Doctoral Thesis

Biochemical Study on Lectins from Calcareous Green Algae of the Genus *Halimeda*

(Summary)

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Lectins, or carbohydrate-binding proteins play important roles as sugar recognition molecules within a cell, between cells, and between cell and matrix, and are present in a wide range of organisms from virus to human. Some of them are available as convenient tools in glycomics and clinical area. Recent investigations on lectins have further demonstrated that algae could be a good source for novel and useful lectins because of their unique and algae-inherent properties, including the strict binding specificity for some definite oligosaccharide structures, various biological activities, and novel molecular structures. Especially, the high mannose *N*-glycan (HM)-specific algal lectins have been attracting considerable attention owing to their prevention of virus infection by blocking the entry of viruses, such as influenza viruses and human immunodeficiency viruses, to target cells, as well as their anti-tumor activity. In our recent survey, it was suggested that a *Halimeda* alga might contain an HM-specific lectin. The calcareous green algae of the genus *Halimeda* inhabit in tropical and subtropical sea, 11 species of which are also found in Japan. However, very little information is known concerning lectins from the genus *Halimeda*. These situations addressed me to investigate the *Halimeda* algae for developing new useful lectins. In this study, three novel lectins, including an HM-specific one, were found out from the *Halimeda* algae as described below.

Molecular Screening of Lectins (Hemagglutinins) in Calcareous Green Algae of the Genus Halimeda

The algal samples of the genus *Halimeda* were collected on the coasts of Yakushima and Ishigakijima in summer and autumn seasons of 2007-2016. Prior to screening, species identification was performed based on DNA sequences of the gene *tuf*A (plastid elongation factor), because the *Halimeda* algae exhibit very similar morphological features among the species, making the difficulty only based on morphological identification. Fifteen samples subjected to the DNA sequencing were identified into 8 different species. Of them, the algal samples of 4 species, *H. macroloba*, *H. kanaloana*, *H. renschii* and *H. borneensis,* which were collected in the relatively large amounts, were verified by morphological identification and subjected to screening of lectins.

The screening of lectins was performed by measuring the hemagglutination activity of a salting-out fraction (with 75% saturation ammonium sulfate) prepared from the buffer extract of each algal sample. Six kinds of erythrocytes; intact and enzyme (trypsin or Pronase)-treated rabbit and sheep blood cells were used for hemagglutination assay. Each preparation from the 4 species agglutinated at least one erythrocyte species with the stronger activity toward enzyme-treated ones. The strongest activity was detected with the preparations from *H. renschii* and *H. borneensis*.

Purification and Characterization of an HM-specific Lectin from H. renschii

The lectins of *H. renschii* were purified from a salting-out fraction by a combination of hydrophobic chromatography with stepwise and gradient elution, gel filtration, and ion-exchange chromatography in this order. In hydrophobic chromatography with stepwise elution, hemagglutination activity was detected in both non-adsorbed and adsorbed fractions. From the latter

fraction, a lectin, named HRL40, was purified. HRL40 gave a single protein band of about 40 kDa in non-reducing and 10 kDa in reducing SDS-PAGE. In hemagglutination-inhibition test, the activity of HRL40 was strongly inhibited by HM-linked glycoproteins, but not by any of monosaccharides examined. In direct binding experiment with 29 pyridylaminated (PA-) oligosaccharides, HRL40 exclusively bound to HMs, especially those having an exposed (α1-3) mannose residue in the D2 arm of branched mannosides, and did not have an affinity for other oligosaccharides examined, including complex type *N*-glycans, an *N*-glycan core pentasaccharide, and oligosaccharides from glycolipids. The oligosaccharide-binding profile of HRL40 well resembled those of Type I HM-specific antiviral algal lectins, or the *Oscillatoria agardhii* agglutinin (OAA) family, which were previously isolated from red algae, blue-green algae (cyanobacteria), and bacteria. As expected, HRL40 potently inhibited the infection of influenza virus (A/H3N2/Udorn/72) into NCI-H292 cells with ED_{50} of 2.45 nM through high-affinity binding to a viral envelope hemagglutinin (K_D, 3.69×10^{-11} M). HRL40 consisted of two isolectins (HRL40-1 and HRL40-2) which could be separated by reverse-phase HPLC. Both isolectins had the same molecular weight of 46,564 Da and were an SS-linked tetrameric protein of an 11,641 Da polypeptide containing at least 13 half-cystines. Thus, HRL40, which is the first Type I HM-specific antiviral lectin from the green alga, had the same carbohydrate binding specificity as the OAA family, but a distinct molecular structure from the family. This lectin may contribute to understand the molecular basis of the high-mannose recognition by Type I HM-specific lectins.

Purification and Characterization of a GlcNAc-Specific Lectin from H. renschii

From *H. renschii*, another lectin, named HRL14, was purified by hydrophobic chromatography with stepwise elution (non-adsorbed fraction described above) followed by affinity chromatography on a bovine submaxillary mucin (BSM) column. In the affinity chromatography, the lectin was adsorbed to the BSM column and specifically eluted with *N*-acetyl-D-glucosamine (GlcNAc). The hemagglutination activity of HRL14 was strongly inhibited by GlcNAc and *O*-glycan-linked glycoproteins (BSM and asialo-BSM). In oligosaccharide binding experiment with 25 kinds of PA-oligosaccharides, however, HRL14 showed no significant binding to any of oligosaccharides examined, suggesting the possibility that the lectin may have strict binding nature to other oligosaccharide(s) unknown yet. HRL14 was unable to inhibit the infection of influenza virus (A/H3N2/Udorn/72) into NCI-H292 cells, unlike HM-specific algal lectins. HRL14 gave a single protein band of about 14 kDa in both non-reducing and reducing SDS-PAGE. HRL14 also consisted of two isolectins (HRL14-1 and HRL14-2) which could be separated by reverse-phase HPLC. The molecular weights of HRL14-1 and HRL14-2 were determined to be 15,817 and 15,671 Da by ESI-MS. The 25 and 40 *N*-terminal amino acids of HRL14-1 and HRL14-2 were determined, representing that both isolectins shared almost the same sequences with a few substitutions. However, similar sequences to both lectins were not searched out from databases. Thus, HRL14 may belong to

a new lectin family.

In the purification procedure of *H. renschii* lectins, although the hemagglutination activity of the salting-out fraction was not inhibited by any of sugar compounds examined, those of active fractions after hydrophobic chromatography were inhibited by some sugar compounds. This suggests that there may exist some endogenous inhibitor(s) in the algal tissue, which could be separated from the lectin molecules by hydrophobic chromatography. However, the possible inhibitor(s) have not yet been isolated during this study.

Purification and Characterization of a Complex Type N-*Glycan-Specific Lectin from* H. borneensis

The lectin from *H. borneensis* was purified from a salting-out fraction by hydrophobic chromatography with stepwise elution followed by ion-exchange chromatography. The purified lectin, named HBL40, gave a single protein band of about 40 kDa in non-reducing and 20 kDa in reducing SDS-PAGE, suggesting that it was an SS-linked dimeric protein of a 20 kDa polypeptide. Its hemagglutination activity was inhibited by both complex type *N*-glycan and *O*-glycan linked glycoproteins, but not by any of monosaccharides examined. The inhibitory activities of complex type *N*-glycan linked glycoproteins were significantly higher with asialo-derivatives than the parent sialoglycoproteins. In oligosaccharide-binding experiment, HBL40 exclusively bound to complex type *N*-glycans having bi- and triantennary branched sugar chains, and did not the other oligosaccharides including tetra- and pentaantennary complex *N*-glycans. The lectin showed the highest affinity for asialo-biantennary sugar chain without core (α1-6) fucose. Thus, sialylation, core fucosylation, and the increased number of branching antennae lowered the binding activity with HBL40. Such strict binding nature of HBL40 to complex *N*-glycans has not been reported so far for other lectins. HBL40 also inhibited the infection of influenza virus (A/H3N2/Udorn/72) into NCI-H292 cells with ED_{50} of 8.02 nM (K_D of 1.21 $\times 10^{-6}$ M). HBL40 also consisted of two isolectins which could be separated by reverse-phase HPLC. The molecular weights of two isolectins were determined to be 38,141 Da and 38,451 Da by MALDI-TOF-MS. Both isolectins shared the same 16 *N*-terminal amino acid sequences. However, similar sequences were not found in databases, suggesting that HBL40 is also a novel protein.

In this study, three novel lectins, which were specific for HMs, complex type *N*-glycans, and GlcNAc, were isolated and characterized from the genus *Halimeda* algae. Judged from their unique and strict carbohydrate-binding specificity, they may be usable as valuable biochemical and biomedical reagents.

Key Words: Lectin, glycan, *Halimeda renschii, Halimeda borneensis*, carbohydrate-binding specificity, anti-influenza virus activity