The Serological, Chemical and Physicochemical Analyses of Cryptococcal Capsular Polysaccharides

Takako Shinoda*, Reiko Ikeda, Akemi Nishikawa and
Yoshimura Fukazawa**

Department of Microbiology, Meiji College of Pharmacy, Tanashi, Tokyo 188, Japan and

**Department of Microbiology, Medical University of Yamanashi, Nakakoma-gun,
Yamanashi 409, Japan

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The taxonomic value of cryptococcal capsular polysaccharides was studied by comparing those of *Cryptococcus neoformans* and of nine related species. The characterization of the soluble capsular polysaccharides of these 10 species by their serological specificity, chemical constituents and structures represented by the proton magnetic resonance (PMR) spectra demonstrated a division into 2 groups. Species belonging to one group were closely related to *C. neoformans* and the members of the other group were markedly different from those of the former. It is suggested that the serological, chemical and physicochemical properties of capsular polysaccharides would be useful criteria for the classification of the *Cryptococcus* species.

Tsuchiya *et al.*^{1,2)} carried out antigenic analyses of various species of yeasts and a new system for yeast classification was proposed. The significance of the proton magnetic resonance (PMR) spectra of the yeast alkali-extracted polysaccharides for taxonomic studies and the correlation between PMR spectra and chemical structures of some yeast species have been investigated and summarized by Gorin and Spencer³⁾. They reported that in many instances the PMR spectral types of yeast strains correlated well with the serological types established by Tsuchiya *et al.* Recently chemical and physicochemical characteristics of the polysaccharides have been recognized as one of the important criteria in yeast taxonomy.

Cryptococcus neoformans is one of the clinically important yeast-like fungi. Among *Cryptococcus* species, however, because of poor antigenicity and

the viscosity of polysaccharide solutions, antigenic and physicochemical analyses of their polysaccharides have not been well developed to date.

The purpose of this paper is to show the differences among *Cryptococcus* species based upon their serological, chemical and physicochemical characteristics, including the PMR spectra of their polysaccharides, and to evaluate those properties as criteria for the classification of the *Cryptococcus* species.

MATERIALS AND METHODS

Cultures. Eight strains of *Cryptococcus* and two *Candida* species, which have been shown to be closely related to *Cryptococcus* species serologically and also have minor capsule, were used for the serological and chemical studies. The strains are listed in Table 1.

Physiological characteristics. Physiological characteristics were examined by sugar assimila-

*To whom inquiries should be addressed.

tion and urease tests. The assimilation tests were performed by the use of the 12 sugars in the Minitek identification kit for yeasts (Baltimore Biological Laboratories). Urease reactions were determined by the methods of Seeliger⁴⁾.

Antiserum. Antisera to *C. neoformans* and the other species were prepared as follows: Heat-killed cells were suspended in physiological saline solution (PSS) at 4°C. A solution of 2,4,6-trichloro-*s*-treazine in dimethyl formamide was then added to the cooled solution. The reaction was stirred at 4°C for 1 h before addition of a solution of bovine γ -globulin (BGG) in PSS. The mixture was then stirred at 25°C for 1 h, kept at 4°C for 18 h, and dialyzed against distilled water for 30 h. Rabbits were immunized with cell-BGG conjugate suspended in formalinized saline solution at a concentration equivalent to 3.8×10^7 cells/ml. Rabbits were given intravenous injections with the cell-BGG conjugate of 0.5, 1.0, 2.0, 4.0, 4.0 and 4.0 ml at 4-day intervals. The injected rabbits were bled from the marginal veins of their ears and the sera tested for their agglutination titers against the homologous organisms. When the titer of a given antiserum was 1:640 or higher, the rabbits were bled seven days after the last injection. In cases where their titer did not reach the desired level, injections of 4.0 ml of antigen were continued until the desired titer was obtained.

Serological characteristics. The serological characteristics of the strains were examined by tube agglutination tests, passive haemagglutination (PHA)⁵⁾ tests and precipitin reactions. Precipitin reactions between antiserum and polysaccharide were performed in PSS with 0.1 ml of serum in a final volume of 1.0 ml. Incubation was at 4°C for 48 h. The precipitate was washed twice with cold PSS by centrifugation. Precipitated antibody protein was measured by the method of Lowry *et al.*⁶⁾

Isolation and purification of soluble polysaccharide. To obtain soluble polysaccharide, each strain was grown in yeast nitrogen base broth (Difco) containing 2% glucose, 1% casamino acids and 100 μ g/ml streptomycin at 27°C for 5 days with shaking. The broth culture was sterilized at 100°C for 20 minutes standing

in flowing steam and the supernatant was separated by centrifugation. After concentration and dialysis of the supernatant, 9 volumes of ethanol and 1/10 volume of 10% sodium acetate solution were added. The precipitate was dissolved in water, dialyzed against water and lyophilized. Crude polysaccharide was treated with pronase by the method of Kozel *et al.*⁵⁾ Total carbohydrate was determined by the phenol-sulfuric acid method⁷⁾, and total protein was determined by the method of Lowry *et al.*⁶⁾.

Chemical analysis of polysaccharide. The conditions for acid hydrolysis were similar to those used by Gorin and Spencer⁸⁾. About 5 mg of polysaccharide were dissolved in 2 ml of 1 N H₂SO₄, heated in a sealed glass tube at 100°C for 18 h and neutralized with BaCO₃. The hydrolysate was then evaporated to syrup. Thin layer chromatography and gas liquid chromatography were used to identify the constituent carbohydrates of the polysaccharides. The solvent system used for thin layer chromatography consisted of n-butanol, ethanol and water at a v/v ratio of 40, 11, and 19. Spots were detected with p-anisidine spray reagent. The hydrolyzate was converted into alditol acetates and analyzed quantitatively by gas liquid chromatography. The procedure was as follows: The hydrolyzate was reduced with sodium borohydride for 3 h at room temperature. Excess borohydride was destroyed by addition of Amberlite IR-120 (H⁺ form), and the solution was evaporated to dryness. Methanol was added to the product, and evaporated to dryness repeatedly. Equal amounts of acetic anhydride and pyridine were added and kept at 100°C for 2 h. Water was added to the cooled reaction mixture, and evaporated to syrup. The residue was dissolved in chloroform and injected into the gas chromatograph. Gas liquid chromatography was carried out by a Shimadzu Gas Chromatograph GC-6A (Shimadzu Seisakusho Ltd. Tokyo) using a glass column containing 3% of ECNSS-M on Gas Chrom Q at 190°C. The uronic acid content of the soluble polysaccharide were determined by the carbazole method⁹⁾.

Determination of PMR spectrum of polysaccharide. The PMR spectra were deter-

mined with 1% solutions of polysaccharide in deuterium oxide at 80°C with a Bruker WH-270 MHz nuclear magnetic resonance spectrometer in which sodium 3-trimethylsilylpropionate (TSP) was employed as the internal standard.

RESULTS

Physiological characteristics. The assimilation patterns of the 12 carbohydrates and hydrolysis of urea by a strain of *C. neoformans* and 9 strains of the related species are shown in Table 1. The characteristics of these strains agreed quite well with their standard descriptions as published in "The Yeasts"¹⁰. All strains hydrolyzed urea.

Serological characteristics. To determine the serological relationships among the species, the antibody activities of the anti-*C. neoformans* serum were tested against homologous cells and the cells of other species (Fig. 1). In the direct agglutination tests, the antigens of *C. neoformans*, *C. terreus*, *C. albidus* var. *diffuens*, *C. albidus* var. *albidus*, *Candida curvata* and *C. humicola* reacted very closely, but *C. luteolus*, *C. gastricus*, *C. laurentii* var. *laurentii* and *C. uniguttulatus* reacted weakly with the anti-*C. neoformans* serum. The cross-

reactivity patterns obtained by PHA paralleled those obtained by direct agglutinations. Similarly, in the quantitative precipitation tests of the poly-

Fig. 1. Cross agglutination patterns of *Cryptococcus neoformans* and related species against anti-*C. neoformans*-BGG serum.
C. nef., *C. neoformans*; *C. ter.*, *C. terreus*; *C. alb. var. dif.*, *C. albidus* variety *diffuens*; *C. alb. var. alb.*, *C. albidus* variety *albidus*; *C. cur.*, *Candida curvata*; *C. hum.*, *Candida humicola*; *C. lut.*, *C. luteolus*; *C. gas.*, *C. gastricus*; *C. lar. var. lar.*, *C. laurentii* variety *laurentii*; *C. ung.*, *C. uniguttulatus*.

Table 1. Assimilation of carbohydrates and hydrolysis of urea by *Cryptococcus neoformans* and related species

Strains studied			Assimilation of:											Hydrolysis of urea	
			Glucose	Galactose	Sucrose	Maltose	Cellobiose	Trehalose	Lactose	Melibiose	Raffinose	D-xylose	Galactitol		Inositol
<i>C. neoformans</i>	CBS	132	+	+	+	+	+	+	—	—	+	+	+	+	+
<i>C. terreus</i>	IFO	0717	+	+ ^b _w	—	+	+	+ _w	+ _w	—	—	+	+ _w	+ _w	+
<i>C. albidus</i>	CBS	142	+	+ _w	+	+	+	+ _w	+ _w	—	+ _w	+	+ _w	+ _w	+
var. <i>albidus</i>															
<i>C. albidus</i>	CBS	160	+	—	+	+	+ _w	+	—	—	+ _w	+	+	+ _w	+
var. <i>diffuens</i>															
<i>C. curvata</i> ^a	CBS	570	+	+	+	+ _w	+	+	+	—	+	+	—	+ _w	+
<i>C. humicola</i> ^a	CBS	571	+	+ _w	+	+	+	+	+	+	+ _w	+	+	+	+
<i>C. luteolus</i>	CBS	943	+	+	+	+	+	+	—	+	+	+	+ _w	+	+
<i>C. gastricus</i>	CBS	1927	+	+	—	+	+	+	—	—	—	+	—	+	+
<i>C. laurentii</i>	CBS	139	+	+	+	+	+	+ _w	+	+	+	+	+ _w	+	+
var. <i>laurentii</i>															
<i>C. uniguttulatus</i>	CBS	1730	+	—	+	+	—	+	—	—	—	+	—	+	+

^a *Candida* species
^b weakly positive

Fig. 2. Precipitin curves of anti-*C. neoformans*-BGG serum against polysaccharides of *Cryptococcus neoformans* and related species.

Symbols; ●—●, *C. neoformans*; ●---●, *C. albidus* variety *diffluens*; ○---○, *Candida humicola*; ■—■, *C. terreus*; △—△, *C. albidus* variety *albidus*; ▲---▲, *Candida curvata*; ○—○, *C. gastricus*; △---△, *C. luteolus*; □—□, *C. uniguttulatus*; ▲—▲, *C. laurentii* variety *laurentii*.

Table 2. Molar ratios of sugar components of polysaccharides of *C. neoformans* and related species

Polysaccharides from			Molar ratios of sugar components				
			Man	Xyl	Gal	Glc	GlcUA ^a
<i>C. neoformans</i>	CBS	132	3.3	1.0	0.5	—	1.6
<i>C. terreus</i>	IFO	0717	1.5	1.0	0.6	—	0.6
<i>C. albidus</i> var. <i>albidus</i>	CBS	142	2.0	1.0	0.7	—	0.9
<i>C. albidus</i> var. <i>diffluens</i>	CBS	160	2.1	1.0	—	—	0.8
<i>C. curvata</i> ^b	CBS	570	2.3	1.0	—	—	0.8
<i>C. humicola</i> ^b	CBS	571	2.0	1.0	—	—	0.6
<i>C. luteolus</i>	CBS	943	5.3	1.0	—	—	3.1
<i>C. gastricus</i>	CBS	1927	0.6	1.0	—	—	0.3
<i>C. laurentii</i> var. <i>laurentii</i>	CBS	139	2.9	1.0	1.9	0.9	1.4
<i>C. uniguttulatus</i>	CBS	1730	4.0	1.0	2.2	0.5	0.9

^a Man, mannose; Xyl, xylose; Gal, galactose; Glc, glucose; GlcUA, glucuronic acid
^b *Candida* species

saccharides of *C. neoformans* and other species against anti-*C. neoformans* serum, the polysaccharides from species, which demonstrated strong cross-reactions against anti-*C. neoformans* serum in direct agglutination, precipitated but the other polysaccharides did not precipitate (Fig. 2). These results indicated that the *Cryptococcus* species could be divided into two groups based on their

serological reactivity against the anti-*C. neoformans* serum.

Chemical analysis of soluble polysaccharides. Purified polysaccharides from all species tested contained total protein of less than 1.9%. The qualitative and quantitative analyses of constituent monosaccharides were determined by thin layer and gas liquid chromatography.

Fig. 3. PMR spectra of polysaccharides of *C. neoformans* and *C. uniguttulatus* in D_2O at $80^\circ C$. Arabic numerals indicate chemical shifts of each signal. H-1, H-1 region; -O-Ac, -O-acetyl; TSP, sodium 3-trimethylsilylpropionate.

As shown in Table 2, the compositions of the polysaccharides from *C. neoformans* and the following five species, which were closely related to *C. neoformans* serologically, were shown to be of similar in composition of monosaccharides, except for their galactose content. As to the other four strains the patterns of carbohydrate contents were characteristic for each species. The polysaccharide from *C. luteolus* contained a lot of mannose and glucuronic acid, and the polysaccharide from *C. gastricus* contained a smaller amount of those monosaccharides relatively. The last two polysaccharides obtained from *C. laurentii* var. *laurentii* and *C. uniguttulatus* were shown to have a similar composition of monosaccharides.

PMR spectra of polysaccharides. The PMR spectra at 270 MHz of polysaccharides from

C. neoformans and *C. uniguttulatus* are shown in Fig. 3. As mentioned above, both species were clearly divided into different serological groups. The H-1 region of both polysaccharides demonstrated 3 main signals at 5.01 and 4.99 ppm, 5.15 ppm, 5.23 and 5.21 ppm respectively, showing that only the intensity of the signal at 4.99 ppm differed markedly between the two strains. Further, while the PMR spectrum of the polysaccharide from *C. neoformans* showed a characteristic signal at 2.19 ppm, thus showing the presence of O-acetyl groups, the polysaccharide from *C. uniguttulatus* gave only a very small signal in this field. The signals of the H-1 region of PMR spectra of the polysaccharides from all of the species are shown in Table 3 and Fig. 4. The relative intensity of the signals at 2.2 ppm

Table 3. PMR signals from the polysaccharides of *Cryptococcus neoformans* and related species

Strains studied				Signal (ppm)					Relative intensity of 2.2 ppm ^b	
<i>C. neoformans</i>	CBS	132	5.01	5.15	5.17	5.23			S ^c	
<i>C. terreus</i>	IFO	0717	4.98	5.13	5.18	5.23			M	
<i>C. albidus</i>	CBS	142	4.99	5.14	5.19	5.22			S	
<i>var. albidus</i>										
<i>C. curvata</i> ^a	CBS	570	4.98	5.15		5.24			S	
<i>C. albidus</i>	CBS	160	5.00	5.06		5.26			S	
<i>var. diffluens</i>										
<i>C. humicola</i> ^a	CBS	571		5.08		5.25			S	
<i>C. luteolus</i>	CBS	943				5.23			M	
<i>C. gastricus</i>	CBS	1927	5.00	5.05	5.14	5.18	5.22	5.28	5.32	W
<i>C. laurentii</i>	CBS	139	4.99		5.18	5.23		5.36		W
<i>var. laurentii</i>										
<i>C. uniguttulatus</i>	CBS	1730	4.99	5.15		5.21				W

a *Candida* species
b peak at 2.2 ppm is due to O-acetyl group
c S: strong, M: intermediate, W: weak

Fig. 4. PMR spectra (H-1 region) of polysaccharides of *C. neoformans* and related species in D₂O at 80°C. Arabic numerals indicate chemical shifts of each signal.

are shown in Table 3. The 6 serologically related species were further divided into two subgroups based on the spectral patterns of their H-1 regions. One group consisted of *C. neoformans*, *C. terreus*, *C. albidus* var. *diffuens*, and *Candida curvata*, and the other one consisted of *C. albidus* var. *albidus* and *Candida humicola*. The other four species, that reacted weakly with the anti-*C. neoformans* serum, showed characteristic spectra for each species. The intensity of the signals at 2.2 ppm that showed the presence of O-acetyl groups were observed to be fairly parallel to the serotypes.

DISCUSSION

Recently the chemical structures of the antigenic determinants of *Saccharomyces cerevisiae* and a few *Candida* species have been defined by Fukazawa *et al.*¹¹⁾ The experiments on the serological relationship among *Cryptococcus* species, obtained either by agglutination or by quantitative precipitation, showed that these species could be divided into two groups based on their reactivity against anti-*C. neoformans* serum, although antisera of the other group's species could not be obtained. In contrast to the cell-BGG conjugate of *C. neoformans*¹²⁾, the cell-BGG conjugate and the polysaccharide-BGG conjugate of the species of the other group failed to induce antibody. The reason for the lower immunogenicity of the conjugate of the species is not clear at present. The polysaccharide components of six strains, which were closely related to *C. neoformans* serologically, resembled each other qualitatively and quantitatively. On the other hand, the strains of the other four species, that showed only weak cross-reactivities to *C. neoformans* serologically, showed characteristic patterns for each species. These results, as well as the results obtained by PMR spectra, suggested that the chemical constituents and structures of the polysaccharides of the *Cryptococcus* species are closely related to their serological characteristics. We have not demonstrated that the polysaccharides from the nine species, other than *C. neoformans*, are a single entity, but they were obtained by the same method as the *C. neoformans* polysaccharide which eluted as a single peak on a SG-200 Gel filtration column and as one peak in analytical ultracentrifugation.

As to the *Cryptococcus* species, because of their poor antigenicity and the viscosity of their polysaccharide solutions, antigenic and physicochemical analysis have not been developed as yet. Since the imperfect states of the *Cryptococcus* species have only poor morphological and physiological features, their capsular polysaccharides should be expected to be of significant taxonomic value.

Although the PMR spectra of mannose containing polysaccharides are known to be useful for yeast classification, PMR analysis of the polysaccharides of the *Cryptococcus* strains was very difficult because of the viscosity of their solutions³⁾. In the present study, however, we succeeded in getting their spectra through the use of soluble polysaccharides and also by the use of a 270 MHz nuclear magnetic resonance spectrometer. In the study of the PMR spectra of the polysaccharides of the *Cryptococcus* species, we found O-acetyl groups in the spectra in addition to the characteristic spectrum in the H-1 region. We also found that the amount of O-acetyl groups correlated with serological specificity. Therefore it is suggested that the O-acetyl groups would be one of the important chemical constituents for antigenic determinant.

In 1961, Miyazaki proposed the chemical structures of a repeating unit of capsular polysaccharide of *C. neoformans*. More recently, the chemical structures of the repeating units of serotypes A, C and D of *C. neoformans* have been demonstrated¹³⁻¹⁹⁾. Further studies on the chemical structures responsible for the antigenic specificities of the serotypes of *Cryptococcus neoformans* and related species remain to be elucidated.

Our results suggest that the serological, chemical and physicochemical characteristics of soluble capsular polysaccharides would be useful criteria in the classification of the *Cryptococcus* species, although microheterogeneity is demonstrated in the polysaccharide structures due to episemantic molecules²⁰⁾. For example, *C. uniguttulatus* was first isolated from a diseased nail by Wolfram and Zach and named *Eutorulopsis uniguttulata*²¹⁾. Based on the morphological and physiological similarities of this yeast-like fungus to *C. neoformans*, except for its weak ability to form a capsule,

Lodder *et al.* redescribed it as *C. neoformans* var. *uniguttulatus*²²⁾. Kreger-van Rij and Staib suggested that *C. neoformans* var. *uniguttulatus* should be elevated to the rank of species on the basis of its assimilation pattern and inability to grow at 37°C²³⁾. In 1970 this variety of *C. neoformans* was raised to the rank of a species¹⁰⁾. In 1977, Kwon-Chung discovered the perfect state of *C. uniguttulatus* and named it *Filobasidium uniguttulatum*²⁴⁾. Earlier Kwon-Chung discovered the perfect state of *Cryptococcus neoformans* as a *Filobasidiella* species^{25,26)}. It was placed in a separate genus on the basis of the mechanism of basidiospore formation. These findings support the status of *C. uniguttulatus* as a separate species. Our results, obtained by the characterization of soluble capsular polysaccharides, may support the recent decision to treat *C. uniguttulatus* as a separate species.

In this paper, clinically important *Cryptococcus* species were studied by the use of only one strain, therefore the differences among strains would have to be taken into consideration. In fact, *C. neoformans* have been shown to be divided into four serotypes. Further characterization of the polysaccharides of *C. neoformans* serotypes A, B, C and D as well as those of the other *Cryptococcus* species are in progress in our laboratory.

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