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Isolation, purification, characterization and glycan-binding profile of a D-galactoside specific lectin from the marine sponge, *Halichondria okadai*

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ABSTRACT

A lectin recognizing both Galβ1-3GlcNAc and Galβ1-4GlcNAc was purified from the demosponge *Halichondria okadai* by lactosyl-agarose affinity chromatography. The molecular mass of the lectin was determined to be 30 kDa by SDS-PAGE under reducing and non-reducing conditions and 60 kDa by gel permeation chromatography. The p*I* value of the lectin was 6.7. It was found to agglutinate trypsinized and glutaraldehyde-fixed rabbit and human erythrocytes in the presence and absence of divalent cations. The hemagglutinating activity by the lectin was inhibited by D-galactose, methyl-D-galactopyranoside, *N*-acetyl-D-galactosamine, methyl-*N*-acetyl-D-galactosaminide, lactose, melibiose, and asialofetuin. The K_d of the lectin against *p*-nitrophenyl-β-lactoside was determined to be 2.76×10^{-5} M and its glycan-binding profile given by frontal affinity chromatography was shown to be similar to many other known galectins. Partial primary structure analysis of 7 peptides by cleavage with lysyl endopeptidase indicated that one of the peptides showed significant similarity with galectin purified from the sponge *Geodia cydonium*.

1. Introduction

Lectins are carbohydrate-binding proteins that recognize specific oligo- and/or monosaccharide structures and are widely distributed in many organisms. High numbers of lectins are found in many kinds of marine invertebrates such as tunicates (Nair et al., 2000), echinoderms (Uchida et al., 2004; Kakiuchi et al., 2002; Nakagawa et al., 1999; Matsui et al., 1994; Ozeki et al., 1991; Giga et al., 1987), arthropods (Saito et al., 1995), echiuroid and annelids (Ozeki et al., 1997a; Matsui, 1984), mollusks (Tunkijjanukij et al., 1998a; Ozeki, 1997b), and corals (Jimbo et al., 2000) for example. Lectins have been thought to play important physiological roles in cellular recognition and regulation through protein–carbohydrate interactions, though clear evidence for the physiological functions of lectins still remains unclear. For the innate immune system of marine invertebrates, lectins effectively function that serve to prevent and limit invading microorganisms or its pathogens. Recently, antibacterial activity (Tasumi et al., 2002; Tunkijjanukij and Olafsen, 1998b) and hemolysis (Uchida et al., 2004) which seemed to be functions of the innate immune system have been observed. Indeed, an increase in the expression levels of lectin in hemocytes purified from Manila clam has been specifically observed following infection by parasitic protozoans, indicating that the lectins may function as an opsonin that results in activation of the clam's immune system (Kim et al., 2006).

Specifically, in the oceanic environment, where many diverse organisms are in competition, there is a difficult struggle for existence and the mucus properties derived from glycoconjugates in skin, blood, and eggs in marine organisms comprise a necessary living strategy which serves to prevent the invasion of pathogenic microorganisms, to cure injury, and to protect blood from freezing through specific molecular recognition systems. Considering these points, glycoconjugates are clearly important molecules that are required for the physiological function of marine invertebrates.

Sponges are evolutionarily, the eldest metazoan phylum and they are found widely distributed throughout the marine environment and in fresh water habitats as well. A large number of lectins that possess various biochemical characteristics have been purified, their structures deduced, and various animal lectin families have been identified

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including: galectins, C-type lectins, tachylectins, and lectins from the skin of the puffer fish for example. For example, four proteins and cDNAs coding for galectin, which basically recognizes β-galactoside, were isolated from both demosponges *Geodia cydonium* (Pfeifer et al., 1993) and *Suberites domuncula* (Schröder et al., 2006; Wiens et al., 2003). Two cDNAs coding for a 24 kDa C-type lectin that required divalent cations for carbohydrate binding activity were purified from the hexactinellid sponge, *Aphrocallistes vastus*, and their gene products were found to act as a putative aggregation factor for the sponge (Gundacker et al., 2001). In another case, two cDNAs coding for a similar primary structure with that of tachylectin (Saito et al., 1995) from the plasma cells of the horseshoe crab *Tachypleus tridentatus* were isolated as a 27 kDa lipopolysaccharide (LPS)-binding lectin from *S. domuncula* (Schröder et al., 2003) and a 24 kDa lectin from the fresh water sponge *Ephydatia fluviatilis* (*Ef* lectin) (Funayama et al., 2005), respectively. More recently, a 13 kDa mannose-binding lectin was purified from a fresh water sponge *Lubomirskia baicalensis* (Wiens et al., 2006) and it was determined to be a member of the same family of lectins as those found in the skin mucus of the pufferfish *Takifugu rubripes* (Tsutsui et al., 2003).

Halichondria okadai, of the class Demospongiae, is a black sponge and is commonly found at the inter-tidal zone of many coastal areas on the Pacific Ocean in Japan. Two lectins, HOL-I and HOL-II have also been isolated from the sponge using affinity columns that employed bovine submaxillary mucin-Toyopearl and acid-treated Sepharose 4B, respectively (Kawagishi et al., 1994). On the other hand, we have also observed another type with galactoside-binding activity in the supernatant from the homogenate of *H. okadai* using a biosensor Biacore (GE Healthcare, USA) that detects molecular interactions using the optical principal of surface plasmon resonance (data not shown), whereby the sensor chip is fixed with a neoglycoprotein of lacto *N*-fucopentose-I (α1-2fucosylated *LNT* or *LNFP*-I) having the epitope of ABO blood type substance H. In this paper, a new galactoside-binding lectin was purified from marine sponge *H. okadai* (HOL-30) with respect to its molecular mass, subunit structure, hemagglutination, sugar specificity, and partial primary sequences analysis.

2. Materials and methods

2.1. Chemicals

Lactose, sucrose, melibiose, D-glucose, D-galactose, D-mannose, L-fucose, *N*-acetyl D-galactosamine, *N*-acetyl D-glucosamine and lysyl endopeptidase were purchased from Wako Pure Chemical Ind. Ltd., Tokyo, Japan and were of the highest purity grade. Methyl α-D-galactopyranoside and methyl β-D-galactopyranosides were from Pfanstiehl Laboratories Inc., IL, USA. Methyl α-*N*-acetyl D-galactosaminide (Me-α-GalNAc) and methyl β-*N*-acetyl D-galactosaminide (Me-β-GalNAc) were purchased from Toronto Research Chemicals Inc., Canada. Glycoproteins, fetuin, asialofetuin and standard protein markers for gel permeation chromatography were purchased from Sigma, USA. Lactosyl-agarose and protease inhibitor mixture were purchased from Seikagaku Kogyo Co. Ltd., Japan. A standard protein marker mixture (Daiichi-III) for SDS-PAGE was purchased from Daiichi Pure Chem. Co. Ltd., Japan. Immobiline DryStrip pH 3–10 (size 13 cm), Superdex 75, Sephadex G-75, Sepharose 4B, and Hitrap NHS-activated high performance (HP) columns were obtained from GE Health Sciences and Sigma, USA. Bicinchoninic acid (BCA) kit was purchased from Pierce Co. Ltd., USA. *p*-Nitrophenyl-β-lactoside (Galβ1-4Glcβ-pNP) was obtained from Sigma, USA. Pyridylaminated (PA) oligosaccharides used in this study are listed in Fig. 5A, PA-N-linked glycans numbered 002–014, 103, 105, 107, 108, 307, 313, 314, 323, 405, 410, 418–420, 501–504, and 506 were from Takara Bio Inc., Japan and the others were from Seikagaku Kogyo Co. Ltd., Japan. Glycolipid-type glycans numbered 701–703, 705–713, 715–721, 724, 726, and 728–731 were from Takara Bio Inc., Japan. Other PA-oligosaccharides were pyridylaminated non-labeled glycans (Funakoshi Co. Ltd., Japan; Dextra Laboratories Ltd., Reading, UK; Calbiochem Co. Ltd., USA; and Seikagaku Kogyo Co. Ltd., Japan) using a GlycoTag kit (Takara Bio Inc., Japan). Mightysil RP-18 column, acetonitrile (HPLC grade), and trifluoroacetic acid (TFA) were obtained from Kanto Chemial Co. Inc., Tokyo, Japan.

2.2. Purification of D-galactoside-binding lectin from H. okadai

The marine sponge *H. okadai* was collected from the intertidal zone of the Zushi coast, Kanagawa Prefecture, Japan and the fresh sponges were immediately cut into small pieces and frozen at −80 °C. For general preparation, 200 g (wet weight) of frozen sponge was dice homogenized in a commercial blender (Waring, USA) with 10 vol (w/v) of Tris-buffered saline (TBS) (10 mM Tris(hydroxymethyl)aminomethane–HCl, pH 7.4, containing 150 mM NaCl) containing 10 mM of a protease inhibitor mixture. The homogenates were roughly filtered via a filter bag in a sink to remove debris and the filtrates were centrifuged at 14,720 *g* in 500-mL centrifuge bottles for 1 h at 4 °C with a Suprema 21 centrifuge equipped with an NA-18HS rotor (TOMY Co. Ltd., Japan). The supernatant was centrifuged again at 27,500 *g* for 1 h at 4 °C and the supernatant was applied to the affinity column of lactosyl-agarose (5 mL) being fitted with a Sephadex G-75 pre-column (5 mL). After applying the extracts, the lactosyl-agarose column was extensively washed with TBS. The lectin was eluted with 50 mM lactose in TBS and each 1 mL of elution was collected in tubes with a fraction collector. Each chromatography step during washing and elution was monitored using a UV monitor (ATTO Co. Ltd., Japan) by the measurement of the absorbance at 280 nm. The eluted fractions as identified by UV spectrophotometer at 280 nm and SDS-PAGE (Laemmli, 1970) were combined, and dialyzed against 1000 times volumes of TBS to remove free lactose. The concentration by purified lectin was determined by the BCA protein assay kit with bovine serum albumin as the standard protein (Smith et al., 1985; Wiechelman et al., 1988) by measuring absorbance at 562 nm with spectrophotometer ND-1000 (Nano Drop Tech. Inc., USA).

2.3. Determination of native and polypeptide molecular mass using FPLC and SDS-PAGE

The purified lectin was dissolved in 2.5% glycerol and subjected to gel permeation chromatography (GPC) utilizing a Superdex 75 column (1.0×65 cm) connected to an FPLC system. The elution time of the lectin from the column was detected by UV at an absorbance of 280 nm. We also separated the lectin in 50 mM lactose containing TBS and/or 20 mM $CaCl_2$ in TBS. To determine the polypeptide size of the lectin, it was mixed with an equal volume of sample buffer (20 mM Tris–HCl, pH 6.8; 0.2% SDS, and 20% glycerol in the presence or absence of 2% 2-mercaptoethanol) and then heated at 70 °C for 15 min. Aliquots of 30 μl were applied to the well of a mini-slab gel (gel size: 80 mm×100 mm with 1 mm thickness; 12.5% and 5% polyacrylamide were used in separation and upper gels, respectively). The molecular mass of the polypeptide was determined by SDS-PAGE (constant current at 30 mA for 1 h) according to a previous report (Laemmli, 1970). After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue (CBB) R-250 in 40% methanol and 10% acetic acid followed by discoloration by excessive staining with 40% methanol and 10% acetic acid.

2.4. Isoelectric focusing (IEF)

Isoelectric focusing was performed with ANATECH CoolPhorestar Model 3610 and a 13 cm Immobiline DryStrip pH range 3–10 under conditions with constant voltage 500 V 90 min, 700 V 15 min, 1000 V 15 min, 1500 V 15 min, 2000 V 15 min, 2500 V 15 min, 3000 V 15 min, and 3500 V 5 h. After electrophoresis, strip was stained with 0.02% CBB R-250, 10% methanol, and 0.1% $CuSO_4$ for 1 h and destained with 30% methanol/10% acetic acid. Estimation of isoelectric point (p*I*) value was followed as per manufacturer's protocol.

2.5. Effects of divalent cations, SH-preservating reagent, temperature, and pH

The effects of Ca^{2+}, Mn^{2+} and Mg^{2+} ions on the hemagglutinating activity of the *H. okadai* lectin were evaluated by the addition of serially diluted $CaCl_2$, $MnCl_2$, $MgCl_2$ and EDTA (10 to 100 mM each, all chemicals including the lectin in TBS) with the lectin using 96-well plates. The effect of a sulfhydryl-preservation reagent was also evaluated by the co-presence of 2-mercaptoethanol (3 to 20 mM) in the hemagglutination assay. The temperature effect on lectin activity was determined by incubating each sample at different temperatures over a range of 20 to 80 °C in 10-degree increments for 1 h. The pH effect was determined by the measurement of the titer of the lectin in the range of pH 3 to 10. Lectin was dissolved in 20 mM sodium acetate-acetic acid (pH 3–5), 20 mM sodium phosphate–HCl (pH 6–7), 20 mM Tris–HCl (pH 8–8.5), 20 mM glycine-NaOH (pH 9–10) for 3 h at 25 °C and subsequently dialyzed against 50 mM TBS, pH 7.4 and the differentiation of hemagglutination by the lectin was measured in titer plates.

2.6. Hemagglutination assay

Hemagglutination assay was performed using 1% (w/v) typsinized and 0.25% glutaraldehyde-fixed rabbit and human erythrocytes as described previously (Matsui, 1984). Erythrocytes were suspended at a concentration of 1% (w/v) with TBS. In the general assay, 20 μL each of TBS, TBS containing 1% Triton X-100, and erythrocytes were added with 20 μL of the serially diluted lectin with TBS in 96-well V-shape titer plates for 1 h. The hemagglutination activity of the lectin was expressed as the titer defined as the reciprocal of the highest dilution giving positive hemagglutination. To determine the sugar binding specificity of the lectin, each 20 μL of the sugar (200 mM) and the glycoprotein (5 mg/mL) was serially diluted with TBS and added with lectin to previously diluted TBS to adjust the titer to 16, 1% Triton X-100, and erythrocytes in 96-well V-shape microtiter plates for 1 h. The minimum inhibitory sugar concentration against the lectin was expressed as the negative activity scored.

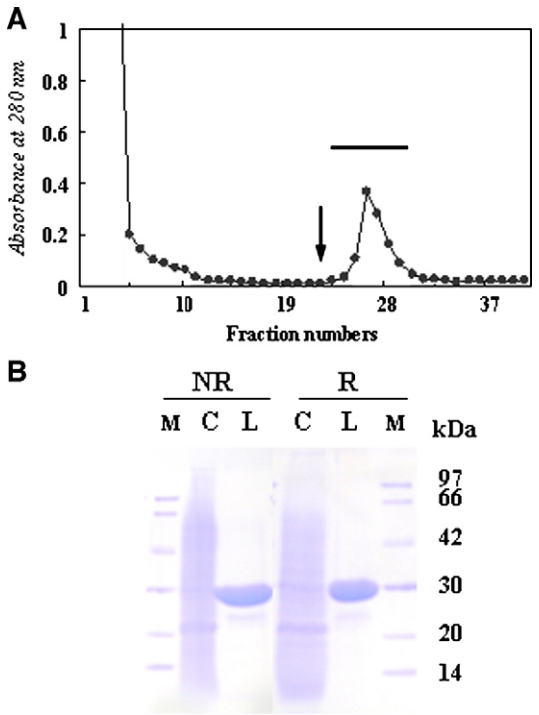

Fig. 1. Purification and SDS-PAGE pattern of HOL-30. A: Crude extract of *H. okadai* (200 mg) was applied to a lactosyl-agarose column equilibrated with TBS. The column was washed with TBS and eluted with TBS containing 50 mM lactose (arrow). B: Purified lectin (L), (10 μg) and crude extract (C), (10 μg) were subjected to SDS-PAGE under non-reducing (NR) and reducing (R) conditions. Standard marker proteins (M) were used as follows, phosphorylase b (97 kDa); bovine serum albumin (66 kDa); ovalbumin (42 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20 kDa); and lysozyme (14 kDa).

Table 1
Purification of lactose binding lectin from *H. okadai*

Fraction	Titer (HU)	Volume (mL)	Total activity[a]	Protein concentration (mg/mL)	Specific activity[b]	Purification ratio (fold)	Recovery of activity (%)
Crude extract	512	200	102,400	2.17	1.2	1	100
Purified lectin	4096	7.5	30,720	0.73	748	623	30

[a] Total activity is shown by titer × volume.
[b] Specific activity was shown by titer/mg of protein.

2.7. Frontal affinity chromatography (FAC)

The purified lectin was dialyzed and dissolved in 0.2 M $NaHCO_3$ buffer, pH 8.3, containing 0.5 M NaCl and coupled to NHS (*N*-hydroxysuccinimide)-activated Sepharose according to the manufacturer's instructions. The lectin-immobilized Sepharose gel was suspended in TBS, packed into a miniature column (2 mm × 10 mm, 31.4 μL), and frontal affinity chromatography was performed with an automated frontal affinity chromatography (FAC) system (model FAC-1) using pNP- and PA-labeled oligosaccharides as previously described (Nakamura et al., 2005). The principle of FAC was previously described (Kasai et al., 1986). Briefly, pNP-glycans (1–100 μM) and PA-glycans (2.5 or 5.0 nM) were continuously injected into the lectin column and the elution of pNP- and PA-glycans was monitored by measuring UV (280 nm) and fluorescence (Ex. 310 nm/Em. 380 nm), respectively. The volume of the elution front relative to that of an appropriate standard oligosaccharide ($V-V_0$) was determined. Bt (effective ligand content) was determined by concentration-dependence analysis using various concentrations of Galβ1-4Glcβ-pNP (0.3–50 μM) as previously described (Nakamura et al., 2005). K_d values of the lectin for each oligosaccharide were calculated from $V-V_0$ and Bt according to the basic equation of FAC (Hirabayashi, 2003).

2.8. Lysyl endopeptidase digestion and separation of peptides

One nanomole of the purified lectin was dissolved in 1 mL of 100 mM Tris–HCl buffer (pH 9) containing 2 M urea and digested with lysyl endopeptidase (Masaki et al., 1981) at a molar ratio of 1:300 at 37 °C for 4 h. Peptides, generated by the enzymatic digestion were separated by reversed phase (RP) HPLC using a Gilson micro preparative HPLC system on a Mightysil RP-18 (2.0 × 50 mm) column. The column was equilibrated with solvent A (acetonitrile:water:TFA, 20:80:0.09, v/v) and peptides were eluted at a flow rate of 200 μL/min using a linear gradient of solvent B (acetonitrile:water:TFA, 80:20:0.075, v/v), at room temperature. Mass spectrometry (MS) of separated peptides was determined by the matrix-associated laser desorption ionization time of flight (MALDI-TOF) MS analysis of each peptide that was performed on an AXIMA-CFR (Shimadzu Corp., Japan). An aliquot (0.5 μL) of each fraction separated by RP-HPLC was spotted onto a target, followed by addition of 0.5 μL 2-mercaptobenzothiazole dissolved in ethanol:water (1:1, v/v) as matrix. The samples mixed with the matrix on the target were dried at room temperature for 5 min, and each spot was desorbed with 80 laser shots for positive-mode analysis.

Table 2
Hemagglutination of rabbit and human erythrocytes by HOL-30

Erythrocytes[a]	Titer (HU)
Rabbit	2048
Human (Type A)	1024
Human (Type B)	1024
Human (Type O)	4096

[a] Trypsinized and glutaraldehyde fixed erythrocytes were used.

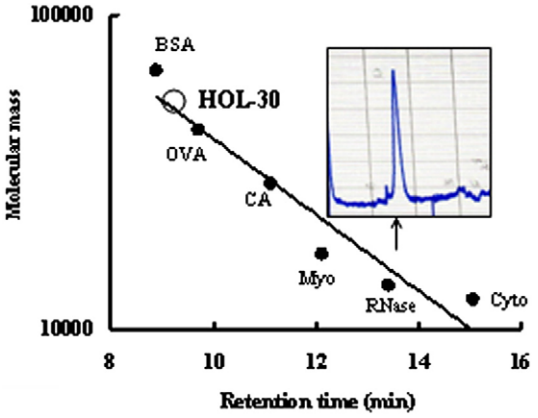

Fig. 2. GPC and calibration curve for the determination of molecular mass of HOL-30. Purified lectin (20 μg) was separated on a Superdex 75 column using an FPLC system at a flow of 0.5 mL/min (chart speed is 0.5 cm/mL). Abbreviations indicate the standard molecular mass marker proteins: bovine serum albumin (BSA: 66 kDa); ovalbumin (OVA: 43 kDa); carbonic anhydrase (CA: 30 kDa); myoglobin (Myo: 17 kDa); ribonuclease (RNase: 14 kDa); cytochrome *c* (Cyto: 12 kDa). The arrow shows the peak of HOL-30 in GPC.

Fig. 3. Isoelectric focusing of HOL-30. A 13 cm Immobiline DryStrip pH range 3–10 was used for the analysis of purified lectin. The positions of cathode and anode are indicated by (−) and (+), respectively. The isoelectric point of the lectin is indicated by an asterisk.

2.9. Peptide sequencing

Automated Edman degradation was carried out with a gas phase protein sequencer, Shimadzu model PPSQ-31A (Shimadzu Corp., Japan). Cleaved PTH-amino acids were separated on-line with an ODS column, Wakosil (2.0×250 mm, Wako Pure Chem. Ind. Ltd., Japan) connected with a model LC-20AT pump, that detected absorbance at 269 nm with a UV–VIS SPD-20A detector. Sequence similarity was searched on-line through the Internet (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTP program in the non-redundant databases of the National Center for Biotechnology Information (NCBI) (Altschul et al., 1997).

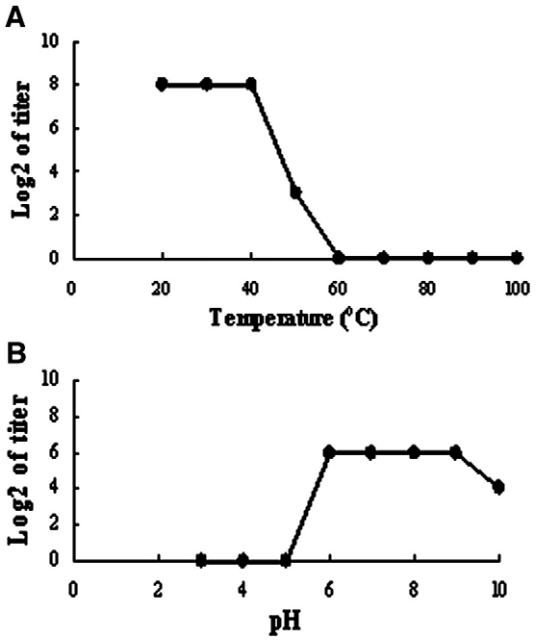

Fig. 4. Thermostability and pH stability of HOL-30. The effects of (A) temperature and (B) pH on the hemagglutination activity.

Table 3
Saccharide and glycoprotein specificity of HOL-30

Saccharides	Minimum inhibitory concentration (mM)
D-Galactose	6.25
Methyl alpha-*N*-acetyl D-galactosaminide	6.25
Methyl beta-*N*-acetyl D-galactosaminide	6.25
N-Acetyl D-galactosamine	50
N-Acetyl D-glucosamine	N.I.[a]
Methyl alpha-D-galactopyranoside	12.5
Methyl beta-D-galactopyranoside	12.5
D-Glucose	N.I.[a]
D-Mannose	N.I.[a]
L-Fucose	N.I.[a]
Lactose	3.13
Melibiose	25
Sucrose	N.I.[a]
Glycoproteins	**Minimum inhibitory concentration (mg/mL)**
Asialofetuin	0.2
Fetuin	N.I.[b]

[a] No inhibition observed, even at 100 mM.
[b] Fetuin did not inhibit even at 2 mg/mL. Titer of lectin was previously diluted to 16.

3. Results

3.1. Purification of lectin (HOL-30) from H. okadai

The supernatant of homogenized *H. okadai* was found to have a strong hemagglutinating activity and therefore it was passed through

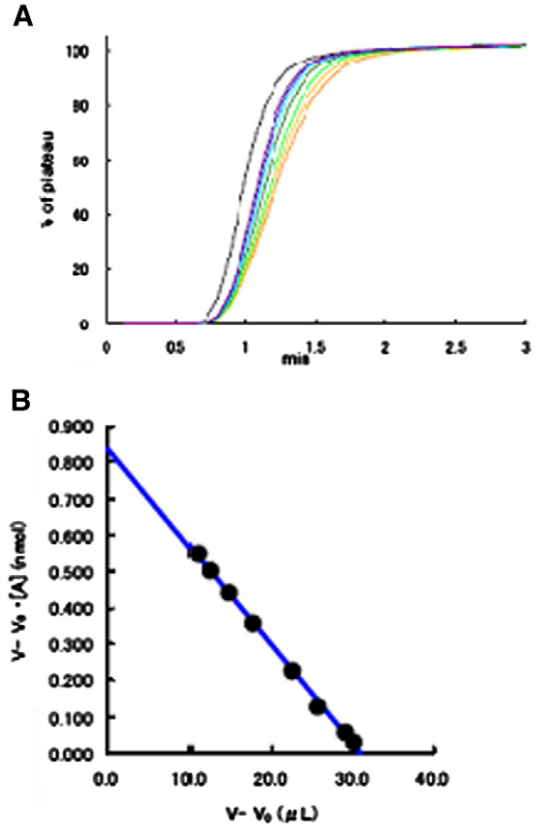

Fig. 5. Determination of Bt values to identify the binding constant of HOL-30. A: The solid lines indicate elution profiles of pNP-β-lactoside. B: The Bt value was determined by the application of various concentrations of pNP-β-lactoside (1–50 μM) to the affinity column containing immobilized HOL-30 (3.0 mg/mL) column (1 mL). Woolf-Hofstee plot was made by using $V-V_0$ values.

a lactosyl-agarose column. After washing the column with TBS, the column binding protein was specifically eluted by the addition of 50 mM lactose in TBS (Fig. 1A). The molecular mass of the lectin appeared to be 30 kDa by SDS-PAGE with and without 10 mM 2-mercaptoethanol in the sample buffer (Fig. 1B). Eluted fractions from the column by TBS containing lactose were collected together and dialyzed against TBS. The purified lectin showed strong hemagglutinating activity against rabbit and human erythrocytes but it did not bind to anything in the acid-treated Sepharose 4B column (size: 1 cm×5 cm, data not shown). The purified lectin is defined as HOL-30 i.e., a galactoside-binding lectin from *H. okadai* with a relative molecular mass of 30 kDa. About 30 mg of the lectin was successfully purified from 1 kg (wet mass) of marine sponge (Table 1). The purified lectin was agglutinated with trypsinized and glutaraldehyde-fixed rabbit and human erythrocytes (Table 2).

3.2. Non-covalent dimeric structure of HOL-30

The relative molecular mass of native HOL-30 as estimated by GPC on a Superdex 75 column connected to an FPLC system using TBS containing 50 mM lactose was shown to be 60 kDa (Fig. 2). Since the target peak also appeared at the same position, when the lectin eluted from the column equilibrated with TBS (data not shown), it was suggesting that the native lectin does not interact with the column

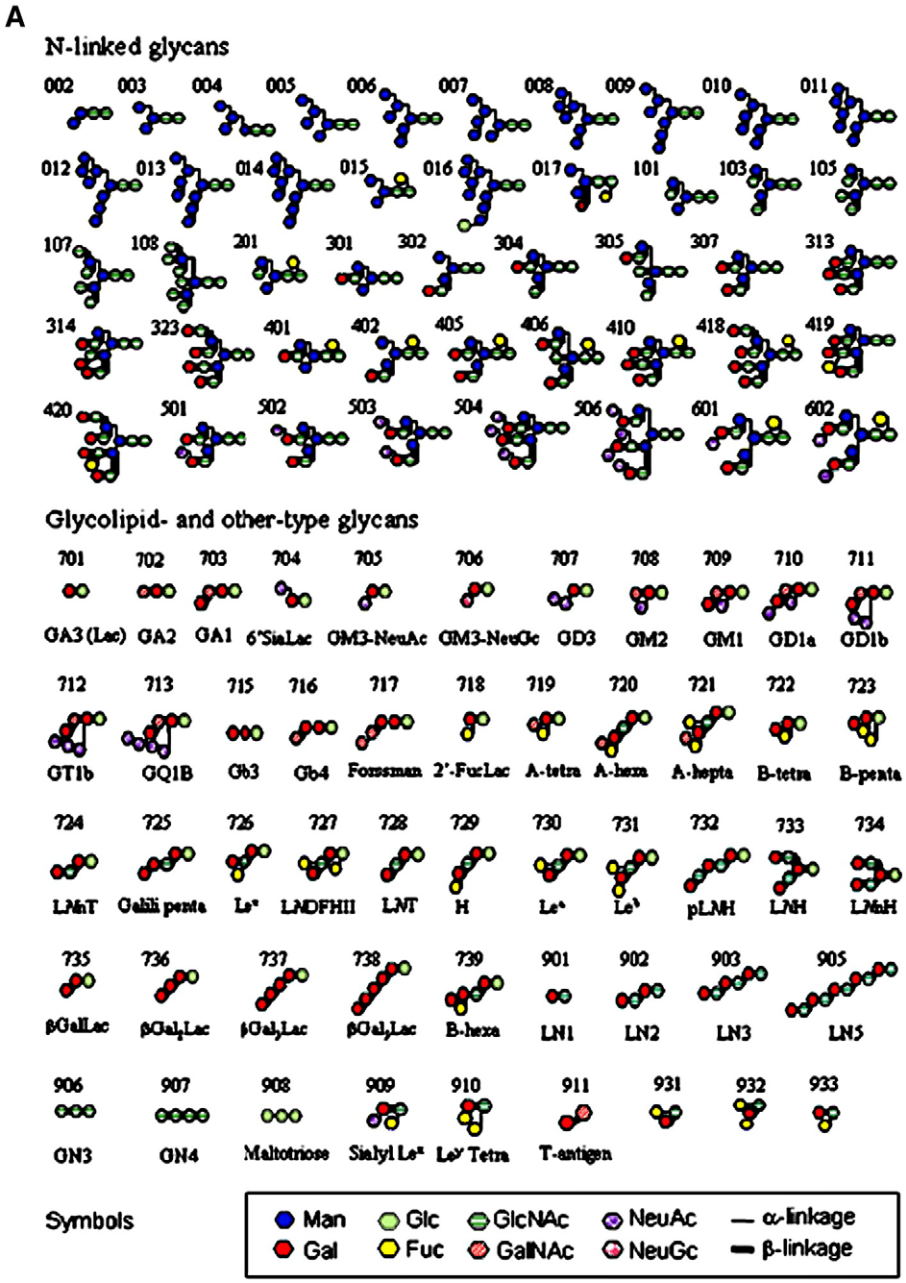

Fig. 6. Glycan binding profile of HOL-30. A: Schematic representation of oligosaccharide structures. Note that the reducing terminal is pyridylaminated for FAC analysis. Symbols used to represent pyranose rings of monosaccharides are shown in the box at the bottom of the figure. Anomeric carbon, i.e. position 1, is placed at the right side, and 2, 3, 4… are placed clockwise. Thin and thick bars represent α and β linkages, respectively. B: Bar graph representation of affinity constants (K_a) of HOL-30 toward N-linked glycans. The small Arabic figures in the below correspond to the sugar numbers indicated in Fig. 5A; whereas large Roman figures on the right side of graphs represent types of glycans: high-mannose-type (I), agalactosylated-type (II), galactosylated-type (III), sialylated-type (IV), glycolipid-type (V) and others (VI).

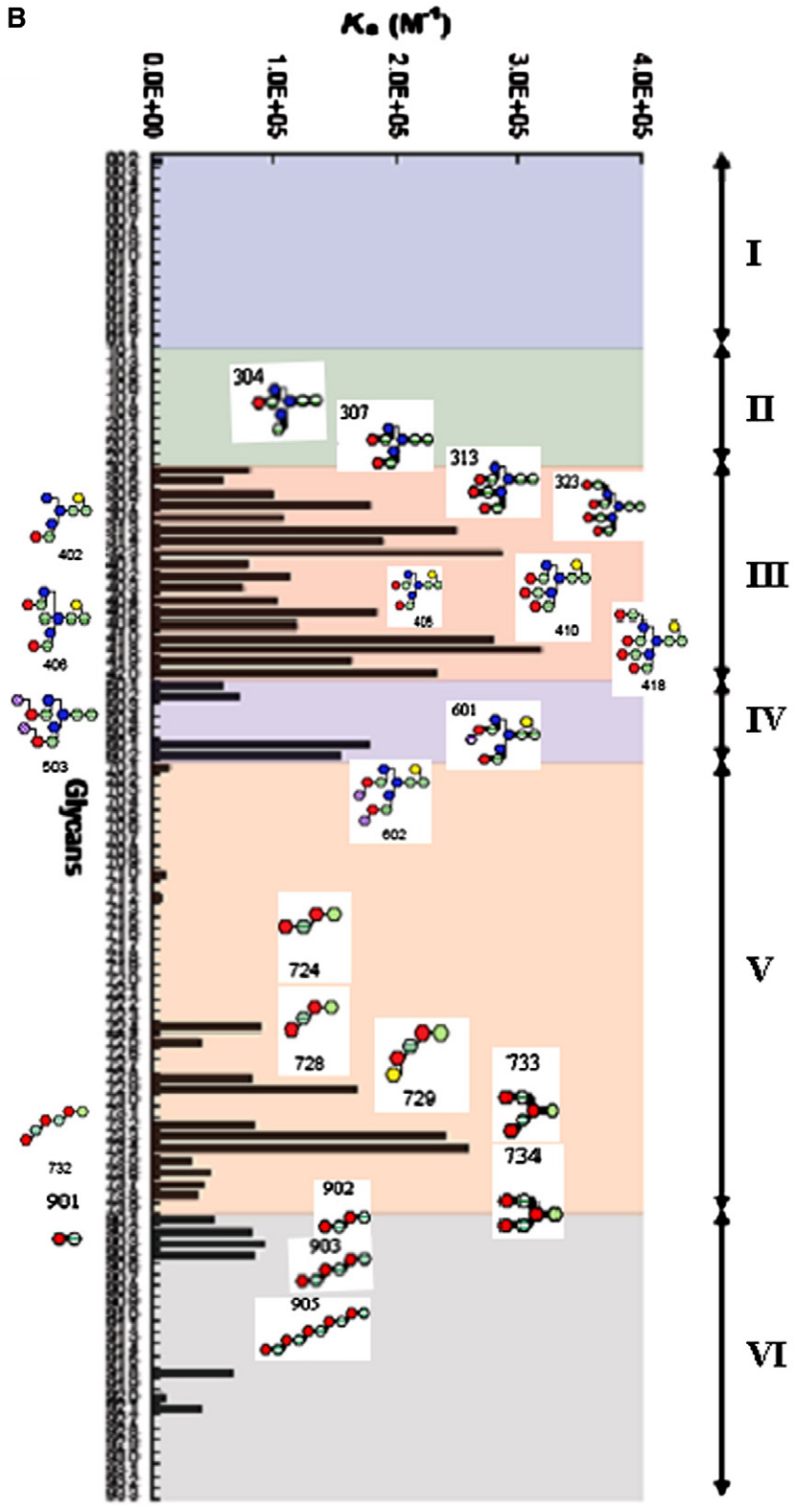

Fig. 6 (*continued*).

medium. The lectin also eluted at the same position by GPC in 20 mM $CaCl_2$ containing TBS.

3.3. Isoelectric point of HOL-30

As shown in Fig. 3, most of the HOL-30 migrated to p*I* 6.7 with faint bands at p*I* 6.4 and 7.2 in isoelectric focusing.

3.4. Thermostability and optimal pH range; divalent cations and sulfhydryl-preserving reagents against the hemagglutination activity

Hemagglutinating activity of HOL-30 decreased to half after incubation at 50 °C for 60 min, and had completely lost all activity by 60 °C for 60 min (Fig. 4A). For pH stability, conditions below pH 5, activity was

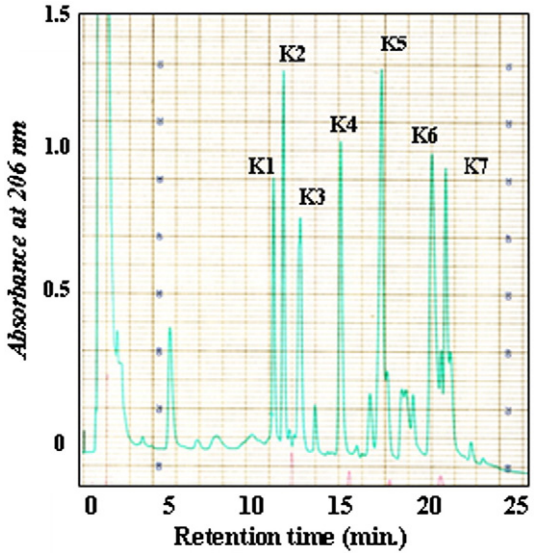

Fig. 7. Elution profile of peptides generated by enzymatic cleavage of HOL-30. Purified peptides are identified with a K prefix in the figure.

totally lost (Fig. 4B). Hemagglutinating activity of HOL-30 in the presence of 20 mM divalent metals was not enhanced compared to a control. The addition of SH-preserving reagents or chilate reagents (EDTA and EGTA) did not interfere with the hemagglutinating activity of HOL-30, indicating that the lectin activity was independent of the presence of Ca^{2+}, Mn^{2+} and Mg^{2+} ions and SH-preserving reagents (data not shown).

3.5. Saccharides inhibit hemagglutinating activity by HOL-30

Hemagglutinating activity of HOL-30 adjusted as hemagglutinating unit into 16 was effectively inhibited by lactose and melibiose. In addition, Gal, methyl α- and β-galactopyranoside, GalNAc, methyl α- and β-GalNAc, and asialofetuin also inhibited the hemagglutination of HOL-30 (Table 3).

3.6. Affinity constant (K_d) and the glycan binding profile of HOL-30 by FAC

HOL-30, 3 mg/mL, was immobilized on NHS-activated Sepharose and the resin was packed into the column (inner diameter, 2 mm; length, 10 mm; bed volume, 31.4 μL). The effective ligand content (Bt) based on concentration-dependent analysis (Hirabayashi et al., 2002, 2003) of the prepared column was determined to be 0.88 nmol using pNP-β-lactoside (1–50 μM; each 800 μL applied) at a flow rate of 125 μL/min. Based upon this data, the K_d value of HOL-30 on pNP-β-lactoside was determined to be 2.76×10^{-5} M (Fig. 5). For the determination of the glycan binding profile of HOL-30, each of 113 pyridylaminated (PA) oligosaccharides is shown in Fig. 6A: (I) high-mannose-type (002–017), (II) agalactosylated-type (101–205), (III) galactosylated-type (304–420), (IV) sialylated-type (501–602), (V) glycolipid-type (701–739), and (VI) others (901–933) were applied to the affinity column. The glycan binding profile for HOL-30 by FAC analysis is given in Fig. 6B. No high-mannose-type and agalactosylated-type oligosaccharides were recognized by the lectin but HOL-30 interacted with complex-type galactosylated-*N*-glycans (III). The increase in number of branched *N*-acetyllactosamine chains to core structure increased with increasing affinity with the lectin, such as: mono-antennary; 402, 306 < bi-antennary; 405, 307 < tri-antennary; 410, 313 < tetra-antennary; 418, 323. The presence of α1-6 fucose to core structure did not contribute to the increase of the affinity as above. On sialylated complex-type N-linked glycans (IV), α2-3 sialylation to biantennary complex-type glycan (i.e. 602) showed the same affinity to the lectin as galactosylated-type biantennary glycan (i.e. 405), however, α2-6 sialylation (i.e. 503) never bound. The addition of bisected GlcNAc to β1-4Man core (i.e. 406) reduced the affinity to HOL-30 compared with the absence of bisected GlcNAc (i.e. 405). In regard to glycolipid-type glycans (V), HOL-30 recognized both Galβ1-3GlcNAc (Type 1, *LNT*; 728 and 733) and Galβ1-4GlcNAc (Type 2, *LNnT*; 724 and 734) structures with the same affinity while HOL-30 had higher affinity for pentaose *LNFP*-I (729), which was twice that of *LNT* (728). On others (VI), the extension of repeating *N*-acetyllactosamine units (901–905) did not result in a significant increase in affinity to HOL-30.

3.7. N-terminus sequence analysis of whole protein and lysyl endopeptidase cleaved peptides of HOL-30

Even though up to 200 pmol of intact HOL-30 was applied, the N-terminus sequence analysis still did not give a sufficient amount of PTH-amino acid and it was concluded that the N-terminal amino acid residue of HOL-30 was blocked. A digest of the protein with 1 nmol of lysyl endopeptidase was separated by reversed-phase HPLC on a C18 column (Fig. 7) and primary structure analyses of seven major peptides were conducted by Edman degradation (Table 4). Each mass of peptides was determined by MALDI-TOF MS (Table 4) and most of the six peptide structures had little similarity with other known proteins. However, Trp^{5}-Asn^{19} in the K6 of the peptide sequence had significant similarity (46%) with the Trp^{116}-Asn^{130} sequence of the 16 kDa sponge galectin purified from *G. cydonium* (Pfeifer et al., 1993).

4. Discussion

HOL-30 showed a 30 kDa band by SDS-PAGE under both reducing and non-reducing conditions, but eluted at a 60 kDa position by GPC, suggesting that the protein is a non-covalently bound dimer. Partial primary structure analysis of 7 peptides generated by cleavage using lysyl endopeptidase showed 160 amino acid sequences of which many parts, contained unique sequences, with the exception of the amino acid sequence of the K6 peptide that showed significant similarity with the sugar recognition motif of *G. cydonium* galectin. In addition to the blocked N-terminus amino acid and the non-metal requirements for sugar-binding activity, the lectin also shared other common

Table 4
Amino acid sequence of peptides by Edman degradation and molecular mass determination by MALDI-TOF MS

Peptides	Sequence																																	MH$^+$	
	1				5					10					15					20					25					30					35	Calculated[a]	Observed[b]
K1	G	S	I	I	S	E	N	N	A	I	H	P	T	V	K																			1679.5	1679
K2	D	Q	L	V	V	F	H	H	D	A	R	A	D	W	G	S	E	E	H	K														2376.7	2376.5
K3	E	I	T	T	Y	N	I	P	A	G	A	N	I	S	D	I	K																	1820.6	1820
K4	E	Q	L	Q	Y	Y	P	T	S	D	V	L	L	N	I	N	P	R	K															2291.6	2291.6
K5	F	A	S	A	D	H	P	L	Y	A	M	I	E	G	V	P	T	L	T	Y	M	A	Y	E	H	S	P	K						3077	3076.5
K6	Y	G	T	I	W	G	T	E	V	R	P	S	G	F	P	F	T	G	N	V	A	V	A	L	G	K								2720.7	2725.1
K7	L	V	S	I	V	S	T	N	V	A	I	H	P	T	V	Q	L	G	V	V	A	H	G	I	A	L	V	G	L	T	P	M	I	Y	K	3603.9	3608.3

[a] Calculated as monoisotopic mass.
[b] Values were calculated for MH$^+$ from multiple charged signal observed.

properties with galectin. The oligosaccharide recognition profile of HOL-30 by FAC analysis showed a similar pattern with that of the general characteristics of galectins, having affinity for free 4-OH and 6-OH of galactose and 3 or 4-OH GlcNAc of lactosamine structure. HOL-30 also shared the following features similar to many galectin types, such as (1) bound to complex-type *N*-glycans and this affinity for binding increased with increase in branching number from mono to tetraantenary *N*-glycans (Fig. 6B III), (2) showed a reduced affinity for complex-type *N*-glycans that possessed a bisecting GlcNAc residue (Fig. 6B III), and (3) recognized α2-3 sialylated non-reducing terminal Gal, but not α2-6 sialylated Gal (Fig. 6B IV). It is therefore concluded from the evidence of the partial primary structure and its glycan binding profile that HOL-30 may be classified as a galectin from the genus *Halichondria*.

Although HOL-30 had a slight structural similarity with *G. cydonium* galectin but it had clearly different oligosaccharide recognition properties with the other lectins. HOL-30 recognized both type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc) *N*-acetyllactosamine glycans, which are both different from the *G. cydonium* galectin, that has a specific affinity for type 1 glycan (Kamekawa et al., 2006) and which is also different from HOL-II that has a specific affinity for type 2 glycan (Kawagishi et al., 1994). The significant affinity of HOL-30 for LNFP-I (Fig. 6B V) also differed from that of *G. cydonium* galectin, having strong affinity for A-tetra/hexasaccharide and Forssman pentasaccharide (Hirabayashi et al., 2003; Hanisch et al., 1996). Interestingly, prototype *G. cydonium* galectin exists as different isomers consisting of 13, 15, and 16 kDa. These isomers also make non-covalent oligomeric complexes as a dimer (30 kDa), a trimer (46 kDa), and a tetramer (62 kDa) as was shown by MALDI-TOF MS, SDS-PAGE, and western blotting when they were not treated with heat under reducing conditions (Staltz et al., 2006). However, when heated under reducing conditions they were dissociated to monomeric lectins. In our case, although we treated HOL-30 under the same dissociation conditions, it showed only a 30 kDa band. So, it is not clear at this moment whether or not HOL-30 will be dissociated further. Many sponge lectins show various molecular sizes, sugar recognition properties and bioactivities, which suggests that there are multiple and varied purposes for these lectins. In *H. okadai*, our purification of HOL-30 adds to the other two previously purified lectins — 21 kDa HOL-I, that recognizes the acetylated amino sugars, GalNAc and GlcNAc, and 42 kDa HOL-II, that recognizes type 2 *N*-acelyllactosamine (Kawagishi et al., 1994). In this study, HOL-30 showed considerably different characteristics including molecular mass and sugar-recognition specificity, and we conclude that at least, three different kinds of lectins (HOL-I, HOL-II, and HOL-30) are present in *H. okadai*. To obtain a clearer information about the primary sequences and physiological role of HOL-30, it has been planned to clone full length cDNA of the lectin.

Porifera is the lowest multicellular animal, but they consist of various characteristic cells such as choanocytes, pinacocytes, sclerocytes, ameboid cells, archaeocytes, and gamete cells embedded in the mesohyl as part of the extracellular matrix. Recently, the mechanism for the promotion of silica formation in the demosponge *S. domuncula* was elucidated as a cooperative relationship between endogenous 35 kDa galectin in the extracellular matrix and 24 kDa silicatein which is an essential component for silica spicule formation (Schröder et al., 2006). After the galectin forms self-aggregating structures thusly forming macromolecules, the hydrophobic C-terminus region of the protein interacts with silicatein, directing silica formation in the presence of Ca^{2+} and endogenous collagen. The relationship between HOL-30 and silica formation in *H. okadai* does not appear to be very strong, however, the amount of HOL-30 in this sponge is high and we conclude that it must serve some important physiological role. As in the case of the lectin from octocoral, it may serve to function as a part of a symbiotic relationship with potentially useful microorganisms (Jimbo et al., 2000). On the other hand, the role of the lectin may be to protect the sponge from enemies for regulating their living environment. Indeed, lectins of the marine sponges may still have many unknown functions. To determine the areas of HOL-30 tissue localization in *H. okadai*, methods such as antibody staining and/or lectin affinity column chromatography may yield valuable information in regard to the roles of HOL-30 in this species and aid in our understanding of its binding properties in relation to endogenous and exogenous ligands.

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