Studies on Fungal Polysaccharides: The Immunological Determinant of the Serologically Active Substances from *Absidia cylindrospora*, *Mucor hiemalis* and *Rhizopus nigricans*

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Six manno-oligosaccharides were isolated from the partial acid hydrolysate of pronase-treated extracellular glycan (pt-ACE-II) of *Absidia cylindrospora* by gel filtration on Bio Gel P-2 and descending paper chromatography. The pentasaccharide and the higher molecular weight oligosaccharides significantly inhibited the precipitation reaction of intracellular glycans from *Absidia cylindrospora* (ACI-B), *Mucor hiemalis* (MhI-B) and *Rhizopus nigricans* (RnI-B) with anti-ACE serum. Structural analysis showed that these six oligosaccharides were composed of linear (1 → 6)- α -linked mannopyranosyl residues.

INTRODUCTION

Previously (Hayashi *et al.*, 1978*a, b*) we have described the isolation, purification and partial characterization of the serologically active fucomannan-peptide of *Absidia cylindrospora*, a member of the Mucorales. Rabbit antiserum (anti-ACE serum) against the extracellular non-dialysable fraction of *A. cylindrospora* reacted not only with this fucomannan-peptide but also with antigenic fractions from four *Mucor* spp. and *Rhizopus nigricans*. However, anti-ACE serum did not cross-react with the purified mannans of *Candida albicans* and *Saccharomyces cerevisiae*, so it would appear that the chemical structure of the serologically active determinant might be different from those of the mannans of *C. albicans* and *S. cerevisiae*.

In the present paper, the purification of six manno-oligosaccharides and the results of precipitation inhibition tests with these oligosaccharides are described. The common determinant in the serologically active substances of *A. cylindrospora*, *M. hiemalis* and *R. nigricans* is discussed.

METHODS

Analytical methods. Quantitative analyses of carbohydrate, protein and phosphate were performed by the methods of Dubois *et al.* (1956), Lowry *et al.* (1951) and of Fiske & Subbarow (1925), respectively, using mannose, bovine serum albumin and KH_2PO_4 as the standards.

For paper and thin-layer chromatography (t.l.c.) the following solvent systems were used: A, ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.); B, 1-butanol/ethanol/water (3:1:1, by vol.). Carbohydrates on paper and thin-layer chromatograms were detected with the alkaline silver nitrate reagent (Trevelyan *et al.*, 1950) or the periodate-benzidine reagent (Cifonelli & Smith, 1954). (The use of benzidine in the UK is prohibited under the Carcinogenic Substances Regulations, 1968). Gas-liquid chromatography (g.l.c.) was performed using a Shimadzu GC-6A chromatograph (Shimadzu, Tokyo, Japan) as described previously (Hayashi *et al.*, 1978*a*). A glass column packed with 3 % (w/w) ECNSS-M on Chromosorb W (AW-DMCS, 60 to 80 mesh) was used for identification of the partially methylated alditol acetates. G.l.c.-mass spectra were recorded with a Shimadzu model LKB-9000 mass spectrometer equipped with a glass

column packed with 3 % (w/w) silicone OV-225 on Chromosorb W (AW-DMCS, 80 to 100 mesh) at 170 °C and operated at an ionization voltage of 70 eV, a trap current of 60 μ A and an ion source at 310 °C.

Infrared spectra were recorded with a Hitachi-215 spectrometer.

Preparation of ACI-B, MhI-B and RnI-B. The serologically active fractions, ACI-B, MhI-B and RnI-B, were prepared from the mycelia of *A. cylindrospora* IFO 4000, *M. hiemalis* IFO 5303 and *R. nigricans* IAM 6070, respectively, as described previously (Hayashi *et al.*, 1978*b*) using affinity chromatography on a concanavalin A-Sepharose column.

Pronase digestion of crude ACE. Crude ACE (10 g), i.e. the non-dialysable fraction of the culture filtrate of *A. cylindrospora*, was dissolved in 150 ml 0.05 M-Tris/HCl buffer (pH 7.6) containing 0.01 M-CaCl₂, and mixed with 25 ml pronase (Kaken Kagaku, Japan) solution (2 mg ml⁻¹ in the same buffer). The mixture was passed through a sterilized Millipore filter (0.45 μ m pore size) and the filtrate was incubated at 37 °C for 48 h. After heating in a boiling water bath for 10 min, the reaction mixture was dialysed in Visking tubing against distilled water at 4 °C for 2 d and then the non-diffusible fraction was lyophilized. The product (pt-ACE-II) was dissolved in a small volume of distilled water and passed through a column of Sephadex G-200 (5 $\times$ 100 cm) to remove the enzyme.

Partial acid hydrolysis of pt-ACE-II and gel filtration of hydrolysates on Bio Gel P-2. Pronase-treated ACE main fraction (pt-ACE-II) was dissolved in 0.05 M-H₂SO₄ at a concentration of 1 % (w/v) and the mixture was heated in a boiling water bath for 4 h. After cooling, the reaction mixture was neutralized with BaCO₃ and the precipitate was filtered off. The filtrate was passed through a Dowex 50 (H⁺) column and the eluate was concentrated to a syrup. To obtain the oligosaccharide fraction, the syrup was added to a 2.5 $\times$ 140 cm column of Bio Gel P-2 (200 to 400 mesh, Bio-Rad) and eluted with distilled water.

Purification of the oligosaccharides by descending paper chromatography. The oligosaccharide fraction from gel filtration on Bio Gel P-2 was purified by descending paper chromatography on Whatman 3MM paper. The papers were washed with distilled water for 1 d and then air-dried before use. Between 30 and 50 mg of the oligosaccharide fraction were chromatographed in solvent system A for 30 to 80 h. Each separated oligosaccharide on the paper chromatograms was extracted with distilled water. Each extract was passed through a Dowex 50 (H⁺) column to remove traces of ionic contaminants and then lyophilized.

Chemical analysis of oligosaccharides. Sugar components of the oligosaccharides were determined by t.l.c. after hydrolysis in 0.5 M-H₂SO₄ at 100 °C for 8 h. G.l.c. analysis was performed on the alditol acetate derivatives of the sugars in the hydrolysate as described previously (Hayashi *et al.*, 1978*a*).

The R_{mannose} value for each purified oligosaccharide was determined by descending paper chromatography using Toyo Roshi no. 50 paper and solvent systems A and B.

Optical rotations of the oligosaccharides were determined in aqueous solutions with a JASCO automatic polarimeter model DIP-SL (Japan Spectroscopic Co., Japan).

The degree of polymerization was determined by the Somogyi-Nelson method (Somogyi, 1952), by reduction with NaBH₄ (McLean *et al.*, 1973) and by g.l.c. of the methylated products (Miyazaki & Naoi, 1974).

For methylation analysis, the oligosaccharides were fully methylated by the method of Hakomori (1964). Completely methylated oligosaccharides which showed no significant absorption band of OH in the 3500 cm⁻¹ region in infrared spectra were extracted with chloroform from the reaction mixture. The permethylated oligosaccharides were hydrolysed with 90 % (w/w) HCOOH at 100 °C for 10 h and then with 0.5 M-H₂SO₄ at 100 °C for 5 h. The partially methylated sugars were converted to their corresponding alditol acetates and analysed by g.l.c.-mass spectrometry and g.l.c. as described previously (Miyazaki & Naoi, 1974).

Immunological procedures. Anti-ACE serum was prepared by immunizing a rabbit with the non-diffusible fraction of the culture filtrate of *A. cylindrospora* and immunodiffusion analysis was carried out as described previously (Hayashi *et al.*, 1978*a*).

Quantitative precipitation inhibition tests were carried out as follows: twofold diluted anti-ACE serum (0.2 ml) and the inhibitor solution (0.1 ml) were mixed in conical tubes and incubated at 37 °C for 1 h. Saline solutions (0.1 ml) of the antigens were added, and the reaction mixtures were incubated at 37 °C for 1 h, then at 4 °C for 40 h. The subsequent procedure followed that used in the quantitative precipitation test (Hayashi *et al.*, 1978*a*). The percentage inhibition at various concentrations of each inhibitor was calculated.

RESULTS

Chemical analysis of ACI-B, MhI-B and RnI-B

Chemical analyses of the serologically active fractions, ACI-B, MhI-B and RnI-B, are shown in Table 1.

Preparation of oligosaccharides from extracellular polysaccharide of A. cylindrospora

A carbohydrate-rich fraction, pt-ACE-II, isolated by Sephadex G-200 gel filtration of pronase-treated ACE, was recovered by lyophilization after dialysis. It constituted about 40 % (w/w) of ACE. Immunodiffusion of pt-ACE-II against anti-ACE serum gave a single precipitin band which was completely fused with that of fucomannan-peptide, the purified serologically active substance of *A. cylindrospora* (Hayashi *et al.*, 1978*a, b*). Chromatography of the partial acid hydrolysate of pt-ACE-II indicated the presence of fucose, a trace of mannose and six oligosaccharides (oligo-1 to oligo-6). An acid-resistant fraction, corresponding to 41.5 % of pt-ACE-II, was obtained in the void volume of the gel filtration on the Bio Gel P-2 column. All six oligosaccharides were homogeneous in descending paper chromatography with solvent systems A and B. No oligosaccharides containing both fucose and mannose were detected under the above conditions.

Chemical properties of the oligosaccharides

All six purified oligosaccharides were composed of mannose alone. Their carbohydrate contents and chromatographic properties are shown in Table 2. Elementary analysis revealed that these oligosaccharides did not contain nitrogen. Determinations of their degree of polymerization (see Methods) showed that they contained between three and eight mannose residues.

The dextrorotatory values of specific rotation suggested that these oligosaccharides contained only α linkages. In the methylation analysis, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-

Table 1. *Chemical analysis of ACI-B, MhI-B and RnI-B*

	Neutral sugar content (%, w/w)	Molar ratio of fucose and mannose†	Protein content (%, w/w)	Phosphate content (%, w/w)	Total (%, w/w)
ACI-B*	63.0	1:2.5	22.7	2.3	88.0
MhI-B	58.7	1:2.9	7.1	0.7	66.5
RnI-B	55.7	1:25.7	19.7	0.4	75.8

* The results for ACI-B are from Hayashi *et al.* (1978*a*).

† Traces of galactose, hexosamine and uronic acid were detected in all fractions. The molar ratio of fucose and mannose was calculated from the results of g.l.c.

Table 2. *Chemical properties of the purified manno-oligosaccharides*

	Sugar content* (%, w/w)	R_{mannose}	$[\alpha]_D^{20}$ (in water)	Degree of polymerization		
				Somogyi	NaB ³ H ₄	Glc†
Oligo-1	97.9	0.39	+64.7	3.04	ND	2.8
Oligo-2	91.2	0.24	+70.8	3.83	ND	3.6
Oligo-3	95.9	0.14	+71.0	5.02	ND	4.5
Oligo-4	95.9	0.09	+71.8	5.88	6.38	ND
Oligo-5	98.2	0.06	+81.1	6.38	7.13	ND
Oligo-6	96.6	0.03	+81.2	7.84	8.60	7.5

ND, Not determined.

* Calculated as anhydrous mannose.

† Calculated from the molar ratios of tetra- and tri-*O*-methyl alditol acetates derived from the methylated oligosaccharides.

Fig. 1. Inhibition by oligosaccharides of the precipitation reaction between ACI-B and anti-ACE serum. The reaction mixture contained 200 μ l anti-ACE serum (diluted twofold), 150 μ g ACI-B and 200 μ l inhibitor, in a total volume of 500 μ l: $\circ$, oligo-1; $\triangle$, oligo-2; $\square$, oligo-3; $\bullet$, oligo-4; $\blacktriangle$, oligo-5; $\blacksquare$, oligo-6. The maximum dose for the tests with oligo-4, -5 and -6 was 4 μ mol because the oligosaccharides were only available in small quantities.

mannitol (m/e 43, 45, 71, 87, 101, 117, 145, 161 and 205) and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol (m/e 43, 87, 99, 101, 117, 129, 161 and 189) were detected. The m/e values in the mass spectra of both alditol acetates agreed well with those in the literature (Björndal *et al.*, 1967). These results showed that the six oligosaccharides were all (1 $\rightarrow$ 6)- α -linked manno-oligosaccharides.

Precipitation inhibition tests

In quantitative precipitation tests on ACI-B, MhI-B and RnI-B using anti-ACE serum, maximum precipitates were obtained with 150 μ g ACI-B, 150 μ g RnI-B and 300 μ g MhI-B. The six oligosaccharides inhibited the precipitation reaction between anti-ACE serum and ACI-B to different extents (Fig. 1). The precipitation reaction was significantly inhibited by oligo-3, -4, -5 and -6, but only slightly inhibited by oligo-1 (trisaccharide) and oligo-2 (tetrasaccharide). Mannose and glucose were not inhibitory. The amounts of hexa-, hepta- and octasaccharide required for 50 % inhibition were approximately 2.5 μ mol and that of pentasaccharide was 4.5 μ mol. These oligosaccharides also significantly inhibited the precipitation reactions of MhI-B and RnI-B with anti-ACE serum (Table 3).

DISCUSSION

Immunochemical analysis of MhI-B and RnI-B by immunodiffusion and their quantitative precipitation reactions against anti-ACE serum showed that the serological reactivities of MhI-B and RnI-B were similar to that of ACI-B (Hayashi *et al.*, 1978*b*). The serologically active site of the fucomannan-peptide isolated from ACI-B might be the carbohydrate moiety rather than the peptide moiety, since periodate oxidation of ACI-B destroyed its reactivity with anti-ACE serum whereas pronase/trypsin digestion did not (Hayashi *et al.*, 1978*a*). The experiment described here indicated that (1 $\rightarrow$ 6)- α -linked linear oligosaccharides of mannose made up the immunological determinant. Peat *et al.* (1961) reported that manno-oligosaccharides containing only (1 $\rightarrow$ 6)- α linkages were obtained from the mannan of *S. cerevisiae* by partial acid hydrolysis, and the optical rotations of the oligomers, tri-, tetra- and pentasaccharides, were +68°, +70° and +67.5° (in water), respectively. In our experiment, the optical rotations of (1 $\rightarrow$ 6)-linked manno-tri-, tetra- and pentasaccharides were +64.7°, +70.8° and +71.0°, respectively. Regularity in the chromatographic mobilities of the six oligosaccharides supports their homogeneity (French & Wild, 1953).

Table 3. *Inhibition of the precipitation reactions of ACI-B, MhI-B and RnI-B with anti-ACE serum by the oligosaccharides*

Reaction mixtures contained 2 μ mol oligosaccharide, twofold diluted anti-ACE serum and 150 μ g ACI-B or 150 μ g RnI-B or 300 μ g MhI-B.

Inhibitor	Inhibition (%)		
	ACI-B	MhI-B	RnI-B
Oligo-1	7.04	15.2	15.0
Oligo-2	8.9	12.6	10.2
Oligo-3	27.0	33.0	22.1
Oligo-4	46.0	67.0	62.7
Oligo-5	46.3	79.3	70.1
Oligo-6	46.9	63.8	59.4

The precipitation reaction of ACI-B with anti-ACE serum was significantly inhibited by the pentasaccharide derived from pt-ACE-II. The higher oligomers, hexa-, hepta- and octasaccharides, showed stronger inhibitory activity. These oligosaccharides also inhibited the reaction of MhI-B and RnI-B with anti-ACE serum; it is not clear why the reaction with ACI-B was inhibited to a lesser extent than the others, but is presumably due to structural differences between the complete antigens. Our observations indicate that the (1 $\rightarrow$ 6)- α -linked mannopyranosyl residues might play an important role as the common determinant of the serologically active substances of *A. cylindrospora*, *M. hiemalis* and *R. nigricans*. Previously, Miyazaki (1962) reported that the mannan isolated from *Candida albicans* had mainly (1 $\rightarrow$ 2)- α and (1 $\rightarrow$ 6)- α linkages, and Suzuki & Sunayama (1968) reported that (1 $\rightarrow$ 6)- α -linked manno-oligosaccharides (di-, tri- and tetrasaccharide) prepared from the mannan of *S. cerevisiae* were not inhibitory against the antigen-antibody system of *C. albicans*. Mitchell & Hansenclever (1970) also suggested that (1 $\rightarrow$ 2)- α and (1 $\rightarrow$ 3)- α linkages in the mannan of *Candida stellatoidea* were important factors in the antigen-antibody interaction but that (1 $\rightarrow$ 6)- α linkages were not significant. In the mannan of *S. cerevisiae*, (1 $\rightarrow$ 6)- α linkages participated much less than (1 $\rightarrow$ 2)- α linkages in the combining site of the antibody (Suzuki & Sunayama, 1968). The failure of anti-ACE serum to react with mannans of *C. albicans* and *S. cerevisiae* (Hayashi *et al.*, 1978*b*) might therefore be reasonable.

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