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Characterization of Extracellular Mannoheteroglycans from *Absidia cylindrospora*

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The extracellular polysaccharide fraction of *Absidia cylindrospora* (ACE), which has been used as an immunogen in immunochemical studies of *Mucorales* polysaccharide, showed marked heterogeneity of its mannoheteroglycans. ACE was classified into mannan-type and fucomannan-type fractions of sequential separation using affinity chromatography on concanavalin-A (con-A) Sepharose, ion exchange chromatography on diethylaminoethyl Sephadex, zone electrophoresis and gel filtration on Sephadex G-100. Serological reactivity to anti-ACE appeared in the con-A bound and high-mannose content fraction. By methylation analysis and proton magnetic resonance studies of each polysaccharide, it was confirmed that the fucomannan-type fraction mainly consisted of $\alpha(1\rightarrow6)$ linked mannopyranosyl residues and fucopyranosyl non-reducing ends, whereas the mannan-type fraction consisted of $(1\rightarrow2)$ linked mannopyranosyl residues and mannopyranosyl non-reducing ends. These results were similar to those for mannoheteroglycans from *A. cylindrospora* mycelium. These results indicated that ACE of the immunogen contained serologically active mannan and fucomannan.

Keywords——*Absidia cylindrospora*; *Mucorales*; mannoheteroglycan; characterization of extracellular polysaccharide; fucomannan; serologically active mannan

Previously, we have described the isolation, purification and characterization of the serologically active mannose-containing polymer of *Absidia cylindrospora* mycelium, a member of *Mucorales*.[2–5] Rabbit antiserum (anti-ACE) against the extracellular non-dialyzable fraction of *A. cylindrospora* reacted with antigenic fractions from other *Mucorales* fungi, but the antiserum did not cross-react with the mannans of *Candida albicans* and *Saccharomyces cerevisiae*.[6] The antigen fraction from *A. cylindrospora* cells contained heterogeneous mannoheteroglycans, and these polymers were classified into mannoproteins (minor fraction) and fucomannopeptide (major fraction).[5] Serological activities were shown by mannoproteins and some fucomannopeptide of high mannose content, but fucoman-nopeptide of low mannose content showed negligible antibody-precipitating activity.[5] We used anti-ACE serum to test the antigenic activities of these polysaccharides, because anti-sera against cell wall or whole cells of *A. cylindrospora* were of lower titer than anti-ACE against the antigen from *A. cylindrospora* mycelium.[3,4] When *A. cylindrospora* mycelium was treated with fluorescein-conjugated anti-ACE, fluorescein stained the mycelial surface.[7] This result indicated that ACE contained an antigen with the same immunodeterminant as cell surface antigen. The mannoheteroglycans from *A. cylindrospora* mycelium were charaterized in a previous paper[5] but the extracellular mannoheteroglycan has not yet been fully characterized.

In this work, we characterized the extracellular mannoheteroglycans as an immunogen in order to compare them with the mannoheteroglycans from *A. cylindrospora* mycelium and to examine the serological specificity of anti-ACE.

Materials and Methods

Materials——*A. cylindrospora* IFO 4000 was obtained from the Institute of Fermentation, Osaka, Japan. The cells were cultured in modified Sabouraud's liquid medium at 27°C until the stationary phase.[2] Extracellular polysaccharide fraction (ACE) from *A. cylindrospora* was prepared as lyophilizate of the non-

dialyzable culture filtrate.[2] Concanavalin-A (con-A) Sepharose, DEAE-Sephadex A-50, and Sephadex G-100 were obtained from Pharmacia Co. Ltd. Pevikon (polyvinyl resin) was purchased from M and S Instrument, Osaka, Japan.

Fraction of Mannoheteroglycans from Non-dialyzable Extracellular Polysaccharide Fraction of *A. cylindrospora* ——The non-dialyzable extracellular polysaccharide fraction (ACE; 617 mg as carbohydrate) was dissolved in water (25 ml) and treated with an aqueous solution (2 g/25 ml) of cetyltrimethyl ammonium bromide (cetavlon). After standing at 20°C for 20 h, the precipitate was collected by centrifugation and redissolved in 10% NaCl. This solution was dialyzed against water for 3 d. The non-dialyzable fraction was obtained as the lyophilizate (ACE-I; 47 mg as carbohydrate). The supernatant was added to 25 ml of 1% boric acid. The solution was stirred and the pH was adjusted to 8.8 by the addition of 2 N NaOH. However, the solution did not precipitate significantly even after 12 h. Acetic acid was added to the supernatant, followed by potassium acetate and 3 volumes of ethanol. The precipitate was washed with acetone and dried with ethyl ether, yielding ACE-II (470 mg as carbohydrate). ACE-I and -II were fractionated by sequential chromatographic and electrophoretic techniques as shown in Fig. 1.

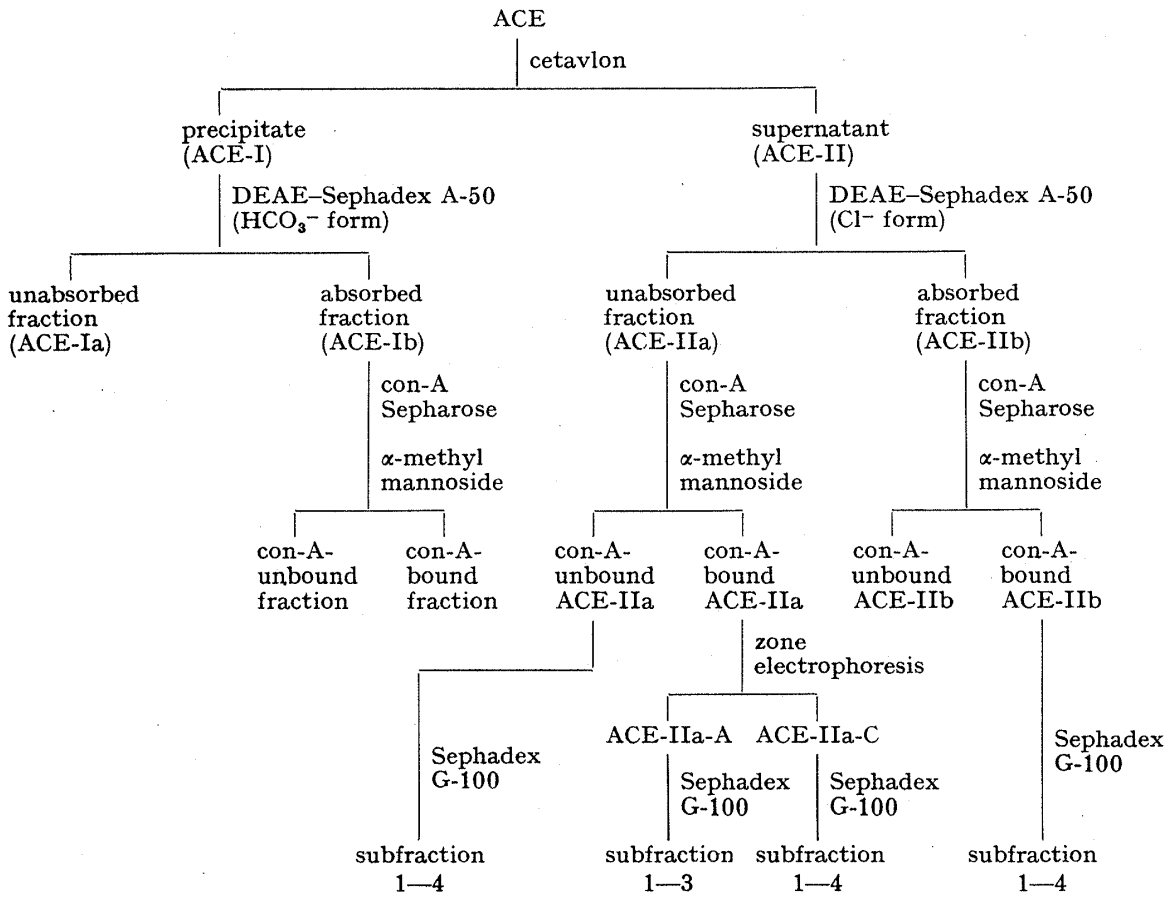

Fig. 1. Flow Diagram of the Fractionation of ACE

Con-A Sepharose Chromatography——ACE-Ib, ACE-IIa and ACE-IIb were fractionated to con-A-unbound and bound fractions by affinity chromatography on con-A Sepharose, by the published procedure.[4-6] Each sample was dissolved in 0.1 M NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$ containing 50 mM phosphate buffer, pH 6.9. The solutions of ACE-Ib (16 mg), ACE-IIa (250 mg) and ACE-IIb (120 mg) were each applied to the column (1.5 × 40 cm) and unbound components were eluted with the same buffer. Con-A-bound components were then specifically eluted with 50 ml of 0.5 M α-methyl mannoside solution in 0.1 M NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$ containing 50 mM phosphate buffer, pH 6.9, and con-A bound ACE-Ib (1.4 mg), ACE-IIa (82 mg) and ACE-IIb (31 mg) were obtained. Each con-A-unbound fraction was reapplied to the con-A Sepharose column, but no bound component was obtained even when the column was washed with 0.1 M borate buffer, pH 6.5.

Analytical Procedure——The total carbohydrate and fucose contents were determined by the phenol–sulfuric acid[8] and the cysteine–sulfuric acid,[9] respectively, using mannose and fucose as standards. Protein was assayed by the method of Lowry *et al.*[10] Phosphorus content was measured as described by Chen *et al.*[11]

Chromatography——Thin layer chromatography was done on Merck cellulose-coated plastic sheet (layer thickness; 0.1 mm) in a solvent system of ethyl acetate/pyridine/acetic acid/water (5: 5: 1: 3, v/v). Reducing sugars were detected with alkaline silver nitrate,[12] amino sugar and amino acid with ninhydrin,[13] and uronic acid with *p*-anisidine hydrochloride.[14] Ascending paper chromatography on Toyo filter paper No. 50 was done with the same solvent system. The acid hydrolyzates of polysaccharides (500 μg) obtained by 1 M trifluoroacetic acid treatment (at 105°C for 4 h) were reduced with $NaBH_4$ for 5 h at 23°C, and the reaction was stopped by neutralization. After the borate ions had been removed by co-distillation with methanol, the reduced material was acetylated with pyridine/acetic anhydride (1: 1, v/v) at 100°C for 1 h. Gas – liquid chromatography (GLC) was performed at 180—250°C on a Shimadzu GC-6A instrument equipped with a flame ionization detector and a glass column (0.3×200 cm) of 3% OV-225 on Gas Chrom Q, with a nitrogen flow rate of 50 ml/min.

Methylation Analysis——Methylation of the mannoheteroglycans was performed by Hakomori's procedure,[15] and complete methylation of the polysaccharides was checked by the use of triphenylmethane as an indicator of the remaining carbanion.[16] Acid hydrolysates of fully methylated polysaccharides were converted into alditol acetates.[17] Combined gas chromatography–mass spectrometry was performed on a Shimadzu LKB-9000 machine, equipped with a glass column packed with 3% Silicone OV-225 on Chromosorb W, operated at 170°C; electron energy 70 eV; trap current 60 μA; temperature of the ion source, 310 °C.

Nuclear Magnetic Resonance Spectroscopy——Proton magnetic resonance (PMR) spectra of polysaccharides were obtained at 100 MHz on an instrument equipped with a JEOL computer operated in the Fourier transform mode. Spectra were obtained at a probe temperature of 80°C. Chemical shifts were expressed relative to sodium (trimethylsilan)-1-propanesulfonate (TSP). The polysaccharides were dissolved in D_2O as a 0.5% solution.

Immunological Method——Anti-ACE was prepared by immunizing rabbits with ACE by the published procedure.[2] Antibody precipitating activity was detected by the agar gel double diffusion method.[18]

Results

Purification of Mannoheteroglycan Antigens from ACE-I and II

When ACE-I was applied on a column of DEAE-Sephadex A-50 (Cl^- form), all the carbohydrate-containing fractions were recovered in the unbound fraction. Then ACE-I was fractionated to obtain an unabsorbed fraction (ACE-Ia) and an absorbed fraction (ACE-Ib) by column chromatography on DEAE-Sephadex A-50 (HCO_3^- form) as shown in Fig. 2a. Only the absorbed fraction reacted with anti-ACE. On the other hand, ACE-II was fraction-

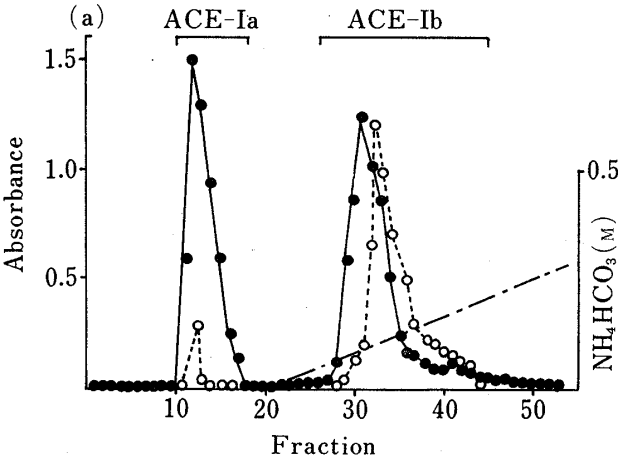

Fig. 2a. Elution Profile of ACE-I from a DEAE-Sephadex A-50 (HCO_3^- form) Column

ACE-I was dissolved in distilled water. Linear gradient elution was carried out with 0—1 M NH_4HCO_3 (– – –). Column size (2×25 cm). Carbohydrate, 490 nm (—●—); protein, 280 nm (···○···).

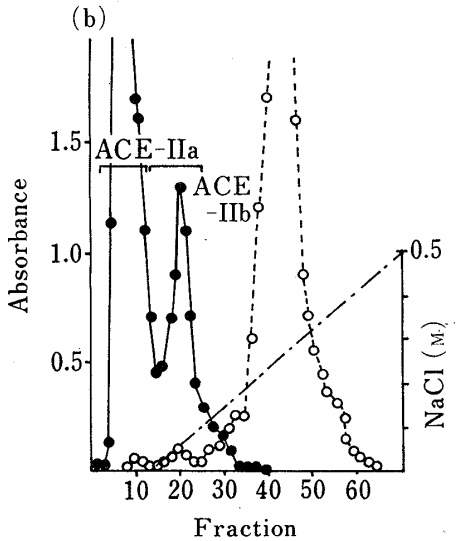

Fig. 2b. DEAE Sephadex A-50 (Cl^- form) Chromatography of ACE-II

ACE-II was dissolved in 50 mM potassium phosphate buffer, pH 6.8. Elution of the unabsorbed fraction was carried out with this buffer,and the absorbed fraction was eluted with a linear gradient of 0—1 M NaCl (– – –) in the same buffer. Column size (2×26 cm). Carbohydrate, 490 nm (—●—); 280 nm (···○···)

ated by column chromatography on DEAE-Sephadex A-50 (Cl^- form), and the unabsorbed (ACE-IIa) and absorbed ACE-IIb fractions were obtained as serologically active carbohydrate-containing substances (Fig. 2b). ACE-Ib and ACE-IIa and -IIb were subjected to affinity chromatography on con-A Sepharose, and all of the active substances were recovered in the bound fraction. The purification of con-A-bound ACE-Ib was not carried further because of the small quantity available. When paper electrophoresis of con-A-bound ACE-IIa was carried out in 0.026 M sodium borate buffer (pH 9.2), two periodate-Schiff reagent positive spots were obtained, but con-A-bound ACE-IIb showed a single spot. The con-A-bound ACE-IIa was further separated into minor fraction (ACE-IIa-A) and a major fraction (ACE-IIa-C) by preparative zone electrophoresis using Pevikon as the supporting medium, as shown

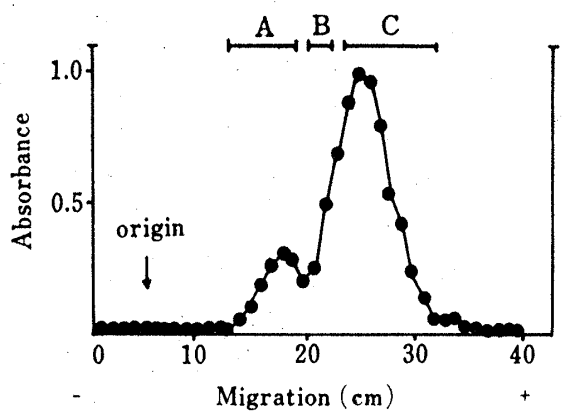

Fig. 3. Zone Electrophoresis of Con-A-bound ACE-IIa

Pevikon (the supporting medium) was equilibrated with 0.026 M sodium borate buffer (pH 9.2). A block (1.5×2.5×40 cm) was prepared and the sample in the same buffer was placed 5 cm from the cathode and allowed to migrate at a constant current of 1 mA/cm² for 15 h at 5°C. After migration, the block was divided into 1 cm portions and each segment was extracted with distilled water (15 ml). The carbohydrate (—●—) contents were determined by the phenol-sulfuric acid method.[8]

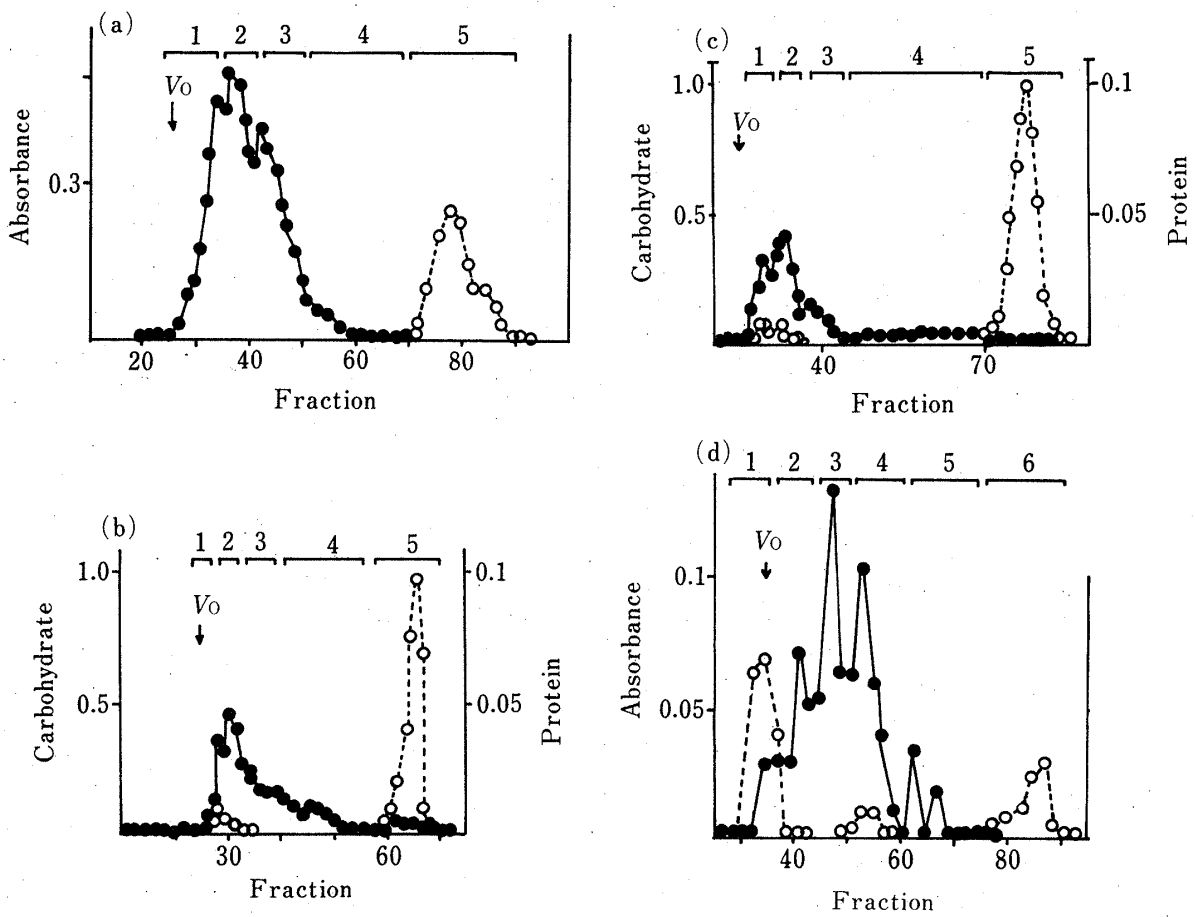

Fig. 4. Sephadex G-100 Column Chromatography of Con-A-unbound Fraction from ACE-IIa [from Fig. 2b (a)], Con-A-bound Fraction from ACE-IIa-A [from Fig. 3 (b)], Con-A-bound Fraction from ACE-IIa-C [from Fig. 3 (c)] and Con-A-bound Fraction from ACE-IIb [from Fig. 2b(d)].

The Sephadex G-100 column (1.9×100 cm for a, b and c, 1.9×110 cm for d) was equilibrated with 0.1 M NaCl and elution was performed with this salt solution. Carbohydrate, 490 nm (—●—); protein, 280 nm (···○···).

Table I. Chemical Analysis of ACE Mannoheteroglycans

Subfraction	Component sugar	Sugar content(%)[a]	Protein content(%)[b]	Phosphorus content(%)[c]	Mannose/ fucose[d]	Reactivity to anti-ACE[e]
Con-A-unbound	Fuc, Man					
ACE-IIa-1	(Gal, GlcN)	85.8	1.0	nd	2.1	—
ACE-IIa-2	Fuc, Man					
	(Gal, GlcN)	85.5	Trace	nd	1.9	—
ACE-IIa-3	Fuc, Man					
	(Gal, GlcN)	90.2	Trace	nd	2.1	—
ACE-IIa-4	Fuc, Man					
	(Gal, GlcN)	74.2	2.6	nd	1.8	—
Con-A-bound						
ACE-IIa-A-1	Man (Fuc, Gal)	52.1	44.6	nd	8.4	+
ACE-IIa-A-2	Man (Fuc, Gal)	56.3	40.6	nd	21.7	+
ACE-IIa-A-3	Man (Fuc, Gal)	48.6	46.0	nd	53.0	+
Con-A-bound						
ACE-IIa-C-1	Fuc, Man					
	(Gal, GlcN)	84.0	2.2	nd	2.3	+
ACE-IIa-C-2	Fuc, Man					
	(Gal, GlcN)	83.1	1.9	nd	2.2	+
ACE-IIa-C-3	Fuc, Man					
	(Gal, GlcN)	78.5	2.8	nd	2.5	+
ACE-IIa-C-4	Fuc, Man					
	(Gal, GlcN)	76.5	5.6	nd	2.3	+
Con-A-bound						
ACE-IIb-1	Fuc, Man					
	(Gal, GlcN)	77.1	6.0	—	2.8	+
ACE-IIb-2	Fuc, Man					
	(Gal, GlcN)	73.2	2.0	0.1	2.5	+
ACE-IIb-3	Fuc, Man					
	(Gal, GlcN)	81.1	3.1	0.1	3.7	+
ACE-IIb-4	Fuc, Man					
	(Gal, GlcN)	74.1	10.7	0.1	3.3	+
Con-A-bound						
ACE-Ib	Fuc, Man	63.2	2.3	1.8	1.7	+

a) Total carbohydrate content was measured by the reported method.[6]
b) Determined by the Folin method of Lowry *et al.*[10]
c) Determined by the method of Chen *et al.*[11]
d) Values were determined by gas-liquid chromatography (GLC) of acid hydrolyzates.
e) Reactivities to anti-ACE serum were detected by the agar gel double diffusion method.[18]
−; no precipitin line was observed. +; precipitin line was observed.
Parenthesis indicate trace components. nd; not detected.
Sugar, protein and phosphorus contents are calculated percentages per weight of sample.

in Fig. 3. Both fractions reacted with anti-ACE serum. Furthermore, con-A-unbound ACE-IIa and con-A-bound-ACE-IIa-A, -ACE-IIa-C and -ACE-IIb were fractionated by gel filtration on Sephadex G-100 as shown in Fig. 4. Component sugars and their molar ratios, contents of protein, carbohydrate and phosphorus, and reactivity to anti-ACE of these subfractions are summarized in Table I. Con-A-bound ACE-IIa-A was characterized as a high-mannose type polymer, whereas other mannoheteroglycans were classified as fucomannan-type polymers. The listed constituents of fucomannan-type polymer account for about 80—90% of the total. The nature of the remaining 5—15% has not yet been elucidated, but may include hexosamine, acetate, and/or trace contaminants.

Proton Magnetic Resonance Spectroscopy of Con-A-bound ACE-IIa-C and IIb-3

Con-A-bound ACE-IIa-C and ACE-IIb-3 showed PMR spectra similar to those of fucomannopeptide of *A. cylindrospora* mycelium (Fig. 5). The signal at 4.96 ppm was represented as the anomeric proton of $\alpha(1\rightarrow6)$-linked mannopyranosyl residues, like those observed in

α(1→6)-linked mannan.[19] PMR spectra of these polysaccharides showed the signals characteristic of C-methyl and O-acetyl groups at 1.3 ppm and 2.2 ppm, and the formar signal was assigned to the methyl group of fucose. The signal at 5.14 ppm in these polysaccharides may be due to the anomeric proton of α-linked fucopyranosyl residues on the basis of previous results.[19] The only difference between the PMR spectra of ACE-IIa-C and ACE-IIb-3 was in the intensity of the signal at 2.1 ppm, which may be due to *N*-acetyl groups, because this signal was unaffected by mild alkaline treatment. The PMR spectrum of con-A-unbound ACE showed the presence of equal amounts of C-methyl and O-acetyl protons (data not shown). The PMR spectrum of con-A-bound ACE-IIa-A was not taken because an insufficient quantity was available.

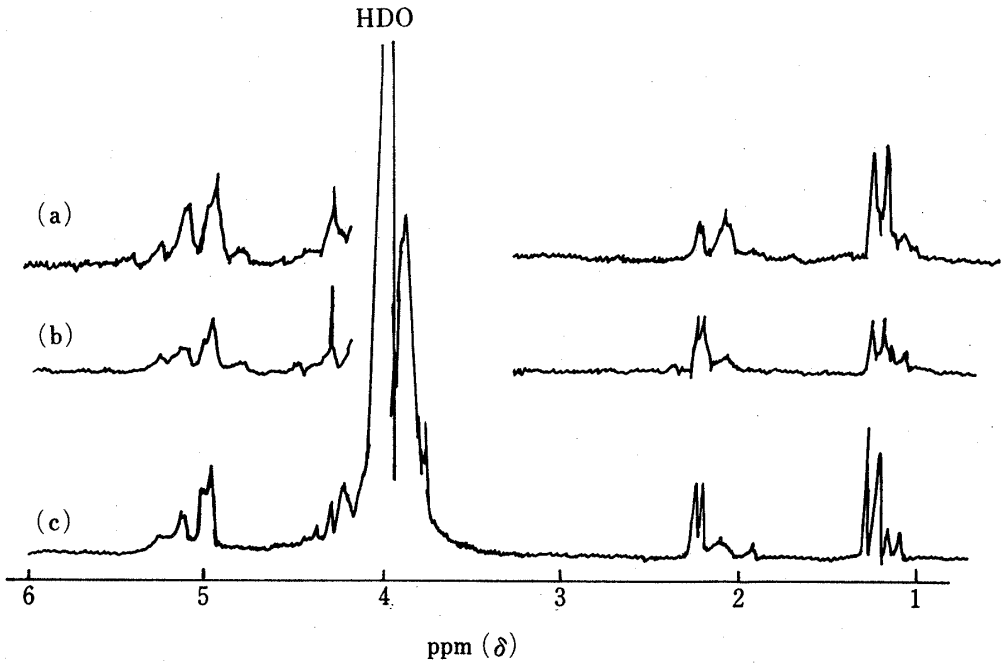

Fig. 5. PMR Spectra of Con-A-bound ACE-IIa-C (a), Con-A-bound ACE-IIb-3 (b) and Fucomannopeptide of *A. cylindrospora* Mycelium (c)

HDO is the water peak.

Methylation Analysis of Mannoheteroglycan Antigens

Major subfractions of each mannoheteroglycan fractionated by gel filtration on Sephadex G-100 were methylated and then converted into alditol acetates. Each partially methylated alditol acetate was identified from its retention time on gas–liquid chromatography and its fragmentation patterm in a mass spectrometer (Table II). The predominant peaks of the derivatives of con-A-bound ACE-IIa-A-2 (mannan-type) were assigned to 2,3,4,6-tetra-*O*- and 3,4,6-tri-*O*-methyl mannitol derivatives, respectively. On the other hand, con-A bound

Table II. Methylation Analysis of the Main Subfractions of ACE Mannoheteroglycan Antigens

Methylated mannoheteroglycans	Methylated alditol acetate derivatives (molar ratios)[a]				
	2,3,4 Fuc	2,3,4,6 Man	3,4,6 Man	2,3,4 Man	2,4 Man
1) Con-A-bound ACE-IIa-C-2 (fucomannan-type)	3.6	1.0	0.72	8.6	3.6
2) Con-A-bound ACE-IIb 2 plus 3 (fucomannan-type)	1.7	1.0	0.31	4.4	3.8
3) Con-A-bound ACE-IIa-A-2 (mannan-type)	—	1.0	1.2	—	—

a) Calculated from peak areas and molecular weights of derivatives. Fuc=fucose, Man=mannose.

ACE-IIa-C-2 and con-A-bound ACE-IIb-2 plus -3 (fucomannan-type) showed predominant peaks due to 2,3,4-tri-*O*-methyl fucitol, 2,3,4,6-tetra-*O*-methyl mannitol, 2,3,4,-tri-*O*-methyl mannitol and 2,4-di-*O*-methyl mannitol. Thus, the fucomannan-type polysaccharide contained fucopyranosyl residues and mannopyranosyl residues at nonreducing ends, and 6-*O*-, 3,6-di-*O*-substituted and a trace of 2-*O*-substituted mannopyranosyl units in the chains. Mannan-type polysaccharide contained mannopyranosyl residues at the non-reducing terminal and 2-*O*-substituted mannopyranosyl units in the chains.

Discussion

Extracellular mannoheteroglycans from *A. cylindrospora* (ACE) showed high heterogeneity and were classified into mannan-type and fucomannan-type fractions like the mycleium mannoheteroglycans from *A. cylindrospora*.[5] When the subfraction of ACE was tested for reactivity to anti-ACE serum, only the con-A-bound fraction showed antibody precipitating activity. ACE-Ib precipitated in the presence of cetavlon. ACE-IIb and ACE-IIa-C were classified as fucomannan -type fractions, but showed different acidity. Con-A-bound ACE-IIa was separated into a small amount of mannan and a large amount of fucomannan by zone electrophoresis. The result was similar to that of the separation of the mannoheteroglycans from *A. cylindrospora* mycelium. Mannan from ACE consisted of almost equal proportions of mannopyranosyl non-reducing ends and (1→2) linked mannopyranosyl residues, and this ACE-mannan was suggested to be a linear structure. However, it is suggested that chemical structure of fucomannan from ACE is composed of linear α(1→6) linked mannan with non-reducing terminal fucopyranosyl residues or mannopyranosyl residues at C-3 on a part of the mannopyranosyl main chain. These structures were very similar to the carbohydrate structures of mannoprotein and fucomannopetide from *A. cylindrospora* mycelium.[19] Mannoprotein from mycelium contained a large amount of *O*-glycoside sugars, because α(1→2)-linked oligosaccharide was released from the polymer by β elimination.[19] Therefore, it is suggested that ACE mannan may be an α(1→2)-linked mannobiose-substituted *O*-glycosyl protein. Previously, we observed that fucomannan type polymer from *A. cylindrospora* mycelium showed high heterogeneity due to the presence of fucomannan with different degrees of fucosylation.[5] It contained serologically active and weakly active or inactive fucomannan, and the latter was fucosylated at C-3 of every other α(1→6) mannan main chain.[5,19] However, it was suggested that serologically active fucomannan contained a more exposed α(1→6) mannan main chain than the inactive one because of the low fucosylation.[19] We have used anti-ACE serum prepared by the immunization of rabbits with ACE in immunochemical studies of mannoheteroglycans from *A. cylindrospora*.[3,4] The precipitin reaction of anti-ACE with the mannoheteroglycan antigen from *A. cylindrospora* mycelium was 55—70% inhibited by the addition of α(1→6)-linked mannopentaose or higher molecular weight α(1→6)-linked mannoligosaccharides prepared by partial acid hydrolysis of Pronase-digested ACE.[20] It was expected that a part of the immunodeterminant of *A. cylindrospora* antigen might be α(1→6)-linked mannopyranosyl oligosaccharide. In the previous studies, we found that the mannoheteroglycan from *A. cylindrospora* mycelium contained mannoprotein antigen and fucomannan antigens.[5] The present results also suggested that ACE as an immunogen contained two kinds of antigenic determinant which may be due to mannan and fucomannan of high mannose content. Both ACE and mannoheteroglycan antigen (mixture of mannoprotein and fucomannopeptide) from mycelium cross-reacted with each other against anti-ACE,[3,4] and fucomannopeptide and α(1→6)-linked mannan which was prepared by partial acid hydrolysis of ACE formed a fused precipitin line with anti-ACE,[5,19] but α(1→6)-linked mannan and the same mannoheteroglycan antigen from mycelium formed a spur with anti-ACE upon agar gel double diffusion (H. Yamada, Y. Ohshima and T. Miyazaki, unpublished results). These results suggested that ACE and mannoheteroglycan from mycelium may contain some other im-

munodeterminant besides $\alpha(1\rightarrow6)$ mannopyranosyl residues, and mannan-type polymer may contain a different immunodeterminant, because the content of $\alpha(1\rightarrow6)$-linked mannopyranosyl residues in this polymer was negligible. Indeed, when mannoheteroglycan antigen (mixture of con-A bound mannoprotein and fucomannopeptide) was subjected to a β-elimination reaction, β-elimination-resistant polymer showed lower antibody-precipitating activity than the original antigen (H. Yamada and T. Miyazaki, unpublished result). However, when the purified serologically active fucomannan-type polymer was subjected to β-elimination, the release of oligosaccharide from the polymer was negligible, and the β-elimination-resistant fraction did not show decreased antibody precipitating activity with anti-ACE in spite of the removal of *O*-acetyl groups from the polymer. These observations suggest that an immunodeterminant of mannan-type polymer might be $(1\rightarrow2)$-linked mannobiose. However, we cannot exclude the possibility of a contribution of a protein moiety as an immunodeterminant of mannan-type polymer. ACE-Ib contained a higher amount of phosphorus, which may be responsible for the acidity. It is known that some microorganisms such as *Saccharomyces cerevisiae*, *Kloeckera brevis* and *Candida albicans* contain phosphorylated mannan,[21-24] but the precipitin reaction of ACE-I with anti-ACE was not inhibited in the presence of mannose 1-phosphate, mannose 6-phosphate or fucose 1-phosphate (Y. Ohshima, H. Yamada and T. Miyazaki, unpublished results). Previously, we found that a mannoheteroglycan antigen from mycelium is acidic due to the presence of uronic acid, and that this acidity was related to the heterogeneity of the mannoheteroglycan, but did not participate as an immunodeterminant group.[5] Phosphate groups of ACE-I may also be related to the heterogeneity of the mannoheteroglycan but unrelated to the immunodeterminant group, because ACE-I cross-reacted with neutral ACE-IIa antigen against anti-ACE.

Further studies on the immunodeterminant of the mannose-containing polymer of *A. cylindrospora* are in progress.

References and Notes

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