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LECTINS AND LECTIN RECEPTORS FROM *ACANTHAMOEBA* SP.

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ABSTRACT

Acanthamoeba isolates from soil and water samples have been characterized for the presence of lectins and lectin receptors. Crude soluble protein extracts have been shown to agglutinate neuraminidase-treated and non-treated fixed horse erythrocytes and fixed rabbit erythrocytes. Purification was done using alpha-methyl-mannoside-agarose column, with a protein peak that showed one band in native PAGE and two bands in SDS-PAGE slightly above the 66 Kd region. Con A-FITC stained the plasma membrane, and binding was inhibited by alpha-methyl-mannopyranoside. By Con-A blotting of the soluble and membrane-bound proteins on nitrocellulose, molecular weights of the glycoconjugates were determined.

INTRODUCTION

The increasing frequency of chronic amebic keratitis among contact lens wearers and a number of cases of granulomatous amebic encephalitis infections have led to the cell and molecular research of the bioactive molecules in *Acanthamoeba* (Ma et al., 1990; Matias, et al., 1991). A significant class of recognition molecule called lectins has been the subject of studies in many biological systems including the parasitic protozoa, bacteria, viruses, plants and higher animals (Sharon and Lis, 1989).

Lectins are proteins or glycoproteins of non-immune origin that agglutinate cells and precipitate complex carbohydrates or both (Goldstein, et al., 1980). Lectins have been implicated in many biological activities. For instance, the phagocytosis of mammalian erythrocytes and yeasts by *Acanthamoeba castellanii* has been shown to be mediated by the mannose-sensitive, carbohydrate-binding sites on the amoeba cell surface (Brown et al., 1975; Allen and Dawidowicz, 1990). So far, glycoproteins have been detected in the plasma membrane of *A. castellanii* (Paatero and Gahmberg, 1988). But the precise nature of this lectin-like activity in *A. castellanii* has yet to be understood.

Are these suspected carbohydrate-binding sites on *Acanthamoeba* cell surface which may be present in the cytoplasm indeed lectins? Hence, there is a need to characterize this kind of recognition molecules in *Acanthamoeba*. Moreover, the detection and characterization of the endogenous lectin receptors have been shown to serve as indirect evidence of the occurrence of lectins in *Naegleria* (Matias et al., 1990). Likewise, studies on lectin receptors may provide important clues and advance our present knowledge on the biological functions of lectins in nature (Rademacher et al., 1988).

The present study endeavors to: 1) isolate and characterize lectins from the Philippine *Acanthamoeba* isolate; and 2) detect the presence and distribution patterns of endogenous lectin receptors in trophozoites and cysts.

The significance of this work is focused primarily on the biochemical and cytochemical characterization of these ubiquitous free-living amoebae which are potentially pathogenic to man. Among others, it will provide a basis for further studies on the exact nature of the carbohydrate-binding sites of *Acanthamoeba* which have been suggested to act as lectins. Furthermore, the identification of a local source of lectins, which can be mass produced and which can be developed as direct probes for the detection and localization of the cytoplasmic or cell surface glycoconjugates, will be of commercial importance.

MATERIALS AND METHODS

Acanthamoeba isolation and cultivation

The *Acanthamoebae* were isolated from the different water samples at the University of the Philippines, Diliman, Quezon City; Ateneo de Manila University; and Novaliches according to the standard method for isolating these amoebae from natural sources (Page, 1988). The isolation was done by inoculating water filtrate to non-nutrient agar plates lawned with 24-hour culture plasmid free *Escherichia coli* (DH1). The isolates were cloned using a Skermann micromanipulator. The cloned isolates were cultured axenically in Page's proteose-peptone-glucose liquid medium (PPG). The cells were harvested after 72 hours incubation (log phase) at 37°C. The cells were washed thrice with amoeba saline (Page, 1988). A final wash was done using 0.1 M Tris-CHl and the resulting cell pellet was stored at -20°C until use.

Lectins

Protein Extraction. The cells were disrupted using a sonicator. This was done by dipping the probe 10 times in the chilled test tube with the cells in extraction buffer (1 mM Phenyl methyl sulfonyl fluoride, 1 mM dithiotreitol, 1 mM ethylene diamine tetra-acetic acid and 1 mM a-amino hexanoic acid in .1M Tris-HCl) for 10 sec, after which the cells were checked for 100% cell disruption. The cell lysate was centrifuged at 32,000 rpm for one hour at 4°C using Beckman Tl-100 Ultracentrifuge. The supernatant was collected and labeled as soluble cytoplasmic extract and stored at -20° C. The membrane-bound proteins were isolated according to the method used by Matthews et al. (1986). Total protein concentration was determined according to the method of Bradford (1976).

Hemagglutination Assay. Glutaraldehyde fixed and neuraminidase-treated rabbit and horse RBC were used. Five ml of fresh erythrocytes were washed three times in PBS (150 mM NaCl, 10 mM phosphate buffer, pH 7.2) and centrifuged after each washing at 1000 rpm for 5 min and resuspended in the same buffer. Two percent RBC suspension was used in the assay. The extracts were serially diluted with PBS buffer in micro-titer V-plates in a total volume of 100 ul. Twenty-five microliters of the 2% RBC was added to each well and incubated in 37°C for one hour. (If the extract has a hemagglutination activity, a flat carpet of RBC will be formed at the bottom of the plate. In case of negative reaction, a clear red dot will be formed at the center of the plate. The reciprocal of the maximum effective dilution rate was expressed as the titer. Determination of the sugar specificity was performed by serially diluting the sugar in PBS with the extract (already diluted to at least 8 times) before adding the RBC to the hemagglutination mixture.

Affinity chromatography. Cell extracts that showed positive lectin activity were purified using alpha-methyl- mannoside-agarose (Sigma) column. The lectin extract was applied directly to a 5 ml column and washed with PBS. The lectin was eluted with PBS containing 0.2 M of the corresponding carbohydrate. The fractions containing the lectin were pooled, dialyzed against PBS and tested for hemagglutination activity. The purity of the lectin was determined by native PAGE and SDS-PAGE analysis according to the modified method of Laemlli (Matias, 1991).

Lectin Receptors

Lectin blotting. The proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane (S&S, 0.45 um) using an LKB (2117) Multiphor II Electrophoresis System (1986). After which the strips of nitrocellulose membrane were washed five times for 5 min with PBS (with 0.1% Tween-20) and blocked with 3% BSA in PBS-Tween 20 for one hour. Blots were washed five times and incubated with 3 ml of peroxidase-labeled lectin (10ug/ml) overnight at 4°C and were washed as above. For the control, the peroxidase-labeled lectin was pre-incubated with the alpha-methyl-mannose at 0.5 M for one hour. The blots were visualized with 10 mg

diaminobenzidine, 0.5 ml 1% $CoCl_2$, 19.5 ml 0.5 M PBS and 50 ul 30% H_2O_2 which was added prior to use. After color development the blots were washed with distilled H_2O, air dried and photographed immediately.

Lectin fluorescence assay. (Hill et al., 1981). For lectin receptor localization, the cells were cultured in petri dishes for 72 hours. The cells were harvested and then washed with PBS three times. The cells were incubated with fluorescein isothiocyanate (FITC)-labeled lectins (100 ug/ml) for one hour and washed three times. The cells were suspended in 1 ml PBS and mounted onto glass slides and sealed with nail polish. Observations were done using a Carl Zeiss fluorescence microscope.

The control sugar inhibition of lectin-FITC binding to *Acanthamoebae* was done by incubating both lectin and *Acanthamoeba* trophozoites with 100 mM of monosaccharides for 30 min before performing the fluorescence assay.

RESULTS AND DISCUSSION

Soluble protein extracts from 11 strains of *Acanthamoeba spp.* were tested for hemagglutination activity (HA). Strains W_4 and W_3 gave positive agglutination results against 1.5% rabbit erythrocytes. These *Acanthamoeba* strains that were positive for hemagglutination test were the isolates from an artesian well in Novaliches. Cyst morphology, isoenzyme patterns and mtDNA digestion phenotypes showed that these two strains could be of the same species (Natividad et al., 1993, unpublished data) indicating that lectin activity could be used to verify inter-strain characteristics.

The soluble cytoplasmic protein extracts of the W_4 strain tested for HA against neuraminidase treated horse erythrocytes showed strong hemagglutination activity (Fig. 1). Neuraminidase is a hydrolytic enzyme that cleaves the terminal N-acetyl-neuraminic acid (sialic acid), thus, exposing the neutral sugars of the glycoconjugates of the erythrocyte plasma membrane. Lectins that may be specific to these sugars could freely bind and exhibit strong hemagglutination reaction. The presence of lectins, which showed specificity to mannose fructose, was demonstrated in the phagocytosis of horse erythrocytes and yeasts by *A. castellanii* (Brown et al., 1975). The occurrence of intracellular lectins with mannose-receptors in *A. castellanii* has been suggested to mediate the delivery of soluble mannose-rich molecules to the degradative compartments, particularly the lysosomes (Allen and Dawidowicz, 1990). The present work on the local isolation of lectins in *Acanthamoeba* has been based in this study, especially on the purification step. The alpha-methyl-mannoside agarose matrix was used as affinity column.

Successful elution of an *Acanthamoeba* lectin with a protein concentration of 2.87 ug/ml was obtained (Fig. 2). The protein was eluted with 0.2 M alpha-methyl-mannoside. One band was obtained under 6% non-denaturing polyacrylamide gel electrophoresis (Fig. 3A). These data suggest that a homogenous protein was

obtained. Further analysis of the protein by 12% SDS-PAGE electrophoresis revealed two slightly separated bands above the 66 kd region (Fig. 3B). These were suspected to be the subunits of eluted lectin.

The specificity of binding between the Con A-FITC and the cell surface-mannose moieties could demonstrate the topographical distribution of the lectin receptor sites (Stevens and Kaufman, 1974; Gonatas and Avrameas, 1977). Lectin receptor studies using fluorescein isothiocyante-labeled Con A showed fluorescent patches around the plasma membrane and cytoplasm (Fig. 4). The fluorescence could be quenched completely with 0.5M alpha-methyl-D-mannopyranoside. Brighter fluorescence was observed on the sites of adherence between the *Acanthamoeba* (W_4) cysts and trophozoites.

The clustering of lectin receptors at the site of aggregation of cells has been demonstrated at the ultrastructural level using ferritin-labeled Con A by Stevens and Kaufman (1974). High concentration of the conjugate was observed routinely on the membrane areas immediately adjacent to and at contact points of both agglutinated avirulent (*A. castellanii* Neff) and virulent (*A. culbertsoni* A-3) strains; but the presence of an electron dense, amorphous material with associated ferritin-labeled Con A was occasionally found in agglutinated avirulent amoebae. Areas more distal to the contact regions were relatively free of conjugate (Stevens and Kaufman 1974). It was also observed that these lectin receptors are much concentrated at the uroidal region of the trophozoite and they adhere to the substrate, leaving a fluorescent trailing. They are completely absent in the pseudopodia and hyaline cap of the cell (data not shown).

Surface lectin receptors on a *Naegleria philippinensis* were localized using Con A-FITC to be uniformly distributed throughout the surface of the trophozoite, cyst wall and cyst pores. It was suggested that lectins can aid in understanding some of the taxonomic and morphogenetic problems within this group of free-living amoebae (Matias et al., 1990).

For the Western blot analysis of lectin receptors, the soluble cytoplasmic extracts showed three major bands between 29 to 66 kd molecular weights (Fig. 5). On the other hand, the plasma membrane extract also contains three major bands at the 40 to 80 kd molecular weight range. The appearance of these bands supports the occurrence of lectin receptors as evidenced by the strong fluorescence observed upon whole cell incubation with Con A-FITC. If purified and labeled with peroxidase or FITC, it may be used to detect the presence of intrinsic *Acanthamoeba* lectins and demonstrate the lectin-lectin receptor interaction.

Con A-peroxidase detection method was completely inhibited by the addition of 0.5M alpha-methyl-D-mannopyranoside. These lectin receptors are believed to be glyco-conjugates in the form of glycoproteins or glycolipids. The presence of glycoproteins in *Acanthamoeba* plasma membrane was demonstrated using periodate/NaB^3H_4 and galactose oxidase/NaB^3H_4 radiolabeling techniques (Paatero and Gahmberg, 1988). In addition, a diffusely labeled region with Mr of 55,000 to 75,000 seen on electrophoresis was suggested as glycolipids (Paatero, 1989).

Several bands for lectin receptors have been observed in the soluble cytoplasmic extracts of other *Acanthamoeba* strains used such as *A. Lenticulata,* C_{13} (an enviromental strain) and H-1 (pathogenic strain) (Fig. 6). The qualitative and quantitative similarities and differences exhibited in their Con A receptor patterns obtained through the lectin-peroxidase conjugate could serve as "glycogram" of the isolate to a particular lectin (Gonatas and Avrameas, 1986). Similar studies showed that glycoprotein patterns of highly pathogenic and weakly pathogenic *Naegleria fowleri* were distinctly different upon detection with Con A/WGA-peroxidase (Scott et al., 1989). Indeed, the great diversity of carbohydrate structures associated with the soluble and surface-bound glyco-conjugates poses the same diversity and significance in their biological function. The evidences show that carbodydrates may serve as markers of cell differentiation, development, pathological states and potential modification of the activities of proteins to which they are attached (Rademacher et al., 1988).

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Figure 1. Neuraminidase-treated horse erythrocytes strongly agglutinated by the soluble cytoplasmic protein extract from *Acanthamoeba* sp. (W-4). (Bar=20 um)

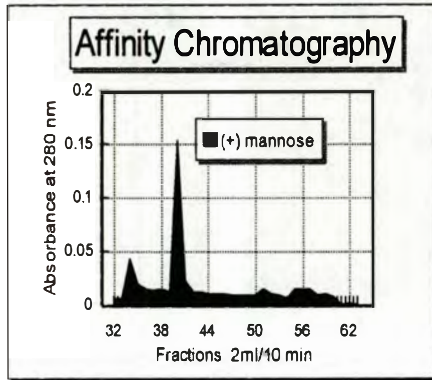


Figure 2. Protein peak eluted from an affinity column for mannose-binding lectins. Soluble cytoplasmic proteins were loaded and bound proteins were eluted with 0.2 M alpha-methyl-mannopyranoside in PBS (.15 M NaCl, 0.01M phosphate buffer, pH 7.2).

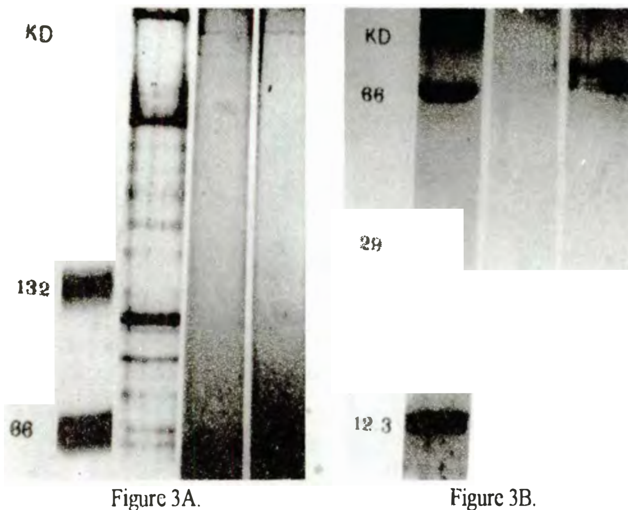


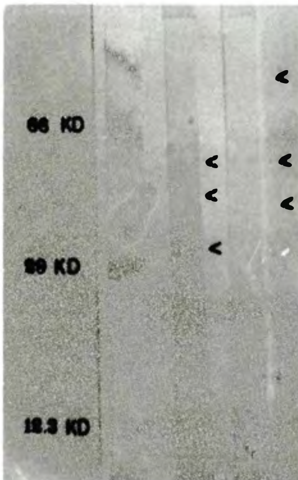
Figure 3A. Native PAGE profile of the *Acanthamoeba* sp. (W-4) protein eluted from mannose agarose affinity column. Lane 1- Molecular weight markers (BSA dimer, 132 kd; BSA monomer, 66 kd); Lane 2-Soluble cytoplasmic protein extract; Lane 3 – Single band of the eluted mannose binding protein of *Acathamoeba* sp. (W-4) (Visualized by silver staining)

Figure 3B. SDS-PAGE profile of the protein eluted from mannoside-agarose affinity column. Lane 1- Molecular weight markers (BSA, 66 kd; carbonic anhydrase, 29 kd; lysozyme, 12.3 kd); Lanes 2-3- *Acanthamoeba* sp. (W-4) mannose-binding protein sub-units at the 66-70 kd region (Visualized by silver staining)

Figure 4A. Figure 4B.

Figure 4A. *Acanthamoeba* sp. (W-4) trophozoites stained with Concannavalin A-fluorescein isothiocyanate (Con A-FITC) showing the lectin receptors concentrated at the plasma membrane particularly at the point of adhesion between the two cells

Figure 4B. The same cells viewed under phase contrast light microscope (bar = 10 um)



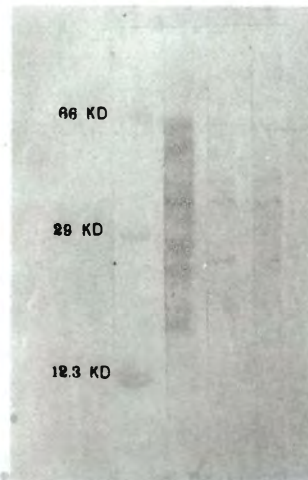


Figure 5. Figure 6.

Figure 5. Lectin (Con A) receptors of the *Acanthamoeba* sp. (W-4) soluble proteins (Lane 2) and plasma membrane proteins (Lane 3) separated by 12% SDS-PAGE visualized by Con A-peroxidase on nitrocellulose membrane; Molecular weight markers stained with India Ink: BSA, 66kd; carbonic anhydrase, 29 kd; lysozyme, 12.3 kd (Lane 1)

Figure 6. Lectin (Con A) receptors of *A. lenticulata*, C-13, and H-1 from the soluble cytoplasmic proteins separated by 12% SDS-PAGE and visualized by Con A-peroxidase on nitrocellulose membrane. Lane 1: Molecular markers; BSA, 66 kd; carbonic anhydrase, 29 kd; lysozyme, 12.3 kd; Lane 2: *A. lenticulata;* Lane 3: C-13 (UP, Diliman); Lane 4:H-1 (Hamburg, Germany)

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