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Molecular characterization of a lectin, BPL-4, from the marine green alga *Bryopsis plumosa* (Chlorophyta)

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A novel lectin specific to N-acetyl-D-galactosamine as well as N-acetyl-D-glucosamine was isolated from *Bryopsis plumosa* and named as BPL-4. Sodium dodecyl sulfate polyacrylamide gel electrophorese (SDS-PAGE) and matrix-assisted laser desorption / ionization-time of flight (MALDI-TOF) mass spectrometry data showed that this lectin was a monomeric protein with molecular weight 12.9 kDa. The N-terminal amino acid sequences of the lectin were determined by Edman degradation and the full cDNA sequence encoding this lectin was obtained using the degenerate primers designed from the amino acid sequence. The size of the cDNA was 414 bp containing single open reading frame (ORF) encoding the lectin precursor. The homology analysis showed that this lectin might belong to H lectin group. BPL-4 showed high sequence similarity (60.6%) to BPL-3, which is a previously reported lectin from the same species. The comparative analysis on the lectin's primary structure showed two conserved domains including one possible active domain of H lectin group.

Key Words: active domain; BPL-4; *Bryopsis plumosa*; lectin; N-acetyl-D-galactosamine

INTRODUCTION

Lectins have the capacity to serve as recognition molecules between cells or organisms because of their non-catalytic sugar binding properties (Sharon 2008). Lectins are proteins that bind reversibly to carbohydrates, agglutinate cells, or precipitate polysaccharides and glycoproteins (Goldstein et al. 1980). Carbohydrate-binding properties of lectins have been applied in the fields of immunology, cell biology, cancer research, and genetic engineering (e.g., Sharon and Lis 2004).

Recently, the medical use of lectins has been studied extensively and some lectins exhibiting high anti-HIV (Sato et al. 2007) and antibiotic activities (Liao et al. 2003) were isolated. The use of lectins as pesticide agents (Wellman-Labadie et al. 2008) and potential drug delivery systems were also explored (e.g., Jung et al. 2010). Lectin-based cell-cell recognition systems have been proposed in many algal groups and in some cases the corresponding lectins have been isolated. However, the number of characterized lectins is still considered too small (Yoon et al. 2008, Han et al. 2011).

Marine green alga *Bryopsis plumosa* (Hudson) Agardh possesses unique ability to regenerate new functional cells from small droplets of protoplasm extruded in seawater (Kim et al. 2001). When protoplasm is released in the seawater, the organelles aggregate *in vitro* and form protoplasts. Then, the protoplasts develop into individual

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plants. Possible involvement of lectins in the aggregation of cell organelles was previously suggested (Pak et al. 1991, Kim et al. 2001, 2002, Klotchkova et al. 2003) and three lectins (Bryohealin, BPL-2, and BPL-3) were isolated in this species (Yoon et al. 2008, Han et al. 2010, 2011).

For the successful aggregation of cell organelles during protoplast formation in *B. plumosa,* the existence of additional lectins was suggested (Kim et al. 2006, Han et al. 2011). In this study, we describe the biochemical and molecular characterization of another lectin from *B. plumosa*, BPL-4, which might also be involved in the protoplast regeneration of this species.

MATERIALS AND METHODS

Organism and laboratory culture

The vegetative plants of *B. plumosa* were collected from Kachon, southern coast of Korea and maintained as unialgal cultures in IMR medium (Han et al. 2011) at 20-23°C in 16 : 8 h light and dark cycles with >20 μmol photons m^{-2} s^{-1} provided by cool-white fluorescent lamps.

Preparation of concentrated algal extract and purification of BPL-4

The concentrated algal extract was prepared as described by Han et al. (2011). It was loaded on N-acetyl-D-galactosamine (GalNAc)-agarose affinity column equilibrated with phosphate-buffered saline (PBS) of pH 7.3. The non-bound materials were removed with PBS until the absorbance at 280 nm was lowered to 0.001, and the bound protein was eluted with the same solution containing 0.05 M N-acetyl-D-glucosamine (GlcNAc). The fractions of 2 mL were collected and analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The fractions showing protein band and high hemagglutinating activity were pooled, concentrated, and dialyzed against PBS. The protein contents were determined by the method of Bradford (1976). SDS-PAGE was performed according to Laemmli (1970) with 15% gel. Samples were treated with 4% 2-mercaptoethanol to reduce disulfide bonds. Proteins were stained with 0.12% Coomassie brilliant blue R-250.

Two-dimensional gel electrophoresis (2-DE)

A mixture of elutes from GalNAc agarose affinity column was dialyzed in distilled water and lyophilized. Dried proteins were dissolved in 150 μL of rehydration buffer (7 M urea, 2 M thio-urea, 4% CHAPS, 2% ampholyte of pH 4-10) and centrifuged at 12,000 g for 15 min to remove insoluble materials. IPG dry strip (pH 4-10, 7 cm; Bio-Rad, Bedford, CA, USA) was rehydrated with 125 μL of sample in rehydration buffer for overnight at 20°C. Isoelectric focusing (IEF) was performed at 20°C using a PROTEAN IEF Cell (Bio-Rad). The voltage was linearly increased from 250 to 4,000 V during 2 h, followed by constant 4,000 V with complete focusing after 10 kVh. After IEF, strip was incubated for 15 min in equilibration buffer (50 mM Tris-HCl at pH 6.8, containing 6 M urea, 2% SDS, and 30% glycerol), first with 1% dithiothreitol and second with 2.5% iodoacetamide. Equilibrated strip was inserted onto SDS-PAGE gel (7 × 7 cm, 15%) and run at 200 V, 20 mA constant. The gels were stained with Coomassie brilliant blue R-250 as described by Han et al. (2011).

Amino acid sequence analysis

The amino acid sequencing was carried out with an Applied Biosystems Precise Sequencer (Applied Biosystems, Foster City, CA, USA) in Korea Basic Science Institute (KBSI, Daejeon, Korea). The protein was electrophoresed on 15% SDS-PAGE and electroblotted onto a polyvinylidine fluoride membrane for 2 h at 4°C. Blotted membrane was stained with ponceau S staining solution. Internal amino acid sequence was obtained using chemically assisted fragmentation-matrix-assisted laser desorption / ionization (CAF-MALDI-TOF; Amersham Biosciences, Piscataway, NY, USA). Protein band was excised and digested with trypsin (Promega, Madison, WI, USA) and digested peptide was sent to Genomine Company (Pohang, Korea). The sonar in CAF-MALDI-TOF and MASCOT program was used for the identification of peptide sequence.

Construction of *Bryopsis plumosa* cDNA library

Double stranded cDNA was synthesized using 5 μg of poly (A) RNA as a template, directionally cloned into a UniZAP-XR vector phage (ZAP-cDNA synthesis Kit; Stratagene, La Jolla, CA, USA), and packaged using the ZAP-cDNA Gigapack III Gold packaging extract (Stratagene). Approximately 1.8 and 1.5 million recombinants were represented in the cDNA libraries, respectively.

RNA isolation and cDNA cloning

Complete BPL-4 cDNA was cloned in two steps. First, a degenerate forward primer [BPL4-NDF1, 5′-CA(A/G)

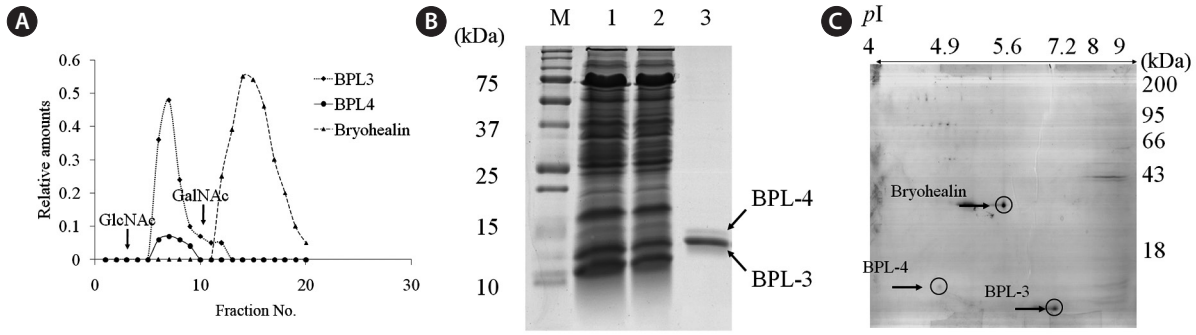

Fig. 1. Purification of BPL-4 using N-acetyl-D-galactosamine affinity chromatography. (A) Chromatogram. (B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (M, molecular weight marker; lane 1, crude extract; lane 2, flow-through fraction; lane 3, purified BPL-3 and BPL-4). (C) Two-dimensional gel electrophoresis (2-DE) gel electrophoresis of eluted fraction from GalNAc affinity column. GalNAc, N-acetyl-D-galactosamine; GlcNAC, N-acetyl-D-glucosamine.

GC(A/T/G/C)GG(A/T/G/C)CTIGT(A/T/G/C)AA-3′] was designed from the amino acid sequence Gln-Ala-Gly-Leu-Val-Leu of a part of N-terminal sequence determined by Edman degradation and then a DNA fragment (~500 bp) was amplified from phage DNA with primers BPL4-NDF1 and T7 (in the lambda ZAP vector) under the following conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min, and then final extension at 72°C for 10 min. This amplified DNA fragment was cloned into a QIAGEN PCR Cloning kit (Qiagen, Valencia, CA, USA) and sequenced. Second, specific reverse primer [BPL4-SF1, 5′-ATAGATATCAGGGTGTGCTG-3′] was designed according to the cDNA sequence and then another DNA fragment was amplified from the phage DNA with primers BPL4-SF1 and SK (in the lambda ZAP vector) under the same conditions as described above. The nucleotide sequences of both polymerase chain reaction (PCR) products were assembled and complete sequence of BPL-4 cDNA was obtained.

Computational sequence analysis

Nucleotide and amino acid sequence homology searches and comparison were carried out using BLAST on GenBank (http://www.ncbi.nlm.nih.gov/genbank), EMBL (http://www.ebi.ac.uk/embl), PDB (http://www.rcsb.org/databases.html), and Uniprot (http://www.uniprot.org). Post-translational modification of protein was identified using the CBS prediction server (http://www.cbs.dtu.dk/services). Secondary structure was predicted using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred). Multiple alignment of protein sequence was performed using BioEdit program (ver. 7.0.9.0) with BLOSUM62 matrix.

RESULTS

Purification of BPL-4

We used two-step elution method to purify BPL-4 because *B. plumosa* has three lectins (Bryohealin, BPL-3, and BPL-4), which could bind to the same sugars, GlcNAc and GalNAc. When the crude extract of *B. plumosa* was loaded to GalNAc affinity column and eluted with a different sugar, GlcNAc BPL-3 and BPL-4 came out first and Bryohealin was eluted later (Fig. 1A). SDS-PAGE result of this first elute showed two protein bands, one strong band at 11.5 kDa and another faint band at 12.8 kDa (Fig. 1B). The faint band on the top of BPL-3 looked like an artifact because two bands located very closely on the gel and the protein band of BPL-3 was much stronger and thicker than that of BPL-4 (Han et al. 2011). However, BPL-3 and BPL-4 were separated on the 2-DE gel (Fig. 1C) and CAF-MALDI analysis of BPL-4 showed that it had different amino acid sequence with BPL-3 (Fig. 2).

Molecular cloning

The N-terminal amino acid sequence of BPL-4 was determined using Edman degradation method; Thr-Gln-Ala-Gly-Leu-Val-Asn-Val-Arg-Gly (BPL4-NT1, Fig. 3). To obtain more sequence information, the lectin was cleaved with trypsin and the resulting fragments were subjected to CAF-MALDI sequencing. The unique sequences Val-[Asp or Asn]-Pro-Glu-[Asp or Asn]-Val-Thr-Pro-Glu-[unknown]-Arg and Thr-[Gln-Ala or Pro-Thr]-Gly-[Asp or Asn or Leu]-Val-[Asp or Asn or Leu]-Val-Arg (Figs 2 & 3) were obtained. The parent masses of isolated peptides were 1,583 and 957 Da, respectively (Fig. 2). The degen-

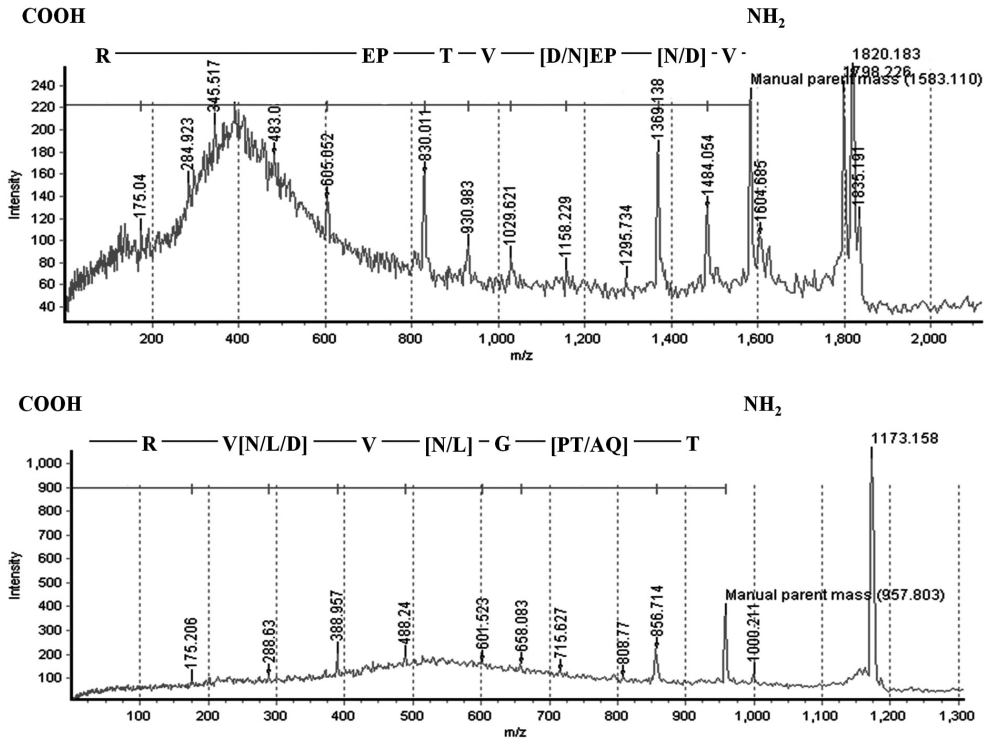

Fig. 2. Amino acid sequences obtained from chemically assisted fragmentation-matrixassisted laser desorption / ionization (CAF-MALDI) mass spectrometry.

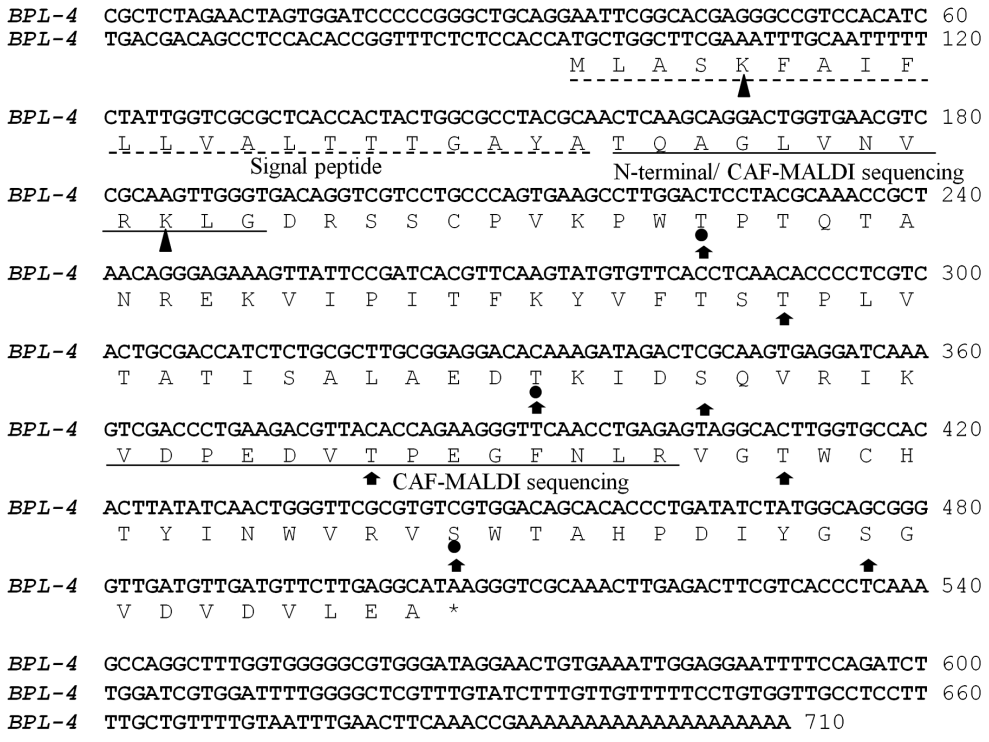

Fig. 3. Full cDNA sequence and deduced amino acid sequence of BPL-4. Underlines, amino acid sequences determined by Edman degradation method and chemically assisted fragmentation-matrix-assisted laser desorption / ionization (CAF-MALDI) sequencing; dashed line, signal peptide; filled triangle, glycate site; filled circles, Yin-Yang sites; arrows, phosphorylation sites; asterisk, termination codon.

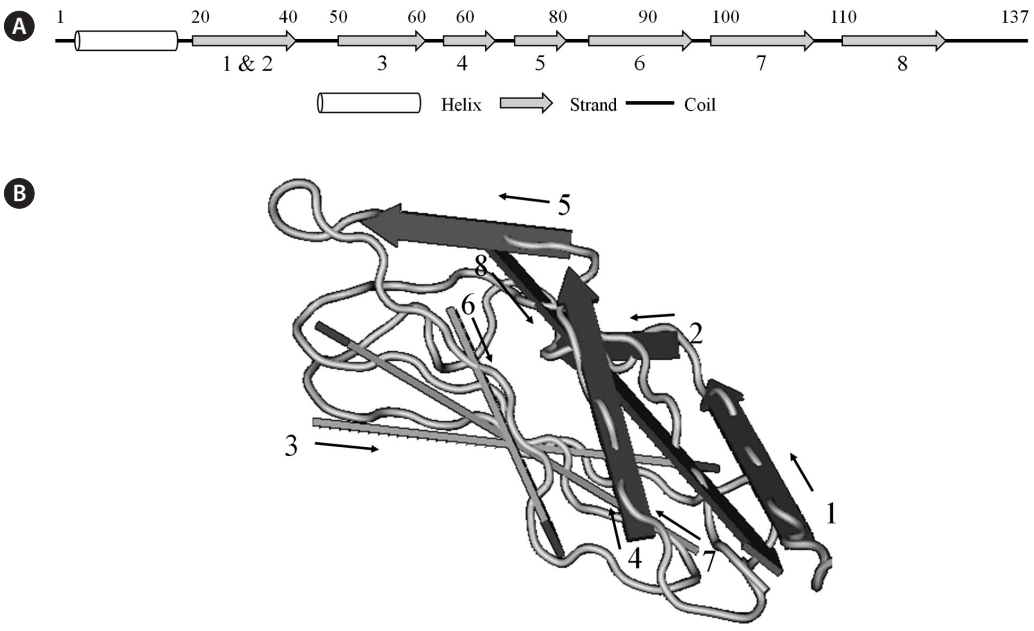

Fig. 4. Analysis of secondary structure of BPL-4 using PSIPRED prediction program (http://bioinf.cs.ucl.ac.uk/psipred). (A) Predicted secondary structure of BPL-4. (B) Tertiary structure of the subunit of H lectin (Conserved domain database [CDD] number, pfam09458). The number and position of ß-strand wall structure of BPL-4 match with those of H lectin.

erate primers for further molecular characterization of BPL-4 were designed from above amino acid sequences.

Full cDNA sequence (710 bp) of BPL-4 was obtained using PCR method based on the degenerate primers of the N-terminal amino acid sequence and specific primers from partial cDNA sequence. The BPL-4 cDNA consisted of 414 bp of open reading frame (ORF) and 203 bp / 93 bp of 3′ / 5′ untranslated regions. The deduced amino acid sequence of BPL-4 contained two known internal tryptic peptide sequences and N-terminal amino acid sequence obtained from the protein. The calculated mass of tryptic digested peptides agreed well with CAF-MALDI data (Figs 2 & 3).

Molecular properties of BPL-4

The protein consisted of 137 amino acids including N-terminal signal peptide (Fig. 3). Molecular weight without the signal peptide was calculated to 12.9 kDa and the isoelectric point was predicted to p*I* 6.04. According to the analysis of signal peptide using SignalP program, a 21 amino acid signal peptide with a cleavage site between Ala^{21} and Thr^{22} was identified. The analysis of deduced amino acid sequence using TargetP program (CBS Prediction Servers, URGI, Unité de Recherche Génomique-Info, http://urgi.versailles.inra.fr) showed that BPL-4 might be a secretory protein targeted to the vacuole (TargetP prediction value, 0.794).

Two possible glycate sites (Lys^{5} and Lys^{31}; score, 0.854 and 0.745) were predicted by the calculation with web-based prediction program (CBS Prediction Servers, http://www.cbs.dtu.dk/services), but the weak potential value (<0.593) of BPL-4 suggested that there was no glycosylation site. During the analysis of Yin-Yang and phosphotylation sites, 3 possible Yin-Yang sites (Thr^{44}, Thr^{80}, and Ser^{118}) and 8 phosphorylation sites (Thr^{44}, Thr^{66}, Thr^{80}, Ser^{84}, Thr^{96}, Thr^{106}, Ser^{118}, and Ser^{128}) were found in BPL-4 sequence (Fig. 3). Analysis of secondary structure of BPL-4 using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred) showed α-helix structure at the N-terminus and following eight ß-stranded wall structures. The predicted secondary structure of BPL-4 conformed well to the tertiary structure of the subunit of H lectin (*Helix pomatia* lectin, Conserved domain database, CDD number, pfam09458). The number and position of ß-strand in BPL-4 matched with those of H lectin (Fig. 4).

BPL-4 showed significant similarity with BPL-3, but it was clearly a different protein (Table 1, Fig. 5). BPL-3 and BPL-4 shared two well conserved domains including putative active domain of H lectin group, which is also an GalNAc and D-galactose binding lectin (Fig. 5). The similarity values ranged from 40 to 51% with other H lectins.

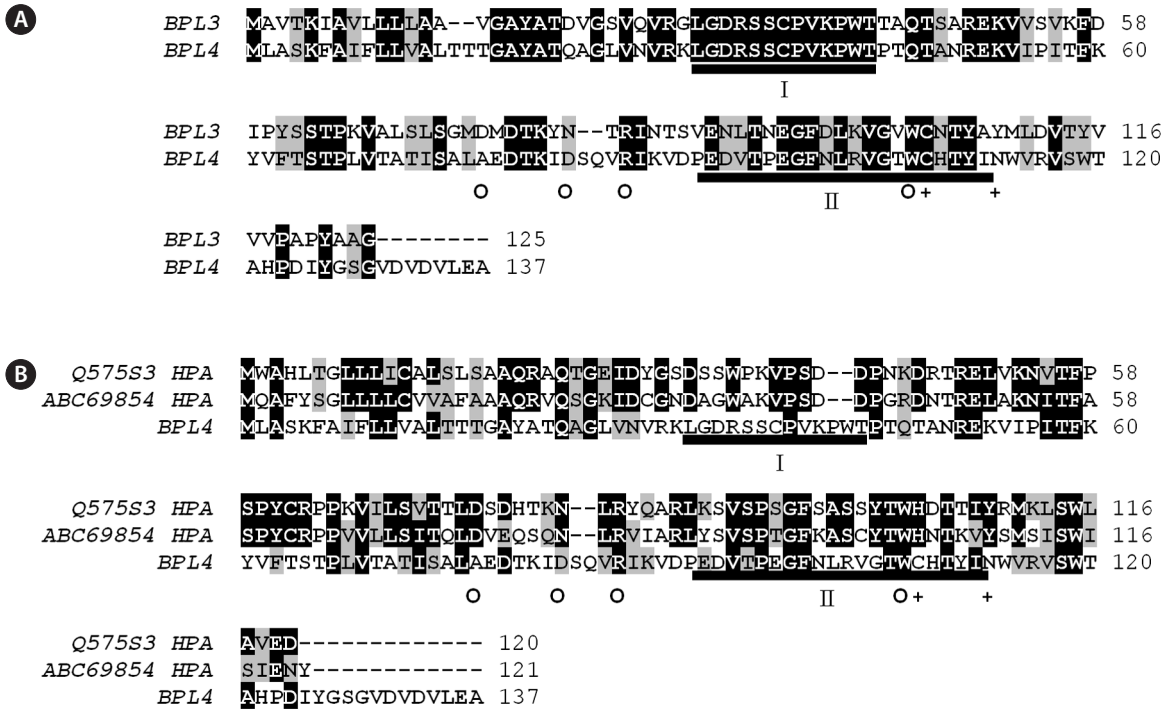

Fig. 5. Sequence comparison of BPL-4 with BPL-3 (A) and related H group lectins (B). Two conserved domains between BPL-3 and BPL-4 were underlined. Similarities between the sequences are shown with shaded background. Predicted amino acids involved in hydrogen bonding through their side chain (o) and hydrophobic contracts to the ligand (+) are marked.

The amino acids involved in hydrogen bonding through their side chain were identical between *Helix pomatia* agglutinin (HPA) and BPL-4, but the hydrophobic contact amino acids were less conserved (Fig. 5B).

DISCUSSION

Possible involvement of lectins has been suggested for the protoplast regeneration of the marine coenocytic green alga *B. plumosa* (Pak et al. 1991, Kim et al. 2001). A lectin-carbohydrate complementary system was proposed for the aggregation mechanism of the extruded cell organelles in seawater (Kim et al. 2006). So far, three lectins (Bryohealin, BPL-2, and BPL-3) have been isolated in this species (Yoon et al. 2008, Han et al. 2010, 2011). The presence of additional lectin(s) specific to the sugars, GalNAc and GlcNAc was suggested because 1) the aggregation of cell organelles occurred at 2 different optimum pH values and 2) some residual lectin activity was remained in the column after purification of Bryohealin and BPL-3 (Han et al. 2011).

In this study, we purified another lectin, BPL-4, possibly involved in the protoplast formation of *B. plumosa.*

Table 1. Sequence identity and similarity between BPL-4 and related proteins

Species / Protein	GenBank accession No.	Identity (%)	Similarity (%)
BPL-3	-	44.5	60.6
Helix pomatia (Roman snail) / H lectin	Q575S3	24.6	45.0
Helix pomatia (Roman snail) / H lectin	ABC69854	21.2	40.0
Sinularia lochmodes (soft coral) / galactose binding lectin	BAD97420	21.0	49.0
Sagittula stellata E-37 (seawater bacterium) / hypothetical protein	ZP_01744098	29.2	51.0
Dictyostelium discoideum (soil-living amoeba) / disc-i(5.6) gene, H lectin	AAA33196	19.0	50.0

The lectin was isolated using GalNAc affinity chromatography and two-step elution by a different sugar, GlcNAc 2-DE gel electrophoresis was used to separate BPL-4 from BPL-3. Although BPL-3 and BPL-4 showed similar sugar specificity with Bryohealin, the binding affinities to the complementary sugars were different as evident from the chromatography results (for N-acetyl-D-glucosamine, Bryohealin << BPL-3 = BPL-4; for N-acetyl-D-galactosamine, BPL-3 = BPL-4 << Bryohealin). Possessing several different types of lectins specific to the same sugars would be helpful for the successful aggregation of cell organelles in ever changing seawater environment.

Although BPL-3 and BPL-4 showed the same sugar specificity and similar primary structure they are clearly different lectins. The sequence similarity of two lectins was just 60.6% which was slightly higher than that (50%) between BPL-4 and an H lectin from amoeba *Dictyostelium discoideum*. The sequence identity between BPL-3 and BPL-4 was 44.5%, which is too small to regard them as isoforms. However, two lectins shared two well conserved domains including putative active domain of H lectin group (*Helix pomatia* lectin group), which is also an GalNAc specific lectin (e.g., Koike et al. 2004). The amino acids involved in hydrogen bonding through their side chain were identical between BPL-4 and H lectin. BPL-3 and BPL-4 shared common putative sugar binding domain of H group lectin (domain II) but the sequence similarity of two lectins at the binding domain was lower than that of domain I which showed 100% match. Analysis of secondary structure of BPL-4 using prediction software, PSIPRED, showed α-helix structure at the N-terminus and following eight ß-stranded wall structures. The predicted secondary structure of BPL-4 conformed well to those of the tertiary structure of H lectin. The number and position of ß-strand in BPL-4 matched with those of H lectin. However, the results from protein crystallography are necessary to confirm this. Further comparative analysis on the tertiary structure of BPL-3 and BPL-4 may reveal interesting information of the binding domains of H lectin group.

The evolutionary origin of BPL-4 is very interesting. Although BPL-3 and BPL-4 share many molecular characteristics in common, the analysis of their primary structure suggested that two lectins might have evolved separately. BPL-4 does not belong to any category of the terrestrial plant lectins just like BPL-3. But these two lectins show similarity with H lectins, which appear in octocoral and invertebrate animals and play a role in immunity and can bind to GalNAc and D-galactose (Koike et al. 2004, Jimbo et al. 2005, Sanchez et al. 2006). H lectins have been used as a research tool in the various fields of biochemical and medical sciences, as marker for tumor cells, for the antimicrobial and antiviral activities and hormone binding ability (Osborne and Brooks 2006, Sanchez et al. 2006, Bogoeva and Russev 2008). BPL-3 and BPL-4 are different from H lectin group in having slightly different sugar-binding specificity: H lectin show specificity to GalNAc and D-galactose, but BPL-3 and BPL-4 bind to GalNAc and GlcNAc not to D-galactose. The difference of amino acids in binding domains of these lectins may explain the difference of sugar binding specificity and hemagglutinating activity between BPL-3 and other H type lectins (Han et al. 2011).

Accumulating evidences show that algal lectins are involved in many biological processes, including wound response and sexual reproduction (Maier and Müller 1986, Kim et al. 1995, Ross et al. 2005, Yoon et al. 2008). However, the evolutionary origin of algal lectins is still in question. The result from molecular studies on algal lectins failed to show any direct relationship between algal and higher plant lectins (Yoon et al. 2008, Han et al. 2011). Instead, three lectins isolated from *B. plumosa* showed similarity to animal lectins (Bryohealin to F-type lectins and BPL-3 and BPL-4 to H lectin group). Considering the long evolutionary distance between this green alga and invertebrate animals, three lectins from *B. plumosa* may provide an interesting example of the parallel evolution across species boundaries.

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