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# Architecture of the Yeast Cell Wall

$\beta(1\rightarrow6)$ -GLUCAN INTERCONNECTS MANNOPROTEIN,  $\beta(1\rightarrow3)$ -GLUCAN, AND CHITIN\*

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In a previous study (Kollár, R., Petráková, E., Ashwell, G., Robbins, P. W., and Cabib, E. (1995) *J. Biol. Chem.* 270, 1170–1178), the linkage region between chitin and  $\beta(1\rightarrow3)$ -glucan was solubilized and isolated in the form of oligosaccharides, after digestion of yeast cell walls with  $\beta(1\rightarrow3)$ -glucanase, reduction with borotritide, and subsequent incubation with chitinase. In addition to the oligosaccharides, the solubilized fraction contained tritium-labeled high molecular weight material. We have now investigated the nature of this material and found that it represents areas in which all four structural components of the cell wall,  $\beta(1\rightarrow3)$ -glucan,  $\beta(1\rightarrow6)$ -glucan, chitin, and mannoprotein are linked together. Mannoprotein, with a protein moiety about 100 kDa in apparent size, is attached to  $\beta(1\rightarrow6)$ -glucan through a remnant of a glycosylphosphatidylinositol anchor containing five  $\alpha$ -linked mannosyl residues. The  $\beta(1\rightarrow6)$ -glucan has some  $\beta(1\rightarrow3)$ -linked branches, and it is to these branches that the reducing terminus of chitin chains appears to be attached in a  $\beta(1\rightarrow4)$  or  $\beta(1\rightarrow2)$  linkage. Finally, the reducing end of  $\beta(1\rightarrow6)$ -glucan is connected to the non-reducing terminal glucose of  $\beta(1\rightarrow3)$ -glucan through a linkage that remains to be established. A fraction of the isolated material has three of the main components but lacks mannoprotein. From these results and previous findings on the linkage between mannoproteins and  $\beta(1\rightarrow6)$ -glucan, it is concluded that the latter polysaccharide has a central role in the organization of the yeast cell wall. The possible mechanism of synthesis and physiological significance of the cross-links is discussed.

Cell walls are essential for the survival of fungal cells. Digestion of cell walls in the absence of an osmotic protector leads to cell lysis due to the high internal turgor pressure. Thus, substances that interfere with cell wall synthesis may be considered as potential antifungal agents (1). Because of its rigidity, the cell wall determines the shape of fungal cells. For that reason, cell wall formation has been used as a model for morphogenesis (1).

The major components of fungal cell walls are polysaccharides and glycoproteins (2). In the yeast, *Saccharomyces cerevisiae*, the cell wall contains  $\beta(1\rightarrow3)$ -D-glucan,  $\beta(1\rightarrow6)$ -D-glucan, chitin, and mannoprotein(s) (3). The polysaccharides appear to have a structural function, whereas the mannoprotein(s) may

act as “filler” and are important for the permeability of the cell wall (4, 5). How can one explain the strength and resilience of the fungal cell wall? Recent results with *S. cerevisiae* suggest that the answer may be found in the existence of covalent linkages between the different components of the wall that would give rise to a continuous and consequently stronger fabric. Thus, previous studies in our laboratories showed the presence of linkages between chitin and  $\beta(1\rightarrow3)$ -glucan (6) as well as among glycoproteins,  $\beta(1\rightarrow6)$ -glucan, and  $\beta(1\rightarrow3)$ -glucan (7).

The strategy for the investigation of interconnections between chitin and  $\beta(1\rightarrow3)$ -glucan consisted in the digestion of cell walls with  $\beta(1\rightarrow3)$ -glucanase, followed by labeling of the exposed reducing ends with borotritide and enzymatic hydrolysis of the chitin. This procedure led to the isolation of a family of tritiated oligosaccharides that contained the sought after connection, i.e. a  $\beta(1\rightarrow4)$ -linkage between the reducing end of a chitin chain and the nonreducing end of a  $\beta(1\rightarrow3)$ -glucan chain (6). The oligosaccharides were separated on a Bio-Gel P-2 sizing column. The void volume fraction of this column contained a fairly large amount of tritium-labeled material of high molecular weight (6). We have now studied the structure of this material. The results described below show that it represents a region or regions of the cell wall where all four major components,  $\beta(1\rightarrow3)$ -glucan,  $\beta(1\rightarrow6)$ -glucan, chitin, and mannoprotein, are linked together. In this complex, chitin is directly attached to a branch of  $\beta(1\rightarrow6)$ -glucan.

## EXPERIMENTAL PROCEDURES

**Materials**— $\beta$ -N-Acetylhexosaminidase,  $\beta$ -galactosidase, and  $\alpha$ -mannosidase (all from jack bean) were from Oxford Glycosystems.  $\beta$ -Glucosidase from sweet almonds and recombinant endoglycosidase-H and proteinase K were purchased from Boehringer Mannheim; glycopeptidase F was from Life Technologies, Inc.; Zymolyase 100 T was from Seikagaku; and proteinase E (Pronase) was from Sigma. Sodium [ $^3$ H]borohydride (100 mCi/mmol) was obtained from ICN; [ $1\text{-}^{14}\text{C}$ ]glucose (50–60 mCi/mmol) was from American Radiolabeled Chemicals; and uridine diphospho-[ $U\text{-}^{14}\text{C}$ ]galactose (305 mCi/mmol) was from Amersham Corp. Bio-Gel P-2 (fine and extra fine) and Bio-Gel P-4 (extra fine) were from Bio-Rad; concanavalin A-Sepharose and Sephadex G-100 were from Pharmacia Biotech Inc.; *Erythrina cristagalli* lectin-agarose was from Vector; and Rezex RSO-Oligosaccharides HPLC<sup>1</sup> column was from Phenomenex. Polyacrylamide gels were from Novex, and PVDF

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<sup>1</sup> The abbreviations used are: HPLC, high performance liquid chromatography; HPAEC, high performance anion exchange chromatography; ConA, concanavalin A; ConA<sup>+</sup>, cell wall fraction that binds to ConA-Sepharose; ConA<sup>-</sup>, cell wall fraction that does not bind to ConA-Sepharose; fraction M, high molecular weight material remaining after  $\beta(1\rightarrow6)$ -glucanase digestion of ConA<sup>+</sup>; endo-H, endo- $\beta$ -N-acetylglucosaminidase H; GPI, glycosylphosphatidylinositol; ES-MS, electrospray mass spectrometry; CID, collision-induced decomposition; ER, endoplasmic reticulum; PVDF, polyvinylidene difluoride; Manp, mannopyranosyl.

membranes were from Millipore Corp. Chitinase from *S. marcescens* was prepared as described (8), and endo- $\beta$ (1 $\rightarrow$ 6)-glucanase was prepared from *B. circulans* WL-12 as described (9).

**Yeast Strains and Yeast Growth**—The *S. cerevisiae* strain used was ECY36-3C (MAT a *chs1-23 chs2::LEU2 trp1-1 ura3-52 leu2-2*). Cells were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) at 30 °C.

**Preparation of Cell Walls and Digestion with Glucanase and Chitinase**—The procedure was as described previously (6), except that, after cell disintegration with glass beads, cell walls were sedimented by centrifugation, washed twice with 50 mM Tris-chloride, pH 7.5, suspended in the same buffer, and incubated in a boiling water bath for 10 min, followed by five additional washings with 50 mM Tris. The isolated cell walls from 30 g (wet weight) of yeast were suspended in 860 ml of 50 mM Tris-chloride buffer, pH 7.5, and incubated with 163 ml of phenylmethylsulfonyl fluoride-treated Zymolyase (56 mg of Zymolyase 100 T was dissolved in 154 ml of 66 mM sodium phosphate, pH 7.5, containing 0.8 mM mannitol to which 250 mg of phenylmethylsulfonyl fluoride in 9.4 ml of isopropyl alcohol was added and incubated 1 h at 30 °C) for 4 h at 37 °C until the absorbance at 660 nm had decreased to about 15% of the original value. The insoluble residue was recovered by centrifugation. Washing of this material with Tris buffer, 1% SDS, and water, reduction with sodium borotritide, and digestion with chitinase were as described previously (6). Any insoluble residue after chitinase digestion was removed by centrifugation, and the supernatant fluid was used directly for Bio-Gel P-2 column chromatography (6). The material eluting in the void volume (fraction  $V_0$ ) was used for further analysis.

**Affinity Chromatography of Fraction  $V_0$  on Sepharose-bound Concanavalin A Column**—Tritiated  $V_0$  fraction ( $5.6 \times 10^6$  cpm) was evaporated to dryness, dissolved in 500  $\mu$ l of 50 mM Tris-chloride, pH 7.4, containing 0.15 M NaCl (binding buffer), and applied to a concanavalin A (ConA)-Sepharose column (0.9  $\times$  9 cm). Elution was with binding buffer, and 0.45-ml fractions were collected. At fraction 34, the eluting solution was changed to 0.5 M  $\alpha$ -methylmannoside in 50 mM Tris-chloride containing 0.25 M NaCl to displace the bound material. A portion of each fraction (10  $\mu$ l) was counted. The fractions containing radioactivity and corresponding either to the unretained (ConA<sup>-</sup>) or to the bound (ConA<sup>+</sup>) material were pooled and concentrated by evaporation.

**Chromatography**—High performance anionic exchange chromatography (HPAEC) and paper chromatography were performed as described previously (6).

**SDS-Polyacrylamide Gel Electrophoresis**—Cell wall proteins were separated by either 18% or linear gradient (2.2–20%) polyacrylamide gels according to Laemmli (10). For Western analysis, gels (2.2–20%) were blotted onto PVDF membrane, and antibody staining was carried out as described by Montijn *et al.* (11).

**Carbohydrate Determinations**—Total carbohydrate was measured with the anthrone reagent (12) or by a modified phenol sulfuric method (13). Samples were diluted to 40  $\mu$ l with water, mixed with 30  $\mu$ l of 5% phenol and 250  $\mu$ l of concentrated sulfuric acid in 96-well microtitration plates, and incubated for 10 min at room temperature. Absorbance at 490 nm was measured in a Bio-Tek EL309 autoreader. Free GlcNAc was quantified by the method of Reissig *et al.* (14), and combined GlcNAc was quantified by the same method after digestion of sample with chitinase and Glusulase (6).

**Transgalactosylation onto Nonreducing GlcNAc Residues**—Different fractions were transgalactosylated by a modification (15) of the procedure described by Trayer and Hill (16). Sample, containing 57  $\mu$ mol of total carbohydrate (as glucose) was evaporated to dryness, diluted to 3 ml with 20 mM Hepes, pH 7.3, containing 20 mM galactose, 0.5% Nonidet P-40, and 10 mM MnCl<sub>2</sub>, and incubated with 15  $\mu$ l of UDP-[U-<sup>14</sup>C]galactose (0.375  $\mu$ Ci; specific activity, 305 mCi/mmol) and 17.1  $\mu$ l (0.8 units) of autogalactosylated (17) bovine galactosyltransferase for 2 h at 37 °C in the presence of 0.02% NaN<sub>3</sub>. After the addition of unlabeled UDP-galactose (92  $\mu$ l, containing 0.92 mg in 2% ethanol) incubation was continued for an additional 4 h. The volume of the incubation mixture was reduced to ~500  $\mu$ l by evaporation, and the sample was applied to an appropriate gel chromatography column. The second incubation with unlabeled UDP-galactose was omitted in the case of the endo-H-released material from fraction M.

**Acetolysis of Mannan**—[<sup>14</sup>C]Galactose-labeled ConA<sup>+</sup> (1.2  $\times$  10<sup>6</sup> cpm of <sup>14</sup>C) was diluted to 200  $\mu$ l with a solution containing 50 mM Tris-chloride, pH 8, and 5 mM CaCl<sub>2</sub>. After adding 15 mg of proteinase E in 4 ml of the same buffer, the mixture was incubated 24 h at 37 °C in the presence of 0.02% sodium azide. The incubated mixture was concentrated to ~500  $\mu$ l by evaporation and applied to a Sephadex G-100 column (1  $\times$  85 cm). The fraction eluting in the void volume, which was

almost devoid of <sup>14</sup>C radioactivity, was collected. A portion (6.3 mg as hexose) of this fraction was acetylated by a modification (18) of the method described by Kocourek and Ballou (19), followed by deacetylation with sodium methoxide (18).

**Protein Determination**—Protein was measured either spectrophotometrically or by the procedure of Lowry *et al.* (20).

**NMR Spectroscopy**—<sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Varian XL-300 spectrometer, operating at 300 MHz (<sup>1</sup>H) and 75 MHz (<sup>13</sup>C) at a probe temperature of 35 °C. Each sample was dissolved in D<sub>2</sub>O, and a trace of acetone was used as an internal standard (for <sup>1</sup>H-NMR spectra  $\delta$  2.217 ppm; for <sup>13</sup>C-NMR spectra  $\delta$  31.07 ppm).

**Preparation of Oligosaccharide to Be Analyzed by Mass Spectrometry**—The ConA<sup>+</sup> fraction was labeled with [<sup>14</sup>C]galactose as described above and digested with  $\beta$ (1 $\rightarrow$ 6)-glucanase (see legend of Fig. 6). Low molecular weight material (Fig. 6b) was fractionated on an *E. crista-galli* lectin-agarose column (Fig. 7a), and the [<sup>14</sup>C]galactose-containing fraction (fraction B) was further chromatographed on a Bio-Gel P-2 column (Fig. 7c). Peak III (Fig. 7c, 13 nmol of carbohydrate as glucose) was evaporated to dryness, dissolved in 100  $\mu$ l of 0.01 M NaOH, and reduced with 10  $\mu$ l of sodium borotritide (1 mCi of NaB<sup>3</sup>H<sub>4</sub> in 100  $\mu$ l of dimethylformamide) for 2 h at room temperature, followed by an additional 2 h with 0.7 mg of unlabeled NaBH<sub>4</sub> in 0.01 M NaOH. The excess of borohydride was eliminated by the addition of 20  $\mu$ l of 1 M acetic acid followed by evaporation of the sample with methanol three times. The sample was applied to an *E. crista-galli* lectin-agarose column (conditions as in Fig. 2), and lectin-retarded radioactivity was applied to a Bio-Gel P-2 column (1  $\times$  85 cm), which was eluted with 0.1 M acetic acid. The radioactive fractions were pooled and evaporated to dryness. The residue was dissolved in 100  $\mu$ l of 50 mM sodium citrate, pH 3.5, and digested with 1 unit of jack bean  $\beta$ -galactosidase for 16 h at 37 °C in the presence of 0.02% NaN<sub>3</sub>. The incubated mixture was applied to a Bio-Gel P-2 column and eluted as above. The tritium-containing fractions, corresponding to degalactosylated peak III, were pooled and concentrated by evaporation. This material was analyzed by mass spectrometry as outlined below. A portion of the oligosaccharide (1 nmol) was evaporated to dryness, dissolved in 25  $\mu$ l of 2 M trifluoroacetic acid, and hydrolyzed for 2 h at 100 °C. After new evaporation to dryness, the residue was dissolved in 50  $\mu$ l of water, and a 25- $\mu$ l sample was analyzed by HPAEC.

**Methylation**—Vacuum-desiccated samples of degalactosylated peak III (see above) were dissolved in 50–200  $\mu$ l of an NaOH/Me<sub>2</sub>SO suspension prepared by vortex mixing of Me<sub>2</sub>SO and dry, finely ground NaOH pellets. After 1 h at room temperature, 10–50  $\mu$ l of methyl iodide were added, and the solution was set for 1 h at room temperature with occasional vortex mixing (21). Samples were partitioned by adding 1 ml of chloroform, and the suspension was back-extracted twice with 2–3 ml of 30% acetic acid. The chloroform layer was dried, and the samples were stored at –20 °C.

**Periodate Oxidation and Reduction**—Periodate oxidation (22) of degalactosylated peak III was performed by using a 9 mM solution of NaIO<sub>4</sub> (50  $\mu$ l) buffered with 0.1 M sodium acetate at pH 5.5 in the dark at 4 °C for 3 days. The reaction was quenched with 1  $\mu$ l of ethylene glycol, and incubation continued overnight under the same conditions. The sample was neutralized with 0.1 M NaOH and reduced by the direct addition of solid NaB<sup>3</sup>H<sub>4</sub> (1 mg) at room temperature for 16 h. Excess reducing agent was destroyed by the addition of 5  $\mu$ l of acetic acid, and the solution was dried in a vacuum centrifuge. Borate was removed by the repeated addition and drying with methanol. Periodate-oxidized and reduced oligosaccharides were then methylated as above.

**Electrospray Ionization Mass Spectrometry**—The instrument used was a TSQ 700 (Finnigan-MAT, San Jose, CA) equipped with an Analytica (Analytica of Branford, CT) electrospray ion source. Methylated oligosaccharides were analyzed by direct infusion at flow rates of 0.85  $\mu$ l/min of a 6:4 methanol:water electrospray buffer with 0.25 mM NaOH.

## RESULTS

**Preparation and General Properties of Cell Wall Complex (Fraction  $V_0$ )**—Yeast cell walls were digested with  $\beta$ (1 $\rightarrow$ 3)-glucanase, labeled by reduction with borotritide, and further hydrolyzed with chitinase as described previously (6). After chromatography of the material solubilized by chitinase on a Bio-Gel P-2 column (6), the fraction emerging at the void volume (fraction  $V_0$ ) was collected. This early fraction, as well as later peaks, contained radioactivity (6). Initial experiments were directed to ascertain the general composition of the material and its relationships with known cell wall components.

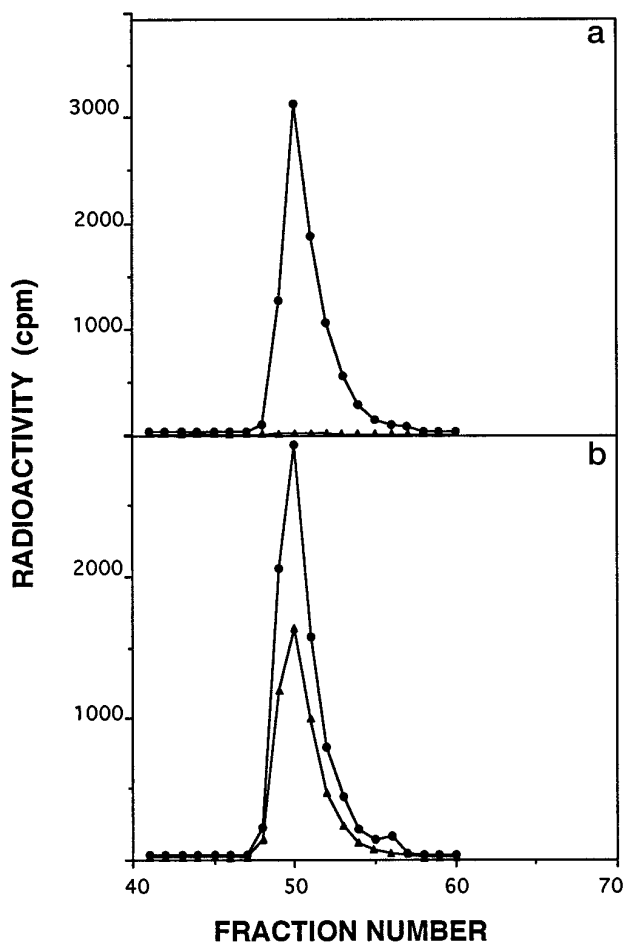


FIG. 1. Labeling of fraction  $V_0$  with  $^{14}\text{C}$ -galactose. The chitinase-solubilized fraction  $V_0$ , obtained by digestion of cell walls and gel chromatography (see "Experimental Procedures") was incubated with UDP- $[U-^{14}\text{C}]$ galactose and bovine galactosyltransferase, with (a) or without (b) preincubation with jack bean  $\beta$ -*N*-acetylhexosaminidase. The incubated fraction was applied to an extra fine Bio-Gel P-2 column ( $2 \times 90$  cm) and eluted with 0.1 M acetic acid. Fractions of 1.8 ml were collected, and a 10- $\mu\text{l}$  portion of each sample was counted using a program that allowed determination of tritium ( $\bullet$ ) and  $^{14}\text{C}$  ( $\blacktriangle$ ) isotope in separate channels.

Labeling with borotritide required prior treatment with  $\beta(1\rightarrow3)$ -glucanase. When borotritide was used on the intact walls, only 24% of the radioactivity was incorporated into fraction  $V_0$ . This result suggested that most substances in the void volume peak had been attached to  $\beta(1\rightarrow3)$ -glucan in the intact cell wall. Similarly, the solubilization of the material upon chitinase treatment clearly indicated that it had been linked to chitin. In our previous study of the chitin- $\beta(1\rightarrow3)$ -glucan connection, it had been easy to identify remaining GlcNAc in the bridge region, which was represented by relatively small oligosaccharides. In the present case, however, chromatography of fraction  $V_0$  on Sephacryl S-400 indicated a size in the range of 200–250 kDa (results not shown), whereas sugar determinations showed the presence of glucose and mannose with very little if any *N*-acetylglucosamine (see below). To look for the presumably few GlcNAc groups that may have been involved in the linkage to chitin, we availed ourselves of a sensitive and specific technique that consists in labeling nonreducing GlcNAc residues with  $[^{14}\text{C}]$ galactose by incubation with UDP- $[^{14}\text{C}]$ Gal in the presence of bovine galactosyltransferase (16). Treatment of fraction  $V_0$  in this fashion resulted in the incorporation of radioactivity (Fig. 1b). A control in which fraction  $V_0$  was pretreated with  $\beta$ -*N*-acetylglucosaminidase showed no incorporation (Fig. 1a). This result confirms the covalent link-

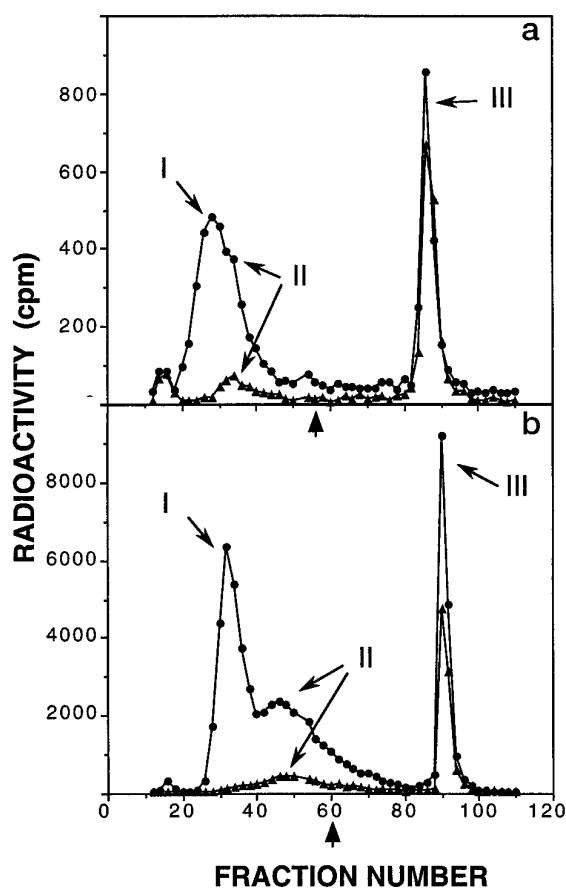


FIG. 2. Affinity chromatography of  $^{14}\text{C}$ -Gal-labeled  $\text{ConA}^-$  and  $^{14}\text{C}$ -Gal-labeled  $\text{ConA}^+$  on *E. cristagalli* lectin-agarose. a, a portion of  $[^{14}\text{C}]$ Gal-labeled  $\text{ConA}^+$  fraction (52,000 cpm of tritium and 20,500 cpm of  $^{14}\text{C}$ ) was diluted to 500  $\mu\text{l}$  with a solution containing 50 mM HEPES, pH 8.0, 100 mM KCl, 5 mM  $\text{MgCl}_2$ , and 2 mM  $\beta$ -mercaptoethanol (binding buffer) and applied to a column of *E. cristagalli* lectin-agarose ( $1 \times 20$  cm). Elution was done with the loading buffer, and 0.45-ml fractions were collected. At fraction 55 (arrow) the eluent was changed to a solution with the same composition of the binding buffer plus 0.2 M lactose and 0.5 M GlcNAc to displace the bound material. Of each fraction, a 50- $\mu\text{l}$  sample was counted in separate channels for tritium ( $\bullet$ ) and  $^{14}\text{C}$  ( $\blacktriangle$ ). b, a portion of  $[^{14}\text{C}]$ Gal-labeled  $\text{ConA}^-$  ( $3.6 \times 10^5$  cpm of tritium and  $7.5 \times 10^4$  cpm of  $^{14}\text{C}$ ) was separated under the same conditions as in a, except that 200  $\mu\text{l}$  of each fraction was counted, and the elution buffer was changed at fraction 60 (arrow).

ages of substances in fraction  $V_0$  to chitin. It should be kept in mind that, because *Serratia* chitinase cleaves two GlcNAc residues at a time, even-numbered chains may be excised completely without leaving any amino sugar, as was found for the chitin- $\beta(1\rightarrow3)$ -glucan linkage (6). Therefore, assuming a random chain length, the residues labeled in the experiment of Fig. 1 may correspond to only half of the connecting chitin chains, those with an odd number of GlcNAc residues.

The evidence outlined above points to a linkage of the material in fraction  $V_0$  to both  $\beta(1\rightarrow3)$ -glucan and chitin but does not address the question of how many different substances are present in fraction  $V_0$ . Further fractionation was required to deal with this problem. Because of the presence of mannose (see below) in fraction  $V_0$ , we used chromatography on ConA-Sepharose columns (see "Experimental Procedures"). Somewhat more than half of the tritium label went through the column without binding, whereas the remainder was attached and required  $\alpha$ -methylmannoside for elution. The two fractions were designated  $\text{ConA}^-$  and  $\text{ConA}^+$ , respectively. Both fractions could be labeled with  $[^{14}\text{C}]$ galactose in the manner outlined above. To ascertain whether the galactose (hence the





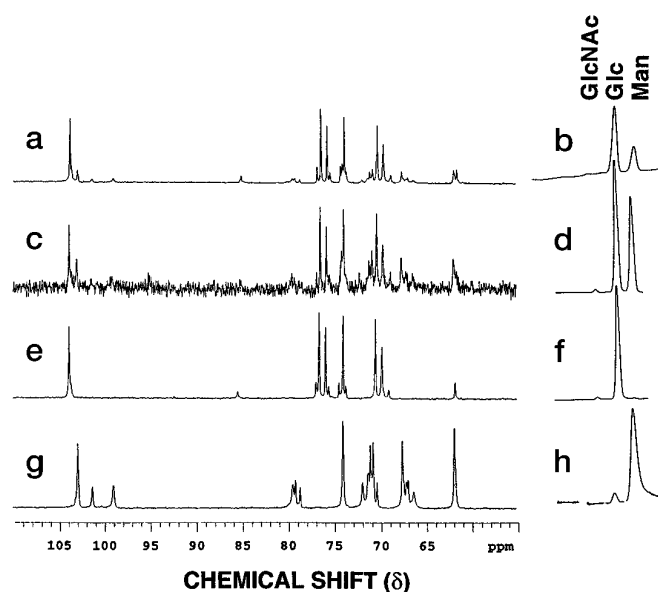


FIG. 4.  $^{13}\text{C}$  NMR spectra of different fractions and corresponding HPAEC chromatograms of their acid hydrolysates.  $^{13}\text{C}$  NMR spectra of fraction  $V_0$ ,  $\text{ConA}^+$ ,  $\text{ConA}^-$ , and fraction M are shown in a, c, e, and g, respectively; the corresponding HPAEC profiles of their acid hydrolysates are presented in b, d, f, and h. For hydrolysis, a sample of each fraction (10–20 nmol of carbohydrate, as glucose) was evaporated to dryness, dissolved in 50  $\mu\text{l}$  of trifluoroacetic acid, and heated for 2 h at 100  $^\circ\text{C}$ . After a new evaporation to dryness, the residue was redissolved in water (5  $\mu\text{l}/\text{nmol}$ ) and analyzed by HPAEC.

nals. There are two sets of signals in this spectrum (Table I). One set of high intensity signals corresponds well with signals for pustulan ( $\beta(1\rightarrow6)$ -linked glucopyranosyl residues). The signals of the second set, of lower intensity, are in very good agreement with the  $^{13}\text{C}$  chemical shifts of laminarin; therefore, they correspond to  $\beta(1\rightarrow3)$ -linked glucopyranosyl units. There are two more signals in the spectrum of  $\text{ConA}^-$ , at  $\delta$  75.46 and 73.65 ppm, which may be assigned to  $\beta(1\rightarrow6)$ -linked glucopyranosyl units of the main chain that are carrying branches composed by  $\beta(1\rightarrow3)$ -linked glucose residues. We arrived at this assignment by exclusion of all other possibilities, as follows: a branch linked at C-4 of the polysaccharide backbone would have resulted in signals either at  $\delta$  79.5 ppm ( $\beta$ -linked) or 78.4 ppm ( $\alpha$ -linked); a branch at C-2 would have yielded signals at  $\delta$  82.1 ppm ( $\beta$ -linked) or 79.5 ppm ( $\alpha$ -linked); finally, the signal of an  $\alpha$ -linked branch at C-3 would have been at  $\delta$  99.8 ppm (26). The only remaining possibility is a side chain composed of  $\beta(1\rightarrow3)$ -linked glucopyranosyl units linked to a C-3 position of the main  $\beta(1\rightarrow6)$  chain. The signals at  $\delta$  75.46 and 73.65 ppm can be assigned to C-5 and C-2 of the branched unit, in very good agreement with the 75.4 ppm signal in spectra of the 3,6-di-*O*-substituted  $\beta$ -D-glucopyranosyl residue of a variety of branched  $\beta(1\rightarrow3)$ -glucans (27). The difference between the two chemical shifts ( $\delta$  75.46 and 73.65 ppm) and those belonging to unbranched glucose units ( $\delta$  75.78 and 73.93 ppm) is probably caused by greater shielding due to the presence of the side chain.

The results of the  $^1\text{H}$  NMR spectrum of  $\text{ConA}^-$  (Fig. 5) were in agreement with those of the  $^{13}\text{C}$  spectrum. In the anomeric region of this spectrum there are two resolved doublets. The first one, at  $\delta$  4.73 ppm ( $J_{1,2}$  7.6 Hz) was assigned to the H-1 proton of the side chain ( $(1\rightarrow3)$ -glucopyranosyl unit), the second one, at  $\delta$  4.52 ppm ( $J_{1,2}$  7.9 Hz), belongs to the H-1 proton of the main chain,  $(1\rightarrow6)$ -linked. Their coupling constants are in good agreement with those published for  $\beta$ -linked D-glucopyranoses (28, 29). There is a third doublet in the anomeric region, partially overlapped by the doublet at  $\delta$  4.52 ppm. We

TABLE I  
 $^{13}\text{C}$  NMR chemical shifts for  $\text{ConA}^-$

	C-1	C-2	C-3	C-4	C-5	C-6
Strong signals						
$\text{ConA}^-$	103.83	73.93	76.49	70.44	75.78	69.74
Pustulan <sup>a</sup>	103.83	73.93	76.50	70.44	75.78	69.72
Weak signals						
$\text{ConA}^-$	103.58	74.35	85.35	68.99	76.86	61.64
Laminarin <sup>b</sup>	103.39	74.10	85.18	68.99	76.49	61.58

<sup>a</sup> Our measurements.

<sup>b</sup> Our measurements, which coincide with published results (24, 25).

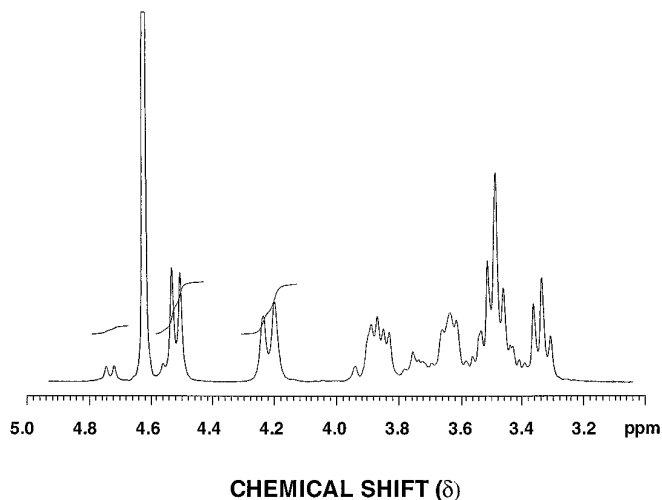


FIG. 5. Proton NMR spectrum of  $\text{ConA}^-$  fraction.

assume that this doublet belongs to the branched glucopyranosyl residues (3,6-linked). The sum of the integrals (22.6 at  $\delta$  4.52 ppm) was compared with the integral (22.2) of the doublet at  $\delta$  4.22 ppm, which represents H-6<sub>a</sub> of the main chain (30). Since they are almost identical, one may conclude that the doublet at  $\delta$  4.52 ppm represents all H-1 protons from the backbone, either from branched or unbranched units.

All these data indicate the presence in  $\text{ConA}^-$  of a  $\beta(1\rightarrow6)$ -linked glucopyranose polysaccharide with  $\beta(1\rightarrow3)$  branches. All of the signals in the NMR spectra of  $\text{ConA}^-$  could be found in those of  $\text{ConA}^+$  and of fraction  $V_0$ ; therefore, these fractions contain the same or a similar polysaccharide.

In confirmation of these results, digestion of [ $^{14}\text{C}$ ]Gal-labeled  $\text{ConA}^+$  or  $\text{ConA}^-$  with bacterial  $\beta(1\rightarrow6)$ -glucanase followed by chromatography on Sephadex G-100 showed extensive degradation of the material, with all of the  $^{14}\text{C}$  and part of the tritium in a low molecular weight wide peak, whereas the remainder of the  $^3\text{H}$  was still in the void volume fraction (Fig. 6b). When the three fractions isolated by chromatography of  $\text{ConA}^+$  on the *E. cristagalli* lectin-agarose column (Fig. 2a) were individually incubated with the glucanase, all of them showed a similar pattern of degradation, with part of the tritium eluted in the void volume fraction (results not shown). Since the  $^{14}\text{C}$  represents galactose attached to GlcNAc, the generation of  $^{14}\text{C}$ -labeled low molecular substances by  $\beta(1\rightarrow6)$ -glucanase treatment indicates that chitin is linked to  $\beta(1\rightarrow6)$ -glucan.

*Chitin Is Directly Attached to  $\beta(1\rightarrow6)$ -Glucan, Probably through a  $\beta(1\rightarrow3)$  Branch*—Fractionation of the low molecular weight material released by  $\beta(1\rightarrow6)$ -glucanase on a *E. cristagalli* lectin-agarose column completely separated the  $^{14}\text{C}$ -containing material (B), which was retarded, from the tritium-labeled substances (A), which were not absorbed by the column (Fig. 7a). Each one of the *E. cristagalli* lectin-agarose fractions was rechromatographed on a Bio-Rad P-2 column (Fig. 7, b and c). The tritium-containing fraction gave rise to several labeled

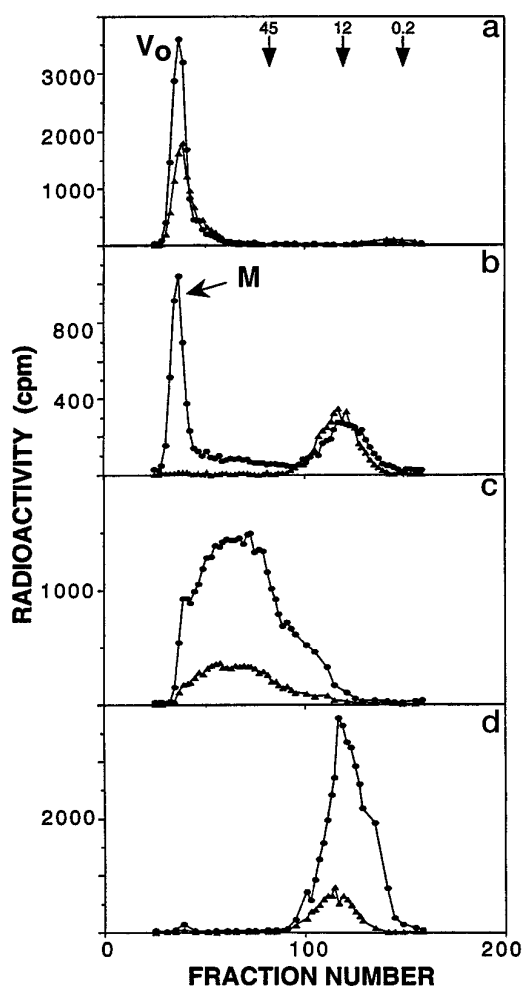


FIG. 6. Gel chromatography of  $^{14}\text{C}$ -Gal-labeled  $\text{ConA}^+$  and  $^{14}\text{C}$ -Gal-labeled  $\text{ConA}^-$  after incubation with endo- $\beta(1\rightarrow6)$  glucanase. *a*, [ $^{14}\text{C}$ ]Gal-labeled  $\text{ConA}^+$  ( $1.5 \times 10^6$  cpm of tritium and  $6.9 \times 10^5$  cpm of  $^{14}\text{C}$ ) was applied to a Sephadex G-100 column ( $1 \times 85$  cm) and eluted with 25 mM Tris-chloride, pH 7.5, containing 0.02% sodium azide. Fractions of 0.45 ml were collected, and samples were counted in separate channels for tritium ( $\bullet$ ) and  $^{14}\text{C}$  ( $\blacktriangle$ ). *b*, [ $^{14}\text{C}$ ]Gal-labeled  $\text{ConA}^+$  ( $5.5 \times 10^5$  cpm of tritium and  $2.5 \times 10^5$  cpm of  $^{14}\text{C}$ ) was evaporated to dryness, dissolved in 0.5 ml of sodium succinate, pH 5.0, and digested with 360  $\mu\text{l}$  (7.9 units) of endo- $\beta(1\rightarrow6)$ -glucanase from *B. circulans* for 16 h at 30  $^\circ\text{C}$  in the presence of 0.02%  $\text{NaN}_3$ . After incubation, the mixture was chromatographed on Sephadex G-100 as in *a*. The tritiated material eluting in the void volume is designated as fraction M. *c*, [ $^{14}\text{C}$ ]Gal-labeled  $\text{ConA}^-$  ( $1.8 \times 10^5$  cpm of tritium and  $3.7 \times 10^4$  cpm of  $^{14}\text{C}$ ) was subjected to Sephadex G-100 chromatography as in *a*. *d*, [ $^{14}\text{C}$ ]Gal-labeled  $\text{ConA}^-$  fraction ( $1.8 \times 10^5$  cpm of tritium and  $3.7 \times 10^4$  cpm of  $^{14}\text{C}$ ) was digested with endo- $\beta(1\rightarrow6)$ -glucanase and separated by gel filtration as in *b*. Samples used for counting were 10, 15, 100, and 200  $\mu\text{l}$  for *a*, *b*, *c*, and *d*, respectively. Positions of molecular mass standards are indicated in *a* in kDa.

peaks, at positions corresponding to oligosaccharides in the 2–6 hexose residue range (Fig. 7*b*). Carbohydrate determinations revealed the presence of unlabeled oligosaccharides in a similar range of sizes but eluting at slightly different positions (Fig. 7*b*). The unlabeled oligosaccharides presumably originate in inner portions of the  $\beta(1\rightarrow6)$ -glucan chain (see Fig. 3) and consist exclusively of glucose, whereas the tritiated compounds are derived from the reducing terminus previously attached to  $\beta(1\rightarrow3)$ -glucan and contain sorbitol. This difference in composition explains the slight variation in elution volume (6).

The  $^{14}\text{C}$ -labeled oligosaccharides fractionated in the P-2 column into three main peaks, I, II, and III (Fig. 7*c*). These oligosaccharides represent linkage points of  $\beta(1\rightarrow6)$ -glucan to chitin, because they contain  $\text{GlcNAc}$ . Since the linkage points

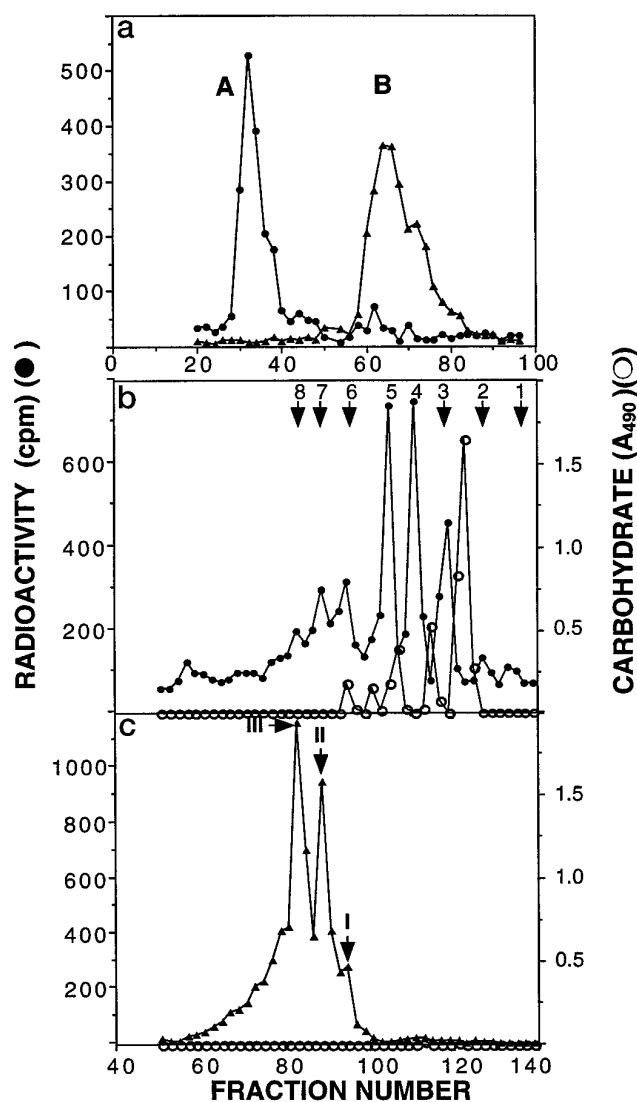


FIG. 7. Separation of tritiated and  $^{14}\text{C}$ -labeled oligosaccharides released from  $^{14}\text{C}$ -Gal-labeled  $\text{ConA}^+$  fraction by endo- $\beta(1\rightarrow6)$ -glucanase. *a*, the low molecular weight peak, isolated from Sephadex G-100 (Fig. 6*b*) after glucanase digestion of [ $^{14}\text{C}$ ]Gal-labeled  $\text{ConA}^+$  fraction ( $2.8 \times 10^5$  cpm of tritium and  $1.6 \times 10^5$  cpm of  $^{14}\text{C}$ ), was fractionated on an *E. cristagalli* lectin-agarose column to separate galactose-containing ( $^{14}\text{C}$ -labeled, B) from galactose-free (tritium-labeled, A) oligosaccharides. Conditions were as in Fig. 2. Since the  $^{14}\text{C}$ -labeled material was retarded rather than bound, the elution buffer was not required. Samples were counted in channels for tritium ( $\bullet$ ) or  $^{14}\text{C}$  ( $\blacktriangle$ ). *b*, fraction A was concentrated by evaporation to  $\sim 1$  ml, applied to an extra fine Bio-Gel P-2 column, and eluted as in Fig. 1. In addition to radioactivity, total carbohydrate (see "Experimental Procedures") was measured in fractions ( $\circ$ ). Positions of standards are indicated with arrows. 1, [ $^{14}\text{C}$ ]glucose (internal standard); 2, hexitol; 3–8, diol to heptaol obtained by reduction of a dextran ladder (Oxford Glycosystems). *c*, fraction B was concentrated and chromatographed as fraction A. Standards were the same as in *b*, except that the internal standard was [ $^3\text{H}$ ]mannose. In all cases, 0.45-ml fractions were collected. Samples of 10  $\mu\text{l}$  were used for counting, and samples of 40  $\mu\text{l}$  were used for carbohydrate determinations.

are very few relative to the total length of the glucan chain, this fraction contains very little carbohydrate, which was undetectable under our conditions (Fig. 7*c*). Peak III was subjected to further analysis. After labeling the reducing end by reduction with borotritide, the [ $^{14}\text{C}$ ]galactose at the nonreducing end was cleaved off with  $\beta$ -galactosidase (see "Experimental Procedures"). The liberated tritiated oligosaccharide emerged from the P-2 column at a position consistent with the composition  $\text{GlcNAcGlc}_3\text{Glc}$ . The same composition was indicated by sep-

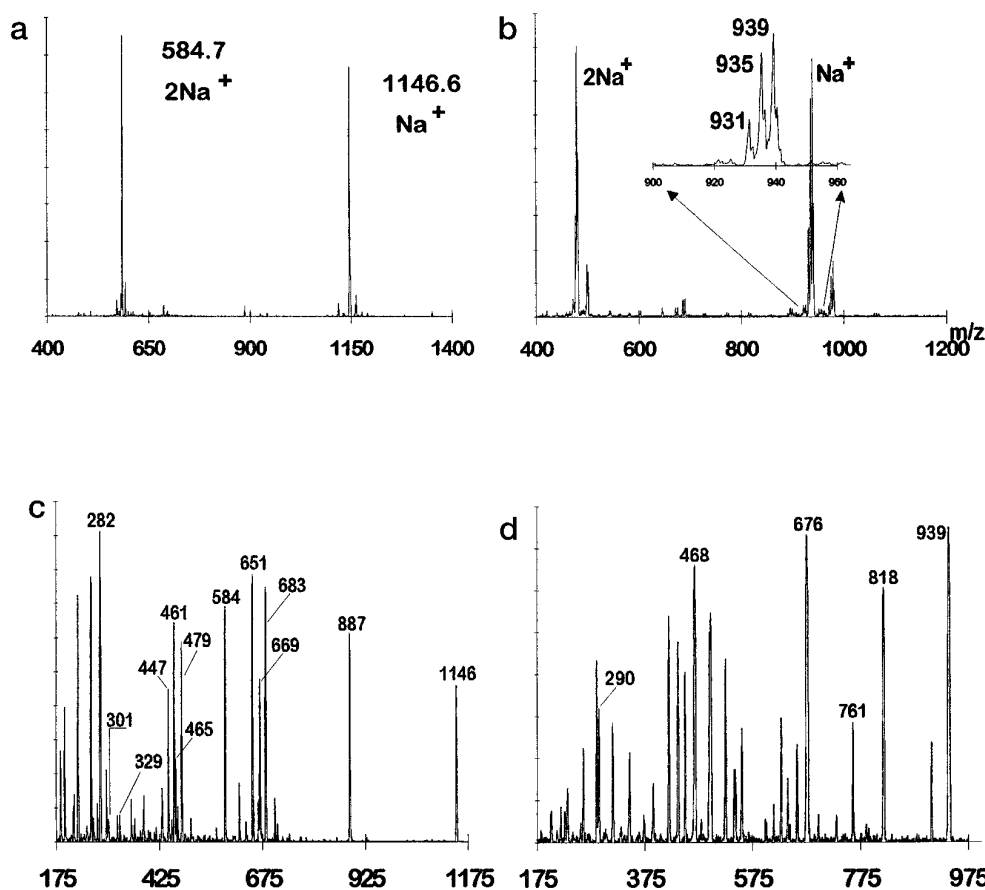
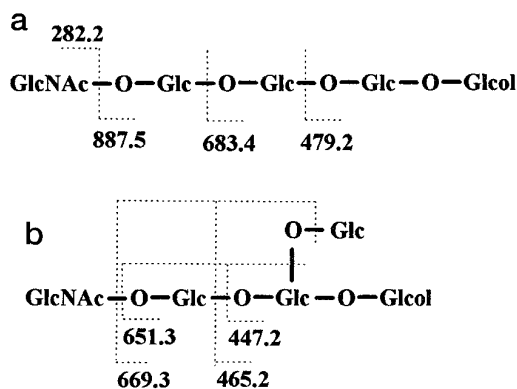


FIG. 8. Mass spectrometry of degalactosylated peak III. *a*, ES-MS spectrum of permethylated sample. The  $m/z$  584.7 peak is doubly charged, adducting two sodium ions, and the  $m/z$  1146.6 peak is singly charged, adducting a single sodium. The mass is consistent with a permethylated oligosaccharide alditol having one *N*-acetyl hexosamine and 4 hexose residues. *b*, ES-MS spectrum of the periodate-oxidized,  $\text{NaB}^2\text{H}_4$ -reduced and permethylated degalactosylated peak III. A triplet of ion peaks is observed in the single and double charge states. *c*, ES-MS-CID-MS spectrum of the doubly natriated, permethylated oligosaccharide alditol at  $m/z$  584.7. The spectrum is plotted as a relative entropy surface (34), which suppresses noise spikes and allows the graphical presentation of high and low amplitude peaks in a single scale, similar to a log scale. *d*, ES-MS-CID-MS of the singly natriated peak at  $m/z$  939 from the periodate-oxidized,  $\text{NaB}^2\text{H}_4$ -reduced and permethylated oligosaccharide. The spectrum is again an entropy surface.

aration of the sugars on HPAEC after acid hydrolysis. Incubation of the oligosaccharide with  $\beta$ -*N*-acetylglucosaminidase, followed by  $\beta$ -glucosidase, resulted in the liberation of tritiated sorbitol (results not shown).

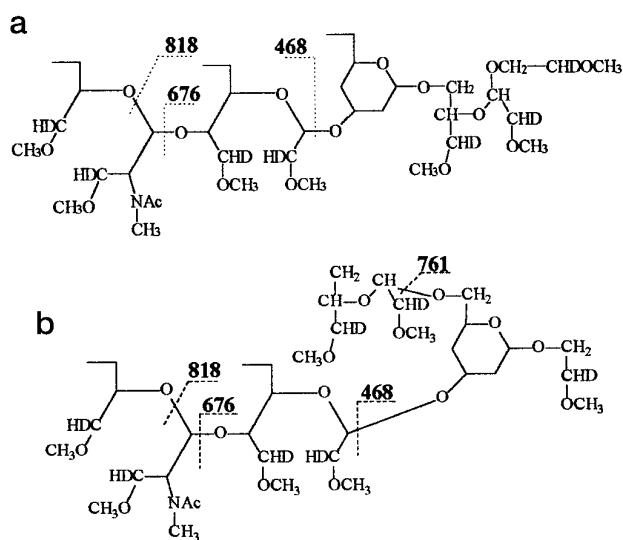
Further studies of degalactosylated peak III were carried out by mass spectrometry before and after derivatization, a technique that provides structural information with very small amounts of material. Electrospray mass spectrometry (ES-MS) of methylated degalactosylated peak III produced two major peaks at  $m/z$  584.7 and 1146.6 (Fig. 8a) corresponding to doubly and singly charged ions (charge arising by the adduction of sodium), with a mass that is consistent with a methylated oligosaccharide alditol containing one *N*-acetyl hexosamine and four hexose residues. Since oligosaccharide permethylation eliminates acyl modifications, the underivatized oligosaccharide was also examined by ES-MS in the negative ionization mode, and this was also consistent with a composition of an oligosaccharide alditol containing one *N*-acetyl hexosamine and four hexose residues (results not shown). The CID spectrum of the derivatized oligosaccharide was examined to obtain information about the sequencing and branching structure. The collision spectrum of the doubly charged molecular ion at  $m/z$  584 (Fig. 8c) produced a prominent  $\text{B}_1/\text{Y}_3$  ion pair (31) at  $m/z$  282 and 887 (Scheme 1a, Fig. 8c). The loss of an additional hexose from the  $m/z$  887  $\text{Y}_4$  ion is observed ( $m/z$  683,  $\text{Y}_3$ ), as is another hexose loss ( $m/z$  479,  $\text{Y}_2$ ), to form a partial set of sequence ions containing the reducing end. The



SCHEME 1

final  $\text{Y}_1$  ion is not observed, since the reducing end glucose had been reduced, and the pyranose ring structure that would stabilize the adduction of a sodium cation on this fragment was destroyed. The set of Y ions at  $m/z$  887, 683, and 479 indicates a linear topology; however, there is another sequence of Y ions that indicates a branched structure. These are the tri- and disaccharide Y/Z fragments at  $m/z$  669/651 and  $m/z$  465/447, showing multiple glycosidic losses (Scheme 1b, Fig. 8c). Although the fragmentation pattern does indicate both a linear and branched structure, it provides a poor quantification of the relative amounts of these structures.

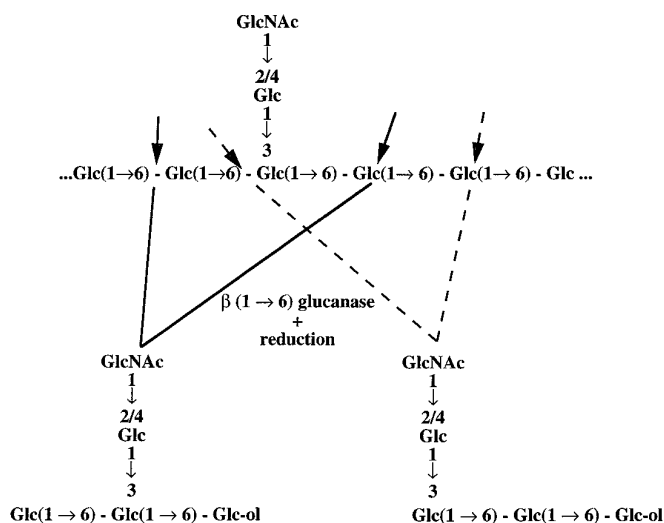




SCHEME 2

The glycosidic linkages in the oligosaccharide were also investigated by CID. The glycosidic bond on the reducing side of *N*-acetyl hexosamine residues is rapidly cleaved under the collision conditions of the triple quadrupole, and this results in the prompt loss of a nonreducing terminal GlcNAc. This, unfortunately, largely prevented the formation of ring-opening fragments that can be used for linkage assignment with tandem mass spectrometry of permethylated oligosaccharides (32, 33). The only ring-opening fragments observed were a minor  $m/z$  301 ( $^{0.4}A$ ) and 329 ( $^{3.5}A$ ) pair (Fig. 8c), and these suggest a terminal 6-linked hexose.

Periodate oxidation, reduction, and permethylation of the oligosaccharide can be used to obtain linkage information at high sensitivity (22). The ES-MS spectrum of the periodate-treated and methylated oligosaccharide (Fig. 8b) shows a triplet of ions, adducting one or two sodium cations. The increments of 4 atomic mass units exhibited by the oxidation products (e.g. the singly adducted triplet at  $m/z$  931, 935, and 939 (Fig. 8b, inset) correspond to the oxidation of additional diols by the periodate anion (4 atomic mass units, since  $\text{NaB}^2\text{H}_4$  was used as the reducing agent). The  $m/z$  939 peak is consistent with the presence of both a branched and a linear structure, and in this case periodate oxidation cannot directly assign the molar fraction of the two structures by a specific mass shift. The oxidation product total mass, together with the knowledge that the reducing end is a hexose alditol, does indicate specific combinations of oxidized and unoxidized residues; however, their sequence in the oligosaccharide requires tandem mass spectrometry (Scheme 2: a, linear structure,  $m/z$  939; b, branched structure,  $m/z$  939). The  $m/z$  939 ion was dissociated by collisional activation, and the mass spectrum of the product ions was taken (Fig. 8d). The ion fragments at  $m/z$  818, 676, and 468 are common to both structures and indicate an oxidized nonreducing terminal *N*-acetyl hexosamine, 2- or 4-linked to a hexose (Scheme 2). The  $m/z$  761 fragment corresponds to the loss of an oxidized hexose and could only arise from the branched structure. The hexose linked to the sorbitol was not oxidized, and this is consistent with it being linked at the 3-position (or branched). The tandem mass spectrometry spectra of the  $m/z$  935 and 931 peaks show that the 4-atomic mass unit decrement arises from a failure to oxidize either the terminal *N*-acetyl hexosamine or the adjacent hexose ( $m/z$  935) or both ( $m/z$  931). Failure to oxidize the terminal *N*-acetyl hexosamine suggests that incomplete oxidation chemistry and not linkage arrangement is responsible for these peaks.



SCHEME 3

Both the mass spectrometry results and the chemical and enzymatic determinations indicate the presence in degalactosylated peak III of a pentasaccharide, containing one GlcNAc and four hexose residues, one of which had been reduced to the corresponding alditol. The fragmentation pattern shows the GlcNAc at the nonreducing terminus, as confirmed by its cleavage by  $\beta$ -*N*-acetylhexosaminidase. All hexoses are  $\beta$ -linked glucose residues, as shown by the action of  $\beta$ -glucosidase and by HPAEC (see above). The acetylhexosamine is attached to the next glucose by a  $\beta(1\rightarrow2)$  or a  $\beta(1\rightarrow4)$  linkage. CID data, both of the permethylated material and of the periodate-oxidized, reduced, and permethylated material indicate the presence of two isomers, one linear and the other branched (Schemes 1 and 2). In both structures, one of the hexoses appears to be  $\beta(1\rightarrow3)$ -linked. In the branched structure, there is a  $\beta(1\rightarrow6)$ -linked glucose. All of these results are compatible with a linkage of the GlcNAc at the reducing end of a chitin chain to a glucose linked  $\beta(1\rightarrow3)$  to the main chain of  $\beta(1\rightarrow6)$ -glucan. Cutting at different locations by the  $\beta(1\rightarrow6)$ -glucanase used in the preparation of peak III would give rise either to the branched or to the linear isomer (Scheme 3; arrows show two possible cutting patterns). Notice that the mass spectrometry results would also be compatible with the  $(1\rightarrow3)$ -linked glucose in the linear isomer being directly attached to sorbitol. However, this would imply that  $\beta(1\rightarrow3)$  linkages would be occasionally present in the main  $\beta(1\rightarrow6)$  chain. There is no evidence for this; in fact, all four internal oligosaccharides from  $\beta(1\rightarrow6)$ -glucan that we analyzed after reduction were completely hydrolyzed to glucose and sorbitol by  $\beta$ -glucosidase (see example below in Fig. 9b), an indication that there were no  $\beta(1\rightarrow3)$  linkages between glucose and sorbitol (6). Furthermore,  $^{13}\text{C}$  NMR spectra indicate the presence of branches attached to the main  $\beta(1\rightarrow6)$  chain by a  $\beta(1\rightarrow3)$  linkage, as discussed above. Another piece of evidence for attachment of chitin to a  $\beta(1\rightarrow3)$  branch comes from a study of oligosaccharide I (Fig. 7c), a tetrasaccharide. After reduction with borohydride and incubation with  $\beta$ -galactosidase and  $\beta$ -*N*-acetylglucosaminidase,  $\beta$ -glucosidase was unable to hydrolyze the residual reduced disaccharide (data not shown), a result suggestive of a  $\beta(1\rightarrow3)$  linkage between glucose and sorbitol (6). We also considered the possibility that the GlcNAc was directly attached to a glucose residue of the main  $\beta(1\rightarrow6)$  chain, but we found that all structures that would have resulted in that case were incompatible with the mass spectrometry results.

*The Reducing End of the  $\beta(1\rightarrow6)$  Chain Is Attached to  $\beta(1\rightarrow3)$ -Glucan*—In a previous study on the chitin- $\beta(1\rightarrow3)$ -glu-

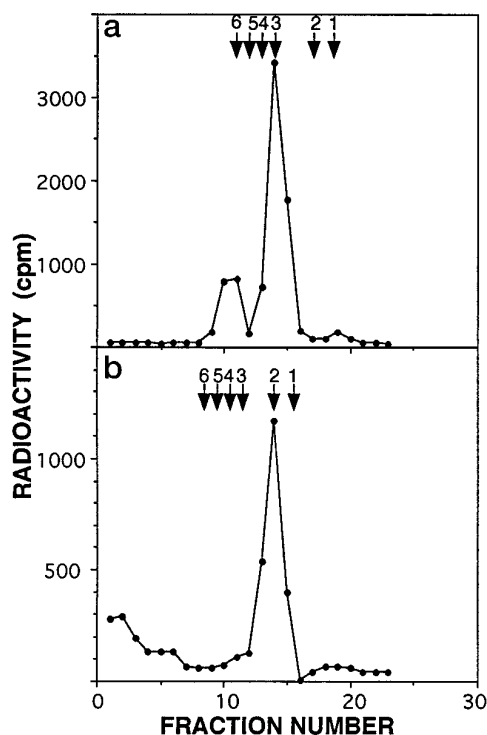


FIG. 9.  $\beta$ -Glucosidase digestion of oligosaccharides released from ConA<sup>+</sup> by  $\beta(1\rightarrow6)$ -glucanase. *a*, a sample of tritiated pentasaccharide ( $2 \times 10^4$  cpm) isolated by Bio-Gel P-2 (Fig. 7*b*) was evaporated to dryness, dissolved in 280  $\mu$ l of 50 mM sodium acetate, pH 5.0, and digested with 0.3 mg of sweet almond  $\beta$ -glucosidase for 16 h at 37 °C in the presence of 0.02% NaN<sub>3</sub>. After incubation, the sample was applied to a Bio-Gel P-2 column, and the digestion product, eluting at the position of a reduced hexose disaccharide, was recovered. Pooled fractions were evaporated to dryness, dissolved in 30  $\mu$ l of water, and subjected to paper chromatography. Segments (1 cm) of the paper were counted. Standards were glucose (<sup>14</sup>C]glucose, internal standard) (1), glucitol (2), laminaribiitol (3), sophoritol (4), cellobiitol (5), and gentiobiitol (6). *b*, a sample of nonreduced tetrasaccharide, isolated from the same chromatography (Fig. 7*b*), was labeled by reduction with sodium borotritide. Part of the recovered reduced tetrasaccharide ( $1 \times 10^5$  cpm) was evaporated to dryness, dissolved in 200  $\mu$ l of 50 mM sodium acetate, pH 5.0, and digested with 1.2 mg of sweet almond  $\beta$ -glucosidase as in *a*. A portion of incubation mixture (20,000 cpm) was subjected to paper chromatography as in *a*. Standards were the same for both panels.

can linkage, we showed that treatment of cell wall with  $\beta(1\rightarrow3)$ -glucanase followed by reduction of the exposed ends with borotritide left stubs consisting of sorbitol and one or a few glucoses attached to chitin (6). These stubs could then be released by chitinase, with or without an attached GlcNAc residue, depending on the even or odd numbering of the chitin chain. The liberation of tritiated oligosaccharides from ConA<sup>+</sup> by  $\beta(1\rightarrow6)$ -glucanase suggested that we had here a similar situation, but with the reducing end of  $\beta(1\rightarrow6)$ -glucan, rather than of chitin, attached directly to  $\beta(1\rightarrow3)$ -glucan. To investigate this possibility, we made use of the previous finding that sweet almond  $\beta$ -glucosidase can hydrolyze a glucose residue attached to sorbitol in a  $\beta(1\rightarrow6)$  but not in a  $\beta(1\rightarrow3)$  linkage (6). When tritiated oligosaccharides from the  $\beta(1\rightarrow6)$ -glucanase digest of ConA<sup>+</sup> were treated with  $\beta$ -glucosidase, the final labeled product was laminaribiitol, as determined by paper chromatography. On the other hand, when unlabeled oligosaccharides from the  $\beta(1\rightarrow6)$ -glucanase digest, originated from internal chains of the polysaccharide, were reduced with borotritide and then subjected to the glucosidase treatment, the tritiated product was sorbitol. Examples of both experiments are shown in Fig. 9. We conclude that  $\beta(1\rightarrow6)$ -glucan chains are directly attached to  $\beta(1\rightarrow3)$ -glucan. The type of linkage between the last glucose of one of the polysaccharides and the

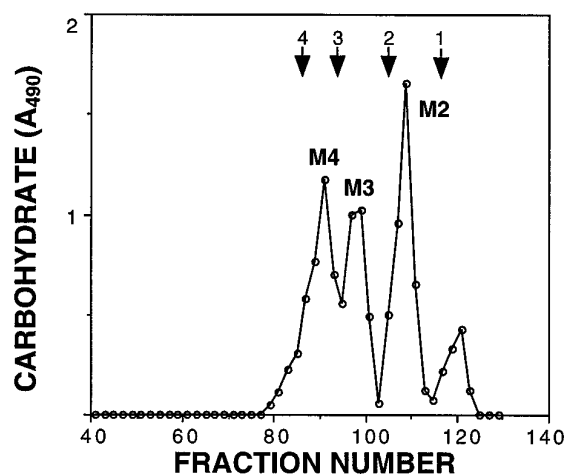


FIG. 10. Acetolysis of fraction M. Fraction M was subjected to acetolysis (see "Experimental Procedures") and subsequently applied to a Bio-Gel P-2 column (1  $\times$  85 cm) and eluted with 0.1 M acetic acid. Fractions of 0.45 ml were collected, and a 40- $\mu$ l portion of each fraction was used for assay of total carbohydrates by the phenol-sulfuric acid method (see "Experimental Procedures"). Standards were glucose (<sup>14</sup>C]glucose, internal standard) (1), laminaribiitol (2), laminaritriitol (3), and laminaritetraitol (4).

first glucose of the other has not been established.

*The High Molecular Weight Material Remaining after  $\beta(1\rightarrow6)$ -Glucanase Digestion Contains Mannan*—The material eluting in the void volume of the Sephadex G-100 column after  $\beta(1\rightarrow6)$ -glucanase digestion (fraction M, Fig. 6*b*) contains all of the mannose and very little of the glucose associated with ConA<sup>+</sup> (Fig. 4*h*). This fraction also contains part of the original tritium label (Fig. 6*b*). The nature of the radioactive component will be discussed below.

Preliminary experiments on digestion of ConA<sup>+</sup> with Pronase<sup>2</sup> followed by Sephadex G-100 chromatography suggested that the mannose could be a component of a large polysaccharide, such as yeast cell wall mannan. This mannan consists of a core region that includes the *N*-linkage to protein and of an extended  $\alpha(1\rightarrow6)$ -linked mannosyl chain with  $\alpha(1\rightarrow2)$  and  $\alpha(1\rightarrow3)$  branches 1–4 mannoses long (35). Acetolysis of mannan results in breakage of the main chain  $\alpha(1\rightarrow6)$  bonds and release of the short side chains (35). High molecular weight carbohydrate-containing material was isolated from a Pronase digest of ConA<sup>+</sup> by Sephadex G-100 chromatography and subjected to acetolysis followed by deacetylation (see "Experimental Procedures"). The products were separated by chromatography on a Bio-Gel P-2 column into four sugar peaks, eluting at the position of free hexose and di-, tri-, and tetrasaccharide (M2, M3, and M4, respectively; see Fig. 10). The structure of the oligosaccharides was studied by <sup>1</sup>H NMR and <sup>13</sup>C NMR. Comparison of spectra with those reported in the literature (36–44) resulted in the assignment of the structure Manp- $\alpha$ -1 $\rightarrow$ 2-Manp- $\alpha$  for M2, Manp- $\alpha$ -1 $\rightarrow$ 2-Manp- $\alpha$ -1 $\rightarrow$ 2-Manp- $\alpha$  for M3, and Manp- $\alpha$ -1 $\rightarrow$ 3-Manp- $\alpha$ -1 $\rightarrow$ 2-Manp- $\alpha$ -1 $\rightarrow$ 2-Manp- $\alpha$  for M4 (data not shown). Signals for the same linkages were found in the <sup>1</sup>H NMR spectrum (not shown) and <sup>13</sup>C NMR spectrum (Fig. 4*g*) of fraction M.

Based on all of these results, it is clear that fraction M contains a high molecular weight mannose polysaccharide of the type normally found in yeast cell walls.

<sup>2</sup> In the course of this study it was found that the commercial preparation of Pronase used contained endo- $\beta(1\rightarrow6)$ -glucanase activity. Therefore, Pronase could not be used in most experiments. Here, however, the contaminating activity was beneficial, because it allowed us to isolate mannan free of  $\beta(1\rightarrow6)$ -glucan by collecting the void volume fraction in gel columns.

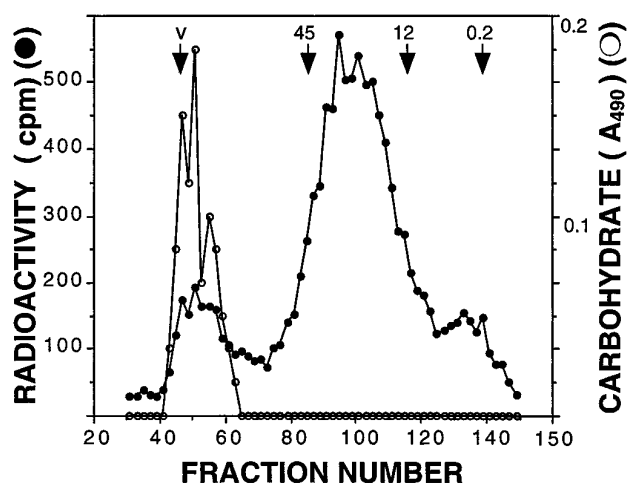


FIG. 11. Treatment of  $^3\text{H}$ -labeled fraction M with glycopeptidase F. A portion of fraction M ( $40\ \mu\text{g}$  of protein) was evaporated to dryness, dissolved in  $40\ \mu\text{l}$  of  $0.01\ \text{M}$  NaOH, and reduced with  $100\ \mu\text{Ci}$  of  $\text{NaB}^3\text{H}_4$ . The excess  $\text{NaB}^3\text{H}_4$  was eliminated by concanavalin A and Sephadex G-100 chromatography. After new evaporation to dryness, the sample ( $2.5 \times 10^5\ \text{cpm}$ ) was incubated with  $8\ \mu\text{l}$  (4000 units) of glycopeptidase F under the conditions specified by the supplier (Life Technologies, Inc.). The mixture was incubated for 16 h at  $37\ ^\circ\text{C}$  in the presence of  $0.02\%$   $\text{NaN}_3$  and subsequently applied to a Sephadex G-100 column ( $1 \times 85\ \text{cm}$ ) and eluted with  $0.1\ \text{M}$  acetic acid. Fractions of  $0.45\ \text{ml}$  were collected, and a  $40\text{-}\mu\text{l}$  portion of every second fraction was either counted ( $\bullet$ ) or used for the assay of total carbohydrates ( $\circ$ ) (see "Experimental Procedures"). Positions of molecular mass standards (in kDa) are indicated.

**ConA<sup>+</sup> Contains Mannoprotein**—Since yeast mannan is invariably found attached to protein, the presence of mannan in fraction M suggested that protein should also be a component of this fraction. Mannan is *N*-linked to protein, and it can be cleaved off with either glycopeptidase F or endoglycosidase H. When fraction M was relabeled by borotritide reduction and treated with glycopeptidase F, chromatography of the digest on Sephadex G-100 separated unlabeled carbohydrate that emerged in the void volume from most of the tritiated material that was eluted in later fractions and presumably contained the protein (Fig. 11). Similar results were obtained with endoglycosidase H (data not shown). The latter finding suggested a procedure to label specifically the protein, by transgalactosidation onto the GlcNAc that remains attached to protein after endo-H hydrolysis. Accordingly, fraction M was treated with endo-H (45) and fractionated on Sephadex G-100. The liberated material containing the intrinsic tritium label (similar to the tritiated peak of Fig. 11) was concentrated and incubated with  $\text{UDP}[^{14}\text{C}]\text{Gal}$  in the presence of galactosyltransferase (see "Experimental Procedures"). Upon Sephadex G-100 chromatography, the incorporated  $^{14}\text{C}$  radioactivity eluted in the same position as the tritiated material. Electrophoresis of the  $^{14}\text{C}$ -labeled material on polyacrylamide in the presence of SDS, followed by autoradiography, showed a broad band at about 100 kDa and some material that trailed behind (Fig. 12a). Fraction M or ConA<sup>+</sup> remained at the origin.

**Mannoprotein in ConA<sup>+</sup> Is Attached to  $\beta(1\rightarrow6)$ -Glucan through a Portion of a GPI Anchor**—The simultaneous binding of the mannoprotein and of  $\beta(1\rightarrow6)$ -glucan to ConA-Sepharose indicated that these two components are covalently linked, because ConA has no affinity for  $\beta$ -linked glucose. The nature of the linkage remained to be determined. Several cell wall proteins are attached to  $\beta(1\rightarrow6)$ -glucan through what seems to be a remnant of a former GPI anchor (7), part of which was lost in a putative transglycosylation. To verify whether that was the case here, fraction M was relabeled by reduction with borotritide and treated in the cold with hydrofluoric acid, under

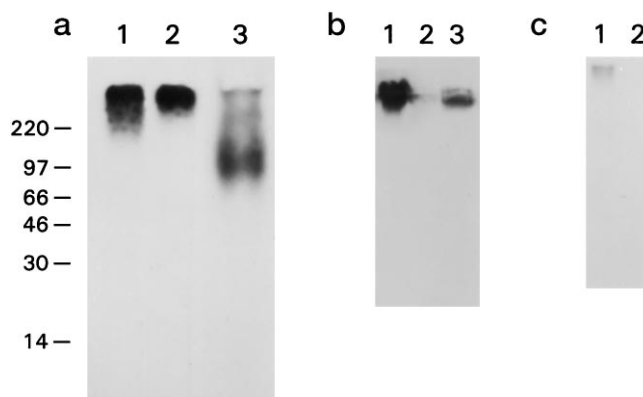
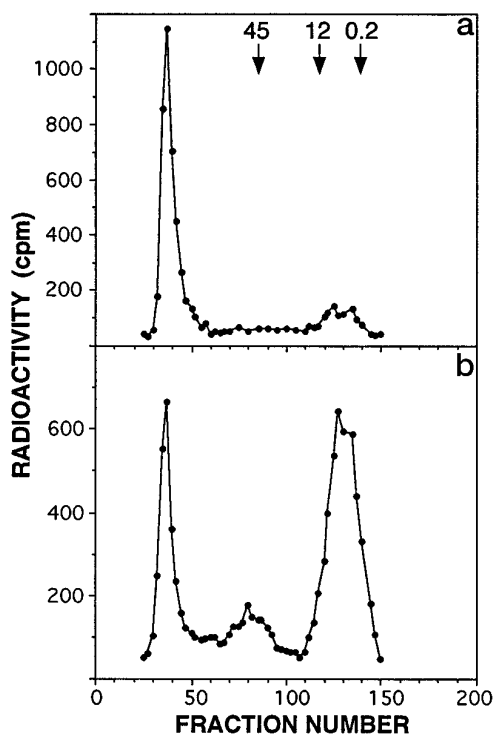


FIG. 12. Electrophoresis and Western blots of different fractions. *a*, SDS-polyacrylamide gel electrophoresis of  $^{14}\text{C}$ -Gal-labeled ConA<sup>+</sup> of fraction M and of material released from fraction M by endo-H and relabeled with  $^{14}\text{C}$ -Gal. Samples ( $20,000\text{--}30,000\ \text{cpm}$ ) were evaporated to dryness, dissolved in sample buffer containing  $0.5\%$   $\beta$ -mercaptoethanol, incubated for 5 min in a boiling water bath, and separated on an 18% gel, together with  $^{14}\text{C}$ -labeled molecular mass standards, in Tris-glycine running buffer. The gel was fixed for 45 min in  $15\%$  trichloroacetic acid, washed 3 times for 30 min in  $\text{Me}_2\text{SO}$ , and soaked for 45 min in  $100\ \text{ml}$  of  $\text{Me}_2\text{SO}$  containing  $50\ \text{g}$  of 2,5-diphenyl-oxazole. After overnight washing in water and drying, the gel was subjected to fluorography with 1 week of exposure. Lane 1,  $^{14}\text{C}$ -labeled ConA<sup>+</sup> fraction; lane 2, fraction M; lane 3,  $^{14}\text{C}$ -labeled endo-H-released material from fraction M. Positions of molecular mass standards (in kDa) are indicated. *b* and *c*, Western blot and autoradiography of the [ $^{14}\text{C}$ ]Gal-labeled ConA<sup>+</sup> fraction after incubation with  $\beta(1\rightarrow6)$ -glucanase or hydrofluoric acid. *b*, portions ( $4 \times 10^4\ \text{cpm}$ ) of [ $^{14}\text{C}$ ]Gal-labeled ConA<sup>+</sup> were either incubated with endo- $\beta(1\rightarrow6)$  glucanase in  $45\ \mu\text{l}$  of  $111\ \text{mM}$  sodium acetate with 20 milliunits of enzyme for 17 h at  $37\ ^\circ\text{C}$  (lane 2) or with  $50\ \mu\text{l}$  of  $50\%$  hydrofluoric acid for 24 h on ice (lane 3). Lane 1, unincubated control. After incubation, samples were separated by SDS-polyacrylamide gel electrophoresis, transferred to a PVDF membrane, and blotted with antibody against  $\beta(1\rightarrow6)$ -glucan (11). *c*, lane 2, a portion ( $3 \times 10^4\ \text{cpm}$ ) of [ $^{14}\text{C}$ ]Gal-labeled ConA<sup>+</sup> fraction was treated with  $50\%$  HF under the same conditions as in the legend of Fig. 13 and separated on SDS-polyacrylamide gel. Lane 1, unincubated control. The gel was transferred to a PVDF membrane. The membrane was dried and exposed for autoradiography for 96 h.

conditions that lead to hydrolysis of the linkage between phosphate and mannose in a GPI anchor (46, 47). When the reaction mixture was applied to a Sephadex G-100 column, a large portion of the tritium label was eluted at a position corresponding to low molecular weight material (Fig. 13b). This material contained the tritium introduced in the relabeling of fraction M, because when the original fraction M was treated with hydrofluoric acid, almost all of the tritium remained in the void volume fraction (Fig. 13a). If the anchor hypothesis were correct, the low molecular weight material released by HF would consist of a mannose oligosaccharide attached to a stub of  $\beta(1\rightarrow6)$ -linked glucoses left over after  $\beta(1\rightarrow6)$ -glucanase hydrolysis of ConA<sup>+</sup> (Fig. 3). This proved to be the case. The tritiated material yielded one peak, corresponding to an oligosaccharide of 8 residues upon HPLC on a Rezex RSO-Oligosaccharides column (Fig. 14a). However, when this material was rechromatographed on a Bio-Gel P-4 column, it split into two peaks eluting at positions corresponding to oligosaccharides of 7 and 8 residues (results not shown). These substances were resistant to  $\beta$ -glucosidase (Fig. 14b). They were, however, hydrolyzed by  $\alpha$ -mannosidase to compounds with the size of oligosaccharides containing 3 (A) and 2 (B) hexoses, respectively (Fig. 14c). Both products were now degraded by  $\beta$ -glucosidase, giving rise to a free hexitol (Fig. 14d). The hexitol was identified as sorbitol by paper chromatography (results not shown). These results are consistent with the HF-released material being made of a chain of five  $\alpha$ -linked mannosyl units (a number found in yeast GPI anchors (47)), attached to a reduced



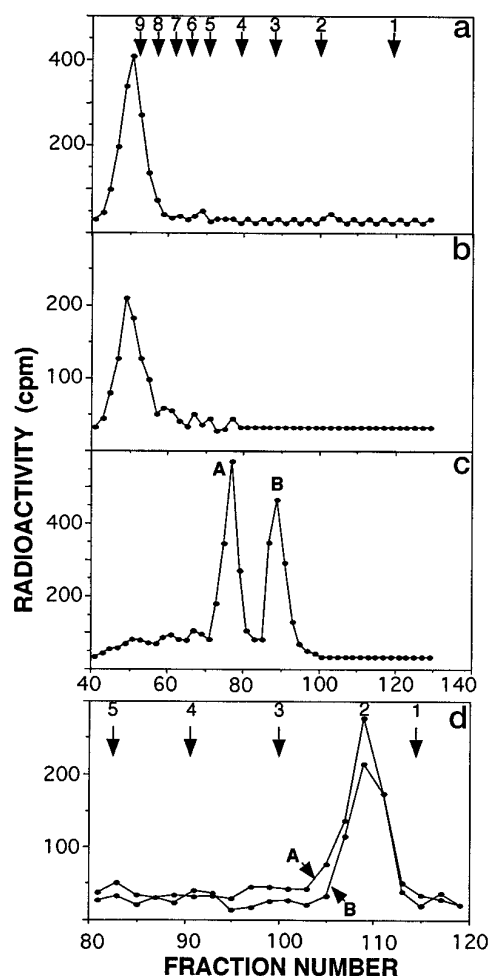


**FIG. 13. Release of an oligosaccharide from fraction M by hydrofluoric acid.** *a*, a portion of fraction M ( $3 \times 10^4$  cpm) was evaporated to dryness, dissolved in 200  $\mu$ l of precooled 50% hydrofluoric acid, and incubated at 0  $^{\circ}$ C for 72 h. The digest was pipetted onto frozen 10 M NaOH (400  $\mu$ l) in a microcentrifuge tube placed in ice. The resulting suspension was spun down, and the NaF precipitate was washed twice with 100  $\mu$ l of water. Supernatants were combined and applied to a Sephadex G-100 column (1  $\times$  85 cm), which was eluted with 25 mM Tris-chloride, pH 7.5, containing 0.02%  $\text{NaN}_3$ . Fractions of 0.45 ml were collected, and a 400- $\mu$ l portion of every second fraction was counted. Positions of molecular mass standards (in kDa) are indicated. *b*, a portion ( $3 \times 10^4$  cpm) of  $^3\text{H}$ -reabeled (see legend of Fig. 14) fraction M was treated with hydrofluoric acid and chromatographed as in *a*.

glucose disaccharide or trisaccharide, respectively. The latter was left over after hydrolysis with the  $\beta(1\rightarrow6)$ -endoglucanase.

The hydrofluoric acid treatment was also applied to the intact  $\text{ConA}^+$ . After HF hydrolysis, the material was subjected to polyacrylamide electrophoresis and Western blotting with an antibody against  $\beta(1\rightarrow6)$ -glucan (11). The amount of immunoreactive material was much diminished after the HF treatment and completely eliminated by incubation with  $\beta(1\rightarrow6)$ -glucanase (Fig. 12*b*). Similarly,  $\text{ConA}^+$  that had been labeled with [ $^{14}\text{C}$ ]galactose was incubated with HF, subjected to electrophoresis on polyacrylamide gel, and transferred to a PVDF membrane. The membrane was exposed for autoradiography under conditions in which only  $^{14}\text{C}$  but not tritium would be detected. The sample treated with HF yielded a negative result, whereas an intact sample showed a band at about the same position as found before for intact  $\text{ConA}^+$  (Fig. 12*c*). In both cases, the results indicate that the  $\beta(1\rightarrow6)$ -glucan had been separated from the protein and therefore was lost in the transfer to the PVDF membrane. The split between the two moieties of the complex could also be shown by  $\text{ConA}$ -Sephadex chromatography, but it was found that hydrofluoric acid, even at the low temperature used in the experiment, led to rather extensive degradation of  $\beta(1\rightarrow6)$ -glucan (results not shown).

**Hexitol Residues Are Linked to Protein in  $\text{ConA}^+$** —After degradation of  $\text{ConA}^+$  with  $\beta(1\rightarrow6)$ -glucanase, tritiated material was still found in the Sephadex G-100 void volume peak (fraction M; see Fig. 6*b*). This result was unexpected, because most of the glucose had been liberated by the glucanase (Fig. 4*h*);



**FIG. 14. Treatment of HF-released oligosaccharides from  $^3\text{H}$ -reabeled fraction M with  $\beta$ -glucosidase and  $\alpha$ -mannosidase.** *a*, a portion (10,000 cpm of  $^3\text{H}$ ) of HF-released oligosaccharide (Fig. 13*b*) was chromatographed on a Rezex RSO-Oligosaccharides column (10  $\times$  200 mm, guard column 10  $\times$  20 mm) at 75  $^{\circ}$ C with a Gilson model 303 HPLC pump at 0.2 ml/min. Fractions of 100  $\mu$ l were collected, and a 25- $\mu$ l portion of every second sample was counted. *b*, a similar portion of oligosaccharide was incubated with 0.1 mg of  $\beta$ -glucosidase in 50  $\mu$ l of 50 mM sodium acetate, pH 5.0, for 16 h at 37  $^{\circ}$ C in the presence of 0.02%  $\text{NaN}_3$ , followed by chromatography on the Rezex column. *c*, another portion of oligosaccharide was incubated with  $\alpha$ -mannosidase (250 milliunits) in 50  $\mu$ l of a reaction mixture containing 100 mM sodium acetate, pH 5.0, 2 mM  $\text{Zn}^{2+}$ , and 0.02%  $\text{NaN}_3$  for 16 h at 37  $^{\circ}$ C and applied to the Rezex column. *d*, peaks A and B from *c* were individually pooled and incubated with  $\beta$ -glucosidase as in *b*, followed by chromatography on a Bio-Gel P-2 column (see legend of Fig. 12). Standards were [ $^{14}\text{C}$ ]glucose (internal standard) (1); hexitol (2); diol to octaol obtained by reduction of a dextran ladder (Oxford Glycosystems) (3–9).

therefore, all of the labeled sorbitol attached to the glucose should be in the low molecular weight fraction. The puzzle was solved when fraction M was hydrolyzed with trifluoroacetic acid and the labeled material was tentatively identified by paper chromatography as mannitol, rather than sorbitol (results not shown). The alditol appears to be directly or indirectly attached to protein, because it remains with the protein after treatment of fraction M with glycopeptidase F (Fig. 11), endo-H, or HF (Fig. 13). Treatment of fraction M with proteinase K, followed by Sephadex G-100 chromatography, yielded a peak containing about 45% of the radioactivity in a position corresponding to low molecular weight material, whereas the remainder was still in the void volume fraction (results not shown). The structure of the alditol-labeled material and the manner in which it is attached to protein remain undeter-



mined. From the practical point of view, it served as a convenient label during the isolation of the protein moiety of ConA<sup>+</sup>.

*ConA<sup>-</sup> Is Similar to ConA<sup>+</sup> but Lacks Mannoprotein*—Although most of the emphasis of this study was on the ConA<sup>+</sup> fraction, many experiments were also done with ConA<sup>-</sup>. The structure of ConA<sup>-</sup> appears to be similar to that of ConA<sup>+</sup>, except for the absence of mannoprotein in ConA<sup>-</sup>, on the strength of the following evidence. (a) ConA<sup>-</sup> is linked to chitin and to  $\beta(1\rightarrow3)$ -glucan, based on the incorporation of [<sup>14</sup>C]galactose and binding to the *E. cristagalli* lectin-agarose column (Fig. 2b) as well as on the production of both <sup>14</sup>C- and tritium-labeled oligosaccharides after incubation with  $\beta(1\rightarrow6)$ -glucanase (Fig. 6, c and d). (b) The <sup>13</sup>C NMR spectrum of ConA<sup>-</sup> shows the presence of  $\beta(1\rightarrow6)$ -glucan, but the signals for mannan are missing (Fig. 4e). Accordingly, ConA<sup>-</sup> contains no mannose (Fig. 4f). The absence of this sugar is also consistent with the inability of ConA<sup>-</sup> to bind to ConA-Sepharose. (c) No “mannitol tritium” was found after  $\beta(1\rightarrow6)$ -glucanase digestion of ConA<sup>-</sup> (Fig. 6d).

From the Sephadex G-100 elution pattern of intact ConA<sup>-</sup> it is clear that this material has a lower average molecular weight than ConA<sup>+</sup> and is quite heterogeneous in size. This heterogeneity presumably reflects variability in length of the  $\beta(1\rightarrow6)$ -glucan chains.

#### DISCUSSION

*A Family of Compounds in the Isolated Complex*—The fraction solubilized from the cell wall by successive treatments with  $\beta(1\rightarrow3)$ -glucanase and chitinase and isolated in the void volume of a P-2 column turned out to be surprisingly complex. This material, expected to represent a linkage region between chitin and  $\beta(1\rightarrow3)$ -glucan, was separated on a ConA-Sepharose column into the ConA<sup>+</sup> and ConA<sup>-</sup> fractions. After transferring [<sup>14</sup>C]galactose to terminal GlcNAc residues in ConA<sup>+</sup> and ConA<sup>-</sup>, each one of them could be further fractionated on an *E. cristagalli* lectin-agarose column into portions containing different amounts of galactose, hence of GlcNAc. Added to this complexity is a certain variability in size evidenced during chromatography of ConA<sup>+</sup> on Sephacryl S-400 or of ConA<sup>-</sup> on Sephadex G-100. Despite all these signs of heterogeneity, there are many indications that the substances comprising either the ConA<sup>+</sup> or the ConA<sup>-</sup> fraction share the same basic structure. Thus, the NMR spectra are relatively uncomplicated and of straightforward interpretation, the sugar composition is simple, and all fractions are degraded by  $\beta(1\rightarrow6)$ -glucanase in a similar fashion. Furthermore, some of the fractions obtained by chromatography of ConA<sup>+</sup> contain mannoprotein,  $\beta(1\rightarrow6)$ -glucan, tritiated sorbitol residues, and GlcNAc (indicated by the presence of [<sup>14</sup>C]galactose): these components could be separated only after treatment of the complex with either enzymes or hydrofluoric acid. The heterogeneity in size can be accounted for by variability in length of the  $\beta(1\rightarrow6)$ -glucan chains. Still, the complexity of the material, together with its scarcity, reflected often in samples at the nanomolar level, severely limited the range of usable methodologies and prevented the complete determination of some of the sugar to sugar linkages.

*Components of the Complex*—The main components of the ConA<sup>+</sup> fraction are  $\beta(1\rightarrow6)$ -glucan and mannoprotein.  $\beta(1\rightarrow6)$ -glucan was identified by the NMR spectrum and by its susceptibility to hydrolysis by  $\beta(1\rightarrow6)$ -glucanase. The proton NMR spectrum provided evidence that  $\beta(1\rightarrow3)$  branches are attached to the main  $\beta(1\rightarrow6)$  chain. The size of the glucan molecules is somewhat in doubt. In ConA<sup>-</sup> only  $\beta(1\rightarrow6)$ -glucan makes a significant contribution to the molecular weight, because the mannoprotein is missing and chitin and  $\beta(1\rightarrow3)$ -glucan are represented by only one or a few sugar residues. Chromatography of ConA<sup>-</sup> on Sephadex G-100 (Fig. 6c) shows a quite

heterogeneous distribution, with sizes between approximately 10 and 100 kDa, which would correspond to chains of between 60 and 600 glucose residues, with an average of perhaps 300–350 residues. By comparison,  $\beta(1\rightarrow6)$ -glucan isolated from yeast by alkali and acetic acid extraction seems to consist on average of 140 glucose units (48). For ConA<sup>+</sup>, it is more difficult to estimate the glucan size because of the presence of mannoprotein. Hydrofluoric acid cuts the linkage between glucan and protein but also partially hydrolyzes the glucan (see “Results”). An attempt was made to degrade the mannoprotein by sequential incubation of ConA<sup>+</sup> with glycopeptidase F and proteinase K, followed by chromatography on Sephadex G-100. The treatment caused a decrease in size, with part of the material now entering the included volume (results not shown). However, we lacked criteria to determine whether the action of the enzymes used had been complete; therefore, the size of  $\beta(1\rightarrow6)$ -glucan in ConA<sup>+</sup> remains in doubt.

The presence of protein in ConA<sup>+</sup> was inferred indirectly from the finding that mannan was a component. The polysaccharide is a typical yeast cell wall mannan, as judged from its NMR spectrum, the oligosaccharides generated by acetolysis and their NMR spectra, and the size shown in gel filtration after excision from protein with glycopeptidase F or endo-H. Use of the latter enzyme allowed us to label the protein by transfer of [<sup>14</sup>C]galactose onto the residual GlcNAc and thus detect it after the subsequent gel electrophoresis. The apparent molecular weight of the deglycosylated protein, in the vicinity of 100,000, is remarkably similar to that we previously found (94,000) for the main component of cell wall proteins after  $\beta(1\rightarrow3)$ -glucanase and endo-H treatment (4). In addition to the *N*-linked mannan, it is possible that the protein has *O*-linked mannose oligosaccharides, but the small amounts of material available did not allow investigation of this point. The radioactivity incorporated into the protein upon reduction of the complex with borotritide was found to be in an alditol residue tentatively identified as mannitol, an indication that mannose with its reducing group exposed was attached, either directly or indirectly, to the untreated protein. This is a very surprising result, because in glycoproteins both *N*-linked and *O*-linked sugars are attached through their reducing group, making the latter unavailable to borohydride. The exceptions are GPI anchors (see below), but the alditol could not be part of an anchor because it was not released by hydrofluoric acid (Fig. 13). Preliminary results indicate that a substantial portion of the tritium incorporated by reduction of intact cell walls is in the alditol, confirming that the reducing carbon of the original sugar, presumably mannose, was unlinked (results not shown).

*Cross-links between the Components*—As found in other cases (7), the connection between mannoprotein and  $\beta(1\rightarrow6)$ -glucan is through a portion of GPI anchor. Previously, the presence of anchor material was inferred somewhat indirectly, from the susceptibility of the protein-glucan linkage to hydrofluoric acid and phosphodiesterase (7) and from the attachment to cell wall of hybrid proteins containing the anchor-bearing domain of  $\alpha$ -agglutinin (49). In the present study, ConA<sup>+</sup> was digested with  $\beta(1\rightarrow6)$ -glucanase, reduced with borotritide, and hydrolyzed with HF. The resulting oligosaccharides were isolated and shown to consist of five  $\alpha$ -linked mannose residues, attached to the nonreducing end of  $\beta(1\rightarrow6)$ -linked reduced disaccharide and trisaccharide, respectively. This result identifies the residual portion of the original GPI anchor as ethanolamine-phosphate-Man<sub>5</sub>. The glucosaminyl residue was eliminated together with the phosphatidylinositol group in the putative transglycosylation reaction that originated the protein-glucan connection.

Connections of the mannoprotein- $\beta(1\rightarrow6)$ -glucan complex to  $\beta(1\rightarrow3)$ -glucan and chitin were postulated on account of the need of both  $\beta(1\rightarrow3)$ -glucanase and chitinase for solubilization of the complex and were confirmed by the presence of remnants of both polysaccharides in the complex. Thus,  $\beta(1\rightarrow3)$ -linked oligosaccharides were connected to the reducing end of  $\beta(1\rightarrow6)$ -glucan, and GlcNAc that survived chitin degradation by chitinase was found to be attached to the same glucan, probably onto  $\beta(1\rightarrow3)$  side branches. The GlcNAc at the reducing end of the chitin chain is connected to glucose either by a  $\beta(1\rightarrow2)$  or a  $\beta(1\rightarrow4)$  linkage. We favor the latter possibility by analogy to the bond between chitin and  $\beta(1\rightarrow3)$ -glucan (6).

If our hypothesis that chitin is attached to a  $\beta(1\rightarrow3)$ -linked glucose residue is correct, one may ask whether the previously found linkage of chitin to glucan might not have been to a  $\beta(1\rightarrow3)$  side chain of  $\beta(1\rightarrow6)$ -glucan rather than to the long  $\beta(1\rightarrow3)$ -linked polysaccharide. This notion seems unlikely for the following reasons: first, the endo- $\beta(1\rightarrow3)$ -glucanase activity in Zymolyase requires a minimum of 5 glucose residues to cut the chain (50), whereas  $\beta(1\rightarrow3)$  branches of  $\beta(1\rightarrow6)$ -glucan are quite short, probably not more than one or two residues in length (48); second, we found that mutant *kre5*, despite its low content of  $\beta(1\rightarrow6)$ -glucan (51), shows a normal complement of oligosaccharides indicative of a chitin- $\beta(1\rightarrow3)$ -glucan linkage (results not shown).

The ConA<sup>-</sup> material is similar to ConA<sup>+</sup>, except for the absence of mannoprotein. Thus, it may be either a precursor or a degradation product of ConA<sup>+</sup>.

*The role of  $\beta(1\rightarrow6)$ -Glucan in the Organization of the Yeast Cell Wall*—From the foregoing analysis, it appears that  $\beta(1\rightarrow6)$ -glucan is the central molecule or “glue” that keeps together the other components of the cell wall, including  $\beta(1\rightarrow3)$ -glucan, mannoprotein, and part of the chitin (Fig. 3). Thus, it is not surprising that defects in  $\beta(1\rightarrow6)$ -glucan formation, as found in several mutants (52, 53), can interfere with cell wall assembly and have severe effects on cell growth (52, 53). For this reason, it is possible that compounds that specifically inhibit  $\beta(1\rightarrow6)$ -glucan synthesis, if such can be found, would behave as antifungal agents.

The chitin participating in linkages to both  $\beta(1\rightarrow3)$ - and  $\beta(1\rightarrow6)$ -glucan is synthesized by chitin synthase 3 (6). This chitin is found both in the ring formed at the base of an emerging bud and, in dispersed form, throughout the cell wall (54). It is of interest to compare the results of the present investigation with those of an earlier study on the distribution of chitin in the cell wall (55). In that study, we found that most of the chitin present in lateral walls and part of the bud scar chitin could be solubilized by incubation of alkali-treated cell ghosts with a  $\beta(1\rightarrow6)$ -glucanase preparation that was contaminated with a small amount of chitinase. The same amount of chitinase without the glucanase had no effect. Those results are in good agreement with the present findings about chitin synthase 3 involvement in chitin synthesis and about the role of  $\beta(1\rightarrow6)$ -glucan in chitin cross-linking. From both sets of results it may be concluded that the chitin interspersed in the cell wall and possibly part of that in the ring are attached to  $\beta(1\rightarrow6)$ -glucan, whereas another portion of the ring chitin is directly linked to  $\beta(1\rightarrow3)$ -glucan (6). It is possible that the  $\beta(1\rightarrow6)$ -glucan molecules attached to a single chitin chain come from a different region than those bearing several chains.

It is difficult to estimate what proportion of the cell wall is involved in the linkages studied in this work. Fraction  $V_0$  contained about 4% of the total cell wall carbohydrate. To this, one should add the chitin and  $\beta(1\rightarrow3)$ -glucan chains that had been eliminated by treatment with the corresponding hydrolytic enzymes.

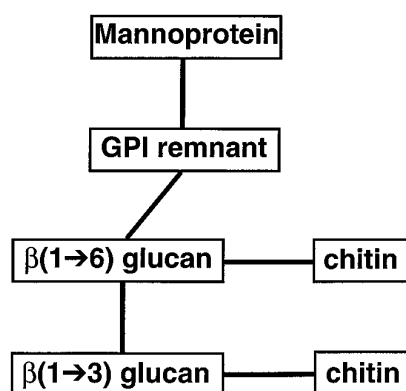


FIG. 15. **Proposed flexible building block of the yeast cell wall.** Some of the components may be missing at different locations; e.g. absence of mannoprotein would result in a ConA<sup>-</sup>-like structure, absence of mannoprotein and  $\beta(1\rightarrow6)$ -glucan would leave a chitin- $\beta(1\rightarrow3)$ -glucan polymer, and so on.

From this and previous studies (6, 7), the concept emerges of a “flexible building block” of the yeast cell wall (Fig. 15). The complete structure would comprise mannoprotein,  $\beta(1\rightarrow6)$ -glucan,  $\beta(1\rightarrow3)$ -glucan, and chitin. Part of the blocks (ConA<sup>-</sup>) would miss mannoprotein, especially in inner layers of the wall (4), or chitin, mostly in the outer layer (7).

*Biosynthesis of Cross-linkages*—How are the components of the yeast wall building blocks joined together in the cell? From the available evidence it appears that chitin (54) and  $\beta(1\rightarrow3)$ -glucan (56) are synthesized at the plasma membrane with simultaneous secretion into the periplasmic region. On the other hand, mannoprotein is synthesized at the ER and modified during its transport through the secretory pathway (57). Finally, at least part of  $\beta(1\rightarrow6)$ -glucan synthesis may occur in the ER or Golgi (53). It is clear that all of the components will only meet in the periplasmic region, which is then the location where the linkage reactions must take place. Because it is unlikely that high energy compounds are present in that location and on account of the nature of the connecting bonds, transglycosidation is the most likely reaction leading to formation of the cross-linkages. Identification of the enzymatic activities involved in these reactions awaits further work.

Although the mechanism of biosynthesis of the cross-linkages remains obscure, we have some information about its timing in the cell cycle. The chitin interspersed in the cell wall that is synthesized by chitin synthase 3 is laid down after septum formation, in the final phase of bud growth (58). As discussed before (6), that must be the phase of the cell cycle in which cross-links involving chitin are formed. Walls of mutants lacking chitin synthase 3 have been found to be less resistant to osmotic shock than those of wild type (59). This invites speculation that the cross-links are not created during most of the period of active growth lest they interfere with the plasticity of the developing wall, but are instead added at the end to increase rigidity of the final product.

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