***Physarum polycephalum* haemagglutinins: effect of nutrition on synthesis, and their possible role in nature**

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The activity of haemagglutinins in plasmodia of *Physarum polycephalum* was measured under different culture conditions. The activity was markedly increased when the plasmodia were incubated in a non-nutrient salt medium. During starvation, significant amounts of haemagglutinins were found in the slime layer on the surface of the plasmodia. An increase in activity was not observed in the presence of actinomycin D or cycloheximide. Under starvation conditions, plasmodia are known to differentiate into either sclerotia (spherules) or fruiting bodies. Acceleration of haemagglutinin synthesis, however, was not always observed during spherulation and fruiting-body formation. Attempts to detect endogenous glycoconjugates that bind to the haemagglutinins were unsuccessful but we found that the haemagglutinins could bind to acidic polysaccharides produced by *Escherichia coli* K12. The bacterial glycoconjugates were purified and partially characterized. They contained *N*-acetylhexosamine residues which appeared to be important for binding with the haemagglutinins. It is possible that the haemagglutinins play a physiological role in the interaction with these organisms.

Keywords: haemagglutinin, *Physarum polycephalum*, sclerotium (spherule)

INTRODUCTION

A number of lectins are distributed in many species of organisms ranging from bacteria to higher animals. The ability of lectins to bind specific glycoconjugates is of great interest in physiological cellular events and many plant lectins have been used as tools to distinguish saccharide structures on tumour cells, human blood cells or bacteria (Lis & Sharon, 1986). In fungi, many lectins have been identified and characterized, but the physiological role of these lectins remains unclear. Ishikawa & Oishi (1989) found that the lectins in *Neurospora sitophila* interact with *O*-glycosidic glycoconjugates in their wall proteoglycans and suggested that they participate in cell wall biosynthesis. In *Dictyostelium discoideum*, lectins termed discoidin I and II have been studied in detail (Barondes & Springer, 1987). The carbohydrate-binding sites of discoidin I interact with bacterial glycoconjugates and are thought to function in the compartmentalization of this protein in multilamellar bodies and its secretion from the cells in these packets (Barondes *et al.*, 1985; Cooper *et al.*, 1986), while cell-binding sites of the lectin

function in cell–substratum attachment and ordered cell migration (Springer *et al.*, 1984). On the other hand, discoidin II, which becomes prominent in the later phase of development, is thought to have a role in spore coat formation (Cooper *et al.*, 1983).

Physarum polycephalum, a true slime mould, shows several discrete phases in its life cycle (Sauer, 1982). Myxamoebae fuse in pairs and form a multi-nucleate plasmodium. Plasmodium is a naked mass of protoplasm surrounded by mucus polysaccharides which grows until nutrients are consumed. As a response to unfavourable conditions, such as starvation, plasmodia differentiate into either sclerotia (spherules) or fruiting bodies, depending on the light conditions (Schreckenbach *et al.*, 1981).

Recently, we isolated soluble haemagglutinins termed haemagglutinins I and II from the plasmodia of *P. polycephalum*, each of which have a molecular mass of 6 and 11 kDa, respectively (Morita & Nishi, 1992). Their haemagglutinating activities were inhibited by certain glycoproteins such as thyroglobulin but not by various monosaccharides and homopolysaccharides. Most of them existed in the intracellular soluble fraction of plasmodia but, after maximal growth, a significant amount of these proteins appeared in the slime layer on the

Abbreviation: HAI, haemagglutination inhibitor.

plasmodial surface. In this paper, our attention was focused on the change in haemagglutinating activity in different phases of this organism, and the paper also reports the interaction of these haemagglutinins with bacterial glycoconjugates.

METHODS

Cultivation of *P. polycephalum*. Microplasmodia of *P. polycephalum* were grown in a semi-defined medium, containing glucose, yeast extract and tryptone, with shaking (Daniel & Baldwin, 1964). The axenic cultures were maintained in 100 ml liquid medium at 26 °C in the dark. In some experiments, microplasmodia were incubated in a synthetic medium (Daniel & Baldwin, 1964; Morita & Nishi, 1993) or a non-nutrient salt medium (Polanshek *et al.*, 1978) which contained 0.04 g HCl, 0.059 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.082 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.033 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3.96 g citric acid, 1.18 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.59 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.39 g KH_2PO_4 per litre. Spherulation was induced by transferring microplasmodia to a semi-defined medium containing 0.5 M mannitol (Chet & Rusch, 1969).

Cultivation of myxamoebae was carried out according to the method of Dee & Anderson (1984). Stock cultures of the myxamoebae were maintained in a two-member culture with *Escherichia coli* in the dark at 26 °C. The myxamoebae were cultured on SM-1 agar plates (7 cm × 7 cm) with 0.1 ml live bacteria (about 1.6×10^7 cells ml^{-1}). Plates became confluent within 3–4 d. The amoebae were harvested from the agar plate by washing them with 10 mM sodium phosphate buffer (pH 6.4). The amoebae were collected by centrifugation at 1500 g for 15 min and washed repeatedly with the same buffer to remove bacteria. SM-1 medium contained 0.7 g tryptone, 0.2 g yeast extract, 0.6 g glucose, 0.8 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.7 g Na_2HPO_4 and 10.7 g agar (1 distilled water) $^{-1}$.

Cultivation of bacteria. *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus subtilis* were cultured in Luria-Bertani (LB) medium with shaking at 37 °C and *Salmonella typhimurium* was grown in nutrient broth (Difco). In some experiments, bacteria were cultured in M9 minimal medium (Maniatis *et al.*, 1982). The number of viable bacteria cells was monitored by plating diluted bacterial cultures on LB-agar plates.

Induction of sporangia formation. Induction of sporangia formation was carried out according to the method of Daniel & Rusch (1962) with some modifications (Morita *et al.*, 1992). Microplasmodia were allowed to fuse and grown as macroplasmodia on oatmeal-agar plates. After 3 d, the cultures were transferred onto a sporulation medium containing inorganic salts and niacin. They were further cultured for 2 d in the dark. Sporulation was induced by exposure of the starved plasmodia to white fluorescent lamps for 12 h. Mature sporangia were observed 11–12 h after the end of illumination.

Purification of haemagglutinins. Plasmodial haemagglutinins were purified from the intracellular soluble fraction by successive chromatography using affinity, ion-exchange and hydroxylapatite columns as described previously (Morita & Nishi, 1991).

Haemagglutinating assay. Plasmodia and myxamoebae were harvested and ruptured in PBS (75 mM NaCl, 75 mM $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.2) with a Teflon homogenizer. The supernatant was obtained by centrifugation at 15000 g for 15 min. Haemagglutinating activity was measured with trypsin-treated, formalin-fixed rabbit erythrocytes by a serial twofold dilution method with microtitre V-plates as described previously (Morita & Nishi, 1991).

Haemagglutination inhibitor (HAI) assay. Glycoconjugates to be tested were dissolved in 0.15 M NaCl at appropriate concentrations. The solutions (25 μl) were mixed with 25 μl haemagglutinin solution in PBS containing four minimum haemagglutinating doses. Unless stated otherwise, partially purified haemagglutinin which contained both haemagglutinin I and II was used in this assay. After incubation for 30 min at room temperature, 25 μl trypsin-treated, formalin-fixed rabbit erythrocyte suspension (3%, v/v) was added to the mixture to test the haemagglutinating activity. Inhibition activity was expressed as the minimum amount of carbohydrate required for 100% inhibition. Lipopolysaccharide (from *E. coli*), 2-keto-3-deoxyocturonic acid, *N*-acetylglucosamine-3-sulfate and dextran sulfate were purchased from Sigma. The acid mucopolysaccharide kit, *N*-acetylgalactosamine, ethylene glycol chitin, xylane and chitoooligosaccharide were purchased from Seikagaku Kogyo, and *N*-acetyllactosamine from Dextra laboratories.

Purification of HAI. *E. coli* K12 cells cultured in M9 liquid medium were harvested by centrifugation at 15000 g for 15 min at 4 °C. The suspension was filtered with a MF-Millipore filter (0.45 μm pore-size, Millipore) to remove cell debris. The filtrate was concentrated by ultrafiltration (XM 300, Amicon), and solid urea was added to the solution to give a final concentration of 8 M, which improved the recovery of HAI. The mixture (9 ml) was then applied on a gel-filtration column (1.7 × 92 cm, Tosoh) of Toyopearl HW-65 equilibrated with 20 mM Tris/HCl, pH 8.0, containing 4 M urea. Fractions of 4.5 ml were collected. A portion of each fraction was removed to measure A_{280} . Sugar content was determined by the phenol/sulphuric acid method using glucose as standard, and the HAI activity was measured as above.

The active fractions were collected and applied on an ion-exchange column (0.8 × 10.5 cm, Pharmacia) of DEAE-Sephacrose CL-6B equilibrated with the same buffer. The adsorbed materials were eluted with a linear gradient of 0–1 M NaCl in the same buffer. Fractions of 3 ml were collected. The active fractions were collected and dialysed against the same buffer for 24 h at 4 °C. The dialysate was re-chromatographed on the same column. Fractions of 1.5 ml were collected.

Periodate oxidation. This was performed according to the method of Green *et al.* (1985). About 5 mg glucose equivalent ml^{-1} of bacterial saccharides was dissolved in 0.05 M sodium acetate buffer (pH 4.6) containing 0.05 M sodium metaperiodate. The mixture was incubated in the dark at 4 °C for 24 h. The reaction was stopped by the addition of an excess amount of ethylene glycol.

Enzyme digestion. Glycoconjugates to be tested were dissolved in 0.1 M Tris/HCl (pH 8.3) at a final concentration of 250 μg glucose equivalent ml^{-1} . Lysozyme (60 μg) (egg white, Seikagaku Kogyo) was added to the suspension (600 μl) and incubated at 37 °C for 24 h under a toluene atmosphere. The reaction was terminated by boiling for 3 min. β -*N*-Acetylglucosaminidase (Jack bean, Seikagaku Kogyo) digestion was performed with 10 U enzyme in 300 μl 0.1 M phosphate/citrate buffer (pH 5.0) at 37 °C for 24 h. Actinase E digestion was performed as follows. Glycoconjugates were suspended in 50 μl 0.1 M ammonium carbonate and the suspension was incubated with 500 μg actinase E (Kaken Chemical) at 37 °C for 24 h. Another 250 μg actinase E was added to the suspension after 24 h, and another at 48 h. After 72 h of incubation, the reaction was terminated by boiling for 5 min.

PAGE. The purified glycoconjugates were electrophoresed on agarose-containing polyacrylamide gels according to the method of Carney (1986) as described in a previous paper

(Morita & Nishi, 1991). The gel was polymerized with 1.2% (w/v) acrylamide and 0.6% (w/v) agarose. After electrophoresis, the gel was stained with toluidine blue.

Paper chromatography and TLC. All procedures were performed as described by Carney (1986). The purified polysaccharide (2–3 mg) was suspended in 100–250 μ l 2 M HCl. The air was replaced by N_2 gas and the mixture heated at 100 °C for 4 h in a screw-capped tube. The hydrolysate was fractionated by descending-paper chromatography for 25 h on Whatman no. 1 paper in a solvent system of *n*-butanol/pyridine/water (10:3:3, by vol.). After development, sugars were revealed by the alkaline silver nitrate method. Glucose, galactose, mannose, fucose and rhamnose were used as standards and spotted on the same paper. In another experiment, the hydrolysate was spotted on a cellulose-coated TLC plate (Merck) and developed in a solvent system of *n*-butanol/pyridine/0.1 M HCl (5:3:2, by vol.). Spots of aldoses, ketoses and oligosaccharides were detected by spraying with a reagent which contained diphenylamine, aniline and phosphoric acid in acetone. Glucose, galactose, mannose, fucose, glucosamine and galactosamine were used as standards.

Protein assay. Protein concentration was estimated by the Lowry method using BSA as standard.

RESULTS

Changes in haemagglutinating activity in different media

Our previous work demonstrated that microplasmodia of *P. polycephalum* produce two soluble haemagglutinins, haemagglutinins I and II (Morita & Nishi, 1992). Most of the haemagglutinins were found in the intracellular soluble fraction, though small amounts of them were associated with the slime layer on the plasmodial surface. The haemagglutinating activity of plasmodia grown in a semi-defined medium was about 100 titre⁻¹ (mg plasmodial protein)⁻¹ (Table 1). When the plasmodia were

Table 1. Changes in haemagglutinating activity of *P. polycephalum* grown in different media

Microplasmodia grown in a semi-defined medium were harvested and transferred into various media. After incubation for 48 h, plasmodia were collected by centrifugation and disrupted by sonic oscillation. The homogenates were centrifuged at 15000 *g* for 15 min, and the haemagglutinating activities of the supernatants were assayed with trypsin-treated, formalin-fixed rabbit erythrocytes as outlined in Methods. The activity was determined by a serial twofold dilution method and expressed as titre⁻¹ (mg plasmodial protein)⁻¹.

Medium	Haemagglutinating activity	
	[Titre ⁻¹ (mg protein) ⁻¹]	Ratio
Semi-defined	100 ± 33	1
Glucose-free semi-defined	322 ± 69	3
Synthetic	410 ± 150	4
Non-nutrient salt	1078 ± 106	11

Fig. 1. Changes in haemagglutinating activity during starvation and spherulation. Plasmodia grown in semi-defined medium were transferred into non-nutrient salt medium and incubated to induce spherulation. Aliquots of the culture were removed at the time indicated, and the haemagglutinating activity (●) in the cell homogenate was measured as in Table 1. The rate of spherulation (open bar) was measured under a microscope after fixing with glutaraldehyde.

cultured in the semi-defined medium lacking glucose or in a complete synthetic medium, a three- to fourfold increase in the activity was generally observed. As described previously (Morita & Nishi, 1993), the doubling times for plasmodial growth in the semi-defined, glucose-free semi-defined and synthetic media were about 9 h, 14 h and 30 h, respectively. The activity (mg plasmodial protein)⁻¹ appeared to increase when the plasmodia were cultured in nutrient-poor media. As the synthetic medium contains glucose and various amino acids, the production of haemagglutinins might not be controlled by glucose catabolites. When the plasmodia grown in the synthetic medium were transferred into the same medium lacking glucose or an amino acid mixture, about a three- or fivefold increase of the activity was observed after 48 h, respectively. As expected, the highest activity [1078 titre⁻¹ (mg protein)⁻¹] was observed when the plasmodia were incubated in a non-nutrient salt medium.

Increase of the activity was inhibited by adding cycloheximide (30 μ g ml⁻¹) or actinomycin D (200 μ g ml⁻¹), suggesting that *de novo* protein and RNA syntheses were required for haemagglutinin synthesis (data not shown).

Haemagglutinating activity during spherule and fruiting-body formation

Plasmodia grown in a non-nutrient salt medium are destined to differentiate into spherules in the dark. More than 90% of the plasmodia were transformed into the structure characteristic of spherules within 48 h. Concomitantly with the spherulation, a marked increase in the haemagglutinating activity was observed (Fig. 1). It is therefore possible that the haemagglutinins may participate in the morphological changes. To test this,

Table 2. Effect of 0.5 M mannitol on haemagglutinating activity

Plasmodia grown in a semi-defined medium were transferred into the same medium in the absence or presence of 0.5 M mannitol, or into non-nutrient salt medium. Haemagglutinating activity was measured after 48 h.

Medium	Haemagglutinating activity		Spherulation (%)
	[Titre ⁻¹ (mg protein) ⁻¹]	Ratio	
Semi-defined	128 ± 5	1.0	0
Semi-defined + 0.5 M mannitol	144 ± 9	1.1	50
Non-nutrient salt	1739 ± 107	13.6	90

Fig. 2. Changes in haemagglutinating activity during fruiting-body formation. Microplasmodia were pipetted onto a MF-Millipore filter on an oatmeal agar medium and cultured in the dark (growth phase; A). After 48 h, the plasmodia were transferred onto a sporulation agar medium and starved for 2 d in the dark (starvation phase; B). Fruiting body formation was induced by exposure of the plasmodia to the light for 12 h (fruiting body formation phase; C). Complete fruiting bodies (D) were harvested 12 h after the end of illumination. Myxamoebae (E) from *P. polycephalum* were cultured on an agar plate with live *E. coli* in the dark at 26 °C. The amoebae were scraped from the plate and separated from bacteria by repeated centrifugation at 1500 g for 15 min. After homogenization, the cells were centrifuged and the haemagglutinating activity in the supernatant was measured.

spherulation was induced by incubating microplasmodia in the semi-defined medium containing 0.5 M mannitol, in which plasmodia likewise differentiated into spherules without starvation (Chet & Rusch, 1969). After incubation for 48 h, spherulation was observed in about 50% of the plasmodia and, at that time, the haemagglutinating activity was about 144 titre⁻¹ (mg protein)⁻¹ (Table 2). This activity was significantly less than that of plasmodia

cultured in the non-nutrient salt medium [1739 titre⁻¹ (mg protein)⁻¹]. These results suggest that spherulation was not necessarily accompanied by an increase of the haemagglutinating activity.

Fig. 2 shows the activity during fruiting-body formation. The haemagglutinating activity in growing plasmodia was about 124 titre⁻¹ (mg protein)⁻¹, which was increased up to 1424 titre⁻¹ (mg protein)⁻¹ after 2 d starvation. Once plasmodia became differentiated into fruiting bodies, the activity was gradually decreased [448 titre⁻¹ (mg protein)⁻¹ at young fruiting bodies], though an appreciable amount of the haemagglutinating activity still remained in the mature fruiting bodies [255 titre⁻¹ (mg protein)⁻¹]. It is questionable, however, whether or not the spores in the sporangia contain haemagglutinins because no detectable haemagglutinating activity was found in myxamoebae. Myxamoebae did not show any haemagglutinating activities even when they were co-cultivated with a bacterium lacking HAI activity. It thus appears that the production of haemagglutinins is characteristically related to the plasmodial phase and is induced by starvation.

Glycoconjugates that bind to haemagglutinin

In an attempt to clarify the physiological role of the haemagglutinins, various cell fractions of the slime mould were examined for their haemagglutinin-binding activity. Endogenous glycoproteins or polysaccharides, in some cases, serve as the natural ligands of carbohydrate-binding proteins. Lectins in the multicellular slime moulds *D. discoideum* and *Polysphondylium pallidum* were reported to bind to membrane or wall-associated glycoconjugates (Cooper *et al.*, 1983; Drake & Rosen, 1982). Our previous reports showed that the plasmodial surface of *P. polycephalum* is also rich in glycoconjugates (Kuroda *et al.*, 1989; Morita *et al.*, 1986; Morita & Nishi, 1989). However, preliminary studies indicated that none of the cellular fractions including the slime substance (McCormic *et al.*, 1970a), plasma membrane (Kuroda *et al.*, 1989), spherule wall (Zaar *et al.*, 1979) and spore coat (McCormic *et al.*, 1970b) inhibit the haemagglutinating activity (data not shown).

Binding of the haemagglutinins to microbial glycoconjugates was also examined by measuring HAI activity. Bacteria grown in LB medium were pelleted by centrifugation and resuspended in H₂O. The suspension was boiled for 1 h and supernatant was used as boiled extract for the HAI assay. High HAI activity was found in the boiled extract from *E. coli* K12. The minimum cell number inhibiting four haemagglutinating doses was 2 × 10⁹ cells. In contrast, the activity from other bacteria such as *K. pneumoniae*, *S. aureus*, *B. subtilis* and *S. typhimurium*, was negligible even at 7 × 10¹⁰, 2 × 10¹¹, 4 × 10¹⁰ and 2 × 10¹⁰ cells, respectively. However, very low HAI activity was sometimes observed in the culture fluid of the latter three bacteria when they were incubated in M9 minimal medium (data not shown). The HAI activity from *E. coli* K12 was resistant to exhaustive proteolytic digestion with actinase E but completely

Fig. 3. Purification of *E. coli* K12 polysaccharides with HAI activity by column chromatography. (a) Gel-filtration chromatography of polysaccharides from *E. coli* K12. *E. coli* K12 was cultured in M9 medium, and the extracellular fluid was obtained by centrifugation. The supernatant was filtered through a MF-Millipore filter (0.45 µm pore-size) to remove cell debris, and solid urea was added to the solution to give a final concentration of 8 M. This solution was then applied onto a Toyopearl HW-65 column (Tosoh, 1.7 × 92 cm), and the column was eluted with 20 mM Tris/HCl (pH 8.0) containing 4 M urea (4.5 ml fractions). Each fraction was assayed for HAI activity (shaded bar), protein (●, A₂₈₀) and sugar (○, phenol/sulfuric acid method measured at A₄₉₀). Standard proteins, thyroglobulin (669 kDa) and carbonic anhydrase (29 kDa), were eluted at fraction nos 23 and 67, respectively. (b) DEAE-Sephacel column chromatography of polysaccharides from *E. coli* K12. The active fractions (nos 43–52) in (a) were collected and applied onto a DEAE-Sephacel CL-6B column (Pharmacia, 0.8 × 10.5 cm). The materials were eluted with a linear gradient of 0–1 M NaCl. The active fractions (nos 49–55) were combined and dialysed, and they were chromatographed on the same column for further purification (data not shown).

destroyed by periodate oxidation, suggesting that the essential structure in HAI from *E. coli* K12 contained a polysaccharide moiety. The molecular mass was more than 300 kDa as judged by gel-filtration on Toyopearl HW-75. When *E. coli* K12 was cultured in M9 minimal medium, more than 95 % of the HAI activity was found in an extracellular fraction. The HAI activity was easily released from *E. coli* K12 cells by boiling with H₂O, suggesting that the HAI is not integral but is a peripheral component of the bacterial cell wall. Until now, however, our efforts to detect HAI activity in the known constituents of bacterial extracellular and wall polysaccharides have been unsuccessful. These compounds include lipopolysaccharide, 2-keto-3-deoxyocturonate, teichoic acid, xylan, dextran sulfate, *N*-acetylmuramic acid (at 5 mg ml⁻¹), *N*-acetylglucosamine, *N*-acetylgalactosamine, glucose, galactose, fucose, rhamnose and glucuronic acid (at 0.2 M). Preliminary experiments also indicated that plasmodial haemagglutinins neither aggregate *E. coli* K12 cells nor influence the bacterial growth at the concentration which caused complete agglutination of rabbit erythrocytes.

Some properties of glycoconjugates with HAI activity

The HAI activity of polysaccharides from *E. coli* K12 disappeared after the treatment with β-*N*-acetylglucosaminidase. This result suggested that the oligosaccharide structure contains terminal β-linked *N*-acetylhexosamine residues required for binding to the haemagglutinins. Similar results were obtained when thyroglobulin, which is a potent HAI, was subjected to mild acid treatment followed by β-*N*-acetylglucosaminidase digestion. Digestion of the asialo-thyroglobulin with this enzyme resulted in a complete disappearance of its capacity to inhibit the haemagglutinating activity. However, none of the other glycoconjugates hitherto tested, such as chito-oligosaccharide (10 mg ml⁻¹), *N*-acetyllactosamine (5 mg ml⁻¹), *N*-acetylglucosamine-3-sulfate (10 mg ml⁻¹) or ethylene glycol chitin (10 mg ml⁻¹), showed any detectable HAI activity.

The HAI activity of the polysaccharides from *E. coli* K12 markedly reduced (25 %) after the digestion with lysozyme, supporting the view that the HAI may be associated with cell wall polysaccharides. To characterize it in more

Fig. 4. Electrophoretogram of the purified inhibitor on a polyacrylamide gel containing agarose. HAI purified from the culture fluid of *E. coli* K12 was dissolved in 10 mM Tris-acetate (pH 6.8) containing 0.25 mM sodium sulfate and about 5 µg glucose equivalent of the inhibitor was loaded on the gel. After the electrophoresis, the gel was stained with toluidine blue. The gel was cut into 3 mm strips and the HAI activity was extracted with 0.15 M NaCl.

detail, we attempted to purify HAI from the culture fluid of *E. coli* K12 by column chromatography. As shown in Fig. 3(a), HAI activity appeared in diverse fractions after gel-filtration. The active fractions (fraction nos 43–52) were then applied on a column of DEAE-Sephacel (Fig. 3b). The ion-exchange chromatography was repeated once more. Based on the minimum amount (µg glucose equivalent ml⁻¹) required to inhibit four haemagglutinin doses, the specific activity of the purified HAI (0.8 µg ml⁻¹) was about fivefold higher than that of the culture fluid (3.8 µg ml⁻¹). The purified HAI was a more potent inhibitor of plasmodial haemagglutinins than thyroglobulin (6.8 µg ml⁻¹). Fig. 4 shows an electrophoretogram of purified HAI. It revealed that the band with HAI activity was positive to the toluidine blue staining suggesting that the active substance is an acidic carbohydrate. Digestion of HAI with actinase E did not affect the electrophoretic mobility. The results of paper chromatography and TLC indicated that the purified HAI was composed of *N*-acetylglucosamine, *N*-acetylgalactosamine, glucose and galactose.

DISCUSSION

In the present study, we found that haemagglutinin synthesis was markedly accelerated in plasmodia grown in nutrient-poor media, in which they are destined to differentiate into dormant forms called spherules or sclerotia. In *Rhizoctonia solani*, the lectin of the sclerotia was reported to serve as a storage protein in the resting structure of the fungus (Kellens & Peumans, 1990) because it increased during sclerotia formation and disappeared after germination. In contrast, a similar result was not observed in *P. polycephalum*. It thus seems unlikely that plasmodial haemagglutinins are functioning as storage proteins in their dormant structures. However, it should be noted that the haemagglutinins were found only in the plasmodial stage (diploid) and not detected in myxamoebae (haploid). As with discoidin I and II, lectins

produced by *D. discoideum* (Barondes & Springer, 1987), the production of *Physarum* haemagglutinins is thought to be controlled in a developmentally regulated fashion.

High haemagglutinating activity in starved plasmodia suggested that the haemagglutinins function under particular environmental conditions in this organism. One approach to determine the functions of these haemagglutinins is to detect glycoconjugates which interact with them since the haemagglutinins may act by binding to such ligands. In *D. discoideum*, an endogenous lectin, discoidin I, has been thought to be involved in cell-substratum attachment and ordered cell migration during aggregation. Discoidins were reported to bind to endogenous cell surface oligosaccharide with a structure Man α 3(Man α 6)(Xyl β 2)Man β 4GlcNAc (Amatayakul-Chantler *et al.*, 1991). Eitle *et al.* (1993) also reported the presence of lectins on the *Dictyostelium* cell surface which react with anionic polysaccharides. Drake & Rosen (1982) have purified a sulfated glycoprotein from the membrane fraction of the cellular slime mould *Pol. pallidum* which was interacting with pallidin, a lectin in this mould. We have previously shown that plasmodial membrane proteins are generally glycosylated (Kuroda *et al.*, 1989; Morita *et al.*, 1986; Morita & Nishi, 1989) and their surface is covered by a slime layer with haemagglutinating activity (Morita & Nishi, 1991). It is possible, therefore, that some of these glycoconjugates may serve as endogenous ligands for plasmodial haemagglutinins. However, various plasmodial fractions did not show any detectable haemagglutinin-binding activity. A more sensitive assay method may be required for detecting the endogenous ligands of the haemagglutinins, if there are any. A search for endogenous glycoconjugates with HAI activity is still in progress in our laboratory.

In the natural environment, starving plasmodia migrate on a substratum in search for a nutrient such as fungi and bacteria. A lectin in *R. solani* hyphae was believed to play a role in the recognition and adherence of the mycoparasite *Trichoderma* (Elad *et al.*, 1983; Inbar & Chet, 1992). It has also been reported that capture of nematodes by the nematophagous fungus *Arthrobotrys oligospora* is mediated by a lectin on the fungal surface (Rosen *et al.*, 1992). The haemagglutinins in the slime layer on migrating *Physarum* plasmodia may interact with polysaccharides derived from bacteria. The present study has shown that plasmodial haemagglutinins recognized wall-associated acidic polysaccharides from *E. coli* K12 and *N*-acetylhexosamine residues in their moiety are responsible for the binding. The HAI activity of the polysaccharide was eight times higher than that of thyroglobulin, suggesting the possibility that certain bacterial polysaccharides are the natural ligand of *Physarum* haemagglutinins. A number of lectins or some lectin-like proteins have been reported to be reactive with bacteria by binding mannans, teichoic acids or lipopolysaccharides (Pistole, 1981). The specificity of symbiotic association between *Rhizobium* and legumes would be determined by the host plant lectin because the plant lectin recognized lipopolysaccharides (Kalsi *et al.*, 1992) or capsular polysaccharides (Calvert *et al.*, 1978) on the *Rhizobium* cell

surface. In some insects (Komano *et al.*, 1980; Jomori & Natori, 1992), a lectin-like protein in haemolymph was shown to be responsible for the process of phagocytosis. This lectin may bind to the glycoconjugates on foreign bacteria and facilitate ingestion of bacteria by haemocytes. It seems unlikely, however, that the plasmodial haemagglutinins have a role in the ingestion of bacteria by phagocytosis because we did not detect any haemagglutinating activity in myxamoebae, which also grow by ingesting bacteria. A similar result was also reported in *D. discoideum*. Although discoidin I is known to interact with bacterial polysaccharides, vegetative amoebae without the lectin activity can ingest bacteria by phagocytosis. At present, the fact that plasmodial haemagglutinins bind exclusively to glycoconjugates from *E. coli* K12 is very puzzling when considering the role of haemagglutinins in the intake of nutrition. The structural properties of capsules, however, vary between bacteria, and wall composition may be altered according to the growth conditions (Sutherland, 1985). Therefore, we do not rule out the possibility that various bacteria commonly possess the polysaccharides that bind to the haemagglutinins.

At present, it is difficult to explain the function of the *Physarum* haemagglutinins, but the present study suggested that their physiological roles in nature should be investigated. In order to elucidate the biological role of plasmodial haemagglutinins, an immunological investigation of relevant endogenous ligands is now in progress.

REFERENCES

- Amatayakul-Chantler, S., Ferguson, M. A., Dwek, R. A., Rademacher, T. W., Parekh, R. B., Crandall, I. E. & Newell, P. C. (1991). Cell surface oligosaccharides on *Dictyostelium* during development. *J Cell Sci* **99**, 485–495.
- Barondes, S. H. & Springer, W. R. (1987). An endogenous lectin and an oligosaccharide participate in adhesion mechanisms in *Dictyostelium*. In *Genetic Regulation of Development*, pp. 129–140. Edited by W. F. Loomis. New York: Alan. R. Liss.
- Barondes, S. H., Haywood-Reid, P. L. & Cooper, D. N. W. (1985). Discoidin I, an endogenous lectin, is externalized from *Dictyostelium discoideum* in multilamellar bodies. *J Cell Biol* **100**, 1825–1833.
- Calvert, H. E., Lalonde, M., Bhuvaneshwari, T. V. & Bauer, W. D. (1978). Role of lectins in plant-microorganism interactions. IV. Ultrastructural localization of soybean lectin binding sites on *Rhizobium japonicum*. *Can J Microbiol* **24**, 785–793.
- Carney, S. L. (1986). Monosaccharides. In *Carbohydrate Analysis*, pp. 1–36. Edited by M. F. Chaplin & J. F. Kennedy. Oxford & Washington, DC: IRL Press.
- Chet, I. & Rusch, H. P. (1969). Induction of spherule formation in *Physarum polycephalum* by polyols. *J Bacteriol* **100**, 673–678.
- Cooper, D. N., Lee, S.-C. & Barondes, S. H. (1983). Discoidin-binding polysaccharide from *Dictyostelium discoideum*. *J Biol Chem* **258**, 8745–8750.
- Cooper, D. N. W., Haywood-Reid, P. L., Springer, W. R. & Barondes, S. H. (1986). Bacterial glycoconjugates are natural ligands for the carbohydrate binding site of discoidin I and influence its cellular compartmentalization. *Dev Biol* **114**, 416–425.
- Daniel, J. W. & Baldwin, H. H. (1964). Axenic culture of *Physarum polycephalum* on soluble media. *Methods Cell Physiol* **1**, 15–29.
- Daniel, J. W. & Rusch, H. P. (1962). Methods for inducing sporulation of pure cultures of the myxomycete *Physarum polycephalum*. *J Bacteriol* **83**, 234–240.
- Dee, J. & Anderson, R. W. (1984). The effect of ploidy on the stability of plasmodial heterokaryons in *Physarum polycephalum*. *J Gen Microbiol* **131**, 1167–1179.
- Drake, D. K. & Rosen, S. D. (1982). Identification and purification of an endogenous receptor for the lectin pallidin from *Polysphondylium pallidum*. *J Cell Biol* **93**, 383–389.
- Eitle, E., Keller, T., Parish, C. R. & Parish, R. W. (1993). Polysaccharides influence the aggregation of *Dictyostelium discoideum* cells and bind to developmentally regulated cell surface proteins. *Exp Cell Res* **205**, 374–382.
- Elad, Y., Barak, R. & Chet, I. (1983). Possible role of lectins in mycoparasitism. *J Bacteriol* **154**, 1431–1435.
- Green, E. D., van Halbeek, H., Boime, I. & Baenziger, J. U. (1985). Structural elucidation of the disulfated oligosaccharide from bovine leutropin. *J Biol Chem* **260**, 15623–15630.
- Inbar, J. & Chet, I. (1992). Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. *J Bacteriol* **174**, 1055–1059.
- Ishikawa, F. & Oishi, K. (1989). Production, purification, and characterization of *Neurospora sitophila* lectin. *Agric Biol Chem* **53**, 1769–1776.
- Jomori, T. & Natori, S. (1992). Function of the lipopolysaccharide-binding protein of *Periplaneta americana* as an opsonin. *FEBS Lett* **296**, 283–286.
- Kalsi, G., Das, H. R., Babu, C. R. & Das, R. H. (1992). Isolation and characterization of a lectin from peanut roots. *Biochim Biophys Acta* **1117**, 114–119.
- Kellens, J. T. C. & Peumans, W. J. (1990). Developmental accumulation of lectin in *Rhizoctonia solani*: a potential role as a storage protein. *J Gen Microbiol* **136**, 2489–2495.
- Komano, H., Mizuno, D. & Natori, S. (1980). Purification of lectin induced in the hemolymph of *Sarcophaga peregrina* larvae on injury. *J Biol Chem* **255**, 2919–2924.
- Kuroda, T., Morita, M., Okai, K., Okamura, S. & Nishi, A. (1989). Isolation of plasma membrane and analysis of membrane glycoproteins from the slime mould *Physarum polycephalum*. *J Gen Microbiol* **135**, 873–881.
- Lis, H. & Sharon, N. (1986). Lectins as molecules and as tools. *Annu Rev Biochem* **55**, 35–67.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). In *Molecular Cloning: a Laboratory Manual*, pp. 68–73. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- McCormic, J. J., Blomquist, J. C. & Rusch, H. P. (1970a). Isolation and characterization of a galactosamine wall from spores and spherules of *Physarum polycephalum*. *J Bacteriol* **104**, 1119–1125.
- McCormic, J. J., Blomquist, J. C. & Rusch, H. P. (1970b). Isolation and characterization of an extracellular polysaccharide from *Physarum polycephalum*. *J Bacteriol* **104**, 1110–1118.
- Morita, M. & Nishi, A. (1989). Characterization of glycopeptides from *Physarum polycephalum* labelled with [³H]mannose or [³H]-glucosamine. *J Gen Appl Microbiol* **35**, 413–427.
- Morita, M. & Nishi, A. (1991). Glycoproteins and enzymes associated with the plasmodial membrane and slime layer of *Physarum polycephalum*. *J Gen Appl Microbiol* **37**, 93–109.
- Morita, M. & Nishi, A. (1992). Isolation and partial characterization of haemagglutinins from plasmodia of *Physarum polycephalum*. *J Gen Microbiol* **138**, 619–625.
- Morita, M. & Nishi, A. (1993). Purification and partial characterization of β -glucosidase from plasmodial membrane and culture medium of *Physarum polycephalum*. *J Gen Microbiol* **139**, 1635–1641.

- Morita, M., Kurosaki, F. & Nishi, A. (1986). Lipid-oligosaccharide intermediate of glycoprotein synthesis in *Physarum polycephalum*. *Biochim Biophys Acta* **881**, 148–153.
- Morita, M., Murata, Y., Kaneko, Y., Matsushima, H. & Nishi, A. (1992). Changes in membrane glycoproteins during fruit-body formation in *Physarum polycephalum*. *Bot Mag Tokyo* **105**, 515–527.
- Pistole, T. G. (1981). Interaction of bacteria and fungi with lectins and lectin-like substances. *Annu Rev Biochem* **35**, 85–112.
- Polanshek, M. M., Blomquist, J. C., Evans, T. E. & Rusch, H. P. (1978). Aminopeptidase of *Physarum polycephalum* during growth and differentiation. *Arch Biochem Biophys* **190**, 261–269.
- Rosen, S., Ek, B., Rask, L. & Tunlid, A. (1992). Purification and characterization of a surface lectin from the nematode-trapping fungus *Arthrobotrys oligospora*. *J Gen Microbiol* **138**, 2662–2672.
- Sauer, H. W. (1982). *Developmental Biology of Physarum*. Cambridge: Cambridge University Press.
- Schreckenbach, T., Walckhoff, B. & Verfuherth, C. (1981). Blue-light receptor in a white mutant of *Physarum polycephalum* mediates inhibition of spherulation and regulation of glucose metabolism. *Proc Natl Acad Sci USA* **78**, 1009–1013.
- Springer, W. R., Cooper, D. N. W. & Barondes, S. H. (1984). Discoidin I is implicated in cell-substratum attachment and ordered cell migration of *Dictyostelium discoideum* and resembles fibronectin. *Cell* **39**, 557–564.
- Sutherland, I. W. (1985). Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides. *Annu Rev Microbiol* **39**, 243–270.
- Zaar, K., Beyer, P. & Kleining, H. (1979). The spherule wall of *Physarum polycephalum*: chemical analysis and electron microscopy. *Biochim Biophys Acta* **582**, 21–32.

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