Purification and Partial Characterization of the Serologically Active Substance from *Absidia cylindrospora*

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Affinity chromatography with a concanavalin A–Sephadex column was successfully used to separate the serologically active substance of *Absidia cylindrospora* from the supernatant of disrupted mycelium fluid, the phenol extract of intact washed mycelium and the culture filtrate. The active substance, a fucomannan–peptide, was isolated homogeneously by means of zone electrophoresis using Pevikon. It was composed of fucose and mannose (94.6%, w/w) in the ratio 1:2.2 and a small amount of protein (1.8%, w/w). It may be bound loosely on the surface of the mycelium.

INTRODUCTION

Numerous fungal polysaccharides and glycoproteins were reviewed by Gorin & Spencer (1968), and classification of fungal polysaccharides was described by Bartnicki-Garcia (1968). A number of investigators have studied the chemical and immunological properties of mannans and mannose-containing polysaccharides such as galactomannans and rhamnomannan. These fungal polysaccharides were the major cell wall antigens. The cell wall polysaccharides of *Mucor rouxii* have been studied chemically by Bartnicki-Garcia and co-workers (Bartnicki-Garcia & Reyes, 1968; Bartnicki-Garcia & Lindberg, 1972). Miyazaki & Irino (1970, 1972) have reported the isolation and characterization of acidic polysaccharides from the cell walls and culture filtrates of *Absidia cylindrospora*, *Mucor mucedo* and *Rhizopus nigricans*, which are all members of the Mucorales. Recently, a serologically active substance(s) of *A. cylindrospora* was found in the supernatant obtained by centrifugation of disrupted cells (Miyazaki *et al.*, 1977). The present paper describes the chemical properties and the morphological localization of this serologically active substance from *A. cylindrospora*.

METHODS

Analytical methods. Total carbohydrate was determined by the phenol–sulphuric acid method (Dubois *et al.*, 1956), protein by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard, and phosphate by the method of Fiske & Subbarow (1925). Hexosamine was determined after hydrolysis with 4 M-HCl for 10 h at 100 °C by the methods of Blix (1948) and Gardell (1953) using glucosamine hydrochloride as a standard.

The component sugars were determined qualitatively by paper chromatography. Each sample was hydrolysed with 0.5 M-H₂SO₄ at 100 °C for 8 h. The hydrolysate was neutralized with BaCO₃, filtered and concentrated to a syrup. A portion of the concentrate was examined by the ascending method on a Toyo Roshi no. 50 filter paper using the solvent system ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.). Sugars on the chromatogram were detected by the following reagents: alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950), *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) and ninhydrin (Partridge, 1948).

The molar ratio of neutral sugars in each sample was determined by gas-liquid chromatography (g.l.c.) of the alditol acetate derivatives of the acid hydrolysate (Miyazaki *et al.*, 1976). A Shimadzu GC-6A chromatograph (Shimadzu, Tokyo, Japan) equipped with a glass column (0.3 × 200 cm) containing 5% ECNSS-M on chromosorb-W (AW-DMCS, 60 to 80 mesh) was used in the g.l.c. analysis. The column temperature was 190 °C.

Paper electrophoresis using 0.026 M-sodium borate was performed on a Toyo Roshi no. 50 filter paper at a constant current of 1.5 mA cm⁻¹ for 3 h. After drying the paper, polysaccharides were detected by periodate-Schiff reagent (Köwi & Grönwall, 1952).

Preparation of the purified cell wall and extracellular material. The purified cell wall of *A. cylindrospora* IFO 4000, designated ACW, was obtained by disruption in a French press, followed by washing with sodium dodecyl sulphate (SDS) and distilled water, and centrifugation as described previously (Miyazaki *et al.*, 1977).

The culture filtrate was dialysed in a Visking tube against distilled water for 3 d at 4 °C. The non-dialysable fraction was concentrated to about 500 ml and then lyophilized. The resulting powder was designated ACE.

Preparation of antisera. ACW and ACE were suspended in saline to give 20 mg ml⁻¹ and 10 mg ml⁻¹, respectively. The suspension of ACW was homogenized in a Teflon homogenizer. Emulsions of ACW and ACE were prepared by adding an equal volume of Freund's complete adjuvant (Difco). Male New Zealand white rabbits, each weighing 2.5 to 3.0 kg, were immunized with 0.5 ml of the emulsions by intracutaneous injection once a week for 3 or 6 weeks. One or two weeks after the last injection, the rabbits were bled and the separated sera were stored frozen. These antisera were designated anti-ACW serum and anti-ACE serum. Further antiserum, designated anti-ACWC serum, was prepared by immunizing a rabbit with 0.5 ml of a homogenate of whole cells in saline (100 mg wet wt ml⁻¹) once a week for 7 weeks; this antiserum was also stored frozen.

Immunochemical methods. Immunodiffusion analysis with 1% (w/v) Golden Agar (Kyokuto Seiyaku, Japan) in 0.15 M-phosphate-buffered saline (pH 7.0) was done according to the method of Ouchterlony (1949).

The quantitative precipitin test was carried out as follows. Undiluted rabbit antiserum (0.2 ml) was mixed with 0.1 ml of serial dilutions of antigen (ACI-B) in saline. The mixtures were incubated at 37 °C for 1 h, stored at 4 °C for 90 h with occasional shaking and then centrifuged. The precipitates were washed twice with 6 ml cold saline and the protein in the precipitated complex was determined by the method of Lowry *et al.* (1951).

Immuno-electrophoresis was carried out with 1% (w/v) agar made up in 0.087 M-veronal buffer (pH 8.6). Anti-ACE serum was added after electrophoresis at a constant current of 30 mA for 1 h and diffusion was allowed to take place at 22 °C for 1 to 5 d.

Separation of the serologically active fractions. The supernatant fluid of the cell homogenate was separated from the pellet of the cell wall fraction by centrifugation as described previously (Miyazaki *et al.*, 1977). The residual cell wall fraction was resuspended in cold saline, the suspension was homogenized with a Teflon homogenizer, and the supernatant was separated by centrifugation. The combined supernatants were dialysed against distilled water for 3 d at 4 °C and lyophilized. The resulting powder was resuspended in saline and the solution was centrifuged at 100000 g for 60 min at 4 °C. After dialysis, the soluble supernatant was lyophilized and designated ACI.

Washed mycelium (110 g wet wt) was extracted with 500 ml of 45% (v/v) phenol at 65 to 75 °C for 1 h by the method of Westphal & Jahn (1965). After cooling in an ice bath, the phenol extract was filtered and centrifuged for 30 min at 15000 rev. min⁻¹ at 5 °C. The aqueous layer of the supernatant was lyophilized after extensive dialysis against running tap water and distilled water. The resulting white powder was designated PhW. The yield was 210 mg.

Portions (100 mg) of each of the fractions ACI, PhW and ACE were dissolved in 0.15 M-phosphate buffer (pH 7.0) and the solutions were placed in a boiling water bath for 15 min. After cooling, each solution was applied to a column (1.5 × 45 cm) of concanavalin A (Con A)-Sepharose 4B and eluted with 0.15 M-phosphate buffer (pH 7.0), to give fractions ACI-A, PhW-A and ACE-A, and then with 0.5 M- α -methyl-D-mannoside in the same buffer, to give fractions ACI-B, PhW-B and ACE-B.

Periodate oxidation and pronase/trypsin digestion of ACI-B. ACI-B (15 mg) was oxidized with 0.015 M-NaIO₄ at 4 °C in the dark. After 24 h, the reaction was stopped by adding 1 ml ethylene glycol. After dialysis for 3 d followed by lyophilization, the oxidized ACI-B was obtained in a yield of 10.9 mg. The oxidized ACI-B was then reduced with NaBH₄ overnight at room temperature. The reaction mixture was acidified with acetic acid, dialysed for 2 d and lyophilized.

ACI-B (10 mg) was dissolved in 5 ml 0.05 M-Tris/HCl buffer (pH 7.9) containing 0.01 M-CaCl₂ and 2 mg pronase (Kaken Kagaku, Japan). The mixture was passed through a Millipore filter (0.45 μ m pore size) and then incubated at 37 °C for 48 h. After heating in a boiling water bath for 10 min, the mixture was dialysed against 0.05 M-Tris/HCl buffer (pH 7.5) for 2 d. Trypsin (2 mg; Boehringer) was added to the

non-dialysable solution. The mixture was passed through a Millipore filter and incubated at 37 °C for 24 h. After heating for 10 min the reaction mixture was dialysed against distilled water and then the non-dialysable solution was lyophilized.

Purification of ACI-B by zone electrophoresis. ACI-B (100 mg) was purified by zone electrophoresis using Pevikon C-870 (polyvinyl resin, M & S Instruments, Japan) as a supporting medium (1.5 × 7.0 × 40 cm) equilibrated with 0.026 M-sodium borate. After migration at a constant current of 40 mA for 15 h, the Pevikon block was cut into 40 segments and each segment was quantitatively extracted with 5 ml 0.026 M-sodium borate solution. The sugar content of each extract was determined using the anthrone-H₂SO₄ reagent (Morris, 1948).

RESULTS

Chemical analysis of the purified cell wall

Examination by light and electron microscopy indicated that the purified cell wall of *A. cylindrospora*, prepared as described in Methods, was free from cytoplasmic material. The yield of purified wall was 1.5 g (1 culture)⁻¹. The purified walls contained 23.9% (w/w) neutral sugar comprising fucose, mannose and galactose in the molar ratio 10:1:2. They also contained hexosamine (31.4%, w/w), protein (3.2%, w/w), extractable lipid (1.7%, w/w) and small amounts of glucuronic acid.

Separation of the serologically active fractions

By means of affinity chromatography on Con A-Sepharose, the serologically active fractions, ACI-B, PhW-B and ACE-B, were obtained as adsorbed fractions. ACI-A, PhW-A and ACE-A were obtained as unadsorbed fractions. These fractions were lyophilized after dialysis against distilled water for 3 d. A representative elution pattern of ACI is shown in Fig. 1. The yields of the various fractions are given in Table 1. Fractions ACI-B, PhW-B and ACE-B contained relatively large amounts of carbohydrate and small amounts of protein (Table 2). Paper electrophoresis of ACI-B and ACE-B revealed two components in each, but PhW-B behaved as a single compound with the same mobility as the main component of fractions ACI-B and ACE-B.

Table 1. *Recovery of the serologically active fractions*

	Recovery [% (w/w) of starting material]			
	Fraction A		Fraction B	Total
	A ₁	A ₂		
ACI	7.0	8.0	76.1	91.1
ACE		28.9	44.7	73.6
PhW		13.4	26.4	39.8*

* Other portion was not eluted.

Table 2. *Chemical analysis of ACI-B, PhW-B and ACE-B*

	Neutral sugar content* (%, w/w)	Molar ratio of fucose and mannose†	Protein content (%, w/w)	Phosphorus content (%, w/w)	Total (%, w/w)
ACI-B	63.0	1:2.51	22.7	2.3	88.0
PhW-B	80.7	1:2.31	8.9	0.6	90.2
ACE-B	55.6	1:2.49	7.7	5.0	68.3

* Standards used for the determination of neutral sugar content were prepared on the basis of the molar ratio of fucose and mannose (calculated from the results of g.l.c.) in each sample.

† Traces of galactose, glucosamine, galactosamine and glucuronic acid were also detected in all fractions.

Fig. 1. A representative elution pattern of ACI on a column of Con A-Sepharose: 5 ml fractions were collected, and elution with 0.5 M- α -methyl-D-mannoside commenced at the arrow. $\circ$, A_{280} ; $\bullet$, A_{625} . The elution patterns of PhW and ACE were similar to that of ACI.

Serological reactivities of anti-ACWC, anti-ACW and anti-ACE sera with ACI-B and other fractions

The maximum dilutions of anti-ACWC, anti-ACW and anti-ACE sera that formed precipitates with ACI-B ($312.5 \mu\text{g ml}^{-1}$ in saline) in a fine glass tube were 1:1, 1:1 and 1:8, respectively. Quantitative precipitin curves of these three antisera with ACI-B showed that precipitation of anti-ACE serum with ACI-B occurred to a larger extent than did precipitation of anti-ACWC and anti-ACW sera with ACI-B (about one-eighth of the reactivity of anti-ACE serum). The immunodiffusion pattern of these antisera with ACI-B showed a fused single precipitin line (Fig. 2). These antisera also reacted with PhW-B and ACE-B to give a precipitin band in a fine glass tube. Sera absorbed with ACI-B did not react with ACI-B, PhW-B or ACE-B.

Immunochemical properties of ACI-B, PhW-B and ACE-B

ACI-B, PhW-B and ACE-B, which had affinity for Con A, reacted strongly with anti-ACE serum. Immunodiffusion of ACI-B, PhW-B and ACE-B with the antiserum gave distinct precipitin lines which fused with each other (Fig. 3). The results of immunoelectrophoresis showed that these fractions gave precipitin arcs with anti-ACE serum which were similar to each other in their mobilities (Fig. 4).

To elucidate whether the serologically reactive site is a carbohydrate or a peptide moiety, ACI-B was subjected to periodate oxidation and digestion with proteolytic enzymes. Portions ($25 \mu\text{g}$) of periodate-oxidized ACI-B and pronase/trypsin-digested ACI-B were examined by immunodiffusion with anti-ACE serum ($10 \mu\text{l}$). Periodate-oxidized ACI-B (3 mg ml^{-1}) and its serial dilutions in saline were also used for the precipitin reaction in a fine glass tube. Periodate-oxidized ACI-B did not react with anti-ACE serum, but digestion of ACI-B with pronase and trypsin did not affect its serological reactivity.

ACI-A and ACE-A also reacted with anti-ACE serum, but the precipitin lines after immunodiffusion were different from those of ACI-B, PhW-B and ACE-B. However, the reaction only occurred to a slight degree.

Further purification of the fucomannan-peptide fraction

Zone electrophoresis of ACI-B gave two peaks, ACI-B-I and ACI-B-III (Fig. 5). The homogeneity of the major peak, ACI-B-III, was confirmed by paper electrophoresis and by

Fig. 2

Fig. 3

Fig. 2. Immunodiffusion pattern of anti-ACWC serum (well 1, 10 μ l), anti-ACE serum (well 2, 10 μ l) and anti-ACW serum (well 3, 10 μ l) with ACI-B (centre well, 12.5 μ g).

Fig. 3. Immunodiffusion pattern of the serologically active fractions ACI-B (well 1, 12.5 μ g), PhW-B (well 2, 12.5 μ g) and ACE-B (well 3, 12.5 μ g) with anti-ACE serum (centre well, 10 μ l).

Fig. 4. Immunoelectrophoresis of ACI-B (upper well, 30 μ g), PhW-B (centre well, 30 μ g) and ACE-B (lower well, 30 μ g), with anti-ACE serum (30 μ l) in the troughs.

Fig. 5. Zone electrophoresis of ACI-B: $\circ$, A_{280} ; $\bullet$, A_{625} .

Fig. 6. (Left) Paper electrophoresis of ACI-B-I, ACI-B-II and ACI-B-III in 0.026 M-borate buffer at a constant current of 1.5 mA cm^{-1} for 3 h. Polysaccharides were detected by the periodate-Schiff reagent. (Right) Immunoelectrophoresis of ACI-B-I (upper well, $75 \mu\text{g}$), ACI-B-III (centre well, $95 \mu\text{g}$) and ACI-B (lower well, $105 \mu\text{g}$), with anti-ACE serum ($30 \mu\text{l}$) in the troughs.

immunoelectrophoresis against anti-ACE serum using 0.087 M-veronal buffer (pH 8.6) (Fig. 6). ACI-B-III contained carbohydrate [94.6% (w/w) as mannose], which was composed of fucose and mannose (in the molar ratio 1:2.2), and a small amount of protein (1.8%, w/w). The minor peak, ACI-B-I, was too small to allow chemical analysis. In immunoelectrophoresis, the precipitin arc of ACI-B-I with anti-ACE serum was different from that of ACI-B-III in its mobility but the precipitin lines of these in the immuno-diffusion pattern fused completely.

DISCUSSION

Miyazaki *et al.* (1977) showed that the serologically active substance of *A. cylindrospora* was found mainly in the supernatant obtained from the disrupted cells by centrifugation and that it was obtained only in a low yield from the purified cell wall. The antibody titre of anti-ACE serum was higher than that of anti-ACW serum. As shown in Table 2 and Figs 3 and 4, it appears that fractions ACI-B, PhW-B and ACE-B contain a common active substance. These observations suggest that the serologically active substance of *A. cylindrospora* may be loosely bound on the surface of the mycelium and easily released from the surface. This active substance was isolated homogeneously as the major peak, ACI-B-III, by means of zone electrophoresis on Pevikon. It was a fucomannan-peptide composed of fucose and mannose in a 1:2.2 molar ratio and a very small amount of protein. The serologically active site of this polymer may be a carbohydrate rather than a peptide moiety since periodate oxidation of ACI-B destroyed its reactivity with anti-ACE serum whereas pronase/trypsin digestion did not.

Miyazaki & Irino (1970) isolated an acidic polysaccharide composed of L-fucose, D-galactose and D-glucuronic acid from an alkali extract of the cell wall of *A. cylindrospora*. The alkali extract had no serological reactivity with anti-wall serum in spite of the presence of the fucose-containing polysaccharide (Miyazaki *et al.*, 1977). Therefore, it appears that the fucomannan-peptide is different from the polysaccharides in the cell wall such as mucoric acid and mucoran (Bartnicki-Garcia & Reyes, 1968) and the acidic polysaccharide.

Mannans and mannose-containing heteropolysaccharides in yeast and other fungi are well known as fungal antigens. Galactomannans have been obtained from many species of fungi such as *Histoplasma* (Azuma *et al.*, 1974), *Trichophyton*, *Cladosporium werneckii*, *Penicillium* and *Aspergillus* (Gander, 1974). Furthermore, a peptido-rhamnomannan has been isolated from *Sporothrix schenckii* and studied immunochemically (Lloyd & Travassos, 1975). Fucose is an uncommon constituent of fungal polysaccharides except in the Mucorales and Basidiomycetes (Bartnicki-Garcia, 1968; Gorin & Spencer, 1968). In the Mucorales,

it remains to be seen whether the fucomannan-peptide is an antigenic substance common to many species or specific to *A. cylindrospora*. Studies on the cross-reactivity of the anti-ACE serum with other Mucorales or fungi are now in progress.

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