

Surface polymers of the nematode-trapping fungus *Arthrobotrys oligospora*

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The nematophagous fungus *Arthrobotrys oligospora* captures nematodes using adhesive polymers present on special hyphae (traps) which form a three-dimensional network. To understand further the adhesion mechanisms, *A. oligospora* surface polymers were visualized by transmission electron microscopy and characterized by chemical methods. Both traps and hyphae were surrounded by a fibrillar layer of extracellular polymers which stained with ruthenium red. The polymer layer was resistant to most of the chemicals and enzymes tested. However, part of the layer was removed by sonication in a Tris-buffer or by extraction in a chaotropic salt solution (LiCl), and the structure of the polymers was modified by treatment with Pronase E. Chemical analysis showed that the crude extracts of surface polymers removed by sonication or LiCl solution contained neutral sugars, uronic acids and proteins. Gel chromatography of the extracts revealed that the major carbohydrate-containing polymer(s) had a molecular mass of at least 100 kDa, containing neutral sugars (75% by weight, including glucose, mannose and galactose), uronic acids (6%) and proteins (19%). There was more polymer in mycelium containing trap-bearing cells than in vegetative hyphae. SDS-PAGE of the extracted polymers showed that the trap-forming cells contained at least one protein, with a molecular mass of approx. 32 kDa, not present on vegetative hyphae. Examining the capture of nematodes by traps of *A. oligospora* in which the layer of surface polymers was modified, or removed by chemical or enzymic treatments, showed that both proteins and carbohydrate surface polymers were involved in the adhesion process.

Introduction

Adhesion of fungi to surfaces is considered to be an important event in the colonization and pathogenesis of humans, animals and plants (Nicholson & Epstein, 1990; Kennedy, 1990). Adhesion might be involved in the recognition of the host, in the firm binding of the pathogen to the host surface, and in serving as the site for penetration and start of the infection of the host cells. In contrast to the adhesion mechanisms of pathogenic bacteria, these processes have not been well characterized in fungi. No fungal surface molecules mediating adhesion to surfaces have been described in detail, and the molecular interactions between such compounds and the host surface are not understood.

Numerous electron microscopic studies suggest that adhesion of fungi to surfaces is mediated by extracellular polymers (EP) (Gold & Mendgen, 1984; Latgé *et al.*, 1986; Veenhuis *et al.*, 1985; Mims & Richardson, 1989;

Onyile *et al.*, 1982; Hyde & Jones, 1989). According to these studies, the structure of such polymers varies from amorphous liquids to fibrillar polymers, and cytochemical and chemical analysis indicates that EP layers consist of polysaccharides and/or proteins or glycoproteins. Similar kinds of polymers have also been found in fungal structures not involved in adhesion to surfaces (Wessels & Sietsma, 1981), which suggests that they might have other functions as well (Nicholson & Epstein, 1991). A few studies have shown that the ultrastructure of surface polymers can change during adhesion. *Gymnosporangium juniperi-virginianae* basidiospore germlings produce a diffuse, filamentous network of material that subsequently changes to an electron-dense layer binding the entire germling to the host surface (Mims & Richardson, 1989). The adhesion of *Candida albicans* to epithelial cells appears to involve spatial rearrangements of their cell wall surface (Tronchin *et al.*, 1984). However, the chemical interactions between the

fungus EP layer and the host surface, and the chemical changes occurring during the adhesion, are not known.

The adhesion of nematodes to the nematophagous fungus *Arthrobotrys oligospora* is a well-studied model system for examining the molecular mechanisms of fungal adhesion to host surfaces (Nordbring-Hertz, 1988). This fungus captures nematodes using adhesive polymers present on special hyphae (traps), which form a three-dimensional network. Previous studies have demonstrated that the adhesion of nematodes to the traps is mediated by a lectin present in trap-containing mycelium (Nordbring-Hertz & Mattiasson, 1979; Borrebaeck *et al.*, 1984). Detailed EM studies have also shown that the cell walls of the traps are surrounded by an extracellular layer of fibrillar polymers, and that these fibrils become oriented in one direction during the first hour of capture (Veenhuis *et al.*, 1985).

To understand further the molecular mechanisms of adhesion in this system, we have extracted and chemically characterized the components in the EP layer from both vegetative hyphae and mycelium containing traps of *A. oligospora*. The function of the components in the EP layer during adhesion was examined by studying the capture of nematodes to traps treated with various chemicals and enzymes shown to affect the chemical structure of the EP layer.

Methods

Culture of organisms. *Arthrobotrys oligospora* Fres. (ATCC 24927) was maintained on corn meal agar (CMA, Difco), supplemented with 2 g l^{-1} K_2HPO_4 . Liquid cultures of *A. oligospora* were obtained by inoculating 400 ml or 4000 ml media with a water suspension of conidia prepared from a 1–2 week old culture grown on CMA. Trap-containing mycelium [T(+)] was grown in a medium containing 0.01% soya peptone (neutralized, Oxoid) supplemented with 0.05 g phenylalanine and 0.05 g valine per litre (Friman *et al.*, 1985). For the production of hyphae without traps [T(-)], the fungus was grown in the same medium but supplemented with 12 mM-phosphate buffer (pH 7.0). Mycelia used for the extraction of surface polymers from T(+) and T(-) cultures were harvested by filtering through a nylon mesh. The mycelia were then washed with $5 \times 10\text{ ml}$ of ice-cold (4°C) distilled water or buffer. For the adhesion bioassay, a conidial suspension of *A. oligospora* was inoculated onto dialysis membranes, which were placed on agar plates supplemented with low-nutrient medium (LNM) and phenylalanyl-valine (Nordbring-Hertz, 1973).

The nematode *Panagrellus redivivus* (Goodey) was grown axenically in a soya-peptone-liver extract medium (Nordbring-Hertz, 1977). The nematodes were washed extensively with distilled water before being used in the adhesion assays.

Transmission electron microscopy (TEM). Treated and untreated mycelia were stained with ruthenium red (Fluka AG, Buchs, Switzerland) as described by Luft (1971) and modified by Handley *et al.* (1988). Control samples were prepared by substituting distilled water for ruthenium red solutions. After dehydrating the samples in a graded series of ethanol and water, they were embedded in Polarbed (Johnsson

Lab System, Solna, Sweden), cut on a Reichert-Jung Supernova ultramicrotome, and examined in a Jeol 100CX electron microscope (located at the Electron Microscopy Unit, Faculty of Medicine, Lund University).

Extraction of surface polymers. Washed T(+) and T(-) mycelia from the liquid cultures were transferred to 50 ml flasks, 10 ml of the solutions used for the extractions was added, and the flasks were incubated on a rotary shaker. The following solutions and conditions were used to extract the surface polymers. (1) A chaotropic salt solution containing 1 M- or 5 M- LiCl in a 10 mM-Tris/HCl buffer (pH 7.4). The mycelia were extracted at 4°C for 10 min. This protocol is a modification of a procedure used to isolate surface polymers from the zygomycete *Absidia glauca* (Teepe *et al.*, 1988). (2) A buffer containing the thiol reagent dithiothreitol (DTT) which dissociates disulphide bridges in proteins. The mycelia were extracted at 4°C for 10 min in 12 mM-DTT and 20 mM-Tris/HCl buffer pH 7.4. Buffers with DTT have been used to extract surface polymers from *Candida* sp. (Chattaway *et al.*, 1974; Ponton & Jones, 1986). (3) A 0.1 M-sodium phosphate buffer (pH 6.5) containing urea (8 M), mercaptoethanol (1%, v/v) and sodium dodecyl sulphate (SDS) (1% w/v). The mycelia were extracted at room temperature for 4 h. Buffers with urea, mercaptoethanol and SDS have been used in numerous studies to extract proteins from fungal cell walls (Wu-Yuan & Hashimoto, 1977; Chaffin & Stocco, 1983). (4) Sonication at 4°C in a 20 mM-Tris/HCl buffer (pH 7.5). The mycelia were first mixed in 10 ml Tris buffer with an Ultra-Turrax homogenizer (type T 18/10; Janka Kunkel, IKA) for 10 s, followed by sonication (Bransonic 220 unit) for 20 s. The mycelia were centrifuged (5000 g, 20 min) and the supernatants retained for chemical analysis. The sonication and centrifugation steps were repeated twice with $2 \times 10\text{ ml}$ Tris buffer. Sonication in water or buffer solutions has been used to extract cell wall polymers from *Aspergillus nidulans* (Claverie-Martin *et al.*, 1986) and from *Neurospora crassa* (Beever *et al.*, 1979). (5) Cold (4°C) 0.1M-NaOH for 10 min. Cold alkali has been used to extract cell wall proteins from a variety of fungi (Taylor & Cameron, 1973).

After the incubations, the cultures were filtered through a nylon mesh. The mycelia were recovered and either prepared for TEM analysis, or lyophilized for dry weight measurements. Before chemical analysis, the polymer extracts were filtered ($0.22\ \mu\text{m}$) and concentrated by ultrafiltration using a Centriprep 10 or a Centricon 10 unit (Amicon Corp.).

Enzymic treatments. Washed mycelia of T(+) cultures were transferred to 10 ml test tubes and incubated at 30°C with various enzymes. Pronase E (Merck) was used at a concentration of 2.9 or 0.3 mg ml^{-1} in a 10 mM-Tris/HCl buffer (pH 7.4) with an incubation time of 1 h. Lyticase (Sigma, a $\beta(1,3)$ -glucanase) at 2.7 or 0.3 mg ml^{-1} ; α -mannosidase (EC 3.2.1.24, Sigma) at 1.0 mg ml^{-1} ; chitinase (EC 3.2.1.14, Sigma) at 2.8 mg ml^{-1} ; glucuronidase (EC 3.2.1.31, Sigma) at a final concentration of 5% (v/v) corresponding to about 10000 Fishman Units and Novozym 234, a commercial preparation of *Trichoderma harzianum* enzymes (Novo Biolabs, Bagvaerd, Denmark) containing $\beta(1,3)$ -glucanase and chitinase activity (Peberdy, 1985) were all incubated for 4 h in a 10 mM-sodium phosphate buffer (pH 5.8). Control samples were treated with the corresponding buffer only. The incubations were stopped by transferring the test tubes to an ice-bath. The samples were centrifuged (4°C , 5000 g, 20 min) and the supernatant discarded. The pellets were washed twice with water and prepared for TEM analysis.

Preparations of cell walls. To study whether the extracellular polymers could be recovered from prepared cell walls of *A. oligospora*, washed liquid-grown mycelia were mixed in an ice-cold 20 mM-Tris/HCl buffer (pH 7.5) with a Buhler mixer (Buhler, Tubingen) for $3 \times 60\text{ s}$. Following homogenization, the hyphal fragments were centrifuged

(4 °C, 5000 g, 20 min) and washed three times in the Tris-buffer. The final pellet was transferred to a test tube. Acid-washed glass beads (0.5 mm diameter) were added and the test tube was agitated with a vortex mixer for 10 × 30 s. The glass beads were allowed to settle (4 °C) and the supernatants were recovered (Catley, 1988). The cell wall fragments were washed three times with 7 M-urea, 1.0 M-NaCl, 0.5 M-NaCl, and distilled water.

Adhesion assay. A biological assay using a dialysis membrane technique (Nordbring-Hertz *et al.*, 1984) was used to examine the effects of extracting the surface polymers from the traps on the capture of nematodes. Pieces of dialysis membranes (1–2 cm²) containing trap-bearing mycelium (50–100 traps cm⁻²) were transferred to Petri dishes and incubated with the chemical solutions or enzymes used to extract polymers from the liquid-grown cultures. The concentrations and conditions were as described above. In one experiment the mycelia were treated with heat-denatured (90 °C, 30 min) enzymes. Following the extractions, the membrane pieces were washed extensively with distilled water, or the appropriate buffer, and transferred to a new set of Petri dishes. The nematodes (20–30 per membrane) were added, and captured, and free living nematodes were counted under a light microscope after 1 h and 20–22 h of interaction. The experiments were performed with 5–10 parallels and repeated at least twice. Controls consisted of trap-bearing mycelium washed with distilled water or the appropriate buffer. The statistical significance of the differences in frequencies of captured and free nematodes in treated samples versus controls were tested by χ^2 -analysis. The viability of *A. oligospora* after treating the mycelia with the extraction solutions and enzymes was examined by transferring one piece of the extracted membrane culture from each treatment to an agar plate with the LMN medium. The mycelia were incubated and the radial growth of the colonies was observed after 1–7 d.

Chemical analysis. Neutral sugars were estimated by the phenol/sulphuric acid procedure (Dubois *et al.*, 1956) using glucose as a standard. The carbazole assay (Bitter & Muir, 1962) was used to determine the concentration of uronic acids, using glucuronolactone as a standard. Hexosamines were measured by the method of Elson & Morgan (1933) using glucosamine as a standard. Proteins were determined by the method of Bradford (1976) using bovine albumin as a standard. The UV-VIS measurements were performed on a Hitachi U-3210 spectrophotometer.

Sugar composition. Monosaccharides were released by hydrolysis in 2M-trifluoroacetic acid for 3 h at 110 °C under nitrogen (Albersheim *et al.*, 1967). Myo-inositol was added as an internal standard, and alditol acetates were prepared as described by Blakeney *et al.* (1983). The derivatives were determined by gas chromatography using a Hewlett Packard 5890 GC equipped with a capillary fused silica column (30 m × 0.2 mm i.d.) coated with a phenylmethyl silicone phase (DB-5, J&W Scientific, Folsom, CA).

Gel chromatography. Surface polymers extracted with 5 M-LiCl or sonication in a Tris-buffer, were applied to a column of Sephacryl S-200 HR (Pharmacia, 1.5 × 70 cm) which had been equilibrated with 50 mM-Tris/HCl buffer (pH 7.2) containing 1 M-LiCl. The column was eluted with the same buffer at a flow rate of 0.5 ml min⁻¹. Fractions of 3.0 ml were collected. Fractions in peaks identified by analysis of neutral sugars were pooled and concentrated by ultrafiltration (Centriprep 10). The gel chromatography was performed at 4 °C.

Pronase digestion. The method was that of Spiro (1966). A polymer extract, obtained from T(+) mycelium after sonication in Tris/HCl buffer, was concentrated by ultrafiltration (Centriprep 10). Pronase E (Merck) was added at a concentration of about 30% of the estimated weight of glycoproteins, and the samples were incubated in test tubes at 30 °C for 10 h, then transferred to an ice-bath. The mixture was fractionated by gel chromatography as described above.

SDS-PAGE. Samples for gel electrophoresis were first desalted on a Sephadex G-25 Superfine (Pharmacia), packed into a HR 10/10 column, using 10 mM-NH₄HCO₃ as the elution buffer. Samples of 1–2 ml were applied to the column and eluted at a flow rate of 2 ml min⁻¹. The PhastSystem (Pharmacia) was used for electrophoresis and gel staining, and was performed according to the manufacturer's instructions. For SDS-PAGE, the PhastGel Gradient 8–25% and LMW Calibration Kit (Pharmacia) were used. The gels were stained with Coomassie Brilliant Blue. Molecular masses used for standard proteins were as follows: phosphorylase b (rabbit), 97.4 kDa; bovine serum albumin, 66.0 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase (bovine), 29.0 kDa; trypsin inhibitor (soybean), 20.1 kDa; α -lactalbumin, 14.2 kDa.

Results

Electron microscopy

TEM showed that the adhesion of *A. oligospora* to the nematode *P. redivivus* was mediated by a layer of extracellular polymers staining with ruthenium red (Fig. 1a). The layer seemed to consist of densely packed fibrils. A layer of extracellular fibrils was also present on trap-forming cells without adhering nematodes (Fig. 1b) as well as on vegetative mycelium (not shown). The EP fibrils on these cells were more loosely packed than in the EP layer bridging the trap and nematode surfaces. Furthermore, the EP fibrils were not present in a uniform layer on the cell surface, but appeared to be more concentrated in areas of cell aggregates, while a few cells seemed to lack most of these polymers. The uneven distribution of EP fibrils might, however, be an artifact introduced during the preparation of the TEM samples. The EP fibrils were not observed in the control samples prepared without ruthenium red.

Extractions of surface polymers

Attempts were made to extract the ruthenium-red-staining EP layer from *A. oligospora* by treating liquid-grown mycelia of the fungus with various solutions or enzymes followed by TEM examination. Most of the ruthenium-red-staining EP fibrils were removed by treating the mycelia with a mild sonication (3 × 20 s, 4 °C) in a Tris buffer (Fig. 1c). No lysis of the sonicated cells was observed. Washing the mycelium with a strong chaotropic salt solution (5 M-LiCl) also seemed to remove some of the polymers in the EP layer. However, at least some of the extracellular fibrils remained on the cell surface after this treatment, and they were more densely packed than those in the untreated samples. Washing the mycelia with a buffer containing the thiol reagent DTT did not remove or modify the ultrastructure of the EP layer, as visualized by TEM. Treating the mycelia with 0.1 M-NaOH or with a mixture of SDS-mercaptoethanol-

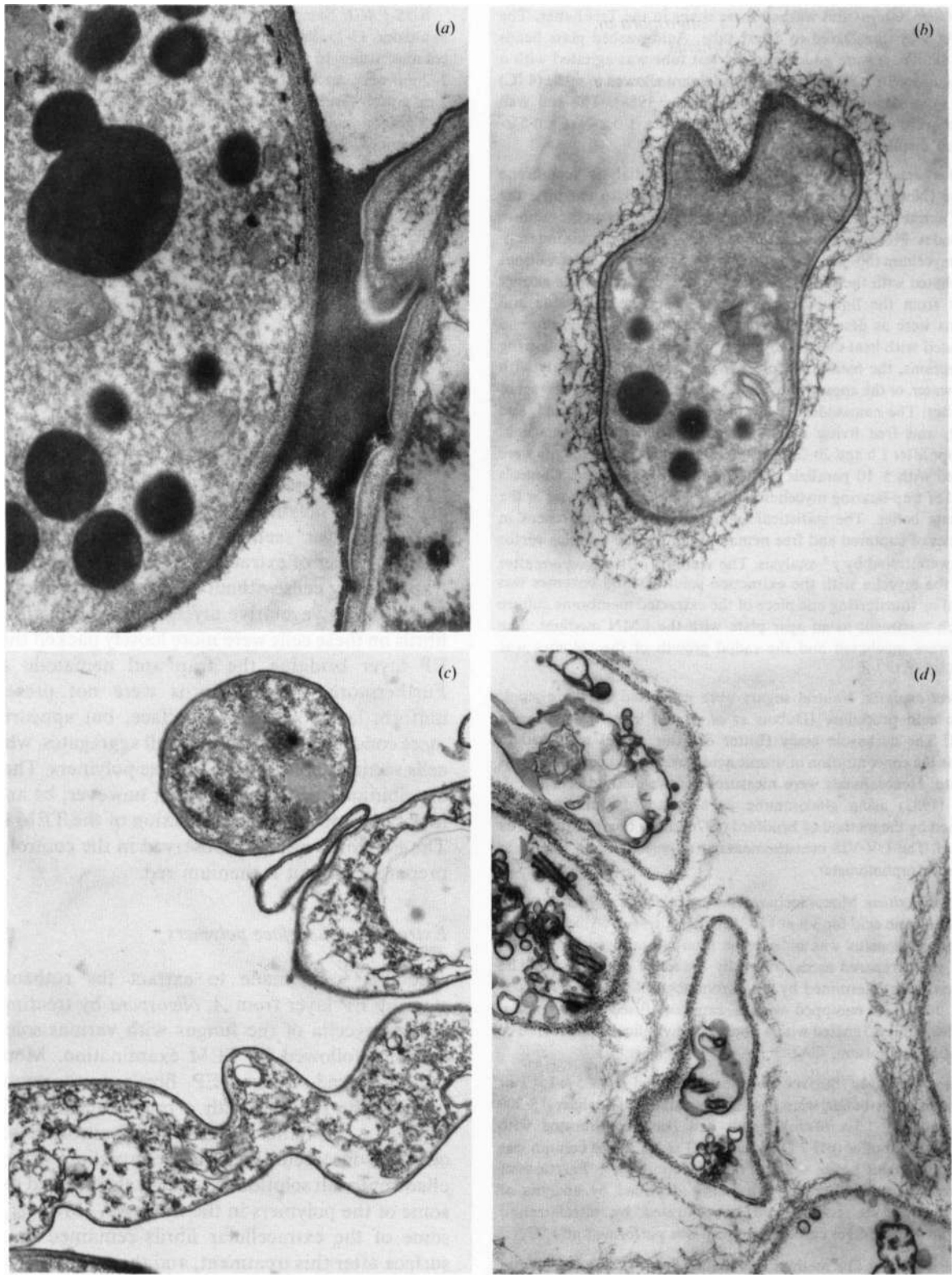


Fig. 1. Transmission electron microscopy using ruthenium red staining of surface polymers of *Arthrobotrys oligospora*. (a) The fungus (left) adhering to the surface of the nematode *Panagrellus redivivus* (right). Magnification $\times 20000$. (b) Polymer layer on trap cells of *A. oligospora* present in the absence of the nematode. Magnification $\times 16000$. (c) Removal of extracellular polymers by treating the mycelia with a mild sonication in a Tris-buffer. Magnification $\times 13000$. (d) Modification of the surface polymers by treating the mycelia with Pronase E. Magnification $\times 13000$.

Table 1. Nematode capture by *A. oligospora* after various mycelium treatments

A bioassay using a dialysis membrane technique was used. Pieces of dialysis membrane containing trap-bearing mycelium were incubated with the solutions in the table. The membranes were washed with a buffer and nematodes were added. Captured and free nematodes were counted under a light microscope after 1 h and 20–22 h of interaction. Controls, using mycelium washed with the appropriate buffer, were in the range of 42–58% capture at time 1 h, and 87–97% at 20–22 h. Experiments used 5–10 parallel incubations and were repeated twice.

Treatment	% Captured nematodes						Viability*
	1 h			20–22 h			
	Mean	SD	P†	Mean	SD	P	
DTT	33.3	11.8	NS	93.4	6.6	NS	+
1 M-LiCl	33.1	22.0	**	53.2	32.7	**	+
5 M-LiCl	2.4	1.9	***	2.3	1.9	***	(+)
0.1 M-NaOH	5.1	4.9	***	0.2	0.4	***	–
SDS-Urea-mercaptoethanol	0.6	1.3	***	0.4	0.9	***	–
Sonication	15.0	14.3	**	52.0	23.5	***	+

* Viability was tested by observing the growth of washed mycelia on agar plates. +, no differences in growth rate compared to control sample, (+); growth delayed; –, no growth.

† *P* values from χ^2 -tests (*df* = 1) comparing the frequencies of captured and free nematodes in treated versus control samples. NS = not significant; *, 0.01 < *P* < 0.1; **, 0.001 < *P* ≤ 0.01; ***, *P* ≤ 0.001.

urea seemed to remove some of the EP polymers. However, these chemicals also extracted most of the cytoplasm from the cells. None of the enzymes used completely removed the ruthenium red EP layer, but some of them seemed to affect the ultrastructure of the fibrillar polymers. The most notable effect was observed in cells treated with Pronase E. The EP fibrils in those samples came loose from the cell surface (Fig. 1*d*). The effects of treatment with cellulase, lyticase or Novozym 234 were less prominent, and the latter two enzymes also led to the degradation of some cell walls. Treatment with mannosidase, glucuronidase and chitinase did not affect the ultrastructure of the EP layer as observed in TEM. The ruthenium-red-staining EP layer was not present on the isolated cell walls of *A. oligospora*. Thus, the EP fibrils were lost during the preparation of cell walls.

Effects on adhesion

The effects of treating the mycelia of *A. oligospora* with the solutions used to remove the surface polymers on the capture of nematodes were also examined (Table 1). Adhesion of nematodes significantly decreased when the fungus was washed with LiCl, 0.1 M-NaOH, SDS-mercaptoethanol-urea, or when sonicated. However,

Table 2. Nematode capture by *A. oligospora* after further mycelium treatments

See Table 1 for descriptions of the bioassay, viability test and statistics.

Treatment	% Captured nematodes						Viability
	1 h			20–22 h			
	Mean	SD	<i>P</i>	Mean	SD	<i>P</i>	
Pronase E 2.9 mg ml ⁻¹	11.3	7.2	***	39.0	29.3	***	+
Pronase E 0.3 mg ml ⁻¹	27.8	14.7	***	75.7	10.3	NS	+
Pronase E 2.9 mg ml ⁻¹ (denatured)†	77.2	8.5	NS	96.9	3.1	NS	ND‡
Lyticase 2.7 mg ml ⁻¹	39.3	18.1	**	85.0	9.9	**	+
Lyticase 0.3 mg ml ⁻¹	48.2	20.2	*	93.8	4.9	NS	+
Lyticase 2.7 mg ml ⁻¹ (denatured)	65.7	19.5	NS	89.9	4.5	NS	ND
Glucuronidase	76.3	11.0	**	97.8	2.4	NS	+
Glucuronidase (denatured)	75.7	4.7	**	99.3	1.5	*	ND
Mannosidase	40.0	10.9	**	75.2	14.8	**	(+)
Mannosidase (denatured)	17.2	5.2	***	74.7	7.3	**	ND

† Enzymes denatured by heating at 90 °C for 30 min.

‡ ND, not determined.

experiments observing the growth of the washed mycelium showed that mycelia treated with NaOH or the SDS mixture were not viable.

Treating the mycelia with Pronase E and lyticase significantly decreased the adhesion of nematodes to the traps of *A. oligospora* (Table 2). No effects were observed using the heat-denatured enzymes, indicating that the effects were due to the activity of the enzymes. The capture of nematodes was also affected after treating the traps with mannosidase or glucuronidase. However, these effects were also observed using the corresponding heat-denatured enzymes.

Chemical composition of surface polymers

The chemical composition of the surface polymers from *A. oligospora* extracted with 5 M-LiCl or sonication, from T(+) and T(–) mycelia, was analysed by UV-VIS spectroscopy. All extracts contained neutral sugars, uronic acids and proteins (Table 3). The concentrations and proportions of uronic acids (of the total amount of sugars) were somewhat higher in the extracts from the T(+) than from the T(–) mycelia. No amino sugars were detected in these extracts.

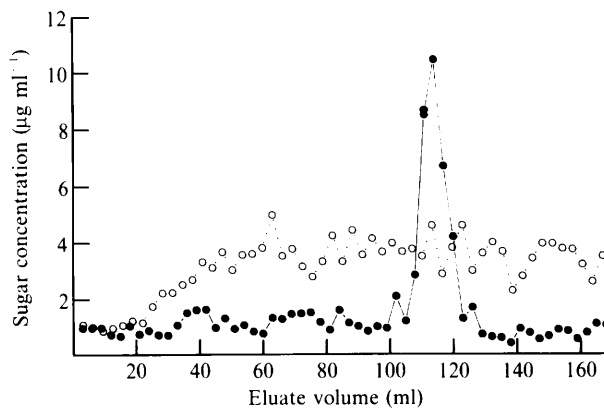


Fig. 2. Fractionation of surface polymers extracted from *A. oligospora* using 5 M-LiCl, according to molecular mass on a Sephacryl S-200 HR column. Extracts were from trap-containing mycelium [T(+)] (●) or from vegetative hyphae [T(-)] (○). The neutral sugar content was determined by the method of Dubois *et al.*, 1956.

Table 3. Contents of neutral sugars, uronic acids and proteins in surface polymer from *A. oligospora*

The fungus was grown in a liquid culture, with T(+) and T(-) designating mycelium with and without nematode-trapping networks, respectively. The polymers were extracted with 5 M-LiCl or by sonication in a Tris/HCl buffer, and were concentrated by ultrafiltration. Values are expressed as µg (mg dry wt)⁻¹.

Compounds	5 M-LiCl		Sonication	
	T(+)	T(-)	T(+)	T(-)
Neutral sugars	1.43	1.70	2.82	2.87
Uronic acids	0.53	0.49	0.47	0.22
Proteins	0.10	0.10	6.32	5.77

Chromatographic separations

Gel chromatography revealed major differences in the composition of carbohydrate-containing polymers in extracts from T(+) and T(-) mycelia. The LiCl extracts from the T(+) culture contained a fraction of carbohydrate-containing polymers with an apparent molecular mass of 22 kDa (estimated from selectivity curves using protein standards) (Fig. 2). This polymer fraction was not detected in LiCl extracts from T(-) cells.

The major carbohydrate-containing polymers in the sonication extracts from T(+) mycelia eluted in the void volume of the column (peak 1, Fig. 3). The molecular masses of the polymers in this fraction are not known, but the exclusion limit for a Dextran polymer of the Sephacryl S-200 gel medium is about 100 kDa (Pharmacia). The overall chemical composition of this polymer fraction was 75% neutral sugars, 6% uronic acids and 19% proteins (by weight), as analysed by UV-VIS spectroscopy. Apart from this fraction, two other carbohydrate-containing fractions (peaks 2a and 2b in Fig. 3), with chromatographic retention times close to

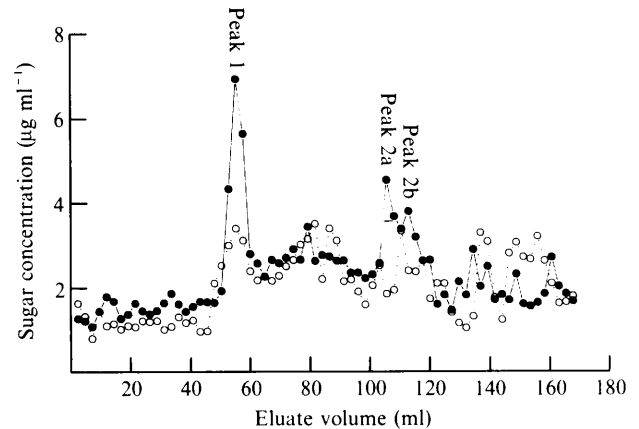


Fig. 3. Fractionation of surface polymers extracted from *A. oligospora* using sonication in a Tris buffer, according to molecular mass, on a Sephacryl S-200 HR column. Extracts were from trap-containing mycelium [T(+)] (●) or from vegetative hyphae [T(-)] (○). The neutral sugar content was determined by the method of Dubois *et al.*, 1956.

Table 4. Composition of neutral sugars in surface polymers from *A. oligospora*

The fungus was grown in a liquid culture with T(+) and T(-) designating mycelium with and without nematode-trapping networks, respectively. The polymers were extracted with 5 M-LiCl or by sonication in a Tris/HCl buffer and concentrated by ultrafiltration. Values are expressed as mol%.

Samples	Mannose	Glucose	Galactose
5 M-LiCl extracts			
T(+), total extract	11.2	88.8	ND†
T(+), peak*	ND	100.0	ND
T(-), total extract	11.7	88.3	ND
Sonication extracts			
T(+), total extract	4.8	92.2	3.3
T(+), peak 1*	8.3	85.5	3.3
T(+), peak 2a	4.7	90.2	5.1
T(+), peak 2b	4.6	92.4	3.0
T(-), total extract	11.3	82.5	6.2
T(-), peak 1	25.9	56.3	17.8
T(-), peak 2a	8.8	74.0	17.1
T(-), peak 2b	8.9	67.5	23.5

* Peaks are shown in gel chromatograms, Figs. 2 and 3.

† ND, not detected.

the major peak in the T(+) LiCl extract, were also detected in the T(+) extracts. Sonication extracts from the T(-) mycelium contained considerably lower amounts of the carbohydrate containing polymers detected in the T(+) extracts.

Carbohydrate analysis

Gas chromatographic analysis showed that glucose was the major neutral sugar component of the surface polymers extracted with 5 M-LiCl (Table 4). The crude LiCl extract also contained minor amounts of mannose. There were no differences in the composition of neutral

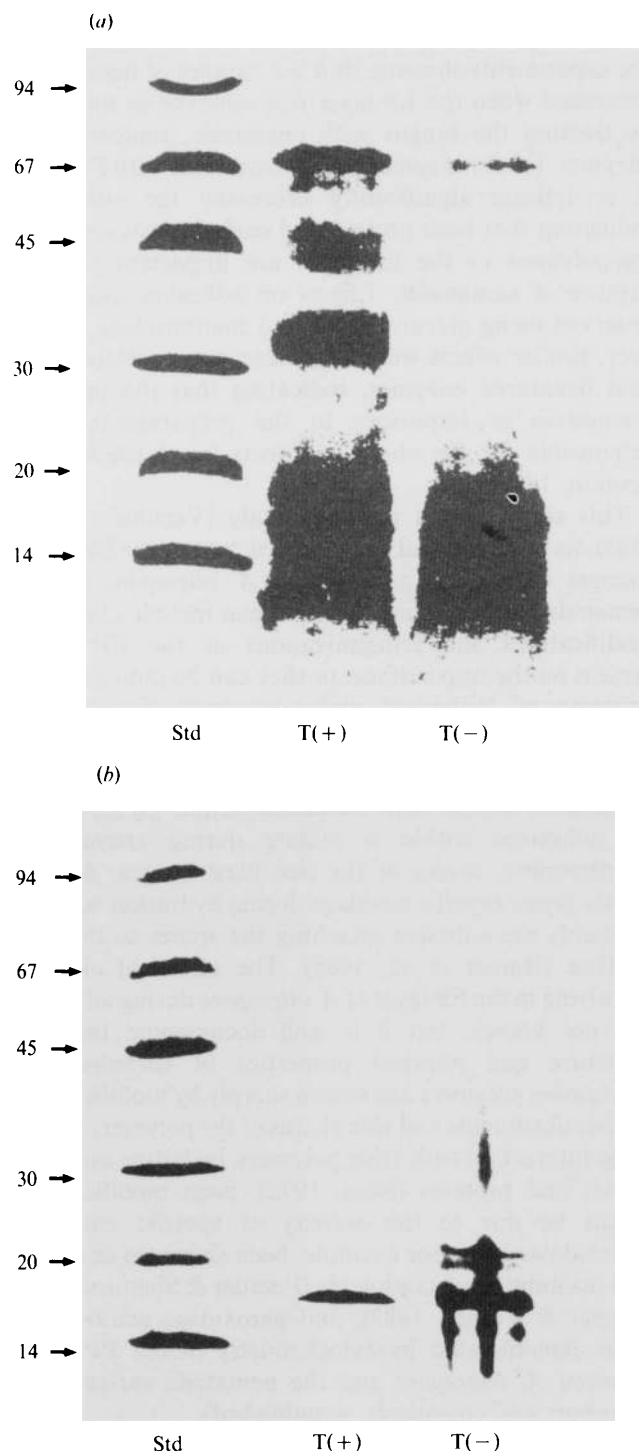


Fig. 4. SDS-PAGE of surface polymers extracted from *A. oligospora* using (a) 5 M-LiCl; and (b) sonication in Tris buffer. Extracts were from trap-containing mycelium [T(+)] or from vegetative hyphae [T(-)]. The gels were stained with Coomassie Brilliant Blue, and molecular masses (kDa) of standard proteins (Std) are indicated.

sugars in the extracts from the T(+) and T(-) mycelium. The carbohydrate polymer in the LiCl extract separated by gel chromatography contained glucose as the only detected neutral sugar.

The crude sonication extracts as well as the polymers fractionated by gel chromatography contained glucose, mannose and galactose as neutral sugars (Table 3). The major peak from the gel filtration had a somewhat lower proportion of glucose than the unfractionated (crude) extracts. The polymers extracted from the T(+) mycelium had a higher proportion of glucose than the corresponding fractions from the T(-) mycelium.

Pronase digestion

Pronase E treatment of surface polymers, extracted by sonication from T(+) mycelium, indicated that the high molecular weight polymer(s) eluting in the void volume was resistant to protease degradation. Minor changes were, however, observed in the chemical composition of the polymer(s) after the treatment, since chemical analysis revealed a content of 74% (by wt) neutral sugars, 13% uronic acids and 13% proteins (cf. above). The two other peaks present in the untreated T(+) extracts (peak 2a and 2b, in Fig. 3) were degraded by the added protease.

Gel electrophoresis

SDS-PAGE of surface polymers extracted with 5 M-LiCl showed the presence of several proteins with molecular masses below 70 kDa (Fig. 4a). At least one of the major bands, with an estimated molecular mass of 32 kDa in extracts from T(+) mycelium was not detected in the T(-) mycelium. The patterns of proteins were less complex in the sonication extracts, as visualized by the Coomassie Blue staining (Fig. 4b). The major band in both T(+) and T(-) sonication extracts corresponded to a protein with a molecular mass of 16 kDa. Notably, the total amount of protein (as estimated by UV absorption) in the T(+) lane was about four times less than in the T(-) lane, which indicates that the amount of the 16 kDa protein was higher in the T(+) compared to the T(-) sample.

Discussion

The mycelium of *A. oligospora* was surrounded by a layer of extracellular polymer which stained with ruthenium red, indicating that the layer consisted of carbohydrate-containing polymers. Ruthenium red complexes with carboxyl or hydroxyl groups in polymers carrying a high charge density, usually acidic polysaccharides, and has been widely used to locate polysaccharides in microorganisms (Luft, 1971; Handley *et al.*, 1988; Marrie & Costerton, 1981). However, it has also been reported that

neutral polysaccharides such as dextran stains with ruthenium red (Brooker, 1979).

Sonication of the mycelium removed most of the ruthenium-red-staining fibrils from the cell surface. It is not known whether this treatment dissolves the EP layer by breaking strategic bonds between the polymers and the cell walls or by disentangling the large macromolecules. Sonication can degrade large molecules (Freifelder, 1982), so polymers extracted with this method might not be intact. Extraction with LiCl seemed to remove different surface polymers from sonication, since the chemical composition of these extracts was quite different, and TEM analysis showed that at least some of the long chain carbohydrate fibrils remained on the cell surface after LiCl extraction. The polymers extracted with LiCl might represent more tightly bound surface polymers not extracted or dissolved by sonication. However, the exact localization of the proteins and carbohydrate polymers in the two extracts cannot be determined with the methods used.

Gel chromatography of the extracted surface polymers showed that the main carbohydrate-containing polymers in the EP layer were long chains of neutral sugars, uronic acids and proteins. It is not known whether this fraction contained one or several carbohydrate polymers. Since this polymer fraction was almost completely resistant to Pronase E treatment, it probably makes up the Pronase-E-resistant fibrils observed in the TEM samples treated with this enzyme. The high-molecular-weight polymers could either be polysaccharides or glycoproteins. Glucan microfibrils have been identified in the EP (mucus) layer of e.g. *Schizophyllum commune* (Wessels *et al.*, 1972) and *Conidiobolus obscurus* (Latzg  *et al.*, 1986), but the EP fibrils of *A. oligospora* are probably not glucans, since the fibrils were resistant to treatments with enzyme preparations containing glucanase activity. The main component in the mucus isolated from the spores of *Colletotrichum graminicola* was a glycoprotein eluting in the void volume of a Sephacryl S-200 medium (Ramadoss *et al.*, 1985). However, the proportion of proteins in this polymer fraction was considerably higher, and the composition of neutral sugars was different from that of the corresponding polymers isolated from *A. oligospora*. Furthermore, in contrast to the polymers isolated from *A. oligospora*, the polymers from *C. graminicola* were sensitive to Pronase E digestion. Further purification of the high-molecular-weight polymers is, however, needed before the chemical structure can be examined in more detail.

The differences in carbohydrate and protein composition between surface polymers extracted from trap-bearing mycelium and vegetative hyphae of *A. oligospora* suggest that these polymers are involved in the adhesion and infection of nematodes. The importance of EP

components in adhesion was more directly suggested in the experiments showing that the capture of nematodes decreased when the EP layer was removed or modified by treating the fungus with chemicals, sonication or enzymes. Of the enzymes tested, treatment with Pronase E or lyticase significantly decreased the adhesion, indicating that both protein and carbohydrate-containing-polymers in the EP layer are important for the capture of nematodes. Effects on adhesion were also observed using glucuronidase and mannosidase. However, similar effects were also present in controls using heat denatured enzymes, indicating that the proteins themselves or impurities in the preparations were responsible for the observed effects (cf. Nicholson & Epstein, 1990).

This study, and a previous study (Veenhuis *et al.*, 1985), have shown that the ultrastructure of the EP layer changes during the adhesion of *A. oligospora* to the nematode surface. These changes can include chemical modifications and reorganizations of the EP layer present on the trap surface, or they can be induced by a secretion of 'adhesive' molecules from the fungus. Gubler & Hardham (1988), using monoclonal antibodies, elegantly demonstrated that zoospores of *Phytophthora cinnamomi* secrete high-molecular-weight glycoproteins as adhesives within a minute during encystment. Furthermore, spores of the rice blast fungus, *Magnaporthe grisea* expell a mucilage during hydration which is probably the adhesive attaching the spores to the host surface (Hamer *et al.*, 1988). The chemical changes occurring in the EP layer of *A. oligospora* during adhesion are not known, but it is well documented that the structure and physical properties of carbohydrate-containing polymers can switch sharply by modifications of the substituents and side chains of the polymer, as well as by interaction with other polymers, including carbohydrates and proteins (Rees, 1972). Such modifications might be due to the activity of specific enzymes. Peroxidases have, for example, been shown to crosslink and insolubilize glycoproteins (Foerder & Shapiro, 1977; Cooper & Varner, 1983), and peroxidase activity has been demonstrated by cytochemistry in the EP layer between *A. oligospora* and the nematode surface (M. Veenhuis and coworkers, unpublished).

Previous studies have shown that the adhesion of nematodes to *A. oligospora* can be inhibited by treating the fungus with *N*-acetylgalactosamine (GalNAc)- (Nordbring-Hertz & Mattiasson, 1979). The lectin has been purified (Borrebaeck *et al.*, 1984), and localized by immunogold techniques to the cell surface of the fungus (B. Nordbring-Hertz and coworkers, unpublished). The question arises as to how the lectin is involved in the adhesion and in the ultrastructural changes occurring in the EP layer. It is possible that the adhesion between the

nematode and *A. oligospora* is a two step process. The initial step could be a recognition event involving an interaction between the lectin and a receptor on the nematode surface (Borrebaeck *et al.*, 1985). This interaction could trigger the reorganisation of the surface polymer layer of the trap cells, leading to the firm binding of the nematode.

The presence of an EP layer on vegetative hyphae with no trap-bearing cells suggests that such polymers might have biological functions apart from the adhesion to, and infection of, nematodes. It has been shown that the mucus layer of some plant pathogenic fungi contain extracellular enzymes that might be important for the penetration of the host cells (Ramadoss *et al.*, 1985; Nicholson *et al.*, 1988). The presence of uronic acids and the high affinity for ruthenium red indicate that the EP layer of *A. oligospora* is anionic, as are many extracellular polymers isolated from bacteria (Sutherland, 1989). Such polymers can both attract cations and charged organic molecules from the surrounding environment and protect the cells from hydrolytic enzymes and other antimicrobial agents (Costerton *et al.*, 1981). It has been shown that EP layers of spores from *C. graminicola* contain compounds that protect fungal mycelium from desiccation and the toxic effects of phenolic compounds (Nicholson & Moraes, 1980; Nicholson *et al.*, 1986).

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