Heterogeneity and Antigenic Properties of Mannoheteroglycan from *Absidia cylindrospora*

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An antigenic fraction extracted from mycelium of *Absidia cylindrospora* contained several mannose-containing glycoproteins which differed in molecular weight and in their affinities to concanavalin A (Con A) and DEAE-Sephadex A-50. The fraction of the extract that bound to concanavalin A was separated into high-mannoproteins (minor fraction) and fucomannopeptides (major fraction). The former showed higher antibody-precipitating activity than the latter. Subfractions of the fucomannopeptides obtained by DEAE-Sephadex A-50 chromatography showed increased serological reactivity and an increased mannose to fucose molar ratio with increasing acidity, but neutral and acidic fractions of the mannoproteins showed the same degree of serological reactivity. The results indicate that the mannose-containing antigenic fractions are highly heterogeneous and can be classified into high-mannose type (mol. wt 15 000–35 000) and fucomannan type (mol. wt 15 000–70 000).

INTRODUCTION

Mannan and mannose-containing heteroglycans are known to be somatic antigens of the fungal cell envelope (Ballou, 1976). The detailed chemical structures of these fungal mannans depend on the particular strain, species or genus (Ballou & Raschke, 1974; Ballou, 1974). They may participate in morphogenesis, sexual agglutination and some other recognition processes (Ballou, 1976; Crandall & Brock, 1968).

Previous work has shown that mechanical disruption of mycelium of *Absidia cylindrospora*, a species of the Mucorales, releases a water-soluble antigenic substance(s) (Miyazaki *et al.*, 1977, 1980; Hayashi *et al.*, 1978*a*). This antigen is probably a common antigen among Mucorales such as *Rhizopus nigricans*, *Mucor mucedo* and *Mucor hiemalis* (Hayashi *et al.*, 1978*b*), and may be distributed on the cell surface, because the mycelium surface was stained by fluorescein-conjugated antiserum (Hayashi *et al.*, 1979). The antigen(s) was isolated from the saline extract of the disrupted mycelium and was characterized as a fucomannopeptide after partial purification by affinity chromatography on concanavalin A (Con A)-Sephadex and preparative zone electrophoresis (Hayashi *et al.*, 1978*a*). The fraction that bound to Con A was further separated into two kinds of electrophoretically distinct, but immunochemically identical, antigens; a major glycopeptide [fucomannopeptide (FMP)] and a minor glycoprotein (Hayashi *et al.*, 1978*a*). Preliminary experiments showed that manno-oligosaccharides with more than five (1→6)- α -linkages formed part of the immunological determinant of the antigen (Miyazaki *et al.*, 1979). When FMP with affinity to Con A-Sephadex was used as an antigen in a quantitative precipitin reaction, more than 100 μ g of it was required for the antigen-antibody reaction (Hayashi *et al.*, 1978*b*). When the FMP fraction was further separated by DEAE-Sephadex A-50 (Cl⁻

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form) column chromatography, the fraction adsorbed to the gel revealed antibody-precipitating activity, whereas most of the unadsorbed FMP was obtained as serologically inactive fractions, after further fractionation by Sephadex G-150 gel filtration. Only fractions in the low molecular weight region showed precipitin activity. These observations suggest that the mannose-containing heteroglycans of *A. cylindrospora* are highly heterogeneous.

The present paper describes the heterogeneity of the mannoheteroglycans from *A. cylindrospora*, and the characterization of the antigens with regard to the relationship between heterogeneity and antibody-precipitating activity.

METHODS

Organism. *Absidia cylindrospora* IFO 4000 was obtained from the Institute for Fermentation, Osaka, Japan. The cells were cultured in modified Sabouraud's liquid medium at 27 °C until the stationary growth phase was reached (Miyazaki *et al.*, 1977). Extracellular polysaccharides from *A. cylindrospora* were prepared according to the procedure previously described (Miyazaki *et al.*, 1977; Hayashi *et al.*, 1978a).

Materials. Con A-Sepharose, DEAE-Sephadex A-50, Sephadex G-150 and Sephadex G-200 were obtained from Pharmacia. Pevikon C-870 (polyvinyl resin) was purchased from M & S Instrument, Osaka, Japan.

Fractionation of high-mannoproteins and fucomannopeptides in the mycelium extract of *A. cylindrospora*. Mycelium in saline was disrupted in a French press, and the extract was prepared as previously described (Hayashi *et al.*, 1978a). The extract was fractionated by sequential chromatographic and electrophoretic techniques as shown in Fig. 1. The extract was first fractionated by affinity chromatography on Con A-Sepharose by stepwise elution with α -methyl-D-mannoside (Hayashi *et al.*, 1978a). The fraction not bound to Con A was collected by washing with 0.15 M-sodium phosphate buffer (pH 7.0) containing 0.1 M-CaCl₂ and 0.1 M-MgCl₂. The fraction interacting with the lectin was obtained by elution with 0.5 M- α -methyl-D-mannoside in the same buffer. Fractionation of the Con A-bound fraction by preparative zone electrophoresis using Pevikon C-870 as a supporting medium equilibrated with 0.026 M-sodium borate buffer, pH 9.2 (Hayashi *et al.*, 1978a) gave a minor high-mannoprotein (MP) fraction and a major fucomannopeptide (FMP) fraction. These two fractions were each applied to a column (1.9 × 20 cm) of DEAE-Sephadex A-50 (Cl⁻ form) and eluted with a linear NaCl gradient from 0 to 0.5 M in 50 mM-sodium phosphate buffer, pH 6.8 (Fig. 2). The resulting fractions were further purified by gel filtration on a column (1.9 × 95 cm) of Sephadex G-150 or G-200.

The MP and FMP fractions were also applied to a column (1.9 × 25 cm) of DEAE-Sephadex A-50 (HCO₃⁻ form) in order to isolate their acidic components. The elution of FMP was carried out in a stepwise manner with water and 0.05, 0.1, and 0.25 M-NH₄HCO₃ (Fig. 7a). Since MP was a minor component in the Con A-bound fraction, the acidic fraction in MP was eluted with a 0 to 1 M-NH₄HCO₃ gradient after removal of the neutral fraction with water (Fig. 6a). Preparations from MP or FMP, after chromatography and electrophoresis, were dialysed against water and lyophilized.

Analytical procedures. The total carbohydrate and fucose contents were determined by the phenol-sulphuric acid method (Dubois *et al.*, 1956) and the cysteine-sulphuric acid method (Dische, 1962), respectively, using mannose and fucose as standards. Protein was assayed by the Lowry method. Phosphorus was measured as described by Chen *et al.* (1956).

Chromatography. Thin-layer chromatography (t.l.c.) was performed on Merck cellulose-coated plastic sheet (layer thickness; 0.1 mm) in the solvent system, ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.). Reducing sugars were detected with alkaline silver nitrate (Trevelyan *et al.*, 1950), amino sugar and amino acid with ninhydrin (Partridge, 1948), and uronic acid with *p*-anisidine hydrochloride (Hough *et al.*, 1950). Ascending paper chromatography on Toyo filter paper no. 50 was done using the same solvent system as described above. The acid hydrolysates of polysaccharides (500 µg) obtained after treatment by 1 M-trifluoroacetic acid (at 105 °C for 4 h) were reduced with NaBH₄ for 5 h at 23 °C and the reaction was then stopped by neutralization. After removal of the borate ions 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 (g.l.c.) was performed at 180–250 °C on a Shimadzu GC-6A instrument equipped with a flame-ionization detector, a glass column (0.3 × 200 cm) of 3% (w/w) OV-225 on GasChrom Q, and with a nitrogen flow rate of 50 ml min⁻¹.

Immunological methods. Antiserum was prepared by immunization of male New Zealand white rabbits with extracellular polysaccharides of *A. cylindrospora* as previously described (Miyazaki *et al.*, 1977; Hayashi *et al.*, 1978a). Antisera to whole cells, to cell walls and to extracellular polysaccharides all reacted with the Con A-binding fraction, and their precipitin lines fused with each other completely (Hayashi *et al.*, 1978a). Because of the low antigen-precipitating activities of the antisera to whole cells and to cell walls, the antiserum to extracellular polysaccharide was used as an antiserum against *A. cylindrospora* cells in this study.

Fig. 1. Procedure for the fractionation of mannoheteroglycans from *A. cylindrospora*.

The quantitative precipitin reaction was carried out as follows. Serial dilutions were made using 0.1 ml of antiserum and 0.4 ml of polysaccharides in saline. The mixtures were then incubated at 37 °C for 1 h, and allowed to stand at 4 °C for 72 h. The resulting precipitates were each collected by centrifugation at 3500 rev. min⁻¹ at 4 °C for 30 min, washed and resuspended in 1% Na₂CO₃ for protein determination by the Lowry method using bovine γ -globulin as a standard.

RESULTS

Heterogeneity of Con A-bound fucomannopeptide and high-mannoprotein fractions

When the serologically active Con A-bound fraction extracted from mycelium of *A. cylindrospora* was fractionated by zone electrophoresis, a minor component was obtained together with a major component previously characterized as a fucomannopeptide (FMP) antigen (Hayashi *et al.*, 1978a). Both fractions had the same antigenic determinants, as their precipitin lines with the antiserum in the immunodiffusion assay fused completely, but they differed in their electrophoretic mobility. The minor fraction contained mannose with a trace of fucose and was designated as high-mannoprotein (MP). The FMP fraction was further separated on a column of DEAE-Sephadex A-50 (Cl⁻ form) into an unbound fraction (FMP-I) and bound fractions (FMP-IIa, b, c) by elution with a linear gradient of NaCl (Fig. 2). FMP-I showed negligible serological reactivity compared with the serologically active FMP-IIa, b and c, but all showed the fused precipitin line in agar gel double diffusion (data not shown). Sephadex G-200 column chromatography of each fraction resulted in a broad

Fig. 2

Fig. 3

Fig. 2. DEAE-Sephadex A-50 (Cl^- form) chromatography of fucomannopeptide (FMP). The unadsorbed fraction (FMP-I) was eluted with 50 mM-potassium phosphate buffer, pH 6.8, and then the adsorbed fraction (FMP-II) was eluted with a linear gradient of 0 to 0.1 M-NaCl (---). Fractions of 2 ml were collected. Carbohydrate, A_{490} (○) was determined by the phenol-sulphuric acid procedure.

Fig. 3. Evidence for heterogeneity in fucomannopeptide (FMP-IIb). (a) Peak FMP-IIb (see Fig. 2) was fractionated on a Sephadex G-200 column (1.9×95 cm) by elution with 0.1 M-NaCl. Carbohydrate, A_{490} (●); protein, A_{280} (○). (b) Mannose:fucose molar ratio of fractions indicated in (a).

Fig. 4

Fig. 5

Fig. 4. Sephadex G-150 column chromatography of fractions not adsorbed to DEAE-Sephadex A-50 (Cl^- form): (a) unadsorbed high-mannoprotein fraction (MP-I) and (b) unadsorbed fucomannopeptide fraction (FMP-I, see Fig. 2). Carbohydrate, A_{490} (●); protein, A_{280} (○).

Fig. 5. Quantitative precipitin curves of mannose-containing polymer fractions against antiserum to *A. cylindrospora*: MP-I3 (○), FMP-I3 (□) and FMP-I2 (△). The amount of antigen was expressed as carbohydrate content.

Fig. 6. (a) Elution profile of high-mannoprotein (MP) on a DEAE-Sephadex A-50 (HCO_3^- form) column, eluted with a linear gradient of 0 to 1 M- NH_4HCO_3 (—). Carbohydrate, A_{490} (●); protein, A_{280} (○). (b) Quantitative precipitin curves of high-mannoprotein neutral and acidic fractions against antiserum: MP-i (○), MP-ii (●).

Fig. 7. (a) Elution profile of fucomannopeptide (FMP) on a DEAE-Sephadex A-50 (HCO_3^- form) column, eluted stepwise with water, 0.05, 0.1, and 0.25 M- NH_4HCO_3 (---). Carbohydrate, A_{490} (○). (b) Quantitative precipitin curves of four fucomannopeptide subfractions against antiserum: FMP-i (○), FMP-iii (●), FMP-ii (■), FMP-i (□).

elution pattern, indicating the presence of fucomannans of different molecular weights (data not shown). Only the low molecular weight fraction of FMP-I contained a trace amount of a serologically active component. The subfractions of FMP-IIb, which showed the broadest elution profile by Sephadex G-200 gel filtration, were analysed for mannose and fucose content (Fig. 3). The mannose:fucose ratios varied from 2:1 to 7:1, suggesting that the heterogeneity of fucomannan was directly related to the amount of mannose in the polymer. The broad peak of carbohydrates was separated into eight fractions (see Fig. 3). Fractions 4, 5, 6, 7 and 8 reacted strongly with antiserum in agar gel double diffusion (data not shown). Thus, the serological reactivity appeared to be in the low molecular weight region with higher mannose content (mol. wt 15 000–35 000).

Fractions of MP and FMP that did not bind to DEAE-Sephadex A-50 (Cl^- form) were purified by gel filtration on Sephadex G-150 (Fig. 4a, b). Only subfractions MP-I3 and FMP-I3 formed precipitin lines with antiserum by the Ouchterlony method. However, the quantitative precipitin curves for these two subfractions were different (Fig. 5). The peak for FMP-I3 was shifted towards higher antigen concentrations in this reaction system, and the curve was broader than that for MP-I3. FMP-I2 showed negligible antibody-precipitating activity. The molecular weights of FMP-I2 and MP-I3 were estimated to be 15 000–70 000 and 15 000–35 000, respectively, on the basis of the gel filtration on Sephadex G-150. MP-I3 was composed of mannose and a trace of fucose (molar ratio 13:1), whereas in FMP-I2 the molar ratio was 2:1. On the other hand, FMP-I3 adsorbed to DEAE-Sephadex A-50 in the

Table 1. *Chemical properties of the subfractions obtained by DEAE-Sephadex A-50 (HCO_3^- form) column chromatography of high-mannoprotein (MP) and fucomannopeptide (FMP)*

Component sugars were analysed by t.l.c. and g.l.c.; trace components are shown in parentheses.

Ratios of mannose to fucose in MP subfractions were based on determinations by the phenol-sulphuric acid (Dubois *et al.*, 1956) and cysteine-sulphuric acid (Dische, 1962) procedures. In FMP subfractions the ratios were determined by g.l.c. of acid hydrolysates.

Total carbohydrate content was calculated as the total quantity of mannose and fucose. Fucose content was measured by the cysteine-sulphuric acid method which is specific to methylpentose. The absorbance at 490 nm obtained by the phenol-sulphuric acid method was corrected for the absorbance at 490 nm due to the amount of fucose in the sample as determined by the cysteine-sulphuric acid method, and then the mannose content was determined from the corrected absorbance using mannose as a standard.

Protein content was determined by the Lowry method and phosphorus content was determined by the method of Chen *et al.* (1956).

Subfraction	Component sugars*	Molar ratio of mannose and fucose	Carbohydrate content (% w/w)	Protein content (% w/w)	Phosphorus content (% w/w)
MP-i	Man (Fuc)	6.7†	50.9	31.7	0.24
MP-ii	Man (Fuc, Uro)	4.8†	53.4	26.5	0.12
FMP-i	Man, Fuc (Gal, GlcN)	2.8	97.5	2.0	0.22
FMP-ii	Man, Fuc (Gal, GlcN, Uro)	1.9	90.3	1.2	0.22
FMP-iii	Man, Fuc (Gal, GlcN, Uro)	2.7	85.3	2.0	0.25
FMP-iv	Man, Fuc (Gal, GlcN, Uro)	3.0	60.1	6.2	0.22

* Fuc, fucose; Gal, galactose; GlcN, glucosamine; Man, mannose; Uro, uronic acid.

† These values are lower than expected. However, MP comprised only 5 to 10% (v/v) of the antigen extract and the extract was not well resolved by zone electrophoresis, so both MP subfractions may have been contaminated with FMP.

HCO_3^- form, which is able to bind all acidic substances (data not shown). This suggested that the antigenic determinant in fucomannan-type polysaccharides is acidic in nature. To confirm this, MP and FMP were applied to DEAE-Sephadex A-50 (HCO_3^- form) columns to separate the acidic fractions. MP was separated into almost equal amounts of unbound (MP-i) and bound (MP-ii) fractions (Fig. 6*a*), but both fractions showed strong serological reactivities (Fig. 6*b*). FMP was fractionated into four subfractions, designated FMP-i, ii, iii and iv, with most carbohydrate being found in the weakly acidic fraction FMP-ii (Fig. 7*a*). The phosphorus contents of these subfractions were similar, but the ratio of mannose to fucose was dependent on the concentration of NH_4HCO_3 solutions eluting each fraction (Table 1). The order of the antibody-precipitating activities of these fucomannans was $\text{iv} > \text{iii} > \text{ii} > \text{i}$ (Fig. 7*b*), which was proportional to their mannose contents except for FMP-i (Table 1). Uronic acid could not be detected in neutral FMP-i and MP-i, but traces of it were detected in all of the acidic FMP and MP fractions by thin-layer chromatography after acid hydrolysis.

Characterization of Con A-unbound fraction

When the Con A-unbound fraction of the extract from mycelium was applied to a column of DEAE-Sephadex A-50 (Cl^- form), two unadsorbed fractions (U-FMP-I and -II) and an adsorbed fraction (U-FMP-III) were obtained (Fig. 8). The unadsorbed fractions were fucomannopeptides of different protein contents (Table 2), but they did not react with antiserum. On agar gel double diffusion, only U-FMP-III formed a precipitin line with antiserum. This weak antigen, U-FMP-III, possessed different antigenic determinants from

Fig. 8. Elution profile of Con A-unbound fucomannopeptide (U-FMP) on a DEAE-Sephadex A-50 column (Cl^- form). After removal of the unadsorbed fraction with 50 mM-phosphate buffer, pH 6.8, adsorbed fraction was eluted with a linear gradient of 0 to 0.1 M-NaCl (—). Carbohydrate, A_{490} (O).

Table 2. Chemical properties of the subfractions obtained by DEAE-Sephadex A-50 (Cl^- form) column chromatography of the Con A-unbound fraction

Methods of analysis and abbreviations were as described in Table 1.

Subfraction	Component sugars	Molar ratio of mannose and fucose	Carbohydrate content (% w/w)	Protein content (% w/w)
U-FMP-I	Fuc, Man (Gal, GlcN)	2.34	77.35	1.2
U-FMP-II	Fuc, Man (Gal, GlcN)	2.42	62.85	4.4

the main antigens, MP-I3 and FMP-I3, since the precipitin line formed by U-FMP-III clearly crossed those of MP-I3 and FMP-I3 (data not shown). The chemical nature of U-FMP-III could not be analysed because of the low yield.

DISCUSSION

Antigenic extracts from mechanically disrupted *A. cylindrospora* mycelium contain several kinds of mannose-containing polysaccharides which are highly heterogeneous with respect to molecular weight. They can be classified as high-mannoproteins (minor fraction) and fucomannopeptides (major fraction). The high-mannoprotein fraction exhibits high serological reactivity with antiserum to *A. cylindrospora*, while the fucomannopeptide fraction has little reactivity.

The antigenic extract was fractionated on the basis of a difference in affinity to Con A. Most of the Con A-bound and -unbound fractions were characterized as fucomannopeptide, but only the Con A-bound fraction showed serological reactivity. Mannoprotein was found in the Con A-bound fraction, together with a large amount of fucomannopeptide. It is known that Con A binds specifically with the non-reducing terminal of two mannopyranosyl residues or two sugar residues containing (1→2)- α -linked mannobiose (Ogata *et al.*, 1975). This implies that *A. cylindrospora* antigen may have mannopyranosyl residues at the non-reducing terminal or (1→2)- α -linked mannobiosyl residues. The Con A-bound fucomannopeptide contained some acidic fractions with differing affinities to DEAE-Sephadex (HCO_3^- form).

While the neutral fucomannopeptide showed negligible serological reactivity, the antibody-precipitating activity of the acidic fucomannopeptide fractions varied with their affinity to DEAE-Sephadex and was proportional to their acidity. This phenomenon is similar to the relationship between acidity and antibody-precipitating activity of mannan from Baker's yeast, as reported by Okubo *et al.* (1978). They found that the activities of mannan

subfractions which were fractionated on a column of DEAE-Sephadex were proportional to their phosphate contents. Since inorganic phosphate was unable to inhibit the homologous antigen-antibody system, they concluded that phosphate groups did not participate in the serological activity. Neutral and acidic subfractions in the Con A-bound fucomannopeptides had similar phosphorus and protein contents, but only the acidic fraction contained uronic acid. Thus the acidity of *A. cylindrospora* fucomannopeptide may be due to the presence of uronic acid. However, the acidic group itself of Con A-bound fucomannopeptide did not contribute a primary part of the immunodeterminant, because Con A-bound high-mannoprotein, which has an antigenic determinant in common with fucomannopeptide antigen, possessed strong serological reactivity in both the acidic and neutral fractions. The density of the longest side-chain corresponding to the antigenic determinant in an acidic subfraction of mannan from Baker's yeast was proportional to its phosphate content, and it was suggested that the phosphate group may contribute to the microheterogeneity of mannan in Baker's yeast (Okubo *et al.*, 1978). In the case of the high-mannoprotein and fucomannopeptide of *A. cylindrospora*, an acidic group such as a uronic acid residue may affect their heterogeneity. The ratio of mannose to fucose was proportional to the acidity in fucomannopeptide, and high-mannose type polymers of molecular weight around 15 000–35 000 showed serological reactivity. Differences in molecular weight were also related to the heterogeneity of fucomannopeptides since the percentage of fucose in the molecule decreased in the low molecular weight region. Galactomannan of *Penicillium charlesii* showed similar heterogeneity and was also fractionated into polymers of different molecular weights (Preston *et al.*, 1969; Rietschel-Berst *et al.*, 1977). The percentage of galactofuranose decreased in the low molecular weight region (Rietschel-Berst *et al.*, 1977). Possibly this heterogeneity is due to the degradation of fucomannopeptide into high-mannose type polymer by fucosidase. If this is correct, partially defucosylated products should have some serological reactivity. However, the product obtained by mild acid hydrolysis did not react with antiserum to *A. cylindrospora* (unpublished result) suggesting that the serological reactivities of mannoheteroglycans are based on the structural difference of the mannan moiety. Since a higher amount of fucomannopeptide than of high-mannoprotein was needed for antibody-precipitating activity, it is suggested that the immunodeterminant group of high-mannoprotein has a higher density than that of fucomannopeptide. Because growth of *A. cylindrospora* is slow in dialysable Sabouraud's medium (Miyazaki *et al.*, 1977), it was cultured for 28 d in the present study. Thus it was possible that the heterogeneity was a result of the long period of incubation. To investigate this possibility we cultured *A. cylindrospora* in yeast extract/peptone/glucose medium, in which stationary growth was obtained after 3–5 d. When these cells were extracted with saline or neutral buffer, similar mannose-containing polymers were obtained with the same high heterogeneity and the same immunological properties. Thus, the heterogeneity of mannose-containing polymers from *A. cylindrospora* may have some important significance. The serological activity of *A. cylindrospora* is closely related to the heterogeneity of the mannose-containing polymers, and thus the relationship between the biosynthetic pathways of high-mannoprotein and fucomannopeptide may be of interest, because both had the same immunodeterminants. A comparison of the carbohydrate structures of high-mannoprotein and fucomannopeptide is now in progress.

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