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Structural Analysis of Water-soluble Fractions Obtained from *Aspergillus Fumigatus* Mycelium

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Fractions were prepared from the water-soluble components of *Aspergillus fumigatus* mycelium either by lectin-affinity chromatography or salt precipitation. While they varied considerably in their amino-acid composition, each contained a preponderance of aspartic and glutamic acids. ^{13}C-NMR spectroscopy of these fractions, compared with that of polysaccharide obtained by alkaline extraction, indicated the presence of glycoproteins, the polysaccharide components of which contained β-D-Gal*f* units that are part of structures chemically different from those obtained by alkali treatment. In two of the three fractions examined, gas-liquid chromatography - mass spectrometry showed marked differences in the contents of non-reducing end-units of α-D-Man*p* and β-D-Gal*f*. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the preparations revealed an array of components, which stained to differing extents with silver stain and with Coomassie Blue and many of which were bound by lectins with specificity for different sugars.

Aspergillus species are among the most common causal agents of the deep mycoses in the developed world. They have been implicated as aetiological agents of different lung diseases including allergic asthma, allergic bronchopulmonary aspergillosis, aspergilloma (fungus ball) and invasive aspergillosis. While a variety of Aspergillus species may be implicated, the chief agent of the disease is *Aspergillus fumigatus.*

The detection of Aspergillus-specific antibodies in a patient's serum represents a significant aid in the diagnosis of the various forms of aspergillosis. Detector antigens with comparable sensitivity include unfractionated, water-soluble supernatants from ruptured mycelium, partially purified mixtures of glycoproteins, proteins and polysaccharides, and galactomannans (GM), the only antigenically-active compounds of *A.fumigatus* which have been chemically defined [1-7]. To date, no standardized detector antigens are available for *A. fumigatus.*

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One commonly used purification method for crude antigens relies on the affinity of Concanavalin A(Con A) for specific sugar residues, present in crude *A. fumigatus* extracts [8-10]. Many of these Con A-binding substances have been partially characterized as acidic glycoproteins of high molecular weight with strong immunological reactivity [11-13]. Another purification method separates water-soluble components by fractional precipitation with ammonium sulphate [1, 14, 15]. Little is known about the composition of these fractions, although it is possible that some of them contain galactomannans.

SDS-PAGE and blotting methods are being investigated as tools to detect specific antigens to Aspergillus antibodies [16, 17]. Both high- and low-mol.wt. components of these mixtures have been shown to have high immunological reactivity with 20-30 separated bands showing antibody-binding properties [18, 19; Hearn, unpublished results]. While serum from patients with antibodies to Aspergillus show similar reaction patterns on immunoblot analysis, there is no agreement among published reports on a specific, immuno-dominant antigen [17, 19, 20]. Some of these fractions have been shown to contain several bands which were bound by Con A [3]. However, further analysis of the composition of these antigenically-reactive molecules appears to be lacking.

In the work presented here, we have selected two methods of antigen preparation which have been used in a number of laboratories, *viz.*, Con A affinity chromatography and salt precipitation. The methods have the advantages of ease and speed of preparation. They constitute mild conditions of fractionation and yield partially-purified preparations of high antigenic reactivity. A major objective in such studies is the production of a diagnostic antigen which is more reproducible than an unfractionated extract and which can more readily be standardized, both chemically and immunologically. To date, detailed chemical structure of such reagents remains undetermined. We report on the total amino-acid composition, together with analysis of the polysaccharide components of these fractions. The mixtures were subjected to SDS-PAGE: those molecules which penetrated the gel matrix were studied. The separated components were stained for protein with the silver/Coomassie Blue reagent and for specific sugars with lectins, following transfer to nitrocellulose membranes.

Materials and Methods

Preparation of A. Fumigatus Antigens

A three-day preparation of *A. fumigatus* mycelium (strain no. NCPF 2109), grown in neutral glucose/peptone medium at 30°C, was harvested by filtration and disrupted in a Dynomill cell disintegrator (Glen Creston, U.K.). Cell debris was removed by centrifugation and the supernatant concentrated before additional centrifugation (at 100 000 x *g*) to remove insoluble material. This supernatant constituted the total, water-soluble material (WS) of the mycelium [21].

Two fractions were separated from the WS material on the basis of their attachment or non-attachment to Con A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). The material that did not bind to Con A was protein rich and designated the UBF fraction. Material which was eluted from the column by 0.2 M methyl α-D-mannopyranoside, was

carbohydrate-rich and designated the BF fraction. From previous experiments these constituted 30 ±6 % and 17 ±4 % respectively, of WS material [9]. [Elution with higher concentrations of methyl α-D-mannopyranoside (0.6 M), increased the yield from Con A-Sepharose by only a further 3% of the applied material. Borate buffer eluted additional carbohydrate-containing material (Hearn, unpublished results), but these fractions have not been studied further].

Two additional fractions were separated from the WS material by salt fractionation. Components were isolated that were insoluble in the range 50-75% w/v ammonium sulphate (AS75 fraction) and which constituted approximately 6.2% of WS. Also retained were those components that were non-precipitable in a saturated solution of the salt (ASS fraction), and which constituted approximately 13.8% of WS. (Fractions precipitable in the 0-50% w/v ammonium sulphate range and which account for 42% of the starting material have not been studied further because of the relatively low content of carbohydrate in these fractions [15]).

Anti-A. Fumigatus Conjugate

Anti-*A. fumigatus* rabbit IgG was purified by affinity chromatography on cyanogen bromide-activated Sepharose 4B (Pharmacia) which had been coupled to WS antigen [22]. The purified IgG was pooled and conjugated to horseradish peroxidase (Sigma Chemical Co., U.K.) according to the method of Avrameas [23].

Polyacrylamide Gel Electrophoresis (PAGE)

Vertical PAGE using gel slabs 1.5 mm thick with a separating gradient of 5-15% (w/v) polyacrylamide was performed according to the method of Laemmli [24]. Samples for analysis were taken up in "dissolving buffer" [2% SDS (w/v); 10% glycerol (v/v); 5% mercaptoethanol (v/v) plus a trace of bromophenol blue in 64 mM-Tris/HCl buffer, pH 6.8] and boiled for 3 min. before the application of a 50 µl aliquot to each well. Sodium dodecyl sulphate (SDS) was added at a concentration of 0.1% (w/v) to the tank buffer (Tris-glycine, pH 8.3), the separating gel buffer (Tris/HCl, pH 8.8) and to the stack gel (3.75% polyacrylamide) buffer (Tris/HCl, pH 6.8). Gels were subjected to electrophoresis at a current of approximately 30 mA and a final voltage of 190 V until the dye marker reached the bottom of the gel. High mol. wt. protein standards (Gibco) were run in parallel.

Protein and Glycoprotein Detection

Separated components were stained for protein using the Bio-Rad silver stain and counter-stained in 0.1% (w/v) Coomassie Brilliant Blue R-250 in 25% methanol (v/v), 7.5% acetic acid (v/v) for 1 h., at room temperature and destained in the same solvent without dye [25]. Component molecules were stained for carbohydrate by the periodic acid-Schiff (PAS) method using a commercial reagent (Sigma).

Electroblotting

Protein and glycoprotein molecules resolved by SDS-PAGE were transferred electrophoretically to nitrocellulose membranes (Hybond C, Amersham, UK) in a transblotting chamber

Table 1. Protocols used to study lectin binding to *Aspergillus fumigatus* fractions and the principal sugars which may be involved in these reactions.

Lectin	Principal Specificity	Reagent Used	Lectin Concentration (time)	Blockers[a]
Con A	α-D-Man*p*/α-D-Glc*p*	Peroxidase conjugate[b]	2.0 µg/ml (0.5 h.)	3% BSA/TBS
WGA	β-D-Glc*p*NAc/Sialic acid	Peroxidase conjugate	2.5 mg/ml (2 h)	2% PVP/TBS
PNA	β-D-Gal*p*-(1,3)-D-GalNAc	Peroxidase conjugate	10.0 mg/ml (2 h.)	3% BSA/TBS
APA	α-D-Fuc*p*	Peroxidase conjugate	10.0 mg/ml (2 h.)	3% BSA/TBS
LCA	α-D-Man*p*/α-D-Glc*p*	Biotin-labelled[c]	10.0 µg/ml (0.5 h.)	0.15% PBS/T-20
SBA	β-D-GalNAc/β-D-Gal*p*	Biotin-labelled	10.0 µg/ml (2 h.)	3% BSA/TBS

[a] All blots were routinely blocked for 1 h at 40°C except for Con A treatment, which was blocked for 1 h at room temperature and for LCA treatment, where blocking was done overnight at 4°C.

[b] The peroxidase substrate used in all cases was DAB

[c] Incubation with streptavidin-biotinylated peroxidase complex was for 1 h at room temperature at a dilution of 1:300 in PBS/T-20.

(LKB 2005 Transphor), using the method of Towbin *et al.*, [26]. Transfer was done in 25 mM Tris/192 mM glycine (pH 8.3) containing 20% methanol (v/v) at 80 V, 350 mA for 2 h. at 8°C. Efficiency of transfer was determined by staining blots with Indian ink, 0.1% (v/v) in PBS-Tween-20, as described by Hancock and Tsang [27], except the time of incubation was reduced to 1 h. The antigenic reactivity of the separated *A. fumigatus* fractions was determined by incubation of the transblotted material with anti-*A. fumigatus* IgG conjugated to peroxidase (1:25 dilution for 2 h at room temperature). Prior to this, free sites were blocked (1 h at 40°C) with 3% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS: 150 mM NaCl in 50 mM Tris/HCl, pH 7.5). After extensive washing, sites of binding of antibody and peroxidase conjugate were determined by development with 3, 3'-diaminobenzidine (DAB) in 50 mM Tris/HCl, pH 7.5 [0.4 mg/ml + 0.005% (v/v) H_2O_2].

Lectin Binding

Blots were air dried before saturation of free sites with an appropriate blocking agent which varied, depending on the lectin to be tested. The lectins were then applied in the blocking solutions at the concentrations and for the times specified in Table 1. After extensive washing, sites of binding of lectin-peroxidase conjugate were detected as above using DAB.

Lectins

Lectins were chosen as probes on the basis of their specificity for selected sugar residues, known to occur in *A. fumigatus* mycelium. Con A, wheat germ agglutinin (WGA), peanut agglutinin (PNA) and *Tetragonolobus purpurpeas* agglutinin, (asparagus pea, APA) were all peroxidase conjugates; *Lens culinaris* agglutinin (LCA) and *Glycine max* agglutinin (SBA) were biotin labelled. All lectins were purchased from Sigma. Lectin-peroxidase conjugates were detected with DAB. Biotin-labelled lectins were detected *via* a Streptavidin "bridge" obtainable as a preformed complex of Streptavidin linked to biotinylated horseradish peroxidase (Amersham), and the peroxidase detected with DAB.

Enzyme Treatment

Some samples were incubated with neuraminidase (EC 3.2.1.18: Type VI from *C. perfringens*, 1.25 units/ml, Sigma), prior to electrophoresis and treatment of blots with PNA or WGA. Incubation (2.5 vol sample : 1 vol enzyme) was for 2 h. at 37°C in 10 mM sodium phosphate buffer, adjusted to pH 5.0 with citric acid.

Protein Analysis

Total protein of the *A. fumigatus* fractions was estimated by the Coomassie Blue dye-binding method of Read and Northcote [28] with BSA as a standard.

Analysis of Amino-acid and Amino-sugar Composition

Samples were heated under nitrogen with 6 M HCl at 110°C for 24 h. Amino-acids in the BF, AS75 and ASS preparations were estimated on an amino-acid analyzer.

Iodine Coloration of Polysaccharides

Polysaccharides were dissolved in hot water, dilute aqueous iodine - KI solution was added and the iodine allowed to evaporate at room temperature. Iodine-glycogen complexes gave a red-brown colour.

Component Sugars of Polysaccharides

Polysaccharides were hydrolysed with 0.5 M H_2SO_4 for 18 h at 100°C, hydrolysates neutralized ($BaCO_3$), filtered and evaporated. Resulting monosaccharides were reduced with sodium borohydride and the alditols converted to their acetates. They were characterized and quantified, relative to each other by GLC according to the method of Sawardeker *et al* [29].

Methylation of Polysaccharides

Polysaccharides were methylated by the method of Haworth [30]. The partially methylated products were then isolated by dialysis and evaporation and completely methylated by the procedure of Kuhn *et al.* [31]. A solution of the per-*O*-methylated polysaccharide in methanol containing 3% HCl was refluxed for 18 h, the acid neutralized (Ag_2CO_3), the

Table 2. Amino-acid composition of *Aspergillus fumigatus* mycelial fractions[a]

Amino-acid	*A. fumigatus fractions*		
	BF (%)	ASS (%)	AS75 (%)
Aspartic acid	10.6	11.9	11.7
Threonine	14.5	5.9	4.4
Serine	13.2	7.4	6.3
Alanine	9.2	10.5	9.1
Glutamic acid	7.9	12.5	10.6
Glycine	7.9	21.8	18.5
Proline	6.6	8.4	8.8
Valine	5.3	3.1	4.3
Leucine	5.3	3.9	6.2
Cystine	0.3	0.4	trace
Methionine	0.8	1.2	0.8
Iso-leucine	4.0	2.3	3.9
Tyrosine	2.6	1.4	2.3
Phenylalanine	4.0	1.9	3.3
Histidine	1.3	1.6	2.1
Lysine	4.0	3.3	4.2
Arginine	2.6	2.5	3.4
Glucosamine	trace	trace	trace
Galactosamine	N.D.[b]	trace	trace
Total amino-acid (μmol/mg fraction)	0.76	8.13	8.55

[a] Expressed as % of the total recovered amino-acids from each fraction.

[b] N.D. = Not detected.

suspension filtered and the filtrate evaporated to a syrup. This was completely hydrolysed with 10% aqueous H_2SO_4 for 18 h at 100°C, the solution neutralized with $BaCO_3$, filtered and evaporated. The product was reduced with sodium borohydride and acetylated. Partially *O*-methylated alditol acetates were identified by use of a GLC unit coupled to a mass spectrometer in the electron impact mode, using the method of Björndal *et al.* [32]. The GLC was equipped with a 30 m x 0.25 mm glass capillary column coated either with a blend of OV-17 + OV-225 (3:1) or with OV-225. These were programmed from 50°C (40°C/min) to 182°C (hold) as described by Barreto-Bergter *et al.* [33].

Partial Acetolysis

This procedure was performed as described by Lee and Ballou [34]. The sugars obtained on *O*-deacetylation were examined by paper chromatography with *n*-butanol/ethanol/water, 2/1/1 by vol, and the spots detected with *p*-anisidine hydrochloride and ammoniacal silver nitrate sprays.

Table 3. Composition of fractions of *Aspergillus fumigatus* mycelium.

Fraction[a]	P/C[b] ratio	Monosaccharide (%) D-Man	D-Gal	D-Glc	Specific rotation $[\alpha]_D^{25°}$(c.0.4 H_2O)
BF	0.07	35	13	52	+68°
AS75	2.50	39	16	46	N.D.[c]
ASS	0.47	68	24	8	+13°

[a] Fractions were obtained as described in the text.

[b] Protein:neutral polysaccharide ratios (adjusted to the base of 1).

[c] Not determined.

^{13}C-NMR Spectroscopy

Spectra were obtained as previously described [35] from polysaccharide samples dissolved in deuterium oxide (2 ml) at 70°C in a 12 mm diameter tube. Chemical shifts are expressed as δ in ppm relative to an external standard of tetramethylsilane, the resonance of which was determined in a separate experiment.

Results

Amino-acid Composition

The amino-acid composition of the BF, AS75 and ASS fractions prepared from WS material is shown in Table 2. The content of the acidic amino acids, glutamic and aspartic acids, together with alanine, is high in all fractions. The predominant amino-acids in the BF fraction are serine and threonine while glycine is the major component of the AS75 and ASS fractions.

Carbohydrate Composition of A. fumigatus Fractions

The protein/carbohydrate ratios of BF, AS75 and ASS fractions of *A. fumigatus* are detailed in Table 3. Their neutral sugar composition and specific rotation measurements are also shown. While glucose is the predominant sugar in the BF and AS75 fractions, mannose is the major component of ASS. Analyses of the *O*-methylalditol acetates derived *via* methylation analysis of the two carbohydrate-enriched fractions of *A. fumigatus* are shown in Table 4. As the AS75 fraction contains relatively little carbohydrate, the methylation data are not highly significant.

BF Antigen

As indicated by the methylation data (Table 4), predominant structures include non-reducing end groups (26%) and 2-*O*-substituted units (21%) of mannopyranose. The large

Table 4. Percentages of *O*-methylalditol acetates obtained from fractions of *A. fumigatus* mycelium.

O-Methyl alditol acetate	Mycelial Fraction[a]	
	BF	ASS
2,3,4,6-Me_4-Man	26	
2,3,5,6-Me_4-Gal	4	23
2,3,4,6-Me_4-Gal		1
2,3,4,6-Me_4-Glc	0	2
3,4,6-Me_3-Man	21	35
2,3,6-Me_3-Gal	2	10
2,3,5-Me_3-Gal		1
2,3,4-Me_3-Man	1	0
2,3,6-Me_3-Glc	31	11
3,4-Me_2-Man	6	18
2,3-Me_2-Gal	6	
2,6-Me_2-Glc	1	

[a] Peak areas (% of total) of *O*-methylalditol acetates.

proportion of 4-*O*-substituted units of glucopyranose (31%) is evidence for the presence of a (1-4)-linked glucan; that glycogen may be present is indicated by the formation of a red-brown colouration on treating BF with iodine solution.

The ^{13}C-NMR spectrum of BF (Fig. 1a), revealed a surprisingly complex C-1 region with signals at δ =109.5, 108.5, 107.6, and 106.9 corresponding to four different -D-Gal*f* structures. These may be attributed respectively to β-D-Gal*f*-(1-6)-α-D-Man*p*, β-D-Gal*f*-(1-5)-β-D-Gal*f*-, and β-D-Gal*f*— units linked (1-2) and (1-3) to α-D-Man*p* units [36, 37].

Partial acetolysis produced mannobiose and mannotetraose with α-(1-2)-linkages, whose structures are consistent with their mobilities on paper chromatograms and their stability to the degradation procedure. The C-1 signal of BF at 102.2 confirms these structures, but elucidation of the nature of the non-reducing end group could not be made.

Structure 1: α-D-Man*p*-[(1-2)-α-D-Man*p*]$_n$

δ =103.7 δ =102.2

Although Structure 1 is common in fungal polysaccharides, it is present here in only small quantities as indicated by the small signal at 103.7 [38]. Thus, most of the 26% of non-reducing end units of mannopyranose, determined by methylation analysis, must arise from other structures.

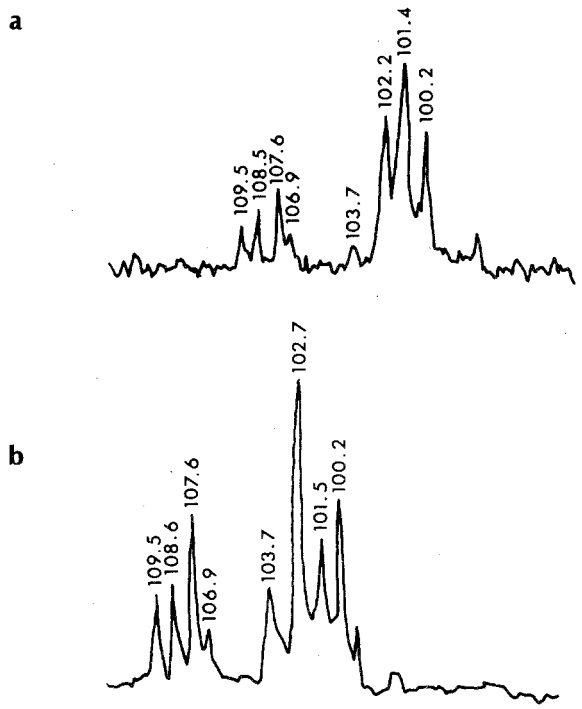

Figure 1. C-1 region of ^{13}C-NMR spectrum in $^{2}H_2O$ at 70°C of *A. fumigatus* fractions. a) BF fraction; b) ASS fraction. Numerical values are in ppm.

ASS Antigen

The methylation and ^{13}C-NMR data are shown in (Table 4 and Fig. 1b, respectively). The major difference between this fraction and the BF fraction resides in the ASS showing an elevated proportion of non-reducing end groups of galactofuranose and an absence of those of mannopyranose. The conjunction of non-reducing end groups (2%) and 4-*O*-substituted units (11%) of glucopyranose suggest the presence of a glycogen-like structure. ASS (with a glucose content of 8%), did not give a colouration with iodine solution comparable to that of BF (glucose content = 52%).

AS75 Antigen

Using the usual weight of sample for a ^{13}C-NMR analysis, no carbohydrate signals were detected for the AS75 fraction of *A. fumigatus*. Nevertheless, analysis of the methylated derivatives of this fraction was attempted but proved unsatisfactory in that the proportion of galactose derivatives observed was much greater than that present in the original hydrolysate. The predominant constitutents were 3,4,6-tri-*O*-methylmannitol acetate (22%) and 2,3,6-tri-*O*-methylgalactitol acetate (53%); 2,3,4,6-tetra-*O*-methylmannitol acetate (10%) and 2,3,5,6-tetra-*O*-methylgalactitol acetate (5%) were also detected.

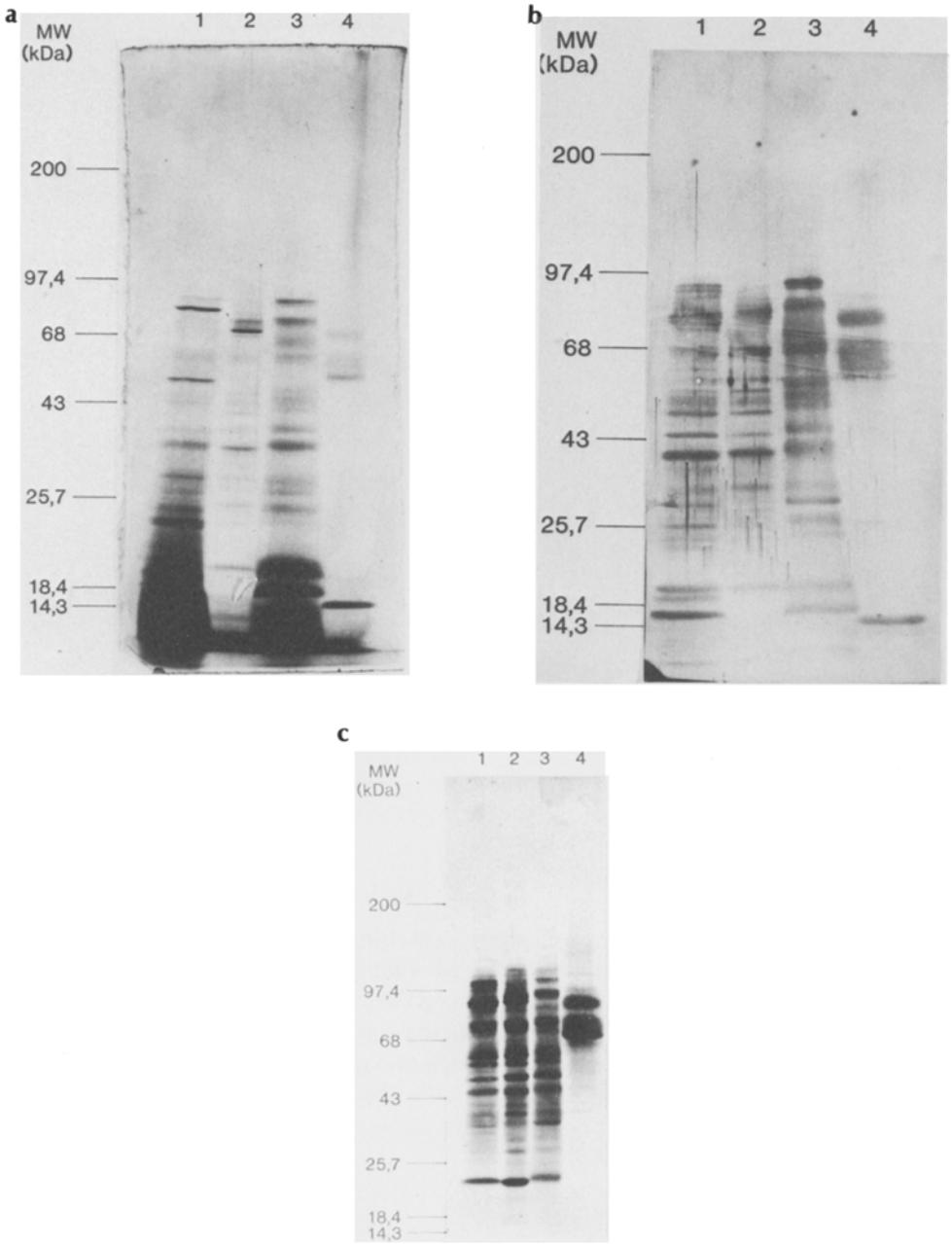

Figure 2. SDS-PAGE on a 5-15% gradient gel of water-soluble fractions from *A. fumigatus* mycelium.
a) The gels were subjected to the combined silver-Coomassie Blue-staining procedures; b) separated components were transblotted and stained for protein with Indian ink; c) transblotted sub-units were probed with a rabbit anti-Aspergillus, affinity purified IgG fraction which had been coupled to horseradish peroxidase. Binding was visualised with diaminobenzidine substrate. Lanes: 1, WS; 2, BF; 3, AS75 and 4, ASS. Molecular weights of protein standards are shown in the left margin.

Gel Electrophoresis

The components of *A.fumigatus* water-soluble material and of three sub-fractions were separated electrophoretically by SDS-PAGE and stained for protein with a combined silver stain and Coomassie Blue reagent (Fig. 2a). Bands in these samples migrated with apparent molecular weights (M_r) in the range 10-100,000. The WS and AS75 preparations showed more than 30 bands, the BF fraction more than 20 bands and the ASS fraction only 5-6 relatively high M_r moieties and one with an apparent M_r of 14,000. While the smaller molecules present in the WS and AS75 preparations gave intense staining with the silver reagent, some of the larger molecules and also the bulk of the components present in the BF fraction, bound only to Coomassie Blue. This can be readily seen in a coloured photographic print of the gel but is not visible in black and white as reproduced here. Very little staining of any of the preparations was seen with PAS.

Electroblotting

Proteins and glycoproteins transferred from PAGE to nitrocellulose membranes were stained with Indian ink [27]. The band profile observed with each fraction was similar to that seen with Coomassie Blue and silver stained gels, indicating an adequate transfer of both high and low molecular weight species from the gels to the nitrocellulose (Fig. 2b).

Detection with Rabbit Anti-A. fumigatus Antibodies

Each of the *A. fumigatus* fractions showed a complex array of bands when probed with an anti-*A. fumigatus* IgG preparation conjugated to peroxidase (Fig. 2c) [22]. The band profile observed with each fraction when compared with protein staining covered a similar range of M_r units, only the lower M_r constituents (<20,000), showing a lack of antigenic activity.

Detection with Lectins

The binding of the *A. fumigatus* preparations by Con A is shown in Fig. 3a. Detection of many lectin staining bands in each fraction, indicated the glycoprotein structure of the separated molecules. Profiles obtained with Con A were somewhat similar, but not identical to those seen with Coomassie Blue. Lectin staining was virtually abolished when methyl α-D-mannopyranoside was included in the incubation mixture (results not shown). WGA-binding capacities towards *A. fumigatus* antigens were similar to those obtained with Con A (Fig. 3b). Inclusion of *N*-acetylglucosamine in the system gave some reduction in lectin binding (results not shown). While SBA bound to many high and low molecular weight molecules present in *A. fumigatus* fractions, binding was much less than that with WGA (results not shown). An *A. fumigatus* UBF fraction which did not bind to Con A Sepharose gave a negative result when probed on blots with Con A conjugated to peroxidase. It also failed to bind to WGA (Fig. 3b). The most intense binding reactivities to LCA were given by the high molecular weight constituents of each fraction (Fig. 3c). Only the low molecular weight components of WS showed any substantial binding to APA (Fig. 3d). No binding to PNA was seen with any of the preparations. Pre-treatment with neuraminidase gave no increase in PNA binding while WGA binding remained unaltered, following similar enzyme treatment.

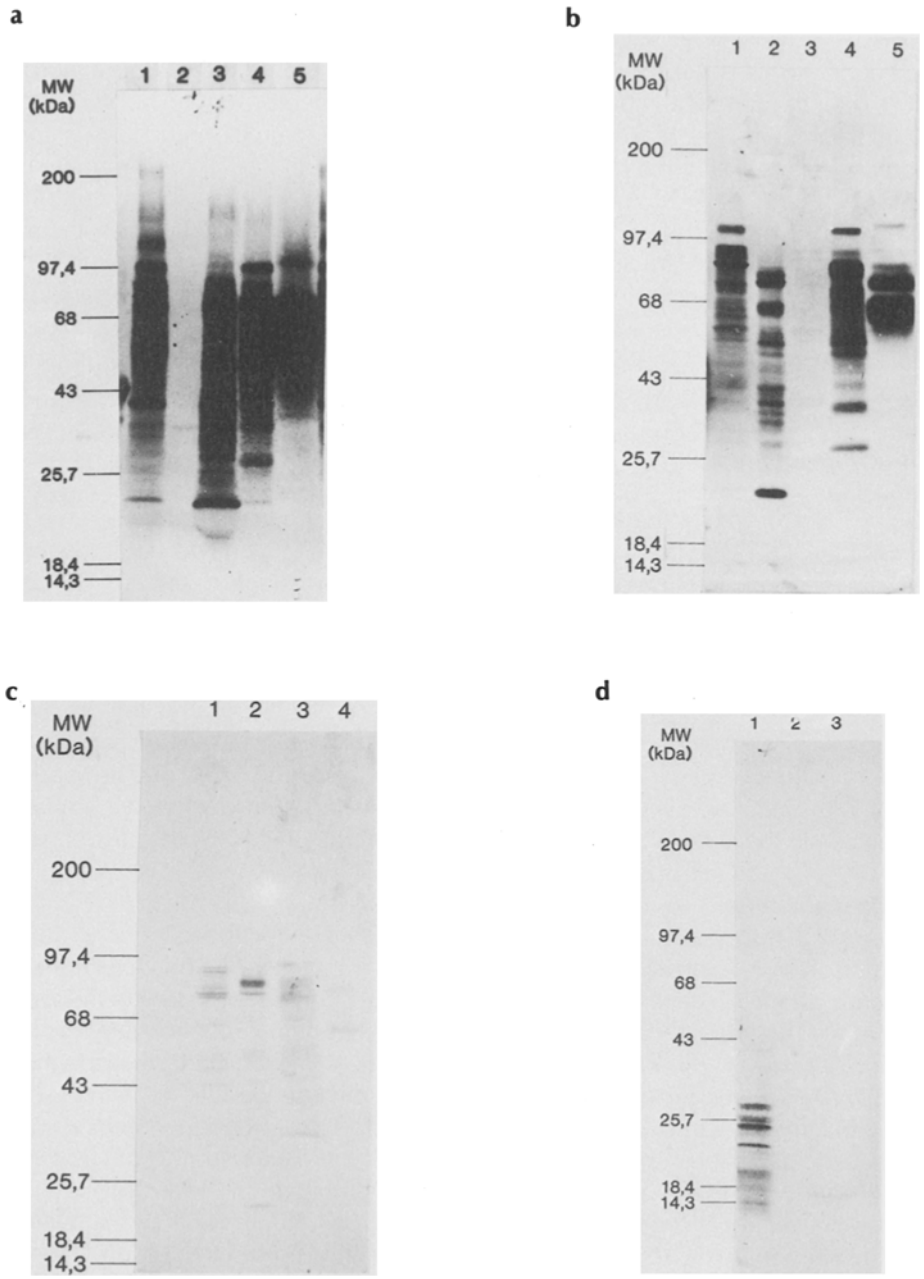

Figure 3. *A. fumigatus* water-soluble mycelial fractions separated by SDS-PAGE and transferred to nitrocellulose for detection of carbohydrate residues with specific lectins. (a) Con A-peroxidase reagent. Lanes: 1, WS; 2, UBF; 3, BF; 4, AS75 and 5, ASS. (b) WGA-peroxidase reagent. Lanes: 1, WS; 2, BF; 3, UBF; 4, AS75 and 5, ASS. (c) LCA-biotin reagent. Lanes: 1, WS; 2, BF; 3, AS75 and 4, ASS. (d) APA-peroxidase reagent. Lanes: 1, WS; 2, AS75 and 3, ASS. Molecular weights of protein standards are shown in the left margin.

Discussion

GMs of related, but not identical, molecular structure have been isolated by a variety of procedures from Aspergillus wall and cytosol [7, 37, 39, 40]. In one instance, a fraction prepared by pyridine extraction of mycelium was characterized as a GM-protein with immunological activity [41]. Current evidence implicates the Gal*f* residues of GM structures as the immunodominant sugar [7, 39, 40, 42]. It is of interest to establish whether any of the fractions described here contain GMs, either free or complexed to protein molecules, and how closely these molecules are related to those already characterized.

Polysaccharides obtained by hot, aqueous alkali extraction of *A. fumigatus* have previously been found to contain a galactomannan [37]. Its main chain was found to consist of (1-6)-linked α-D-mannopyranosyl units, substituted at O-2 by one, two and three consecutive α-D-mannopyranosyl residues which are (1-2)-interlinked. This contrasts with BF which contains one, two and four of such structural units. The mannan core of the alkali extract was also substituted at O-6 by as many as six units of β-D-galactofuranose which are (1-5)-interlinked. Such structures were represented by C-1 NMR signals at 109.5 and 108.5, respectively. While these signals are present in ^{13}C-NMR spectra of BF and ASS, that of 108.5 is smaller than that of the alkaline extract, indicative of relatively short β-D-Gal*f*-(1-5)-linked side chains. In this respect it resembles more closely the GM isolated by Reiss and Lehmann [7] which is reported to have short β-D-Gal*f* side chains and which also contains a substantial amount of terminal, non-reducing mannose residues. The marked complexity of β-D-Gal*f*-containing structures in BF is indicated by the presence in the ^{13}C-NMR spectrum of two additional signals at 107.5 and 106.9, probably arising from oligosaccharides linked to protein. As glycoproteins would have been converted, under alkaline conditions to low mol. wt. structures, not precipitable with ethanol, these signals are absent from the spectrum given by this extract [37].

Glucans have also been isolated from Aspergillus [7, 41, 43, 44]. A glucan with a glycogen-like structure has been described; it was co-extracted from *A. fumigatus* with GM [37]. Fragments corresponding to non-reducing end groups were not detected in a methylation analysis of the BF fraction, despite its high glucose content (52%) and a typical iodine colouration for glycogen. However, with the GLC coating used, acetates of 2,3,4,6-tetra-*O*-methylglucitol and mannitol are not easily resolved. The amount of 2,3-di-*O*-methyl glucose arising from glycogen is small (approximately 6%) and disaccharide residues are readily destroyed under the harsh acid conditions used in the procedure.

The ASS material did not react positively with iodine, however the glucan constitutes a very small proportion of this fraction (8% glucose). In this instance 2,3,4,6-tetra-*O*-methylglucitol acetate was detected in ASS. AS75 contained 46% of glucose, but this largely disappeared on methylation analysis, possibly because it was present predominantly in low molecular weight structures. Only 9% of 2,3,6-tri-*O*-methylglucitol acetate, 2% of 2,3,4,6-tetra-*O*-and 4% of 2,3-di-*O*-methyl derivatives were detected. These data are again possibly indicative of a glycogen-like structure.

A ^{13}C-NMR signal at δ =55 corresponds with the C-2 of hexosaminyl residues. Its absence demonstrates that these sugar units are, at most, present in only trace amounts in the BF and ASS fractions (Figs. 1a and b). This was also the finding from the analysis of the amino-acids

and amino-sugars (Table 2). This makes it unlikely that the link between carbohydrate and protein is an aspartamide-amino sugar linkage, despite the relatively high proportion of aspartic and glutamic acids present in all fractions. These (especially the BF), because of their high content of serine and threonine may resemble other glycoproteins in which a large number of short carbohydrate chains are linked glycosidically to the hydroxyl group of these residues in a peptide backbone. Hydroxy-amino-acids may be linked glycosidically to oligomannosyl residues as components of galactomannan-proteins; glucan-proteins may also occur. The peptidogalactomannan extracted by Azuma *et al* [41] from *A. fumigatus* mycelium had serine and threonine as the dominant amino-acids.

SDS-PAGE revealed a complex array of molecules in fractions obtained from *A. fumigatus* mycelium. Their protein moieties could be differentiated on the basis of their chemical reactivities towards the Coomassie Blue stain, which bound predominantly to BF components, and the silver reagent, which bound selectively to the AS75 and ASS fractions, especially their low M_r constituents. Reactivity towards Coomassie Blue is strongly indicative of the presence of the basic amino-acids; hydrophobic and aromatic amino-acids show slight reactivity [45]. Proteins without cysteine residues have been reported to stain negatively with the silver reagent [46]. The apparent absence of some of these amino-acids in the parent fractions may be due to their chemical destruction, under the conditions used for hydrolysis.

PAS, the generalized carbohydrate stain, gave little colour with *A. fumigatus* fractions separated on SDS-PAGE. It should be borne in mind that gel patterns seen on SDS-PAGE may not be strictly representative of the total preparation in that any uncharged polysaccharides present will not enter the gel. Thus the detection of *N*-acetyl-D-glucosaminyl residues, indicated by the WGA-binding experiments, may represent an enrichment of this sugar in the electrophoresed fractions. The results do not necessarily contradict the GLC-MS. and amino-acid/sugar analysis which were performed on the whole sample.

That *N*-acetyl-D-glucosamine, not sialic acid, was bound by this lectin was indicated by partial inhibition of binding by this sugar and by the failure of neuraminidase to reduce WGA-binding to these fractions. That the overall amounts of sialic acid in the fractions must be low is supported by the non-detection of signals corresponding to *N*-acetyl and methylene groups. High and low-molecular weight components of these fractions also bound to SBA with affinity for *N*-acetyl-D-galactosaminyl and/or β-D-galactosyl residues. However, the reaction was much weaker than that with WGA. The hexosamine detected most likely represents a very small proportion of total carbohydrate of BF, AS75 and ASS. Con A was found to bind readily to a wide range of molecules present in each fraction. LCA, which has a primary sugar requirement similar to Con A has, in addition, a preference for mannose attached in an amino-sugar-asparagine structure [47]. This lectin bound to far fewer components of *A. fumigatus* fractions than did Con A.

The bulk of the evidence presented here indicates that the polysaccharides isolated from the water-soluble fraction of *A. fumigatus* mycelium are predominantly galactomannans and glucans, some of which are presumably complexed to protein moieties and are separable on SDS-PAGE. Many of these glycoproteins proved antigenically reactive when tested with a specific anti-*A. fumigatus* IgG.

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