

Structure of Cell Wall and Extracellular Mannans from *Saccharomyces rouxii* and Their Relationship to a High Concentration of NaCl in the Growth Medium

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Cells of *Saccharomyces rouxii* (a salt-tolerant yeast) were grown in the presence of two levels of NaCl, 0 and 15%. Mannans obtained from both the cell walls and culture filtrates (extracellular) were examined. Yields based on the dry weight of cells demonstrated that the levels of both cell wall and extracellular mannans were lower when cells were grown in the presence of 15% NaCl. However, the carbohydrate and protein contents of the mannan preparations were not altered. The cell wall mannans obtained from the two growth conditions had similar molecular weights, whereas the extracellular mannans had different molecular weight distributions. Structural analyses showed that the cell wall and extracellular mannans had similar structures. Both had an α 1-6-linked backbone to which single mannose and mannosyl units were connected as side chains, predominantly by α 1-2 linkages. The mannans also contained mannosyloligosaccharides, mannotriose, mannobiose, and mannose attached to protein through an *O*-glycosidic bond. The molecular structure of the cell wall mannans remained unchanged at both levels of NaCl. However, in the presence of 15% NaCl, the side chains consisting of a mannobiose unit were slightly reduced.

Saccharomyces rouxii, which is important for the development of flavor in soy sauce fermentation, has tolerance to high concentrations of NaCl in the growth medium. It has been found that *S. rouxii* accumulates glycerol or other polyols or both in response to increasing concentrations of extracellular solutes (2, 5, 14). These polyols are considered necessary for osmotolerance as they may attain high levels intracellularly without causing damage to the cellular enzyme function (3). However, the cell wall structure of osmotolerant yeasts, which may play an important role in the physical protection of the cells, has not been investigated yet. In this paper we describe the structure of the cell wall and extracellular mannans from *S. rouxii* and their relationship to a high concentration of NaCl in the growth medium.

MATERIALS AND METHODS

Materials. Bio-Gel P-2 (200 to 400 mesh) was obtained from Bio-Rad Laboratories and Sepharose 6-B was obtained from Pharmacia. All other chemicals were of reagent grade and were purchased from commercial sources.

Organism and growth conditions. *S. rouxii* ATCC 13356 was grown aerobically at 30°C in a medium of following composition (per liter): glucose, 50 g; vitamin-free Casamino Acids, 9 g; KH_2PO_4 , 0.6 g; KCl, 0.4 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.15 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g; MnSO_4 , 2.5 mg; FeCl_3 , 2.5 mg; thiamine hydrochloride, 0.25 mg; riboflavin, 0.1 mg; pyridoxine hydrochloride, 1 mg; niacin, 5 mg; *p*-aminobenzoic acid, 0.5 mg; biotin, 10 μg ; inositol, 25 mg; calcium pantothenate, 0.5 mg; and NaCl, 0 or 150 g (pH 5.0). Cells were harvested at early stationary growth phase by centrifugation, washed with water, and lyophilized.

Scanning electron microscopy. The cells were fixed with 2% glutaraldehyde at 4°C for 2 h and postfixed with 1%

osmium tetroxide at 4°C for 1 h. They were dehydrated through an ethanol series and then critical point dried. The specimens were observed with a JEOL model JSM-T20 scanning electron microscope.

Preparation of mannans. Lyophilized cells were extracted by autoclaving at 120°C for 90 min in 0.1 M citrate buffer (pH 7.0) (17). After centrifugation, the residue was reextracted in the same manner, and the extracts were combined. The cell wall mannan was separated from the extracts by Cetavlon (cetyltrimethylammonium bromide) fractionation (7, 12). The extracellular mannan was isolated from the culture filtrate as follows: (i) The culture filtrate was dialyzed, concentrated, and precipitated by using 3 volumes of ethanol; and (ii) the extracellular mannan was purified from the ethanol precipitate by Cetavlon fractionation (7, 12).

General methods. Carbohydrate was determined by the phenol-sulfuric acid method (4). Protein was determined by the method of Lowry et al. (13), and phosphate was determined by the method of Bartlett (1). Paper chromatography was carried out on no. 50 filter paper (Toyo Roshi Co. Ltd.) with the following solvent system: pyridine-*n*-butanol-water (4:6:3). Sugars on paper chromatograms were detected with alkaline silver nitrate reagent. Gas chromatography of the sugar derivatives was carried out with a Shimadzu model GC-4M gas chromatograph equipped with a flame ionization detector. The alditol trifluoroacetates were chromatographed on a glass column (0.3 by 200 cm) of 1.5% QF-1 on Chromosorb W (AW-DMCS) at 135°C at a nitrogen flow rate of 18 ml/min. The alditol acetates were chromatographed on a glass column (0.3 by 200 cm) of 3% OV 210 Chromosorb W (AW-DMCS) at 185°C at a nitrogen flow rate of 20 ml/min.

Acid hydrolysis. Complete acid hydrolysis of mannan was carried out with 1.5 N trifluoroacetic acid at 100°C for 3 h. The hydrolysates were characterized by paper chromatography and gas chromatography as alditol trifluoroacetates (8).

Acetylation. Mannans (100 mg) were dissolved in formamide (2 ml) and acetylated in a mixture containing 5 ml of dry

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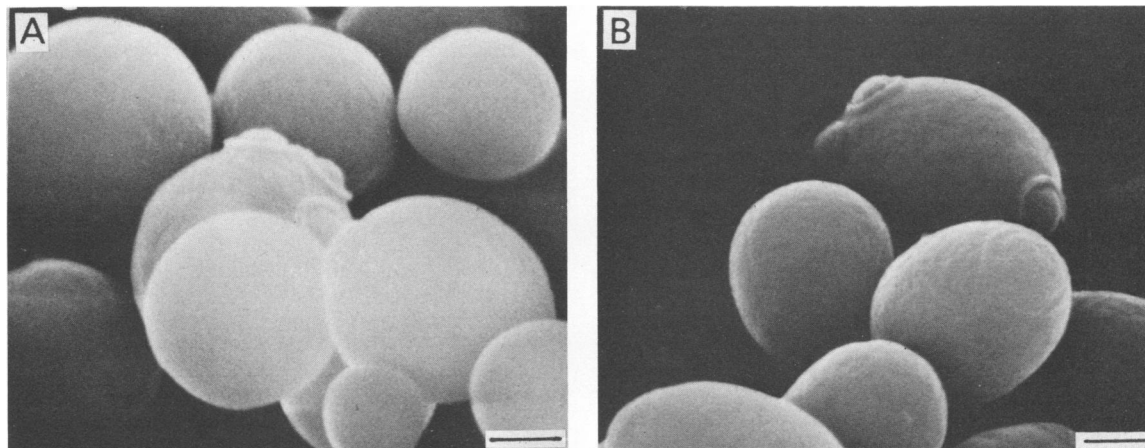


FIG. 1. Scanning electron micrographs of *S. rouxii* cells grown in the absence of NaCl (A) and in the presence of 15% NaCl (B). Bar = 1 μ m.

pyridine and 5 ml of acetic anhydride at 30°C overnight. The solution was poured into water (50 ml) and centrifuged. The precipitate was dried and acetylated in 10 ml of acetolysis mixture (acetic anhydride-acetic acid-sulfuric acid, 10:10:1) at 40°C for 13 h by using the procedure of Kocourek and Ballou (9). The acetolysis products were deacetylated with 0.1 N sodium methoxide and neutralized with Dowex 50(H⁺). The deacetylated products were separated by gel filtration on a column of Bio-Gel P-2 (1.9 by 150 cm). The column was eluted with water at a rate of 15 ml/h; 2-ml fractions were collected.

Methylation. Acetolysis products were methylated by the method of Hakomori (6), as described by Lindberg (11). Methylated samples were hydrolyzed with 90% formic acid at 100°C for 2 h. After evaporation at 45°C to remove formic acid, the residue was dissolved in 0.5 N H₂SO₄ and kept at 100°C for 12 h. The solution was neutralized with barium carbonate, filtered, and concentrated. The hydrolysates were converted to alditol acetates by reduction with sodium borohydride, followed acetylation with acetic anhydride-pyridine. The characterization of these derivatives was done by gas chromatography as described above.

β -Elimination. Mannans (50 mg) were dissolved in 4 ml of 0.1 N NaOH and kept at 25°C for 24 h. The solution was neutralized with 1 N HCl and concentrated to dryness. The β -elimination products were separated on a Bio-Gel P-2 column by elution with water at a rate of 15 ml/h; 2-ml fractions were collected.

RESULTS

Scanning electron microscopy of *S. rouxii* cells. Figure 1 shows scanning electron micrographs of *S. rouxii* cells

TABLE 1. Analyses of cell wall and extracellular mannans

Mannan	Concn of NaCl in growth medium (%)	Yield ^a	Composition (%) ^b		
			Protein	Carbo-hydrate	Phos-phorus
Cell wall	0	151.0	4.9	83.2	0.41
	15	121.2	5.6	85.3	0.53
Extracellular	0	76.1	7.8	82.1	0.23
	15	50.1	10.1	79.1	0.45

^a Expressed as milligrams of mannan per gram (dry weight) of cells.

^b Based on weights of mannans.

grown in the presence of 15% NaCl and in the absence of NaCl. We observed that the cells grown in a medium containing no NaCl were spherical, whereas those grown in the presence of 15% NaCl were spheroidal.

Properties of cell wall and extracellular mannans. Table 1 shows some properties of the mannans prepared from cells and culture filtrates. The yields of mannans based on the weight of dried cells were slightly different in the presence of 15% NaCl and in the absence of NaCl. The high concentration of NaCl in the medium gave a lower yield of mannans. On the other hand, the protein and carbohydrate contents of the mannans were not significantly different between the two growing conditions. The distributions of the molecular weights were compared by gel filtration on a Sepharose 6-B column (Fig. 2). The cell wall mannans showed no difference under the two conditions, whereas the extracellular mannans showed different molecular weight distributions. The extracellular mannan obtained from the medium containing 15%

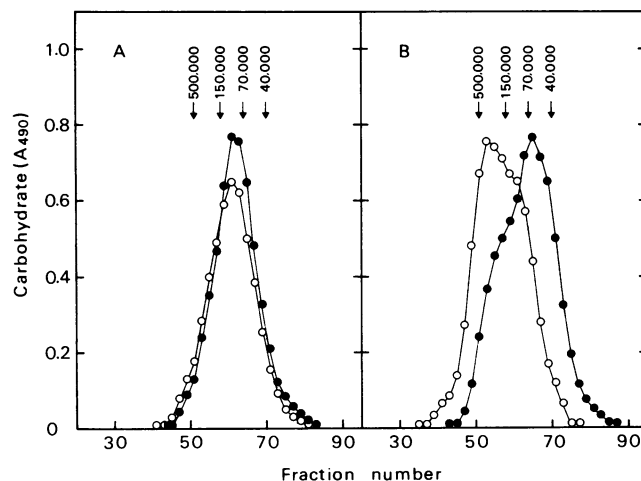


FIG. 2. Gel filtration patterns of cell wall (A) and extracellular (B) mannans on a Sepharose 6-B column. Each mannan obtained from cells grown in the absence of NaCl (●) and in the presence of 15% NaCl (○) and from culture filtrates was applied to a column (1.5 by 80 cm) and eluted with 0.2 M KCl (8 ml/h); 1.5-ml fractions were collected. Dextran T-500, Dextran T-150, Dextran T-70, and Dextran T-40 were used as standard polysaccharides. A₄₉₀, Absorbance at 490 nm.

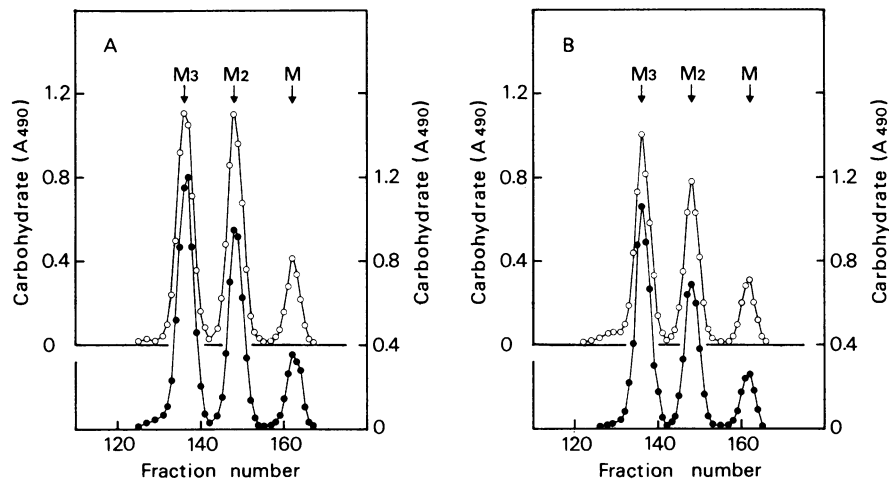


FIG. 3. Gel filtration profiles of the acetolysis products from cell wall (A) and extracellular (B) mannans on a Bio-Gel P-2 column. Each mannan was obtained from cells grown in the absence of NaCl (●) and in the presence of 15% NaCl (○) and from culture filtrates. M, Mannose; M2, mannobiose; M3, mannotriose; A_{490} , absorbance at 490 nm.

NaCl had a higher molecular weight than that obtained from the NaCl-free medium.

Acetolysis studies. To define the structure of the cell wall and extracellular mannans from *S. rouxii*, controlled acetolysis, which cleaves the 1-6 linkage preferentially and yields an oligosaccharide mixture of side chains, was performed. The deacetylated acetolysis products were separated on a Bio-Gel P-2 column (Fig. 3). The elution position of each saccharide was confirmed relative to that obtained from standard oligosaccharides. The acetolysis fingerprints (9) of the cell wall and extracellular mannans were almost identical to each other and showed that the maximum length of the acetolysis products was a trisaccharide unit. From this result we concluded that both the cell wall and extracellular mannans had 1-6-linked backbones with side chains of single mannose and mannobiose units. The molar ratio of each component is shown in Table 2. For both mannans the amount of side chains consisting of mannobiose units was slightly reduced in the medium containing 15% NaCl compared with the medium containing no NaCl.

Methylation analysis. To determine the linkages of the side chains, acetolysis products of the cell wall and extracellular mannans were subjected to methylation analysis. Each component was identified by its retention time and by cochromatography with authentic reference compounds (Table 3). Mannotriose and mannobiose obtained by acetolysis of the cell wall and extracellular mannans gave mostly 3,4,6-tri-*O*-methylmannose which was derived from 1-2-linked oligosaccharides. This indicates that the side chains of both cell wall and extracellular mannans consist predominantly of 1-2 linkages.

TABLE 2. Molar ratios of acetolysis products

Mannan	Concn of NaCl in growth medium (%)	Molar ratio		
		Mannotriose	Mannobiose	Mannose
Cell wall	0	34.8	38.6	26.6
	15	30.8	42.7	26.5
Extra-cellular	0	38.0	36.7	25.3
	15	34.0	38.4	27.6

β -Elimination. To determine whether the oligosaccharides were directly linked to serine or threonine residues in the protein part of the molecule through O-glycosidic bonds, mild alkaline degradation of the cell wall and extracellular mannans was carried out. The oligosaccharides released by β -elimination were separated by gel filtration on a column of Bio-Gel P-2 (Fig. 4). β -Elimination gave the same products (namely, mannotriose, mannobiose, and mannose) for both cell wall and extracellular mannans, although the molar ratios of these oligosaccharides were different from each other (Table 4). With a medium containing 15% NaCl, the content of the alkaline-labile mannose residues was increased. However, most of the carbohydrates of both the cell wall and extracellular mannans were not released from the protein parts by β -elimination and appeared in the void volume fraction on Bio-Gel P-2. This suggests that the polymannose chains attached through di-*N*-acetylchitobiose to asparagine by N-glycosidic bonds are major components of both mannans. The linkage of the oligosaccharides released by this reaction was determined by methylation analysis. β -Elimination products gave 3,4,6-tri-*O*-methylmannose, similar to the products obtained by acetolysis.

DISCUSSION

The highly salt-tolerant yeast *S. rouxii* can grow at NaCl concentrations ranging from 0 to about 20%. The cell wall structure of this salt-tolerant yeast has not been well clarified yet.

First, we examined whether morphological changes in *S. rouxii* cells occurred when they were grown in a medium containing 0 or 15% NaCl. Electron microscopic observations showed that the shapes of the cells grown in the presence of 0 and 15% NaCl were slightly different from each other. The cells grown in the absence of NaCl were spherical, whereas those grown in a medium containing 15% NaCl were spheroidal. This result suggests that the cell wall structure of *S. rouxii* might undergo some changes at higher concentrations of NaCl.

Second, we studied the effect of NaCl concentration in the growth medium on the structure of *S. rouxii* cell wall mannan. Slight decreases in the cell wall mannan content with a higher NaCl concentration in the growth medium were observed. On the other hand, no significant change was

TABLE 3. Methylation analysis of acetolysis products

Acetylated alditol derivatives	Relative retention time	Molar ratio ^a							
		Cell wall mannan				Extracellular mannan			
		0% NaCl		15% NaCl		0% NaCl		15% NaCl	
		Manno-triose	Manno-biose	Manno-triose	Manno-biose	Manno-triose	Manno-biose	Manno-triose	Manno-biose
2,3,4,6-tetra- <i>O</i> -methyl	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2,4,6-tri- <i>O</i> -methyl	1.67	0.11	0.06	0.05	0.07	0.08	0.07	0.13	0.12
3,4,6-tri- <i>O</i> -methyl	1.93	2.01	0.94	1.91	0.95	2.04	0.87	1.69	0.75

^a Values represent molar ratios normalized to 2,3,4,6-tetra-*O*-methylmannose.

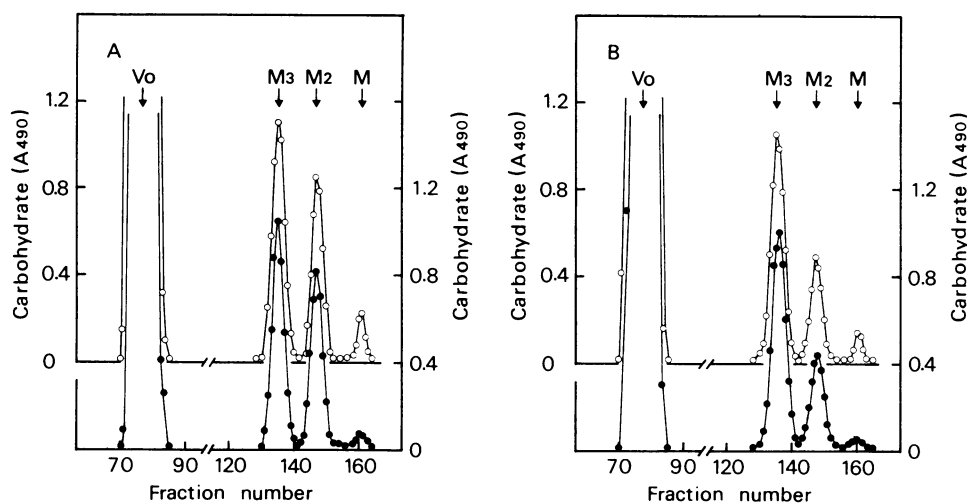


FIG. 4. Gel filtration profiles of the β -elimination products from cell wall (A) and extracellular (B) mannans on a Bio-Gel P-2 column. Each mannan was obtained from cells grown in the absence of NaCl (●) and in the presence of 15% NaCl (○) and from culture filtrates. M, Mannose; M2, mannobiose; M3, mannotriose; A_{490} , absorbance at 490 nm.

seen in the chemical properties of each mannan preparation between 0 and 15% NaCl. Results from acetolysis, methylation and β -elimination studies suggest that the cell wall mannans from cells grown under the two different conditions have an α 1-6-linked main chain to which single mannose and mannobiose units are connected, predominantly by α 1-2 linkages, as side chains. They also contain mannosyloligosaccharides attached to protein parts through O-glycosidic bonds. The structure of this mannan is quite different from that of the non-salt-tolerant yeast *Saccharomyces cerevisiae* (15) with respect to the length and linkage of the side chains.

It has not been clarified whether the difference in the chemical structures of cell wall mannans has any relationship to the mechanisms of salt tolerance of the yeasts. Therefore, further studies are needed.

TABLE 4. Composition of β -elimination products

Mannan	Concn of NaCl in growth medium (%)	Void vol of oligosaccharides ^a	Molar ratio		
			Manno-triose	Manno-biose	Mannose
Cell wall	0	11.3	58.4	36.5	5.1
	15	9.4	52.6	32.9	14.5
Extra-cellular	0	13.9	42.7	50.3	7.0
	15	12.0	40.7	44.3	15.0

^a Values represent weight ratios normalized to oligosaccharides.

The extracellular mannans have quite similar structures to the cell wall mannans, except for molecular weight. Krátký et al. detected a progressive release of mannan into the growth medium of *S. cerevisiae*, but they reported that the cell wall mannan was not subject to turnover or release into the medium (10). Recently, it was demonstrated that two independent mannan pools were associated with the *S. cerevisiae* cell envelop (16). One of these was formed by cell wall mannan, and the other was formed by periplasmic mannan. The former was stable, whereas the latter showed significant turnover. From these results it appears that the extracellular mannans from *S. rouxii* may originate from the mannans located in the periplasmic space.

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