ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 7, pp. 709-716. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 7, pp. 877-885.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-299, April 12, 2009.

Purification and Biochemical Characterization of a D-Galactose Binding Lectin from Japanese Sea Hare (*Aplysia kurodai*) Eggs

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Received September 19, 2008
Revision received February 18, 2009

Abstract—A lectin was purified from Japanese sea hare *Aplysia kurodai* by lactosyl-agarose affinity chromatography. The molecular mass of the lectin was determined to be 56 and 32 kDa by SDS-PAGE under non-reducing and reducing conditions, respectively. It was found to agglutinate trypsinized and glutaraldehyde-fixed rabbit and human erythrocytes in the absence of divalent cations. The lectin exhibited stable thermo-tolerance as it retained hemagglutinating activity for 1 h even at 80°C and showed stability at pH 10. By contrast, it was very sensitive at pH less than 5 and in the presence of the sulfhydryl-group preserving reagent, β-mercaptoethanol. The hemagglutinating activity by the lectin was specifically inhibited by D-galactose, galacturonic acid, methyl-α- and methyl-β-D-galactopyranoside, lactose, melibiose, and asialofetuin. The association rate constant (k_{ass}) and dissociation rate constant (k_{diss}) were determined for the lectin to be $4.3 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$ and $2.2 \cdot 10^{-3}$ sec^{-1}, respectively, using a surface plasmon resonance biosensor. The lectin moderately inhibited cell proliferation in the P388 cell line dose dependently. Interestingly, lectin-treated cells did not show a fragmented DNA ladder as is caused by apoptosis, suggesting that the cell proliferation inhibition was caused by another unknown mechanism.

DOI: 10.1134/S0006297909070025

Key words: *Aplysia kurodai*, cell proliferating inhibition, D-galactose-binding lectin, galacturonic acid, sea hare, surface plasmon resonance

Lectins are carbohydrate-binding proteins that recognize specific oligo- and/or monosaccharide structures and are widely distributed in many organisms. In marine invertebrates, they may function in immunity as antibacterials, for self and non-self recognition, for gamete recognition in fertilization, and as protective elements as toxins. Many animal lectins with various carbohydrate-binding specificities have been discovered from eggs or reproductive organs [1-4], the carbohydrate recognition mechanisms seeming to be important for biological events such as fertilization or early development. In phylum Mollusca, some lectins with antibacterial, opsonizing, and cytotoxic activities were found from their organs [5, 6]. The hermaphroditic sea hare belonging to class Opisthobranchia, species *Aplysia kurodai*, is common at Japanese tidal zones and they spawn eggs like noodle strings in spring to early summer season. From the

Abbreviations: AGL, *Aplysia depilans* gonad lectin; AKL, *Aplysia kurodai* egg lectin; BCA, bicinchoninic acid; BSA, bovine serum albumin; Con A, concanavalin A; EDC, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide; HOL, *Halichondria okadai* lectin; NHS, *N*-hydroxysuccinimide; RCA, *Ricin communis* agglutinin; SBL, sialic acid binding lectin; SPR, surface plasmon resonance; TBS, Tris-buffered saline containing 150 mM NaCl, pH 7.4; WGA, wheat germ agglutinin.

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species, 70 kDa hexameric galacturonic acid-binding lectin consisting of 13 kDa subunits has been isolated from eggs using galacturonic acid-conjugated Sepharose gel [7]. Two 28 and 26 kDa D-galactose binding lectins with cell attachment potency against human sarcoma cells were purified from the mantle [8]. Gilboa-Garber and colleagues discovered a galactophylic lectin from gonad of *Aplysia depilans* (*Aplysia* gonad lectin; AGL) [9] having antibacterial activity and cytotoxicity for carcinoma cells [10]. They characterized the precise saccharide binding specificity with strong affinity against galacturonic acid and developed medical applications [11, 12]. Wilson and colleagues found a dimeric lectin consisting of 34 kDa subunits from gonad of *Aplysia californica* and they showed that the lectin grows neurons of the sea hair [13]. In this study, we have purified a lectin from ovulated eggs of *A. kurodai* presenting a different molecular species from lectins purified from same animal, and showed its general biochemical properties. Sea hare eggs remain in tide pools until hatch, so they need to oppose enemies biochemically. Sometime, lectins and toxins are closely related as shown as a galactose-binding lectin RCA 120 presents together with harmful toxin ricin in beans and many lectins are present in snake venom. As seen in bullfrog eggs, a sialic acid-binding lectin (SBL) has activity as a ribonuclease and apoptotic activity against mouse lymphoma cells P388 [14].

We evaluated the effect of the lectin from sea hare eggs for cell proliferation of lymphoma cells and determined the kinetics against the glycoprotein recognized by the lectin using surface plasmon resonance (SPR).

MATERIALS AND METHODS

Chemicals. Lactose, sucrose, melibiose, D-glucose, D-galactose, D-mannose, L-fucose, *N*-acetyl D-galactosamine, *N*-acetyl D-glucosamine, D-galacturonic acid, and D-glucuronic acid were purchased from Wako Pure Chemical Ind. Ltd. (Japan) and were of the highest purity grade. Lactosyl-agarose, concanavalin A (Con A) from jack bean *Canavalia ensiformis*, and wheat germ agglutinin (WGA) were purchased from Seikagaku Kogyo Co. Ltd. (Japan). Methyl-α-D-galactopyranoside and methyl-β-D-galactopyranoside were from Pfanstiehl Laboratories Inc. (USA). Proteins asialofetuin and fetuin were purchased from Sigma (USA). Sephadex G-75, *N*-hydroxysuccinimide (NHS)-activated Sepharose 4 Fast Flow, Sensor chip CM5, and Amine coupling kit (including 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC), NHS, and 1 M ethanolamine-HCl, pH 8.5) were from GE Health Sciences (USA). Bicinchoninic acid (BCA) kits were purchased from Pierce Co. Ltd. (USA). Alamar blue was from BioSource International Inc. (USA).

Animals. Sea hare *Aplysia kurodai* and its eggs were collected in the tidal zone at the Zushi coast, Kanagawa prefecture, Japan from spring to summer season. Eggs and animals were stored at −80°C or used after collection according to the situation.

Protein purification. Two hundred grams (wet weight) frozen sea hare *A. kurodai* eggs as yellow string noodles was crushed into particles in a mortar, then mixed with 10 volumes (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 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 two times and was applied to a lactosyl-agarose affinity column (5 ml) that was fitted with a Sephadex G-75 pre-column (5 ml). After application of the extracts, the column was washed extensively 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 chromatographic 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 a UV spectrophotometer at 280 nm and SDS-PAGE [15] were combined and dialyzed against 1000 volumes of TBS to remove free sugar. The concentration of purified lectin was determined using a BCA protein assay kit with bovine serum albumin as the standard protein [16, 17] by measuring absorbance at 562 nm.

Physical and chemical characteristics: molecular mass determination by electrophoresis. 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% sodium dodecyl sulfate (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 × 100 × 1 mm; 12.5 and 5% polyacrylamide was used in separation and upper gels, respectively). The molecular mass of the polypeptide was determined by SDS-polyacrylamide gel electrophoresis (PAGE) (constant current at 30 mA for 1 h) according to a previous report [15]. After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid followed by destaining with 40% methanol and 10% acetic acid.

Carbohydrate analysis. The sugar content of the purified lectin was determined by the phenol–sulfuric acid method with glucose as the standard [18] by measuring absorbance at 340 nm. To characterize the oligosaccharide moiety of the lectin, 0.5 mg lectin was dissolved in 100 mM sodium bicarbonate containing 150 mM NaCl and conjugated with NHS-activated Sepharose (0.5 ml) overnight at 4°C. Non-reacted groups on the medium were masked with 1 M ethanolamine, pH 8, overnight.

The lectin-conjugated Sepharose was packed in a column and washed with TBS.

HOL-30, a lectin purified from Japanese black sponge *Halichondria okadai* that has affinity to branched-complex type *N*-linked oligosaccharides was prepared as previously reported [19] for the characterization of glycan on the *A. kurodai* eggs lectin. After dissolving 100 μg Con A, WGA, HOL-30, and the *A. kurodai* egg lectin in 500 μl of TBS containing 10 mM $CaCl_2$, they were applied to the lectin-conjugated Sepharose gel, respectively. During the analysis by Con A and WGA, 50 mM galactose was co-present in every step to avoid the lectin-conjugated Sepharose binding galactose residue of glycans on these plant lectins. After washing the column with TBS containing 10 mM $CaCl_2$, the column-bound materials were eluted using TBS containing 50 mM D-mannose (for Con A), *N*-acetyl D-glucosamine (for WGA), lactose (for HOL-30), and D-galactose (for *A. kurodai* eggs lectin), respectively.

Hemagglutination and carbohydrate binding specificity. The hemagglutination assay was performed using 1% (w/v) trypsinized and 0.25% glutaraldehyde-fixed rabbit and human erythrocytes as modified previously [20]. Erythrocytes were suspended at a concentration of 1% (w/v) with TBS. In the general assay, 20 μl each of TBS, TBS containing 1% BSA, and erythrocytes were added with 20 μl of the serially-diluted lectin with TBS in 96-well V-shaped 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 (4 mg/ml) was serially diluted with TBS and added with the lectin to previously diluted TBS to adjust the titer to 16, 1% BSA, and erythrocytes in 96-well V-shaped titer plates for 1 h. The minimum inhibitory sugar concentration against the lectin was expressed as a score of negative activity. The effects of Ca^{2+}, Mn^{2+}, and Mg^{2+} ions on the hemagglutinating activity of the *A. kurodai* eggs 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 the range of 20 to 100°C in 10°C increments for 1 h.

The pH effect was determined by the measurement of the titer of the lectin in the range pH 3 to 12. The 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 8-12), for 3 h at 25°C and subsequently dialyzed against 50 mM TBS, pH 7.4, and the hemagglutinating activity by the lectin was measured in titer plates.

Kinetic analysis of the lectin using surface plasmon resonance (SPR). Asialofetuin dissolved in 10 mM citrate buffer (pH 5) was manually immobilized on a CM5 sensor chip using an amine coupling kit according to protocol after the activation of carboxymethyl groups with dextran on the chip with a mixture of NHS (100 μl of 12 mg/ml) and EDC (100 μl of 75 mg/ml). Analysis was performed with a BIAcore 3000 instrument (Biacore Life Science, GE Healthcare, USA) whereby 90 μl of *A. kurodai* lectin (0 to 12 nM) as an analyte was applied to the sensor chip for injection (association) and washing (dissociation) for 2.5 min each at a flow rate of 20 μl/min. The association rate constant (k_{ass}) and dissociation rate constant (k_{diss}) were determined with BIAevaluation software v.3.0 [21].

Anti-cell proliferation activity of the lectin. Anti-cell proliferation activity was measured with P388 cells according to the method of Nitta et al. [14] whereby sarcoma cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum. The cells were collected by centrifugation, suspended in the medium, diluted to $2 \cdot 10^5$ cells/ml, and applied to a 96-well plate with each well containing 200 μl of cell suspension, 10 μl of various concentrations of *A. kurodai* lectin, and sialic acid-binding lectin (SBL) purified from an amphibian American bullfrog (*Rana catesbeiana*) as positive control [4]. The number of proliferated cells was counted after 24 and 48 h of incubation at 37°C in 5% CO_2. Cells were gently mixed with Alamar blue (10%) in each well for 2 h after cell culture. Colored medium in each well was measured using a plate reader by the absorbance at 570 nm [22]. The percentage decrease was calculated as the increase in cell number against the increase in the control cell number.

RESULTS

Purification of *Aplysia kurodai* egg lectin (AKL). Crude extraction from sea hare *Aplysia kurodai* eggs showed strong hemagglutination activity against human red blood cells, and the activity was cancelled by the presence of saccharides such as galactose and lactose. *Aplysia kurodai* egg lectin was purified on a lactosyl-agarose column via elution with 50 mM lactose containing TBS (Fig. 1), and it was shown to be a single polypeptide with molecular masses 56 and 32 kDa under non-reducing (NR) and reducing (R) conditions by SDS-PAGE, respectively (Fig. 2, lanes *1*). On the other hand, crude extracts of eggs contained various proteins by SDS-PAGE (Fig. 2, lanes *2*), indicating that the lectin was present as a disulfide bonded dimeric protein consisting of two 32 kDa polypeptide subunits. Fifteen milligrams of AKL was purified from 200 g of eggs (wet weight) and it was concentrated 172 times by affinity purification (Table 1). AKL was suggested to be a glycoprotein containing sac-

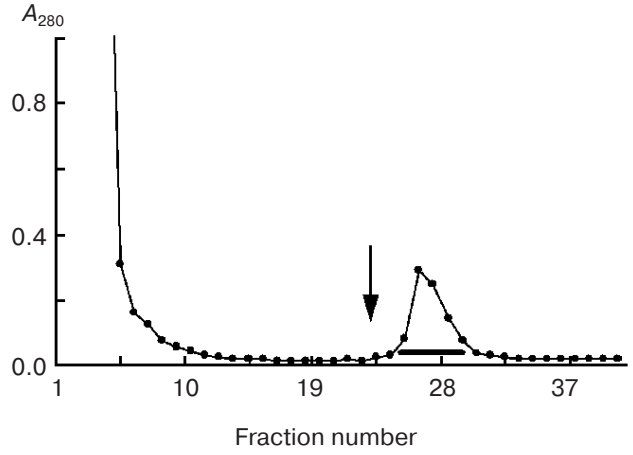

Fig. 1. Purification of AKL. Crude extract of *A. kurodai* was applied to a lactosyl-agarose column equilibrated with TBS. The column was washed with TBS and eluted with TBS containing 50 mM lactose (arrow).

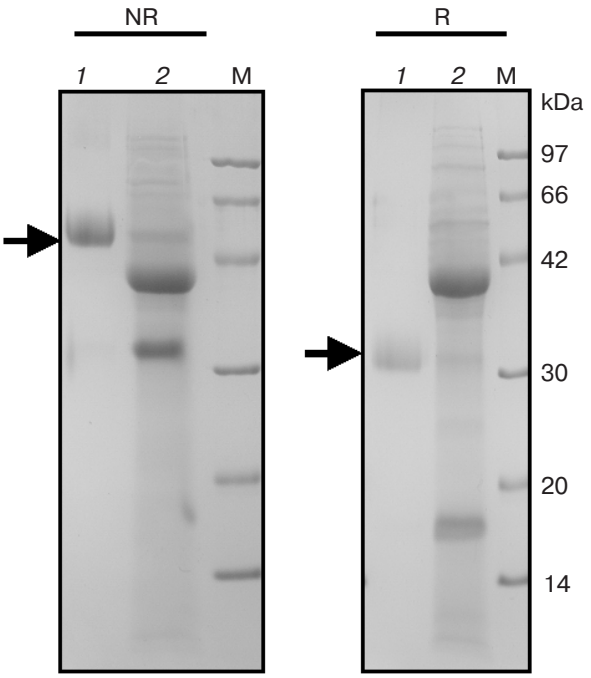

Fig. 2. SDS-PAGE pattern of AKL under non-reducing (NR) and reducing (R) conditions: *1*) purified lectin (10 μg); *2*) crude extract (10 μg); M, standard marker proteins (phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 42 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa; lysozyme, 14 kDa). Arrows designate the lectin position.

charides as 6-14% molar ratio depending on the sample lots of the lectin detected by the phenol–sulfuric acid method. Con A was bound to AKL conjugated-Sepharose column and it eluted selectively by TBS containing 50 mM D-mannose (Table 2). However, WGA, HOL-30, and AKL passed through the column. AKL was also present in the gonad of the adult sea hare (data not shown).

Saccharides inhibit hemagglutination activity of AKL. Hemagglutination activity of AKL adjusted as hemagglutinating units (HU) into 16 was effectively inhibited by D-galactose, D-galacturonic acid, methyl-α- and methyl-β-D-galactopyranoside, lactose, melibiose, asialofetuin, and asialo bovine submaxillary mucin indicating that AKL has D-galactose, D-galacturonic acid, galactosides, and glycoproteins which containing these saccharides. However, D-glucuronic acid, *N*-acetyl D-galactosamine, *N*-acetyl D-glucosamine, and fetuin did not inhibit the activity even at over 100 mM (over 1 mg/ml in the case of fetuin) (Table 3).

Hemagglutinating activity of AKL was retained even at the incubation temperature of 80°C for 60 min but almost lost activity at 90°C for 60 min (Fig. 3a). In regard to pH stability, it was stable at pH 7-12 but lost about half of its hemagglutinating activity at pH 5-6 (Fig. 3b).

Hemagglutinating activity of AKL in the presence of 20 mM divalent cations was not enhanced compared to a control. The addition of chelating reagents such as EDTA and EGTA did not interfere with the hemagglutinating activity of AKL, indicating that the lectin activity was independent of the presence of Ca^{2+}, Mn^{2+}, and Mg^{2+} ions (data not shown).

The addition of sulfhydryl-preserving reagent 2-mercaptoethanol (10 mM) for 1 h caused a loss in the hemagglutination activity of AKL (data not shown), suggesting that the lectin contains a disulfide bonds within and between molecules.

Association and dissociation rate constants of AKL. SPR analysis showed that the AKL bound asialofetuin on the sensor chip dose-dependently (Fig. 4). Kinetic parameters k_{ass} and k_{diss} for AKL were $4.3 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$ and $2.2 \cdot 10^{-3}$ sec^{-1}, respectively, by the fitting 1 : 1 affinity model using BIAevaluation 3.0 software.

Inhibition of cell proliferation by AKL. Cell proliferation of P388 was dose-dependently inhibited by the presence of AKL in the culture media (Fig. 5a). AKL ~200 μg/ml reduced cellular proliferation to 40%. However, this cell proliferation inhibition by AKL was only one tenth of that of SBL. The laddering of cellular DNA after treatment with 5 μM of SBL (as seen by agarose gel electrophoresis according to the concentration of lectin) indicated that the action of SBL was apoptosis (Fig. 5b, lanes *5-7*), but the fragmentation of DNA in P388 did not occur when cells were incubated with 10-200 μM of AKL (Fig. 5b, lanes *1-4*).

DISCUSSION

AKL is a dimeric molecule of disulfide-bound 32 kDa subunits; it has physicochemical properties similar to a hexameric 13 kDa-subunit lectin [7] purified from

Table 1. Purification of lactose binding lectin from *A. kurodai*

Fraction	Titer, HU	Volume, ml	Total activity*	Protein concentration, mg/ml	Specific activity**	Purification, fold	Recovery of activity, %
Crude extract	512	80	40 960	4.1	1.56	1	100
Purified lectin	4096	8.5	34 816	1.8	267.8	172	74

Note: HU, hemagglutinating units.
* Total activity is shown by titer × volume.
** Specific activity is shown by titer/mg protein.

Table 2. Lectin binding to AKL-Sepharose

Lectin*	Interaction with sorbent	Eluent	Oligosaccharide binding specificity of each lectin
Con A	bound**	D-Man	high mannose type N-linked
WGA	passed through***	D-GlcNAc	complex type N-linked with GlcNAc
HOL-30	–"–	lactose	complex type N-linked with lactosamine
AKL	–"–	D-Gal	D-galactose and D-galacturonic acid

* Lectins were dissolved in TBS containing 10 mM $CaCl_2$.
** After washing the column, the lectin was specifically eluted by sugar containing buffer.
*** Lectins were passed through the column when it was applied, as confirmed by SDS-PAGE.

eggs of the same species in affinity to galacturonic acid and lack of dependence on divalent cations for its activity. Thus *A. kurodai* eggs contain more than one lectin. The high temperature tolerance for hemagglutinating activity of AKL was similar with that of some other animal lectins as another *Aplysia* lectin [7, 9] and sponge lectin (HOL-I) [23]. The facts of high thermal tolerance and broad spectrum of pH stability of AKL (Fig. 3) are curious because in general, sea hare eggs are placed in mild tide pool environments.

Many animal lectins belong to representative structural families such as C-type lectin and galectin (S-type lectin), which have characteristic biochemical properties as divalent cation and SH-preserving reagent requirement, respectively. On the other hand, AKL does not require either divalent cations or SH-preservation reagent for activity, suggesting that the lectin is different from those of these families. AKL had similar biochemical properties with AGL purified from other species and the 34-kDa lectin purified from *A. californica* with primary

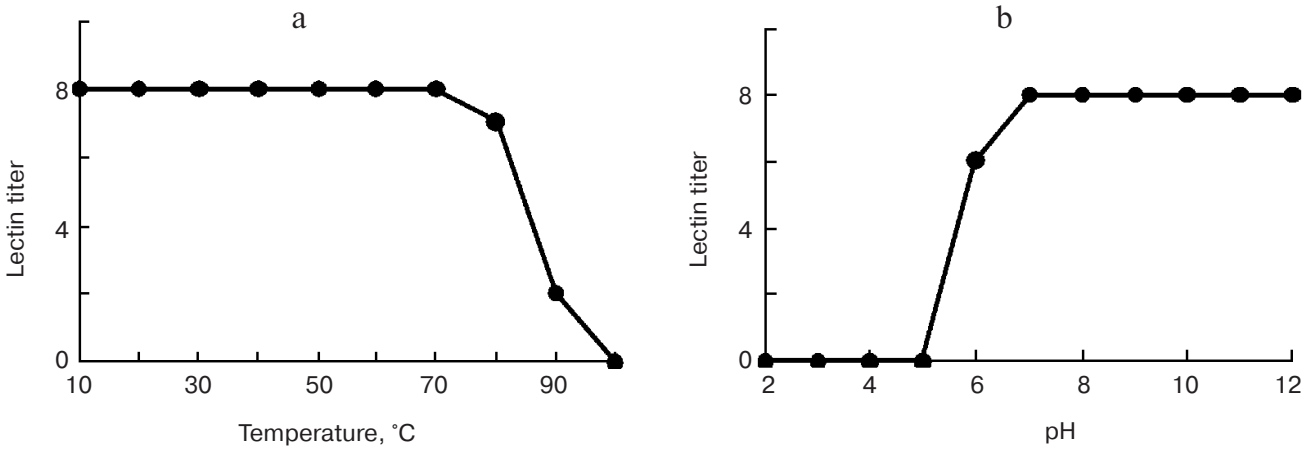

Fig. 3. Thermostability and pH stability of AKL. Effects of temperature (a) and effects of pH (b) on hemagglutination activity.

Table 3. Saccharide and glycoprotein specificity of AKL

Saccharide	Minimum inhibitory concentration, mM
D-Galactose	0.39
N-Acetyl-D-galactosamine*	–
N-Acetyl-D-glucosamine*	–
Methyl-α-D-galactopyranoside	0.39
D-Glucose*	–
D-Mannose*	–
L-Fucose*	–
Lactose (Galβ1-4Glc)	0.78
Melibiose (Galα1-6Glc)	0.39
N-Acetyllactosamine (Galβ1-4GlcNAc)	0.78
Sucrose*	–
D-Galacturonic acid	0.2
D-Glucuronic acid*	–
Glycoprotein	**Minimum inhibitory concentration, mg/ml**
Asialofetuin	0.1
Fetuin**	–
Porcine stomach mucin	0.3
Bovine submaxillary asialomucin**	–
Galα1-3Gal-HAS	0.3

Note: Titer of lectin was previously diluted to 16.
* Inhibition did not occur even at 100 mM.
** Glycoproteins did not inhibit even at 1 mg/ml.

structure of N-terminal sequence similar with that of a plant lectin, PHA [13]. Further protein chemical study of the lectins provides useful information of the structural diversity of animal lectins.

AKL strongly recognizes both α-galactosides as melibiose (Galα1-6Glc), methyl α-D-galactopyranoside, and Galα1-3Gal-HAS and β-galactosides as lactose (Galβ1-4Glc), *N*-acetyllactosamine (Galβ1-4GlcNAc), methyl β-D-galactopyranoside, and asialofetuin. *N*-Acetyl neuraminic acid (Neu5Ac) was not recognized by the lectin since fetuin did not inhibit hemagglutinating activity of AKL (Table 3) and the terminal D-galactose of saccharides was important for the binding with AKL. The inhibition by the saccharide was abolished if *N*-acetyl D-galactosamine (GalNAc) was substituted for D-galactose (Table 3). It was also unique as the lectin was inhibited by galacturonic acid, suggesting that C-6 position of the sugar is unimportant for the lectin recognition. From these results, AKL has been characterized as a D-galactose-binding or galactophilic lectin like AGL purified from *Aplysia depilans* gonad [9]. However, the carbohydrate recognition moiety of lectins slightly differs from the case among monosaccharides and oligosaccharides. Our future plan is to determine the detail of carbohydrate binding moiety of AKL. The frontal affinity chromatography (FAC) technology profiling the glycan-binding of lectin will be an excellent tool for the analysis [19].

The inhibition of hemagglutinating activity of AKL by porcine stomach mucin and asialofetuin was curious, since they contain T antigen (Galβ1-3GalNAc). Another *Aplysia* lectin, AGL, was shown to have affinity with T antigen in addition to galacturonic acid by ELISA [24]. AKL is a glycoprotein by the results from phenol–sulfuric acid and lectin binding analysis using AKL-conjugated Sepharose gel. Con A binds to D-mannose and high mannose type *N*-glycan [25]. The result that Con A was bound by AKL gel suggested AKL has N-type glycans. On the other hand, the result that two galactose-binding lectins, HOL-30 (which binds to complex type *N*-linked oligosaccharides [22]) and AKL, did not interact with the column suggests that AKL does not contain galactose residues at the terminal.

By SPR study of AKL to the asialofetuin-conjugated sensor chip we determined both association (k_a) and dissociation (k_d) rate constant in addition to the equilibrium constant (K_D), showing that slowly interacting patterns

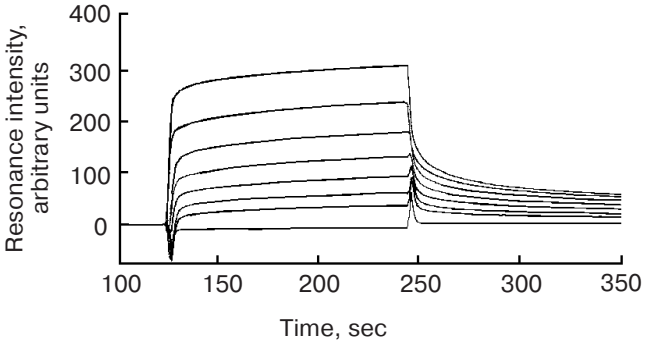

Fig. 4. Kinetic analysis of AKL by SPR. AKL was applied onto the CM5 sensor chip coupled with asialofetuin at 6.8 ng/mm^2. AKL (0-12 nM) was applied onto the asialofetuin conjugated sensor chip with 20 μl/min for 2.5 min. The chip was washed with TBS for 2.5 min. Kinetics were analyzed by BIAevaluation software version 3.0.

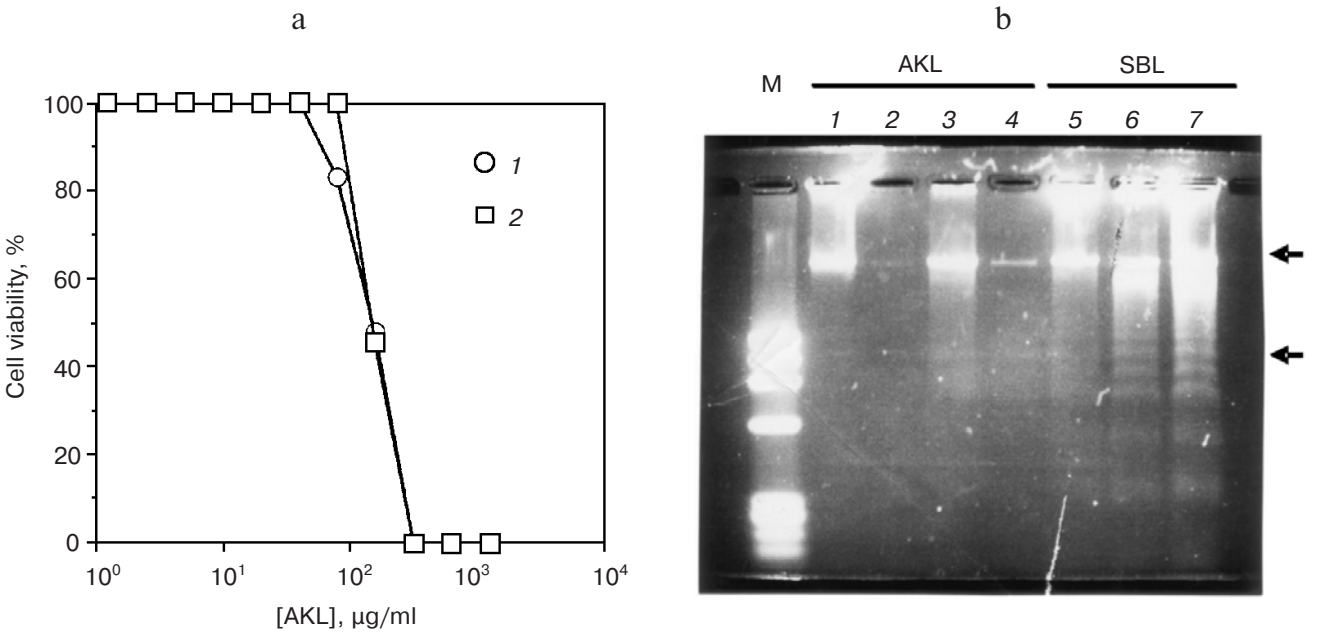

Fig. 5. Cell proliferation-inhibiting potency of AKL. a) AKL was mixed with mouse P388 cells. The growth of cells was counted after staining with 10% Alamar blue dye after incubation for 24 (*1*) and 48 h (*2*). b) Agarose gel electrophoresis to detect fragmentation of DNA by the lectins. After incubation of cells with each lectin, extracted DNA from harvested cells was analyzed by agarose-gel electrophoresis and ladders detected at wavelength 260 nm after staining with 2% ethidium bromide. Lanes: M, molecular marker; *1-4*) 10, 50, 100, and 200 μM AKL; *5-7*) 1, 2, and 5 μM SBL. Arrows designate positions of non-fragmented and fragmented DNA.

both of association and dissociation were similar to other galactose-binding lectin, RCA 120 [21]. The lower K_D value of AKL suggests oligomeric effect by the dimeric structure to straight affinity with ligand.

The inhibition of cell proliferation of mouse lymphoma P388 cells by the lectin was detected by Alamar blue assay. The dye colors proliferating cells blue by a reduction-oxidation reaction similar to the mechanism of MTT dye [22]. Alamar blue is water-soluble (so it is not necessary to solubilize cells by detergent after reaction) and is available for floating cells. The cell proliferation inhibiting activity seen in AKL was characteristic though the activity of AKL to P388 cells was only one tenth of that of SBL. The cell proliferation regulation activity by AKL seemed not to be apoptosis because we did not observe DNA fragmentation when the lectin was applied to the cells (Fig. 5b). For this reason, it is hard to explain at present how AKL induced down regulation of the cell proliferation. The mechanisms of the cell proliferative regulation activity mediated by lectin–carbohydrate interaction are still unclear.

The production of AKL-conjugated Sepharose seemed to be useful for identifying the glycan binding profiling of AKL as a further study. Using the column, we will also try to isolate endo- and exogenous ligand for the lectin as a specific glycoprotein for use as biochemical tools.

Authors are thankful to Dr. Robert A. Kanaly for critical reading of this manuscript.

This work was supported in part by 2007 Grants-in Aid for Scientific Research (No. 0760110001 to Y. O.) from JSPS (Japan Society for the Promotion of Science) and Strategic Research Project No. K20002 from Yokohama City University. This work was also supported by the Ph.D. scholarship program, Ministry of Education, Culture, Science, Sports, and Technology (MEXT), Japan.

REFERENCES

1. Ozeki, Y., Matsui, T., Nitta, K., Kawauchi, H., Takayanagi, Y., and Titani, K. (1991) *Biochem. Biophys. Res. Commun.*, **178**, 407-413.
2. Ozeki, Y., Matsui, T., Suzuki, M., and Titani, K. (1991) *Biochemistry*, **30**, 2391-2394.
3. Hosono, M., Ishikawa, K., Mineki, R., Murayama, K., Numata, C., Ogawa, Y., Takayanagi, Y., and Nitta, K. (1999) *Biochim. Biophys. Acta*, **1472**, 668-675.
4. Titani, K., Takio, K., Kuwada, M., Nitta, K., Sakakibara, F., Kawauchi, H., Takayanagi, G., and Hakomori, S.-I. (1987) *Biochemistry*, **26**, 2189-2194.
5. Melo, V. M. M., Duarte, A. B. G., Carvalho, A. F. F. U., Siebra, E. A., and Vasconcelos, I. M. (2000) *Toxicon*, **38**, 1415-1427.

6. Banerjee, S., Chaki, S., Bhowal, J., and Chatterjee, B. P. (2004) *Arch. Biochem. Biophys.*, **421**, 125-134.
7. Kamiya, H., and Shimizu, Y. (1981) *Bull. Japan Soc. Sci. Fish.*, **47**, 255-259.
8. Ozeki, Y. (1998) *Mol. Biol. Int.*, **45**, 989-995.
9. Gilboa-Garber, N., Susswein, A. J., Mizrahi, L., and Avichezer, D. (1985) *FEBS Lett.*, **181**, 267-270.
10. Zipris, D., Gilboa-Garber, N., and Susswein, A. J. (1986) *Microbios.*, **46**, 193-198.
11. Gilboa-Garber, N., Sudakevitz, D., Levene, C., Rahimi-Levene, N., and Yahalom, V. (2006) *Immunohematology*, **22**, 15-22.
12. Gilboa-Garber, N., and Sudakevitz, D. (2001) *FEMS Immunol. Med. Microbiol.*, **30**, 235-240.
13. Wils, M. P., Garrow, G. M., and Levitan, I. B. (1992) *J. Neurobiol.*, **23**, 739-750.
14. Nitta, K., Ozaki, K., Ishikawa, M., Furusawa, S., Hosono, M., Kawauchi, H., Sasaki, K., Takayanagi, Y., Tsuiki, S., and Hakomori, S.-I. (1994) *Cancer Res.*, **54**, 920-927.
15. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
16. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.*, **150**, 76-85.
17. Wiechelman, K. J., Braun, R. D., and Fitzpatrick, J. D. (1988) *Anal. Biochem.*, **175**, 231-237.
18. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) *Anal. Chem.*, **28**, 350-356.
19. Kawsar, S. M. A., Fujii, Y., Matsumoto, R., Ichikawa, T., Tateno, H., Hirabayashi, J., Yasumitusu, H., Dogasaki, C., Hosono, M., Nitta, K., Hamako, J., Matsui, T., and Ozeki, Y. (2008) *Comp. Biochem. Physiol.*, **150B**, 349-357.
20. Gourdine, J.-P., Cioci, G., Miguet, L., Unverzagt, C., Silva, D. V., Varrot, A., Gautier, C., Smith-Ravin, E. J., and Imberty, A. (2008) *J. Biol. Chem.*, **283**, 30112-30120.
21. Shinohara, Y., Kim, F., Shimizu, M., Goto, M., Tosu, M., and Hasegawa, Y. (1994) *Eur. J. Biochem.*, **223**, 189-194.
22. Ahmed, S. A., Gogal, R. M., Jr., and Walsh, J. E. (1994) *J. Immun. Meth.*, **170**, 211-224.
23. Kawagishi, H., Yamawaki, M., Isobe, S., Usui, T., Kimura, A., and Chiba, S. (1994) *J. Biol. Chem.*, **269**, 1375-1379.
24. Wu, A. M., Song, S. C., Chen, Y. Y., and Gilboa-Garber, N. (2000) *J. Biol. Chem.*, **275**, 14017-14024.
25. Pashov, A., MacLeod, S., Saha Rinku, Perry, M., vanCott, T. C., and Kieber-Emmons, T. (2005) *Glycobiology*, **15**, 994-1001.