Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 330 (2005) 157–162
BBRC
www.elsevier.com/locate/ybbrc

Cloning and characterization of a lectin from the octocoral *Sinularia lochmodes*

Mitsuru Jimbo [a,*], Kazuhiko Koike [a], Ryuichi Sakai [a], Koji Muramoto [b], Hisao Kamiya [a]

[a] *Department of Marine Biosciences, School of Fisheries Sciences, Kitasato University, Oofunato, Iwate 022-0101, Japan*
[b] *Department of Molecular Life Science, Graduate School of Life Sciences, Tohoku University, Sendai, Miyagi 981-8555, Japan*

Received 16 February 2005

Abstract

In the present study, the entire amino acid sequence and cDNA structure encoding the D-galactose-binding lectin, SLL-2, isolated from the octocoral *Sinularia lochmodes*, were determined. SLL-2 regulates the morphology of symbiotic dinoflagellates *Symbiodinium* spp. through unknown mechanisms. Here, three cDNAs that encode SLL-2 were cloned and characterized. All the SLL-2 cDNAs encoded 142 amino acids with high similarity to each other. The mature subunit of SLL-2 was found to be composed of 94 amino acids and to contain one putative glycosylation site common to all three SLL-2. *N*-Glycopeptidase F treatment of SLL-2 resulted in a protein band shift from 16.5 to 9.5 kDa in SDS–PAGE, confirming that SLL-2s are glycoproteins. Two-dimensional polyacrylamide gel electrophoresis analysis of the deglycosylated SLL-2 indicated a presence of three polypeptides as encoded in SLL-2 cDNAs. The deduced sequences of SLL-2 cDNAs had a similarity to the C-terminal region of discoidin I, the slime mold *Dictyostelium discoideum* lectin.

Keywords: Octocoral; *Sinularia lochmodes*; D-Galactose-binding lectin; Discoidin; Symbiotic dinoflagellates; Symbiosis; *Symbiodinium*

A number of lectins, carbohydrate-binding proteins, are known to be present in a variety of animals and plants, and their roles in various biological processes have been characterized. In marine animals, lectins, especially invertebrate humoral lectins, are believed to contribute as non-self recognition factors to the defense mechanism [1,2]. Moreover, there is a collective body of evidence supporting that marine invertebrate lectins are also involved in various endogenous biological events such as biomineralization [3] and embryonic development [4–6]. Interestingly, it has been theorized that some marine animal lectins mediate the interaction between symbiont and host. For example, a lectin isolated from the marine sponge *Halichondria panacea* has been reported to have a growth-promoting effect on symbiotic bacteria *Pseudomonas insolita* [7]. It has also been proposed that the symbiosis between the tunicate *Didemnum molle* and the microalga *Prochloron* sp. is also mediated by *D. molle* lectins [8]. Tridacnin, a mitogenic D-galactose-binding lectin that is present in the hemolymph of the giant clam *Tridacna maxima*, reacts with various galactans, which are constituents of symbiotic algae, and is regarded as essential for elimination and utilization of their symbionts, which have exposed galactan structures on their surface during degeneration [9]. It has also been reported that glycoprotein or glycoconjugate on the symbiont surface is important in symbiont acquisition by hosts, since glycosidase or lectin treatment of symbiont surface showed ill effects on the acquisition [10–12]. These observations strongly suggest the presence of a chemical substance, which mediates the establishment of symbiosis between symbiotic algae and host. We previously isolated a D-galactose-binding lectin, SLL-2, from the octocoral *Sinularia lochmodes* and found that the lectin was distributed densely on

* Corresponding author. Fax: +81 192 44 3932.
E-mail address: mjinbo@kitasato-u.ac.jp (M. Jimbo).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.02.137

the surface of symbiotic dinoflagellate *Symbiodinium* sp. cells [13]. Recently, SLL-2 was confirmed to transform free-swimming stage (motile stage) *Symbiodinium* cells into non-motile stage (coccoid stage) *Symbiodinium* cells and keep them in their non-motile stage. These results indicated that SLL-2 is a chemical cue in the symbiosis between dinoflagellates and coral [14]. This is the first experimental evidence that marine invertebrate lectin mediates the symbiosis between symbiont and host. In the present study, we cloned the genes encoding SLL-2 and found that SLL-2 comprised of three glycosylated subunits named SLL-2a, -2b, and -2c. Here we describe the entire amino acid sequence and some physicochemical properties of *S. lochmodes* lectin SLL-2, which has highly unique biological activity.

Materials and methods

Materials. Specimens of *S. lochmodes*, collected off Akajima Island, Okinawa, in 2002, were immediately frozen with dry ice at the collection site and kept at −70 °C until use.

Hemagglutination assay. Serial twofold dilutions of the test solution (20 μl) were made in multi well microtiter plates using 150 mM NaCl/50 mM Tris–HCl, pH 8.0 (THB). Agglutinating activity against rabbit erythrocytes was assayed using a 4% suspension (20 μl). After allowing the microtiter plates to stand at 37 °C for 30 min, the titer of the maximum dilution showing positive agglutination was recorded.

Purification of SLL-2. S. lochmodes was extracted with three volumes of THB with 0.5% L-ascorbic acid and 0.1% kojic acid, and centrifuged at 12,000*g* for 20 min at 4 °C. The supernatant was applied to a column (1 ml bed volume) of D-galactosamine-bound HiTrap, which was prepared by conjugating D-galactosamine with HiTrap NHS-activated HP (Amersham Bioscience, NJ, USA) following the manufacturer's protocol. After washing with THB, the lectin was eluted with THB containing 0.2 M D-galactose using a peristaltic pump. A single protein peak was obtained, applied to a Superdex 200 HR pg column (1.6 × 60 cm, Amersham Bioscience), developed with a buffer containing THB and 0.2 M D-galactose, and dialyzed against distilled water to test for hemagglutination activity. The major peak obtained was dialyzed and lyophilized to give SLL-2 in pure form.

Analytical method. Matrix-assisted laser desorption induction time-of-flight mass spectrometry (MALDI-TOF MS) of SLL-2 and its chemical degradation products were carried out with Voyger DE-STR (Applied Biosystems, CA, USA) mass spectrometer using sinapic acid as a matrix. The amino terminal sequence was analyzed using either a Shimadzu gas-phase PSQ-1 sequencer or PPSQ-20 sequencer. For internal sequence determination, SLL-2 fragments were digested with *Achromobacter* proteinase I (Roche diagnostics, Basel, Switzerland) and purified with reversed phase high performance liquid chromatography and then the amino terminal sequence was analyzed. The fragments obtained by cyanogen bromide (CNBr) cleavage of SLL-2 [15] were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), blotted to polyvinylidene fluoride membrane Fluorotrans (PALL, USA), and then analyzed for the amino terminal sequence. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was carried out according to the manufacturer's protocol. Digestion of SLL-2 with *N*-glycopeptidase F (TaKaRa Bio, Shiga, Japan) was then carried out according to the manufacturer's protocol with both the intact SLL-2 and denatured SLL-2. The product obtained was then analyzed by SDS–PAGE [16] and 2D-PAGE. Following SDS–PAGE and 2D-PAGE, the gel was stained by zinc reverse staining [17].

Table 1
PCR primer used for SLL-2 cloning

Name	sequence
SLL5	TGYGARATGGGNACNWSNAC
SLL3	GGCCARAANSWDATNGCYTCRTC
SLL-RACE5	TGGAACACCGATTTAATCACAGGCAATC
SLL-RACE3	GTTGTTGGCCTCGCGAGTGCGACAC

Cloning of SLL-2. Degenerated primers for PCR amplification (SLL5, SLL3 in Table 1) were synthesized according to the amino terminal amino acid sequences (CEMGTSTH, DEAISFWP). DNA amplification was performed using KOD Dash polymerase (Toyobo, Osaka, Japan) under the following conditions: 96 °C for 15 s, 45 °C for 30 s, and 72 °C for 30 s for 35 cycles. PCR products were subjected to agarose gel electrophoresis and were purified with the QIAEX II DNA purification kit (Qiagen, Hilden, Germany). The fragments were cloned into a pCR2.1-TOPO vector (Invitrogen, California, USA) and sequenced. To determine the 5′ terminal of SLL-2 cDNAs, 5′ cDNAs were amplified by Advantage 2 polymerase (BD Biosciences, CA, USA) and 5′ RACE was performed using a Marathon cDNA amplification kit (BD Biosciences). The PCR primers used were the specific primers of SLL-RACE5 (Table 1) and the cassette primer AP1. The amplified PCR products were cloned in pCR2.1-TOPO cloning plasmid (Invitrogen) and were then sequenced. Next, to determine the full length SLL-2 cDNAs, 3′ cDNAs were determined by the 3′ RACE method with SLL-RACE3 (Table 1). The nucleotide sequence obtained was deposited in the DDBJ, nucleotide sequence databases under Accession Nos. AB195426, AB195427, and AB195428. Obtained sequences were aligned using ClustalW [18].

Results and discussion

Isolation and properties of SLL-2

Both the octocoral itself and the extract, prepared by squeezing the fresh animal, rapidly changed to a black color. This phenomenon is frequently accompanied by insolubilization of the extract and results in poor recovery of hemagglutination activity during the purification procedures. We postulate that the 'blackening,' which hampers the efficient purification of SLL-2, is due to melanin formation since the presence of dopamine is evident in this ocotocoral. Additions of kojic acid, a tyrosinase inhibitor, and L-ascorbic acid, an antioxidant, to the extract indeed suppressed the blackening and were effective in preventing insolubilization. The extract was applied to galactosamine-bound HiTrap affinity chromatography. Further separation of the affinity purified specimen fraction by FPLC on Superdex 200 HR pg revealed one major active protein peak. Typically, 30 mg of SLL-2 is obtained from a 100 g specimen which is the yield three times higher than that of our previous research [13]. In SDS–PAGE, the purified specimen gave a single protein band at 16.5 kDa. In 2D-PAGE, however, the component of 16.5 kDa was further separated into many spots with different pI values ranging from 3 to 5, indicating the heterogeneity of SLL-2 subunits [19] (Fig. 1).

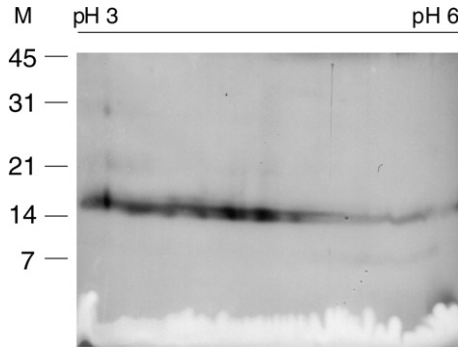

Fig. 1. Purification of SLL-2. Two-dimensional-PAGE of SLL-2. Purified SLL-2 was applied to 2D-PAGE. The isoelectric focusing of the first dimension was over a pH range of 3.0–6.0. The second dimension was SDS–PAGE in a 15% polyacrylamide gel.

Table 2
Amino terminal sequence analysis of SLL-2

Fragment	Sequence
N-terminal	RLIHVSRXEMGTSTHRXWPREXD TSSDEAISFWPPFEN
Achromobacter protease I digests	LIWIAXD
CNBr degradation	LDVDNSNNLRVXSSA

Cloning of cDNA encoding SLL-2

Despite the complex profile indicated by 2D-PAGE, up to 38 residues of the amino terminal of SLL-2 were successfully sequenced [13]. *Achromobacter* proteinase I digestion and CNBr degradation of SLL-2 also allowed us to determine some internal amino acid sequences of SLL-2 (Table 2). On the basis of these amino acid sequences, the primers were designed, and RT-PCR and RACE were performed. As a result, three closely related cDNA sequences were obtained (Fig. 2), indicating that the SLL-2 molecule comprises three kinds of subunits, which we denoted as SLL-2a, b, and c. All the clones contained poly(A)$^+$ signal sequence AATAAA at position 585–590. Each open reading frame was composed of 420 bp encoding 46 and 94 residues of each hydrophobic signal sequence and the mature protein, respectively (Fig. 2). The deduced amino acid sequences were found to have more than 78% identity. The N-glycosylation site (NSS) at position 60 was present in each of the subunits. The calculated molecular weights for each of SLL-2a, -2b, and -2c were 10,827, 10,641, and 10,786, respectively. The deduced N-terminal amino acid sequences for SLL-2a–c corresponded mostly with that determined by amino acid sequencer.

Glycosylation of SLL-2

In MALDI-TOF MS, SLL-2 showed a cluster of molecular ions at about *m/z* 13,200 (Fig. 4), while the calculated molecular weight for SLL-2 subunits was around 10,800, with a difference of approximately 2500 Da. It is strongly suggested that the SLL-2 molecule is N-glycosylated because this site is present in all of three subunits of SLL-2 along with a 7% sugar content estimated by the phenol–sulfuric acid method.

```
                                                    CATTGGAAAGATTGGAGTTGTAAGTTTAAATTGTGTAAGACAAATTTAAAAGATTTCGGAC

ATG AAG TTG ATC TGG GGA ATT GTA ATT GCT GTT TTT GTC GCA AAT TGT GCC GTA   115
Met Lys Leu Ile Trp Gly Ile Val Ile Ala Val Phe Val Ala Asn Cys Ala Val   -29

AAT CAA GGT GCA CGG ATA ACT TTC CAT GAG ATG CCA AAG ACG CTT GGA AAG ACT   169
Asn Gln Gly Ala Arg Ile Thr Phe His Glu Met Pro Lys Thr Leu Gly Lys Thr   -11

GTT GGG GAT TTT GAA ACG CTT TCC AAA CGT CGA CTA ATT CAT GTT TCG CGT TGC   223
Val Gly Asp Phe Glu Arg Leu Ser Lys Arg Arg Leu Ile His Val Ser Arg Cys     8

GAG ATG GGA ACT TCC AGT CAC CGT TGT TGG CCT CGC GAG TGC GAC ACT AGC AGT   277
Glu Met Gly Thr Ser Ser His Arg Cys Trp Pro Arg Glu Cys Asp Thr Ser Ser    26

GAT GAA GCA ATC AGC TTT TGG CCA CCA TTC GAA AAC ACA CCG AAG GTT ATC GTA   331
Asp Glu Ala Ile Ser Phe Trp Pro Pro Phe Glu Asn Thr Pro Lys Val Ile Val    44

AGC TTT GGT ATG CTG GAT GTG GAC AAT TCC CAT AAT CTT CGT GTC AAT AGC AGC   385
Ser Phe Gly Met Leu Asp Val Asp Asn Ser His Asn Leu Arg Val Asn Ser Ser    62

GCA GAT GAT GTG ACT GTA GGC GGC TTT ACA CTC CAC TAC AAT AGC TGG TAT ACA   439
Ala Asp Asp Val Thr Val Gly Gly Phe Thr Leu His Tyr Asn Ser Trp Tyr Thr    80

ACC ATA GTC TGG AAT TAC AAG CTT ATT TGG ATT GCC TGT GAT TAA               484
Thr Ile Val Trp Asn Tyr Lys Leu Ile Trp Ile Ala Cys Asp ***               94

ATCGGTGTTCCAGATTTGGACTACTTTAATCTGAATTAAGTTTAGTTTTATTTGTGACATATAAAGAAAC
ATAAGTCAGTGACATGTCATGTGAAATGCTAATAAATTTCAATGGCAAAAAAAAAAAAAAAA
```

Fig. 2. The nucleotide sequences of SLL-2 cDNA. A representative SLL-2 cDNA sequence is shown. The deduced amino acids of SLL-2a are shown below the nucleotide sequences. Position 1 of the deduced amino acid sequence indicates the first amino acid of the mature SLL-2 as determined by N-terminal sequence analysis. An arrow indicates the starting point of the mature SLL-2. A thick line indicates poly(A)$^+$ signal. A thin line indicates the corresponding regions determined by amino terminal sequence analysis of the digested SLL-2. A box indicates the N-glycosylation site. Asterisk indicates a stop codon.

Thus, we treated samples of SLL-2 with *N*-glycopeptidase F. Only denatured SLL-2 was digested with the glycopeptidase while the intact protein was resistant to the treatment. As expected, the *N*-glycopeptidase F-treated SLL-2 showed a protein band shift from 16.5 to 9.5 kDa, which was close to the molecular weight of subunits (10.8 kDa) observed on SDS–PAGE (Fig. 4A).

Conversely, the glycopeptidase-treated SLL-2 gave three protein spots at p*I*s of 4.7, 5.0, and 5.3 with a molecular weight of 9.5 kDa under reduced conditions on 2D-PAGE (Fig. 4B). They presumably corresponded to SLL2a, b, and c (theoretical pIs of 4.2, 4.9, and 5.3, respectively). Although actual and theoretical pI for SLLa was somewhat different, this is within allowance considering the experimental settings as reported by O'Farrell [20]. The size of each spot in the 2D-PAGE was almost identical, suggesting that three subunits were equally expressed in *S. lochmodes*. This result supported the observation in the above amino acid sequencing.

The fact that native protein gave many spots in 2D-PAGE may reflect a microheterogeneity of SLL-2 glycosylation. The low pI of some SLL-2 could be a result from sialic acid content in the glycomoiety. Since some glycoproteins such as α_1-acid glycoprotein and invasive trophoblast antigen have low p*I* due to sialic acids containing carbohydrate chain [21], a further detailed study on carbohydrate moiety attached to SLL-2 molecules is necessary.

These results, along with the relative molecular weight 122 kDa of SLL-2 estimated by gel filtration [13], suggest that functioning SLL-2 is composed of nine subunits containing equimolar of the isoforms SLL-2a, b, and c.

Similarity search

PSI-blast analysis with three iterations [22] showed that SLL-2 has amino acid sequence similarities to hypothetical proteins encoded by an open reading frame

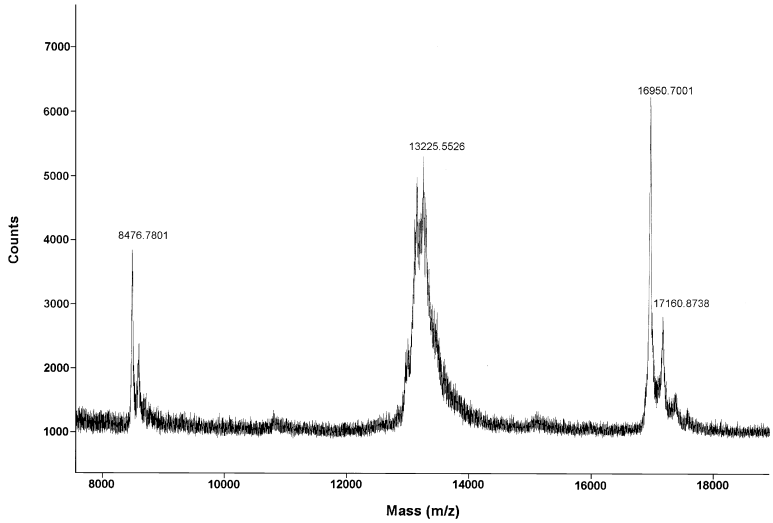

Fig. 3. Mass spectral analysis of SLL-2. Mass spectrometry was carried out by MALDI-TOF MS with sinapic acid as a matrix, using apomyoglobin as an internal control. A cluster of peaks was observed centered at *m*/*z* 13,200. Myoglobin is indicated by a peak at *m*/*z* 16950.7 and 8476.7.

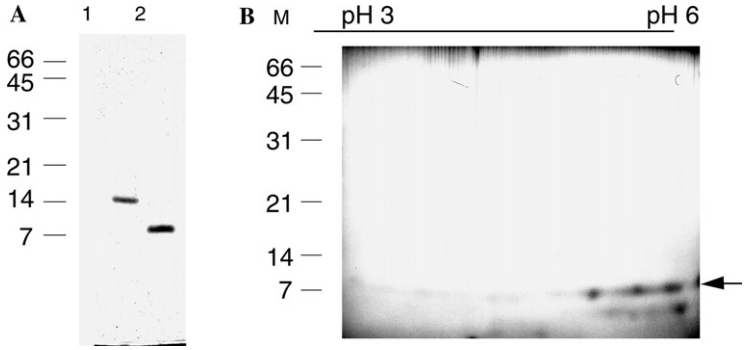

Fig. 4. Polyacrylamide gel electrophoresis of deglycosylated SLL-2. Denatured SLL-2 was incubated with *N*-glycopeptidase F at 37 °C for 16 h and analyzed by 2D-PAGE using a 15% polyacrylamide gel. (A) SDS–PAGE on 15% polyacrylamide gel. Lane 1, native SLL-2; lane 2, the digested SLL-2. (B) 2D-PAGE. *N*-glycopeptidase F treated SLL-2 was analyzed by 2D-PAGE. The method was the same as in Fig. 1.

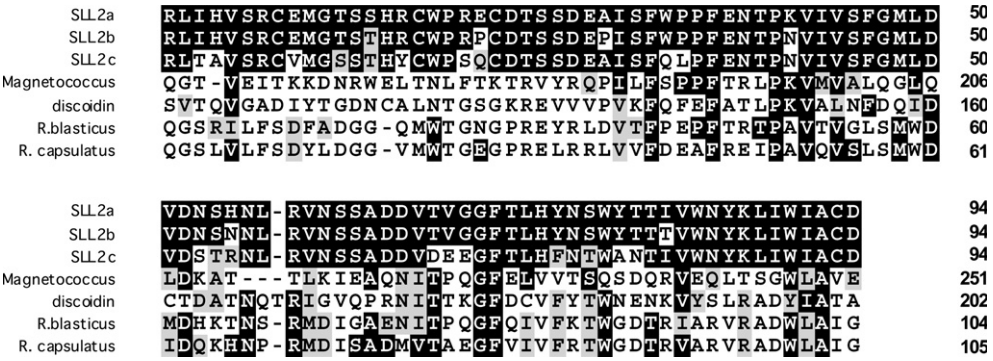

Fig. 5. Multiple alignment of SLL-2 and homologous proteins. Conserved residues and identical residues against SLL-2a are indicated with gray and black boxes, respectively. R. capsulatus *R. capsulatus* hypothetical protein (Accession No. CAA67912), R. blasticus; ATP synthase subunit region ORF 7 of *R. blasticus* (Accession No. P05450), discoidin: Discoidin I, A chain of D. discoideum (Accession No. P02886), and Magnetococcus; hypothetical protein of *Magnetococcus* sp. MC-1 (Accession No. ZP_00042742).

of ATP synthase operon in *Rhodobacter capsulatus* (40.9% similarity against SLL2a identity), *R. blasticus* (41.9% similarity against SLL2a identity), and *Magnetococcus* sp. (35.2% similarity against SLL2a identity) as shown in Fig. 3. The only biochemically characterized protein which showed identity to SLL-2 was discoidin I. SLL-2 has 33.0% similarity to the C-terminal region of this protein, which is a D-galactose-binding lectin from slime mold *Dictyostelium discoideum* and is reported to be expressed in a developmentally regulated manner [23]. The tripeptide Arg-Gly-Asp, which mediates cell–substratum adhesion, is present in discoidin I in a region distinct from the carbohydrate-binding region [24]. However, to date, no carbohydrate recognition domain or biological role of discoidin I in slime mold has been identified. Our results suggested that the carbohydrate recognition domain of discoidin I may be present in the C-terminal region, since the amino acid sequence and the sugar-binding properties of these proteins share considerable similarity. The series of proteins known as discoidin domain-containing proteins are of interest in the study of cell adhesion mechanisms in animals, however this ''discoidin domain'' resides in the N-terminal region of the protein. SLL-2 is the first example of a protein that has similarity to the C-terminal of discoidin. The biological importance of these structural units in both proteins must be the subject of further studies (Fig. 5).

In a biological assay of various microalgae, differential activities of SLL-2 to symbiotic and non-symbiotic microalgae were observed. To non-symbiotic microalgae such as *Gymnodinium catenatum* and *Prorocentrum lima*, SLL-2 showed highly toxic effects, including bursting the cells. In contrast, SLL-2 arrested motile *Symbiodinium* cells in the non-motile stage without any inhibition of cell division [14]. At present, the molecular mechanisms of the physiological modulation of *Symbiodinium* and non-symbiotic dinoflagellate by SLL-2 are not known. Structural and genetic information permits not only the study of the structure–function relationship of SLL-2 but also the survey of related proteins in various soft corals by genetic means to determine which general chemical symbiosis cues exist between corals and dinoflagellates.

Acknowledgment

This study was supported in part by Grants-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, and Science, Japan, to H.K. (80011964 and 15380145).

References

[1] G.R. Vasta, J.D. Lambris, Innate immunity in the Aegean: ancient pathways for today's survival, Dev. Comp. Immunol. 26 (2002) 217–225.
[2] D.C. Kilpatrick, Animal lectins: a historical introduction and overview, Biochim. Biophys. Acta 1572 (2002) 187–197.
[3] H. Kamiya, M. Jimbo, H. Yako, K. Muramoto, O. Nakamura, R. Kado, T. Watanabe, Participation of the C-type hemolymph lectin in mineralization of the acorn barnacle *Megabalanus rosa*, Mar. Biol. 140 (2002) 1235–1240.
[4] H. Ahmed, S.J. Du, N. O'Leary, G.R. Vasta, Biochemical and molecular characterization of galectins from zebrafish (*Danio rerio*): notochord-specific expression of a prototype galectin during early embryogenesis, Glycobiology 14 (2004) 219–232.
[5] C.H. Dong, S.T. Yang, Z.A. Yang, L. Zhang, J.F. Gui, A C-type lectin associated and translocated with cortical granules during oocyte maturation and egg fertilization in fish, Dev. Biol. 265 (2004) 341–354.
[6] M.C. Alliegro, M.A. Alliegro, The structure and activities of echinonectin: a developmentally regulated cell adhesion glycoprotein with galactose-specific lectin activity, Glycobiology 1 (1991) 253–256.
[7] W.E. Müller, R.K. Zahn, B. Kurelec, C. Lucu, I. Müller, G. Uhlenbruck, Lectin, a possible basis for symbiosis between bacteria and sponges, J. Bacteriol. 145 (1981) 548–558.

[8] W.E. Müller, A. Maidhof, R.K. Zahn, J. Conrad, T. Rose, P. Stefanovich, L. Müller, U. Friese, G. Uhlenbruck, Biochemical basis for the symbiotic relationship *Didemnum–Prochloron* (Prochlorophyta). Biol. Cell 51 (1984) 381–388.
[9] G. Uhlenbruck, G. Steinhausen, Tridacnins: symbiosis-profit or defense-purpose?, Dev. Comp. Immunol. 1 (1977) 183–192.
[10] D. Weis, Correlation of infectivity and concanavalin A agglutinability of algae exsymbiotic from *Paramecium bursaria*, J. Protozool. 26 (1978) 117–119.
[11] W. Reisser, A. Radunz, W. Wiessner, Participation of algal surface structures in the cell recognition process during infection of aposymbiotic *Paramecium bursaria* with symbiotic chlorellae, Cytobios 33 (1982) 39–50.
[12] K.-L. Lin, J.-T. Wang, L.-S. Fang, Participation of glycoproteins on zooxanthella cell walls in the establishment of a symbiotic relationship with the sea anemone, *Aiptasia pulchella*, Zool. Stud. 39 (2000) 172–178.
[13] M. Jimbo, T. Yanohara, K. Koike, R. Sakai, K. Muramoto, H. Kamiya, The D-galactose-binding lectin of the octocoral *Sinularia lochmodes*: characterization and possible relationship to the symbiotic dinoflagellates, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 125 (2000) 227–236.
[14] K. Koike, M. Jimbo, R. Sakai, M. Kaeriyama, K. Muramoto, T. Ogata, T. Maruyama, H. Kamiya, Octocoral chemical signaling selects and controls dinoflagellate symbionts, Biol. Bull. 207 (2004) 80–86.
[15] E. Grosse, The cyanogen bromide reaction, in: Methods Enzymology, Academic Press, New York, 1967, pp. 238–255.
[16] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[17] C. Fernández-Patrón, L. Castellanos-Serra, P. Rodríguez, Reverse-staining of sodium dodecyl sulfate polyacrylamide gels by imidazole-zinc salts: sensitive detection of Coomassie blue undetected proteins, BioTechniques 12 (1992) 564–573.
[18] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[19] T.W. Rademacher, R.B. Parekh, R.A. Dwek, Glycobiology, Annu. Rev. Biochem. 57 (1988) 785–838.
[20] P.H. O'Farrell, High resolution two-dimensional electrophoresis of proteins, J. Biol. Chem. 250 (1975) 4007–4021.
[21] M.S. Jaime, A.C. Laurence, Sialic acid-deficient invasive trophoblast antigen (sd-ITA): a new urinary variant for gestational Down syndrome screening, Prenat. Diagn. 24 (2004) 194–197.
[22] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[23] S.H. Barondes, D.N. Cooper, P.L. Haywood-Reid, Discoidin I and discoidin II are localized differently in developing *Dictyostelium discoideum*, J. Cell Biol. 96 (1983) 291–296.
[24] W.R. Springer, D.N. Cooper, S.H. Barondes, Discoidin I is implicated in cell–substratum attachment and ordered cell migration of *Dictyostelium discoideum* and resembles fibronectin, Cell 39 (1984) 557–564.