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Isolation of a Water-soluble Polysaccharide from the Mycelium of *Penicillium chrysogenum*[1] (Studies on Fungal Polysaccharides. V[2])

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Main water-soluble intracellular polysaccharide of *P. chrysogenum* is a galactomannan consists of D-galactose and D-mannose in an approximate ratio of 2:3 with trace of D-glucose. Assay of formic acid and formaldehyde released by periodate oxidation and that of Smith type degradation products showed that the polysaccharide has a linear structure and contains 1→2 main linkage with 1→3 linkage also 1→4 linked galactopyranose or 1→5 or 1→6 linked galactofuranose residue.

Although several polysaccharides from *Penicillium* species have been examined in detail,[4] the structural feature of water-soluble intracellular polysaccharide from *P. chrysogenum* remains to be elucidated.

In 1957, Eckart[5] proved the presences of two intracellular polysaccharides, namely a limited dextrin and a heteroglycan designated as endopenicillan A, by twenty percent hot alkaline extraction from the mycelium of a *Penicillium* strain, and described that endopenicillan A was composed of D-mannose, D-galactose and D-glucose in the approximate ratio of 3:2:0.74.

Recently, Kawakami, Watanabe, *et al.* and Nakayama[6] reported that some water-soluble intracellular polysaccharides from several species of air-borne filamentous fungi including *Penicillium* showed a positive skin-test to a case of eczema or asthatic. It is therefore of interest to examine whether the intracellular water-soluble polysaccharides of these fungi are similar or different in their structures.

In the present paper, isolation and some structural investigations of a water-soluble polysaccharide from the mycelium of *P. chrysogenum* 976 strain, which is being used for industrial penicillin production, are described.

In order to avoid decomposition by strong alkaline extraction, the polysaccharide was extracted with 3% saline in an autoclave at 120° from defatted mycelium, and the crude polysaccharide obtained was purified by protease-digestion and Sevag's treatment, and finally by DEAE-cellulose column chromato-

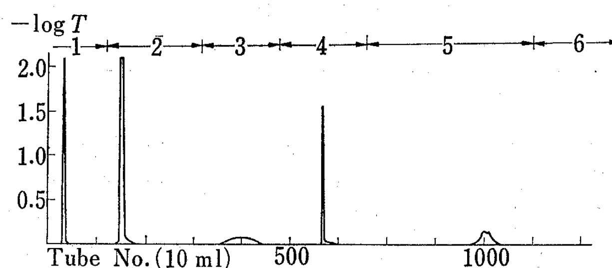

Fig. 1. Elution Pattern of the Crude Polysaccharide on DEAE-Cellulose

1: water elution
2: 0.05 M $NaHCO_3$ elution
3: 0.1 M $NaHCO_3$ elution
4: 0.2 M $NaHCO_3$ elution
5: 0.01 N NaOH elution
6: 0.1 N NaOH elution

1) This work was presented at the 86 th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, Oct 1966.
2) Part IV: T. Miyazaki, *Yakugaku Zasshi*, **82**, 1678 (1962).
3) Location: *600 Kashiwagi 4-chome, Shinjuku-ku, Tokyo.*
4) M. Stacey and S.A. Barker, "Polysaccharides of Microorganisms," Oxford Press, London, 1960, p. 174.
5) E. Eckart, *Z. Physiol. Chem.*, **308**, 225 (1957).
6) Y. Kawakami, *Shinkin to Shinkinsho*, **6**, 274 (1965); **7**, 65 (1966); S. Watanabe and S. Fujisawa, *ibid*, **7**, 73 (1966); Y. Nakayama, *ibid*, **7**, 156 (1966).

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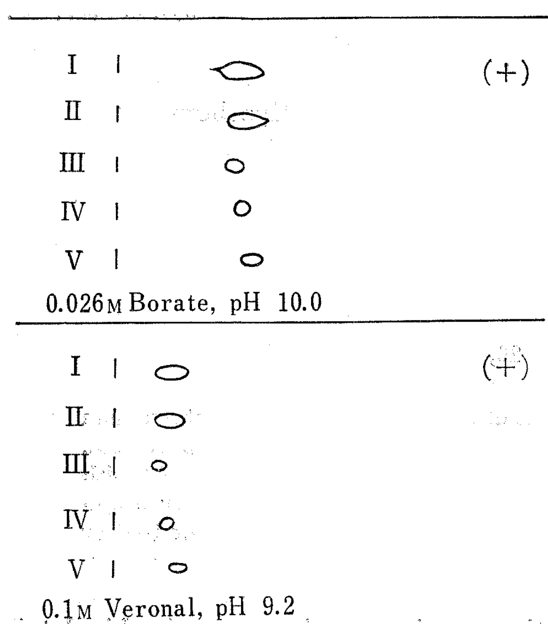

Fig. 2. Paper Electrophoresis of Each Fraction Separated on DEAE-Cellulose

I: crude polysaccharide
II: polysaccharide after treatment of protease
III: water fraction on DEAE-cellulose
IV: 0.05 M $NaHCO_3$ fraction on DEAE-cellulose
V: 0.2 M $NaHCO_3$ fraction on DEAE-cellulose

graphy. Elution was effected stepwise with water, sodium bicarbonate and sodium hydroxide. As it is shown in the Fig. 1 the main fraction was eluted by 0.05 M sodium bicarbonate.

The main polysaccharide, $[\alpha]_D^{21}$ $-8.4°$ (c=3.07, l=0.5, water), gave a singlespot on a paper electrophoresis using veronal buffer (0.1 M, pH 9.2) or borate buffer (0.026 M, pH 10.0), and did not give blue color with iodine, and contains 0.85% of phosphorous and 1.4% of nitrogen but no sulfur.

This polysaccharide was considered to be pure enough to carry out the structural studies.

The component sugars of the polysaccharide were identified as D-mannose, D-galactose and trace of D-glucose by paper chromatography of the hydrolysate, and the componental molar ratio of mannose and galactose was estimated approximately as 3:2 by the procedure of Dubois, *et al.*[7]

Estimation of the reducing power of the material by the modified Somogyi method[8] revealed the respective ratio of reducing end group to monosaccharide unit of 1:0.034, calculated as mannose, but the method by hypoiodite oxidation[9] gave an indefinite data.

Table I. Molar Ratio of the Componental Sugars

	Fraction	Component Sugar	Mol. ratio
I	water	glc.-gal.-man.	0.12 : 1.00 : 1.53
II	0.05 M $NaHCO_3$	glc.-gal.-man.	0.08 : 1.00 : 1.50
III	0.2 M $NaHCO_3$	glc.-gal.-man.	0.06 : 1.00 : 1.08

On periodate oxidation of the polysaccharide, the consumption of periodate from anhydrohexose unit was 1.03 mole (extrapolated to zero time from the periodate consumption curve), the value of formic acid liberated from the unit was 0.09 mole, and that of formaldehyde was 0.05 mole after 24 hr respectively. The periodate-oxidized polysaccharide was treated with sodium borohydride followed by mild acid hydrolysis (Smith type degradation). Paper chromatographic analysis of the hydrolysate revealed the presences of mannose and galactose in addition to threitol and glycerol. The molar ratios, approximately in 1.0:1.6:2.2:7.2 (gal.-man.-thr.-gly.), were estimated by the procedures of Dubois, *et al.*[7] (for hexoses) and of Lambert-Neish[10] (for polyalcohols) after the hydrolysate was separated by paper chromatography.

7) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
8) T. Miyazaki, *Chem. Pharm. Bull.* (Tokyo), **9**, 826 (1961).
9) M. Tomoda, *Yakugaku Zasshi*, **80**, 1696 (1960).
10) M. Lambert and A.C. Neish, *Can. J. Research.*, **28B**, 83 (1956).

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Table II. Products of Smith Type Degradation

Component	Gal.	Man.	Threitol	Glycerol
Content (μg)	661.5	890.5	851	1991
Mol. ratio	1.0	1.6	2.2	7.2

From the above results, it is concluded that the main intracellular polysaccharide is composed of mannose and galactose in the molar ratio of 3:2 with trace of glucose and has a linear structure to consist of about 30 hexose units from the low formic acid value and the value of reducing end assay. When Smith type degradation was carried out on the polysaccharide, remarkable quantity of glycerol, some quantity of unoxidized component sugars, and reasonable quantity of threitol were given. For that reason, it is apparent that the polysaccharide is composed of 1→2 linked pyranose residue which is able to give glycerol and no formic acid by the degradation, and smaller amount of unoxidized linkage which should be 1→3 pyranose residues of the component sugars, also 1→4 linked galactopyranose or 1→5 or 1→6 linked galactofuranose residue which is able to give threitol.

On the other hand, the crude water-soluble polysaccharide solution gave blue color with iodine, it may be caused by the water-eluted fraction which is glucose-rich than other fractions. Therefore, it seems that at least two kinds of water-soluble polysaccharide are contained in a mycelium of *P. chrysogenum*.

The reducing end of the main polysaccharide seems to be C_2-linked mannose because of its strange behavior on the alkaline hypoiodite oxidation.[11]

Detail of the structure of the main polysaccharide will be discussed in the following paper.

Experimental

Isolation of Polysaccharide from Mycelium——The dried mycelium of *P. chrysogenum* 976 which was used for preparation of the polysaccharide was kindly supplied by Nippon Kayaku Co. Ltd. through Dr. A. Matsuda.

To the mycelium, 2-volumes of EtOH-ether (1:1) was added and the mixture was mildly refluxed for 6 hr. The mycelium separated from the mixture by filteration was washed with the same solvent, and dried *in vacuo*. The defatted mycelium immersed in 2-volumes of 3% NaCl aqueous solution and the mixture was heated in autoclave at 120° for 15 min, the suspension was centrifuged. After the same procedure was repeated 3-times, the combined supernatant was dialyzed in a Visking cellophane tubing against running water for 3 days.

Solution of the material in the tube was concentrated to a small volume and 3-volumes of EtOH containing 0.1% AcOK was added to the concentrate. The yellowish-brown precipitate appeared was collected by centrifugation, dissolved in about 4-volumes of water, the shaken vigorously for a half hr with 0.5 volume of $CHCl_3$-BuOH (4:1). The same procedure was repeated until the gelatinous substances in the mixture solution was not formed. After centrifugation, the supernatant was concentrated to a small volume *in vacuo*, and 3-volumes of EtOH containing 0.1% AcOK was added to this concentrate. The greyish-brown precipitate was collected by centrifugation, washed with EtOH, acetone and ether, then dried *in vacuo*. Yield: *ca.* 1.46%.

Protease Digestion of the Crude Polysaccharide——Crude polysaccharide (2.03 g) was dissolved in 50 ml of water, adjusted to pH 7.7 with $NaHCO_3$. To the solution was added pronase (100 mg), the mixture was kept standing for 55 hr at 37°, and then dialyzed in a Visking cellophane tubing against running water for 3 days. Solution in the tube was diluted to 200 ml, and was shaken with $CHCl_3$-BuOH (4:1) as described above. After centrifugation, the supernatant was concentrated to a small volume *in vacuo*, and 3-volumes of EtOH containing 0.1% AcOK was added.

The precipitate was collected by centrifugation, washed with EtOH, acetone and ether, and dried *in vacuo*. Yield: 37.5% to the crude polysaccharide.

Purification on DEAE-Cellulose Column——DEAE-cellulose used was made by Brown Co., Ltd. The resolution of the polysaccharide was achieved on a DEAE-cellulose column using water, aqueous sodium bicarbonate and sodium hydroxide.

11) T. Miyazaki and T. Yadomae, unpublished result.

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The crude polysaccharide (500 mg) in water (10 ml) was applied to a column (3×45 cm). Stepwise elution was effected by water, 0.05 M, 0.1 M, 0.2 M $NaHCO_3$, 0.01 N and 0.1 N NaOH. The rate of flow through the column was 100 ml/hr, each fraction (10 ml) was collected automatically, and aliquots of each fraction (0.5 ml) were mixed with 1.5 ml of water followed by 4 ml of 0.2% anthrone reagent and the optical densities at 625 mμ were read on a colorimeter. The results were given in Fig. 1.

Each fractions (except the water–eluted fraction) were dialyzed in a Visking cellophane tubing against running water and then distilled water for 3 days. Internal solutions of the tubes were concentrated to a small volume *in vacuo*, and 6– volumes of EtOH was added to these concentrates. Yielded precipitates were collected by centrifugation, washed with EtOH, ether and then dried *in vacuo*. Yields were as follows: Water–eluate, 76.4 mg (15.3%); 0.05 M $NaHCO_3$ eluate, 242.9 mg (48.5%); 0.1 M $NaHCO_3$ eluate, 31.7 mg (6.3%); 0.2 M $NaHCO_3$ eluate, 40.2 mg (8.0%); 0.01 N NaOH eluate, 25.2 mg (5.0%); 0.1 N NaOH eluate; trace. Recoverly: 83.1%.

Component Sugars of Each Fractions——Each fractions (*ca.* 10 mg) in 2 ml of 2 N H_2SO_4 sealed in a tube were heated in a boiling water bath for 13 hr. After neutralization ($BaCO_3$) and filtration, a portion of the concentrate was examined by ascending method on Toyo Roshi No. 50 filter paper using the following solvent systems: (1) ethyl acetate–pyridine–water (10:4:3), (2) butanol–acetic acid–water (4:1:5), (3) phenol–water (3:1).

In the solvent systems (1) and (2), three spots corresponding to mannose, galactose and glucose (trace) were detected with the spray reagents of *p*–anisidine–HCl[1,2] and silver nitrate–NaOH.[13]

Quantitative Estimation of the Component Sugars——A portion of hydrolysate of the each fractions was spoted on a filter paper (Toyo Roshi No. 50, 20×40 cm) and developed in the solvent system (1) for 8 hr. Developing procedure was repeated 4 times. After air drying, the areas containing sugars on the paper chromatogram were cut out and quantitatively extracted with constant volume of distilled water, and the amount of sugar in 2 ml of the extracts were estimated colorimetrically by the method of Dubois, *et al.* Results were given in Table I.

Properties of the Main Polysaccharide——The main water–soluble polysaccharide (0.05 M $NaHCO_3$ eluted fraction), white powder, $[\alpha]_D^{21}$ $-8.4°$ (c=3.07, l=0.5, water), containing 0.85% of phosphorus and 1.4% of nitrogen, was neutral in a aqueous solution and gave no color with iodine.

Paper electrophoresis using 0.1 M veronal buffer (pH 9.2) or 0.026 M borate buffer (pH 10.0), showed a single spot (detected with periodate–Schiff's reagent[14]).

Determination of reducing power of this polysaccharide was carried out as follows; A solution of 5 ml of 3.084 mg of the polysaccharide was heated with 5 ml of the alkaline copper reagent for 40 min at 100°, cooled in ice, and the mixture was acidified with 2 N H_2SO_4 (1.5 ml).

The liberated I_2 was titrated with 0.005 N $Na_2S_2O_3$ and the consumption of 0.60 ml of 0.005 N $Na_2S_2O_3$ was observed. The control experiment was conducted in the same way using 0.3001 mg of mannose and it consumed 1.71 ml of 0.005 N $Na_2S_2O_3$.

Periodate Oxidation of the Polysaccharide——The polysaccharide (12.2 mg) was dissolved in 10 ml of distilled water and its total volume made up to 25 ml with 2 ml of 0.21 M $NaIO_4$ and distilled water. The mixture was allowed to stand in dark at room temperature and determinations of the consumption of $NaIO_4$, HCOOH and HCHO produced were carried out with this solution by the procedures of Maraprade,[15] Whilstler[16] and O'dea & Gibbons.[17]

The number of moles of $NaIO_4$ consumed per anhydrohexose unit of polysaccharide were as follows; 0.57 (1 hr), 0.70 (3 hr), 0.80 (6 hr), 0.89 (12 hr), 1.05 (24 hr), 1.24 (48 hr), 1.32 (72 hr), the value of formic acid was 0.05 (12 hr), 0.09 (24 hr), 0.22 (72 hr), and the formaldehyde was 0.05 (24 hr), 0.13 (96 hr).

Smith Type Degradation of the Polysaccharide——The polysaccharide (94.0 mg) was oxidized with $NaIO_4$ as described above. After 24 hr, ethylene glycol (1.2 g) was added to destroy the excess periodate and the reaction mixture was dialyzed against running water. To non–dialyzable solution concentrated to *ca.* 100 ml *in vacuo*, was added $NaBH_4$ with vigorous stirring and the stirring was continued overnight. The excess $NaBH_4$ was destroyed by acidification with AcOH, the reaction mixture was concentrated to dryness, and was heated with 0.1 N H_2SO_4 (2 ml) on a boiling water bath for 10 hr. The hydrolysate was passed through a column of ion–exchange resin, Amberlite IR–45, and the effluent was concentrated to a small volume *in vacuo* and examined by paper chromatography using the solvent systems (1) and (2). As main products on the paper chromatograms, four spots corresponding to glycerol, threitol, mannose and galactose of standard substances were detected. In order to estimate relative molar ratio in those main

12) L. Hough, J.K.N. Jones and W.H. Wadman, *J. Chem. Soc.*, 1702 (1950).
13) W.E. Trevelyan, D.P. Protector and J.S. Harrison, *Nature*, **166**, 444 (1950).
14) E. Koiwa and A. Gronwall, *Scand. J. Clin. Lab. Invest.*, **4**, 244 (1952).
15) L. Maraprade, *Bull. Soc. Chim. France*, **1**, [5], 833 (1934).
16) R.L. Whilstler and J.L. Hickson, *J. Am. Chem. Soc.*, **76**, 1671 (1954).
17) J.F. O'dea and R.A. Gibbons, *Biochem. J.*, **55**, 580 (1953).

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products, the syrup was spoted on a filter paper, and multiple developing was carried out 3- times in the solvent system (1). After air-drying, corresponding areas to the spots on a paper chromatogram were quantitatively extracted with constant volume of distilled water, filtered through sintered glass filter. The determinations of mannose and galactose were performed by the method of Dubois, *et al.*, of glycerol and threitol by the method of Lambert-Neish. The results were given in Table II.

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