The production and partial characterization of a monoclonal IgG antibody specific for moulds belonging to the order Mucorales

GERHARD A. DE RUITER,¹ ANDREA W. VAN BRUGGEN-VAN DER LUGT,¹ WILHELM BOS,² SERVÉ H. W. NOTERMANS,³ FRANK M. ROMBOUTS^{1*} and HARMEN HOFSTRA²

¹Wageningen Agricultural University, Department of Food Science, PO Box 8129, 6700 EV Wageningen, The Netherlands

²Institute for Biotechnology and Chemistry, TNO-Food Division, PO Box 360, 3700 AJ Zeist, The Netherlands

³National Institute of Public Health and Environmental Protection, Laboratory for Water and Food Microbiology, PO Box 1, 3720 BA Bilthoven, The Netherlands

(Received 11 December 1992; revised 22 February 1993; accepted 8 March 1993)

A monoclonal antibody (mAb) was raised against extracellular polysaccharides from *Mucor racemosus* after intrasplenic immunization of mice. An indirect ELISA and a dot-blot assay were developed with this mAb. The IgG antibody was found to be very specific for all mould species tested belonging to the order of Mucorales, except species belonging to the genus *Mortierella sensu stricto*. No cross-reactions were observed with other moulds or yeasts. The immunoreactivity of the polysaccharides of these moulds with this mAb is based on carbohydrate epitopes, in which fucose residues probably play an important role. The mAb may be suited for specific detection of species of the genera *Mucor*, *Rhizopus*, *Rhizomucor*, *Thamnidium*, *Absidia*, *Syncephalastrum* and species belonging to the *Mortierella isabellina* group in food, and possibly for diagnosis of mucormycosis in humans.

Introduction

Moulds belonging to the order Mucorales (Zygomycetes), including the genera *Mucor*, *Rhizopus*, *Rhizomucor*, *Syncephalastrum*, *Absidia* and *Thamnidium*, are found in many parts of the world. Some species are important as casual agents of mucormycosis in man (Sugar, 1992). Some are pathogenic for crop plants and many can cause food spoilage resulting in a huge economic loss (Pitt & Hocking, 1985). In general, the presence of moulds in food is often associated with loss of quality; therefore early detection of moulds is important.

Modern detection methods in food mycology are based on the recognition of fungal antigenic polysaccharides by specific immunoassays (Notermans & Heuvelman, 1985; De Ruiter *et al.*, 1993*b*). Recently, the

development of a specific ELISA for species of Mucorales was described and successfully used to detect these species in food (De Ruiter *et al.*, 1992*a*).

Polyclonal IgG antibodies (pAbs) raised in rabbits against the extracellular polysaccharides (EPSs) of *Mucor racemosus* were recently characterized as being almost specific for moulds belonging to the order of Mucorales, as cross-reactivity was only observed towards the yeast *Pichia membranaefaciens* but not to any other species of mould or yeast tested (De Ruiter *et al.*, 1992*d*). To enhance the specificity and the feasibility to commercialize specific test kits, monoclonal antibodies (mAbs) are necessary (Dewey, 1992). Recently, mAbs specific for *Penicillium*, *Aspergillus* and *Botrytis* species have been described and used in the analysis of foods and feeds (Dewey *et al.*, 1990; Stynen *et al.*, 1992; Bossi & Dewey, 1992) but until now, no mAbs specific for mould species belonging to the order Mucorales have been reported.

The EPSs of Mucorales consist mainly of carbohydrates and protein, with fucose, galactose, mannose, glucose and glucuronic acid as the main sugar residues (Bartnicki-García & Reyes, 1968; Miyazaki *et al.*, 1980; De Ruiter *et al.*, 1991*a, b*). Glucuronic acid is present as $\beta(1-4)$ -linked D-glucuronic acid polymers, an important

* Author for correspondence. Tel. 8370 83786; fax 8370 84893.

Abbreviations: mAb, monoclonal antibody; pAb, polyclonal antibody; EPS, extracellular polysaccharide; PBS, phosphate-buffered saline; PBST, PBS with 0.05% Tween 20; NC, nitrocellulose; DEAE I, fraction 1 of a DEAE anion-exchange column; TFA, trifluoroacetic acid.

structural entity of these EPSs (De Ruiter *et al.*, 1992*b*). Furthermore these EPS preparations contain 2-*O*-methyl-mannose residues, which are immunoreactive with polyclonal rabbit antibodies (G. A. De Ruiter, A. W. Van Bruggen-van der Lugt, P. Smid, P. Mischnick, J. H. Van Boom & F. M. Rombouts, unpublished data).

In the present report, we describe the production of a monoclonal IgG antibody against EPS of *Mucor racemosus* and the development of an indirect ELISA and a dot-blot assay based on this antibody. These assays were used to determine the specificity of the mAb and to compare its activity with that of polyclonal IgG antibodies. Furthermore, the structure of the epitopes of the antigenic EPSs reactive with this mAb was studied by periodate treatment, by specific enzymic degradation and by partial acid hydrolysis.

Methods

Extracellular polysaccharides. EPSs of 29 strains of moulds (Table 1) were produced, isolated and purified by ethanol precipitation as described by De Ruiter *et al.* (1991*b*). Also, 39 yeast strains were tested in this study as described by De Ruiter *et al.* (1992*d*).

Preparation of hybridomas. Mice (BALB/c, female, 6 weeks old) were immunized by intrasplenic injection of 50 µg EPS from *Mucor racemosus* in PBS (70 mM-sodium phosphate, pH 7.2, 150 mM-NaCl) according to the method of Spitz (1986). Spleen cells were collected and fused with cells of the mouse myeloma cell line P3 × 63Ag8.653 (ATCC: CRL 1580) according to the polyethylene glycol method as described by De St Groth & Scheidegger (1980).

The fused cells were distributed at a density of 1×10^5 viable cells per well in 96-well sterile culture trays (Intermed Nunclon), and tested for antibody production by indirect ELISA as described below. Wells positive for antibody production were twice subcloned and expanded by transferring them to 24-well sterile cell culture plates, and subsequently expanded further to 50 ml flasks and 500 ml culture flasks (Davis *et al.*, 1982). Cells in exponential growth were frozen by control rate freezing in Dulbecco's modified Eagle's medium (Gibco) with 10% (v/v) dimethyl sulphoxide (DMSO) and stored in the vapour phase of a liquid nitrogen freezer.

Determination of the immunoglobulin subclass and concentration of the mAb. Isotyping of the mAbs produced by these lines was performed using a mouse mAb isotyping kit (Life Technologies) according to the supplier's instructions.

The concentration of mAb IgG in the supernatant was determined using an ELISA, by coating 100 µl of a $10 \mu\text{g ml}^{-1}$ solution of goat anti-mouse IgG + IgM (4153; Tago Inc.) in PBS for 3 h at 37 °C. The microtitre plates were washed three times with PBS containing 0.05% (w/v) Tween 20 (PBST). Next, the wells were filled with 50 µl PBST, 100 µl of the mAb-containing supernatant was added to the first well and subsequently diluted serially by transferring 50 µl to the next well each time. After incubation for 1 h at 37 °C, the plates were washed three times with PBST, and 100 µl peroxidase conjugated to goat anti-mouse IgG (Zymed Laboratories) was added to each well (1:2000 in PBST with 1%, v/v, bovine calf serum). Following incubation for 1 h at 37 °C, the plates were washed three times with PBST and incubated with 100 µl of 3,3',5,5'-tetramethylbenzidine in DMSO containing

H₂O₂ prepared according to Bos *et al.* (1981). The peroxidase reaction was stopped after incubation for 30 min at 25 °C by adding 50 µl 2 M-H₂SO₄ to each well. The absorbance of the yellow colour was measured spectrophotometrically at 450 nm. The concentration of mAb IgG1 was determined by comparing the ELISA titration curve of the supernatant with those of a sample of mouse IgG1 (Southern Biotechnology Associates), which was diluted from a 1.0 mg ml^{-1} solution on the same microtitre plate for calibration.

Indirect ELISA for polyclonal and monoclonal IgG. Polyclonal IgG antibodies (pAb 1000/1201) were obtained by immunization of rabbits with EPS isolated from *Mucor racemosus* as described by Notermans & Heuvelman (1985). The IgG fraction of the serum was purified using selective precipitation with octanoic acid as described by Steinbuch & Audran (1969). ELISA was carried out essentially as described by Notermans *et al.* (1988). For this, wells of polyvinyl chloride microtitre plates (Dynatech) were coated with 100 µl of a $5 \mu\text{g ml}^{-1}$ preparation of EPS derived from *Mucor racemosus* in PBS for 16 h at 25 °C. After washing three times in PBST, 100 µl of different dilutions of either the pAb diluted in PBS containing 1% (w/v) bovine serum albumin (BSA) or the mAb diluted in PBS were added and incubated for 1.5 h at 25 °C. In the pAb-ELISA, peroxidase-conjugated goat anti-rabbit antibodies (Sigma A-8275) were used and in case of the mAb-ELISA, peroxidase-conjugated goat anti-mouse antibodies (Sigma A-4416) were used. The respective conjugates were diluted 1000-fold in PBST containing 1% (w/v) BSA and 100 µl was subsequently added to each well. Incubation was performed for 1.5 h at 25 °C then the plates were rinsed three times with PBST. Finally, 100 µl of 3,3',5,5'-tetramethylbenzidine substrate was added as described above. The ELISA reactivity was expressed as the titre, defined as the reciprocal dilution of a solution of $10 \mu\text{g ml}^{-1}$ antibody just giving a positive reaction, i.e. an absorbance ≥ 0.1 above that of a blank.

Dot-blot analysis. Antigenic fungal polysaccharides were bound to nitrocellulose membranes (NC; 0.1 µm; Schleicher & Schuell) by spotting droplets (2 µl) of 50 mg ml^{-1} preparations of various EPSs in PBS. After the antigen had been allowed to air-dry onto the membrane for 30 min at 25 °C, it was blocked with a 0.1% (w/v) solution of sodium caseinate (DMV Campina) in PBS for 1 h at 25 °C. The resulting membrane was then exposed to solutions of either the mAb 12.8 or the pAb 1000/1201 in PBS containing 0.5% (w/v) BSA. Prior to use, the mAb-containing supernatant was diluted ten times while a pAb solution with a similar concentration of protein was used. Both were incubated for 16 h at 25 °C. After washing the membrane three times with PBST (2 min each wash), the nitrocellulose membranes were incubated for 1.5 h at 25 °C with the anti-mouse or anti-rabbit antibodies conjugated with peroxidase as described above for the indirect ELISAs. Finally, the nitrocellulose membranes were washed (PBST, 3 × 2 min) and coloured by dipping for 30 min in a ready-to-use substrate solution of 4-chloro-1-naphthol (PrestoSol BL; Janssen Biotech) which is converted by the peroxidase enzyme to a compound with a dense blue colour. The optical density (OD) of the spots was measured using a laser densitometer (Computing densitometer 300A, Molecular Dynamics) equipped with a helium-neon laser (λ 672 nm) using ImageQuant 3.15 software. The values were corrected by subtracting the OD of the nitrocellulose paper alone.

Periodate oxidation of the antigens. A polysaccharide sample (5 mg) was treated for 24 h at 4 °C with 2 ml of a solution of 50 mM-NaIO₄ in 0.25 M-formic acid, adjusted to pH 3.7 with 1 M-NaOH. The excess periodate was inactivated by the addition of 400 µl 1,2-ethane diol and incubation for 1 h. The oxidized polysaccharide was reduced by adding 4 ml 0.5 M-sodium borohydride in 1 M-NaOH and incubating for 16 h at 25 °C. The mixture was cooled on ice and 300 µl acetic acid was added twice to destroy the excess NaBH₄. The polysaccharide was recovered after overnight dialysis against running tap-water followed

by dialysis against several batches of distilled water for 24 h, and finally lyophilized.

Enzyme treatment of the EPSs. An exo- α -D-mannanase was purified from an enzyme preparation of the mould *Trichoderma harzianum*, and an α -mannosidase (EC 3.2.1.24) isolated from Jack beans (*Canavalia ensiformis*) was obtained from Sigma (M 7257), and used without further purification. Ten microlitres 2 M-sodium acetate buffer (pH 5.0) was added to 175 μ l EPS solution (1 mg ml⁻¹) and 65 μ l (0.75 mU) exo- α -D-mannanase or, alternatively, 0.75 mU α -mannosidase. Incubation was performed at 30 °C for 16 h and the enzymes were subsequently inactivated by heat treatment (5 min, 100 °C). The immunoreactivities of the native and the different enzyme-treated EPSs were measured using indirect ELISA as described above.

Separation of the EPSs. The EPSs were separated by differences in charge into three different fractions by anion-exchange chromatography using a DEAE-Sepharose CL-6B column (Pharmacia) as described by De Ruiter *et al.* (1991b). The fractions were designated as DEAE I, DEAE II and DEAE III according to the order of elution from this column using a gradient of 0.05–1 M-sodium acetate buffer (pH 5.0).

Partial acid hydrolysis of the antigens. Partial acid hydrolysis of the immunoreactive polysaccharide fraction of *M. racemosus* was performed on 250 μ g material in a screw-cap tube. Solutions of different concentrations of trifluoroacetic acid (TFA) in distilled water were prepared in the range 0.5–250 mM. An aliquot (0.5 ml) of the respective TFA solution was added to each tube which was subsequently closed, mixed and heated in an oil bath at different temperatures. After incubation for 1 h the tubes were cooled and the content was evaporated to dryness by a stream of air (25 °C). Distilled water (100 μ l) was added to each tube and mixed thoroughly and finally the mixture was transferred to an HPLC vial.

The inhibitory capacity of the resulting mixture was determined in the inhibition ELISA as described above. The amount of liberated monosaccharide residues was estimated by high-performance anion-exchange chromatography using a Dionex Bio-LC high-performance liquid chromatography system (Dionex) equipped with a CarboPac PA 1 column (4 $\times$ 250 mm) and pulsed-amperometric detection using a gold working electrode and an Ag-AgCl reference electrode as described by De Ruiter *et al.* (1992c).

Results

Production of a mAb against EPS from Mucor racemosus

Fusion of spleen cells of mice immunized intrasplenically with EPS from the mould *Mucor racemosus* and 8-azaguanine-resistant myeloma P3 $\times$ 63. Ag8 cells, yielded 490 hybridomas of which 9 were reactive with EPS of *Mucor racemosus*. The hybridomas were tested for antibody production in the indirect ELISA in which EPS of *Mucor racemosus* was coated to the microtitre plates. Supernatants from these wells were tested for cross-reactivity with EPS preparations from a number of moulds, including representatives of *Penicillium*, *Aspergillus*, *Fusarium*, *Botrytis*, *Alternaria* and *Cladosporium*. Only one of these hybridomas (12.8) showed a strong reaction with EPS from *Mucor racemosus* and no reaction with EPSs from moulds

belonging to other genera. Cells from this well were cloned, and wells positive for growth and antibody production were subcloned once more. The resulting clone (designated mAb 12.8) was grown as described and the supernatant was used for all further experiments. mAb 12.8 was typed as IgG of subclass 1 (kappa). The concentration of the IgG1 mAb in the supernatant was estimated by ELISA to be 25 μ g IgG ml⁻¹.

Specificity of mAb 12.8

The specificity of mAb 12.8 was studied by determination of the titres of 29 different EPS preparations of 13 mould genera (Table 1). mAb 12.8 gave a positive immunoreaction with the EPS preparations from all moulds tested belonging to the order Mucorales except species of the genus *Mortierella sensu stricto*, which includes the species *reticulata*, *hyalina* and *polycephala* (De Ruiter *et al.*, 1993a). No reactivity was observed with other mould species often occurring in food such as *Penicillium*, *Aspergillus* and *Fusarium*. Furthermore, 39 yeast species belonging to 20 different genera (see Table 1 from De Ruiter *et al.*, 1992d) were tested for cross-reactivity with mAb 12.8. These yeasts did not show any reactivity. Even the yeast *Pichia membranaefaciens*, which gave a clear positive immunoreaction with pAb 1000/1201, did not show cross-reactivity with mAb 12.8 (Table 1).

Reactivity of polyclonal and monoclonal antibodies raised against M. racemosus

The reactivity of mAb 12.8 was compared with the reactivity of pAb 1000/1201 antibodies raised in rabbits against the same EPS preparation of *M. racemosus* as described previously (De Ruiter *et al.*, 1992d). This was performed by determination of the titration curves in the respective indirect ELISAs after binding 5 μ g ml⁻¹ of the same EPS preparation of *M. racemosus* to the wall of microtitre plates. As shown in Fig. 1, both pAb 1000/1201 and mAb 12.8 were reactive with this EPS preparation although slightly higher extinctions could be obtained with the pAbs. A more detailed comparison of the selectivity of each antibody was made by determination of the titres using different EPS preparations obtained from moulds belonging to Mucorales listed in Table 1. Both antibodies reacted with all EPSs but their reactivity differed significantly. pAb 1000/1201 gave the highest reactivity with the EPS preparation of *Mucor circinelloides* whereas mAb 12.8 gave only a weak immunoreaction. In contrast, the EPSs from *Rhizomucor* species showed a much higher reaction with mAb 12.8 than with pAb 1000/1201. To exclude the possibility that these differences in reactivity were caused by differences

Table 1. Reactivity of mAb 12.8 and pAb 1000/1201 raised against the EPS of *Mucor racemosus* with EPSs of different moulds

Microtitre plates were coated with $5 \mu\text{g ml}^{-1}$ of the respective EPS preparation. ELISA reactivity is expressed as the titre, defined as the reciprocal dilution of a solution of $10 \mu\text{g ml}^{-1}$ antibody just giving a positive reaction. —, ≤ 10 ; +, $10 < \text{titre} \leq 100$; ++, $100 < \text{titre} \leq 1000$; + + +, titre > 1000 . Experiments were carried out in triplicate.

Mould strain	Indirect ELISA titre	
	mAb 12.8	pAb 1000/1201
<i>Mucor racemosus</i> Fres., CBS 222.81	+++	+++
<i>Mucor hiemalis</i> Wehmer CBS 201.28	+++	++
<i>Mucor circinelloides</i> van Tieghem RIVM M 40	+	+++
<i>Rhizopus stolonifer</i> (Ehrenb.) Lind CBS 609.82	++	++
<i>Rhizopus oryzae</i> Went & Prinsen Geerlings LU 581	++	++
<i>Rhizomucor pusillus</i> (Lindt) Schipper CBS 432.78	+++	++
<i>Rhizomucor miehei</i> (Cooney & Emerson) Schipper CBS 371.71	+++	++
<i>Absidia corymbifera</i> (Cohn) Sacc. & Trotter LU 017	+	++
<i>Thamnidium elegans</i> Link CBS 342.55	+	++
<i>Syncephalastrum racemosum</i> Cohn CBS 443.59	+	++
<i>Mortierella reticulata</i> van Tieghem CBS 452.74	—	—
<i>Mortierella hyalina</i> (Harz) W. Gams CBS 654.68	—	—
<i>Mortierella polycephala</i> Coemans CBS 327.72	—	—
<i>Mortierella roseonana</i> W. Gams CBS 473.74	++	++
<i>Mortierella nana</i> Linnem, CBS 730.70	++	++
<i>Mortierella ramanniana</i> (Möller) Linnem. var. <i>ramanniana</i> CBS 243.58	++	+
<i>Mortierella ovata</i> Yip CBS 499.82	+	+
<i>Mortierella isabellina</i> Oudem. CBS 560.63	+	+
<i>Penicillium citrinum</i> Thom CBS 117.64	—	—
<i>Penicillium dierckxi</i> Biourge RIVM M 90	—	—
<i>Penicillium aurantiogriseum</i> Dierckx CBS 342.51	—	—
<i>Penicillium digitatum</i> Sacc. RIVM M58	—	—
<i>Aspergillus fumigatus</i> Fres. RIVM M3	—	—
<i>Aspergillus niger</i> van Tieghem CBS 553.65	—	—
<i>Fusarium solani</i> (Mart.) Sacc. CBS 165.87	—	—
<i>Fusarium poae</i> (Peck) Wollenweber CBS 446.67	—	—
<i>Alternaria alternata</i> (Fr.) Keissler RIVM M 13	—	—
<i>Botrytis cinerea</i> Pers. RIVM M 17	—	—
<i>Cladosporium cladosporioides</i> (Fres.) de Vries CBS 143.65	—	—
<i>Pichia membranaefaciens</i> (Hansen) Hansen	—	++

Fig. 1. Titration curves of mAb 12.8 (Δ) and pAb 1000/1201 ($\circ$) in an indirect ELISA coated with a preparation of EPS ($5 \mu\text{g ml}^{-1}$) from *Mucor racemosus*. The values represent the dilution of a $10 \mu\text{g ml}^{-1}$ solution of the respective antibodies.

in binding properties of the EPS preparations to the wall of the microtitre plates, a dot-blot assay was developed for both the polyclonal and monoclonal antibodies.

Development of a dot-blot assay

A dot-blot assay in which the polysaccharide antigens were directly bound to nitrocellulose (NC) paper was developed by testing various conditions for binding the EPSs to the NC, using different buffers with pH values in the range 5–9. Maximum binding was obtained by spotting $2 \mu\text{l}$ droplets containing $100 \mu\text{g}$ EPS in PBS (pH 7.2) followed by air-drying for 30 min. Optimal blocking of the NC was performed with a solution of 0.1% (w/v) sodium caseinate. Both BSA and a crude milk protein were not very effective blockers as concentrations of more than 1% (w/v) were needed for complete

Fig. 2. Dot-blot analysis of various EPSs isolated from species belonging to the order Mucorales with mAb 12.8 and pAb 1000/1201. After correction for the optical density of the nitrocellulose paper itself, the limits for black were set at OD 0.22 and 0.70 for mAb 12.8 and pAb 1000/1201, respectively.

blocking. After washing, the NC was incubated with the respective antibodies in similar concentration and subsequently incubated with the respective peroxidase conjugates. The NC membranes were developed with the 4-chloro-1-naphthol substrate, PrestoSol.

The EPS preparations used in this dot-blot assay revealed a difference in reactivity of mAb 12.8 and pAb 1000/1201 as shown in Fig. 2. EPS from *Mucor racemosus* gave the strongest immunoreaction with pAb 1000/1201 whereas *Rhizomucor pusillus* gave the strongest reaction with mAb 12.8.

Isolation of the immunoreactive fraction of the EPSs

The role of the sugar residues in the immunoreactivity of EPSs with the mAb was studied by periodate oxidation. Mild periodate oxidation, which is reported to be specific for sugar residues containing vicinal -OH groups, completely destroyed the reactivity of both the pAb and the mAb with EPSs from Mucorales (Table 2).

The pAbs raised against EPS of *M. racemosus* reacted with two specific polysaccharide fractions: the first (DEAE I) did not bind to a DEAE anion-exchange column; and the second (DEAE II) could be eluted from this column with 200–300 mM buffer salt, as also shown for the EPS of *Mucor hiemalis* (De Ruiter *et al.*, 1991b). As shown in Table 2, mAb 12.8 did not react with these

pAb-reactive fractions but reacted with the major polysaccharide fraction (DEAE III). This immunoreactive DEAE III fraction was isolated by anion-exchange chromatography using 0.9–1 M-sodium acetate buffer, pH 5.0. This fraction, sometimes called mucoran, is characteristic of moulds belonging to Mucorales and contains approximately 50% glucuronic acid (Bartnicki-García & Lindberg, 1972; De Ruiter *et al.* 1991b). Recently, we isolated the $\beta(1-4)$ -linked D-glucuronic acid polymeric backbone of this polysaccharide (De Ruiter *et al.*, 1992b), but mAb 12.8 was not reactive with this preparation.

Partial determination of the mAb epitopes

The antigenic determinants of the immunoreactive polysaccharide fraction were partly characterized using specific enzymes and partial acid hydrolysis to degrade the polysaccharide. As shown in Table 2, treatment of various EPSs isolated from species of Mucorales with an exo- α -D-mannanase, which is able to degrade specifically the epitopes reactive with pAb 1000/1201, did not degrade the epitopes reactive with mAb 12.8. Treatment of the EPSs with a α -mannosidase, which hydrolyses non-reducing terminal mannose residues, did not have any effect on the immunoreactivity of the EPSs with mAb 12.8 and pAb 1000/1201.

Table 2. Reactivity of mAb 12.8 and pAb 1000/1201 IgG antibodies with different fractions of the EPSs from Mucorales and with EPSs subjected to various treatments

EPS from	Fraction	Treatment	Indirect ELISA titre*	
			mAb	pAb
<i>Mucor racemosus</i>	DEAE I†		—	+++
	DEAE II		—	++
	DEAE III		+++	—
<i>Rhizopus stolonifer</i>	DEAE I		—	+++
	DEAE II		—	++
	DEAE III		+++	—
<i>Rhizomucor pusillus</i>	DEAE I		—	+++
	DEAE II		—	+++
	DEAE III		+++	—
<i>Mucor hiemalis</i>	Polyglucuronic acid‡		—	—
<i>Mucor hiemalis</i>		Periodate oxidation	—	—
<i>Rhizopus oryzae</i>		Periodate oxidation	—	—
<i>Absidia corymbifera</i>		Periodate oxidation	—	—
<i>Mucor racemosus</i>		Exo- α -D-mannanase§	+++	—
<i>Rhizopus stolonifer</i>		Exo- α -D-mannanase	+++	—
<i>Mucor racemosus</i>		α -Mannosidase	+++	+++
<i>Rhizopus stolonifer</i>		α -Mannosidase	+++	+++

* See legend Table 1.

† Fractions of a DEAE anion-exchange chromatography similar to those shown for *M. hiemalis* in Fig. 3 of De Ruiter *et al.* (1991b).‡ Polymer of β (1-4)-linked D-glucuronic acid as described in De Ruiter *et al.* (1992b).§ Purified from an enzyme preparation from *Trichoderma harzianum*.

Partial acid hydrolysis was performed on the immuno-reactive polysaccharide fraction (DEAE III) using increasing concentrations of TFA. The reaction products were analysed and the inhibitory capacity of the remaining polysaccharide fraction was established by indirect mAb-ELISA. As shown in Table 3, fucose residues were liberated in increasing amounts with the use of up to 5 mM-TFA and heating for 1 h at 100 °C. This also resulted in a considerable decrease in the inhibitory capacity of the remaining partially hydrolysed immunoreactive polysaccharide. At higher concentrations of acid more fucose and gradually also some galactose, glucose and mannose residues were liberated. The inhibitory capacity of the remaining polysaccharide decreased to only 1% of the initial inhibitory capacity after hydrolysis with 250 mM-TFA. Additional partial hydrolysis experiments were performed with 5 mM-TFA using different temperatures. These experiments revealed that at 94 °C approx. 40% of the inhibitory capacity was abolished, with fucose as the only hydrolysis product (results not shown).

Discussion

The mAb raised against EPS isolated from *M. racemosus* was specific for all mould species belonging to the order Mucorales. No cross-reactions were observed with EPSs from mould or yeast species tested other than Mucorales.

This mAb was found to be more specific for these moulds than the pAbs raised against the same EPS, as the latter showed cross-reactivity with the yeast *Pichia membranaefaciens* (De Ruiter *et al.*, 1992d). With these antibodies, an indirect ELISA and a dot-blot assay were developed which may be used to detect these mould species.

mAb 12.8 and pAb 1000/1201 differ in their reactivity with the different EPS preparations from Mucorales. pAb 1000/1201 reacted best with EPS from *Mucor racemosus* and *M. circinelloides* whereas mAb 12.8 showed the highest activity towards *M. racemosus* and the *Rhizomucor* spp. mAb 12.8 and pAb 1000/1201 reacted in a similar way with the EPS preparations obtained from species from the *Mortierella isabellina* group. This supports the recently described affinity of this group of moulds to the Mucoraceae, while *Mortierella sensu stricto* falls outside this affinity (De Ruiter *et al.*, 1993a).

The immunological reaction of these fungal carbohydrate antigens with mAb 12.8 is based on carbohydrate epitopes, as mild periodate treatment completely destroyed their antigenic activity. The pAb-reactive fractions were not reactive with mAb 12.8, and the exo- α -D-mannanase, which specifically degrades the pAb-epitopes, did not have any influence on the immunoreactivity of mAb 12.8. This monoclonal antibody reacted exclusively with the DEAE III fraction containing mucoran. It can be assumed that the antigenicity

Table 3. Influence of partial hydrolysis of the immunoreactive polysaccharide fraction from *Mucor racemosus* with TFA (1 h, 100 °C) on its capacity to inhibit the reaction between the EPS of *M. racemosus* and mAb 12.8 raised against this EPS*

TFA concn (mM)	Liberated carbohydrate monomers†				Inhibitory capacity‡ (%)
	Fuc	Gal	Man	Glc	
250	16	10	1	4	1
50	7	3	0	3	10
25	5	2	—	1	17
12.5	4	1	—	1	37
5	3	0	—	—	58
2.5	2	—	—	—	75
1.25	1	—	—	—	83
0.5	0	—	—	—	86
0	—	—	—	—	100

* Values are the average of duplicate experiments.

† Amount (values in µg; 0, present but < 0.5 µg; —, not present) of carbohydrate monomers liberated during incubation of 250 µg of the immunoreactive polysaccharide fraction (DEAE III) with the respective TFA solution, as estimated by high-performance anion-exchange chromatography (De Ruiter *et al.*, 1992c). Abbreviations: Fuc, fucose; Gal, galactose; Man, mannose; Glc, glucose.

‡ Inhibitory capacity of 150 µg ml⁻¹ of the acid-treated polysaccharide as tested in an indirect ELISA with 5 µg ml⁻¹ of immunoreactive polysaccharide fraction coated to the wall of a microtitre plate and expressed as the amount of inhibition remaining.

resides in the neutral sugar side-chains of mucoran, as the β(1-4)-linked D-glucuronic acid backbone of this polysaccharide was not reactive. The partial acid hydrolysis experiments in which fucose as a 6-deoxy-type sugar was hydrolysed preferentially, indicated that the fucose residues may play an important role in the epitopes. More detailed analysis of this fraction should be performed to elucidate the structure of these epitopes. Specific enzymes can be valuable for this purpose as recently shown by Van Bruggen-van der Lugt *et al.* (1992).

Apparently, different carbohydrate residues are immunoreactive with the rabbit-raised monospecific pAbs and mouse-raised mAbs. This can be attributed either to the different immunization procedures used or to different host animal species. Intrasplenic immunization was used for raising mAb 12.8 in mice. It can be assumed that in this particular case the species difference might be the major factor for the difference in immune response.

As mAb 12.8 was highly specific for moulds belonging to the order Mucorales, it may be used to develop diagnostic assays for the detection of these species. mAb-based immunoassays may be very useful to monitor mould contamination in food production processes and to monitor fungal spoilage. With this mAb it may be feasible to test raw materials, food and feed in industrial

control laboratories and food inspection services for the presence of moulds belonging to the genera *Mucor*, *Rhizopus*, *Rhizomucor*, *Absidia*, *Thamnidium* and *Syncephalastrum* (De Ruiter *et al.*, 1993b). Furthermore, in medicine these assays may be useful for diagnosis of mucormycosis in humans, by detection of circulating antigens (Kaufman *et al.*, 1989).

We gratefully acknowledge Dr H. J. Kamphuis (Gerkens Cacao Industrie, Wormer, The Netherlands) for carrying out some initial screening experiments and critical reading of the manuscript; Dr G. Keizer (Holland Biotechnology, Leiden, The Netherlands) for valuable discussions about the dot-blot assay and about the manuscript; and Dr W. Gams and Dr R. A. Samson (Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) for providing some of the strains of moulds used in this study and critical reading of the manuscript. The investigations were supported by the Netherlands' Foundation for Chemical Research (SON) with financial aid from The Netherlands' Technology Foundation (STW).

References

- BARTNICKI-GARCÍA, S. & LINDBERG, B. (1972). Partial characterization of mucoran: the glucuronomannan component. *Carbohydrate Research* **23**, 75–85.
- BARTNICKI-GARCÍA, S. & REYES, E. (1968). Polyuronides in the cell walls of *Mucor rouxii*. *Biochimica et Biophysica Acta* **170**, 54–62.
- BOS, E. S., VAN DER DOELEN, A. A., VAN ROOY, N. & SCHUURS, A. H. W. M. (1981). 3,3',5,5'-tetramethylbenzidine as an Ames test negative chromogen for horse-radish peroxidase in enzyme-immunoassay. *Journal of Immunoassay* **2**, 187–203.
- BOSSI, R. & DEWEY, F. M. (1992). Development of a monoclonal antibody-based immunodetection assay for *Botrytis cinerea*. *Plant Pathology* **41**, 472–482.
- DAVIS, J. M., PENNINGTON, J. E., KUBLER, A. M. & CONSCIENCE, J. F. (1982). A simple, single-step technique for selecting and cloning hybridomas for the production of monoclonal antibodies. *Journal of Immunological Methods* **50**, 161–171.
- DE RUITER, G. A., SMID, P., VAN DER LUGT, A. W., VAN BOOM, J. H., NOTERMANS, S. H. W. & ROMBOUTS, F. M. (1991a). Immunogenic extracellular polysaccharides of Mucorales. In *Fungal Cell Wall and Immune Response*, NATO ASI Series vol. H 53, pp. 169–180. Edited by J.-P. Latgé & D. Boucias. Berlin & Heidelberg: Springer Verlag.
- DE RUITER, G. A., VAN DER LUGT, A. W., VORAGEN, A. G. J., ROMBOUTS, F. M. & NOTERMANS, S. H. W. (1991b). High-performance size-exclusion chromatography and ELISA detection of extracellular polysaccharides from Mucorales. *Carbohydrate Research* **215**, 47–57.
- DE RUITER, G. A., HOOPMAN, T., VAN DER LUGT, A. W., NOTERMANS, S. H. W. & NOUT, M. J. R. (1992a). Immunochemical detection of Mucorales species in food. In *Modern Methods in Food Mycology*, pp. 221–227. Edited by R. A. Samson, A. D. Hocking, J. I. Pitt & A. D. King. Amsterdam: Elsevier.
- DE RUITER, G. A., JOSSE, S. L., COLQUHOUN, I. J., VORAGEN, A. G. J. & ROMBOUTS, F. M. (1992b). Isolation and characterization of β(1-4)-D-glucuronans from extracellular polysaccharides of moulds belonging to Mucorales. *Carbohydrate Polymers* **18**, 1–7.
- DE RUITER, G. A., SCHOLS, H. A., VORAGEN, A. G. J. & ROMBOUTS, F. M. (1992c). Carbohydrate analysis of water-soluble uronic acid-containing polysaccharides with high-performance anion-exchange chromatography using methanolysis combined with TFA hydrolysis is superior to four other methods. *Analytical Biochemistry* **207**, 176–185.
- DE RUITER, G. A., VAN BRUGGEN-VAN DER LUGT, A. W., NOUT, M. J. R., MIDDELHOVEN, W. J., SOENTORO, P. S. S., NOTERMANS, S. H. W. & ROMBOUTS, F. M. (1992d). Formation of antigenic extracellular polysaccharides by selected strains of *Mucor* spp.,

- Rhizopus* spp., *Rhizomucor* spp., *Absidia corymbifera* and *Syncephalastrum racemosum*. *Antonie van Leeuwenhoek* **62**, 189–199.
- DE RUITER, G. A., VAN BRUGGEN-VAN DER LUGT, A. W., ROMBOUTS, F. M. & GAMS, W. (1993*a*). Approaches to the classification of the *Mortierella isabellina* group (Mucorales): antigenic extracellular polysaccharides. *Mycological Research* **97**, 690–696.
- DE RUITER, G. A., NOTERMANS, S. H. W. & ROMBOUTS, F. M. (1993*b*). Review: new methods in food mycology. *Trends in Food Science and Technology* **4**, 91–97.
- DE ST GROTH, S. F. & SCHEIDEGGER, D. (1980). Production of monoclonal antibodies: strategy and tactics. *Journal of Immunological Methods* **35**, 1–21.
- DEWEY, F. M. (1992). Detection of plant-invading fungi by monoclonal antibodies. In *Techniques for the Rapid Detection of Plant Pathogens*, pp. 47–62. Edited by J. M. Duncan & L. Torrance. Oxford: Blackwell Scientific Publications.
- DEWEY, F. M., MACDONALD, M. M., PHILLIPS, S. I. & PRIESTLEY, R. A. (1990). Development of monoclonal-antibody-ELISA and -DIP-stick immunoassays for *Penicillium islandicum* in rice grains. *Journal of General Microbiology* **136**, 753–760.
- KAUFMAN, L., TURNER, L. F. & McLAUGHLIN, D. W. (1989). Indirect enzyme-linked immunosorbent assay for zygomycosis. *Journal of Clinical Microbiology* **27**, 1979–1982.
- MIYAZAKI, T., YADOMAE, T., YAMADA, H., HAYASHI, O., SUZUKI, I. & OHSHIMA, Y. (1980). Immunochemical examination of the polysaccharides of Mucorales. In *Fungal Polysaccharides*, ACS Symposium Series no. 126, pp. 81–94. Edited by P. A. Sandford & K. Matsuda. Washington DC: American Chemical Society.
- NOTERMANS, S. & HEUVELMAN, C. J. (1985). Immunological detection of moulds in food by using the enzyme-linked immunosorbent assay (ELISA); preparation of antigens. *International Journal of Food Microbiology* **2**, 247–258.
- NOTERMANS, S., VEENEMAN, G. H., VAN ZUYLEN, C. W. E. M., HOOGHERHOUT, P. & VAN BOOM, J. H. (1988). (1→5)-linked β -D-galactofuranosides are immunodominant in extracellular polysaccharides of *Penicillium* and *Aspergillus* species. *Molecular Immunology* **25**, 975–979.
- PITT, J. I. & HOCKING, A. D. (1985). *Fungi and Food Spoilage*. Sydney: Academic Press.
- SPITZ, M. (1986). ‘Single-shot’ intrasplenic immunization for the production of monoclonal antibodies. *Methods in Enzymology* **121**, 33–41.
- STEINBUCH, M. & AUDRAN, R. (1969). The isolation of IgG from mammalian sera with the aid of caprylic acid. *Archives of Biochemistry and Biophysics* **134**, 279–284.
- STYNEN, D., SARFATI, J., GORIS, A., PRÉVOST, M.-C., LESOURD, M., KAMPHUIS, H., DARRAS, V. & LATGÉ, J.-P. (1992). Rat monoclonal antibodies against *Aspergillus* galactomannan. *Infection and Immunity* **60**, 2237–2245.
- SUGAR, A. M. (1992). Mucormycosis. *Clinical Infectious Disease* **14** (Suppl 1), S126–129.
- VAN BRUGGEN-VAN DER LUGT, A. W., KAMPHUIS, H. J., DE RUITER, G. A., MISCHNICK, P., VAN BOOM, J. H. & ROMBOUTS, F. M. (1992). New structural features of the antigenic extracellular polysaccharides of *Penicillium* and *Aspergillus* species revealed with exo- β -D-galactofuranosidase. *Journal of Bacteriology* **174**, 6096–6102.