Analysis of the feeding behavior and related proteins in the heliozoon Actinophrys sol

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Analysis of the feeding behavior and related proteins in the heliozoon *Actinophrys sol*

原生動物 *Actinophrys sol* における捕食行動と関連タンパク質の解析

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Contents

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Abstract

The heliozoon *Actinophrys sol* is a predatory protozoa. *A. sol* has stiff pseudopodia, which are called axopodia, radiating from the cell body. The feeding behavior of *A. sol* has been studied for a long time. The axopodia and extrusomes, which are secretory granules beneath the cell membrane, are considered to play an important role in the prey capture by *A. sol*. The exocytosis of extrusomes is occurred when prey cells make contact with the axopodia. In early studies, it was suggested that a 40-kDa glycoprotein (gp40) was present in the extrusome, and gp40 had multiple functions in prey capture, such as prey adhesion and recognition. The purpose of the present study is to characterize the feeding mechanism and associated proteins in *A. sol*.

In Chapter 2, a deduced peptidase has been cloned and characterized. By the microscopic observation, it was found that the deduced peptidase was localized in the lysosome. Taken together with the localization, the sequence similarity indicates that the peptidase is a lysosomal peptidase classified in the serine proteinase family, sedolisin.

In Chapter 3, it has been shown that prey capture by *A. so*l was extracellular Ca^{2+}-dependent. The results indicate that secretion of extrusomes is necessary for prey capture and suggests that the mechanism of exocytosis is conserved from protozoa to metazoa. Moreover, prey capture was specifically inhibited by concanavalin A. The results suggest that concanavalin A-binding glycoconjugates are involved in prey capture, which agree with the suggestion that gp40 is responsible for prey capture.

In Chapter 4, gp40 has been cloned and characterized. The amino acid sequence of gp40 has significant homology to invertebrate β-1,3-glucan recognition proteins, which are pathogen recognition proteins in innate immune system. Furthermore, a protein A-binding protein was identified, which is probably localized in the extrusomes and involved in prey capture. These results possibly suggest that prey capture, or recognition, in protozoa might be evolutionarily linked to the innate immunity, self-nonself recognition system in metazoa.

Chapter 1

Introductory review

The term Heliozoa has been used to classify heterotrophic protozoa that have spherical cell body and radiating stiff pseudopodia supported by microtubules (Fig. 1-1), which are called axopodia (Mikrjukov and Patterson 2001). Heliozoa had been considered to be a polyphyletic group from the viewpoint of ultrastructural studies (Cavalier-Smith and Chao 2003), and recently it was confirmed by molecular phylogenetic studies (Nikolaev et al. 2004). The actinophryid heliozoon *Actinophrys sol* (Patterson 1979) is now classified as stramenopile, or into the kingdom Chromista. Chromista members are characterized in that they have the plastid derived from secondary symbiosis, but *A. sol* lacks plastid and feeds on other protists. *A. sol* and related actinophryid heliozoa have been used for studying cell movements (Grebecki and Hausmann 1993, Kinoshita et al. 2001, Arikawa et al. 2002, Suzaki et al. 2003), cytoskeleton (Macdonald and Kitching 1967, Tilney and Byers 1969, Sugiyama et al. 1992), feeding behavior (Sakaguchi et al. 1998, Hausmann 2002), and so on. The present study is focused on the feeding behavior of *A. sol*.

When a prey organism happens to attach to the axopodia of *A. sol*, the prey becomes immediately trapped, conveyed toward the cell body surface, and ingested by a phagosome. Beneath the cell membrane of *A. sol*, there are many electron dense granules that are called extrusomes (Fig. 1-2). Extrusomes are extrusive organelles that are present in various protozoa and have a variety of functions, such as prey capture and defense against predators (Hausmann 2002, Miyake 2002). In *A. sol*, contents of extrusomes are discharged when a prey is attached to the cell surface of *A. sol*. Hence, it has been considered that the extrusome of *A. sol* has an important role in prey capture. Moreover, a concanavalin A-binding 40-kDa glycoprotein (gp40) was identified, and it was reported that this protein was involved in the feeding behavior (Sakaguchi et al. 2001). The N-terminal amino acid sequence of gp40 was determined, but neither its molecular structure, defined functions of gp40, nor interaction with other proteins had been elucidated. After a prey organism is enclosed in the phagosome, it fuses with lysosomes to become a phagolysosome. Then prey organisms are lysed gradually by lysosomal enzymes. These digestion events have been researched by light microscopy (Patterson and Hausmann 1981) and electron microscopy (Linnernbach et al. 1983). It, however, had not been known what enzyme was in the lysosome until recently.

In this study, I have cloned and characterized two proteins, gp40 and a lysosomal protein, by immunomicroscopy and biochemical methods, and I also investigated factors involved in prey capture. I discuss how these proteins are involved in the feeding mechanism of *A. sol.*

Chapter 2

Lysosomal protein; cDNA cloning and characterization

Introduction

A. sol captures prey organisms with its axopodia, and subsequently prey are conveyed to the cell body and engulfed in phagosomes. The phagosome fuses with lysosomes, and the contents of the phagosome are gradually degraded by lysosomal enzymes. These digestion events have been investigated (Patterson and Hausmann1981, Linnenbach et al. 1983). However, no digestive enzymes have so far been identified in the lysosomes of *A. sol*.

In this chapter, a cDNA of *A. sol* has been cloned and sequenced. The result shows that it has significant homology to sedolisins. Sedolisin, or serine-carboxyl proteinase, is a new family of serine proteases, which is classified as S53 in the MEROPS database (Wlodawer et al. 2001). The type peptidase sedolisin, isolated from *Pseudomonas* sp. 101, is a pepstatin-insensitive carboxyl proteinase (Oda et al. 1987). The enzyme is active at low pH, but it does not contain the consensus sequence of Asp-Thr/Ser-Gly and is not sensitive to the protease inhibitor, pepstatin, unlike other aspartic proteases. Subsequent studies, including a mutational analysis (Oyama et al. 1999) and crystal structural analysis (Wlodawer et al. 2001), have elucidated its catalytic triad and other essential residues. It was described as a serine-carboxyl proteinase and named *Pseudomonas* serine-carboxyl proteinase (PSCP). Recently it was renamed sedolisin because of its catalytic triad of Ser, Asp and Glu (Wlodawer et al. 2003b). In addition to bacteria, sedolisins have been found in metazoa, fungi, and protozoa. The most famous member of the sedolisin family is the CLN2 protein from *Homo sapiens* (Sleat et al. 1997), changes in which cause a fatal neurodegenerative disease, classical late-infantile neuronal ceroid lipofuscinosis (LINCL). CLN2 has been identified as a tripeptidyl-peptidase I (TPPI; Rawlings and Barret 1999). Related proteins have been found in other vertebrates (Wlodawer et al. 2003a), and Lys60 and Lys45 from *Amoeba proteus* (Kwon et al. 1999) also belong to the family S53, although their enzyme activities have not been demonstrated. However, some species, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, apparently lack sedolisin-like genes. Furthermore, no sedolisin-like genes have so far been found in plants, or in protozoa other than Amoebozoa, and the physiological role of sedolisins other than CLN2 are still unclear. Therefore, I have characterized the primary sequence of the protein from *A. sol* and localized it using immunomicroscopic analysis.

Materials and Methods

Organism and culture

A. sol (ATCC 50937) was cultured monoxenically as described by Sakaguchi and Suzaki (1999). The prey flagellate, unicellular green algae, *Chlorogonium* sp. was cultured axenically as described by Sakaguchi et al. (1998). Cells were collected by centrifugation and washed with 10% artificial seawater (47 mM NaCl, 1.1 mM KCl, 1.1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 2.5 mM $MgSO_4$ and 1 mM Tris-HCl at pH 7.8) before they were used for experiments.

cDNA cloning and sequencing

A. sol cells were homogenized in liquid nitrogen and lysed in guanidinium thiocyanate. Total RNA was extracted according to Sambrook et al. (1989) and mRNA was isolated using an mRNA Purification Kit (GE Healthcare UK Ltd). cDNA was synthesized by a Marathon cDNA Amplification Kit (Takara Bio) and amplified by PCR using a primer, 5'-GATTTCGACAC-3', which was designed based on the N-terminal amino acid sequence of gp40 (Sakaguchi et al. 2001). Rapid amplification of cDNA ends (5'-RACE) was used to isolate the full-length cDNA. The PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen). After extraction of plasmid DNA, sequencing was carried out using a Thermo Sequenase Cycle Sequencing Kit (USB) with a DNA auto sequencer LIC-4200L-1 (Aloka). The cDNA sequence is available in the DDBJ/EMBL/GenBank databases under the accession number AB276124. The amino acid sequence deduced from the cDNA was compared to databases using the NCBI BLAST search program. Amino acid sequences of homologous proteins were aligned using Clustal W and BOXSHADE programs.

Expression and purification of protein

The plasmid DNA was digested with EcoRI and resultant fragments were cloned into pGEX-4T1 (GE Healthcare UK Ltd). The plasmids were transformed into *E. coli* XL1-Blue and the expression of the GST-fusion protein was induced by the addition of 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside). After incubation, harvested cells were disrupted and centrifuged. The supernatant was applied to a Glutathione Sepharose 4B column (GE Healthcare UK Ltd). After incubation for 15 h at 4°C, protein was eluted. The purified GST-fusion protein was subjected to SDS-PAGE, and we confirmed that its molecular mass was consistent with the one deduced from its structure.

Preparation of antibody

The recombinant protein, emulsified in Freund's Complete Adjuvant (DIFCO), was injected into a rabbit, and immunization was repeated at two-week intervals. After the 4th immunization, the supernatant of collected blood was stored at –80°C. To confirm antibody activity and specificity, SDS-PAGE and

immunoblotting were carried out. *A. sol* cells were solubilized in a sample buffer containing 170 mM Tris-HCl (pH 6.8), 5.3% SDS, 13.4% 2-mercaptoethanol. Proteins were separated on 12% SDS-polyacrylamide gels with a molecular-weight marker DAIICHI-II (Daiichi Pure Chemicals). Gels were stained by Coomassie Brilliant Blue, or blotted onto polyvinylidene difluoride membranes (Clear Blot Membrane-p, ATTO). Membranes were rinsed with 0.05% Tween 20 in TBS (10 mM Tris-HCl pH 7.4 and 154 mM NaCl), and incubated with the antibody for 1 h at room temperature. After being rinsed, membranes were incubated with HRP-conjugated anti-rabbit IgG (SEIKAGAKU CORPORATION) for 1 h at room temperature. Finally, rinsed membranes were visualized with Konica immunostaining Kit HRP-1000 (Konica).

Immunofluorescence microscopy

Starved *A. sol* were supplied with food *Chlorogonium* sp. At intervals of 30 min after feeding, cells were collected and fixed with 4% formaldehyde for 15 min. After fixation, they were permeabilized with 100% ethanol followed by 3% bovine serum albumin (BSA) for 30 min to prevent nonspecific binding of antibodies. Then they were incubated with the antibody for 1 h at room temperature and rinsed with 0.1% BSA in PBS (pH 7.4, 137 mM NaCl). Finally, they were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes), rinsed, and mounted in 50% glycerol and 0.1% p-phenylenediamine·2HCl. Observation and photography were carried out using a confocal laser scanning microscope FV300-IX71 (OLYMPUS). Thirty cells are randomly chosen from each sample, and fluorescent-labeled vesicles in each cell are counted. The results were expressed as mean ± standard error of the mean (S.E.M.).

Immunoelectron microscopy

A. sol feeding on *Chlorogonium* sp. were fixed for 30 min at room temperature with a pre-fixative containing 4% formaldehyde, 0.4% glutaraldehyde, 1 mM $MgCl_2$, 1 mM EGTA and 50 mM phosphate buffer (pH 6.9). Cells were permeabilized with 1% Triton X-100 in PBS, followed by incubation in 1% BSA for 30 min. Then they were incubated with the antibody for 1 h at room temperature. After being rinsed, they were incubated with gold-conjugated anti-rabbit IgG (5 nm, SIGMA) for 1 h at room temperature. Cells were post-fixed with the same fixative in the same conditions, dehydrated in a graded ethanol series, and embedded in Spurr's low-viscosity embedding resin (Spurr 1969). Ultrathin sections were stained with 3% aqueous uranyl acetate for 15 min and Reynolds' lead citrate stain (Reynolds 1963) for 5 min. Sections were observed and photographs were taken with a transmission electron microscope H-7100 (HITACHI) equipped with a CCD camera system (AMT Advantage HR, HITACHI). For the quantitative analysis, postembedding immunogold labeling was also performed. Cells were fixed at 30

min or 90 min after food supply for 30 min with the fixative that was used for the pre-fixation method as shown above. Cells were dehydrated in a graded ethanol series at 4 ºC and embedded in LR white (London Resin Co.) at -20 ºC. The sample were polymerized with UV irradiation at -20 ºC. Ultrathin sections were blocked with 20% Blocking One (Nacalai Tesque) and 10 mM glycine in PBS for 20 min followed by incubation with the polyclonal antibody for 1 h at room temperature. After being rinsed with PBS, they were incubated with AuroProbe EM Protein A G10 (GE Healthcare UK Ltd) for 1 h at room temperature. Finally sections were stained and observed as described above. Fifteen phagosomes, which contained digested *Chlorogonium*, were randomly chosen from 30 and 90 min-fixed samples, and photographs were taken. Number of gold particles per unit area photographed, about 11 μm^2, was counted. As a control, vesicles other than phagosomes were also examined.

Results

Cloning and sequencing

Using primers designed for the amino acid sequence of gp40, a cDNA was incidentally cloned, and its nucleotide sequence was determined. It contains an open reading frame of 1,722 nucleotides and encodes 574 amino acid residues with a calculated molecular mass of 62,204 Da. The deduced amino acid sequence is not consistent with the N-terminal amino acid sequence of gp40. The first 18 residues are predicted to comprise a signal peptide with a probable cleavage site between residues 18 and 19. Moreover, between residues 490-500, there is a consensus pattern G-T-S-x-[SA]-x-P-x-x-[STAVC]-[AG] for the subtilase family (classified as S8 in the MEROPS database; Siezen et al. 1991). Using the NCBI BLAST program, however, the sequence shows significant similarity to the sedolisin family (MEROPS S53), such as the CLN2 protein (Sleat et al. 1997), physarolisin (Nishii et al. 2003), kumamolysin (Oyama et al. 2002), sedolisin (Oda et al. 1994) and sedolisin B (Oda et al. 1996), but not to subtilases. BLAST results show an identity of 33% for CLN2 protein and physarolisin, 31% for kumamolysin, and 25% for sedolisin and sedolisin B. Additionally, some significant residues, which are conserved among the sedolisin family and considered to be essential for its catalytic activity, are also conserved in this protein (Fig. 2-1). Namely, the so-called catalytic triad, Glu 80, Asp 84 and Ser 287, in sedolisin (Wlodawer et al. 2001) corresponds to Glu 290, Asp 294 and Ser 492 in the *A. sol* protein, respectively. Asp 170, forming the oxyanion hole, and Asp 328, involved in Ca^{2+}-binding, in sedolisin also correspond to Asp 374 and Asp 533 in *A. sol*.

Immunoblotting and immunomicroscopy

In order to characterize the protein, whole *A. sol* proteins were subjected to SDS-PAGE followed

by immunoblotting using a polyclonal antibody raised against a recombinant protein. The antibody reacted specifically with a 48-kDa protein (Fig. 2-2). The molecular mass was lower than the 62 kDa that was predicted from the amino acid sequence.

This antibody was used for immunofluorescence microscopy and immunoelectron microscopy. The results of immunofluorescence microscopy are shown in Figure 2-3. During the first 60 min of feeding, there was no stained area at 0 and 30 min, and only part of cells at 60 min had stained vesicles (Fig. 2-3A, B, C and Table I). At 90 min after the initiation of feeding, parts of cells that correspond to phagosomes were stained in most cells (Fig. 2-3D and Table I). However, as reported by Patterson and Hausmann (1981), phagosomes form within 30 min, so the protein did not appear in early phagosomes. More detailed observations with electron microscopy are shown in Figure 2-4. When a prey organism, *Chlorogonium* sp., was being digested in a phagosome, small vesicles of 0.2–0.4 μm, which appeared to be lysosomes, were observed surrounding the phagosome (Fig. 2-4A). The contents of these vesicles were labeled with gold-conjugated antibodies (Fig. 2-4B). Some vesicles were fused to phagosomes, and their contents were being released into the phagosome (Fig. 2-4C). In the postembedding method, approximately 3 times gold labeling was observed in phagosomes in 90 min-fixed cells than that of 30 min-fixed cells (Table II). These results suggest that the antibodies bind to lysosomal proteins, which gradually accumulate in the phagosomes.

Discussion

I attempted to clone the cDNA of gp40, a glycoprotein that is thought to be involved in the capture of prey by *A. sol* (Sakaguchi et al. 2001). Analysis of the coding region of the cDNA indicated that the cDNA we cloned does not encode gp40. Nevertheless, the incidentally cloned protein seemed to be involved in feeding of *A. sol*, so we conducted further analysis. There is a discrepancy between the molecular mass predicted from amino acid sequence and the result of immunoblotting. This suggests that this protein is synthesized as a precursor protein, and then processed into a mature protein of 48 kDa, in a manner similar to that reported for other peptidases of the sedolisin family (Oda et al. 1994 and 1996). The N-terminal amino acid sequence of the mature protein should be determined in future studies to assess this possibility. The primary structure of the protein shows significant homology to sedolisin peptidases, and it retains important catalytic residues. This suggests that the protein encoded by the cDNA may be a new member of the sedolisin family. Interestingly, the highest score (of 265) using the NCBI BLAST program was with murine tripeptidyl-peptidase I, although this was not significantly greater than the scores for other sedolisins. This suggests that the protein may have TPP activity.

In an earlier study, the process of digestion in *A. sol* was examined by light microscopy (Patterson

and Hausmann 1981). It was observed that lysis of the prey began just after, or sometimes even before, the formation of a phagosome, and the prey was killed by lysis within 1 h after feeding. Additionally, electron microscopy revealed that electron-dense contents were released into the phagosome from vesicles at that time (Linnenbach et al. 1983). Although the function of the material was unclear, it was considered that it might contain lysosomal digestive enzymes. In this chapter, I report a putative peptidase in the lysosomes that is secreted into the phagosomes. Based on all these observations, we speculate that the new protein is a lysosomal digestive enzyme. This is consistent with early studies on sedolisin in *A. proteus* (Kwon et al. 1999, Yoo et al. 1996). Taking into consideration both its sequence homology and the microscopic analysis, the protein appears to be a sedolisin-like peptidase.

Although several peptidases of the sedolisin family have been discovered in protists, all are from Amoebozoa. *A. sol* has recently been classified as a stramenopile (Nikolaev et al. 2004), and is therefore phylogenetically distant from Amoebozoa. The diatom *Thalassiosira pseudonana* and some parasitic protozoa, such as *Plasmodium*, *Trypanosoma* and *Entamoeba*, lack sedolisin-like proteins in their genomes. Among protozoan sedolisin-like peptidases, functions have been examined in only two. In the slime mold, *Physarum polycephalum*, physarolisin was considered to be involved in conjugation and the following morphological changes (Nishii et al. 2003). However, *Amoeba proteus* sedolisin was localized in phagosomes. It seems likely that the *A. sol* peptidase is functionally related to the sedolisin of *A. proteus*. To my knowledge, *A. sol* sedolisin is the first characterized protein in heliozoa, and also the first reported sedolisin from the stramenopiles. Future studies on its enzyme activities, such as substrate specificity and tolerance to protease inhibitors, will shed light on the relationship between *A. sol* sedolisin and other members of the same protein family.

Chapter 3

Ca^{2+}- and oligosaccharide dependent prey capture

Introduction

Secretory granules, or dense-core granules, have been studied extensively in metazoan cells, and the role of Ca^{2+} in secretion of granules is well documented (Burgoyne and Morgan 2003). Appropriate stimuli cause Ca^{2+} influx into cells, where it interacts with Ca^{2+} sensor proteins and induces exocytosis of the granules. Among unicellular organisms, exocytosis of trichocysts, the extrusomes of *Paramecium*, has been shown to be dependent on Ca^{2+} (Plattner and Kissmehl 2003). However, with respect to the function of Ca^{2+} in heliozoa, only Ca^{2+}-dependent contraction of axopodia has so far been reported (Matsuoka and Shigenaka 1984).

In this chapter, I examined the role of extracellular Ca^{2+} in exocytosis and prey capture in *A. sol*. In addition, I investigated the effect of Con A on this process. Taken together, I discuss the possible role of extrusomes and gp40 in prey capture.

Materials and Methods

Organism and culture

A. sol and *Chlorogonium* sp. were cultured as described in Chapter 2. Both cell types were collected by centrifugation and washed with 10% artificial or with Ca^{2+}-free 10% ASW before they were used for experiments.

Prey capture experiments

All experiments were carried out under a stereoscopic microscope (Nikon SMZ1500), and photographs were taken with a DP11 digital camera on a microscope (Olympus BX50). Prior to the experiments on Ca^{2+} dependency, *A. sol* cells were washed twice with Ca^{2+}-free 10% ASW. $CaCl_2$ was added to Ca^{2+}-free 10% ASW to final concentrations of 0, 0.25, 0.50, 0.75, or 1.00 mM. *A. sol* cells that had been washed with Ca^{2+}-free 10% ASW were then mixed with these solutions on depression slides and incubated for 5 min. *Chlorogonium* cells were then added to the solutions which were incubated for another 5 min. Finally, *A. sol* cells were transferred to new depression slides, and the number of cells per 20 cells that had captured prey was counted.

Other experiments were conducted by the same method, with reagents added to 10% ASW containing 1 mM Ca^{2+}. EGTA was added at 1.1 mM in order to remove Ca^{2+}, and the final concentration of free Ca^{2+} was calculated by an iterative procedure (Suzaki and Williamson 1986). Verapamil (50 μM;

Nacalai Tesque), nifedipine (100 μM; Nacalai Tesque), ω-conotoxin GVIA (200 nM; Alomone Labs), $NiCl_2$ (100 μM), $LaCl_3$ (40 μM) and ruthenium red (2 μM) were used as Ca^{2+} channel blockers. The calmodulin antagonists W-7 and W-5 (BIOMOL International, LP) were used at 20 μM. Calcium ionophore A23187 (Nacalai Tesque) was used at 2 μM. Nifedipine, W-7, W-5 and A23187 were dissolved in dimethylsulfoxide (DMSO), the final concentrations of which was less than 0.1%. Chloral hydrate was used at 10 mM to disturb the extrusomes.

The lectin Con A was used at 20 μg/ml, with or without its hapten sugar α-methyl-D-mannoside, at 20 mM. The effects of a pretreatment with Con A were also examined. *A. sol* cells or *Chlorogonium* cells were incubated for 5 min in solution containing 20 μg/ml Con A. The cells were then washed with 10% ASW, and new untreated prey or predator cells, respectively, were added. After 5 min, the number of *A. sol* cells that had captured prey was counted.

The cells did not appear to be damaged by any of these treatments, except chloral hydrate (discussed below). For each experiment, data were taken from at least five experiments and results were expressed as mean ± standard error of the mean (S.E.M.).

Results

Prey capture is dependent on extracellular Ca^{2+}

To determine the effect of extracellular Ca^{2+} concentration on prey capture by *A. sol*, we supplied prey to cells at various concentrations of Ca^{2+}, and counted cells that captured prey. In the Ca^{2+}-free solution, *A. sol* did not capture any prey (Figs. 3-1 and 3-2), and very few prey were captured at 0.25 mM Ca^{2+} (Fig. 3-2). I observed similar Ca^{2+}-dependency for prey capture in a related heliozoon species *Echinosphaerium akamae* (data not shown). The concentration of Ca^{2+} required for prey capture by *A. sol* is relatively high compared to that required for exocytosis of trichocysts of *Paramecium* (Kerboeuf and Cohen 1990). Perhaps this is because the *A. sol* used here was originally collected from brackish water.

Chelation of Ca^{2+} by EGTA also inhibited prey capture (Fig. 3-2), although not completely as in Ca^{2+}-free solution. It is possible that EGTA chelated other cations or intracellular Ca^{2+}, and consequently some extracellular Ca^{2+} would remain even when it was theoretically absent. Capturing ability was incompletely restored by the addition of Ca^{2+} (Fig. 3-2). Because of these effects, I could not use Ca^{2+}/EGTA buffer to examine dose dependency or the threshold value. Nevertheless, these results indicate that extracellular Ca^{2+} is necessary for prey capture in *A. sol*.

Inhibition by drugs

To rule out the possibility that Ca^{2+}-dependent adhesion molecules are responsible for prey capture,

I used Ca^{2+} channel blockers. Verapamil, nifedipine and $LaCl_3$ inhibited prey capture, although conotoxin GVIA, $NiCl_2$ and ruthenium red did not affect it (Fig. 3-3).

The calcium ionophore A23187 inhibited prey capture (Fig. 3-4). In the experiments using calmodulin antagonists, capture was inhibited by W-7, but not by W-5 at the same concentration (Fig. 3-4). These drugs bind to calmodulin and inhibit its activity. W-7 is, however, more specific and effective on calmodulin than W-5 (Hidaka et al. 1981). Chloral hydrate also inhibited prey capture. In the heliozoon *Echinosphaerium akamae*, it was previously observed that extrusomes, described as dense granules, were detached from the plasma membrane by chloral hydrate (Suzaki and Shigenaka 1982). The inhibition by chloral hydrate was therefore possibly due to effects on granule secretion. However, as it was observed that longer incubation in chloral hydrate disturbed the cell surface of *A. sol* (Fig. 3-5), we cannot exclude the possibility that the inhibition effect was a non-specific effect of chloral hydrate.

Concanavalin A treatments

In Con A-containing solution, prey capture was almost completely inhibited, but prey capture was not affected when its specific hapten sugar, α-methyl-D-mannoside, was also present (Fig. 3-6). Furthermore, the addition of mannoside after incubation in Con A allowed *A. sol* to again capture prey (data not shown). In the pretreatment experiments, inhibition was achieved when *A. sol* cells were pretreated with Con A solution (Fig. 3-6). In contrast, pretreatment of the prey did not strongly affect prey capture. Additionally, *A. sol* cell surface was disturbed when it was incubated in Con A together with EGTA at same concentrations above. And then prey cells extremely adhered to axopodia and the cell body of *A. sol* (Fig. 3-7). This phenomenon was not observed when Ca^{2+}-free ASW was used, suggesting the adhesion was Ca^{2+}-dependent.

Discussion

Extrusomes are secretory granules that are observed under the cell membrane of many protozoa. Extrusomes have various roles, such as offensive or defensive functions; those in ciliates have been the most studied. For example, trichocysts of *Paramecium* and cortical granules of *Blepharisma* were reported to have defensive functions (Miyake 2002), while toxicysts of *Didinium* and *Homolozoon* are known to have offensive functions (Hausmann 2002). In the actinophryid heliozoa, the extrusomes have been implicated in prey capture (Patterson and Hausmann 1981, Sakaguchi et al. 2001). Unlike those in ciliates, the contents of the extrusomes in actinophryid heliozoa are not ultrastructurally organized; they have an amorphous appearance, with electron-dense material, and resemble secretory granules of metazoa.

In this study, I have shown Ca^{2+}-dependency of prey capture by *A. sol*. As prey adhesion was also inhibited by Ca^{2+} channel blockers, it is unlikely that prey adhesion is merely due to Ca^{2+}-dependent adhesive substances on the cell surface. The Ca^{2+} channel blockers verapamil and nifedipine that inhibited prey capture in *A. sol* are selective inhibitors for L-type Ca^{2+} channels. Therefore, the results suggest that an L-type Ca^{2+} channel is involved in prey capture. Influx of Ca^{2+} causes release of secretory granules in both metazoa and other protozoa, and earlier studies showed that granule secretion occurred during prey capture by *A. sol* (Patterson and Hausmann 1981). In *Paramecium*, it was observed that L-type Ca^{2+} channel blockers inhibited secretion of trichocysts (Maleki et al. 1987, Satir et al. 1988). In mammalian cells, L-type channels are also known to play crucial roles in granule secretion in some cell types, including dendritic cells, cytotoxic T lymphocytes and pancreatic B cells (Gardella et al. 2000, Lyubchenko et al. 2001, Mears 2004). Ca^{2+} influx is also necessary for granule secretion in invertebrates (e.g. Johansson and Söderhäll 1985). Several proteins that are components of metazoan exocytosis were also found in *Paramecium* (Plattner and Kissmehl 2003). It seems likely that similar mechanisms are employed in exocytosis in both multicellular- and unicellular organisms, including *A. sol*.

The calmodulin antagonist W-7 also inhibited prey capture. A similar inhibition effect was reported in *Paramecium* (Garofalo et al. 1983). Calmodulin is not considered to be essential for exocytosis in metazoan secretory cells; nevertheless it may play a regulatory role in exocytosis (Burgyone and Morgan 1998, Burgoyne and Morgan 2003, Easom 1999). Prey capture was also inhibited by A23187. This could possibly be due to depletion of extrusomes. A23187 might cause Ca^{2+} influx, elevating intracellular Ca^{2+} concentration, and finally resulting in unregulated secretion of extrusomes and inhibition of prey capture. It has been reported that treatment with A-23187 induced contraction of axopodia in *Echinosphaerium akamae* (Matsuoka and Shigenaka 1984), and this was also ascribed to increase in Ca^{2+} concentration in the axopodia. Extracellular Ca^{2+} and its influx seem to be necessary for prey capture by *A. sol*, and the secretion of extrusomes is probably involved. However, the detailed mechanism of prey capture remains unknown. It is possible that intracellular Ca^{2+} stores may also be involved in the secretion of extrusomes, as putative Ca^{2+} stores were observed in *E. akamae* (Matsuoka and Shigenaka 1984). In the slime mold *Dictyostelium discoideum*, it was reported that Ca^{2+} was needed for phagocytosis, although whether influx of Ca^{2+} was also required was not determined (Yuan et al. 2001).

The Con A pretreatment results indicate that the glycoconjugates that bind to Con A are localized on the cell surface of *A. sol*. Considering that gp40 is a dominant protein that binds to Con A (Sakaguchi et al. 2001), it would be reasonable to surmise that gp40 is the targeted protein in these experiments. gp40 is, however, considered to reside in the extrusomes, and it seems unlikely that Con A would penetrate into the extrusomes. There may therefore be other Con A-binding glycoconjugates on the cell surface that

participate in prey capture. Alternatively, parts of extrusomes could be secreted constitutively and adhesive substances might be conveyed to the cell surface of *A. sol*. However, the results from the Ca^{2+} experiments show that regulated exocytosis is probably necessary for prey capture. Therefore, it is possible that cell surface gp40, or some other Con A-binding glycoconjugate, act as receptors for inducing exocytosis of extrusomes and is used for prey recognition. In *Euplotes mutabilis*, it has been implied that lectin-binding regions were involved in food selection (Wilks and Sleigh 2004). To elucidate the secretion and prey capture mechanism in *A. sol*, I raised an antibody against gp40 and to clone the gp40 gene (Chapter 4). Such an antibody would also provide an excellent molecular marker for secretion of extrusomes and prey capture in *A. sol*.

Chapter 4

cDNA cloning and characterization of gp40

Introduction

Likewise other predatory protozoa, *A. sol* needs to recognize prey cells to capture them. Therefore, they should have certain mechanisms of prey recognition and selection. In an early study, it was reported that the amoeba *Acanthamoeba castellanii* had a mannose receptor that was responsible for the binding and phagocytosis of yeasts (Allen and Dawidwicz 1990). Previous studies have also reported self-recognition mechanisms to prevent cannibalism in amoeba. It was found that a peptide in *Amoeba proteus* was responsible for discriminating prey particles from non-prey materials (Kusch 1999) and that the mutant strain of cellular slime mold *Dictyostelium caveatum* showed cannibalism and could not complete multicellular development (Waddell and Duffy 1986).

In *A. sol*, a Con A-binding glycoprotein (gp40) was isolated as a prey recognition protein that localized in the extrusomes (Sakaguchi et al. 2001). It has been observed that gp40-binding beads induced phagocytosis by *A. sol* and adhered to prey flagellates. In addition, I have shown that Con A inhibited prey capture as described in Chapter 3.

In this chapter, I have cloned and sequenced the cDNA of gp40. It showed homology to β-1,3-glucan-binding proteins of invertebrates in their innate immune systems. Subsequently, I have examined its glucan-binding ability and enzyme activity. Furthermore, I have identified another putative prey capture protein, which binds to *Staphylococcus* protein A.

Materials and Methods

Organisms and culture

A. sol and the prey *Chlorogonium* sp. was cultured as described in Chapter 2. Cells were collected by centrifugation and washed with 10% ASW before they were used for experiments.

Protein purification with Con A

A. sol cells were incubated in lysis buffer (0.5% Nonidet P-40, 200 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1mg/ml leupeptin, 1 mM EDTA and 1mM PMSF) for 10 min on ice. Cells were centrifuged at 10,000 g for 5 min at 4ºC, and supernatant was collected. Con A-affinity chromatography was carried out according to the previous study (Sakaguchi et al. 2001). Eluted proteins were used in following experiments.

Deglycosylation

Con A-binding proteins were deglycosylated with N-glycosidase F (Roche) according to the manufacture's instructions. As a control, proteins were incubated without N-glycosidase F. After incubation, proteins were concentrated with trichloroacetic acid (TCA). TCA was added to a final concentration of 6%. After incubation for 2 h at 4ºC, the mixture was centrifuged at 15,000 g for 15 min at 4ºC. Cold acetone was added to the precipitation, and it was centrifuged again. The precipitate was solubilized in a sample buffer containing 170 mM Tris-HCl (pH 6.8), 5.3% SDS, 13.4% 2-mercaptoethanol and analysed by SDS-PAGE and immunoblotting as described below.

SDS-PAGE and immunoblotting

Proteins in the sample buffer were subjected to SDS-PAGE (12% acrylamide gel) with a molecular-weight marker DAIICHI-II (Daiichi Pure Chemicals). Separated proteins were stained with CBB or blotted onto Clear Blot Membrane-p (ATTO). Membranes were blocked with 20% Blocking One (Nacalai Tesque) and 0.05% Tween 20 in PBS followed by incubation with the anti-gp40 antibody (described below). After being rinsed with PBS containing 0.05% Tween 20, membranes were incubated with HRP-conjugated anti-rabbit IgG (Seikagaku Corporation). For Con A-binding glycoconjugates, membranes were incubated with Con A-HRP (Seikagaku Corporation). Finally, immunoreactive bands were visualized with ECL plus Immunoblotting Detection System (GE Healthcare).

Determination of partial amino acid sequences

Proteins were collected with Con A-agarose as described above and subjected to SDS-PAGE. The band corresponding to gp40 was excised, and two internal amino acid sequences were determined by APRO Science.

Antibody production

Polyclonal antibodies were raised against the N-terminus of gp40, which had been determined previously (Sakaguchi et al. 2001), by Sigma Genosys, and also against an internal region of the protein by Thermo Electron Corporation. The antibody against the N-terminus, KVLKFEDDFDTFDL, was used for detection of gp40. The antibody against the internal region, SGEIDIMESRGNSPSC, was used to confirm the deduced amino acid sequence of gp40.

Immunomicroscopy

Immunoelectron- and immunofluorescence microscopy were carried out as described in Chapter 2,

except using the anti-gp40 N-terminus antibody.

cDNA cloning and sequencing

cDNA of *A. sol* were synthesized using Marathon cDNA Amplification Kit (Takara Bio) as described in Chapter 2. PCR was performed using a primer, 5'-GGIGAYGAYGTIGCIYTICARATHG-3', which was designed based on the internal amino acid sequence of gp40. 5'-rapid amplification of cDNA ends (5'-RACE) was used to determine the full-length cDNA sequence. The PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen). After extraction of plasmid DNA, sequencing was carried out using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI 310 PRISM Genetic Analyzer (Applied Biosystems). The cDNA sequences are available in the DDBJ/EMBJ/GenBank databases under the accession number AB368718 and AB368719. The amino acid sequence deduced from the cDNA was analyzed with Pfam databases (www.sanger.ac.uk/Software/Pfam/), PredictProtein (www.predictprotein.org) and SignalIP 3.0 (www.cbs.dtu.dk/services/SignalP/), and was compared to databases, NCBI and TBestDB (tbestdb.bcm.umontreal.ca/), using the BLAST search program. Amino acid sequences of homologous proteins were aligned using Clustal W and BOXSHADE programs.

Glucan binding assay

Insoluble β-1,3-glucan curdlan was suspended in PBS and heated at 90ºC and cooled for gelation. Cells were lyzed as described above and proteins were incubated with 1mg/ml curdlan gel in PBS for 1h at 4ºC. After a rinse with PBS, curdlan-binding proteins were eluted with sample buffer. Soluble β-1,3-glucan laminarin, *E. coli* lipopolysaccharide (LPS), methyl-cellulose, mannan, mannoside or glucose were added in PBS with curdlan for competitive inhibition. Instead of curdlan, cellulose or zymosan were also used to bind to proteins. Eluted proteins were concentrated and subjected to SDS-PAGE and immunoblotting in the same manner as described above.

Glucanase activity assay

gp40 was purified using Con A-agarose and dialyzed with PBS. Dialyzed gp40 was concentrated by VIVASPIN 400 (Sartorium) and used in the following enzyme assay. Enzyme assays were performed using Laminarin azure from *Laminaria digitata* (Sigma) as substrates according to the manufacture's instructions. Laminarinase, from *Trichoderma* species (Sigma) and Albumin Standard (Pierce) were used as positive and negative control, respectively. In brief, the enzyme and proteins were incubated with Laminarin azure at 37ºC for 10 min in 100 mM sodium acetate buffer (pH 5.0 at 37ºC), and the reaction

were stopped by addition of 95% ethanol. Solutions were filtered, and absorbances at 595 nm were recorded.

Protein A-binding experiments

For electron microscopy, cells were prefixed for 20 min with a fixative containing 3% glutaraldehyde, 0.01 mM $MgSo_4$, 1 mM sucrose, and 50 mM cacodylate buffer (pH 7.0). The cells were then postfixed with 0.5% OsO_4 for 30 min, rinsed with same buffer. Cells were dehydrated in a graded ethanol series, and embedded in Spurr's resin. Ultrathin sections were blocked with 20% Blocking One (Nacalai Tesque) and 10 mM glycine in PBST. Sections were then incubated with AuroProbe EM Protein A G10 (GE Healthcare UK Ltd) for 2 h. As control, sections were also incubated with Anti-Mouse IgG (whole molecule) GOLD CONJUGATE, 10nm (Sigma), Immunogold Conjugate EM Goat Anti-Rabbit IgG: 10nm (BBInternational), or AuroProbe EM Protein A G10 with IgG, which was purified from rabbit preimmune serum.

For immunoprecipitation, *A. sol* proteins were incubated with nProtein A Sepharose 4 Fast Flow (GE Healthcare UK Ltd) in PBS containing 1mg/ml leupeptin and 1mM PMSF. As a control, *Chlorogonium* proteins were mixed with *A. sol* proteins and incubated with nProtein A Sepharose. The Sepharose beads were washed with PBS, and binding proteins were eluted with sample buffer followed by SDS-PAGE and silver stain using Silver Stain "DAIICHI" (Daiichi Pure Chemicals Co., LTD.).

Results

Purification and deglycosylation of gp40

Con A-binding proteins were analyzed by SDS-PAGE and immunoblotting (Fig. 4-1). By Con A-chromatography, gp40 was detected as a Con A-binding protein (data not shown). In addition to gp40, a 65 kDa protein was also present as a Con A-binding protein. After treatment with N-glycosidase F, the 40-kDa band disappeared, and instead a 38-kDa band appeared (Fig. 4-1A). In immunoblotting using the anti-gp40 antibody, both 40- and 38- kDa bands were reacted (Fig. 4-1B). Whereas, in the case using Con A-HRP, only the 40-kDa band was stained (Fig. 4-1C). In the control sample, the 40-kDa band appeared in both anti-gp40 and Con A-HRP experiments. These results indicate that gp40 was N-linked glycoprotein and that the 38-kDa protein was deglycosylated gp40.

Partial amino acid sequences

Two internal amino acid sequences of gp40 were determined as PWADTSXIAYLQF and YPTWSGDDVALWID. The latter sequence was used for designing a primer for cDNA cloning.

Immunomicroscopy

In the immunofluorescence microscopy, axopodia and the periphery of the cell body were stained (Fig. 4-2). Each labeled particle probably corresponds to the extrusome, because extrusomes are usually located beneath the cell membranes of axopodia and the cell body. Additionally, it is shown that the contents of extrusomes were labeled with colloidal golds by pre-embedding immunoelectron microscopy (Fig. 4-3). Although golds were not bound to the inner contents of the extrusomes, secreted contents were well labeled. Extrusomes labeled with gold particles were rarely observed, which was possibly due to the fact that extrusomes are seldom extruded under normal conditions. Thus, the results of immunofluorescence microscopy showed that the antibody could bind to gp40, but due to its lager size, colloidal gold particles were not able to access into the interior of the extrusomes. Permeabilization of the plasma membranes with Triton X-100 was performed and smaller colloidal golds (5 nm) were used, which did not prove to be successful. In post-embedding immunoelectron microscopy, no gold-labeling was detected to the extrusomes (data not shown), suggesting that the antigen of gp40 might have been masked or denatured during the process of fixation and embedding. Therefore, techniques for antigen retrieval were employed for unmasking antigen, but again, it did not affect the antigenicity of gp40.

cDNA cloning and sequencing

Two cDNA sequences were acquired using a primer designed from the internal amino acid sequence, both of which contained an open reading frame encoding 372 amino acid residues (DDBJ Accession Number AB368718 and AB368719). Deduced amino acid sequences of the two cDNA contained N-terminal and internal amino acid sequences that correspond to sequences obtained from gp40 protein (Fig. 4-4). Therefore, it was concluded that these cDNA encode gp40, and these sequences are tentatively called gp40-1 and gp40-2. Two amino acid sequences share 90% identity, and most of the variations are in N-terminal region. Neither have predicted transmembrane region, but both have a signal peptide in their N-terminus. It is consistent with that gp40 is deduced to be a secretory protein (Sakaguchi et al. 2001). In comparison with the results of amino acid sequencing, the deduced amino acid sequences have extra 21 residues following to signal peptide. The peptide may be a propeptide, and, if it is the case, the calculated molecular mass of the mature protein is 37.9 kDa, which is consistent with the result of deglycosylation. Residues 75-78 were potential N-glycosylation sites, suggesting glycosylation of the Con A-binding sugar chain. Pfam analysis revealed that residues 123-296 were Glycosyl hydrolases family 16 domain (PF00722, hereafter referred to as glucanase domain), and PredictProtein indicated that residues 193-204 were its active sites (PS01034). For confirmation of the amino acid sequences, immunoblotting using the antibody against internal amino acid sequence was performed, and the antibody

was reacted with gp40 (data not shown).

NCBI Blast results showed significant similarity of gp40 to invertebrate β-1,3-glucan-recognition proteins (GRPs) and its homologs, which contain the glucanase domain. The highest homology was shown to sponge *Suberites domuncula* GRP, with a score of 353 bits (Perović-Ottstadt et al. 2004). Lower homology was shown to proteins of insects such as Gram-negative bacteria-binding proteins (GNBPs) in *Drosophila melanogaster*, with 100 bits (Kim et al. 2000), except GNBP in the mosquito *Anopheles gambiae*, with 300 bits (Dimopoulos et al. 1997). In protozoa, there was no study in GRP-like proteins, and only predicted proteins were found in genomes of protozoa, such as *Dictyostelium discoideum*, *Tetrahymena thermophila*, and *Thalassiosira pseudonana*, although the homology score were less than 100 bits. In TBestDB, several homologous EST sequences were found in *Trimastix pyriformis*, *Histiona aroides*, *Malawimonas jakobiformis*, which were grouped as Excavata (Simpson 2003). The score for these sequences are relatively high as 323 bits for *T. pyriformis*.

The deduced amino acid sequences of gp40-1 and homologous proteins were aligned (Fig. 4-5). The glucanase active sites were conserved between gp40 and homologs other than that of *D. melanogaster*. In addition, a putative polysaccharide-binding motif was also conserved (Dimopoulos et al. 1997, Lee et al. 1996), although another putative biding motif was not conserved (Lee et al. 1996, 2000).

Glucan binding and enzyme assays

After incubation with insoluble β-1,3-glucan curdlan, proteins were subjected to SDS-PAGE (Fig. 4-6). It showed that 40-kDa proteins bound to curdlan, which was confirmed to be gp40 by immunoblotting (data not shown). The binding was inhibited by the addition of laminarin, on the other hand not inhibited by LPS (Fig. 4-6). Methyl-cellulose, mannan, mannoside and glucose also did not inhibit the binding (data not shown). gp40 also bound to zymosan, which contained β-1,3-glucan, but not to cellulose, β-1,4-glucan. In the enzyme assay, no glucanase activity could be detected when gp40 was incubated with Laminarin Azure (data not shown). These results suggest that gp40 is capable of specifically binding to β-1,3-glucan, though might not have a glucanase activity.

Protein A-binding experiments

During the course of immunoelectron microscopy, sections were stained with only protein A gold as a negative control experiment. Intriguingly, it was observed that fibrous structures that were apparently discharged from extrusomes were labeled with gold colloids (Fig. 4-7). In addition, contents of mottled extrusomes were also labeled. On the other hand, condensed extrusomes were not labeled. In the case of incubation with preimmune IgG, the number of gold labeling was significantly decreased. Moreover,

there were no binding of anti-Mouse and–Rabbit IgG golds (data not shown).

In the immunoprecipitation, approximately 50-kDa protein band was detected (Fig. 4-8). The 50-kDa band was disappeared when *A. sol* proteins were incubated together with *Chlorogonium* proteins.

Discussion

For invertebrate animals, the innate immunity plays a crucial role in their defense mechanisms because they lack adaptive immunity unlike vertebrates. Various innate immune components have been developed in invertebrates, which detect and respond to pathogens (Iwanaga and Lee 2005). They discriminate nonself pathogens from themselves by pattern recognition receptors (PRRs). GRPs are known as the members of PRRs, and they recognize β-1,3-glucan, a major component of fungal cell wall (Brown and Gordon 2005). GRPs that contain the glucanase domain have been discovered in major invertebrate groups, such as sponge, echinoderm, annelid, mollusc and arthropod (Perović-Ottstadt et al. 2004, Lee et al. 2000, Bachman and McClay 1996, Beschin et al. 1998, Kozhemyako et al. 2004). Some GRPs also bind to LPS and recognize the outer membrane of Gram-negative bacteria, and so these proteins are called as GNBPs or LPS- and β-1,3-glucan-binding proteins, LBGPs (Kim et al. 2000, Lee et al. 2000). Many resulting immune reactions by GRPs have been reported, such as opsonization (Thörnqvist et al. 1994, Cerenius et al. 1994), coagulation (Seki et al. 1994), and the activation of the prophenoloxidase cascade (Wang and Jiang 2006).

In this chapter, I report cDNA cloning of gp40 that is a putative prey recognition and adhesion protein, and found its homology to GRPs of invertebrates. Interestingly, gp40 shows higher homology to invertebrate GRPs than that of protozoa, except for excavata, although *A. sol* is now classified in stramenopiles and phylogenetically distant from metazoa (Nikolaev et al. 2004). Thus, it seems likely that gp40 retains ancestral character of GRPs. I found two paralogous gp40 like as GNBPs in *Drosophila melanogaster* (Kim et al. 2000). I have no knowledge of the functional difference between two gp40, if any. However, N-terminal differences of amino acid sequences might be involved in functions such as cellular localization of the proteins.

The glucanase domains of GRPs are considered to be derived from β-1,3-glucanase in bacteria (Yahata et al. 1990). Among GRPs, several proteins have been reported to have glucanase activity (Bachman and McClay 1996, Kozhemyako et al. 2004, Kovalchuk et al. 2006), however, other GRPs were considered not to have enzyme activity (Beschin et al. 1998, Cerenius et al. 1994). Notably, it has been known that residues in the glucanase active sites of GRPs in insects, except *Anopheles*, were replaced as shown in Fig. 4-5, and so they lack the enzyme activity (Kim et al. 2000, Dimopoulos et al. 1997, Lee et al. 1996, Ma and Kanost 2000). In the case of protozoa, a diatom *T. pseudonana* is known to

have β-1,3-glucan storage product, called chrysolaminarin or leucosin (Round and Crawford 1990). Therefore, glucanase-like protein of *T. pseudonana* is possibly used for metabolism of the glucan. Such a storage vacuole has not been reported in *A. sol* so far. Together with the result of the glucanase assay here, gp40 is unlikely to be glucanase.

Taking into consideration the functions of GRPs in invertebrate innate immunity, my results imply that gp40 bind to β-1,3-glucan of prey organisms. However, I could not detect β-1,3-glucan in prey *Chlorogonium* and *A. sol* cells by immunoelectron microscopy using anti-β-1,3-glucan antibodies (data not shown). It possibly means that gp40 recognizes other cell surface glycoconjugates like GNBPs and LGBPs. Clearly, further research is required to clarify the molecular mechanism how gp40 recognizes prey cells.

From the results of immunomicroscopy, it was suggested that the extrusome probably contains gp40, though the anti-peptide antibody was not suitable for post-embedding immunoelectron microscopy. It is necessary that other antibodies or improved antigen retrieval techniques are to be used for revealing more detailed localization of gp40 by immunoelectron microscopy.

In the protein A-binding experiments, discharged fibrous materials and mottled extrusomes were labeled with protein A-gold. Until now, such fibrous structures that are secreted from extrusomes have not been observed clearly, although discharge of similar filamentous materials was reported previously (Sakaguchi et al. 2001, Hausmann and Patterson 1982). In actinophryid heliozoa, two types of extrusomes have been observed. In the heliozoon *Echinosphaerium nucleofilum*, mottled dense granules and condensed granules were reported (Suzaki et al. 1980). In *A. sol*, Hausmann and Patterson (1982) have reported that extrusomes converted from a compact condensed form to a large expanded, or dissipated, form when *A. sol* was feeding on prey. Taking the results of gold labeling into consideration, extrusomes probably convert to mottled, dissipated form followed by exocytosis of fibrous contents. Protein A-binding ability is possibly gained during conformational change of the extrusomes. Similar conformation-dependent protein A-binding was suggested in chicken immunoglobulin (Barkas and Watson 1979).

In protein A affinity chromatography, a 50-kDa protein band was detected. The protein is possibly identical with protein A gold-binding components of extrusomes. Moreover, disappearance of the 50-kDa band by the addition of *Chlorogonium* proteins suggests that the protein is responsible for the interaction between *A. sol* and *Chlorogonium*. Taken together with the results of gold labeling and the role of extrusomes, the protein may be involved in prey capture.

I will try to identify the molecule on the prey surface that is interacted with gp40. It would help to understand the interaction between predatory protozoa and prey.

Chapter 5

General Discussion

Until today, few molecular data were reported for *Actinophrys sol*, that is, only a partial sequence of actin gene has been known (Nikolaev et al. 2004). In Chapter 2, I described about the sedolisin-like protein of *A. sol*. The cDNA sequence of the lysosomal protein, which I determined here, was the first report of a complete cDNA sequence of *A. sol*. In this study, the protein was characterized as a lysosomal peptidase of the sedolisin family. Furthermore, it seemed to be related to the CLN2 proteins rather than other sedolisins. Sedolisins were found in limited species. In particular, *D. melanogaster*, *C. elegans*, *S. cerevisiae* and *E. coli* apparently lack sedolisin-like gene from their genomes, thus sedolisins are not conserved proteins. Meanwhile, CLN2-like enzymes are present not only in mammals but also in other vertebrates, such as fishes and amphibians (Wlodawer et al. 2003b). Moreover, physiological roles of CLN2 proteins have been studied well. In contrast, physiological roles of sedolisins, except CLN2 proteins, are still unclear. Therefore, studying the lysosomal peptidase of *A. sol* will lead to understand physiological roles of sedolisins. In addition, it was observed that extrusomes were consumed even during digestion of prey organism (Patterson and Hausmann 1981). Taking into consideration the fact that prey digestion occasionally begins before completion of the phagosome formation, it is implied that some contents of extrusome could play a role of digestive enzyme. Thus, by using gp40 as a marker together with the lysosomal protein, it would become possible to analyze feeding pathway of *A. sol* in more detail.

Many predatory protozoa, including *A. sol*, feed on other protozoa. It is necessary for such protozoa to prevent them from feeding their own clones, although some species are known to cannibalize when they are at risk of starvation. It has been reported that *Amoeba proteus* inhibited a mutual consumption by using a surface protein, which was called A-factor (Kusch 1999). It means that A-factor has a self-recognition function. It is also important for other predators to discriminate prey organisms from themselves, so all the predatory protozoa should have abilities of self-nonself discrimination.

The self-nonself discrimination is also important in the metazoan immune systems for defending them against infection. The immune systems, including adaptive and innate immunity, play an important role in both recognition and elimination of invaded microorganisms. In innate immunity, the complement system involves a specific response performed by pattern recognition, in which receptors recognize pathogen-associated molecular patterns (PAMPs). It contains three pathways, namely classical, alternative and lectin pathway (Endo et al. 2006, Fujita et al. 2004a, b). Recognition of PAMPs by receptors triggers various immune responses, such as the opsonization and agglutination. The accumulated evidences indicate that the lectin pathway is the origin of the complement systems. In the lectin pathway, pattern

recognition is performed by lectins, that is, mannose-binding lectins or ficolins. These complement systems have been found in both vertebrates and invertebrates, but not in protostomes. Arthropods, however, have the nonself recognition system, such as the Toll pathway and the Imd pathway in the fruit fly *Drosophila* using as the host defense mechanism. More recently, a homologue of vertebrate C3, which is a main component of the complement system, and other components, which include lectins, were found in horseshoe crab (Zhu et al. 2004). Furthermore, PAMPs induced protease activation in the horseshoe crab plasma was examined. It strongly suggests that the complement systems originate in the ancestor of deuterostomes and protostomes.

The self-nonself recognition system and lectins were even found in Porifera (sponge), the evolutionarily earliest metazoa. Sponges have species-specific proteoglycans, which were named glyconectins, and isolated glyconectins displayed Ca^{2+}-dependent self-aggregation (Popescu and Misevic 1997). The carbohydrate-carbohydrate interaction was considered to be essential for self-assembly of dissociated single cells and the emergence of multicellularity. Additionally, sponges have an antibacterial lectin that recognizes bacterial polysaccharide and is involved in the defense system of sponge (Schroder et al. 2003).

In contrast, the role of plant lectins are still unclear, despite the first lectin was found in plant. It is, however, believed that the main function of plant lectins is involved in the plant defense system (Chrispeels and Raikhel 1991, Peumans and Van Damme 1995). Various defense responses of plants are initiated by pathogen-produced signals that are called elicitors or effectors. Although some of elicitors are specific, general elicitors are non-specific and conserved components of pathogens (Gomez-Gomez 2004). Receptors of such elicitors include oligosaccharide-binding proteins, so-called lectins (Ebel 1998). It seems that elicitors correspond to PAMPs in the metazoan immune systems. Whereas in seaweed, or Chromista, there are few studies about the defense system. Recently, it has been reported that a lectin-like protein and antimicrobial defense are present in brown algae (Nomura et al. 2000, Kubanek et al. 2003). Although it has been poorly understood about the defense system in Chromista, most multicellular organisms have some kinds of defense systems and self-nonself recognition systems (Fig. 5-1).

In the transition from unicellular protozoa to multicellular metazoa, signaling and adhesion proteins should play important roles, and so these proteins are considered to have evolved in protozoa (King 2004). And the mechanism of nonself recognition in metazoan innate immunity is also inferred to originate at the stage of unicellular organisms (Mushegian and Medzhitov 2001, Danilova 2006). Indeed, in the choanoflagellate, which is a protozoa closely related to metazoa, cell signaling and adhesion proteins that are involved in metazoan cell interactions, such as tyrosine kinases and Ca^{2+}-dependent lectins, have been discovered (King et al. 2003). Furthermore, some candidate proteins are present in the

protozoa. For instance, an amoeboid protozoan *Acanthamoeba castellanii* have a mannose receptor, which have a role for initiating phagocytosis like receptors of the mammalian immune system (Allen and Dawidowicz 1990), and *A. proteus* have self-recognition factor as described above. Only recently, it was revealed that the dinoflagellate recognized prey by Ca^{2+}-dependent mannose-binding lectins, although its evolutionary link to metazoan lectins is not yet known (Wootton et al. 2007). Additionally, it was reported that a Toll/interleukin-1 receptor domain protein was required for feeding on bacteria and for defense against pathogens in the social amoeba *Dictyostelium discoideum* (Chen et al. 2007).

It has been inferred that gp40 is one of the candidates for the link between protozoan and metazoan non-self recognition systems (Sakaguchi et al. 2001, King 2004). As shown in Chapter 4, gp40 is a major Con A-binding glycoprotein in *A. sol*. Sakaguchi et al. (2001) reported that gp40 had multiple functions, in which prey adhesion and induction of phagocytosis seemed to be related with phagocytosis by macrophages in the immune system. As described above, carbohydrates and its receptors play an important role in the self-nonself recognition system. So Con A-binding polysaccharide of gp40 may be responsible for prey recognition and other functions, such as colony formation and self-induced exocytosis of extrusome. All functions of gp40 are analogous to the innate immune system, and especially prey recognition is similar to self-nonself recognition that is the most basic function in the immune system. Therefore, it is implied that prey recognition in *A. sol* might be the ancient origin of self-nonself recognition in the metazoan immune systems.

Results shown here are consistent with the anticipation. As described in Chapter 3, Con A-binding glycoconjugates are possibly implicated in prey recognition and induction of exocytosis of extrusomes. In Chapter 4, gp40 was shown to be homologous to GRPs in invertebrate immune systems. GRPs were known to recognize nonself pathogen, and gp40 was considered to recognize prey cells. The analogy in functions and homology in molecular structures imply the evolutionally link between these proteins. As noted above, *A. sol* is phylogenetically distance from metazoa. The cDNA sequences of the sedolisin-like protein and gp40, however, have homology to proteins in metazoa rather than that in plant or stramenopile. It may indicate that *A. sol* does not have plastid and so retains phagocytic character, which is probably characteristic in common ancestor of eukaryotes. Furthermore, I identified a protein A-binding 50-kDa protein that is probably localized in extrusomes. *Staphylococcus* protein A is known to specifically bind to IgG. Although it has been known that protein A could bind to some other proteins in vertebrates (Sjobring et al. 1989, Vanamala et al. 2003), few protein A-biding proteins have been reported in invertebrates (Chiou et al. 2000, Yang et al. 1994). It suggests that the 50-kDa protein might be related to immunoglobulin or immunoglobulin-like proteins. The discovery of immune-related proteins would provide a clue to clarify the evolutional path from protozoan prey capture to metazoan innate immunity.

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Tables

Table I. Immunofluorescent labeling of phagosomes of *Actinophrys sol*. Thirty cells were randomly chosen, and number of labeled vesicles in each cell were counted.

(min)	0	30	60	90
Labeled vesicles				
Mean (SEM*)	0 (0)	0 (0)	0.5 (0.1)	1.4 (0.2)

* SEM means standard error of the mean.

Table II. Postembedding immunogold labeling in phagosomes and other vesicles of *Actinophrys sol*. Fifteen phagosomes and control vesicles were randomly chosen from cells fixed at 30 min or 90 min after feeding, and labeling gold particles were counted.

	30 min		90 min	
	phagosome	control	phagosome	control
Gold particles				
Mean (SEM*)	6.7 (1.2)	2.2 (0.5)	21.3 (2.5)	3.3 (0.4)

*SEM means standard error of the mean.

Figures and Legends

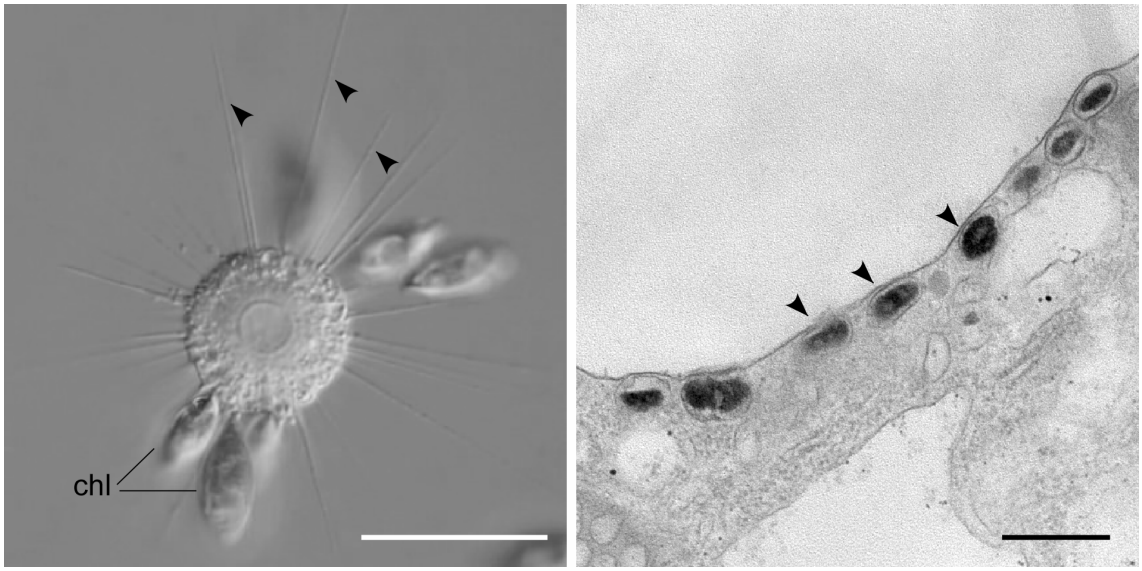

Fig. 1-1. (left) *Actinophrys sol* captures prey *Chlorogonium* sp. (chl) with its axopodia (arrowheads). Bar = 50 μm. Fig. 1-2. (right) Extrusomes (arrowheads) are present under the cell membrane of *A. sol*. Bar = 500 nm.

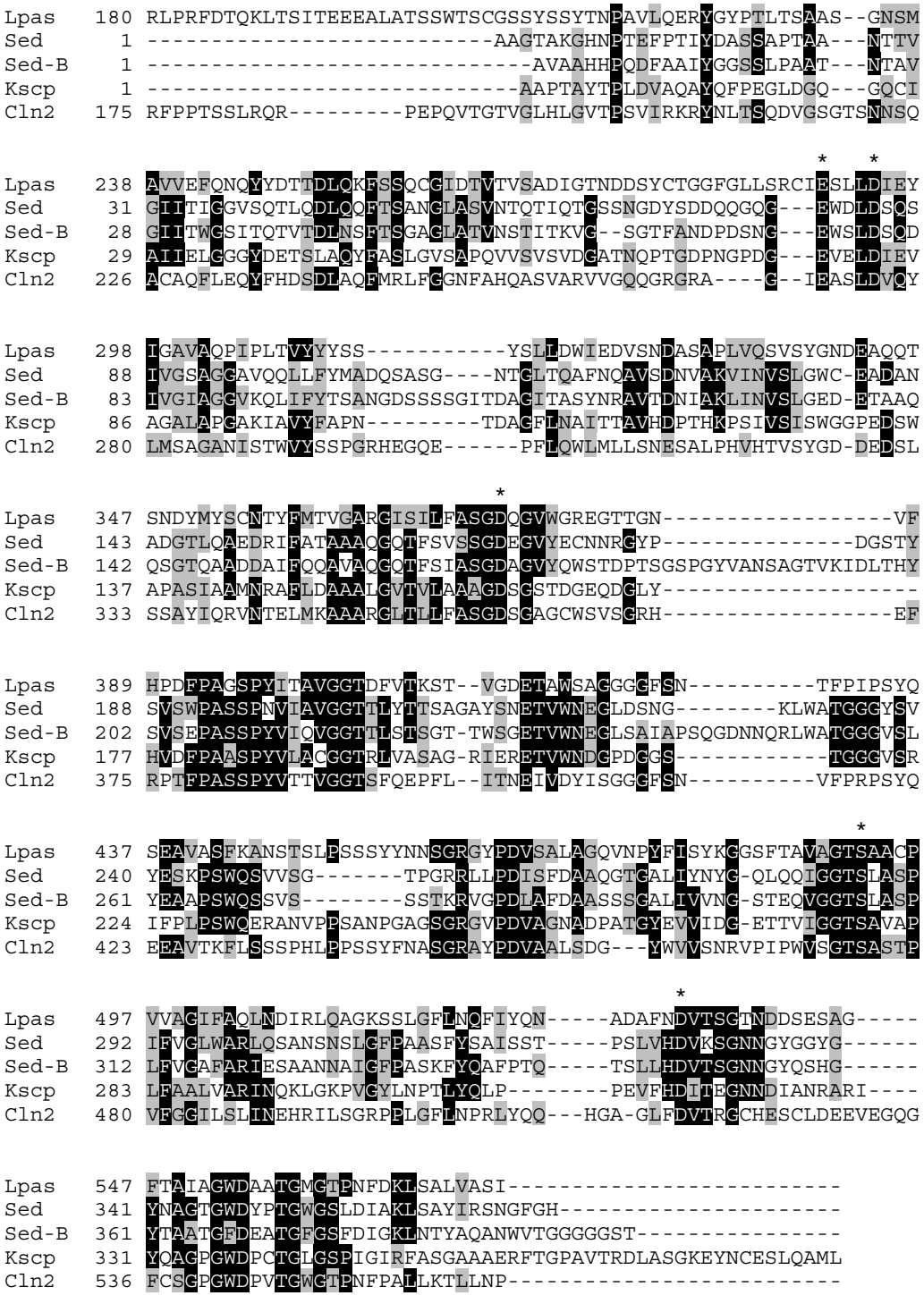

Fig. 2-1. Comparison of the amino acid sequences of sedolisins. Lpas: lysosomal protein of *Actinophrys sol*, Sed: Sedolisin (P42790), Sed-B: Sedolisin B (Q60106), Kscp: kumamolysin (BAB85637), Cln2: CLN2 protein or TPPI (AAQ88866), asterisks: catalytic sites. Heavy and light shadings indicate identical and similar amino acid residues, respectively.

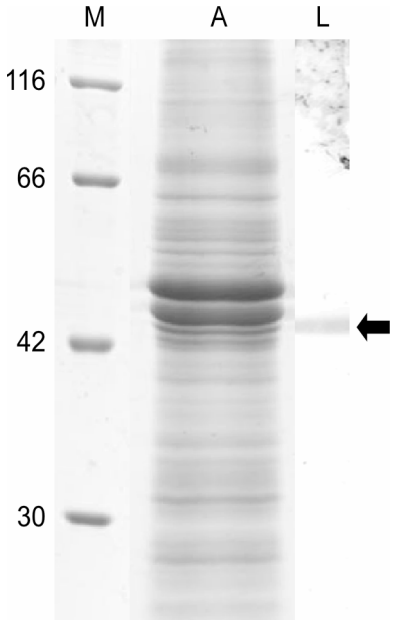

Fig. 2-2. SDS-PAGE and Western blot analysis of the sedolisin-like protein in *Actinophrys sol*. M: Molecular weight standards stained with CBB. Molecular sizes (kDa) are shown at the left side. A: *A. sol* proteins stained with CBB. L: *A. sol* proteins stained with HRP and anti-lysosomal protein antibody, showing only a 48-kDa protein (arrow).

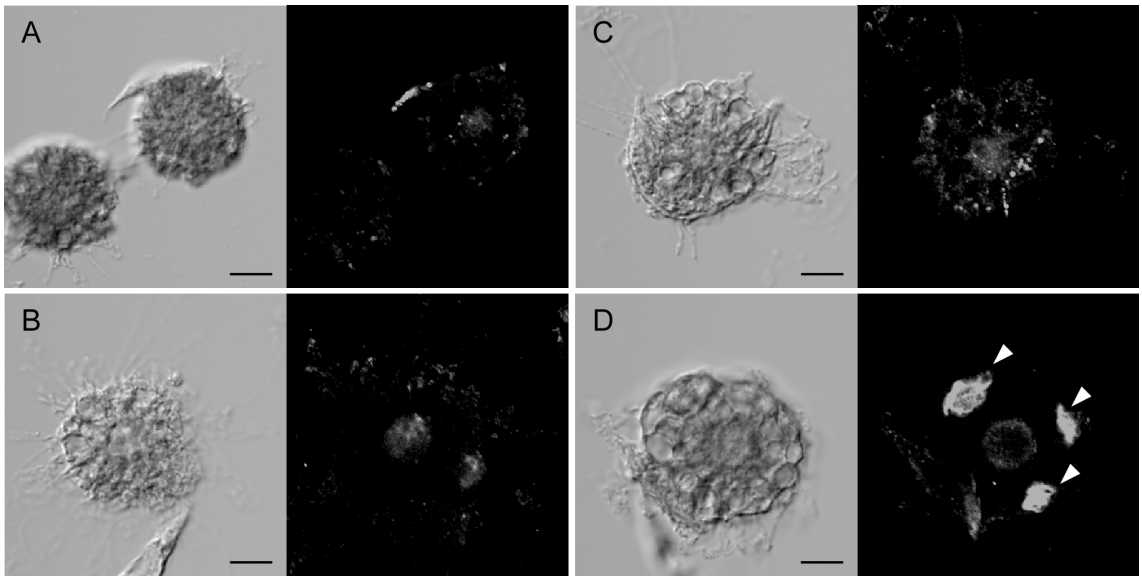

Fig. 2-3 Immunofluorescence localization of the sedolisin-like protein in *Actinophrys sol*. Cells were fixed and stained after initiation of feeding at 0 min (A), 30 min (B), 60 min (C) and 90 min (D). Phagosomes (arrowheads) were stained only at 90 min. The weakly labeled areas located outside the perimeter of the spherical cell body (seen especially in B) correspond to tangled-up axopodia, in which lysosomes may also be present in the cytoplasm. Observation was with differential interference contrast and immunofluorescence microscopy. Bars = 10 μm.

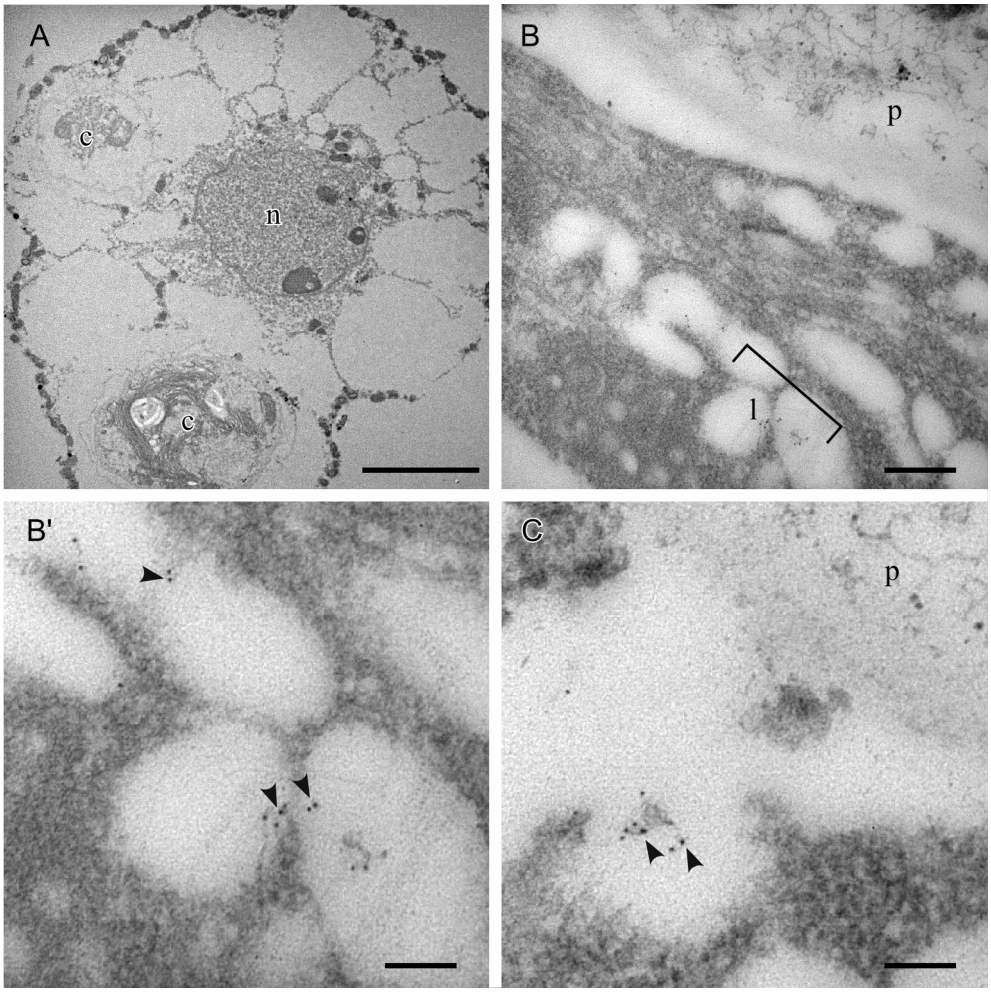

Fig. 2-4. Immunoelectron microscopy. (A) *A. sol* with digested prey organisms in the phagosome. n: nucleus, c: *Chlorogonium* sp. Bar = 5 µm. (B) A phagosome (p) surrounded by small vesicles (l: lysosomes). Bar = 250 nm. (B') An enlarged lysosome from the area marked by the bracket in (B). Colloidal gold particles (arrowheads) are bound to the contents of lysosomes. Bar = 100 nm. (C) Gold-labeled contents are secreted into a phagosome. Bar = 100 nm.

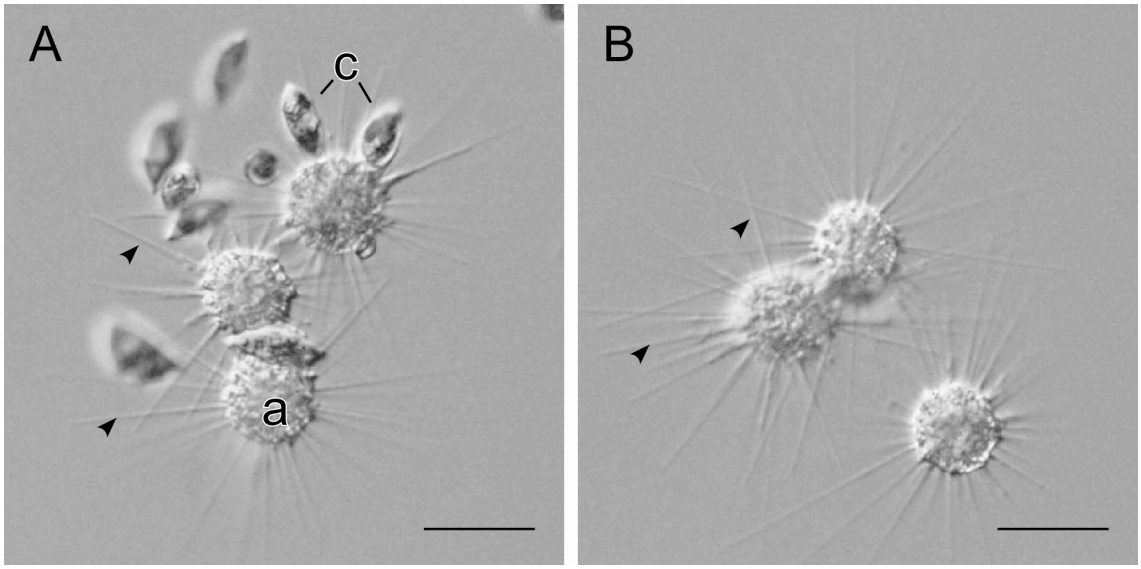

Fig. 3-1. Prey capture by *Actinophrys sol*. (A) In solution containing 1 mM Ca^{2+}, *A. sol* (a) captured prey organisms *Chlorogonium* (c) with its axopodia (arrowheads). (B) In a Ca^{2+}-free solution, *A. sol* did not capture prey. Bars = 50 μm.

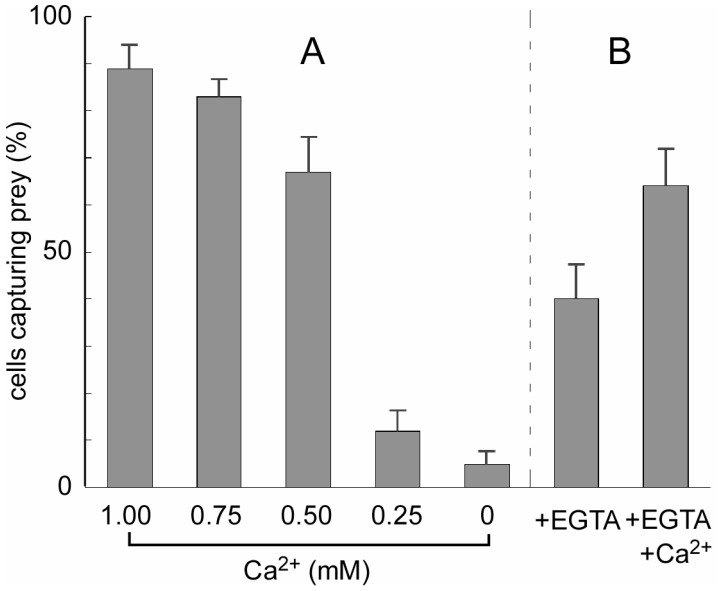

Fig. 3-2. Prey capture was dependent on extracellular Ca^{2+} concentration. (A) Prey cells were supplied to *A. sol* in solutions containing Ca^{2+} at concentrations from 0 to 1 mM. The percentages of cells that captured prey were determined. Prey capture decreased at lower Ca^{2+} concentrations. When the concentration was lower than 0.25 mM, capture was strongly inhibited (n=5). (B) In the presence of EGTA, prey capture was inhibited, although not completely. The addition of Ca^{2+} partially removed the inhibition (n=5). Theoretical Ca^{2+} concentrations were 0 and 1 mM, respectively. Error bars are S.E.M. n indicates number of experiments.

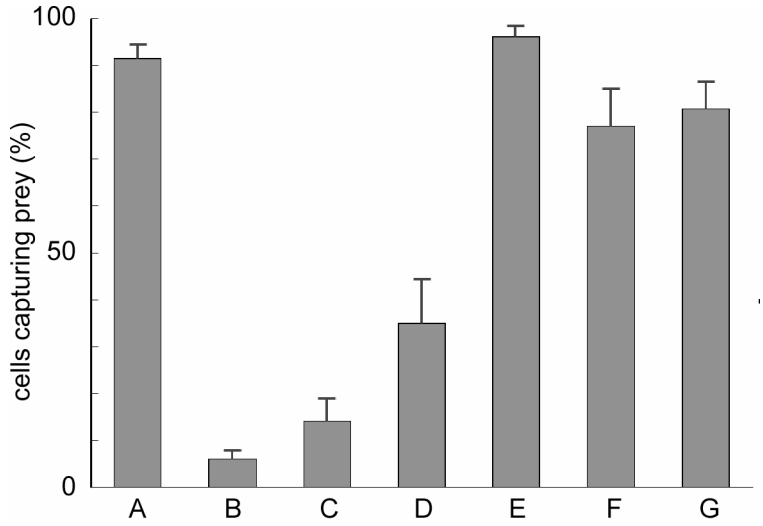

Fig. 3-3. Effect of Ca^{2+} channel blockers on prey capture. Heliozoons were incubated with Ca^{2+} channel blockers and the percentages of cells that captured prey were determined. (A) Control, *n*=10. (B) 50 μM verapamil, *n*=5. (C) 100 μM nifedipine, *n*=5. (D) 40 μM $LaCl_3$, *n*=5. (E) 100 μM $NiCl_2$, *n*=5. (F) 2 μM ruthenium red, *n*=5. (G) 200 nM w-conotoxin GVIA, *n*=6. $LaCl_3$ and the L-type Ca^{2+} channel blockers, verapamil and nifedipine (D, B, C), inhibited capture. Other drugs did not cause significant inhibition. Error bars are S.E.M. *n* indicates number of experiments.

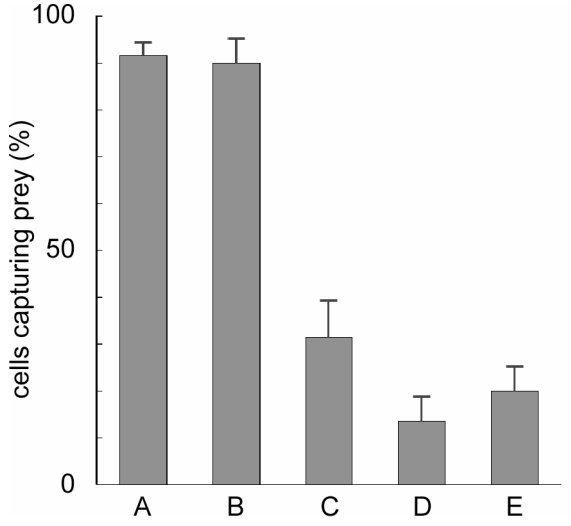

Fig. 3-4. Effect of various drugs on prey capture. Heliozoons were incubated with drugs and the percentages of cells that captured prey were determined. (A) Control, *n*=10. (B) 20 μM W-5, *n*=5. (C) 20 μM W-7, *n*=7. (D) 2 μM A-23187, *n*=7. (E) 10 mM chloral hydrate, *n*=11. The calmodulin antagonist W-7 inhibited prey capture, while its less efficient analog W-5 did not. The calcium ionophore A-23187 and chloral hydrate also inhibited prey capture. Error bars are S.E.M. *n* indicates number of experiments.

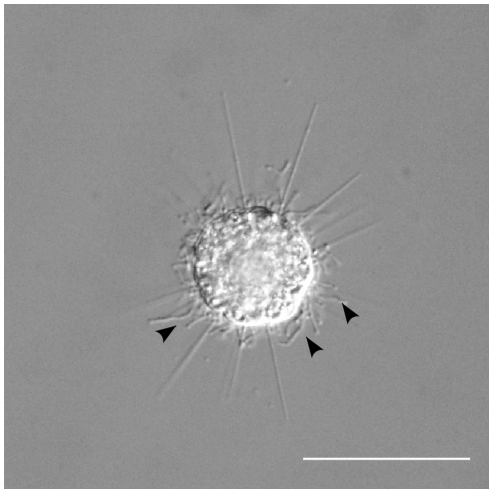

Fig. 3-5. Effect of long time incubation in chloral hydrate solution. After incubation for 30 minutes, *A. sol* cells were appeared to be damaged. The cell surface of *A. sol* was disturbed (arrowheads) although axopodia were not diminished. The effect was not observed within five minutes. Bar = 50 μm.

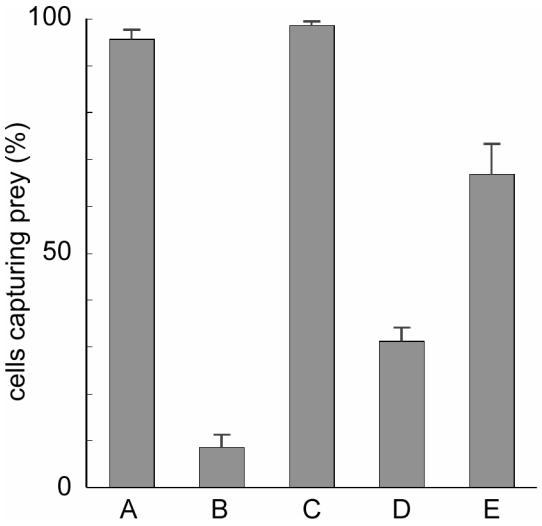

Fig. 3-6. Effect of concanavalin A on prey capture. Control (A, *n*=7). In the presence of 20 μg/ml Con A, prey capture was inhibited (B, *n*=7). Addition of 20 mM mannoside relieved the inhibition completely (C, *n*=7). When *Actinophrys sol* cells were pretreated with 20 μg/ml Con A, prey capture was inhibited (D, *n*=8), whereas the pretreatment of prey cells did not cause strong inhibition (E, *n*=8). Error bars are S.E.M. *n* indicates number of experiments.

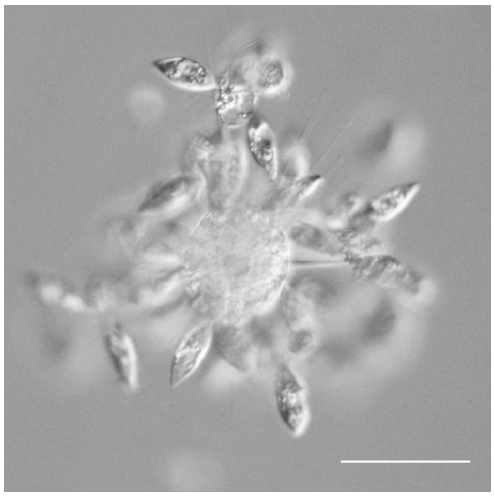

Fig. 3-7. Effect of Concanavalin A with EGTA. A lot of prey cells adhered to the cell surface of *A. sol*. The adherence was not observed without Ca^{2+} in the medium. Bar = 50 μm.

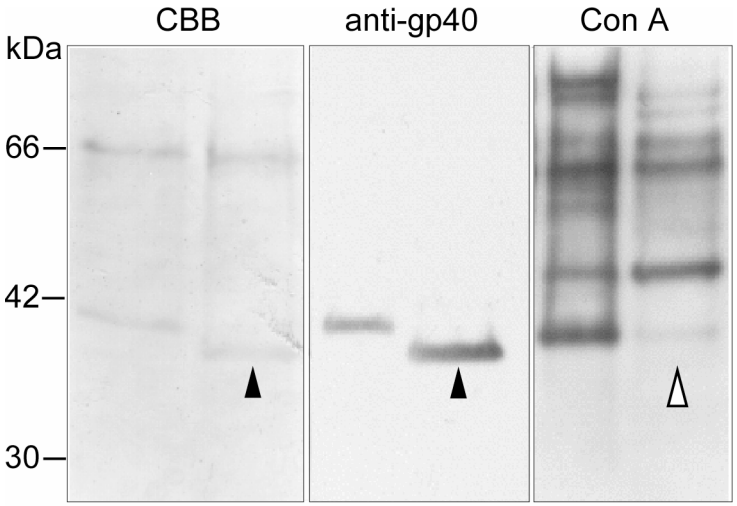

Fig. 4-1. SDS-PAGE and immunoblotting analyses of deglycosylated gp40. Control sample (left lane) and deglycosylated Con A-binding proteins (right lane) were subjected to SDS-PAGE. Proteins were stained with CBB (left column). Blotted proteins were detected with the anti-gp40 antibody (middle) or Con A-HRP (right). After deglycosylation, a 38-kDa band appeared (solid arrowhead), which was not stained with Con A-HRP (open arrowhead). Positions of the molecular size markers are shown on the left side.

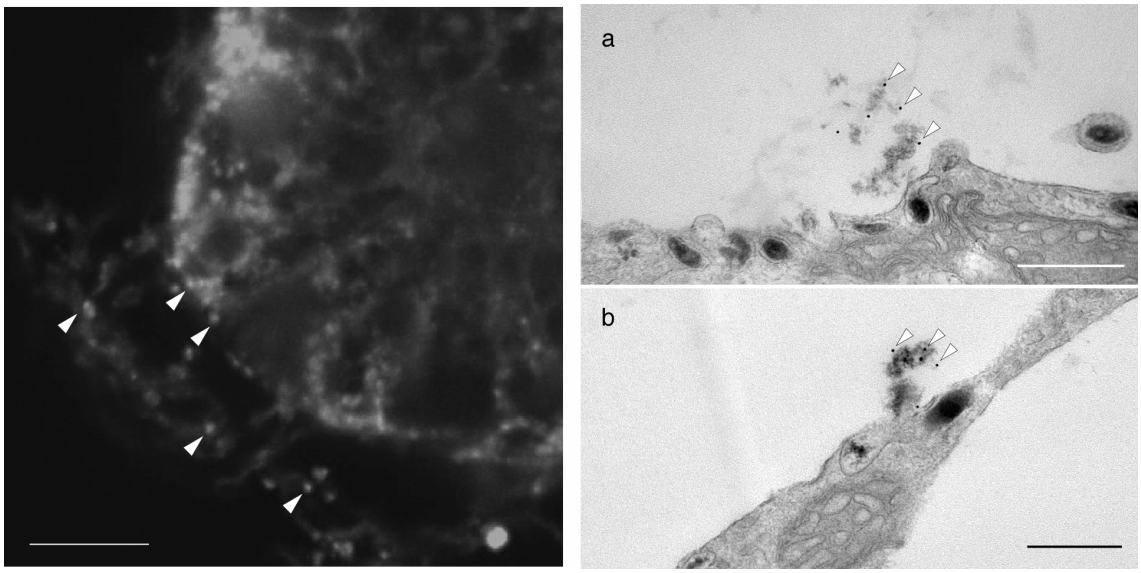

Fig. 4-2. (left) Immunofluorescence microscopy. *A. sol* cells stained with the anti-gp40 antibody and Alexa-488. A lot of particles were labeled, which were located at the periphery of the cell body and axopodia (arrowheads). Bar = 10 μm. Fig. 4-3. (right) Immunoelectron microscopy. Both photographs were taken at the peripheral region of the cell body of *A. sol*. Gold particles (arrowheads) were bound to secreted contents of extrusomes. Bars = 500 nm.

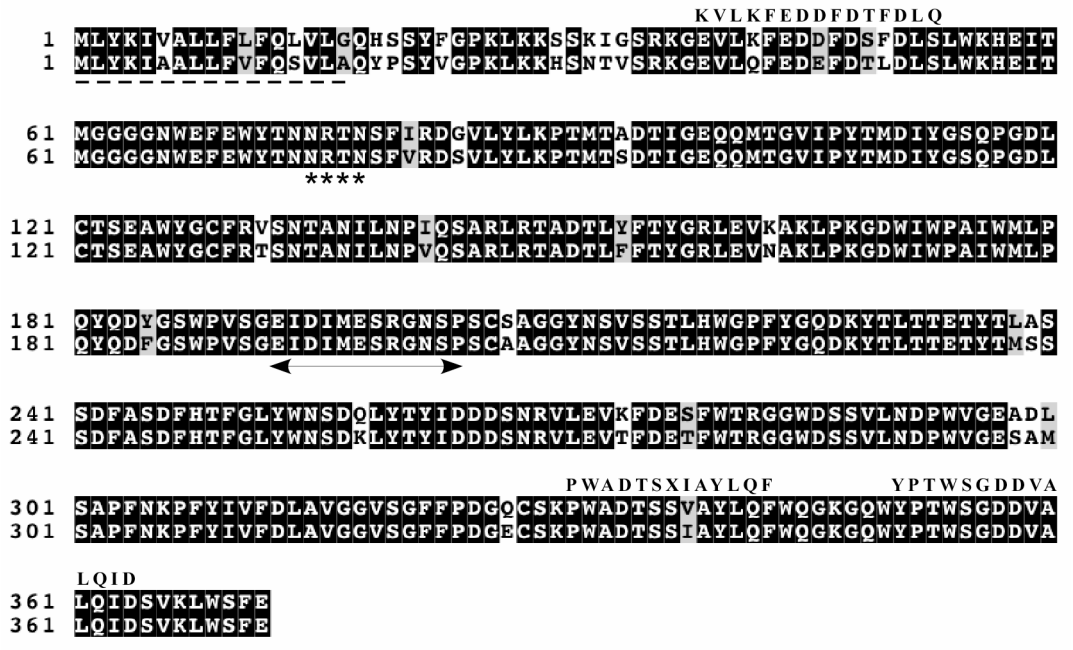

Fig. 4-4. Comparison of deduced amino acid sequences of two gp40, gp40-1 (upper) and gp40-2 (lower). Three partial amino acid sequences are shown upper side of the alignment. Heavy and light shadings indicate identical and similar amino acid residues, respectively. Asterisks indicate a potential N-glycosylation site. Dashed line represents predicted the signal peptide. The active site of glycosyl hydrolases family 16 is shown with a double-headed arrow.

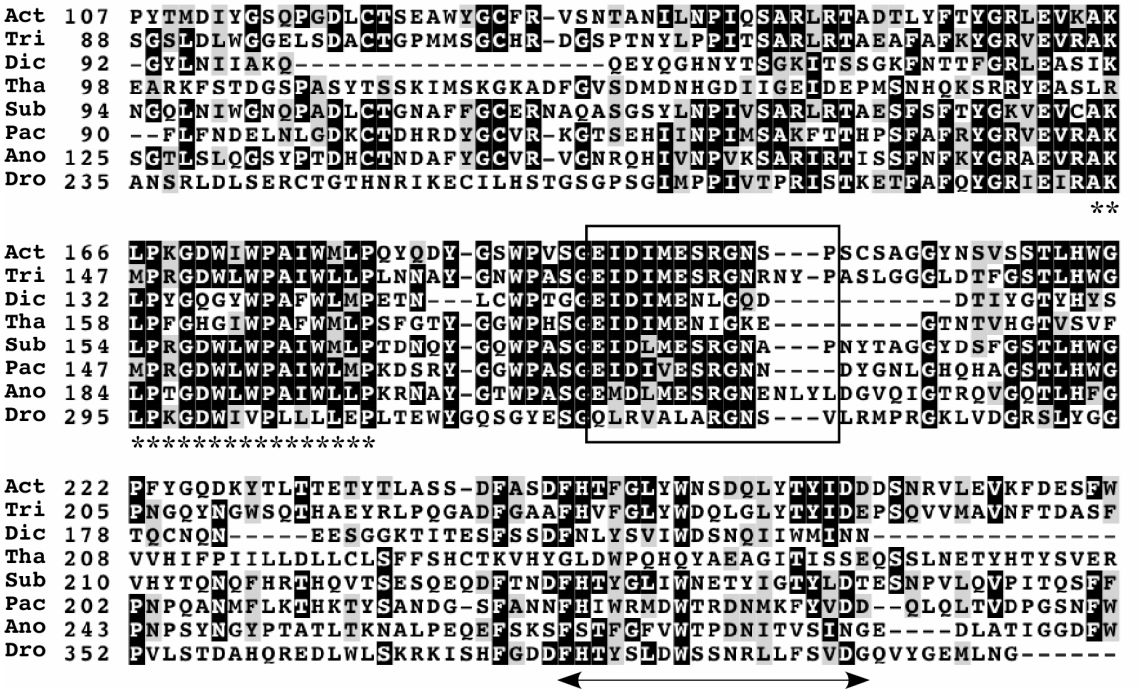

Fig. 4-5. Multiple alignment of amino acid sequences around glucanase active sites of gp40 and homologs. Heavy and light shadings indicate identical and similar amino acid residues, respectively. Active site residues are boxed. Two putative polysaccharide binding motif is shown with asterisks and a double-headed arrow. Act, *Actinophrys sol* gp40; Tri, *Trimastix pyriformis* predicted GRP; Dic, *Dictyostelium discoideum* predicted GRP; Tha, *Thalassiosira pseudonana* predicted GRP; Sub, *Suberites domuncula* GRP (CAE54585); Pac, *Pacifastacus leniusculus* LGBP (CAB65353); Ano, *Anopheles gambiae* GNBP (CAA04496); Dro, *Drosophila melanogaster* GNBP (AAF33849).

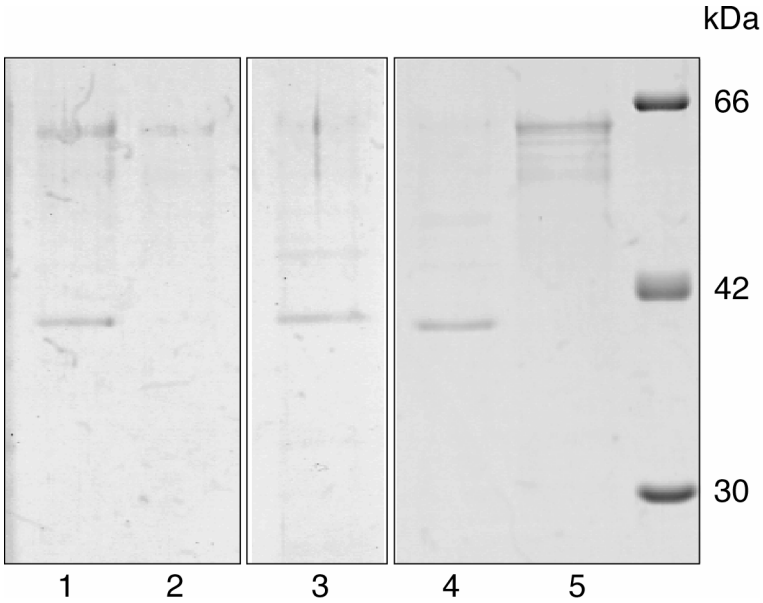

Fig. 4-6. Glucan-binding of gp40. After incubation with curdlan or other glucans, binding proteins were subjected to SDS-PAGE and stained with CBB. Incubation with curdlan only (lane 1); Curdlan and laminarin (lane 2); Zymosan (lane 3); Curdlan and LPS (lane 4); Cellulose (lane 5). Molecular size marker is shown right side.

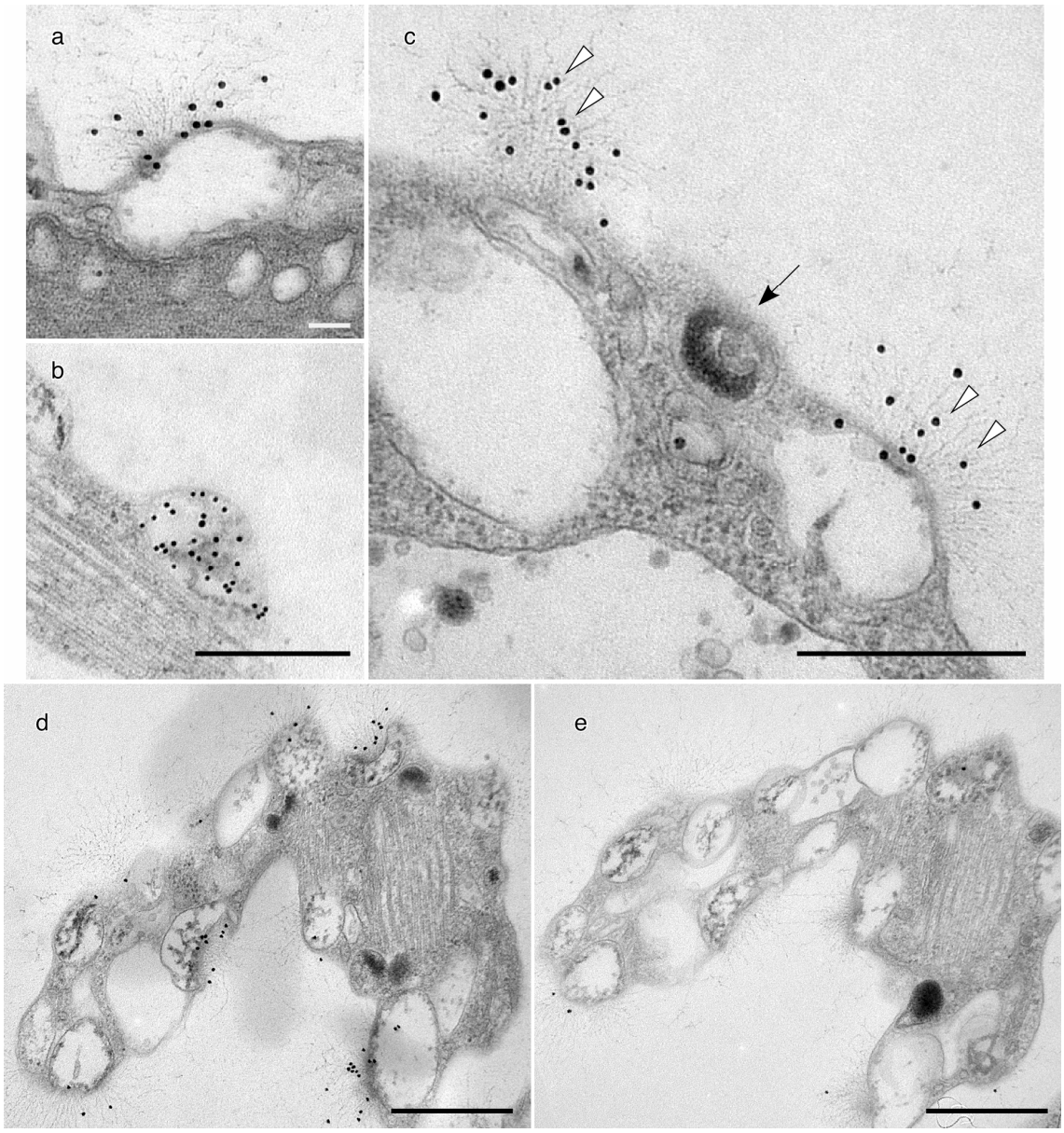

Fig. 4-7. (a) Protein A immunogold labeling. Fibrous structures that was discharged from an extrusome were labeled with gold-conjugated protein A. Bar = 100 nm. (b) Mottled extrusome was labeled. Bar = 500 nm. (c) Extrusive structures were labeled (arrowheads). While, a condensed extrusome was not labeled (arrow). Bar = 500 nm. (d) Incubated with protein A-gold, mottled extrusomes and fibrous structures were labeled. Bar = 500 nm. (e) Incubated with protein A-gold and preimmune IgG, the labeling was inhibited. Bar = 500 nm.

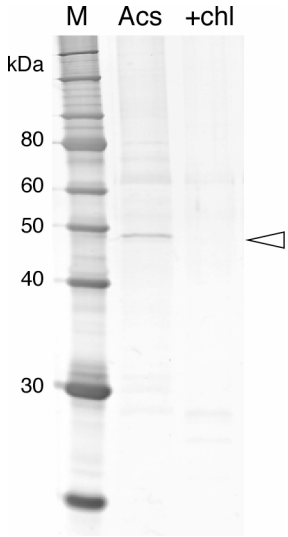

Fig. 4-8. Protein A affinity chromatography. In *A. sol* protein (Acs), a 50-kDa protein band was detected. After incubation with *Chlorogonium* proteins (+chl), the bands disappeared (arrowhead). Molecular size marker is shown on the left side.

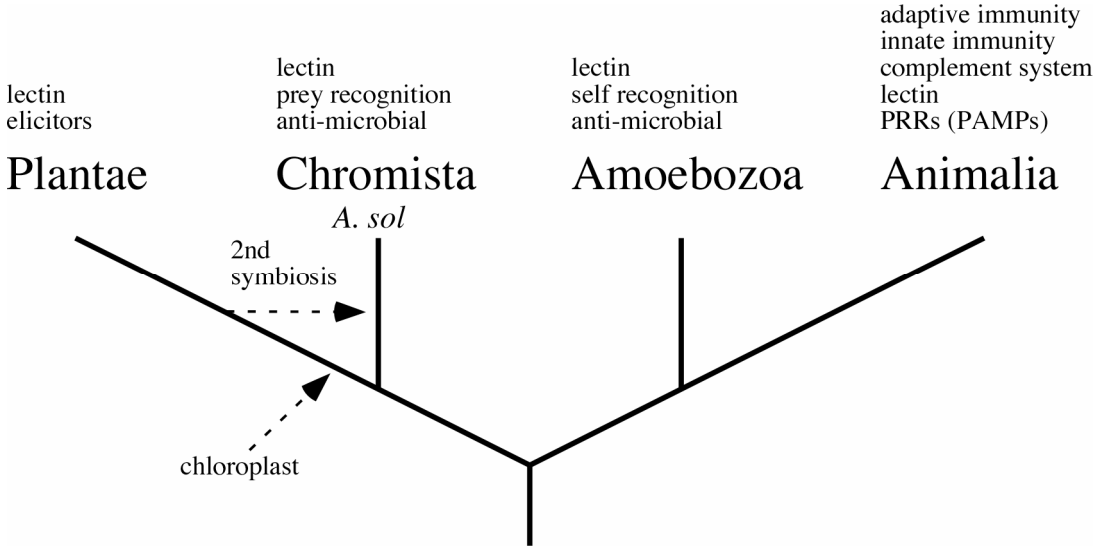

Fig. 5-1 The schematic view of eukaryotic phylogenetic tree, based on Cavalier-Smith (2003), with reference to defense systems and related self-recognition systems. Arrows mean deduced symbiotic events. Some kinds of immune systems or self-nonself recognition systems are present in the kingdom Animalia, Chromista (stramenopile), Plantae and phylum Amoebozoa.