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High-performance size-exclusion chromatography and ELISA detection of extracellular polysaccharides from Mucorales

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ABSTRACT

In this study high-performance size-exclusion chromatography and ELISA detection were used to study the extracellular polysaccharides excreted by mould species belonging to Mucorales. EPS preparations from different species were heterogeneous as detected by refractive index measurements. ELISA detection of fractions collected after high-performance size-exclusion chromatography revealed reactivity of antibodies with a limited number of polysaccharides with a retention time of 27 to 29 min in EPS preparations of all species tested. In addition to the immunochemically active polysaccharide fraction containing glucuronic acid, other neutral polysaccharides were present, depending on species and carbon source used for growth. The method employed allowed one to fractionate EPS preparations and to discriminate between immunochemically active and non-reactive components. All Mucorales tested produced polysaccharides reactive with polyclonal antibodies raised against EPS from *Mucor racemosus*.

INTRODUCTION

The chemistry and immunochemical properties of polysaccharides excreted by fungi belonging to the family of the Mucoraceae of the order Mucorales of the class Zygomycetes have been previously studied[1–5]. Recently, immunological detection of moulds based on the immunochemical properties of the excreted polysaccharides was proposed by Notermans and associates[6,7] using an enzyme-linked immunosorbent assay (ELISA). Reactivity of the extracellular polysaccharide (EPS) preparations of *Mucor* and *Rhizopus* species with antibodies raised against EPS of *Mucor racemosus*, was found, indicating similar immunodominant structures[8].

Until now, no detailed information was available about the composition and molecular weight distribution of the mixture of polysaccharides excreted by Mucorales and its relation to their immunochemical properties. High-performance size-exclusion

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chromatography was used to study the composition of the glucuronic acid-containing extracellular polysaccharides. This method was previously applied for D-galacturonic acid-containing pectins[9–11] and a fungal extracellular polysaccharide scleroglucan[12]. The combined use of high-performance liquid chromatography (h.p.l.c.) and immunochemical assays for screening glycoproteins on their immunological properties was recently proposed[13].

In this study the behaviour of extracellular polysaccharides of mould species belonging to Mucorales in high-performance size-exclusion chromatography and ion-exchange chromatography is presented. The IgG fraction of rabbit polyclonal antibodies raised to EPS of *M. racemosus*, was used in a sandwich ELISA to study immunochemical reactivity of fractions of the EPS of various mould species separated by size-exclusion chromatography.

The method employed allowed us to fractionate EPS preparations and to discriminate between immunochemically active and non-reactive components. All Mucorales tested produced polysaccharides reactive with polyclonal antibodies raised against EPS from *M. racemosus*.

EXPERIMENTAL

Moulds. — The strains of *Mucor hiemalis* (CBS 201.28), *Mucor racemosus* (H473-R5), *Mucor circinelloides* (RIVM M 40), *Rhizopus arrhizus* (RIVM M 64), *Rhizopus stolonifer* (CBS 609.82), *Rhizopus oryzae* (LU 581), *Rhizomucor miehei* (CBS 371.71), *Rhizomucor pusillus* (CBS 432.78), *Absidia corymbifera* (LU 017), *Syncephalastrum racemosus* (CBS 443.59), and *Thamnidium elegans* (CBS 342.55) used in this study were grown on a yeast nitrogen base (YNB, Difco Labs., Detroit, U.S.A.) synthetic culture medium supplemented with glucose (Merck) as carbon source in a concentration of 30 g/L. Incubation was performed at 30° on a rotary shaker at 100 r.p.m. (Gallenkamp Ltd., Loughborough, U.K.), except with *M. hiemalis*, *R. stolonifer*, and *T. elegans*, which were incubated at 25°, and *A. corymbifera*, and *S. racemosum* which were incubated at 37°.

Isolation of extracellular polysaccharides (EPS). — The culture liquid (200 mL) was separated from the mycelium by filtration with pre-weighed filter paper (55 mm, 589 black ribbon; Schleicher & Schüll, Dassel, Germany) on a Büchner funnel.

The filtrate was concentrated five-fold with a vacuum rotary evaporator in a water bath at 40° and heated for 5 min at 100° to inactivate enzymes which might have been present. The filtrate was poured into a dialysis bag (preboiled with distilled water), dialysed against running tap water overnight, and then against distilled water for 24 h. The solution was further concentrated under reduced pressure and lyophilized. The residue was dissolved in 20 mL of distilled water, and any water-insoluble material was removed by centrifugation. Finally, the EPS-containing water fraction was poured into five volumes of ethanol (96%) and stored for 16 h at 4°. The precipitate was separated by centrifugation (30 min, 19 600 *g*), and dissolved in 20 mL of distilled water and precipitated for a second time. This process was repeated once more, and the precipitate

was dissolved in 10 mL of distilled water, freeze-dried, and weighed. This constituted the isolated amount of extracellular polysaccharide (EPS).

Chemical analysis of the EPS. — The neutral sugar composition of the EPS was determined as alditol acetates using g.l.c. Neutral sugars were released by pre-treatment with 72% (w/w) H_2SO_4 for 1 h at 30°, followed by hydrolysis with 1M H_2SO_4 for 3 h at 100°. Next, sugars were converted to their alditol acetates as described[14] and analysed by g.l.c. using inositol as the internal standard.

Molecular weight distribution. — The molecular weight distribution was studied using high-performance size-exclusion chromatography, which was performed on a SP8800 h.p.l.c. system (Spectra Physics, San Jose, CA U.S.A.) equipped with three Bio-Gel TSK columns in series (40XL, 30XL, 20XL; 300 × 7.5 mm; Bio-Rad Labs, Richmond, CA U.S.A.) in combination with a TSK-XL guard column (40 × 6 mm) at 30°. An aliquot of 20 μL of a solution of EPS in distilled water (1 mg/mL) was injected into the system, and 0.4M acetic acid–sodium acetate (pH 3.0) was used as eluent with a flow rate of 0.8 mL/min. The effluent was monitored using a Shodex SE-61 refractive index detector. The mol. wt. calibration of this system was performed by using dextran standards (10k, 40k, 70k, and 500k; Pharmacia, Uppsala, Sweden) using software obtained from Spectra-Physics (San Jose, CA U.S.A.).

The effluent was partitioned in fractions of 0.4 mL (0.5 min) using a Bio-Rad 2110 fraction collector. After adding 0.14 mL of a M solution of NaOH for neutralization, the samples were diluted by adding 1.06 mL of distilled water. These samples were examined by sandwich ELISA and further diluted (2 to 8 times) on microtiter plates. The optimal dilution was determined by comparing the extinctions with the ELISA extinctions of a dilution curve of a standard EPS derived from *Mucor hiemalis*.

Sandwich ELISA for the detection of EPS. — Sandwich ELISA was carried out as described[6], in wells of polyvinyl chloride microtiter plates (Dynatech, Chantilly, VA, U.S.A.). An IgG fraction of polyclonal antibodies was obtained by immunization of rabbits with extracellular polysaccharide antigens produced by *M. racemosus* as described[8]. An aliquot (100 μL), containing 10 μg/mL of rabbit IgG anti *M. racemosus* in 0.07M sodium phosphate buffer (pH 7.2) containing 0.15M NaCl (PBS), was added to each well and incubated overnight at room temperature. After rinsing with PBS containing 0.05% Tween 20 (PBS-T), 100-μL portions of samples diluted in PBS-T were added and incubated for 90 min at room temperature. After another rinse with PBS-T, the wells were incubated with rabbit IgG (anti *M. racemosus*), conjugated to horse radish peroxidase, for 90 min. Finally, the rinsed wells were incubated for 10 min at room temperature with 100 μL of a substrate solution. The substrate solution was 3,3′,5,5′-tetramethylbenzidine in methyl sulfoxide (Me_2SO) containing H_2O_2 prepared according to Bos[15]. A stock solution of 42mM 3,3′,5,5′-tetramethylbenzidine in Me_2SO was prepared, and 1 mL of the solution was added dropwise under gentle shaking to 100 mL of 0.1M sodium acetate–citric acid buffer, pH 6.0. Just before use, 7 μL of 30% H_2O_2 was added to the 10 mL of buffer solution. The enzyme reaction was stopped by adding 50 μL of 2M H_2SO_4 to each well. The absorbance of the yellow color was measured at 450 nm.

Preparative ion-exchange chromatography. — This procedure was performed on a column (12 × 1.6 cm) of DEAE-Sepharose CL-6B[16] (Pharmacia, Uppsala, Sweden), equilibrated with 0.05M sodium acetate buffer (pH 5.0). After loading of the sample of EPS (4 mL of a 2.5 mg/mL solution), the column was washed with 25 mL of buffer and then eluted at 30 mL/h with a linear gradient (100 mL) of 0.05 M sodium acetate buffer, followed by 50 mL of M buffer. Fractions (3 mL) were assayed for both glucuronic acid and neutral sugars.

Analytical methods. — The contents of glucuronic acid of the fractions were determined with the automated *m*-hydroxydiphenyl method[17], using glucuronic acid as standard. Neutral sugars in the eluted fractions were determined with the automated orcinol method[18], using D-glucose as the standard. Corrections in the latter analysis were made for interference from glucuronic acid.

RESULTS

Determination of the EPS of Mucor *species.* — The mol. wt. distribution of the extracellular polysaccharides (EPS) excreted by *M. hiemalis*, *M. racemosus*, and *M. circinelloides* were found to be very similar as revealed in Fig. 1. The EPSs derived from these species had an unimodal mol. wt. distribution and a retention time between 27 and 29 min on this size-exclusion chromatography system using refractive index (r.i.) detection. Calibration of the system with dextran standards indicated that this peak corresponded to a mol. wt. ranging from 10–30k. The peak widths of these EPS preparations were found to be similar to that of the dextrans used for calibration of the system.

The EPS of *M. racemosus* was used to raise polyclonal antibodies in rabbits. A sandwich ELISA using the IgG fraction of these antibodies was used to characterize the EPS immunochemically after chromatographic separation. To this end, the chromatography system was connected to a fraction collector, and fractions of 0.4 mL (0.5 min retention time) were collected. After neutralization and dilution, the sandwich ELISA was performed on these fractions. The ELISA extinctions of the fractions are shown in

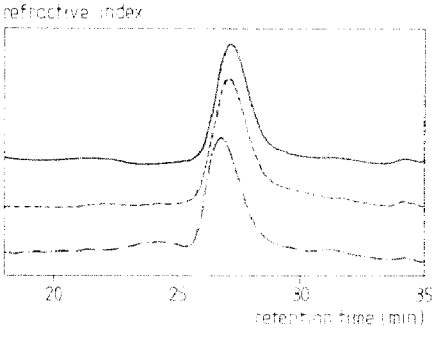

Fig. 1. High-performance size-exclusion chromatograms of EPS of *Mucor* species with refractive index (r.i.) detection. *M. racemosus*, ——; *M. circinelloides*, – – –; *M. hiemalis*, –●–●–.

TABLE I

Sugar composition of EPS preparations of various mould species belonging to Mucorales[a]

Mould species	*Sugar composition (mol%)*[b]				
	Fuc	*Man*	*Gal*	*Glc*	*GlcA*
Mucor hiemalis	18	17	13	4	48
Mucor hiemalis[c]	7	9	4	60	20
Mucor racemosus	13	23	9	8	47
Mucor circinelloides	13	27	8	11	41
Rhizopus oryzae	15	13	4	13	55
Rhizopus arrhizus	10	16	4	21	49
Rhizopus stolonifer	13	9	11	23	44
Rhizomucor miehei	25	15	7	—	53
Rhizomucor pusillus	19	21	8	3	49
Absidia corymbifera	11	11	7	39	32
Syncephalastrum racemosum	22	30	6	—	42
Thamnidium elegans	9	8	6	27	50

[a] Grown on culture media with D-glucose as the carbon source, unless otherwise noted. [b] Values are the average of duplicate experiments. The sugar content of the EPS preparations varied between 60–80% (w/w). [c] Mould grown on a culture medium with maltose as the carbon source.

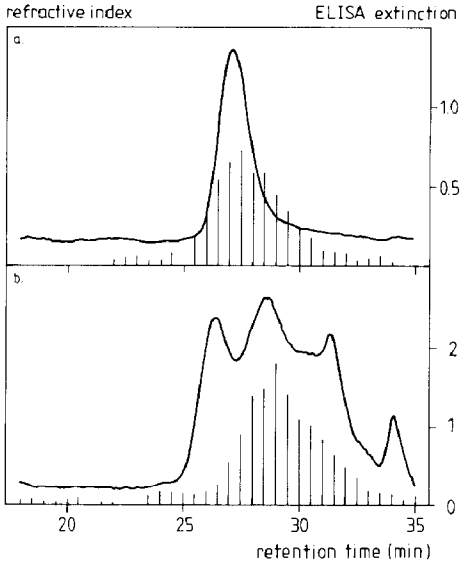

Fig. 2. High-performance size-exclusion chromatograms with ELISA detection of EPS of (a) *M. racemosus* grown with D-glucose and (b) *M. hiemalis* grown on a YNB medium with maltose as the carbon source. R.i. detection, —; ELISA detection, bars.

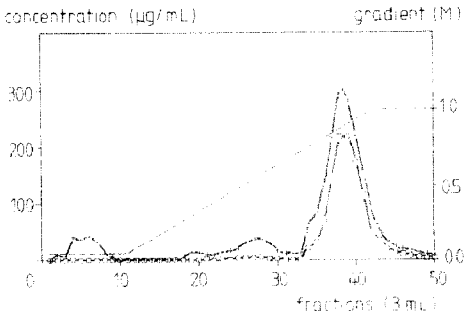

Fig. 3. Chromatography of crude EPS from *M. hiemalis* on DEAE-Sepharose CL-6B with sodium acetate buffer (pH 5.0) gradient (see Experimental section). D-Glucuronic acid, -□-□-; neutral sugars, -+-+-; gradient, ------.

Fig. 2a, in which figure they can be compared with the results of r.i. detection. Very similar graphs (not shown) were obtained using the EPS of *M. hiemalis* and *M. circinelloides* (*cf.* Fig. 1). The sugar composition of EPS of *M. racemosus* resembled that of both other *Mucor* species as shown in Table I.

If D-glucose in the growth and EPS-production medium was replaced by maltose, a more complex EPS was excreted by the mould as indicated by the differences in sugar composition (Table I) and by the chromatogram as given in Fig. 2b.

The composition of the EPS excreted by D-glucose-grown *M. racemosus* was studied further by using preparative ion-exchange chromatography. Fig. 3 shows the elution pattern using a DEAE-Sepharose CL-6B column with a linear gradient of a sodium acetate buffer (pH 5.0). Analysis of the collected fractions with the orcinol assay (neutral sugars) and the *m*-hydroxydiphenyl (*m*-hdp) assay (uronic acids) revealed one major fraction (Fig. 3) and several minor fractions.

Determination of the EPS of Rhizopus *species.* — The EPS preparations of three different D-glucose-grown *Rhizopus* species were found to be much more heterogeneous than the EPS excreted by the *Mucor* species, as revealed by sugar analysis (Table I) and size-exclusion chromatography (Fig. 4). In each of these EPS preparations, a broad peak with a retention time of 27–29 min, containing the antigens against the antibodies raised against EPS from *M. racemosus*, could be detected.

Ion-exchange chromatography was performed on EPS of *R. oryzae* which resulted in the elution pattern shown in Fig. 5. After pooling the fractions (I and II) as indicated in the figure, the sandwich ELISA was performed on each of them. The ELISA-positive part of the EPS of *R. oryzae* was found to be present in fraction II. This fraction, further applied to the high-performance size-exclusion chromatography system, revealed an unimodal mol. wt. distribution, similar to Fig. 2a. The sugar composition of the EPS preparations shown in Fig. 4 are given in Table I.

Determination of the EPS of Rhizomucor *species.* — The size-exclusion chromatograms of two species of the genus *Rhizomucor* are shown in Fig. 6. The elution profiles of *Rhm. pusillus* (Fig. 6a) and *Rhm. miehei* (Fig. 6b) showed marked differences and were

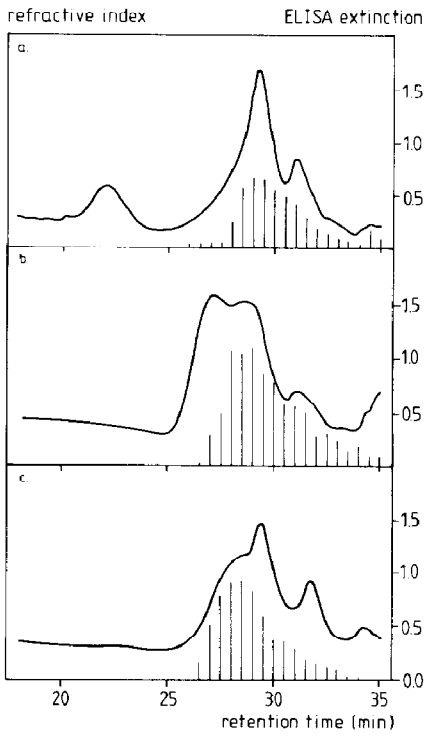

Fig. 4. High-performance size-exclusion chromatograms with ELISA detection of the EPS of (a) *R. oryzae*, (b) *R. stolonifer*, and (c) *R. arrhizus*. R.i. detection, —; ELISA detection, bars.

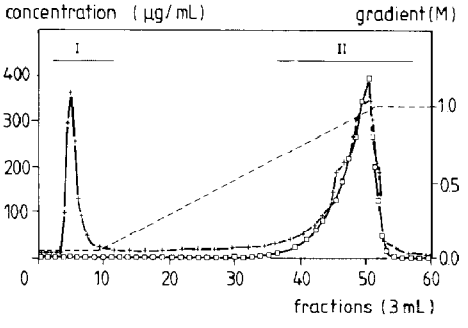

Fig. 5. Separation of crude EPS from *R. oryzae* using DEAE-Sepharose CL-6B with sodium acetate buffer (pH 5.0) gradient (see Experimental section). D-Glucuronic acid, -□-□-; neutral sugars, -+-+-; gradient, -------.

also different from those of the *Mucor* and *Rhizopus* species tested. Also, no or little D-glucose was found in these EPS preparations (Table I). However, a similar unimodal fraction was found to be immunochemically active.

Determination of the EPS of other species belonging to the Mucorales. — The multimodal mol. wt. distribution chromatograms of D-glucose-grown *Absidia corym-*

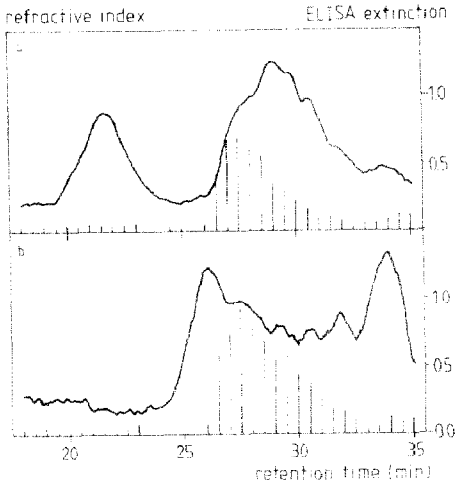

Fig. 6. High-performance size-exclusion chromatograms with ELISA detection of EPS of (a) *Rhizomucor pusillus* and (b) *Rhm. michei*. R.i. detection, ——; ELISA detection, bars.

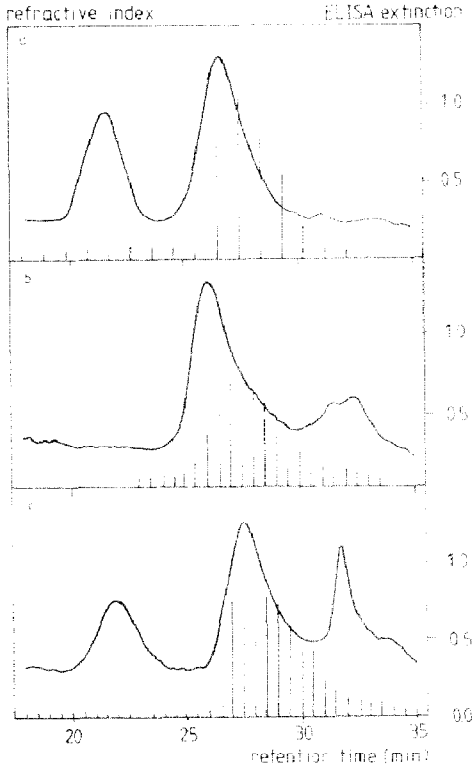

Fig. 7. High-performance size-exclusion chromatograms with ELISA detection of EPS derived from (a) *Absidia corymbifera*, (b) *Syncephalastrum racemosum*, and (c) *Thamnidium elegans*. R.i. detection, ——; ELISA detection, bars.

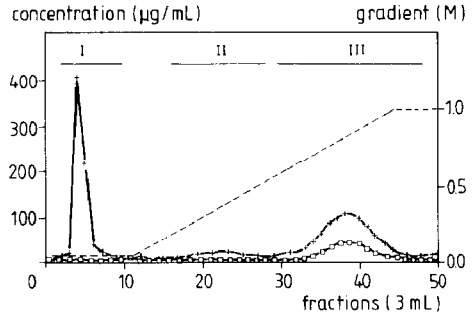

Fig. 8. Separation of the crude EPS from *Absidia corymbifera* with DEAE-Sepharose CL-6B with sodium acetate buffer (pH 5.0) gradient (see Experimental section). D-Glucuronic acid, -□-□-; neutral sugars, -+-+-; gradient, -------.

bifera, *Syncephalastrum racemosum*, and *Thamnidium elegans* EPS preparations are shown in Figs. 7a, 7b, and 7c, respectively. Fig. 7a reveals a bimodal mol. wt. distribution. The high mol. wt. peak ($500–2 \times 10^3$k) did not react with the antibodies raised against *M. racemosus*. The second peak, reactive in the ELISA, had a retention time of 27–29 min, similar to the immunochemically active peak of the other Mucorales.

Separation of the EPS derived from *Absidia corymbifera* by ion-exchange chromatography as shown in Fig. 8 revealed three peaks (I–III) as indicated in the figure. While peak I was not reactive with the IgG antibodies raised against *M. racemosus*, peaks II and III were found to give positive ELISA reactions.

The EPS derived from *Syncephalastrum racemosum* (Fig. 7b) was found to be composed of a broad range of fractions with different hydrodynamic volumes. The epitope-containing part could be detected with the size-exclusion chromatography system with ELISA detection as revealed in the figure. Its mol. wt. distribution was similar to that of the ELISA-positive fraction of *Absidia corymbifera*. The mol. wt. distribution of *Thamnidium elegans* EPS (Fig. 7c) was again different from that of *Absidia corymbifera* and that of *Syncephalastrum racemosum*. However, in this case a similar fraction was also determined to be immunochemically active. The sugar composition of the EPS preparations from these three species differed markedly as shown in Table I.

DISCUSSION

In this study, high-performance size-exclusion chromatography and ion-exchange chromatography were combined with ELISA detection to study the extracellular polysaccharides excreted by species of Mucorales.

Each of the EPS preparations of the mould species belonging to the Mucorales tested contained a 27–29 min size-exclusion chromatography fraction, reactive with the antibodies raised against EPS of *M. racemosus*. In the EPS preparations derived from *Mucor* species, only small portions of neutral polysaccharides were found, if species were grown on D-glucose as the carbon source. In addition to the 27–29 min reactive

material, EPS preparations derived from species belonging to the genera *Rhizopus*, *Rhizomucor*, *Absidia*, *Syncephalastrum*, and *Thamnidium* also produce high mol. wt. polysaccharides which were not reactive with the antibodies. In these EPS preparations, the shape of the peak of the immunochemically active material differs slightly.

Notwithstanding the fact that the low pH of the eluent in the system minimised the number of ionised glucuronic acid residues, it cannot be excluded that the mol. wt. distribution must be corrected for possible differences in hydrodynamic volume due to charge effects, as reported for galacturonic acid containing polymers[21]. Determination of the exact mol. wt. distribution of this fraction requires additional methods, such as laser light scattering and ultracentrifugation. Furthermore, the unimodal mol. wt. distribution of the immunochemically active fraction of EPS preparations tested does not necessarily correspond to a monodispersed, hydrodynamic volume distribution. However, it can be concluded that the fraction contains polysaccharide antigens which appear common to all representatives of Mucorales tested. Therefore the use of the sandwich ELISA previously proposed for the determination of *Mucor* and *Rhizopus*[5], can be extended to more genera of Mucorales.

The sugar composition of D-glucose-grown *Mucor* species (Table I) indicates that the immunochemically active fraction may be similar or identical to mucoran, the cell-wall associated polysaccharide of members of Mucorales as described by Bartnicki-Garcia's group[19,20]. Chemical and biochemical properties of cell-wall polysaccharides of fungi have been proposed as important features in fungal taxonomy[1]. As shown in this paper, extracellular polysaccharides may also be useful for this purpose. The use of high-performance size-exclusion chromatography with ELISA detection is an elegant method to study these polysaccharides.

The method was found to be very sensitive and reproducible. Sandwich ELISA detection was ten times more sensitive than refractive index detection. A disadvantage of the system is the necessity to apply the ELISA batchwise on collected fractions instead of measuring continuously.

No detailed information is available about the composition of the epitopes of the EPS of Mucorales responsible for the immunological reaction in rabbits[5]. As demonstrated for *Penicillium* and *Aspergillus* species by Notermans and associates[22], the reliable ELISA detection of fungi in food or medical samples requires synthetic antigens to eliminate false-positive ELISA reactions. Therefore it will be desirable to reveal the structure of the epitopes to develop specific diagnostic assays enabling rapid detection and identification of the medically and agriculturally important mould species of the Mucorales.

Preliminary hapten-inhibition studies have been recently performed[23], indicating an important role for mannose residues. Further research is in progress to reveal the structure of the EPS preparations of species of Mucorales in general and the immunodominant structures in the 27–29 min fraction in particular.

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