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Participation of Glycoproteins on Zooxanthellal Cell Walls in the Establishment of a Symbiotic Relationship with the Sea Anemone, *Aiptasia pulchella*

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Ku-Lin Lin, Jih-Terng Wang, and Lee-Shing Fang (2000) Participation of glycoproteins on zooxanthellal cell walls in the establishment of a symbiotic relationship with the sea anemone, *Aiptasia pulchella*. *Zoological Studies* **39**(3): 172-178. Recent studies have revealed that glycoproteins on cell membranes are usually involved in the process of cell recognition. This study provides evidence to demonstrate the role of cell surface glycoproteins of zooxanthellae in establishing a symbiotic relationship between algae and a sea anemone host. When freshly isolated zooxanthellae from the sea anemone, *Aiptasia pulchella*, were incubated with trypsin, α-amylase, N-glycosidase F, or O-glycosidase, the algae could not infect bleached animals as efficiently as did the control algae. If the glycoproteins on the surface of the zooxanthellae were masked with lectins, the infection rate also declined significantly. Further, the glycoproteins on the cell walls of zooxanthellae were isolated and characterized with SDS-polyacrylamide gel electrophoresis and Western blotting. There are 10 protein bands in total revealed by Coomassie blue and silver staining, of which 5 are identified as glycoproteins. Two types of terminal sugar residues, mannose-mannose and galactose-β(1-4)-N-acetylglucosamine, were characterized among them. The most abundant glycoprotein with a molecular weight of 64 kDa carries a terminal sugar mannose. These data indicate that the glycoproteins on cell walls of zooxanthellae are a crucial factor in the successful establishment of the symbiotic relationship between zooxanthellae and *A. pulchella*.

Key words: *Symbiodinium*, Cnidarians, Endosymbiosis, Lectin, Recognition.

Many lower invertebrates harbor photosynthetically active unicellular algae in their cells. This symbiotic relationship is very peculiar because the host compartment, an animal cell, allows a plant cell of the zooxanthellae not only to live intracellularly, but also to have complicated physiological interactions. The intracellular symbiotic relationship between zooxanthellae and hosts has been subjected to studies of cell-cell recognition, interaction, and possible communication for many years (Trench 1979 1981, McAuley and Smith 1982, Markell and Trench 1993). The establishment of this relationship requires a certain degree of specificity for the host to select the proper symbiont. Although the mechanism is not yet quite clear, two possibilities are generally proposed. First, the recognition of a symbiont by the host occurs by the interaction of surface molecules between the symbionts and host (Roth 1973, Pool 1979, Meints and Pardy 1980). Second, the recognition process is established not at the first encounter of the host and symbiont, i.e., not derived from the recognition of the surface molecules, but at the process following phagocytosis (Jolley and Smith 1980, Trench 1981, McAuley and Smith 1982, Colley and Trench 1983). Despite several lines of evidence suggesting the existence of symbiont specificity in alga-invertebrate symbiosis, recent studies using 18S rRNA as a phylogenic marker revealed that a single coral host sometimes can harbor at least 3 different clades of *Symbiodinium* (Rowan and Knowlton 1995). Furthermore, symbiotic algae with different host origins can also infect heterogeneous hosts

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(Schoenberg and Trench 1980, Davy et al. 1997). It seems that, in alga-invertebrate symbiosis, the specificity of the host in selecting proper symbionts is not as restricted as generally understood. A certain degree of flexibility may exist.

It was found that the successful symbiosis between the alga *Chlorella* and the host, *Paramecium*, depended on lectin-binding ability of the algae (Weis 1978). However, these recognition sites on the algal surface change according to environmental conditions or interactions with the host (Pool 1979, Colley and Trench 1983). Meints and Pardy (1980) found that lectins, which bind with carbohydrate groups on the cell wall of green algae, decrease the rate of establishing symbiotic relationships between algae and a hydra. Reisser et al. (1982) used antibodies against algal cell wall, lectins, cellulase, pectinase, trypsin, and pepsin to treat *Chlorella* and found that the capability to form symbiotic relationships was greatly reduced. This information suggested that proper exposure and the integrity of surface molecules of symbionts are crucial for the establishment of a symbiotic relationship. However, no specific surface molecules have yet been identified. Recently, surface molecules with carbohydrate groups have been proven to be greatly involved in cell recognition (Varki 1994, Weis and Drickamer 1996, Gabius 1997). In this study, the involvement of cell wall glycoproteins in the establishment of a symbiotic relationship between zooxanthellae and a sea anemone host was examined. We first removed carbohydrate groups from the glycoproteins with deglycosylation enzymes to test whether the infectious capability of zooxanthellae to the sea anemone, *Aiptasia pulchella*, was affected. The infectious capability was further examined by masking these glycoproteins with several types of lectin. Glycoproteins on the cell wall of zooxanthellae were also isolated and characterized. These results indicate that the integrity of surface glycoproteins of zooxanthellae is importantly involved in successful establishment of the symbiotic relationship.

MATERIALS AND METHODS

The animals

Sea anemones (*Aiptasia pulchella*) bearing *Symbiodinium* sp. of clade-b, sensu Rowan and Powers (1991) (A. E. Douglas pers. comm.) were collected from an effluent water channel of a seawater aquaculture farm at Tong-kang, Taiwan (22° N). The animals were maintained in a seawater tank with a closed circulating filtration system. Illumination was provided by fluorescent tubes with a light-dark cycle of 12 h:12 h and an intensity of 80 μmol photons $m^{-2}s^{-1}$ at 25 °C.

The method of Steen and Muscatine (1987) was used to prepare bleached sea anemones. Sea anemones were incubated in aerated seawater at 4 °C for 4 h, then returned to room temperature (25 °C). After the sea anemones regained vitality, released zooxanthellae were removed by the frequent changing of filtered seawater (FSW). The procedure was repeated 5-10 times within a month, and the bleached animals were maintained in the dark for more than 6 mo before the experiment. The remaining zooxanthellae in the tentacles were examined by fluorescence microscope (BX-40F, Olympus; Tokyo, Japan) with excitation at 460-490 nm and emission at 515 nm before the infection test. Zooxanthellae display a bright red color under fluorescence due to the chlorophyll in the plastids. The animals were fed with 1-d-old *Artemia* nauplii (Ocean Star International, Ontario, Canada) every other day.

Preparation of freshly isolated zooxanthellae

Ten sea anemones (approximately 1 cm in oral disc diameter) were shaken with FSW in a 25-ml centrifuge tube to remove mucus. Then, the animals were homogenized with a polytron. The supernatant was decanted, and repeatly passed through several layers of gauze to get rid of nematocysts. The clean homogenate (20 ml) was supplemented with 100 μl of 10% dodecyl sodium sulfate (SDS) to make the final concentration equal to 0.05% SDS (w/v) (McAuley 1986). The solution was vortex mixed for 10 s, and centrifuged immediately at 500 g for 10 min to collect the algal precipitate. The precipitate was washed with FSW 5 times and centrifuged at 22 g for 5 min; the algal precipitate was collected between each wash. Finally, the algae were suspended in FSW, and the numbers of algae were counted in a hemocytometer. The extent of alga cells still enclosed inside the animal cell was estimated by staining the cells with coomassie blue and using light microscopy (x 400). A blue ring around the alga cell indicated that the cell was still enclosed inside the animal cell. Using this method, the animal cell contamination in the alga preparation was proven to be less than 5%.

Modification of surface proteins of isolated zooxanthellae

Glycoproteins on the cell wall of zooxanthellae

were modified by treatment with either deglycosylation enzymes or lectin masking before the infection test. Deglycosylation of the glycoproteins was achieved by incubation of the algae (1.5×10^5) in 50 μl phosphate buffer saline (50 mM potassium phosphate, 50 mM NaCl, pH 6.5) at 25 °C for 2 h, which was supplemented respectively with 5 mg/ml α-amylase (Sigma; St. Louise, MO), 1 U/ml N-glycosidase F (Boehringer Mannheim: Mannheim, Germany), 50×10^{-3} U/ml O-glycosidase (Boehringer Mannheim), or the mixture of the 2 glycosidases described above. A protein digestive enzyme, trypsin (10 U/ml), was also used to examine the contribution of protein moiety of the zooxanthellae cell wall to the capability of re-infection. During the incubation of zooxanthellae, the solution was gently vortex mixed every 20 min. After incubation, the zooxanthellae were washed with 400 μl FSW 5 times, and suspended in 50 μl FSW for further infection study.

For the masking experiment, several types of lectin, i.e., concanavalin A (Con A), *Limulus polyphemus* agglutinin (LPA), *Phaseolus vulgaris* erythroagglutinin (PHA-E), and *Triticum vulgaris* agglutinin (wheat germ, WGA), were used to mask the glycoproteins on the cell wall of zooxanthellae. The lectin supplements were prepared in a stock solution containing 1 mg/ml lectin in 50 mM phosphate buffer (pH 7.0). Lectin masking of glycoproteins was performed by adding 2 μl of lectin stock to 3×10^5 zooxanthellae in 20 μl and incubating at 25 °C for 2 h. After incubation, the zooxanthellae were washed 5 times with 1 ml FSW, and suspended in 50 μl FSW for further infection study.

Infection of zooxanthellae

Each group of the zooxanthellae treated with lectin or deglycosylation enzymes was mixed separately with homogenates of shrimp meat in FSW, and fed to the bleached animals through their oral disc. The algal density applied to bleached animals was adjusted to about 10^4 per 20 μl for the lectin treatment and 5×10^4 per 20 μl for enzyme treatments. The infection tests were performed in 3 sea anemones for each treatment. The isolated zooxanthellae incubated in phosphate buffer saline only (i.e., under the conditions of the experimental treatment described above but without the addition of lectin or enzymes) were also applied to the bleached animals as a positive control, and zooxanthellae killed by boiling (100 °C for 5 min) as a negative control. After infection, the sea anemones were maintained at a standard light-dark regime as described above in aerated seawater at 25 °C. The 3 longest tentacles of each animal were sampled for determining zooxanthellae numbers every 24 h.

The data obtained in these experiments were analyzed by one-way analysis of variance (ANOVA) using a 5% significant level, followed with the Bonferroni/Dunn test to examine the difference of the significance between each treatment.

Isolation and identification of cell wall glycoproteins

Freshly isolated zooxanthellae (ca. 10^9 cells) were concentrated by centrifugation at 10 000 g for 2 min, then supplemented with 300 μl distilled water and a similar amount of silica beads (zirconia/silica beads, 0.5 mm in diameter, BIOSPEC, Bartlesville, OK) in a water-tight centrifuge tube (1.5 ml). The cells of the zooxanthellae were broken using a high-speed mini bead beater (BIOSPEC) at 500 rpm for 60 s (Huang et al. 1994). Then, the cell wall fragments were collected by centrifugation at 22 000 g for 10 min and washed 5 times with distilled water.

To purify cell wall fragments, the samples derived from 10^9-10^{10} algal cells were mixed with 1 ml TES buffer (20 mM N-tris[hydroxy-methyl]-methyl-2-aminoethane-sulfonic acid, 600 mM sucrose, 2 mM ethylene diaminetetraacetic acid) following the method described by Omata and Murata (1983). The cell wall samples were loaded into 5-ml ultracentrifuge tubes containing 3.5 ml of 85% sucrose solution, and centrifuged at 10 000 g for 1 h. The cell wall pellet was further purified by washing with distilled water, and centrifuging in an 85% sucrose solution at 7500 g for 1 h.

According to a preliminary test, typical sampling buffer for SDS-polyacrylamide gel electrophoresis (PAGE) as described by Hames (1990) could only extract a limited amount of proteins from the cell walls of zooxanthellae. When the concentration of the sampling buffer was increased 5 fold, the extraction of cell wall proteins reached its maximum. Therefore, in this study, cell wall proteins from about 10^9-10^{10} zooxanthellae were extracted with 50 μl of 5-fold concentrated sampling buffer in boiling water for 5 min. The extracted portion was dialyzed in dialysis tubing (MW cutoff 10 000) against 1 l of double distilled water twice for 3 h for each operation. Then, the precipitate was removed by centrifugation at 10 000 g and 4 °C for 15 min, and the supernatant was decanted for further concentration using an Amicon Y-10 concentrator (MW cutoff 10 000, Amicon, Bedford, MA). The concentrated proteins were first oxidized and labeled with DIG-3-O-succinyl-ε-aminocaproic acid hydazide following the

manufacturer's instructions (Boehringer Mannheim) and then separated by 13.5% SDS-polyacrylamide gel electrophoresis (Hames 1990). The detection of glycoproteins on the polyacryamide gel was performed using a DIG glycan detection kit (Boehringer Mannheim) after electro-blotting the DIG-labelled proteins onto nitrocellulose paper (Hames 1990). Following manufacturer's instructions (Boehringer Mannheim), the DIG-labeled proteins were revealed by the reaction of anti-DIG antibody-conjugated alkaline phosphatase and the substrates (5-bromo-4-chloro-3-indolyl-phosphate and 4-nitro blue tetrazolium).

The glycoproteins detected in the above method were characterized by a specific glyco-binding protein, agglutinin, to reveal the terminal sugar residue. In this study, several types of agglutinin derived from *Galanthus nivalis, Ambucus nigra, Maackia amurensis, Datura atramonium*, and peanut were used. They could specifically bind to different types of sugar linkages. For example, *G. nivalis* agglutinin (GNA) could bind to the mannose-mannose linkage; *Sambucus nigra* agglutinin (SNA) to the galactose-α-(2-6)-sialic acid linkage; *M. amurensis* agglutinin (MAA) to the galactose-α-(2-3)-sialic acid linkage; peanut agglutinin (PNA) to the galactose-β-(1-3)-N-acetyl galactosamine linkage; and *D. atramonium* agglutinin (DSA) could bind to the galactose-β-(1-4)-N-acetyl galactosamine linkage. The cell wall proteins from zooxanthellae were isolated and electrophoresed as described above. After Western blotting, the nitrocellulose strip was incubated with DIG-3-O-succinyl-ε-aminocaproic acid hydazide-labeled agglutinin, and the agglutinin-binding proteins were visualized by anti-DIG antibody-conjugated alkaline phosphatase and the substrates as mentioned above.

RESULTS

Infection of bleached animals by treated zooxanthellae

As shown in figure 1, when infected with control zooxanthellae, the algal density in tentacles of bleached animals greatly increased from the 24th h after infection. However, with algae treated with trypsin, α-amylase, N-glycosidase, O-glycosidase, or a mixture of N-glycosidase and O-glycosidase, the algal density in the tentacles of bleached animals increased little when compared to the results derived from the control algae. At the end of 72 h after infection, the algal density in the tentacles of bleached animals treated with different enzymes was only 9%-23% that of the control group. The results of Bonferroni/ Dunn post-hoc ANOVA also revealed that the infection rate between the control and enzyme treatments varied significantly ($p < 0.001$). Yet the infection rates were similar between different enzyme treatments ($p > 0.05$).

Consistent with the results of enzyme treatments, when the algae were pre-treated with lectin, the infection rate was reduced by 71%-89% that of the control algae after 72 h of infection (Fig. 2). The test of Bonferroni/Dunn post-hoc ANOVA also revealed a significant difference between lectin treatments and the control algae ($p < 0.05$). Moreover, differences between treatments with different types of lectin were not significant ($p > 0.05$).

The infection rate obtained from heat-killed algae was nearly 0 (Figs. 1, 2), suggesting that the repopulated zooxanthellae in the tentacles of bleached animals were derived exclusively from infected algae, not from endogenous ones.

Glycoproteins on the cell wall of zooxanthellae

The glycoproteins contained in cell walls of zooxanthellae are shown in figure 3. The protein staining with Coomassie brillant blue R-250 displayed 9 major bands on the polyacrylamide gel. The molecular weight of the dominant protein band

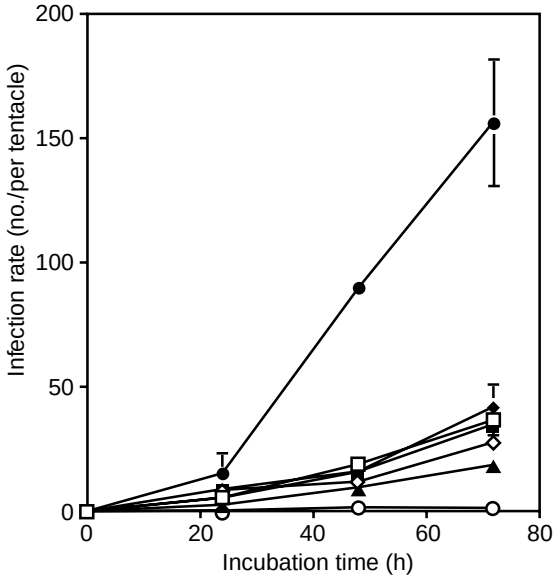

Fig. 1. The infection rate of enzyme-treated zooxanthellae in bleached *Aiptasia pulchella*. Control (●), and zooxanthellae treated with N-glycosidase F (◇), O-glycosidase (◆), N-glycosidase F plus O-glycosidase (▲), α-amylase (□), trypsin (■), or boiling water for 15 min (○) are shown.

was estimated to be about 64 kDa, which is approximately half of the concentration of total detected cell wall proteins from zooxanthellae. Glycan detection showed 5 bands displaying a positive reaction. Their molecular weights were estimated to be about > 150, 64, 62, 44, and 33 kDa, respectively. The band with a molecular weight of 33 kDa, which showed no positive staining with Coomassie brillant blue R-250, was further identified as a protein by silver staining (data not shown). Combining the results of Coomassie blue staining and silver staining, ten protein bands in total could be detected in the extract of zooxanthellae cell wall.

In order to characterize terminal sugar residues of the glycoproteins on zooxanthellae cell wall, specific sugar-binding agglutinins were used. As table 1 shows, there might be at least 2 types of sugar linkages in the glycoproteins on zooxanthellae cell wall: a mannose-mannose linkage and galactose-β(1-4)-N-acetylglucosamine. Note that the major protein (64 kDa) on the zooxanthella cell wall carries a mannose at the terminal sugar residue.

DISCUSSION

Similar to the results obtained from *Chlorella-Paramecium* symbiosis (Reisser et al. 1982), this study demonstrates that the algal infection rate declined when isolated zooxanthellae were treated with several types of glycosidases. In this study, the glycosidase specifically cutting off the linkage between C-N, C-O, or both was used and revealed similar results between different enzyme treatments. Treating the isolated zooxanthellae with trypsin or α-amylase also displayed similar results. These results indicate that glycoproteins are involved in the establishment of the symbiotic association between zooxanthellae and *Aiptasia pulchella*. More recently, glycoproteins have been proven to be the key molecules during cell-to-cell recognition (Varki 1994, Weis and Drickamer 1996, Gabius 1997). This suggests that glycoproteins on the surface of zooxanthellae are likely to be the recognition determinant for the cnidarian host to recognize its specific symbiont. Because different enzymes exhibited similar impacts on reducing the infective capability of treated algae, the recognition determinant on the zooxanthellae surface may be far more complex than our current general understanding of it (Page and Roy 1997, Campbell 1998, Condaminet et al. 1998, Dini and Carla 1998, Maldonado et al. 1998).

Another line of evidence suggesting that surface glycoproteins of zooxanthellae are the recognition determinant of the sea anemone host is derived from the lectin masking experiment. When glycoproteins on the cell walls of freshly isolated zooxanthellae were masked with lectin, the infection rate of treated algae to *A. pulchella* declined dramatically (Fig. 2). In this study, four types of lectin with varied binding affinities to the sugar residues of glycoproteins were

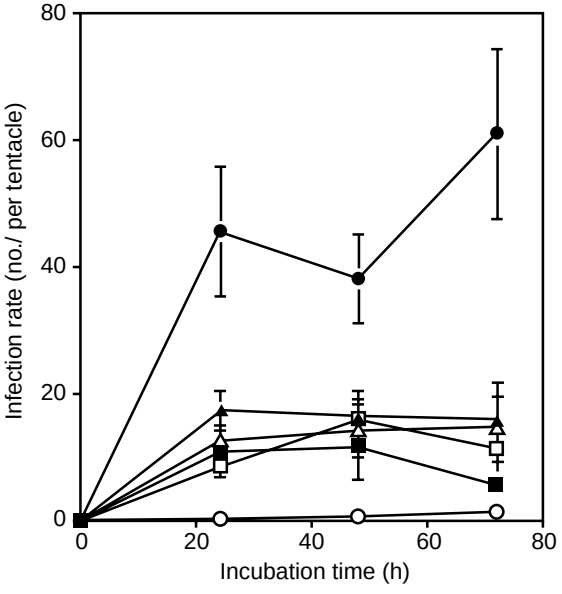

Fig. 2. The infection rate of lectin-treated zooxanthellae in bleached *Aiptasia pulchella*. Control (●), and zooxanthellae treated with Con A (▲), LPA (△), PHA-E (■), WGA (□), or boiling water for 15 min (○) are shown.

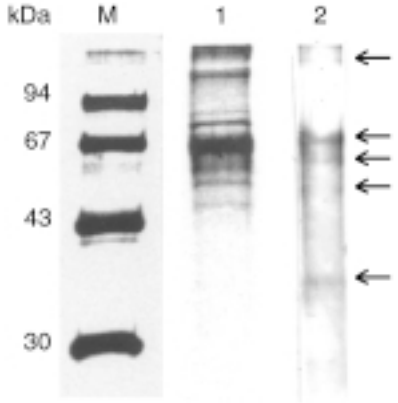

Fig. 3. Analysis of total proteins and glycoproteins from the cell wall of zooxanthellae using SDS-PAGE, followed by Western blotting. Total cell wall proteins were detected by staining with Coomassie brilliant blue R-250, and the carbohydrate groups of separated proteins were detected on nitocellulose paper using DIG derivation, anti-DIG antibody-conjugated alkaline phosphatase, and the substrates. Lane M: protein marker; Lane 1: proteins stained with Coomassie blue; Lane 2: carbohydrate residue staining.

applied separately to isolated zooxanthellae. For example, Con A could bind to α-D-mannosyl and α-D-glucosyl groups; LPA to N-acetyl neuraminic acid, glucuronic acid, and phosphorylcholine analogs; PHA-E to oligosaccharide; and WGA could bind to N-acetyl-β-D-glucosaminyl and N-acetyl-β-D-glucosamine oligomers. All lectin treatments revealed a similar effect of decreasing the infection rate of treated algae. This result is consistent with previous studies on hydra (Meints and Pardy 1980) and *Paramecium* (Reisser et al. 1982), which suggests that the sugar residues described above might be involved in the establishment of symbiosis between algae and cnidarian hosts. However, the zooxanthellae derived from jellyfish showed no affinity to the 4 types of lectin described above (Colley and Trench 1983). Weis (1979) also found that the successful association in *Chlorella-Paramecium* symbiosis strongly relied on a lower affinity of the algae for Con A and the capability of the algae to release photosynthetic products to the host. The ambiguous results obtained from different symbiotic associations indicate that the sugar residues revealed in this study might be crucial only to some specific associations, and the use of lectin masking should be further considered. For example, Jolley and Smith (1980) had argued that the decrease in the algal infection rate resulting from lectin masking should also be considered a side effect of reduced photosynthate release from the symbiotic algae.

Moreover, this study further isolated and characterized these glycoproteins on the algal wall. In the analysis of the proteins associated with the cell wall of zooxanthellae isolated from *Aiptasia pulchella*, five protein bands were identified as glycoproteins (Fig. 3). The analysis of the terminal carbohydrate residue of each protein band revealed that 3 glycoproteins, including the dominant one (MW 64 kDa), contained a mannose-mannose linkage, and the glycoprotein with a molecular weight of 44 kDa contained galactose-β-(1-4)-N-acetylglucosamine at the end of the glyco residue. Mannose has been suggested to be the recognition determinant for several types of cells in humans and other mammals (Page and Roy 1997, Campbell 1998, Condaminet et al. 1998, Dini and Carla 1998, Maldonado et al. 1998). Whether the mannose terminal of the glycoproteins of molecular weight 64, 62 or 33 kDa is the determining site for *A. pulchella* to recognize its symbiont requires further study.

According to the results described above, it is evident that glycoproteins on the cell walls of zooxanthellae are necessary for the sea anemone host to recognize its symbiont during the establishment of the symbiotic relationship. The recognition mechanism probably involves more than a single determinant which, unfortunately, could not be identified or further defined by our experimental design. Determination of these glycoproteins is worthy of further study, to provide more insightful evidence toward our understanding of endosymbiotic associations.

Table 1. Detection of terminal sugar residues of glycoproteins isolated from zooxanthellae cell wall. The proteins blotted onto nitrocellulose strips were incubated with DIG-3-O-succinyl-ε-aminocaproic acid hydazide-labeled agglutinin, and the agglutinin-binding proteins were visualized by anti-DIG antibody-conjugated alkaline phosphatase and the substrates. The symbol "+" indicates positive staining, "–" negative staining, and "+/–" an obscure result

Protein band (kDa)	GNA[a]	SNA[b]	MAA[c]	PNA[d]	DSA[e]
> 150	–	–	–	–	–
64	+	–	–	–	–
62	+/–	–	–	–	–
44	–	–	–	–	+
33	+	–	–	–	–

[a]GNA (*Galanthus nivalis* agglutinin) can bind the mannose-mannose linkage.
[b]SNA (*Sambucus nigra* agglutinin) can bind the galactose-α-(2-6)-sialic acid linkage.
[c]MAA (*Maackia amurensis* agglutinin) can bind the galactose-α-(2-3)-sialic acid linkage.
[d]PNA (peanut agglutinin) can bind the galactose-β-(1-3)-N-acetyl galactosamine linkage.
[e]DSA (*Datura atramonium* agglutinin) can bind the galactose-β-(1-4)-N-acetyl galactosamine linkage.

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共生藻細胞壁醣蛋白參與海葵和其共生藻共生機轉的建立

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一般認為細胞膜上的醣蛋白 (glycoprotein) 是細胞間互相辨識的分子，本研究則進一步研究共生藻細胞壁上的醣蛋白與海葵宿主形成共生時所扮演的角色。當單離之共生藻受 trypsin 、α-amylase 、N-glycosidase F 或 O-glycosidase 處理後，其進入白化海葵細胞中，並恢復共生的能力明顯低於未處理之共生藻，若以 lectin 來遮蔽共生藻表面的醣蛋白時，亦有相同之效果，進一步以 SDS-PAGE 及西式點墨法分析共生藻表面之醣蛋白時，發現 10 條主要蛋白質染色帶中有 5 條是被鑑定為醣蛋白，其中醣基之結合模式，初步鑑定出有二種，即 mannose-mannose 和 galactose-β(1-4)-N-acetyl glucosamine ，而且發現量最多的醣蛋白(64 Kda)具有 mannose 的末端醣基，這些結果顯示，當共生藻與海葵(*A. pulchella*)形成共生關係時，共生藻表面之醣蛋白將扮演重要的角色。

關鍵詞：共生藻，刺絲胞動物，胞內共生，植物凝集素，識別機制。

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