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120. Shigeo Suzuki, Masuko Suzuki, and Osamu Sakaguchi: Biochemical and Immunochemical Studies on Fungi. Ⅶ.*1 Isolation of a Galactomannan produced in the Culture of *Trichophytou rubrum.*

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Isolation of galactomannan from the culture medium of *Trichophyton rubrum* by means of Sephadex gel-filtration and ion-exchange chromatography was described. The galactomannan was not precipitated with Fehling's solution, and the ratio of galactose to mannose was 1:2.24, and had a positive specific rotation, $[\alpha]_D^{18}$: +25°.

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Recently, Bishop, *et al.* revealed that the mycelium of *Trichophyton rubrum* contained a mannan, while the other four species of this genus gave galactomannans by the same extraction method. However, on the extracellular polysaccharides of this, genus, only a few papers which dealt with fractionations of the crude polysaccharides of *T. mentagrophytes* have been published, but no description was made on the polysaccharide constituents in the culture of *T. rubrum.* This paper describes the isolation and the characterization of a galactomannan produced in the culture of *T. rubrum.*

At the first step, the macromolecule constituents were precipitated from the concentrated and dialyzed culture by the addition of methanol. The methanol precipitate was then fractionated by gel-filtration to remove the relatively low molecular constituents. Fig. 1-A shows the elution pattern of the gel-filtration. The top peak which gave strong Molisch's reaction was collected and termed as G-50 Fr. I. The composition of G-50 Fr. I are represented in Table I-A. The paper electrophoreogram (Fig. 2-A) indicated that G-50 Fr. I was consisted of at least five materials, and the presence of a substance which gave both carbohydrate and ninhydrin reactions at slightly anode side from the original point. The isolation of this material is the subject of the present work. From the result of paper electrophoresis, this material was assumed to be a neutral substance. Moreover, no precipitation was occurred by the addition of Fehling's solution into a solution of G-50 Fr. I in spite of mannose was detected as a sugar component. Therefore, an attempt was made to removal of the other basic or acidic components by a combination of ion-exchange column chromatography as follows. An aqueous solution of G-50 Fr. I was passed through a column of diethylaminoethyl(DEAE)-Sephadex A-50 acetate form (Fig. 1-B). The top peak was collected and termed as GD-50 Fr. I. As will be seen in Table I-B, carbohydrate content of this fraction increased remarkedly. In the paper electrophoreogram of GD-50 Fr. I (Fig. 2-B), no materials which gave positive ninhydrin reaction were detected on the anode side. However, on the cathode side, one ninhydrin positive band accompanying with a faintly tailed zone still remained. In order to remove the cationic impurities, GD-50 Fr. I was further fractionated on a column of carboxymethyl(CM)cellulose equilibrated with 0.05*M* acetate, pH 5.6 (Fig. 1-C). The peak which gave the strongest Molisch's reaction was collected and lyophilized after dialyed against distilled water and termed

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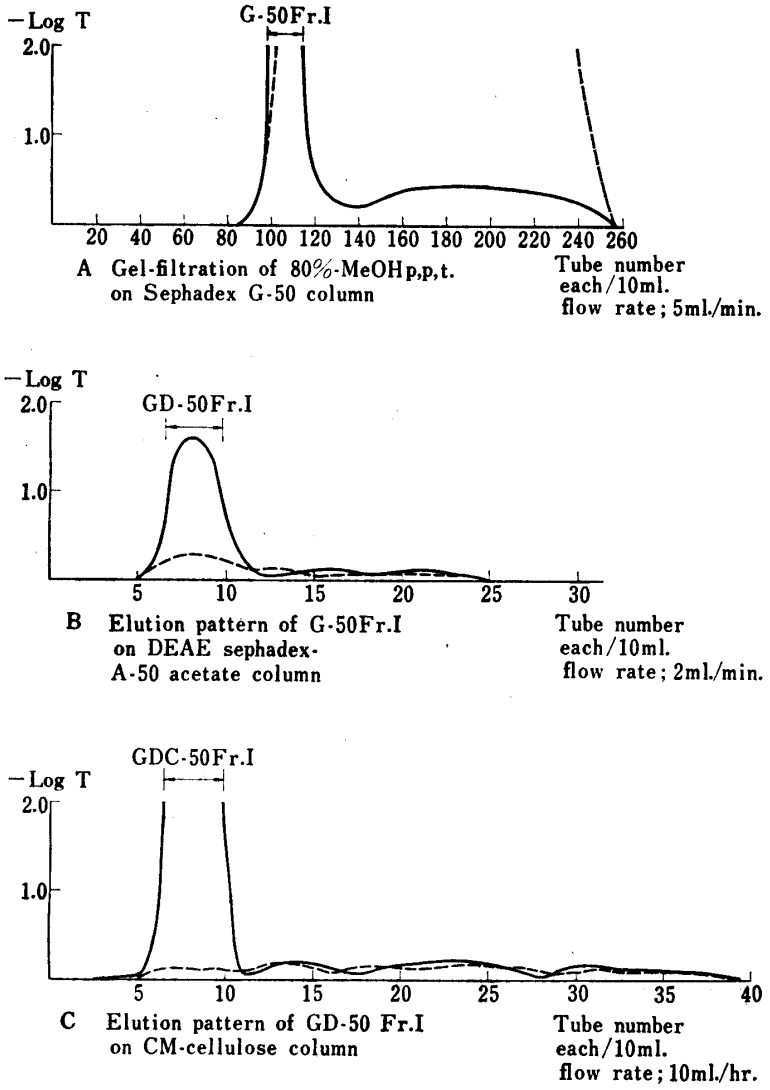

Fig. 1.

——— Molisch value at 550 mμ ------- U.V. at 280 mμ

as GDC-Fr. I. The carbohydrate content of GDC-Fr. I was increased to 95% as mannose, and the ratio of mannose to galactose was 2.24:1 (Table I-C). From the results of free boundary electrophoreses in borate and veronal buffers, a small quantity of impurities still remained in GDC-Fr. I (Fig. 3), although the impurities were not detected on the paper electrophoretic pattern with both ninhydrin and periodate-*o*-tolidine reagents (Fig. 2-C). The larger peak in Fig. 3 was assumed to be a polysaccharide, since migration toward the anode was observed in the borate while no migration occurred in the veronal. Since the carbohydrate components were occupied by mannose and galactose in the ratio of 1:2.24, and no resolution of the larger peaks was observed neither in the veronal nor in the borate, it is obvious that the major

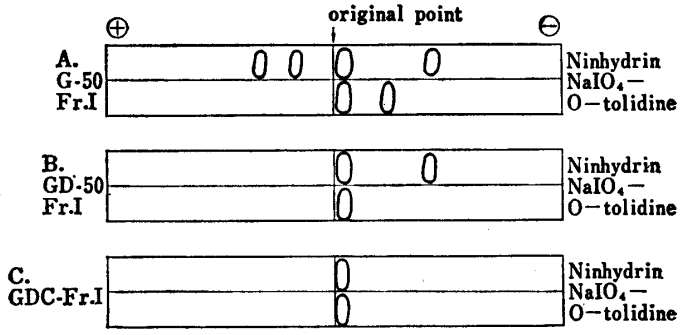

Fig. 2. Paper Electrophoretic Patterns of G-50 Fr. I, GD-50 Fr. I and GDC-Fr. I

Table I. Composition of G-50 Fr. I. GD-Fr. I and GDC-Fr. I

Samples		Total carbohydrate as mannose (%)	Monosaccharide components	Total N (%)	Total P (%)	Appearance
A	G-50 Fr. I	29.4	Mannose Galactose Glucose Xylose	4.9	1.8	brown
B	GD-50 Fr. I	72.0	Mannose Galactose Glucose Xylose	1.1	trace	light yellow
C	GDC-Fr. I	95.5	Mannose 2.24 Galactose 1	<0.5	trace	white

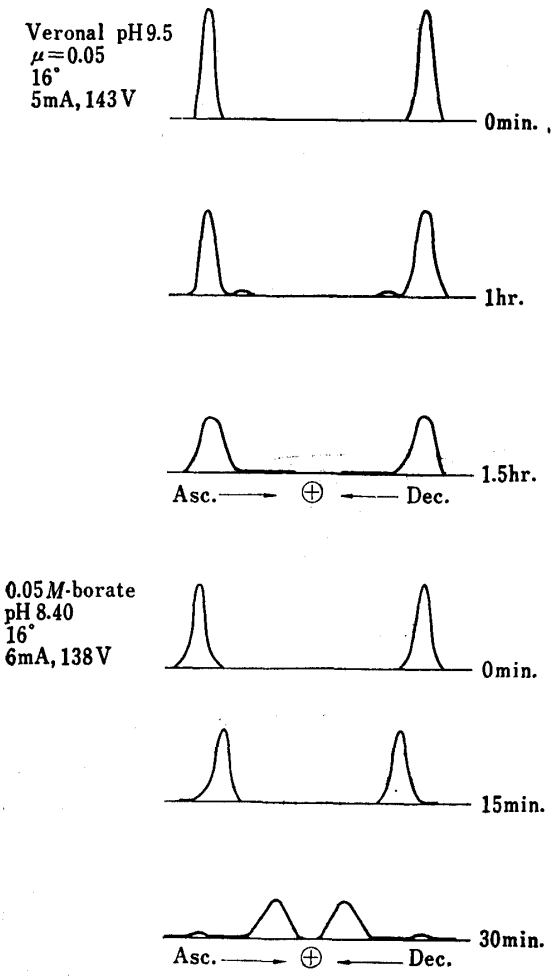

Fig. 3. Electrophoretic Pattern of GDC-Fr. I

components of GDC-Fr. I was a galactomannan. Positive specific rotation, +25°, of GDC-Fr. I suggested that the predominant glycosidic linkage was α-form. Presence of galactofuranose residues was ascertained since 50% of galactose was released by heating of GDC-Fr. I in 0.025*N* oxalic acid solution with 3 hr. From the results above mentioned, chemical structure of the galactomannan produced in the culture of *T. rubrum* was assumed to be analogous to those which were isolated from mycelium of the other species of Trichophyton genus by Bishop, *et al.*,[1] though the galactomannan was not precipitated with alkaline copper solution and showed lower positive value of specific rotation. Structural studies of the galactomannan are in progress and will be published in the early date.

Experimental

General Procedures——Paper chromatography was carried out on Toyo Roshi No. 51A filter paper using the following solvent systems (v/v); (A) *n*-butanol–pyridine–water 5:3:1, (descending method, 48 hr., 17~28°), (B) phenol water 5:1, (ascending method). Before applying to the paper, each 2 mg. of samples were hydrolyzed by heating with 1 ml. of $N H_2SO_4$ for 4 hr. at 96°, and neutralized with powdered $BaCO_3$. Aniline hydrogen phthalate (AHP) spray reagent[4] was used for detecting of monosaccharides.

Paper electrophoreses were done on Toyo Roshi No. 51A filter paper in the following conditions; buffer pyridine, acetic acid, water 10:2:488, (pH 5.85), voltage–75 V/cm, time–50 min, spray reagent, (1) for polysaccharides; 0.5%-$NaIO_4$ solution and 1% *o*-tolidine in 10% acetic acid solution, (2) for peptide or proteins; 0.2%-ninhydrin in water-saturated with *n*-butanol. Quantitative determinations of carbohydrate

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were carried out with Molisch's reagent,[5] which gave identical color extinction to both galactose and mannose. Total nitrogen analyses were carried out by micro-Dumas' method, and phosphorous determinations were by modified Allen-Nakamura's method.[6] Moving boundary electrophoreses were carried out using a Hitachi HTD-I apparatus.

Culture of *Trichophyton rubrum* and Separation of the Macromolecule Constituents of the Culture Medium——A strain of *T. rubrum* was grown in a 1%-thiamine-added Sabouraud medium at 20° for 7 days under shaking. After removal of the mycelium by filtration, the filtrate was concentrated with a rotary evaporator in diminished pressure to 1/50 of the original volume below 40°, and dialyzed against running tap water for 48 hr. The non-dialyzables were then concentrated to 1/100 of the original volume. After cooling at −5°, methanol was added with stirring to the final volume of 80%, and the mixture was kept for 12 hr., at 0°. The brown precipitate was collected by centrifugation and was washed twice with 80%-methanol, then dried *in vacuo* on P_2O_5. The yield of this fraction was approximately 2 g. from 3.6 L. of the culture.

Gel-filtration of 80% Methanol Precipitate (G-50 Fr. I)——5 g. of the 80% methanol p.p.t. was dissolved into 50 ml. of water and the solution was subjected to a column of Sephadex G-50 (medium) 5×50 cm., then eluted with water (Fig. II-A). The first peak which gave strong Molisch's reaction was collected, and lyophilized after concentration in diminished pressure to a small volume. A brown amorphous powder (230~400 mg.) was obtained and termed as G-50 Fr. I. This fraction contained nitrogen and phosphorus.

Fractionation of G-50 Fr. I with DEAE Sephadex A-50 Acetate Column——A solution of 200 mg. of G-50 Fr. I in 10 ml. water solution was applied to a column of DEAE-Sephadex acetate (2×50 cm.) and eluted with water (Fig. II-B). Tube numbers 11 to 13 were combined and lyophilized after dialysis against distilled water for 48 hr., below 4°. Approximately 50 mg. of a light yellow amorphous powder was obtained.

Isolation of the Galactomannan (GDC-Fr. I) from GD-50 Fr. I——To a column of carboxymethyl cellulose (2×50 cm.), equilibrated with 0.05*M* acetate buffer, pH 5.6, a solution of 200 mg. of GD 50-Fr. I in 10 ml. of the same buffer was applied and eluted with the same buffer (Fig. III-C). The top peak was collected and lyophilized by the same method as described above. The yield of GDC-Fr. I was approximately 34% of the weight of GD-50 Fr. I. Carbohydrate content of this fraction was 95% as mannose, and had $[\alpha]_D^{18}$: +25°(c=1.0, l=1.0, H_2O). This polysaccharide did not form and precipitate with Fehling's solution, and gave two spots identical with those of mannose and galactose by qualitative paper chromatography.

Estimation of Constituent Sugars——The hydrolyzate of 100 mg. of GDC-Fr. I, prepared by the same method of the samples of paper chromatography, was dissolved into 2 ml. of 5%-acetic acid and added 0.5 ml. of phenylhydrazine. Crystallization occurred immediately. The crystalline mass was collected by suction after left overnight at 0°, and recrystallized from 50% ethanol, m.p. 198~200°. No depression was observed with admixture of authentic D-mannose phenylhydrazone. The filtrate of D-mannose phenylhydrazone was heated on a boiling water bath for 2 hr. A yellow precipitate formed was collected by centrifugation and recrystallized from 50% ethanol, m.p. 194~196°. No depression was observed with admixture of authentic D-galactose phenylosazone.

Determination of the Galactose-Mannose Ratio——Ten mg. of the acid hydrolyzate of GDC-Fr. I was subjected to paper chromatography (solvent A). The corresponding areas of galactose and mannose on the paper strip were cut off separately, then eluted with water and made up to 10 ml. respectively. The carbohydrate content of each eluate was determined by Molisch's method, and the molar ratio of galactose to mannose, 107:240 or 1:2.24, was obtained.

Partial Hydrolysis of GDC-Fr. I with 0.025 *N* Oxalic Acid—— Ten mg. of GDC-Fr. I was dissolved in 10 ml. of 0.025*N* oxalic acid, and the solution was heated for 3 hr. in a boiling water bath, then neutralized by heating with a small amount of calcium carbonate. The neutral solution was then centrifuged and the clear supernatant was dialyzed in a cellophane tube against 200 ml. of distilled water with stirring for 24 hr. below 4°. The dialyzable fraction was concentrated under diminished pressure and was made up to 10 ml. with water. A small amount of the solution was subjected to paper chromatography. No spot other than galactose was detected on the chromatogram. Quantative determination of the carbohydrate content of the diffusate by Molisch's reagent gave a value 136 μg./ml., or 14.3% of carbohydrate of the original polysaccharide, or 50% of total galactose.

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