Detection of Low- M_r Glycoproteins in Surface Washes of Some Fungal Cultures by Gel-filtration HPLC and by Monoclonal Antibodies

By M. M. MACDONALD,† R. H. DUNSTAN‡ AND F. M. DEWEY*

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RA, UK

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Surface washings were prepared from cultures of fungi grown on agar slants and the wash components were fractionated by gel-filtration HPLC. A range of low- M_r glycoproteins (<10000) were detected in all the washes, and in some species several high- M_r glycoprotein components (>150000) were also present. The HPLC analyses indicated species-specific differences in the profiles. Regions of specific antigenicity within profiles were detected by monoclonal antibodies.

INTRODUCTION

Little is known at a molecular level about the outermost surfaces of fungal hyphae and the processes involved in cell recognition and interaction which must take place at or near their surfaces (Callow *et al.*, 1987; Evans & Cooke, 1982). This is in contrast to the extensive studies on the surface nature of medically important bacteria and viruses which have led to the identification of many of their surface compounds and to a further understanding of how they stimulate host responses (Hadley, 1986; Brubaker, 1985; Roantree, 1967).

Early studies of fungal surface compounds concentrated on the chemical nature of well-washed mycelial walls (see Taylor & Cameron, 1973) whereby any loosely associated compounds on the surface were missed. More recent studies have been concerned with the ability of extracts from heat-treated fungal walls and molecules secreted by fungi in liquid media to elicit host responses (Ebel, 1986; Dixon, 1986); however, very little specific information is available on the biochemistry of these compounds.

To understand fully how fungi initiate infection in potential hosts, or trigger defence mechanisms in non-hosts and other micro-organisms, the surface compounds from the mycelia must be collected in a biologically active form from non-submerged fungi growing on a medium that as far as possible approximates their natural environment, such as a solid plant-based medium.

In the accompanying paper Dewey *et al.* (1989*a*) described the collection of surface molecules from fungal cultures grown on solid media by washing the mycelia with phosphate-buffered saline (PBS). These fresh fungal washes were then successfully used to raise monoclonal antibodies (MAbs), some of which were species-specific. This indicates that loosely associated water-soluble surface molecules are present on the mycelial surface and that some of these molecules are characteristic of the species. Further investigation of these wash components might have been made either by iso-electrophoresis and SDS-PAGE under reducing and non-reducing conditions, or by reverse-phase HPLC. However, these methods have limitations, since some molecules are inevitably changed during the analysis procedure (resulting in potential loss of biological activity) and is not possible to determine whether low- M_r

† Present address: Biotechnology/Research State Chemistry Laboratory, East Melbourne, Victoria 3052, Australia.

‡ Present address: Department of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia.

Abbreviations: PBS, phosphate-buffered saline; PBST, PBS with 0.05% Tween 20; PVDF, polyvinylidene difluoride; TCMC, tissue culture medium control.

compounds observed are natural metabolites produced by the fungus *in vivo* or are products of degradation that have been produced *in vitro* by the experimental conditions.

Gel-filtration HPLC was therefore chosen for this investigation since it allows the separation of sample components in an aqueous mobile phase that closely resembles the surface-wash preparation buffer utilized as the immunogen. The aim of this study was to gain more comprehensive information about the range of surface proteins, glycoproteins and polypeptides which may be present in the fungal washes and to determine which fractions, if any, contained species-specific antigens. The fungi used in this study were those to which genus- and species-specific MABs had already been raised (Dewey *et al.*, 1989*a*, *b*).

METHODS

Culture and growth of fungi. All fungi, both those to which MABs had been raised previously and other test fungi used in this study are listed in Table 1, together with the type of growth medium and incubation temperatures. Each species was grown on a medium and at a temperature that supported good growth. All cultures were grown on slants in 30 ml Universal tubes containing 10 ml agar per tube.

Preparation of surface washings. All fungal cultures were washed briefly with 1 ml PBS (0.8% NaCl, 0.02% KCl, 0.115% Na₂HPO₄, 0.02% KH₂PO₄, pH 7.2). To help wet the hydrophobic surface of each culture, the mycelial mass was gently stroked with a sterile plastic pipette tip. The resulting fluid, approximately 900 µl in volume, was removed by suction, placed in an Eppendorf tube and spun in a Microfuge for 2 min at 13250 *g* to remove cell debris. Supernatants were then either analysed by HPLC as described below or, in the case of *Humicola lanuginosa* and *Penicillium islandicum*, were used immediately as the immunogen without concentration or freezing. These surface washings contained 0.01–1.5 mg protein ml⁻¹ as determined by the micro-Lowry method or protein blot (Table 2, Fig. 1).

Immunization protocol. Female Balb/c mice (6-week-old) were given five intraperitoneal injections of 300 µl of the immunogen freshly prepared for each injection. For mice immunized with *Ophiostoma ulmi*, a suspension of mycelial fragments in PBS (see Dewey & Brasier, 1988) was used but for both *H. lanuginosa* and *P. islandicum* PBS surface washings were used directly (Dewey *et al.*, 1989*a*). Mice were bled from the tail vein 4 and 5 d after injections.

Production of hybridomas. Details of the methods used to raise hybridoma cell lines are reported elsewhere (Dewey *et al.*, 1989*a*, *b*). The protocol used was essentially the same as that described by Kennet *et al.* (1978), which was based on a modification of the original method pioneered by Kohler & Milstein (1975). Cells of the myeloma cell line SP2/0-Ag-14 were fused with splenocytes from an immunized mouse by centrifugation in 33% (w/v) PEG 1500. Fusion products were diluted into 50 ml medium and plated out into ten 96-well trays, at 50 µl per well. Hypoxanthine azaserine (HAZA) was used as a selective medium (Foung *et al.*, 1982).

ELISA. Methods used were based on Dewey & Brasier (1988). All test volumes were 50 µl per well and all solutions were prepared in glass or polypropylene tubes. Wells were coated with antigen solution overnight at 4 °C, washed four times with PBST (PBS with 0.05% Tween 20), once with PBS and rinsed briefly with distilled water. Plates were air-dried in a laminar flow hood and used immediately for assay.

Antigens were either fractions representing peaks of HPLC profiles of surface washings, collected, stored on ice and frozen within 1 h of collection, or surface washings (i.e. immunogen) diluted 1:20 in PBS and plated out immediately.

The primary antibodies were the undiluted supernatants of MABs raised against fungal surface washings and, in the case of *O. ulmi*, mycelial homogenates (Dewey *et al.*, 1989*b*). Tissue culture medium and antiserum diluted 1:1000 into PBST were taken as negative and positive controls respectively.

Secondary antibody conjugate was Sigma goat anti-mouse polyvalent immunoglobulin (IgM + IgG) horseradish peroxidase conjugate (Sigma no. A 0412) diluted 200-fold into PBST. The substrate, tetramethylbenzidine, was prepared as a ×10 stock solution in DMSO and freshly diluted into sodium acetate buffer, pH 5.4, before each assay. Details of the ELISA method and periodate treatment of antigens are given in Dewey *et al.* (1989*a*).

Protein assays. (i) *Micro-Lowry.* The method used is based on Lowry's original method but has been adapted for use in disposable microtitre wells (see Dewey *et al.*, 1989*a*). BSA (Sigma no. A 7030) at doubling dilutions from 8 mg ml⁻¹ in PBS was used as a standard.

(ii) *Protein dot-blot.* A highly sensitive blot method was developed that required only 3 µl of sample. It is an adaptation of the method recommended by Janssen Life Science Products for staining protein bands on Western blots with their protein-specific stain Auro Dye forte. A new polyvinylidene difluoride membrane (PVDF) from Millipore was used instead of nitrocellulose. With this assay system we were able to detect as little as 8 µg BSA ml⁻¹. The membrane was first wetted in AnalaR grade methanol for 2–5 s, and then washed in AnalaR water for 5 min. Excess water was removed by gentle shaking and the membrane was laid in a clean Petri dish. Surface

Table 1. *Source of fungal isolates and growth conditions*

Isolate	Subgroups	Code No.	Source	Culture routine	
				Medium	Temp. (°C)
<i>Ophiostoma ulmi</i>	Non-aggressive	R21	Infected elm, Bozovici, Romania. C. M. Brasier, 1980	MEA	22
	Non-aggressive	P32	Infected elm, Kolbiel Park, Poland. C. M. Brasier, 1980	MEA	22
	NAN aggressive	0160	Infected elm, Orsett, Essex, UK. C. M. Brasier, 1983	MEA	22
	NAN aggressive	H175	Infected elm, Lyons Falls, New York, USA. J. N. Gibbs, 1977	MEA	22
	NAN aggressive	H160	Infected elm, Jersey, New Brunswick, Canada. J. N. Gibbs, 1979	MEA	22
	NAN aggressive	H623	Infected elm, Jersey. N. J. Bradshaw, 1981	MEA	22
	EAN aggressive	H328	Infected elm, Charkov, USSR. H. M. Heybroek, 1979	MEA	22
	EAN aggressive	H323	Infected elm, Palarikova, Czechoslovakia. J. Jamnicky, 1979	MEA	22
	EAN aggressive	H327	Infected elm, Brezno-Nike, Czechoslovakia. J. Jamnicky, 1979	MEA	22
	EAN aggressive	H648	Infected elm, (<i>Ulmus fulva</i>), Botanic Garden, Uzbek Academy of Sciences, Tashkent, USSR. H. Kryukova, 1981	MEA	22
<i>Ophiostoma piceae</i>		H920	Infected elm, Quercus, Chiddingfold, Surrey, UK. J. N. Gibbs, 1985	MEA	22
<i>Ophiostoma piceae</i>		H926	Infected elm, (felled <i>Ulmus carpiniifolia</i>), Rubena, Spain. J. Weber, 1984	MEA	22
<i>Ophiostoma piceae</i>		H916	Infected elm, Picea, France. M. Magema, 1976	MEA	22
<i>Ceratocystis adiposa</i>		A35	University of Bristol culture collection. C. Hawes	MEA	22
<i>Humicola lanuginosa</i>		—	Rice grain, Indonesia. S. Phillips	YSA	45
<i>Pseudocercospora anguioides</i>		—	Wheat stems, Berlin, FRG. Nirenberg	V8 1/10	18
<i>Pseudocercospora herpotrichoides</i>		—	Wheat stems, Berlin, FRG. Nirenberg	V8 1/10	18
var. <i>herpotrichoides</i>		—	Wheat stems, Berlin, FRG. Nirenberg	V8 1/10	18
var. <i>acuformis</i>		—	Wheat stems, Berlin, FRG. Nirenberg	V8 1/10	18
<i>Penicillium islandicum</i>		—	Rice grain, Indonesia. S. Phillips	MEA	37
<i>Penicillium</i> sp.*		X8	Rice grain, Indonesia. S. Phillips	MEA	37
<i>Penicillium waksmanii</i>		—	Red seaweed, <i>Eucheuma striatum</i> , Ponape, East Caroline Islands. F. M. Dewey	MEA	22

* New species of *Penicillium* not yet named (S. Phillips *et al.*, unpublished).

washings, HPLC fractions or BSA standards were applied as 3 µl droplets and allowed to air-dry for 15 min. The membrane was rewetted in methanol for 2 min, washed for 30 min in PBST and thereafter in three, 5 min changes of PBST. Incubation in Auro Dye forte for 1½–2 h was followed by several washes in AnalaR water. The membrane was then dried between filter paper. Protein values of test solutions were determined by comparing by eye test droplets with droplets from standard solutions. A control dilution series of BSA in PBS was always done at the same time using concentrations of 1000 µg ml⁻¹ to 5 µg ml⁻¹ (see Fig. 1).

Gel-filtration HPLC. The surface components of the fungal wash preparations were separated using a Waters 600 multisolvent delivery HPLC system in conjunction with a Waters UV/VIS variable wavelength LC spectrophotometer (model 455) and a Waters 740 data module. The HPLC system was fitted with a Waters Protein Pak 300 SW column (7.5 × 300 mm) and a flow rate of 0.75 ml min⁻¹ was used. The LC spectrophotometer had a 10 µl flow cell and the wavelength was set at 280 nm. All samples were thoroughly mixed for 20 s using a Fisons Whirlmix and centrifuged at 13250 g for 2 min in a Sorval Microspin 125 bench centrifuge prior to injection. A 20 µl injection loop was fitted to the rheodine injector and an injection volume of 30 µl was used. The mobile phase contained 1.15 g Na₂HPO₄ · 12H₂O l⁻¹ and 0.2 g KH₂PO₄ l⁻¹ in water (pH 7.2). The *M_r* values of the various sample components were calculated on the basis of a series of nine protein standards, ranging in *M_r* from 2512–150000, as well as blue dextran (*M_r* 2000000) and guanosine (*M_r* 283).

Chemicals. Unless otherwise stated, all chemicals were obtained from Sigma or BDH. The water used for HPLC was BDH HiPerSolv, HPLC grade, and was filtered immediately prior to use through a Millipore Durapore filter (PVDF) with a mean pore size of 0.22 µm.

RESULTS

Total protein levels measured in some of the wash preparations by the micro-Lowry or protein dot-blot methods are given in Table 2 and Fig. 1.

Gel-filtration HPLC of washes from the fungi tested indicated that each wash had a species-specific profile (Fig. 2). The washes from the various strains of *O. ulmi* could not, however, be distinguished by this technique. Analyses of these strains indicated that there were eight main groups of compounds which could be classified on the basis of their apparent *M_r* values (Table 2). (The components detected in the profiles of the other species generally corresponded to this classification with some obvious qualitative differences.)

The *M_r* fractions which reacted with the species-specific MAbs are also shown in Fig. 2. Control experiments indicated that there were no cross-reactions of these antibodies with components in washes prepared from the respective uninoculated agar slopes. Protein dot-blot analyses of the antigenic fractions from *H. lanuginosa* (i.e. fractions 1, 2, 3, 4 and 7) and *P. islandicum* (i.e. fraction 7) indicated that all these fractions contained protein components. Autoclaving at 15 p.s.i. (≈103.5 kPa) for 20 min destroyed the antigenicity of fractions 1 (partially), and 3, 4 and 7 (totally) from the *H. lanuginosa* wash (see Table 3 and Fig. 3) and fractions 2 and 3 from the *O. ulmi* (NAN 0160) wash. Fractions 1 and 2 contained some heat-stable components. Specificity for carbohydrate was tested by periodate oxidation of *H. lanuginosa* fractions before probing with MAbs. Binding of specific MAbs was affected to differing degrees by periodate treatment (Table 3). The periodate treatments of the antigenic components indicated, by the consequent loss of antigenicity, that all contained carbohydrate moieties. The sodium periodate data, susceptibility of some antigens to heat treatment, absorbance at 280 nm and detection by the specific dot-blot protein assays suggest that the respective antigens in these fractions are glycopeptides or peptidoglycans.

To determine whether these low-*M_r* compounds are normally present on fungal surfaces or are artefacts produced by degradation during the course of the experiment, the *H. lanuginosa* washes were injected for analysis by HPLC (a) immediately after preparation, (b) after incubation at 4 °C for 4 h and (c) after heat treatment by autoclaving (see Fig. 3). The absence of significant changes in the profiles following incubation at 4 °C for 4 h indicated that the wash preparations were not susceptible to either enzyme-catalysed breakdown (e.g. protease activity) or chemical degradation during this time period. Washes were also incubated at 24 °C for 4 h and 26 h; these treatments resulted in no changes in the HPLC profiles. The heat treatment caused some changes in peak shape in fractions 1 and 2 (presumably by denaturation) indicating that some of the high-*M_r* components were heat-sensitive.

Fig. 1. Protein DOT-BLOT on Millipore membrane Immobilon II processed with Janssen Auro Dye forte. Upper two rows (standards): 3 μ l droplets of a dilution series of BSA in PBS starting at (1) 1000 μ g ml⁻¹ and finishing at (10) 1.8 μ g ml⁻¹. Lower two rows (samples): 3 μ l droplets of undiluted PBS surface washings of solid slant cultures of (a) *Ophiostoma ulmi* non-aggressive P32, (b) *O. ulmi* non-aggressive R21, (c) *O. ulmi* EAN H328, (d) *O. ulmi* NAN 0160, (e) *O. piceae* H916, (f) *O. piceae* H920 and (g) *Ceratocystis adiposa* A35.

Table 2. Classification by apparent M_r of surface molecules detected in washes of selected fungi

ND, Not determined; *, large peak detected; +, detection of a peak; (), very small peak detected.

		M_r classification of fractions 1-8							
		8	7	6	5	4	3	2	1
		Protein (mg ml ⁻¹)	1300- 1800	3400- 3700	4400- 4600	5500- 5700	6200- 6500	7500	<150000
<i>Ophiostoma ulmi</i>									
NAN 0160	0.25	+	1700	3500	4400	5500	6300	+	+
NAN H175	ND	+	1600	3500	4500	5600	6300	+	+
NAN 0106	ND	+	1600	3500	4500	5600	6300	+	+
NAN 623	ND	+	1600	3600	4500	5500	6200	+	+
EAN H328	0.25	+	1500	3600	4500	5500	6300	+	+
EAN H323	ND	+	1600	3500	4400	5500	6200	+	+
EAN H327	ND	+	1600	3500	4500	5500	6200	+	()
EAN H648	ND	+	1700	3500	4500	5500	6200	+	+
R21 (non-aggressive)	0.25	+	1700	3500	4500	5500	6200	+	()
P32 (non-aggressive)	ND	+	1700	3500	4400	5500	6200	+	+
<i>Ophiostoma piceae</i>									
H920	ND	+	1700	3700	4600	5700	6400	+	+
H926	ND	+	1700	3700	4600	5800		+	*
H916	ND	+	1600	3500	4500	5500	6300	+	+
<i>Ceratocystis adiposa</i> A35	ND	+	1700	3500	4500	5500	6200	+	*
<i>Humicola lanuginosa</i>	0.30	+	1800	3100- 4300		5200	6200	+	•
<i>Pseudocercospora anguioides</i>	0.01	+	1300- 1700	3700	4600	5700	(6200)	8100	()
<i>Pseudocercospora herpotrichoides</i>									
var. <i>herpotrichoides</i>	ND	+	1700	3600	5300	5700	6400	+	+
var. <i>acuformis</i>	ND	+	1700	3700	4700	5700	6500	+	
<i>Penicillium islandicum</i>	1.50	()	1900	3900	4500	5500	6200	(+)	*
<i>Penicillium waksmanii</i>	0.25	()	1500	3500	4500	5500	(6200)	(+)	+
<i>Penicillium</i> sp. X8	1.00	()	1700	3400	4500	5500	(6200)	(+)	•

Fig. 2. Gel-filtration HPLC profiles of the exogenous material in the wash preparations from a range of fungi. Fractions were collected and the antigenic responses to MAbs, as determined by ELISA, are indicated by shading of the peaks. The MAbs were species-specific and thus did not cross-react with washes from different genera. The cell-lines of the MAbs used are indicated in the Figure.

DISCUSSION

The analyses of fungal wash preparations by gel-filtration HPLC indicated that a range of water-soluble surface molecules could be separated by gel-filtration HPLC and detected by their absorbance at 280 nm. Each species gave a characteristic profile but no differences were observed between profiles of different strains of the same species.

The most significant result from these HPLC studies is the detection, in washes of all fungi tested, of a number of surface molecules with M_r values <10000. Several of these low- M_r components, for example those in the M_r range 6200–8200 were antigenically specific when tested with species-specific MAbs, suggesting that this particular M_r class of surface molecules

Table 3. *Antigenic stability of H. lanuginosa fractions following autoclaving and treatment with sodium periodate*

S, stable (antigenicity fully maintained after treatment); P, partially stable (antigenicity not completely destroyed after treatment period); U, unstable (antigenicity completely destroyed following treatment); -, no response with untreated fractions.

Fraction	MAb . . .	Heat stability*			Periodate stability†		
		EC6	IC1	ID12	EC6	IC1	ID12
1		S ₁₀	S	U	P	U	U
2		S ₁₀	-	-‡	U		
3		U	-	-	U		
4		-	U	-		U	
5		-	-	-			
6		-	-	-			
7		U	-	-	U		
8		-	-	-			

* A positive response was taken to be $\geq 3 \times$ TCMC (tissue culture medium control) value ($S_{10} \geq 10 \times$ TCMC) after autoclaving at 15 p.s.i. for 20 min.

† Those fractions with antigenic properties (i.e. $\geq 3 \times$ TCMC) detected in untreated fractions were tested for periodate stability.

‡ A positive response (i.e. $\geq 3 \times$ TCMC) was detected after autoclaving.

may be characteristic for each species. These low- M_r surface antigens from *H. lanuginosa* were susceptible to periodate oxidation, absorbed at 280 nm, were detected by the highly sensitive protein-specific assays (Auro Dye forte blots), and were heat-labile. This evidence strongly suggests that these antigens are either glycopeptides or peptidoglycans.

The function of these low- M_r surface molecules is not known. It could be argued that they have no function, that they are breakdown products produced during the experiment and are not normally found *in vivo*. However, great care was taken to establish that they are present initially and that they do not increase as degradation products during the experiment.

These low- M_r compounds extracted in the aqueous surface-washes may be secondary metabolites produced *de novo* by the fungi or by protease digestion of larger molecules. Many fungal secondary metabolites have been isolated and characterized as polypeptides (see Turner & Aldridge, 1983), e.g. malformin C (M_r 531) from *Aspergillus niger*, rhodotorucine A (M_r 1509) from *Rhodospiridium toruloides* and sillucin (M_r 3206) from *Mucor pusillus*. Since antigenic specificity was demonstrated in some of the low- M_r components in this investigation, it is tempting to speculate that these low- M_r surface molecules have specific biological functions perhaps involved in host invasion reactions. Tepfer & Taylor (1981) and Cooper (1983) suggest that only globular proteins with M_r values $< 60\,000$ could rapidly penetrate the plant cell wall, and Carpita *et al.* (1979) suggest that the M_r limit is only 17 000. The low- M_r surface components are thus easily small enough to penetrate the plant cell wall matrices for the initiation of plant infection processes. These surface molecules may be able to affect membrane properties and/or cellular metabolism in specific plant hosts, as has been demonstrated for the action of a number of antibiotic polypeptides on pathogenic microbes, e.g. tyrocidin A (M_r 1269) (Franklin & Snow, 1978), nicin (M_r 3500), cecropin A (M_r 4000) and seminal antimicrobial protein (M_r 6385) (see Sahl, 1985). Initial effects on the plant cell may then allow invasion and infection by the fungus to proceed. Alternatively, these low- M_r molecules may have antimicrobial activities, and prevent infection of the fungus itself and/or competitive infection of the plant by other pathogens.

There were at least three different antigenic surface molecules in the high- M_r fraction (fraction 1) of the wash from *H. lanuginosa*. Since binding of the specific MAbs to these antigens was affected by periodate it was concluded that all these high- M_r surface molecules contain carbohydrate epitopes. Binding of MAb EC6 to the respective antigen was, however, only partially affected by periodate treatment, which indicates that this antigen is a glycoprotein.

Fig. 3. Comparison of the gel-filtration HPLC profiles of the *H. lanuginosa* wash to those recorded after a range of incubation treatments. Wash samples were obtained from two similar cultures of *H. lanuginosa*, subsequently combined, mixed and divided in half. A sample from one half of the wash was injected for analysis by gel-filtration HPLC 4 min after the initiation of the wash preparation procedure. After the injection at 4 min the sample was incubated at 4 °C for 4 h. The second half of the wash preparation was immediately heated (20 s after initiating the wash procedure) to 100 °C by immersion of the tube into boiling water and then autoclaved for 15 minutes. The sample was then cooled to 24 °C and injected for analysis by gel-filtration HPLC 30 min after the initiation of the wash preparation procedure. The antigenic responses, as determined by ELISA, to the respective MAbs are indicated by shading of the peaks as follows. ■, ID12; ▨, EC6; ▩, IC1.

The heat-lability of the ID12 antigen and its susceptibility to periodate treatment suggests that it is also a glycoprotein. The IC1 antigen was heat-stable and susceptible to periodate treatment, which suggests that it is a carbohydrate or a peptidoglycan. The presence of glycoproteins in the high- M_r fraction is also consistent with the absorption at 280 nm and detection by the protein specific assays. The function of these high- M_r surface molecules is not known. They may be enzymic and be involved in host invasion (and/or recognition) reactions, perhaps acting secondarily following initial perturbation of the host by the low- M_r surface molecules.

The presence of low- M_r surface glycoproteins as major components of the material extracted by aqueous solution from the fungal cell surfaces warrants further investigation into their chemical characterization, species specificity and their biological activities. In addition, the detection and fractionation, by gel-filtration HPLC, of these molecules in surface washings provides purer antigenic material and is thus a potential method for enhancing the species-specific production of MAbs for the development of fungal diagnostic assays enabling rapid detection and identification of medically and agriculturally important fungi.

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