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Glycan-binding profile of a D-galactose binding lectin purified from the annelid, *Perinereis nuntia* ver. *vallata*

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ABSTRACT

A lectin recognizing D-galactose was purified from the pacific annelid *Perinereis nuntia* ver. *vallata* (Polychaeta) by affinity chromatography. Hemagglutinating activity, with a very low titer suggesting the presence of lectin appeared in the supernatant from the homogenization of body with Tris-buffered saline. However, dialyzed supernatant from the precipitate homogenized by galactose in the buffer revealed strong hemagglutinating activity against human erythrocytes. The crude supernatant was applied onto lactosyl–agarose column, and only the supernatant eluted from precipitate with galactose was obtained a galactose-binding lectin with 32 kDa polypeptide was obtained from the supernatant of the precipitate, extracted in presence of galactose. It suggests that the lectin tightly binds with glycoconjugate as endogenous ligand(s) in the tissue. Hemagglutinating activity against trypsinized and glutaraldehyde-fixed human erythrocytes was specifically inhibited by D-galactose, *N*-acetyl-D-galactosamine, lactose, melibiose, and asialofetuin. Glycan-binding profile of the lectin analyzed by frontal affinity chromatography shows that the lectin recognizes branched complex type N-linked oligosaccharides and both type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc) lactosamine. The surface plasmon resonance study of the lectin against asialofetuin showed the k_{ass} and k_{diss} values are 5.14×10^{4} M^{-1} s^{-1} and 2.9×10^{-3} s^{-1}, respectively. The partial primary structure of the lectin reveals 182 amino acids with novel sequence.

1. Introduction

Lugworm, polychaeta is a large class in phylum annelid. All live in the sea and over 10,000 species are known to locate in the world from tideland to deep sea. They have segmented body cavities (coelom) filled by coelomocytes and well developed organs as sense nerve system, muscled body wall, and guts. Strong regenerative and autonomic abilities are present for the high viability and immense reproduction. A number of carbohydrate-binding protein (lectin) which recognize D-galactose, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine are isolated from families Nereididae; *Neanthes japonica* (Ozeki et al., 1997), Eunicidae; *Marphysa sanguinea* (Ozeki et al., 1997), Terebellidae; *Amphitrite ornate* (Garte and Rissell, 1976), Sabellidae; *Serpula vermicularis* (Molchanova et al., 2007), Chaetopteridae; *Chaetopterus variopedatus* (Mikheyskaya et al., 1995). D-Galactose-binding lectins were purified also from other class oligochaeta (earthworm), *Lumbricus terrestris* (Hirabayashi et al., 1998) and hirudinoidea (leech), *Hirudo medicialis* (Cole and Zipser, 1994a). They were purified from tissues by galactose containing buffer (Hirabayashi et al., 1998; Ozeki et al., 1997; Cole and Zipser, 1994a). The requirement of the haptenic saccharide for lectin extraction from the tissue was the same as the purification of galactose-binding lectin from coelomocytes and body wall of echiuroid (spoon worm) (Matsui, 1984; Ozeki et al., 1997) which is believed to be a phylum closely related to the annelids. This evidence suggests that galactose-binding lectins localize in tissues by binding with endogenous ligand(s). A galactose-binding lectin isolated from leech has been shown to be located in sensory neurons of the embryo and it has abundantly located in the epithelial cells of adult leech in addition to sensory neuron suggesting that it is a developmentally regulated lectin (Cole and Zipser, 1994b). Since the characterizations of primary structure of most of marine invertebrate lectins except the one from earthworm (Hirabayashi et al., 1998) are not understood, it is worth to study their functional role.

Generally the carbohydrate-binding specificity of marine invertebrates is not preciously profiled and believed to be simple than vertebrate, and many marine invertebrate lectins can recognize both anomeric bond α- and β- (Kawsar et al., 2008; Ozeki et al., 1997, 1991). However, precious analysis of marine invertebrate lectins has shown that they recognize specifically characteristic carbohydrates (Kawsar et al., 2008; Kamekawa et al., 2006; Naganuma et al., 2006). Profiling glycan-binding helps diagnostic purposes through carbohydrate moiety of targets and supports useful information for glycobiology. Besides,

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precious bioactivities of lectins from marine organisms also have been found to be similar to mannosebinding lectin from cyanobacteria *Oscillatoria agardhii* having strong anti-human immunodeficiency virus (HIV) activity (Sato et al., 2007). In a similar way, two polychaeta lectins from Chaetopteridae and Sabellidae inhibited the syncytium formation of HIV-I infected C8166 cells (Wang et al., 2006; Molchanova et al., 2007). They are a 30 kDa polypeptide recognizing D-galactose and a disulfide-bounded quartermer consisting of 12.7 kDa polypeptides recognized *N*-acetyl-D-galactosamine, respectively. Finding glycan-binding profile of them will make clear the mechanisms of such bioactivities. Frontal affinity chromatography is established by Kasai and Ishii (1978a,b) as an advanced technology to detect weak interaction between proteins and its ligand. It has been developed by Hirabayashi and colleague (Hirabayashi et al., 2002) as a tool to profile the glycan binding specificities of lectins.

Perinereis nuntia (Nereididae) lives in the sand of tidal zones and the sandy bottom of shallow sea throughout Japan, Micronesia, and Australia. A variation *vallata* reproduces at the tideland. *P. nuntia* var. *vallata* are artificially bred in Japan for the feed of fishing, being able to stably supply for study. In this study, a 32 kDa galactose-binding lectin was purified from the lugworm and characterized with analysis of partial primary structures and the glycan-binding property was profiled by frontal affinity chromatography to compare with other invertebrate lectins.

2. Materials and methods

2.1. Chemicals

Lactose, melibiose, D-galactose, D-glucose, D-mannose, D-fucose, L-fucose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and lysyl endopeptidase were purchased from Wako Pure Chemical Co. Inc., Japan and were of the highest purity grade. Methyl α-D-galactopyranoside and methyl β-D-galactopyranosides were from Pfanstiehl Laboratories Inc., USA. α-Methyl-*N*-acetyl-D-galactosamine (Me-α-GalNAc) and β-methyl-*N*-acetyl-D-galactosamine (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 Chem. Co. Ltd., Japan. Superdex 75, Sephadex G-75, Sepharose 4B, *N*-hydroxysuccinimide (NHS)-activated Sepharose 4 Fast flow, Sensor chip CM5, BIAcore amine coupling kit were obtained from GE Healthcare and Sigma-Aldrich, USA. Bicinchoninic acid (BCA) kit is from Pierce Co. Ltd., USA. Mightysil RP-18 column, acetonitrile (HPLC grade), trifluoroacetic acid (TFA), and sodium bicarbonate buffer (pH 9) obtained from Kanto Chemial Co. Inc., Japan. Twenty pyridylaminated (PA) oligosaccharides used in this study were obtained from Takara Bio. Co. Inc., Japan.

2.2. Purification of D-galactose-binding lectin from P. nuntia

The marine worm *P. nuntia* was supplied from a fishing shop at Yokohama City. In general preparation, 200 g (wet weight) of worms were disrupted to paste with a razor blade and homogenized with 10 vol (w/v) of Tris-buffered saline (TBS) (10 mM Tris(hydroxylmethyl) aminomethane-HCl, pH 7.4, containing 150 mM NaCl) containing 2 mM of a protease inhibitor mixture. The homogenates 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 crude supernatant (C_2) was centrifuged again at 27,500 *g* for 1 h at 4 °C and the precipitate was homogenated again with 3 vol (w/v) of TBES (TBS containing 10 mM EDTA) containing 50 mM galactose for 4 h overnight at 4 °C. It was centrifuged at 27,500 *g* for 1 h at 4 °C and the supernatant was dialyzed against TBS till free from galactose. Centrifuged crude supernatant (C_1) extract was applied to an affinity column of lactosyl–agarose (10 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 galactose in TBS and 1 mL fractions were collected by an auto-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 purified lectin by TBS to remove free galactose. The concentration of 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 Co. Ltd., 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. 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 10% 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; 15% and 5% polyacrylamide were used in separation and stacking gels, respectively). The molecular mass of the polypeptide was determined by SDS–PAGE (constant current at 30 mA for 1 h) according to the method of 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 destaining by washing with 40% methanol and 10% acetic acid.

2.4. Effects of reducing agent, temperature, and pH

The effects of a sulfhydryl-preservation reagent for the hemagglutinating activity of lectin were evaluated by 2-mercaptoethanol (0 to 20 mM) with the lectin using 96-well plates. The temperature stability of the lectin was determined by incubating lectin at different temperatures over a range of 20 to 80 °C with 10° increments for 1 h. The effect of pH 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 buffer (pH 3–6) and 20 mM Tris-HCl buffer (pH 7–12) for 3 h, and the differences of hemagglutinating activity by the lectin were measured in titer plates.

2.5. Hemagglutination assay and carbohydrate-binding specificity

Hemagglutination assay was performed using 1% (w/v) trypsinized and 0.25% glutaraldehyde-fixed rabbit and human erythrocytes as described previously (Matsui, 1984). Erythrocytes were suspended at a concentration of 1% (w/v) in TBS. In the general assay, 20 μL each of TBS, TBS containing 1% Triton X-100, and erythrocytes were added to 20 μL of the two times-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, 20 μL of each of the sugar (200 mM) and the glycoprotein (5 mg/mL) was serially diluted with TBS and added to lectin with the titer of 16, 1% Triton X-100, and erythrocytes