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MANNAN PRODUCED BY *Rhodotorula rubra* STRAIN 14

NICOLAY P. ELINOV, GALINA A. VITOVSKAYA, VACHESLAV A. MARIKHIN, YULINA B. MARJUKHTA, AND TAMARA V. KOZLOVA

Chemical Pharmaceutical Institute, Leningrad (U.S.S.R.)

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ABSTRACT

Some structural features and physicochemical properties of the mannan from *Rhodotorula rubra* strain 14 have been investigated. Chemical analysis indicated the mannan to have alternating β-(1→3) and β-(1→4) lingages. Wide-angle X-ray diffraction of the original mannan showed an intensive, amorphous halo, suggesting short-distance, macromolecular arrangement, as in other amorphous substances. The specimen underwent radical structural changes upon heating the polymer in phosphate buffer solution.

INTRODUCTION

Considering the general biological protective role of natural carbohydrates and especially of polysaccharides having β-links between residues[1,2], we have investigated an extracellular mannan of *Rhodotorula rubra* strain 14 that exhibits interesting biological properties. Thus the ability of similar compounds to activate and stabilize crystalline serum cholinesterase[3] was shown in 1973, and subsequent experiments *in vivo* revealed that the mannan increases the activity of serum cholinesterase, alpha amylase, and aldolase, as well as that of liver and erythrocytic catalase[4–6]. Furthermore, administration of the polysaccharide promoted the normalization of catalase structure in radiation-exposed rats[7]. It also normalized the isoenzymic spectrum and some physicochemical parameters of blood-serum aldolase in tumor-bearing animals[8]. Moreover, the mannan inhibited the development of some experimental tumors in animals[8,9].

The present paper is concerned with some structural features and physicochemical properties of this polymer.

EXPERIMENTAL

Production of polysaccharide. — *Rhodotorula rubra* strain 14 (Department of Microbiology, Chemical Pharmaceutical Institute of Leningrad) was maintained on a

malt–agar medium, in which it retained its morphological–physiological characteristics[10].

The culture was grown for 96 h at 25°, on a rotary shaker operated at 220 r.p.m., in 750-mL Erlenmeyer flasks containing 300 mL of synthetic medium with 5% of glucose and vitamins of the B group[11], or in 100- and 295-L tanks with the same medium. Crude polysaccharide was precipitated from the culture liquor by ethanol. The mannan was isolated from the crude polysaccharide via its water-insoluble copper complex formed with Fehling's solution[12–14].

Characterization of the polysaccharide. — Polymer specimens were assayed for purity: sugars were determined chromatographically[15], protein by the Lang method[16], and phosphorus by the procedure of Chen[17]. The cupric-ion content was also checked[18].

Gel chromatography was performed on Sephadex G-200 with pH 6.6 phosphate buffer[14], and Sepharose 6 B with pH 7.2 phosphate buffer (column 5 × 200 mm, 0.25 mL of 0.15% solution, elution rate 0.6 mL/h); 0.25-mL fractions were collected.

The sedimentation of the preparation (7.5–15.0 mg/mL) was evaluated on the basis of curves taken from a "MOM" centrifuge at 50,000 r.p.m. The diffusivity was measured in a Tsvetkov diffusion meter[19]. The molecular weight was calculated by Svedberg's equation and was determined osmometrically.

Our modification of Vilensky's osmometer was used[20]; the model includes one cell in the form of a Plexiglas ring, i.d. 38 mm, with a 150 mm-long, thick-walled glass capillary tube, i.d. 0.8–1.0 mm, attached to it. A standard UCF-ff type membrane was used, which was pressed against the ring by 55 × 55-mm Plexiglas plates perforated with holes having a diameter of 38 mm. The outer rim of the ring and of the plates were ground to fit one another. As a result, the chamber was airtight and the membranes were readily replaced in the osmometer.

The calculations were performed by using the formula[21]:

$$M = RT/[\pi/C]_{C\to 0}$$

where π = osmotic pressure (atm), C = concentration of solution (g/mL).

Values of $[\pi/C]_{C\to 0}$ were obtained by measuring the osmotic pressure of polysaccharide solutions at concentrations of 0.2–1.0% in the "water–water" system at 20°.

The mannan was completely methylated and analyzed as the free methylated sugars and their methyl glycosides by gas–liquid chromatography[14].

Periodate oxidation was performed as described earlier[22]. The sequences of glycosidic linkages were assessed after the isolation of 2-*O*-β-D-mannopyranosyl-D-erythritol by mild acid hydrolysis of the modified mannan[23] obtained by periodate oxidation and subsequent borohydride reduction of the original preparation[14], as well as by analysis of the mannotriose fraction obtained by partial hydrolysis of the mannan[23].

The viscosity of the mannan solution was determined in a capillary viscometer with a reference solvent (water or phosphate buffer, pH 7.2)[24].

X-Ray structural analysis of polysaccharide specimens was in the wide-angle region in a diffractometer of the DRON-I-type[25].

RESULTS AND DISCUSSION

The yield of crude polysaccharide was about 2–3 g/L. After purification, the yield of mannan comprised 55–60% of the original amount.

The hydrolyzate of the polysaccharide (M sulfuric acid, 4 h), was found to contain a single monosaccharide, corresponding to mannose, by its chromatographic mobility and m.p. of the crystalline phenylhydrazone. The mannan specimens contained no nitrogen or phosphorus. The cupric-ion content of the mannan was <0.003%. Various specimens that differed slightly in the methods of their preparation had V_e/V_0 = 1.00–1.33, as shown by gel chromatography on Sephadex G-200 and Sepharose 6B.

Ultracentrifugation of the mannan having V_e/V_0 = 1.33 gave a single, symmetric peak, having $S_{w_{20}}$ = 1.62. The specific rotation of the mannan from *Rh. rubra* was —83° (*c* 0.25, water). The results of periodate oxidation of the polymer with subsequent borohydride reduction (mannose, erythritol, and traces of glycerol in the hydrolyzate of the reduced product) showed the existence of 1→3 links (51%), 1→4 links (41%), and 1→6 links or non-reducing end-groups (8%).

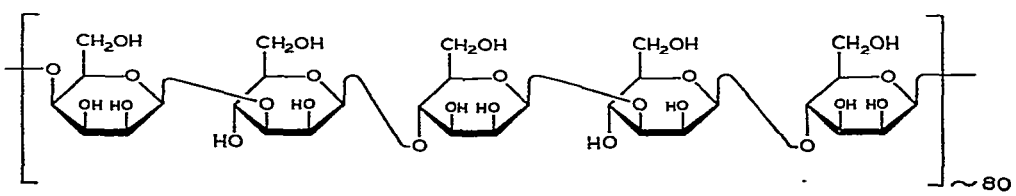
Paper chromatography revealed a tri-*O*-methylmannose and traces of tetra-*O*-methylmannose and a di-*O*-methylmannose in the hydrolyzate of the completely methylated mannan (OMe = 43%). The fraction of tri-*O*-methylmannoses was divided into two portions[14].

Gas–liquid chromatography of the methyl mannosides [15% of 1,4-butanediol succinate in a column of G-22 Celite (100 × 0.5 cm), 175°, nitrogen carrier, 45 mL/min, and 5.5% of OV-17 in a column of Chromosorb W, 80–100 mesh, 186 × 0.2 cm, 160°, helium carrier, 40 mL/min], showed them to include: methyl 2,3,4,6-tetra-*O*-methyl-D-mannoside (6.6%), methyl 2,4,6-tri-*O*-methyl-D-mannoside (49.5%), methyl 2,3,6-tri-*O*-methyl-D-mannoside (43.9%), and methyl di-*O*-methyl-D-mannoside (2.6%).

Analysis of the products of mild acid hydrolysis of the mannan that had been oxidized by periodate and then reduced by borohydride, and study of the mannotriose fraction obtained by partial hydrolysis of the polysaccharide, suggested a provisional structure of the mannan as that of a polymer having alternating β-(1→3) and β-(1→4) links.

The $\overline{M}_w$ value of the mannan, as calculated by the Svedberg formula, was 65 × 10^3; M_n = 63.7 × 10^3 (osmometric data).

Therefore, the structure of the mannan from *Rh. rubra* may be shown as follows:



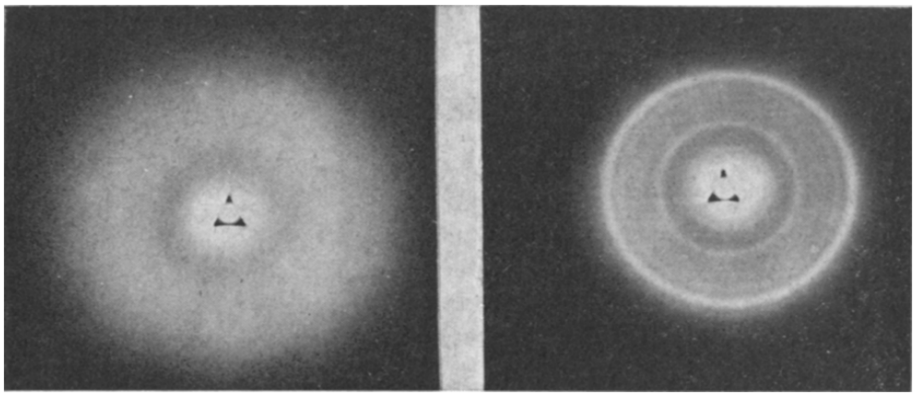


Fig. 1 (left). Diffractogram of an original specimen of mannan.

Fig. 2 (right). Diffractogram of mannan after heating its solution in phosphate buffer for 1.5 h at 120°, followed by dialysis and precipitation of the mannan by ethanol (96°).

As regards the glycosidic linkages and their alternation, the mannan from *Rh. rubra* is close to those from four species isolated[23] from *Rhodotorula* and investigated by Gorin *et al.* However, the mannan from *Rh. glutinis*, the species most studied by those authors, was found to be less polymerized than we observed with the polymer from *Rh. rubra*.

The mannan forms colorless, slightly opalescent solutions, pH 6.0–7.0. The relative viscosity of 0.5–0.6% solutions in water, 0.9% sodium chloride, and phosphate buffer (pH 7.2), generally ranged from 5 to 7. At higher concentrations, the viscosity increases considerably, and shows a rather great decrease when solutions of the mannan are heated.

Some data on the molecular arrangement in mannan specimens were determined by wide-angle X-ray diffraction. Fig. 1 shows a diffractogram of the original specimen of mannan (0.6% solution in water, η_{rel} 6.5, pH 6.75; 0.6% solution in phosphate buffer, η_{rel} 6.1, pH 7.2; V_e/V_0 1.00 through Sepharose 6B). An intense, amorphous halo was observed, suggesting the presence of a short-distance order of macromolecular arrangement, as in other amorphous substances.

If this specimen is dissolved in water or phosphate buffer (pH 7.2), and then reprecipitated with ethanol, the diffractogram shows no change. Fig. 2 shows a diffractogram of the same specimen when a 0.6% solution in phosphate buffer (pH 7.2) was heated for 1.5 h at 120°. The relative viscosity of the solution, cooled to 20°, was 1.6. The solution was then dialyzed against distilled water for 3 days. The mannan was precipitated from the dialyzate with 2 vols of ethanol (96°), washed with ethanol (96°), and dried. The specimen did not contain any phosphorus. In gel chromatography through Sepharose 6B, the V_e/V_0 value rose to 1.33.

The X-ray studies suggested that the specimen underwent radical structural changes: the intense halo being replaced by a series of distinct diffraction patterns corresponding to the following interplanar spacings (Å): d_1 7.55; d_2 4.9; d_3 4.4;

TABLE I

THE METHYL GLYCOSIDES FROM METHYLATED GLUCOMANNANS AND MANNANS OF *Rhodotorula* (%)

Methyl glycosides	*Water-insoluble glucomannans of cell wall*	*Water-soluble glucomannans of cell wall*	*Extracellular mannans*
Methyl 2,3,4,6-tetra-*O*-methyl-α,β-D-glucoside	13.7	13.8–14.3	
Methyl 2,3,4,6-tetra-*O*-methyl-D-mannoside			1.9–6.6
Methyl 2,4,6-tri-*O*-methyl-β-D-glucoside	10.3	1.0–5.5	
Methyl 2,4,6-tri-*O*-methyl-D-mannoside	30.0	37.8–39.4	48.1–49.2
Methyl 2,3,6-tri-*O*-methyl-D-mannoside	31.0	33.2–35.7	43.7–49.5
Methyl di-*O*-methyl-D-mannoside	15.0	8.0–11.3	0.1–2.6

d_4 2.8; and d_5 2.5. The intensities of these diffractions vary greatly, the most intense one corresponding to an interplanar spacing of 2.8 Å. The diffraction patterns point to a correlated packing of mannan molecules in crystalline regions, a field under further study.

In conclusion, the present study has established the ability of all species of *Rhodotorula* to produce extracellular mannans of similar structure at decreased pH values[14,26].

Moreover, our study of the carbohydrate polymers of *Rhodotorula* cells has revealed that the cell wall of these yeasts contains soluble and insoluble branched β-D-gluco-D-mannans (D-glucose:D-mannose ~1:3.) The backbone of the polymer thus contains D-mannosyl residues connected by (1→3)- and (1→4)-links in the approximate ratio[27–29] of 1:1. Table I presents data on the comparison of glucomannans and extracellular mannans. On the basis of these observations, we suggest that the mannans are produced extracellularly because of changes in the enzymic process of assembly of the glucomannans of the cell wall at elevated concentrations of hydrogen ions, as well as changes in the permeability of cytoplasmic membranes and the cell wall caused by the same factors.

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