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114. Toshio Miyazaki : Studies on Fungal Polysaccharides. I. On the Isolation and Chemical Properties of Capsular Polysaccharide from *Cryptococcus neoformans*.

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Cryptococcus neoformans (*Torula histolytica*, *C. hominis*) is a kind of pathogenic yeast-like fungi first isolated from human body by Busse.[1] The disease caused by this fungi happens to spread sporadically all over the world, usually being called cryptococcosis (European blastomycosis or torulosis). In Japan, cryptococcosis was first reported by Watanabe,[2] and Goto.[3]

Generally, the cells of *C. neoformans* are surrounded by a thick viscous capsule and its thickness is sometimes larger than the diameter of the cell body. The capsule or capsular polysaccharide of the fungi has antigenic activity[4~9] and this polysaccharide seems to play some rôle in its serologic cross-reactions[10] between an anticryptococcal serum and *Candida albicans*, *Saccharomyces cerevisiae*, *Pneumococcus* Type II, trichophytin, or tragacanth.

This capsular polysaccharide has been obtained from the cells or its culture medium by Aschner, *et al.*[11,12] Kligman,[13] Hehre, *et al.*,[6] Evans, *et al.*,[8,14,15] Drouhet, *et al.*,[16] Foley, *et al.*,[17] Einbinder, *et al.*[18] and by Yanai.[9] However only a few studies on its chemical property have been reported and those were almost all concerned with several color-reactions or paper chromatography of the componental sugars, and also their data were diverse.

Recently, Reber, *et al.*[19] reported that the polysaccharide, isolated by Evans and Kessel[8] from the culture medium of *C. neoformans* A, was not a single compound but a mixture, from which each componental polysaccharide could not be separated by precipitation with the Type-II antipneumococcal serum or treatment with cetyltrimethylammonium bromide (Cetavlon). The analysis of this polysaccharide indicated the presence of D-xylose, D-mannose, D-galactose, and D-glucurone as its component and fractional precipitation with the Type-XIV antipneumococcal serum resulted in increase of the proportion of D-galactose of the componental monosaccharide.

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1) O. Busse : Centralb. f. Bact., **16**, 175 (1894).
2) S. Watanabe : Fukuoka Idai Zasshi, 8, 470 (1915).
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19) P.A. Reber, *et al.* : J. Am. Chem. Soc., **80**, 1135 (1958).

The present investigation was focused on the purification and chemical structure of the polysaccharide in the capsular substance having serological activity.

For the preparation of the polysaccharide, defatted cells of *C. neoformans* CRD-1(Duke) were used and they were extracted by the procedure reported by Einbinder, *et al.*,[18] using 1% potassium carbonate added to 30% potassium chloride solution as a solvent, and white amorphous powder was obtained in ca. 17% yield.

Cryptococcus neoformans CRD-I (Duke)
↓ Me_2CO, $EtOH \cdot Et_2O$
Defatted cells
↓ shaken for 48 hr. with 20 vol. of 30% KCl-1% K_2CO_3, repeated 3-times, centrifuged
Supernatant
↓ dialyzed in fish skin, concentrated to a small volume, and added with 3 vol. of EtOH
Precipitate
↓ dissolved in water, adjusted to pH 4.0 with AcOH, shaken with kaolin, and centrifuged
Supernatant
↓ shaken with iso-AmOH-$CHCl_3$ (1:4), organic solvents removed. Concentrated to a small volume, and 3 vol. of EtOH added
White stringy precipitate
(crude capsular polysaccharide)

Chart 1. Isolation of the Capsular Polysaccharide

The substance seemed to be identical with capsular polysaccharide obtained by Einbinder, *et al.*, positive to Molisch, Bial, and naphthoresorcinol reactions, but negative to Biuret and Ninhydrin reactions.

The zone electrophoresis using starch gel and borate buffer (pH 10.0) was employed for further purification of this substance.

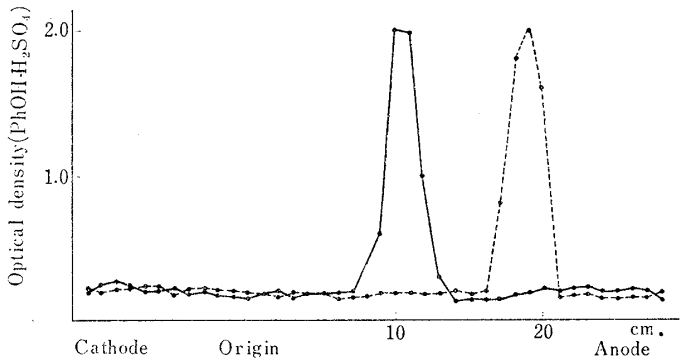

Fig. 1. Zone Electrophoresis of the Polysaccharide
•——• Borate buffer (pH 10.0) 66 hr. 2 mA/cm^2
•----• Acetate buffer (pH 5.0) 74 hr. 2 mA/cm^2

The main fraction thus obtained was extracted with water, sodium and borate ions were removed by treatment with Amberlite IR-120 (H) and by the method of Ziel, *et al.*,[20] followed by the treatment described in the experimental part, to obtain the white powder as most purified state. The material (as potassium salt) showed $[\alpha]_D^{15}$ +15.5° (c=0.775), $[\eta]$=20 (c=g./100 cc.), did not contain nitrogen, phosphorus, or sulfur, and gave negative iodine reaction. This potassium salt was deionized by electrodialysis and was freeze-

20) L. P. Ziel, *et al.* : J. Am. Chem. Soc., **75**, 1339 (1953).

dried to the ash-free polysaccharide. A 0.1% aqueous solution of this ash-free polysaccharide has pH 3.5. This polysaccharide was found to be pure by the ultracentrifugal analysis (Fig. 2). The observed $S^{1}_{20}=2.47$. The infrared spectrum of the potassium salt showed strong absorptions at 1612 and 1410 cm^{-1}, characteristic to carboxylate ion, but no absorption corresponding to free acid and nonionized carboxyl group. The spectrum showed the absorption of the type 2b (893 ± 6 cm^{-1}), but not type 2a (833 ± 8 cm^{-1}).[21] However, it could not be elucidated from the absorption curve whether the hexose units were

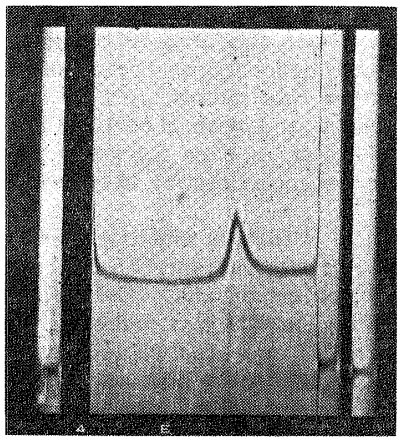

Fig. 2. Sedimentation Diagram of the Polysaccharide

0.4% NaH_2PO_4-Na_2HPO_4 buffer, pH 7.7, $\mu=0.2$, 144 min., Spinco Model-E ultracentrifuge (59,700 r.p.m.)

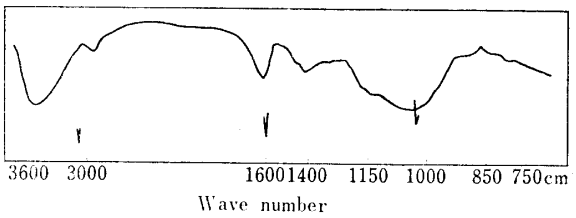

Fig. 3. Infrared Spectra of the Polysaccharide (Potassium salt)

combined in α- or β-configuration, because of the presence of absorption by uronic acid in this region.[22] On the zone electrophoresis run in acetate buffer (pH 5.0) ($2\ mA/cm^2$, 74 hr.), this substance was found to migrate to furnish only a single peak, and it was free from substance which gives phenol-sulfuric acid reaction at both origin and cathode part. This should be sufficient evidence to consider that this substance is not a mixture.

Experimental

Isolation of Polysaccharide from the Capsule——The strain of *C. neoformans* CRD-1 (Duke) was used for preparation of the polysaccharide. The strain was cultured on Sabouraud glucose-agar for 10 days at 37°. The cells cautiously collected were immersed for 24 hr. in a dehyd. Me_2CO, Me_2CO was replaced with 2 volumes of dehyd. Me_2CO, and the mixture was mildly refluxed for 1 hr. When cooled, Me_2CO was decanted off. To the residual cells, 3 volumes of EtOH-Et_2O (1:1) was added and the mixture was kept standing for 45 days at room temperature. The cells separated from the mixture by filtration were powdered, washed with EtOH-Et_2O, and dried *in vacuo*. The defatted material was shaken in an automatic shaking machine for 2 days with 20 volumes of 30% KCl solution containing 1% K_2CO_3 and the suspension was centrifuged. After the same procedure was repeated three times, the sediment was washed twice with the same solvent. The combined supernatant was dialyzed in a tube of fish skin against running water. Solution of the material in the tube was concentrated *in vacuo* and 3 volumes of EtOH containing 1% AcOK was added to this concentrate. The stringy white precipitate appeared was collected by centrifugation, dissolved in 500 volumes of H_2O, and the solution was adjusted to pH 4.0 with AcOH. The solution was shaken for

21) S. A. Barker, E. J. Bourne, D. H. Whiffer: "Methods of Biochemical Analysis." **3**, 213 (1956). Interscience Publishers, Inc., New York.
22) S. A. Barker, A. B. Foster, I. B. Siddiqui, M. Stacy: J. Chem. Soc., **1958**, 2358.

several hours with kaolin (1% solution) and was centrifuged. This treatment was repeated three times. The sediment obtained after final centrifugation, was washed with H_2O. The supernatant, combined with aqueous washings, was shaken vigorously for several hours with 0.5 volume of $CHCl_3$–iso-AmOH mixture (4:1). After centrifugation, the supernatant was concentrated *in vacuo* and 3 volumes of EtOH containing 1% AcOK were added to this concentrate. The white stringy flocculate that appeared was collected by centrifugation, washed with EtOH and Et_2O, and dried *in vacuo*. Yield, ca. 17%.

Zone Electrophoresis of the Polysaccharide——The potato starch gel (1.5×10×40 cm.) was used as the supporting medium, which was pretreated by washing with H_2O and 0.05*M* borate buffer (pH 10.0) until PhOH-H_2SO_4 reaction diminished. A 10% solution of the polysaccharide in borate buffer was packed at the position of 12 cm. from the cathode and allowed to migrate at 2 mA/cm^2 for 27~64 hr. The migrated distance (9~23 cm., 2 mA/cm^2) depended on the condition of packing of the supporting medium. After migration, the starch gel was cut off in 1 cm. portions and each cutting was eluted with 30 cc. of H_2O. Each eluate was treated with a small amount of charcoal, and filtered through a sintered glass filter. A portion of 0.5 cc. of each filtrate was diluted to 2 cc. with H_2O and the concentration of the carbohydrate was measured by the method of Dubois, *et al.*[23] (Fig. 1).

The fractions containing polysaccharide were eluted with H_2O and the combined eluate was treated with Amberlite IR-120 (H) and anhyd. MeOH to remove Na^+ and BO_3^-. After evaporation of the treated eluate, the residue was dissolved in a small volume of 0.5% KOH. After centrifugation and filtration of the solution (through a sintered glass filter), EtOH was added to the filtrate. The precipitate that appeared was centrifuged, washed with EtOH and Me_2CO, and dried *in vacuo*. The material obtained was a white amorphous powder, $[\alpha]_D^{15}$ +15.5°(c=0.775), and its aqueous solution was extremely viscous. This material showed positive Molische, Bial, and naphthoresorcinol reactions, but negative Biuret, Ninhydrin, and iodine reactions.

Electrodialysis of the Polysaccharide——A solution of the potassium salt of the polysaccharide was electrodialyzed against distilled H_2O at 100~130 V, ca. 10 *mA*, for 24 hr., when the current dropped to 1 *mA*. The non-dialysable solution was freeze-dried and a white powder was obtained. The anhydrous polysaccharide, which was further dried *in vacuo* at 70° for 8 hr., consisted of 43.2% of carbon and 6.1% of hydrogen. An 0.1% aqueous solution of this ash-free polysaccharide had pH 3.5.

Viscosity of the Polysaccharide——The potassium salt of the polysaccharide was dissolved in H_2O to a concentration of 0.008%, 0.016%, and 0.032%, and the viscosity of each solution was measured by the Ostwald viscometer (vol. 2.0 cc.) at 26°. Result : η_{sp} 0.163, 0.333, 0.690.

Infrared Spectrum of the Polysaccharide——The iufrared absorption spectrum of the potassium salt of polysaccharide was determined in potassium disk. (Fig. 3).

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Summary

Capsular polysaccharide of *Cryptococcus neoformans* CRD-1 was isolated by Einbinder's method and purified by zone electrophoresis with starch gel and borate buffer (pH 10.0) or acetate buffer (pH 5.0). This material (potassium salt) showed physical constants of $[\alpha]_D^{15}$ +15.5°(c=0.775) and $[\eta]$=20(c=g./100 cc.), and was positive to Molisch, Bial, and naphthoresorcinol reactions, but negative to iodine, biuret and Ninhydrin reactions. On infrared spectrum, its potassium salt showed the presence of carboxylate ion.

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