THE MANNAN FORMED BY THE YEAST *Bullera tsugae*

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The structure of an extracellular mannan formed by the yeast *Bullera tsugae* has been studied. It has been shown that the repeating unit of this polysaccharide contains β-(1 → 3)- and β-(1 → 4)-bound D-mannopyranose residues in equimolar ratio. A comparison has been made of the polymer under investigation with the extracellular mannan from a yeast of the genus *Rhodotorula*.

The yeast *Bullera tsugae* belongs to the family *Sporobolomycetaceae* [1]. Having a strong polysaccharide capsule it may be a promising source of extracellular or capsular polysaccharides. However, the carbohydrate polymers secreted by this organism into the surrounding medium have not been studied. We have shown previously that, depending on the conditions of cultivation, this yeast produces different extracellular polysaccharides: a mannan or an acidic heteropolysaccharide [2].

In the present paper we give the results of a study of the structure of the extracellular mannan formed when *B. tsugae* is grown in a medium containing glucose, mineral salts, and trace amounts of vitamins of the B group [3]. When grown in this medium, the yeast produced the mannan predominantly, and the amount of contaminating heteropolysaccharide was insignificant.

The mannan from the total polysaccharide preparation obtained by precipitating a filtrate of the culture liquid with two volumes of ethanol was fractionated by means of the Fehling reagent [4]. To eliminate traces of the heteropolysaccharide completely, this purification was performed twice.

When subjected to gel chromatography on Sepharose 6B, the mannan samples obtained each gave a single peak with V_e = 12.5 ml (V_0 = 4.5 ml). On liquid chromatography (LC), the polysaccharide likewise issued as a single peak and corresponded to a polymer with a molecular weight of 20,000-40,000. Samles of dextrans with various molecular weights and known molecular-weight characteristics were used for comparison.

The specific rotation of the *B. tsugae* mannan studied was −80° (c 0.25; water).

Complete hydrolysis of the samples of polysaccharides followed by paper chromatography revealed only mannose, $[\alpha]_D^{20}$ +14° (c 0.8; water). The phenylhydrazone obtained from it had $[\alpha]_D^{20}$ +27° (pyridine) and a melting point of 198°C (with decomposition).

The structure of the mannan was established by periodate oxidation [4], methylation [5], and ^{13}C NMR spectrometry [6].

In the process of periodate oxidation, for each anhydro unit 0.46 mole of periodate was consumed and 0.05 mole of formic acid was liberated. In a hydrolysate of the polyalcohol formed in the tetrahydroborate reduction of the products of periodate oxidation, mannose, erythritol, and traces of glycerol were identified with the aid of PC. The formation of mannose indicated the presence of (1 → 3)-bonds between the units of the polymer. The formation of erythritol confirmed the presence of (1 → 4)-bound mannose residues. The glycerol was produced in the oxidation of the terminal mannose units. The numbers of glycosidic bonds calculated from the results of periodate oxidation are given in Table 1.

Two methylations of the mannan by Hakomori's method led to the completely methylated polysaccharide. With the aid of gas-liquid chromatography (GLC), methyl 2,3,4,6-tetra-O-methyl-D-mannoside, methyl 2,4,6-tri-O-methyl-D-mannoside, and methyl 2,3,6-tri-O-methyl-D-mannoside were identified in the methanolysate of the methylated product. The quantitative

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TABLE 1. Comparative Characteristics of the Extracellular Mannans of B. tsugae and of Rh. rubra (rhodexman)

Material	$[\alpha]_D^{20}$ (H_2O)	Periodate oxidation, percent			Methylation products, percent			Molecular wgt. according to TLC
		nonreducing terminal groups	(1 → 4)-bonds	(1 → 3)-bonds	methyl-2,3,4,6-tetra-O-methyl-D-mannoside	methyl 2,3,6-tri-O-methyl-D-mannoside	methyl 2,4,6-tri-O-methyl-D-mannoside	
The B. tsugae mannan	−80°	5.0	45,0	50 0	12.2	43,2	44.6	$20 \cdot 10^3$ — $40 \cdot 10^3$
The Rh. rubra mannan (rhodexman)*	−83÷−86°	4,0—8,0	41,0–43,0	51.0—54.0	1.1 1.6	43,9—48,0	46,9—50,5	$300 \cdot 10^3$ — $500 \cdot 10^3$

*The limiting values for samples of rhodexman obtained under various conditions are given.

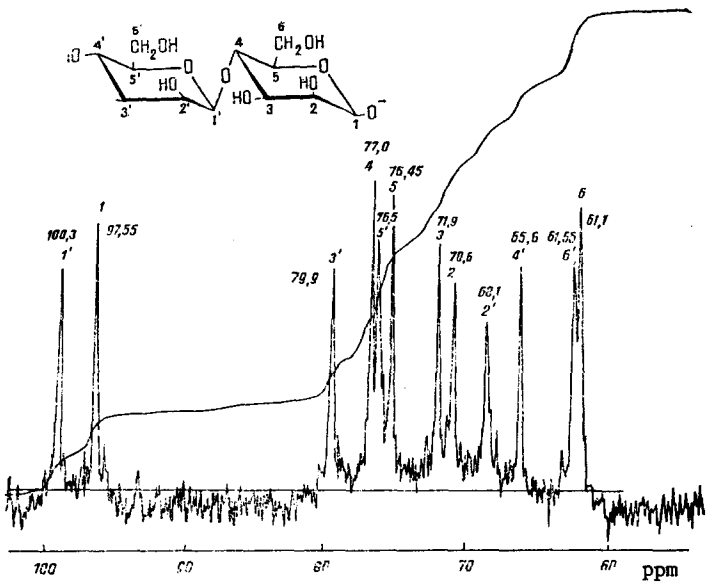

Fig. 1. ^{13}C NMR spectrum of the Bullera tsugae mannan.

TABLE 2. ^{13}C NMR Chemical Shifts Mannan Samples

Mannan sample	Chemical shift, ppm											
	C_1'	C_1	C_2'	C_2	C_3'	C_3	C_4'	C_4	C_5'	C_5	C_6'	C_6
B. tsugae	100,3	97,6	68,1	70,6	79,9	71,9	65,6	77,0	76,5	75,5	61,6	61,1
Rh. rubra	101,1	97,9	68,7	71,4	80,3	72,7	66,3	77,3	77,1	76,1	62,1	62,0
Rh. glutinis	101,6	98,7	69,4	72,0	81,1	73,2	66,8	78,4	77,8	76,7	62,7	62,3

ratios of methyl 2,4,6-tri-O-methyl-D-mannoside and methyl 2,3,6-tri-O-methyl-D-mannoside confirmed the results of periodate oxidation and showed the presence of approximately equal numbers of (1 → 3)- and (1 → 4)-bonds in the polymer molecule while the specific rotation of the polysaccharide indicated the β confirmation between the D-mannopyranose residues (Table 1).

Figure 1 shows the ^{13}C NMR spectrum of the B. tsugae mannan, which is identical with the spectra obtained previously for the mannans of Rh. glutinis [6] and the samples of the Rh. rubra mannans that we have investigated (Table 2). These results confirm the structure of the mannan, the repeating unit of which is constructed of β-(1 → 3)- and β-(1 → 4)-bound mannose residues in equimolecular ratio, which agrees with the results of the structural investigation of the polymer by chemical methods.

In x-ray diffraction, the mannan under investigation gave an intense amorphous halo (Fig. 2), which predicates an unordered arrangement of the macromolecules in the polymer, i.e., the existence of the polysaccharide in the amorphous state.

The results of the investigtion of the structure of the B. tsugae mannan by chemical methods (Table 1) and those obtained by ^{13}C NMR spectrometry and x-ray diffraction indicate a great similarity of this polymer to the linear extracellular mannans formed by various species of yeast of the genus Rhodotorula including the mannan of Rh. rubra (rhodexman) [4, 7-9]. However, the B. tsugae mannan has a lower molecular weight than rhodexman. According to the results of high-pressure LC, its molecular weight was approximately 12-15 times lower than samples of rhodexman investigated by the same method.

EXPERIMENTAL

The chromatographic analysis of the monosaccharides was performed on Filtrak-11 paper in the butan-1-ol–water–ethanol–ammonia (47:49:10:1) system. The chromatograms were revealed with aniline hydrogen phthalate and an ammoniacal solution of silver nitrate.

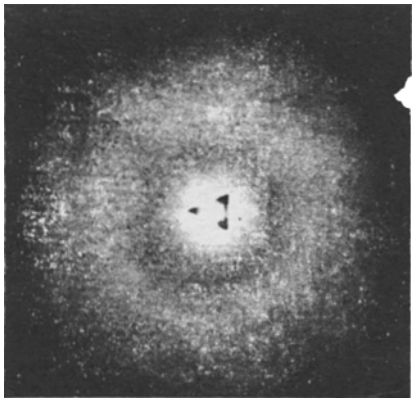

Fig. 2. Debyeogram of the Bullera tsugae mannan.

The gas–liquid chromatography of the methylated sugars in the form of methyl glycosides was performed on a Chrom 4 instrument (Czechoslovakia) using a steel column (0.4 × 120 cm) filled with Chromaton (0.16-0.20 mm) impregnated with 15% of poly(butan-1,4-diyl adipate) at 190°C and a rate of flow of helium of 30 ml/min, with a flame-ionization detector.

For gel chromatography, a column (0.8 × 80 cm) filled with Sepharose 6B was used. The eluent was phosphate buffer, pH 7.2. The polysaccharide in the fractions (0.5 ml) was determined by the reaction with phenol/sulfuric acid [10].

The liquid chromatography of the materials was performed on a Dupont 830 instrument (USA) using two columns (6.2 × 250 mm) with GPC-100 and GPC-500 modified silica gels (pore diameters 100 and 500 μ). The rate of flow of the mobile phase (a 0.05% solution of sodium azide in water) was 0.7 ml/min. A LDC differential refractometer (USA) was used as the detector. The calibration curve for determining molecular weights was plotted from the results for standard samples of dextrans from Pharmacia (Sweden). The amount of polysaccharide introduced into the instrument was 50 μl of a 0.5% solution.

The ^{13}C NMR spectra were taken on a Bruker HX-90 instrument with a working frequency for carbon nuclei of 22.628 MHz (pulsed regime followed by Fourier transformations). The polysaccharide was investigated in the form of a 4% solution in D_2O. DMSO was used as internal standard. The recording temperature was +80°C and the length of a pulse 10 μsec; the pulse repetition frequency was 0.7 sec. Accumulation was carried out in the 4 K cell of the memory of a B-NC 12 computer, the number of accumulations being 80,000, the scale 100 Hz/cm, and the scale in recording 30 Hz/cm (2 ppm/cm).

The diffractogram of the mannan was obtained in a DRON-1 instrument, the samples of polysaccharide being taken in the form of tablets (d = 3 mm; h = 0.5 nm).

The distance from the tube to the x-ray plate was 33 mm. The time of irradiation was 6 h.

IR spectra were recorded on a UR-20 instrument. Specific rotations were measured on a Perkin-Elmer 241 automatic polarimeter.

Isolation of the Polysaccharide. A two-hour culture of B. tsugae BKM Y-1280 previously grown on wort-agar was seeded into a medium with the following composition (g/liter of mains water): glucose – 50; $(NH_4)_2SO_4$ – 2.5; KH_2PO_4 – 1; $MgSO_4 \cdot 7H_2O$ – 0.5; NaCl – 0.5; $CaCl_2 \cdot 2H_2O$ – 0.1; thiamine bromide – 0.0004; riboflavin – 0.0002; pyridoxine hydrocloride – 0.0004 [3]. Inoculation (2 days) and fermentation (6 days) were performed on laboratory shaking machines at 220 rpm and a temperature of 24-25°C in flasks with a volume of 750 ml containing 50 and 150 ml of medium, respectively. The inoculate was seeded into the fermentation flasks in an amount of 8% of the volume of the medium.

The polysaccharide was isolated by the procedure described previously [4], and the mannose was separated from the accompanying polysaccharide impurities by purification with the aid of the Fehling reagent [4, 7].

Complete Hydrolysis. The polysaccharide (10 mg) was heated with 2 N sulfuric acid (0.5 ml) in a sealed tube at 100°C for 4 h. The hydrolysate was neutralized with barium carbonate. The monosaccharide was identified with the aid of PC.

Periodate Oxidation. The mannan (50 mg) was oxidized with a 0.016 M solution of sodium metaperiodate (50 ml) at room temperature for 72 h. The results of the determination of the amount of periodate consumed and the amount of formic acid liberated were used to calculate the numbers of glycosidic bonds [4]. The polyaldehyde (20 ml) was treated with sodium tetrahydroborate at 20°C for 16 h. The excess of tetrahydroborate was decomposed with acetic acid. The residue was evaporated with methanol. The resulting polyalcohol was hydrolyzed with 1 N hydrochloric acid (1 ml) at 100°C for 6 h. The neutralized hydrolysate was investigated with the aid of PC.

Analysis by the Methylation Method. The mannan (20 mg) was methylated by Hakomori's method [5]. The completeness of methylation was established from the absence of the hydroxyl absorption in the IR spectrum. The completely methylated polysaccharide was hydrolyzed in a 5% solution of hydrogen chloride in absolute methanol in a sealed tube at 100°C for 5 h. The methyl glycosides so obtained were analyzed with the aid of GLC (the authors express their gratitude to A. S. Shashkov of the Institute of Organic Chemistry of the Academy of Sciences of the USSR for consulation on the ^{13}C NMR spectra).

SUMMARY

1. When the yeast Bullera tsugae was grown on a nutrient medium containing glucose, mineral salts, and trace amounts of vitamins of the B group an extracellular mannan was obtained.

2. The repeating unit of the polysaccharide contains β-(1 → 3)- and β-(1 → 4)-bound D-mannopyranose residues in equimolar ratio.

3. The B. tsugae mannan is similar in structure to the exomannan formed by yeasts of the genus Rhodotorula. The polymer investigated had a lower molecular weight than the mannan from Rh. rubra.

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