Two Lectins from the Marine Sponge *Halichondria okadai*

**AN N-ACETYL-SUGAR-SPECIFIC LECTIN (HOL-I) AND AN N-ACETYL-LACTOSAMINE-SPECIFIC LECTIN (HOL-II)**

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Two lectins (HOL-I and HOL-II) were isolated from the marine sponge *Halichondria okadai* were affinity chromatography on a bovine submaxillary mucin (BSM)-Toyopearl and an acid-treated Sepharose 4B columns, respectively. In hemagglutination inhibition assays, GlcNAc, GalNAc, and their methyl glycosides were the most potent inhibitors among the monosaccharides tested against the HOL-I-mediated hemagglutination, suggesting that HOL-I can especially recognize the N-acetyl groups of the sugars. This N-acetyl specificity was supported by 1H NMR analyses; the highest field-shifts of the signal of the N-acetyl group among all the signals in Me βGlcNAc were observed in 1H NMR spectra of mixtures of HOL-I and the sugar. Among the oligosaccharides tested, GlcNAcβ1→4GlcNAcβ1→2Manβ1→O(CH2)2CH3 was the most potent inhibitor, and the inhibitory potency of the oligosaccharide was 2 times greater than those of GlcNAc and GalNAc. On the other hand, N-acetyllactosamine (Galβ1→4GlcNAc) and its analogs were the strongest inhibitors toward HOL-II-induced hemagglutination. The agglutination was completely inert to Galβ1→3GlcNAc, Galβ1→6GlcNAc, Galβ1→3GalNAc, Galβ1→4GalNAc, and Galβ1→6GalNAc. Furthermore, HOL-II exhibited no binding ability to BSM, asialo-BSM, fetuin, asialofetuin, α,β-acid glycoprotein, and human transferrin. These results indicate that HOL-II strictly recognizes simple Galβ1→4GlcNAc unit.

Many lectins have been isolated from various sources. However, information is relatively sparse concerning lectins from marine origins as compared with the large variety of purified lectins of the other sources. It is well known that marine organisms produce unique low molecular compounds possessing unique structures and biological activities. It is also possible that novel lectins having unique properties will be found from the organisms.

*Halichondria okadai* is a toxic marine sponge, and potent toxins, okaidiac acid (1), and halichondrins (2, 3) were isolated from this sponge. However, there is no report about highly molecular substances from the sponge. Herein the isolation and characterization of two unique lectins from the sponge are described.

EXPERIMENTAL PROCEDURES

**Materials.** — *H. okadai* was collected at Aburatsubo, Kanagawa, Japan, frozen upon collection, and stored at ~20 °C. Sepharose 4B, Superox 12 HR10/30, and PrepRPC HR5/10 columns were products of Pharmacia (Sweden). Amino-Toyopearl and Toyopearl 55F were purchased from Tosoh (Japan). Spectra/Por 1 was a product of Spectrum Medical Industries. All glycoproteins and lectin for the hemagglutinating inhibition tests were products of Sigma. Galβ1→3GlcNAc, Galβ1→4GlcNAc, Galβ1→3GalNAc, and their methyl glycosides were the most potent inhibitors among the monosaccharides tested, GlcNAcβ1→4GlcNAcβ1→2Manβ1→O(CH2)2CH3 was the most potent inhibitor, and the inhibitory potency of the oligosaccharide was 2 times greater than those of GlcNAc and GalNAc. On the other hand, N-acetyllactosamine (Galβ1→4GlcNAc) and its analogs were the strongest inhibitors toward HOL-II-induced hemagglutination. The agglutination was completely inert to Galβ1→3GlcNAc, Galβ1→6GlcNAc, Galβ1→3GalNAc, Galβ1→4GalNAc, and Galβ1→6GalNAc. Furthermore, HOL-II exhibited no binding ability to BSM, asialo-BSM, fetuin, asialofetuin, α,β-acid glycoprotein, and human transferrin. These results indicate that HOL-II strictly recognizes simple Galβ1→4GlcNAc unit.

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Table I

| Fraction                  | Total protein (mg) | Total agglutination titer | Specific agglutination activity titer/mg protein | Recovery of activity % |
|---------------------------|-------------------|---------------------------|-----------------------------------------------|------------------------|
| PBS extract               | 68.2              | 2800                      | 41.1                                          | 100                    |
| HOL-I                     | 2.92              | 5430                      | 1860                                          | 195                    |
| Affinity chromatography   |                   |                           |                                               |                        |
| with BS-M-Tyopearl        |                   |                           |                                               |                        |
| Gel filtration with       |                   |                           |                                               |                        |
| Tyopearl 55S             | 1.86              | 3760                      | 2020                                          | 134                    |
| Affinity chromatography   |                   |                           |                                               |                        |
| with Sepharose 4B         | 1.98              | 1660                      | 838                                           | 59.3                   |

* Titer defined as the reciprocal of the end point dilution exhibiting the hemagglutination with Pronase-treated type A erythrocytes in PBS.

* Based on the initial PBS extract.

Table II

| Amino acid | HOL-I | HOL-II |
|------------|-------|--------|
| Asx        | 14.0  | 8.80   |
| Gln        | 12.9  | 10.7   |
| Ser        | 6.71  | 5.69   |
| Gly        | 10.6  | 10.2   |
| His        | 1.06  | 1.49   |
| Arg        | 4.68  | 4.71   |
| Thr        | 9.75  | 6.18   |
| Ala        | 10.1  | 14.7   |
| Pro        | 4.23  | 4.63   |
| Tyr        | 3.00  | 2.84   |
| Val        | 5.25  | 6.45   |
| Met        | 0.681 | 1.70   |
| Ile        | 3.84  | 4.11   |
| Leu        | 5.52  | 8.51   |
| Phe        | 3.17  | 4.02   |
| Trp        | 0.580 | 0.259  |
| Lys        | 4.03  | 5.19   |
| Cys        | ND    | ND     |

* ND, not determined.

RESULTS AND DISCUSSION

The purification procedure was summarized in Table I. Since crude saline-extract of H. okadai exhibited no remarkable binding specificity to any simple sugar and showed the specificity against asialo-BSM and BSM in the hemagglutination inhibition assay, BSM-Tyopearl was chosen as the affinity support. The lectin activity of the extract was largely adsorbed to the crude saline-extract of H. okadai but to galactose unlike the crude extract before the chromatography. Therefore, the fraction was applied to an acid-treated Sepharose 4B affinity column, and all the activity was adsorbed. Elution from the column was carried out with distilled water, and another purified lectin, HOL-II, was obtained.
**Table III**

| Inhibitor | Minimum inhibitory conc. a |
|-----------|---------------------------|
| HOL-I     | HOL-II                   |
| GlcNAc    | 0.098                    | NI c |
| GalNAc    | 0.098                    | 50.0 |
| Gal       | NI c                     | 25.0 |
| Me αGlcNAc| 0.098                    | NI c |
| Me βGlcNAc| 0.049                    | NI c |
| Me αGalNAc| 0.049                    | NI c |
| Me βGalNAc| 0.098                    | NI c |
| NeuAc     | 12.5                     | NI f |
| NeuGc     | 12.5                     | NI f |
| Galβ1→4Glc (lactose) | 0.39 | 0.049 |
| Galβ1→4GlcNAc (LacNAc) | 0.39 | 0.049 |
| Galβ1→4GlcNAc βMe (Me β-LacNAc) | ND a | 0.049 |
| Galβ1→4GlcNAc βPNP (PNP β-LacNAc) | ND | 0.049 |
| Galβ1→4GlcNAc β1→2Man | 0.39 | 0.049 |
| Galβ1→4GlcNAc (β1→4GlcNAc) | ND | NI f |
| Galβ1→4GlcNAc | ND | NI f |
| Galβ1→4GlcNAc | ND | NI f |
| Galβ1→6GlcNAc | ND | NI f |
| Galβ1→6GlcNAc βPNP | ND | NI f |
| GlcNAcβ1→2Manα1→6Manβ1→O(CH₂)₂CO₂CH₃ | 0.012 | ND |
| GlcNAcβ1→2Manα1→6Manβ1→O(CH₂)₂CO₂CH₃ | 0.049 | ND |
| Manα1→O(CH₂)₂CH₃ | 0.024 | NI e |
| GlcNAcβ1→2 | ND | ND |
| GlcNAcβ1→6 | Manα1→3Manβ1→O(CH₂)₂CO₂CH₃ | 0.20 | ND |
| GlcNAcβ1→2 | ND | ND |
| GlcNAcβ1→4 | Manα1→O(CH₂)₂CH₃ | 0.006 | NI e |
| GlcNAcβ1→2 | ND | ND |
| GlcNAcβ1→4 | Manα1→3Manβ1→O(CH₂)₂CO₂CH₃ | 0.024 | ND |
| GlcNAcβ1→2 | ND | ND |
| GlcNAcβ1→6 | Manβ1→O(CH₂)₂CO₂CH₃ | 0.012 | ND |
| GlcNAcβ1→2Manα1→3 | NS | ND |
| N-Ac-Glu | 6.25 | NI c |
| N-Ac-Gly | 6.25 | NI c |
| N-Ac-Leu | 12.5 | NI c |
| BSM a | 0.98 | NI f |
| Asialo-BSM b | 0.12 | NI f |
| Fetuin b | 7.81 | NI f |
| Asialofetuin b | 3.91 | NI f |

a α-L-Arabinose, α-L-fucose, ribose, 2-deoxyribose, fructose, glucose, glucosamine, mannosamine, mannose, and xylose exhibited no inhibition at concentrations up to 0.2 M. Human transferrin and α1 acid glycoprotein exhibited no inhibition at concentrations up to 1 mg/ml.

b Minimum concentrations required for inhibition of 4 hemagglutinating doses of the lectins.

c NI, not inhibited at concentrations up to 0.2 M.

d ND, not determined.

e PNP, p-nitrophenyl.

Each purified lectin was appeared as a single band in SDS-polyacrylamide gel electrophoresis (Fig. 1). HOL-I showed a single band corresponding to a molecular weight of M₀ = 21,000 regardless of the presence or absence of 2-mercaptoethanol. But without 2-mercaptoethanol the band was somewhat diffuse. The reason is not clear but the subunit might have some intramolecular S-S linkage(s). The native molecular weight of the lectin was 84,000 on the basis of the result of gel filtration in PBS, suggesting that HOL-I consists of four identical subunits of M₀ = 21,000. On the other hand, HOL-II showed a band of molecular weight 42,000 in SDS-polyacrylamide gel electrophoresis, and the estimation of the molecular weight of native HOL-II by gel filtration gave the same molecular weight. This result indicates that HOL-II exists as a monomeric molecule in its native state.

A comparison of the amino acid compositions of HOL-I and HOL-II (Table II) indicated that the two lectin forms are similar but differed mainly in their amount of Asx, Thr, Met, Leu, and Trp. Although 2 nmol of each protein was applied to the sequencer, N-terminal amino acid could not be detected. This result suggests that N termini of the proteins may be blocked.

In isoelectric focusing of the lectins, both lectins indicated families of bands in pH zone near 4.5 (data not shown). The carbohydrate contents of HOL-I and HOL-II amounted 2.0 and...
7.5%, respectively. The hemagglutination of the lectins was not affected by demetalization, and addition of CaCl₂, MgCl₂, ZnCl₂, or MnCl₂ to the demetalized lectins did not cause any change of the activities. Thermostability of HOL-I and HOL-II was shown in Fig. 2. HOL-I was stable below 50 °C, but HOL-II was sensitive to temperature, and the activity was rapidly decreased over 40 °C. Both lectins were stable under wide range pH regions (Fig. 3).

The two lectins did not agglutinate any type of native human erythrocytes, but Pronase-treated blood cells caused agglutination by the lectins: the titer of HOL-I (100 pg/ml) was 1024 to the treated type A cells, 128 to the type B, and 512 to the type O cells. The titer of HOL-II (100 pg/ml) was 128 to the treated type B cells, and 512 to the type O cells. The titer of HOL-I (100 µg/ml) was 1024 to the treated type A cells, 128 to the type B, and 512 to the type O ones. The titer of HOL-II (100 µg/ml) was 128 to the treated type B cells, and 512 to the type O ones. Both lectins exhibited slight preferences to the Pronase-treated type A erythrocytes.

The results of hemagglutination inhibition assays of HOL-I are shown in Table III. GlcNAc, GalNAc, and both anomers of their methyl glucosides 5-8 were the best inhibitors to the hemagglutination by HOL-I among monosaccharides tested 1-10. N-Acetyleneuraminic acid 9 and N-acetylated amino acids such as N-acetylglutamine 31, N-acetylglycine 32, and N-acetylleucine 33 were also inhibitory at higher concentrations. This result suggests that HOL-I mainly recognizes N-acetyl groups. This suggestion was further supported by NMR analyses of mixtures of HOL-I and Me βGlcNAc (Figs. 4 and 5 and Table IV). In the binding equilibrium between a lectin and its specific binding sugar, resonance of the sugar may shift and/or broaden due to chemical changes between the free and bound environments (12, 13). Furthermore, the binding site in the sugar to the lectin can be deduced by comparison of the chemical shift of each proton in the sugar bound to the lectin with those of free sugar (13).

Fig. 4 shows 1H NMR data for Me β-GlcNAc, which is one of the best haptenic sugars of HOL-I, in the absence or presence of the lectin. The resonances of N-acetyl groups of the sugar in the presence of the lectin (Fig. 4, A-F) caused the highest field shifts among all the signals in the sugar. The chemical shift changes are summarized in Table IV and Fig. 5. The shifts were dependent on the mole ratio of the sugar to the lectin; a lower mole ratio caused higher field shift. The signals of H-2 in the sugar also showed similar but smaller changes than N-acetyl groups. The chemical shifts of the other protons, however, were almost the same as those in the absence of the protein. Almost the same results were obtained in the case of GalNAc (data not shown). These data indicate that HOL-I can specifically bind to the N-acetyl group in GlcNAc or GalNAc, and the binding to the other parts in the sugar was much weaker than that of the acetyl group. Among the oligosaccharides tested (11-30) in the hemagglutination inhibition assays, oligosaccharide 28 was the best inhibitor, and oligosaccharides 24 and 30 were second best. All the saccharides having terminal GlcNAc residues, 24-30, were more inhibitory than the others (11-23). All the results of the hemagglutination inhibition tests and NMR analyses show that HOL-I can specifically recognize the N-acetyl group of the terminal GlcNAc residue. The detailed binding properties to GalNAc residues of HOL-I remains to be determined. The hemagglutination by HOL-I was also inhibited by BSM 34, asialo-BSM 35, fetuin 36, and asialofetuin 37. Among them, asialo-BSM exhibited the most inhibitory effect. Since asialo-BSM has mainly terminal β-galactosyl residues in the sugar chains, this result was inconsistent with those of monosaccharides and oligosaccharides. However, trypsin- or Pronase-digested asialo-BSM showed much weaker inhibitory effect on HOL-I-mediated hemagglutination than native one (data not shown). This result suggests that the inhibitory effect of asialo-BSM is not due to only

| Portion | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | 12.0 |
|---------|-----|-----|-----|-----|------|------|
| Ac      | -0.147 | -0.098 | -0.085 | -0.050 | -0.039 | -0.032 |
| OMe     | 0.003 | -0.002 | -0.001 | -0.002 | -0.002 | -0.002 |
| H1      | 0.001 | 0.001 | 0.001 | 0.001 | 0.002 | 0.002 |
| H2      | -0.086 | -0.050 | -0.030 | -0.025 | -0.020 | -0.015 |
| H3      | 0.013 | 0.009 | 0.007 | 0.002 | 0.002 | 0.003 |
| H4      | 0.000 | 0.000 | 0.002 | 0.002 | 0.002 | 0.002 |
| H5      | 0.007 | 0.007 | 0.005 | 0.002 | 0.002 | 0.002 |
| H6a     | 0.003 | 0.003 | 0.001 | 0.000 | 0.000 | 0.001 |
| H6b     | 0.002 | 0.001 | 0.001 | 0.001 | 0.002 | 0.002 |

* Shift change = chemical shift in the presence of HOL-I - that in the absence of HOL-I.
simple interaction between the lectin and the sugar chains of the glycoprotein.

Binding reactivity of HOL-II is limited to βGal(1→4)GlcNAc (LacNAc) 12 and its analogs 13-15 (Table III). Interestingly, Galβ1→4GlcNAcβ1→4GlcNAc β16 was inert to the lectin at 25 mM. Two regioisomers of LacNAc, Galβ1→3GlcNAc and Galβ1→6GlcNAc, did not affect HOL-II-mediated hemagglutination at all even at 25 mM. Similar disaccharides having GalNAc as subterminal residues, pGal(1→3)GalNAc, pGal(1→4)GalNAc, and βGal(1→4)GalNAc, were also inactive to the hemagglutination. These results show the binding specificity of the lectin is strictly LacNAc unit only. In general, Galβ1→4GlcNAc-specific lectins can bind to Galβ1→4GlcNAc at higher concentrations (14), and Galβ1→3GlcNAc-specific lectins can also recognize Galβ1→4GlcNAc (15). To our knowledge, this is the first lectin which exhibits such strict specificity to Galβ1→4GlcNAc linkage. In addition, the hemagglutination caused by HOL-II was not inhibited by BSM, asialo-BSM, fetuin, asialofetuin, α1-acid glycoprotein, and human transferrin even at 1 mg/ml. Hemagglutination by known lectins is usually inhibited by at least one of the glycoproteins used in this study. Similar lectins (PFA) have been isolated from larvae of the moth Phalera flavescent (16).

Glycoproteins such as fetuin, asialofetuin, human transferrin, ovalbumin, and porcine stomach mucin showed no inhibitory activity to the hemagglutination by PFA, and LacNAc was the best inhibitor like HOL-11.

Some lectins have been isolated from sponge (17-20). A lectin was obtained from Halichondria panicea which is the same genus as H. okadai. The lectin also recognizes β-galactosides like HOL-II. But the lectin activity was dependent on metal cation whereas HOL-II did not need the cation for the activity.

All the data mentioned above allowed us to conclude that HOL-I and HOL-II can be used as valuable tools for sugar chain research.

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