d*) 2,4-Di-*O*-methyl-D-glucose	14.6	12.4	3.3	4.1

[a] Not determined. [b] The relative retention times were 1.00, 1.72, 2.06, and 3.63, respectively, and the results are the average of four g.l.c. analyses.

The methylation results for the acetic acid-soluble glucan (Table III) indicated an extremely highly branched structure with only minor proportions (5–9%) of unbranched sequences of (1→3)- and (1→6)-linked D-glucose residues. No peaks occurred indicating undermethylation, and the 2,3,6-tri-*O*-methyl-D-glucose derivative, which could have indicated glycogen contamination, was not detected. Although traces of galactose and mannose are known to be present in the original glucan, no peaks were present that could be correlated with these monosaccharides.

TABLE III

A COMPARISON OF THE PROPERTIES OF SOME ALKALI-INSOLUBLE, ACETIC ACID-SOLUBLE $(1\rightarrow6)$-β-D-GLUCANS

	Schiz. pombe	S. cerevisiae[4]
Approx. yield from alkali-insoluble residue (%)	23	13
$[\alpha]_D$ (H_2O) (degrees)	−27	−32
D.p.	186	140
Methylation analysis		
Mol. per cent of alditol acetates of		
(*a*) Tetra-*O*-methyl-D-glucose	43.9[a]	16
(*b*) 2,4,6-Tri-*O*-methyl-D-glucose	5.0	5
(*c*) 2,3,4-Tri-*O*-methyl-D-glucose	8.5	65
(*d*) 2,4-Di-*O*-methyl-D-glucose	42.7	14

[a] See footnote *b* in Table II.

Periodate-oxidation analysis. — The extent of periodate oxidation was relatively low for the β-D-glucans from G-1 and C-2, the rate becoming constant after 80 h at 2.5°, and was equivalent to the reduction of 0.31 molecular proportion (mol.) for both samples. Analyses for glycerol[14], after oxidation, reduction, and mild hydrolysis with acid, yielded 0.14 and 0.17 mol., respectively, for the G-1 and C-2 samples. Although the glycerol value for G-1 was slightly low, these results are consistent with the data from the methylation analysis and confirm the presence of one end-group per 7 D-glucose residues. On Smith degradation of the glucans, 48.0 mg of G-1 yielded 32.4 mg of insoluble, resistant material, and 42.5 mg of C-2 gave 36.0 mg. The soluble products were mostly excluded from Bio-Gel P-2 on column chromatography, no distinctive peaks for products of low molecular weight being evident. Paper chromatography of the concentrated, soluble material, prior to application to the column, indicated glycerol, glycolaldehyde and two distinct spots of very low mobility (R_{GLC} 0.28 and 0.15). No major product with the mobility expected for β-D-glucosyl-glycerol was evident, although there were several very weak, unidentified spots between glucose and the origin.

Periodate oxidation of the acetic acid-soluble glucan likewise gave results that were consistent with the methylation analysis; 1.06 mol. of periodate was reduced and 0.52 mol. of glycerol was liberated. Again, the rate of reduction was constant after 80 h.

Analysis of the α-D-glucan. — Although analysis of the α-D-glucan was not the main purpose of this investigation, some observations were made to confirm and extend the previous results of Bush and co-workers[9] (see Introduction). Partial, acid hydrolysis of the fraction obtained by exo-(1→3)-β-D-glucanase digestion of G-1, and the products from the hydrolysis of this fraction by the (1→3)-α-D-glucanase, both yielded the homologous series of nigerosaccharides having d.p. 2–5, as shown by paper chromatography. Similar analysis of the enzymic hydrolysis products from the corresponding fraction from C-2 gave the same oligosaccharides as for G-1, with an additional product having R_{GLC} 0.32 (*cf.* nigerotriose 0.40, and nigerotetraose 0.27); this product was not identified. The d.p. of the (1→3)-α-D-glucan fraction from G-1 was 207 (D-glucitol dehydrogenase method); the d.p. of the α-D-glucan from C-2 was not determined. The extent of periodate oxidation by the G-1 fraction was low (0.12 mol.), which was consistent with the methylation evidence[9] for a high proportion (89%) of (1→3)-linked residues.

Analysis of the alkali-insoluble and acetic acid-insoluble residue (*R-1*). — Partial hydrolysis of R-1 by acid, followed by paper chromatography, produced the expected series of oligosaccharides from a mainly (1→3)-linked β-D-glucan; gentiobiose was also evident. In addition, a product having the mobility of maltose was present. Incubation of R-1 with endo-(1→3)-β-D-glucanase caused rapid hydrolysis, with the formation of glucose, laminaribiose, and laminaritriose. This observation also identifies R-1 as mainly a (1→3)-linked β-D-glucan. However, as the partial, acid hydrolysate indicated, it may still be contaminated by glycogen.

DISCUSSION

The occurrence of alkali-soluble α-D-glucan in the cell walls of the genus *Schizosaccharomyces* has been known for some time[8] and has been the subject of structural investigations[9,22]. However, the nature of the β-D-glucans has not been intensively studied. It had been suggested[5,6] that the chemical structures may be related to those found in *S. cerevisiae*, where two distinct glucans have been characterized; one, insoluble in both alkali and acetic acid and considered to contribute to maintaining the cell-wall shape[2]; and a second, insoluble in alkali, but soluble in acetic acid[4]. Recently, an alkali-soluble β-D-glucan has also been described in this species[3], and confirms earlier reports[23,24] of such glucans in *Saccharomyces*. The presence of alkali-soluble β-D-glucans in other fungi has recently been reported[25,26].

The carbohydrate content of *Schiz. pombe* cell-walls (79%, determined from the recovered fractions) was higher than other investigators have reported[2,27], but lower than that (97%) reported by Bush and co-workers[9]. In this latter instance, however, the walls were conceded to be enriched in carbohydrate by prior solvent extraction of lipid material. Growth conditions and homogeneity of the cell-wall material, as well as inhibition of glucanases associated with cell walls, are factors that may affect the composition of the wall preparation.

Although glucan–mannan–protein complexes have been suggested as forming part of the alkali-soluble material in *S. cerevisiae*[3], no attempt was made to substantiate such a relationship in the present study. The presence of galactose and mannose in the G-1 fraction was eliminated during purification, while the related glucan fraction, C-2, was free from these sugars. This evidence suggests that these monosaccharides were present only as a galactomannan contaminant.

The β-D-glucan contents of both alkali-soluble fractions (G-1 and C-2) accounted for ~29% of the cell wall, while the α-D-glucans account for ~18%. The yield of this latter glucan was lower than previously reported (28%) for this yeast[9].

Structural analysis of the two, alkali-soluble β-D-glucan fractions showed them to be very similar, in being mainly (1→3)-linked with a significant degree of branching (~14%) at C-6. Additionally, a small number (4%) of (1→6)-linked residues occur. Some of these may be interspersed along the main chains of (1→3)-linked D-glucose residues, as Smith degradation results in some solubilization. The soluble products of this degradation suggest that the majority of the (1→6)-linkages are not located in close proximity to each other, as glucosyl-glycerol and laminaribiosyl-glycerol were not evident. The molecular size of the β-D-glucan in either the G-1 or C-2 preparations was quite heterogeneous, as shown by the column chromatography on Sepharose 2B (Fig. 2). Both fractions gave carbohydrate that was eluted throughout the separation range of the gel, with the G-1 fraction weighted slightly towards the higher sizes. The number-average d.p. values of 809 and 544, respectively, for the two fractions suggest that the separation of this glucan into two fractions during extraction was simply a physical phenomenon at the point of neutralization of the alkaline extract, the major portion of the β-D-glucan being entrapped within the rapidly precipitating (1→3)-linked α-D-glucan. The alkali-soluble β-D-glucan thus exists as a family of molecules of similar overall structure, but showing considerable variation in molecular size. The actual size of these molecules within the living cell wall is, of course, not known.

The β-D-glucan qualitatively resembles that isolated from *S. cerevisiae*, in being a branched (1→3)-β-D-glucan containing, in addition, a proportion of residues linked only at positions 1 and 6 (Table II). Quantitatively, the β-D-glucan from *Schiz. pombe* is only about one-third of the size of the β-D-glucan from *S. cerevisiae*, has a much higher degree of branching, and a smaller proportion of (1→6)-linked β-D-glucose residues. It has been suggested[3] that the solubility of these β-D-glucans in alkali arises, in part, from the presence of some (1→6)-linkages along the main chains of (1→3)-linked residues.

The alkali-insoluble, acid-soluble glucan component of *Schiz. pombe*, representing ~2% of the cell wall, shows some differences from the comparable glucan that has been described[4] in *S. cerevisiae* (Table III). The latter was mainly (1→6)-α-D-linked with a high degree of branching (~15%) at position 3; in addition, some (1→3)-linkages (5%) served as inter-residue linkages. The glucan in this study was also mainly (1→6)-linked, but with an extremely high proportion (43%) of triply linked residues at positions 1, 3, and 6. A small (5%) proportion of (1→3)-linked residues were also present. These may be located mainly in the side chains, rather than as inter-residue linkages, as the production of glucose by an exo-(1→3)-β-D-glucanase was almost quantitatively equivalent to the number of (1→3)-linkages present. If the (1→3)-linkages occurred as inter-residue linkages between the branching points, one might have expected some degradation products representing side chains. The high degree of branching may also explain the unexpected resistance of the glucan to any significant action of the endo-(1→6)-β-D-glucanase. The methylation results also indicate that the side branches must be very short, the majority containing one glucose residue, with the remainder having probably not more than two successive (1→3)-linkages. This latter concept is supported by the evidence from partial hydrolysis with acid, where only laminaribiose was found together with the homologous series of gentiosaccharides. Some (1→6)-linkages (8.5%) also occur as inter-residue linkages.

The presence of branched (1→6)-β-D-glucans has been reported in *Candida albicans* (serotypes A and B) and *C. parapsilosis*[28], in addition to *S. cerevisiae*, although none were as highly branched as that in *Schiz. pombe*. Although the last glucan has an unusual structure, it is not unique in the sense that the envelopes of heterocysts and spores of *Anabaena cylindrica* also contains a main chain of hexose residues, all of which are present as branch points[29].

The alkali- and acetic acid-insoluble residue R-1 qualitatively resembles the corresponding component in *S. cerevisiae*[2], as judged by partial enzymic and acidic hydrolysis; R-1 therefore may be a lightly branched (1→3)-β-D-glucan.

The (1→3)-α-D-glucan appears to be a polysaccharide of medium size (d.p. ~200), and the sample obtained from fraction C-2 may contain small amounts of a linkage other than the (1→3)-α-D-type. Other workers have suggested[9] the presence of some (1→4)-linkages in the (1→3)-α-D-glucan from *Schiz. pombe*.

The present work has been mainly concerned with three β-D-glucans from the cell walls of *Schiz. pombe*. It is apparent from the fractionation scheme (Fig. 1) that other polysaccharide samples composed of two or more sugars (*e.g.*, fractions C-3 and C-4) can be isolated. It is not known how far these represent mixtures of the various β-D-glucans with the (1→3)-α-D-glucan and the galactomannan characterized by Bush and co-workers[9], and it is clear that further work is required before a complete description of the molecular architecture of the cell walls from *Schiz. pombe* can be given.

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REFERENCES

1 H. J. Phaff, in A. H. Rose and J. S. Harrison (Eds.), *The Yeasts–Physiology and Biochemistry of Yeasts*, Vol. 2, Academic Press, London and New York, 1971, pp. 135–210.
2 D. J. Manners, A. J. Masson, and J. C. Patterson, *Biochem. J.*, 135 (1973) 19–30.
3 G. H. Fleet and D. J. Manners, *J. Gen. Microbiol.*, 94 (1976) 180–192.
4 D. J. Manners, A. J. Masson, J. C. Patterson, H. Björndal, and B. Lindberg, *Biochem. J.*, 135 (1973) 31–36.

5 D. J. Manners and A. J. Masson, *FEBS Lett.*, 4 (1969) 122–124.
6 D. J. Manners, A. J. Masson, and J. C. Patterson, *J. Gen. Microbiol.*, 80 (1974) 411–417.
7 J. Deshusses, S. Berthoud, and T. Posternak, *Biochim. Biophys. Acta*, 176 (1969) 803–812.
8 D. R. Kreger, *Biochim. Biophys. Acta*, 13 (1954) 1–9.
9 D. A. Bush, M. Horisberger, I. Horman, and P. Wursch, *J. Gen. Microbiol.*, 81 (1974) 199–206.
10 G. H. Fleet and H. J. Phaff in J. R. Villanueva, I. Garcia-Acha, S. Gascon, and F. Uruburu (Eds.), *Yeast, Mould and Plant Protoplasts*, Academic Press, London and New York, 1973, pp. 33–59.
11 D. J. Manners, A. J. Masson, and R. J. Sturgeon, *Carbohydr. Res.*, 17 (1971) 109–114.
12 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
13 B. Lindberg, *Methods Enzymol.*, 28 (1972) 178–195.
14 D. W. Noble and R. J. Sturgeon, *Carbohydr. Res.*, 12 (1970) 448–452.
15 B. Lindberg and J. McPherson, *Acta Chem. Scand.*, 8 (1954) 985–988.
16 G. H. Fleet and H. J. Phaff, *J. Biol. Chem.*, 249 (1974) 1717–1728.
17 D. J. Manners, J. R. Stark, G. Wilson, and J. Y. Brodie, *Carbohydr. Res.*, 49 (1976) 383–388.
18 M. Meyer, Ph. D. Thesis, University of California, Davis, 1975.
19 G. H. Fleet and H. J. Phaff, *J. Bacteriol.*, 119 (1974) 207–219.
20 J. K. N. Jones and R. J. Stoodley, *Methods Carbohydr. Chem.*, 5 (1965) 36–38.
21 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
22 J. S. D. Bacon, D. Jones, V. C. Farmer, and D. M. Webley, *Biochim. Biophys. Acta*, 158 (1968) 313–315.
23 A. A. Eddy and J. S. Woodhead, *FEBS Lett.*, 1 (1968) 67–68.
24 J. K. Bowden and B. Hodgson, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, 36 (1970) 81–108.
25 J. H. Sietsma, J. J. Child, L. R. Nesbitt, and R. H. Haskins, *J. Gen. Microbiol.*, 86 (1975) 29–38.
26 A. F. Cirelli and R. M. de Lederkremer, *Carbohydr. Res.*, 48 (1976) 217–223.
27 R. K. Poole and D. Lloyd, *Arch. Mikrobiol.*, 88 (1973) 257–272.
28 R. J. Yu, C. T. Bishop, F. P. Cooper, F. Blank, and H. F. Hasenclever, *Can. J. Chem.*, 45 (1967) 2264–2267.
29 L. Cardemil and C. P. Wolk, *J. Biol. Chem.*, 251 (1976) 2967–2975.