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Characterization of carbohydrate combining sites of Bryohealin, an algal lectin from *Bryopsis plumosa*

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Abstract Bryohealin is a lectin involved in the wound-healing process of the marine green alga *Bryopsis plumosa*. In the previous purification study, it has been shown that lectin was composed of two identical subunits of 27 kDa, cross-linked by disulfide bond, and showed binding specificity to *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine (GlcNAc and GalNAc, respectively). To determine if the lectin recognize the two different sugars at the same binding domain, the carbohydrate binding sites of Bryohealin was analyzed using chromatography and chemical modification methods. Results showed that the same binding site of the lectin was responsible for the recognition of two sugars, GalNAc as well as GlcNAc. Chemical modification studies showed that hemagglutinating activities of Bryohealin were not affected by modification of histidine, tryptophan, aspartic acid, and glutamic acid. When arginine residues were modified with 1,2-cyclohexanedione, the activity of Bryohealin rapidly decreased. The sugar binding sites remained intact when the lectin was treated with inhibitory sugars (0.2 M GalNAc and/or GlcNAc) prior to 1,2-cyclohexanedione treatment. The sugar binding domain of Bryohealin was predicted from the MALDI-TOF analysis and the full cDNA sequence of the lectin gene.

Keywords *Bryopsis plumosa* · Algae · Bryohealin · Lectin · *N*-acetyl-D-galactosamine · *N*-acetyl-D-glucosamine · Sugar binding site · Chemical modification

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Introduction

Marine coenocytic green algae possess unique ability to regenerate new functional cells from small droplets of protoplasm extruded in seawater (Pak et al. 1991; Kim et al. 2001, 2006; Klochkova et al. 2005; Yoon et al. 2008). To form viable protoplasts, aggregation of all necessary cell organelles in seawater after disruption of the original cell is the most important event. In several coenocytic algal species, a possible involvement of lectins in this step was previously suggested (Pak et al. 1991; Kim et al. 2001; Klotchkova et al. 2003). Recently, a lectin involved in this process was isolated and characterized from *Bryopsis plumosa* and named as Bryohealin (Kim et al. 2006; Yoon et al. 2008).

Algal lectins differ from higher plant lectins in a variety of properties: algal lectins have lower molecular masses (ranging from 4.2 to 43 kDa) than most of higher plant lectins and have higher affinity to complex oligosaccharides and glycoproteins than to monosaccharides (Shiomi et al. 1979, 1981; Kamiya et al. 1980, 1982; Hori et al. 1990; Calvete et al. 2000; Nagano et al. 2002). Most marine algal lectins do not require divalent cations for their biological activity (Rogers and Hori 1993). They occur mainly in monomeric forms and have a high proportion of acidic amino acids, with isoelectric points from 4 to 6 (Shiomi

et al. 1981; Hori et al. 1990; Oliveira et al. 2002). Although the number of isolated lectins from algae increased remarkably for last decades and many of them have been chemically characterized (Yoon et al. 2008), there are few studies on the structure of sugar binding domains of algal lectin.

The structural analysis of sugar binding domain of a lectin is essential to understand the biological and chemical properties of the protein, and the identification of specific amino acid residues within the sugar binding domains is also necessary to develop a drug delivery system using lectins. Molecular structure analysis of plant and animal lectins were studied using crystallography (Beisel et al. 1999), nuclear magnetic resonance (NMR) technique (Hemmi et al. 2009), and chemical modification methods (Privat et al. 1976; Patnjali et al. 1984; Sultan et al. 2004). However, we could not find any reports of those studies on algal lectin except Ziólkowska et al. (2006, 2007). In this study, we analyzed the structure and properties of sugar binding sites of Bryohealin and predicted possible active sites using chemical modification and chromatography methods.

Materials and methods

Vegetative plants of *Bryopsis plumosa* were collected from Kachon, the southern coast of Korea, and maintained as unialgal cultures in autoclaved IMR medium (pH 8.0, Kim et al. 2006). Algae were grown at 20°C in a 16:8 L/D cycle with 20 μmol photon m^{-2} s^{-1} provided by cool white fluorescent lamps.

Purification of Bryohealin

The purification of Bryohealin was performed according to the methods described in the previous paper (Kim et al. 2006) with some modification. Briefly, *B. plumosa* (25 g) was ground in liquid nitrogen with mortar and pestle and transferred to 250 mL of 1x potassium phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 7.4) including 1 mM phenylmethanesulfhonylfluoride as a protease inhibitor. Homogenate was incubated at 4°C, stirred for 2 h, and centrifuged for 20 min at 12,000×*g* to remove cell debris. The supernatant was filtered using 0.45-μm sterile filter and loaded on GalNAc-agarose affinity column (5 mL, Sigma). The column was washed with 10 volumes of 1x PBS and Bryohealin was eluted using 0.1 M GalNAc (Sigma). Each fraction was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and hemagglutinating test and active pure fractions were pooled.

Hemagglutinating assay

A type of human blood was obtained from healthy donor. Erythrocytes were washed four times in 10 volumes of saline by centrifugation at 2,000×*g* for 10 min at 4°C. Erythrocytes were resuspended into 1x PBS as a 3% (*v*/*v*) suspension. Agglutination test were performed in a 96-well microtiter U bottom plate by mixing serial twofold dilution procedure. The proteins were diluted with PBS or suitable buffer and then 25 μL of each was mixed with 25 μL of a 3% (*v*/*v*) erythrocyte. The mixture was allowed to stand at room temperature for 30 min and then hemagglutinating activity (HA) was measured.

Inhibition of lectin-induced hemagglutinating by carbohydrates

Purified lectin (25 μL, 4 HA) was reacted with an equal volume of various concentrations of sugars (GlcNAc, GalNAc, L-fucose, D-galactose, D-glucose, D-mannose, α-methyl-D-mannose, α-lactose, *N*-Acetyl-D-lactosamine, β-D-Gal-(1→3)-D-GalNAc, β-D-Gal-(1→3)-D-GlcNAc) at room temperature for 30 min and then half of the mixture was removed. After pre-incubation, 25 μL of 3% A type erythrocyte was added to the mixture and the HA inhibition was observed after 30 min.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS-PAGE was carried out according to the method of Laemmli (1970) using a 15% gel with 2-mercaptoethanol. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

Determination of protein concentration

Protein concentration was determined using modified Bradford assay (Bradford 1976). Standard curve was prepared with various concentrations of bovine serum albumin (BSA). BSA or samples and Bradford reagent was mixed in 96-well microplate and incubated for 10 min at room temperature. Absorbance was measured at 575 nm using an ELISA reader.

Characterization of binding sites

The experimental scheme is shown in Fig. 1. Optimum sugar concentration needed for the inhibition of Bryohealin was determined by chromatography. Bryohealin (2 μM, 500 μL) was loaded on GalNAc and GlcNAc-agarose affinity column (1.5 mL) and washed with 10 volumes of 1x PBS after incubating at 4°C for 2 h. Bryohealin was eluted using various concentrations of sugars (0.05, 0.1,

0.2, and 0.4 M), and eluted fractions were confirmed using SDS-PAGE.

Bryohealin pre-incubated with 0.2 and 0.4 M of two different sugars (GalNAc and GlcNAc) was loaded on opposite column (GalNAc pre-incubated–GlcNAc affinity column, GlcNAc pre-incubated–GalNAc affinity column) and then incubated at 4°C for 2 h (see Fig. 1). Columns were washed with 1x PBS and unbound materials were analyzed using SDS-PAGE.

Chemical modification of Bryohealin

Possible amino acids were selected using alignment of other GlcNAc or GalNAc specific lectins. Selected amino acids were sequentially modified using chemical modification methods. After modification, decrement of lectin activity was defined using hemagglutinating assay.

Modification of tryptophan using NBS. Bryohealin was dialyzed in 50 mM sodium acetate buffer, pH 4.0 (3x, 1 L), and protein concentration was adjusted to 2.03 μM using dialysis buffer. *N*-bromosuccinimide (NBS) stock solution was prepared in 5 mM by dissolving into double distilled water. NBS stock solution (1 to 40 times concentration compared with protein) was added to the protein solution. The mixtures were incubated at room temperature for 5 min, and 50 μL of portion was used to measuring hemagglutinating activity. The modified tryptophan residues were determined spectrometrically at 280 nm using the equation with extinction coefficient (5,500 M^{-1} cm^{-1}, Spande and Witkop 1967).

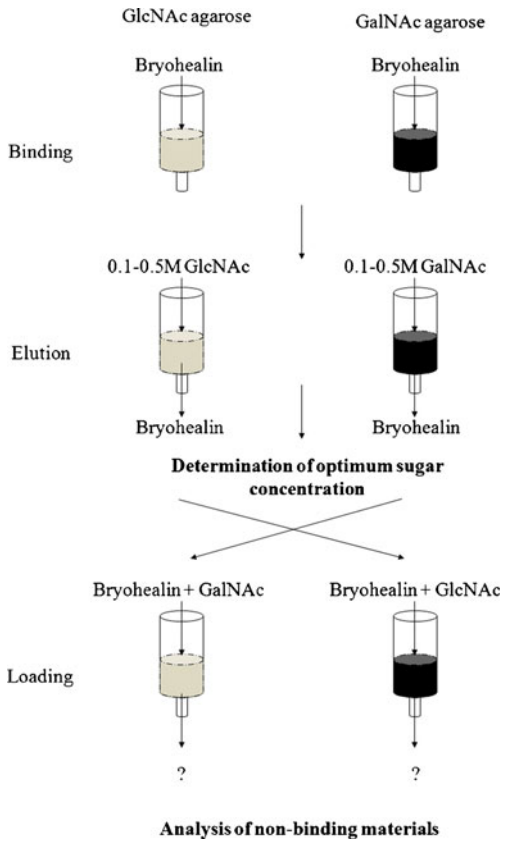

Fig. 1 Scheme of chromatographic methods for the determination of binding sites

Modification of histidine using diethylphyrocarbonate. Histidine modification was performed according to Miles (1977). Bryohealin was dialyzed against 0.1 M potassium phosphate buffer, pH 6.5 (3x, 1 L). Modified histidine residues were determined by changes of absorbance at 240 nm using molar absorption coefficient (3,300 M^{-1} cm^{-1}).

Modification of tyrosine using NAI. N-acetylimidazole (NAI) was added to the protein solution in 0.5 and 5 mM of final concentration and incubated at room temperature for 1 h (Riordan et al. 1965). After reaction, hemagglutinating activity was measured and modified tyrosine residues were calculated by increment of absorbance at 278 nm using molar absorption coefficient (1,160 M^{-1} cm^{-1}).

Modification of carboxyl group using glycine methyl ester. Modified carboxyl group was determined by analysis of amino acid composition after hydrolysis following the procedure described by Carraway and Koshland (1972).

Modification of arginine residues using CHD or phenylglyoxal. Bryohealin was dialyzed against 0.2 M sodium borate buffer at pH 8.0 and then protein concentration adjusted to 3.8 μM using dialysis buffer. 1,2-Cyclohexanedione (CHD) was added to the protein solution in 50 mM of final concentration and incubated at room temperature under nitrogen gas condition. Every 30 min, 50 μL of the reaction mixture was made aliquot and activity was measured. Modified residues were determined by analysis of amino acid composition. For modification by phenylglyoxal, Bryohealin was dialyzed against 0.1 M sodium bicarbonate buffer at pH 8.3. Phenylglyoxal (2, 5, 10, and 50 mM of final concentration) was added to dialyzed Bryohealin and incubated at room temperature for 2 h. Excess reagent was removed by gel filtration (Sephadex G-25, 0.5×25 cm, Amersham Pharmacia) with 1.5 mL min^{-1} of flow rate. Modified residues were determined by spectrometry at 250 nm, and the remaining hemagglutinating activity was observed.

Protection of Bryohealin by GalNAc. Dialyzed Bryohealin (0.1 M sodium bicarbonate buffer, pH 8.3, 4 μM) was incubated with 0.15 M of GalNAc for 30 min to protect binding sites. The protein was reacted with excess phenyl-

Table 1 Purification table of Bryohealin (hemagglutinating activity was defined using A-type human blood)

	Total protein (mg)	Total activity (titer)	Specific activity (titer mg^{-1})	Recovery	Purification fold
Crude extract	17.6	144,000.0	8,181.8	100	1
Affinity chromatography	0.5	8,960.0	17,920.0	6.2	2.2

glyoxal (500 times) or CHD for 3 h and modified amino acids measured at 250 nm after removal of phenylglyoxal.

Analysis of amino acid composition

The analysis of amino acid composition of modified or native Bryohealin was performed by the Pico-Tag method (Kim et al. 2006; Waters, USA) from Peptide Library Support Facility in POSTECH (Pohang, Kyoungbuk, Korea).

HPLC analysis of peptides

Protein samples were prepared in 50 mM ammonium bicarbonate buffer at pH 8.0. Protein solution was incubated at 90°C for 5 min with 5 mM of DTT and cooled to room temperature. Iodoactamide solution was added in 10 mM and incubated at room temperature for 20 min. Protein was digested by addition of trypsin (50:1, *w*/*w*, protein/trypsin) and incubation for 12 h at 37°C.

Tryptic digested peptides were lyophilized using vacuum lyophilizer (Hanil Bioscience, Korea). Dried samples were dissolved with 20 μL of double distilled water and then centrifuged at 12,000×*g* for 20 min to remove insoluble materials.

Peptide samples were injected and analyzed by HPLC (GILSON, USA) using a reverse phase column, Luna-C18 (2) (5 μ, 100 Å, 150×4.6 mm, Phenomenex). Solvent gradient was performed in the following procedure: water and acetonitrile (0.05% TFA); 5% acetonitrile at 0 min, 5–10% for 5 min, 10–20% for 10 min, 20–35% for 8 min, 35% for 12 min, 35–65% for 10 min, 65–100% 5 min, and 100% 20 min with 1 mL min^{-1} flow rate. Absorbance chromatogram was recorded at a wavelength of 214 nm. Differentially appearing peaks were collected and solvent were removed using lyophilizer. Dried samples were stored at −20°C until use.

MALDI-TOF analysis

Collected peptides from HPLC was analyzed for peptide mass using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Applied Biosystem 4700 proteomic analyzer) by Peptide Library Support Facility in POSTECH (Pohang, Kyoungbuk, Korea) based on the manufacturer's method. Each peptide which differentially appeared on chromatogram was embedded in a α-cyano-4-hydroxycinnamic acid matrix. The samples were allowed to air dry at room temperature and then subjected to MALDI-MS analysis. MALDI mass spectrometer used a 200-Hz frequency tripled Nd:YAG laser operating at a wavelength of 355 nm. The calibration was performed using Applied Biosystems 4700 Proteomics Analyzer Mass Standards Kit that included 6 peptides (Part No. 4333604, Applied Biosystems, USA).

Results

Purified Bryohealin was prepared using the methods by Kim et al. (2006) for cross-binding experiment and chemical modification study. About 0.5 mg of the lectin was obtained from 25 g of *B. plumosa* plants (Table 1), and the specific activity of the eluted lectin increased 2.1-fold as compared with that of the crude extract (Fig. 2). Sugar inhibition test showed that HA of the purified fraction was inhibited by 82.5 mM of GlcNAc or 41.3 mM of GalNAc. To profile the carbohydrate combining sites, similar monosaccharides and disaccharides containing *N*-acetyl group as well as GlcNAc and/or GalNAc moieties were used for inhibition experiment. None of them showed HA inhibition up to 250 mM (Table 2).

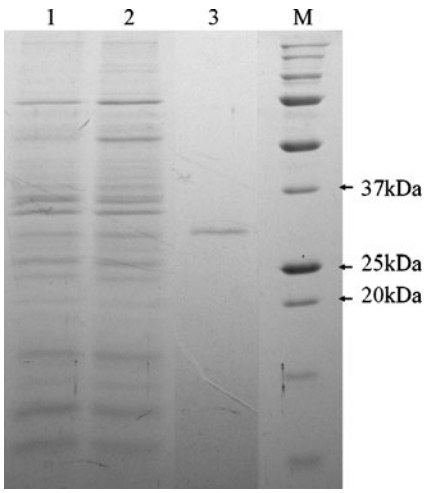

Fig. 2 Purification of Bryohealin from *B. plumosa*. SDS-PAGE analysis. *Lane 1* Crude extract. *Lane 2* Flow-through from GalNAc affinity chromatography. *Lane 3* isolated Bryohealin. *M* molecular weight marker (Bio-Rad, wide range pre-stained marker)

Table 2 Inhibition by mono- and disaccharides of the hemagglutinating activity of Bryohealin

	Minimum concentration (mM)
GlcNAc	82.5
GalNAc	41.3
L-fucose	–[a]
D-galactose	–
D-glucose	–
D-mannose	–
α-Methyl-D-mannose	–
α-Lactose	–
β-D-Gal-(1→4)-D-GlcNAc (*N*-Acetyl-D-lactosamine)	–
β-D-Gal-(1→3)-D-GalNAc (Thomsen-Friedenreich disaccharide)	–
β-D-Gal-(1→3)-D-GlcNAc	–

The titer and dilution used were fractions that showed four hemagglutinating activity

[a] No inhibition up to 250 mM of sugar treatment

Cross-binding experiment was performed to determine if the same sugar binding site of Bryohealin could recognize two different sugars. Purified lectin was loaded again to GlcNAc-agarose column and eluted using two different sugars, GlcNAc and GalNAc (Fig. 3). Over 95% of the lectin was eluted from the column with 0.2 M of GlcNAc.

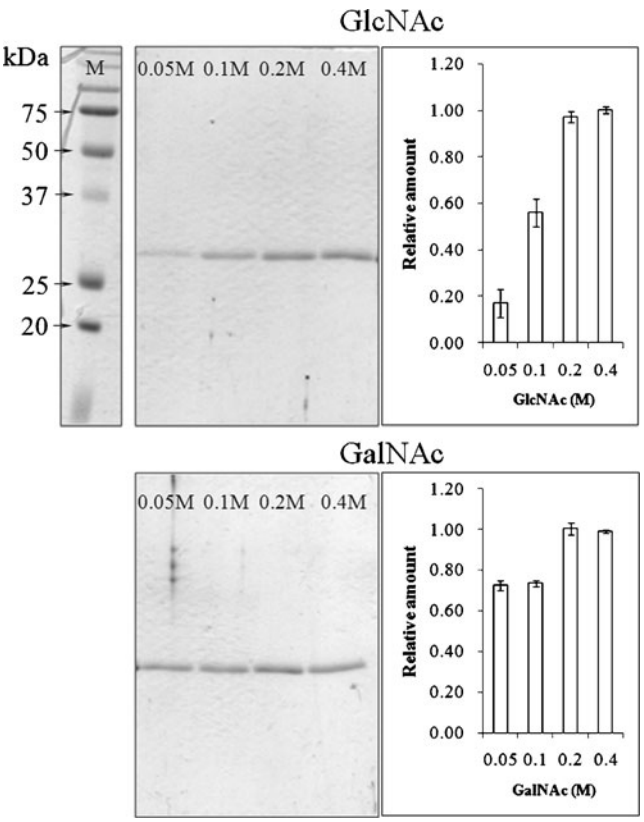

Fig. 3 Determination of optimum sugar concentration to block all of binding sites. SDS-PAGE analysis. *Lane M* Standard molecular weight marker. *Lanes 1–4* Elution of Bryohealin using different concentration of sugar

Although the lectin was bound to the GlcNAc column, it was eluted more easily with the sugar GalNAc. Over 90% of the lectin was eluted from the column with 0.05 M of GalNAc (Fig. 3).

To analyze the sugar binding preference, Bryohealin was pre-incubated with 0.2 M of each sugar and loaded to the opposite agarose column, respectively (Fig. 4). When Bryohealin was pre-incubated with GalNAc and loaded to the GlcNAc agarose column, most of the proteins did not bind to the column and came out as flow-through (Fig. 4). However, when the lectin was pre-incubated with GlcNAc and loaded to the GalNAc agarose column, most of the protein bound to the column (Fig. 4).

In order to determine which amino acids are incorporated to the carbohydrate binding sites, we conducted chemical modification of six different amino acids (tryptophan, histidine, tyrosine, arginine, aspartic acid, and glutamic acid) which was estimated to be involved in the binding sites from the sequence data (Yoon et al. 2008).

When the Bryohealin was treated with 50 mM of CHD, the activity was decreased to 50% after 30 min of incubation, and >95% of the activity disappeared after 2 h. Half of modified arginine residues were observed by amino acid analysis at removal of ~95% lectin activity (Fig. 5a, solid squares). The protein was incubated with 0.2 M GalNAc for 30 min and treated with CHD. All of the hemagglutinating activity remained after dialysis (Fig. 5a, open squares). Another reagent, phenylglyoxal (PGO), was used for arginine modification to confirm the above experiment. Hemagglutinating activity was decreased to 20% by adding 2 mM of PGO. The increase of PGO concentration up to 50 mM did not change the inhibitory activity much (Fig. 5b, solid squares). When the protein was incubated with 2 mM PGO, absorbance at 250 nm was strongly increased. When the lectin was incubated with 0.2 M GalNAc prior to treatment of 2 mM PGO, the

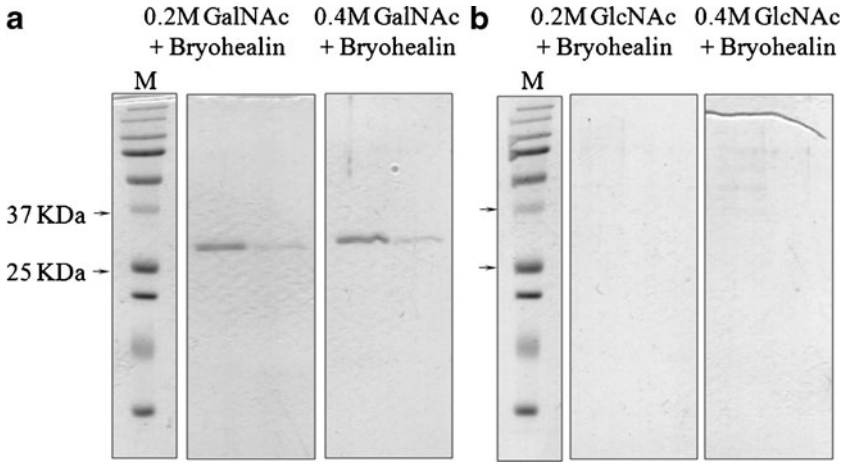

Fig. 4 Analysis of binding sites using chromatographic method. SDS-PAGE analysis of flow-through fractions. *Lane M* Standard molecular weight marker. **a** Flow-through fraction from GlcNAc-agarose after pre-incubation of Bryohealin with 0.2 or 0.4 M GalNAc. **b** Flow-through fraction from GalNAc-agarose after pre-incubation of Bryohealin with 0.2 or 0.4 M GlcNAc

absorbance at 250 nm decreased again (Fig. 6). The changes of amino acid composition of Bryohealin after the treatment of CHD and PGO are summarized in Table 3. When the arginine modification reagents, CHD and PGO, were applied to the lectin pre-incubated with 0.2 M GalNAc, most of the arginine component in the protein remained intact (Table 3).

Modification of other amino acids, histidine, tyrosine, and carboxyl group (aspartic acid and glutamic acid), did not change the hemagglutinating activity of the lectin. As *N*-bromosuccinimide tryptophan modification reagent caused total degradation of the protein, we did not consider the inhibitory effect as a result of the modification of the amino acid (Table 4).

To determine the exact site of modified arginine in the protein, the peptides produced from tryptic digestion of Bryohealin were analyzed before and after arginine modification using HPLC system. The peptides containing modified arginine would disappear in HPLC profile because the modified arginine residues would not be digested by trypsin. HPLC analysis of peptides before and after arginine modification showed that five peaks of peptides disappeared after modification (Fig. 7, solid triangle). Three candidate peaks (Fig. 7, star marks) turn out to be a noise during HPLC analysis. Two major peaks which disappeared after modification were analyzed using MALDI-TOF mass spectrometry (Fig. 7, solid triangles). Mass and amino acid sequence of the first peptide was 1,040.3 Da (EDCCWDR, Cys_CAM), and the second peptide was 928.4 Da (LHNFEIR). Two peptides were located side by side in the protein (first peptide, 175–181th amino acid; second peptide, 182–188th amino acid).

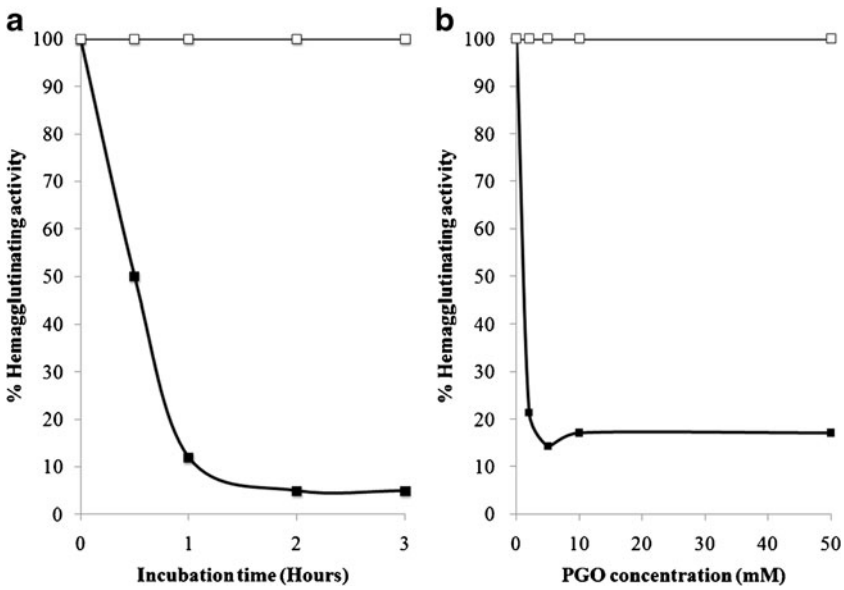

Fig. 5 Effect of 1,2-cyclohexanedione (*CHD*) and phenylglyoxal (*PGO*) on the hemagglutinating activity of Bryohealin. *Solid squares* indicate hemagglutinating activity after modification, and *open squares* is after protection of arginine modification using GalNAc

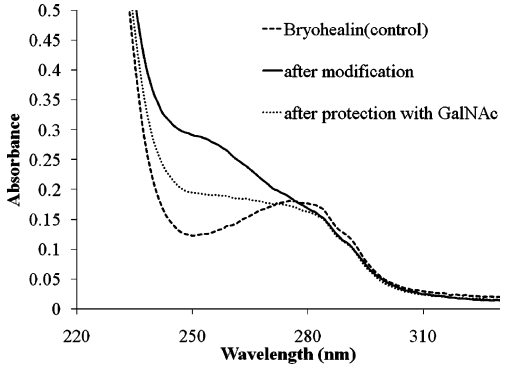

Fig. 6 Light absorbance profile of Bryohealin after the treatment of phenylglyoxal (*PGO*). *Bottom line* natural Bryohealin; *middle line bottom* Bryohealin treated with 0.2 M GalNAc and 2 mM PGO; *top line* Bryohealin treated with 2 mM

Discussion

Our cross-binding experiment showed that Bryohealin could recognize two different sugars, GalNAc and GlcNAc, using the same sugar binding site. The lectin bound to GlcNAc column was easily eluted with GalNAc, and pre-incubation of the lectin with GalNAc could block binding of the protein to GlcNAc column. The lectin showed much higher preference to GalNAc. Even when the binding site was saturated with GlcNAc, the lectin could bind to GalNAc column.

Lectins with similar sugar binding activity to Bryohealin were reported in a mushroom, *Agaricus bisporus* (Carrizo et al. 2006), and a fungus, *Sclerotium rolfsii* (Leonidas et al. 2007). Both lectins bind GalNAc and GlcNAc in different binding sites and obviously with different potency. X-ray structure analysis and molecular biological data showed that there are structural similarity and strong sequence homology between the lectins. However, Bryohealin showed very little (<5%) sequence homology to any of the lectins, suggesting that the carbohydrate combining sites of Bryohealin consisted of different peptides from the fungal lectins.

There are several reports on GlcNAc and/or GalNAc-specific lectins in algae; a GlcNAc-specific lectin has been isolated from *Codium* spp. (Fabregas et al. 1988; Roger et al. 1986; Wu et al. 1997) and some galactose and/or GalNAc-specific lectins were isolated from red algae *Ptilota* spp. (Sampaio et al. 1998, 1999) and *Hypnea* spp. (Nagano et al. 2005). Giraffine, a monomeric lectin isolated

Table 4 Summary of results obtained from the chemical modification studies on Bryohealin

Reagent	Residue modified	Number of residues modified/ percent modification (%)	Agglutination activity after modification
N-bromosuccinimide	Tryptophan	90	–
Diethyl pyrocarbonate	Histidine	50	Unchanged
Glycine methyl ester	Aspartic acid and glutamic acid	40	Unchanged
N-Acetylimidazole	Tyrosine	50	Unchanged
1,2-Cyclohexanedione	Arginine	50	No activity
Phenylglyoxal	Arginine	50	No activity

Table 3 Amino acids composition (percent) of Bryohealin

Amino acid	CHD			PGO		
	Before modification (%)	After modification (%)	After protection of modification (%)	Before modification (%)	After modification (%)	After protection of modification (%)
S	9.23	7.12	10	9.2	8.4	9.2
H	1.73	2.06	1.72	1.7	1.4	1.5
R	3.08	1.54	3.84	2.9	1.4	2.3
T	4.33	4.63	4.14	5.7	7.5	5.4
A	5.19	5.27	5.07	4.6	5.9	4.6
P	3.94	3.6	3.74	3.4	3.0	3.1
Y	2.31	4.63	1.78	3.4	3.0	2.3
V	3.56	3.73	3.8	2.9	3.0	2.3
I	3.46	3.22	3	2.9	4.5	2.3
L	3.94	5.66	3.94	3.4	4.5	3.1
F	2.6	2.19	2.07	2.9	3.0	2.3
K	3.17	4.76	2.32	2.9	3.0	2.3

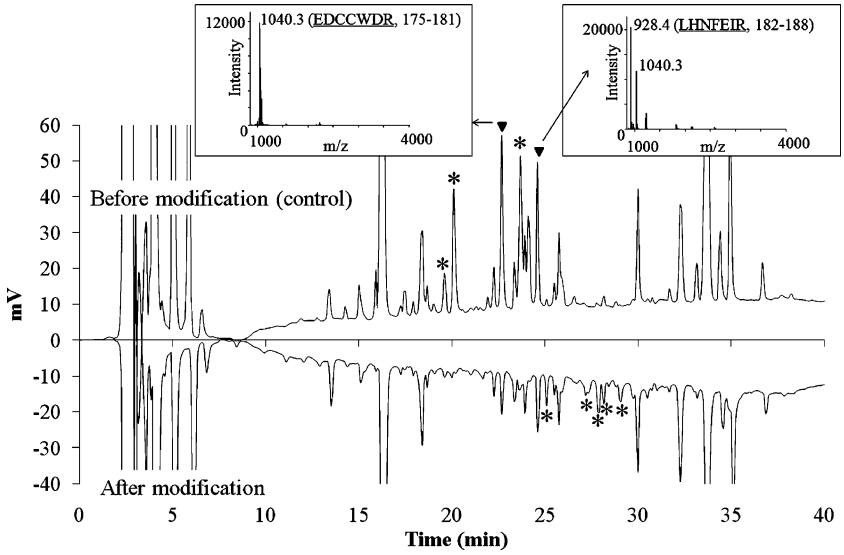

Fig. 7 Isolation and MALDI-TOF analysis of modified peptides. Arginine modification was performed with 50 mM CHD. *Star marks* indicate differently produced peptides comparing between control and modified protein. MALDI-TOF analyzed peptides were marked as *triangles* and MALDI-TOF spectrum was shown in the *boxes*

from *Codium giraffe*, showed similar sugar binding activity to Bryohealin (Alvarez-Hernandez et al. 1999). The agglutinating activity was inhibited by GlcNAc as well as GalNAc and D-raffinose. The monosaccharides showed 15–30 times stronger inhibitory effect than trisaccharide, D-raffinose. The hemagglutinating activity of Bryohealin was strongly inhibited with monosaccharides, but not with any disaccharides up to 250 mM like giraffine. It is interesting because all disaccharides tested contained *N*-acetyl group as well as GlcNAc and/or GalNAc moieties. These results suggest that *N*-acetyl group may not be crucial for the binding activity of Bryohealin. Instead, the hydroxyl group at C3 or C4 would be the combining site for Bryohealin because those hydroxyl groups were removed for disaccharide formation. It is hard to profile the carbohydrate combining sites using indirect evidence from sugar inhibition experiment. Further studies including X-ray structure analysis are necessary to confirm this.

The bioinformatics analysis based on our previous data of full cDNA sequence of Bryohealin (Yoon et al. 2008; Genbank accession no. ACF05191) using Radar program (http://www.ebi.ac.uk/Tools/Radar/) predicted that there are less than three binding domains in each subunit of Bryohealin. These results could be explained by two hypotheses: (1) there is only one sugar binding site at each subunit of Bryohealin which shows specificity to both sugars with strong preference to GalNAc. (2) Two different sugar binding sites are present at each subunit: one for GalNAc and one for GalNAc and GlcNAc, with strong preference to GalNAc. Although cross-binding experiment could not explain which hypothesis is true, it is pretty sure that a sugar binding site in Bryohealin could recognize two different sugars. We assume that the first hypothesis is more probable because most lectins which have multiple binding domains show repeated regions in their cDNA sequence (ex. Tachylectin 2, Beisel et al. 1999; Jacalin-related lectins, Nakamura et al. 2005). Bryohealin did not have any repeated region in its cDNA sequence (Yoon et al. 2008).

Bryohealin showed high sequence homology with the fucolectin family from eel at the conserved domain of C-terminal region where the sugar binding domain of fucolectin exists (Fig. 8; Vasta et al. 2004; Yoon et al.

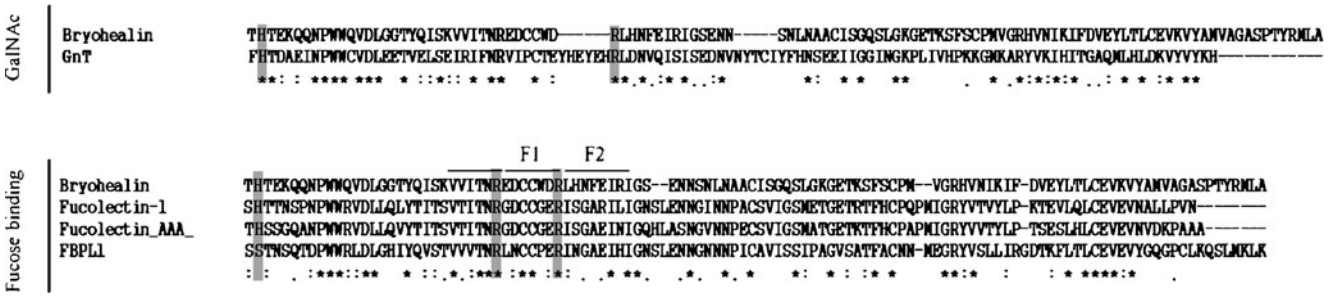

Fig. 8 Multiple alignments of Bryohealin with fucolectin or *N*-acetyl-galactosaminetransferase (*GnT*) domains. *Gray boxes* correspond to amino acids that interact with fucose or GalNAc (Yoon et al. 2008; Vasta et al. 2004). Isolated peptide fragments are F1 and F2. Genbank accession no. GnT (*Acetobacter pasteurianus*, YP_003188332), Fucolectin -1 (*Anguilla japonica*, Q9I931), Fucolectin_AAA (*Anguilla anguilla*, Q7SIC1) and FBPL1 (*Diano rerio*, ABB30000)

2008). Yoon et al. (2008) suggested that this conserved domain might be responsible for the sugar binding activity of Bryohealin too and predicted that histidine and/or arginine might present at the binding pocket. Our data from chemical modification of amino acid in Bryohealin showed that a region containing arginine is responsible for sugar binding of Bryohealin. Bryohealin consisted of 2.7% of arginine and have seven residues in each subunit (Yoon et al. 2008). Our chemical modification data showed that about half of those argentines were modified by the treatment with 50 mM CHD. As sugar binding activity of protein rapidly dropped without sequential reaction, we could assume that only one or two arginine residues are interacting with sugars. To pinpoint the modified site, trypsin digestion was carried out and the protein was divided to peptides. Using HPLC analysis, we found two peptides which contain modified arginine. When we compared the sequences of these peptides with the cDNA sequence of Bryohealin, they are located side by side at the predicted sugar binding domain of Bryohealin (Fig. 8, F1 and F2), which was consistent with that of fucolectin. These results corresponded well with our previous prediction based on alignment data with fucolectin (Yoon et al. 2008).

Questions still remain because the sugar specificity of Bryohealin and fucolectin is different. Fucose-binding lectin, fucolectin, domain have two arginine residues which directly participate in polar interaction with the fucose (Vasta et al. 2004). Involvement of arginine residues which could recognize with hydroxyl group of carbohydrate via polar interaction were reported in *N*-acetyl-b-hexosaminidase (32CBM; Elizabeth and Alisdair 2006) and 16 kDa galectin from *Caenorhabditis elegans* (Ahmed et al. 2002) too. If the arginine residues in the sugar binding domain of Bryohealin also recognize carbohydrate via polar interaction, the sequence difference at the sugar binding motif may explain why it recognizes different sugars.

Although there is a long history of algal lectin studies and many lectins have been isolated successfully over the last decades, there are still very few reports on the sugar binding motif of algal lectins. Recent progress in biomedicine has diversified the application of lectin in many fields, including the development of drug delivery systems, cell surface monitoring, and antibiotic applications etc (e.g., Sharon 2008; Sharon and Lis 1989), and it has become more urgent to understand the structure and mechanism of the sugar binding domain of lectin. Further study using X-ray crystallography or NMR techniques is necessary to characterize the sugar binding site of Bryohealin.

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