Growth inhibitory effects on microorganisms by a D-galactose-binding lectin purified from the sea hare (*Aplysia kurodai*) eggs: An *in vitro* study

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Abstract: A D-galactose specific lectin purified from the eggs of sea hare, *Aplysia kurodai* (AKL) by lactosyl-agarose affinity chromatography has been evaluated for screening of antimicrobial activities. AKL was disulfide bonded dimeric lectin consisted of two 32 kDa polypeptides. This lectin has significant hemagglutinting activity against trypsinized rabbit and human erythrocytes and it was inhibited by galactose and galacturonic acid. AKL has been screened for *in vitro* both antibacterial activity against eleven human pathogenic bacteria and antifungal activity against six phytopathogenic fungi. Antimicrobial evaluation of standard antibiotics, ampicillin and nystatin were used as comparative study. AKL significantly inhibited the growth of gram-positive bacteria. *Staphylococcus aureus* (12 mm) and *Bacillus megaterium* (11 mm) were exhibited the highest zone of inhibition by the addition of the lectin (250 µg/disc). However, AKL did not inhibit the growth of gram-negative bacteria as *Escherichia coli*. On the other hand, AKL (100 µg/mL) has also inhibited the mycelial growth of *Curvularia lunata* (21.53%). These antimicrobial activities by the lectin will provide an effective defense ability of the sea hare eggs against invading microbes. [Nature and Science 2010;8(2):82-89]. (ISSN: 1545-0740).

Key words: *Aplysia kurodai*, lectin, organisms, mycelial growth, SDS-popyacrylamide gel electrophoresis

1. Introduction

Lectins are multivalent carbohydrate-binding proteins that are widely distributed in various organisms from microorganisms to higher vertebrates. The chemical properties of animal lectins, such as the sugar specificity, divalent ion requirement and structure of carbohydrate binding domains, provide the classification of these lectins into several families. By virtue of their sugar-binding property, they are useful candidates for detection of cell-surface carbohydrates (Yu et al., 2001), biomedical applications (Pryme et al., 2002), and purification of glycoconjugates (Yamamoto et al., 1984). Physiologically, animal lectins have been postulated to perform important roles in various endogenous biological processes including self defense, cell-cell recognition, sugar transportation, development, biomineralization, immunity as antibacterials and others (Dong et al., 2004; Kamiya et al., 2002; Kilpatrick, 2002, Suzuki et al., 2003, Iijima et al., 2003). Many animal lectins with various carbohydrate-binding specificities have been discovered from eggs or reproductive organs (Ozeki et al., 1991; Hosono et al., 1999), the carbohydrate recognition mechanisms are seemed to be important for the biological events as fertilization or their early development. In phylum Mollusca, some lectins with anitibacterial, opsonizing, and cytotoxic activities were found from their organs (Banerjee et al., 2004; Melo et al., 2000).

Sea hares of the species *Aplysia kurodai* belong to the subclass Opisthobranchia of the mollusca. They lay yellow eggs in gelatinous strings in their spawning season (May-June). Sea hare species have attracted the interest of many workers investigating the chemical compounds secreted by the purple gland or present in different tissues, possibly involved in the defense of these invertebrates. Thus, some sea hare species have been shown to contain low molecular mass substances with antimicrobial (Ichida & Higa, 1986) and antitumor activities (Usami et al., 2008). From the species, a 70 kDa hexameric galacturonic acid-biniding lectin consisting of 13 kDa subunits has been isolated from eggs using galacturonic acid-conjugated Sepharose gel (Kamiya & Shimizu, 1981) and demonstrated potent agglutinins in extracts of *A. kurodai* egg masses which could agglutinate mammalian erythrocytes and marine bacteria. Two 28 kDa and 26 kDa D-galactose binding

lectins with cell attachment potency against human sarcoma cells were purified from the mantle (Ozeki, 1998). Very recently, two β-1,3-glucanases were purified from the digestive fluid (Kumagai & Ojima, 2010) of *A. kurodai*. *Aplysia* gonad lectin (AGL) has been purified a galactophylic lectin from gonad of *Aplysia depilans* (Gilboa-Garber et al., 1985) as first, appearing antibacterial activity and cytotoxity for carcinoma cells (Zipris et al., 1986). However, *Aplysia juliana* showed the antibacterial and antineoplastic activity against gram-positive bacteria (Kamiya et al., 1989).

Especially, eggs of sea hare are kept in tide pool until hatch, they need to prevent 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 the beans and many lectins present in snake venom. As seen in bullfrog eggs, a sialic acid-binding lectin (SBL) has activity as ribonuclease and apoptotic activity against mouse lymphoma cells P388 (Nitta et al., 1994). Sea hare eggs may possess antibiotic factors, as the egg masses appear to be free of bacteria. Egg mass of sea hare exhibited the antibacterial activity and suggested that antibacterial factors were produced in the albumen gland, such that each egg was coated with antibacterially active albumen before passing down the oviduct (Kamiya et al., 1984). Aplysianin-A with 320 kDa glycoprotein has been purified from the albumen gland of a sea hare, *Aplysia kurodai* and it showed 50% inhibition growth of *Bacillus subtilis* gram-positive bacteria (Kamiya et al., 1986). Also plant pathogens, like animal ones, use protein-carbohydrate interactions in their strategy for host recognition, attachment and invasion (Kostlanova et al., 2005).

We previously evaluated the affect of the lectin from *Aplysia kurodai* eggs for cell proliferation of lymphoma cells and determined the kinetics against the glycoprotein recognized by the lectin using surface plasmon resonance (Kawsar et al., 2009). This study aimed to evaluate the antibacterial and antifungal activity of the AKL purified from the sea hare *Aplysia kurodai* eggs against some human and phytopathogens.

2. Materials and Methods

2.1. Reagents

Lactosyl-agarose was 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. Bicinchoninic acid (BCA) kit was purchased from Pierce Co. Ltd., USA. Agar, dextrose, peptone, beef extract were purchased from Merck Ltd., India and BDH Ltd., Bangladesh.

2.2. 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 -80^0C or used after collection according to the situation.

2.3. Purification of *A. kurodai* lectin (AKL)

A galactose-binding lectin was purified from the sea hare *Aplysia kurodai* (AKL) as previously reported (Kawsar *et al.,* 2009). In brief, two hundred grams freezed sea hare, *A. kurodai* eggs as yellow string noodles was crushed into particles in a mortor, then mixed it 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. The supernatant was centrifuged again at 27,500 *g* for 1 h at 4°C for two times and was applied to a lactosyl-agarose affinity column that was fitted with a Sephadex G-75 pre-column. 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 chromatography step during washing and elution was monitored using a UV monitor by the measurement of the absorbance at 280 nm. The eluted fractions as identified by UV spectrophotometer at 280 nm were combined, and dialyzed against 1,000 times volumes of TBS to remove free from sugar.

2.4. Hemagglutinating activity

Hemagglutinating activity 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 in 96 well V-shape titer plates for 1 h incubation. The minimum inhibitory sugar concentration against the lectin was expressed as negative activity.

2.5. SDS-polyacrylamide gel electrophoresis and molecular mass

The molecular mass of the polypeptide was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Purified lectin was mixed with an equal amount of sample buffer (20 mM Tris-HCl, pH 6.8; 0.2% SDS, and 20% glycerol) and then heated at 70^0C 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% and 5% polyacrylamide were used in separation and upper gels, respectively, constant current at 30 mA for 1 h) according to a previous report (Laemmli, 1970). The following polypeptides were used as molecular mass markers; phosphorylase b (M_r 94 kDa), bovine serum albumin (M_r 66 kDa), ovalbumin (M_r 42 kDa), carbonic anhydrase (M_r 30 kDa), trypsin inhibitor (M_r 20 kDa), and lysozyme (M_r 14 kDa). After SDS-PAGE, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue (CBB) R-250 in 40% (v/v) and 10% acetic acid (v/v) followed by discoloration by excessive staining with 40% methanol and 10% acetic acid.

2.6. Protein determination and sugar content

Protein concentrations were determined using BCA protein assay kit (Smith et al*.,* 1985 and Wiechelman et al., 1988) with bovine serum albumin as the standard by measuring absorbance at 562 nm with spectrophotometer ND-1000 (Nano Drop Tech. Inc., USA). The carbohydrate content of the purified lectin was determined by the phenol-sulfuric acid method (Dubios et al*.,* 1956), using D-glucose as the standard by measuring absorbance at 340 nm.

2.7. Strains

The bacterial and fungal strains used in this study were obtained from the Microbiology Laboratory, Department of Microbiology, University of Chittagong, Bangladesh. Gram-positive bacterial strains were *Bacillus subtilis* BTCC 17, *Bacillus cereus* BTCC 19, *Bacillus megaterium* BTCC 18 and *Staphylococcus aureus* ATCC 6538 and Gram-negative bacterial strains were *Salmonella typhi* AE 14612, *Salmonella paratyphi* AE 146313, *Shigella dysenteriae* AE 14396, *Shigella sonnei* CRL (ICDDR,B), *Escherichia coli* ATCC 25922, *Vibrio cholerae* (CRL (ICDDR,B) and *Pseudomonas sp.* CRL (ICDDR,B). The fungal pathogens were *Alternaria alternata* (Fr.) Kedissler, *Botryodiplodia theobromae* Pat., *Curvularia lunata* (Wakker) Boedijin, *Colletotrichum corcori* Ikata (Yoshida), *Fusarium equiseti* (Corda) Sacc., and *Macrophomina phaseolina* (Tassi) Goid.

2.8. Medium and culture

Standard NA (Nutrient Agar) medium was used for growing bacterial strains throughout the work. A 20 g of agar powder, 5 g of peptone, 3 g of beef extract and 0.5 g of NaCl was added slowly to 1000 mL water and the solution was mixed thoroughly with a glass rod. After 10 minutes of boiling, the medium was transferred into 500 mL conical flask and flask was closed with a cotton plug. The medium was autoclaved for 15 minutes at 121^0C and 15 psi and ready to use bacterial culture. Older cultures were transferred to freshly prepared NA slants separately for each species via sterilized bacterial loop. In such a way, four test tubes were freshly prepared for each bacterial pathogen. These test tubes of inoculated slants were incubated at $(35\pm2)^0$C in incubator for 18-24 hours and each culture was used throughout for antibacterial screening studies. For preservation of the stock culture, one set of culture slants were kept in polythene bag, properly tied and preserved at 10^0C.

2.9. Antibacterial assay

The *in vitro* sensitivity of the bacteria to the test purified lectin was done by disc diffusion method (Bauer *et al*.1996). In this method sterilized paper discs of 4 mm in diameter and petridishes of 150 mm in diameter were used throughout the experiment. The autoclaved Mueller-Hinton agar medium, cooled to 45^0C, was poured into sterilized petridishes to a depth of 3 to 4 mm and after solidification of the agar medium, the plates were transferred to an incubator at 37^0C for 15 to 20 minutes to dry off the moisture that develops on the agar surface. The plates were inoculated with the

standard bacterial suspensions (as of McFarland 0.5 standard) by help of sterilized glass and allowed to dry for three to five minutes. Dried and sterilized filter paper discs were treated separately with 20µL (250 µg/disc) from 5% phosphate buffered saline (PBS) solution of lectin using a micropipette, dried in air under aseptic condition and were placed at equidistance in a circle on the seeded plate. A control plate was also maintained in each case without any test material. These plates were kept for 4-6 hours at low temperature and the test materials diffused from disc to the surrounding medium by this time. The plates were then incubated at 35 ± 2^0C for 24 hours to allow maximum growth of the organisms. The antibacterial activity of the test agent was determined by measuring the mean diameter of zone of inhibitions in millimeter. Each experiment was repeated thrice. Lactose was used as negative control. All the results were compared with the standard antibacterial antibiotic ampicillin [20 µg/disc, BEXIMCO Pharma Bangladesh Ltd.].

2.10. Antifungal activity

The *in vitro* antifungal activity of the purified lectin was determined by the poisoned food technique (Grover & Moore, 1962; Miah et al., 1990). Potato dextrose agar (PDA) medium was used for the culture of fungi. A required amount of PDA was taken in conical flasks separately and was sterilized by autoclave (121^0C, 15 psi) for 15 minutes. Purified lectin (in PBS solution) was mixed with sterilized melted PDA medium to have 100 µg/mL PDA and this was poured (about 20 mL/plate) in sterilized petridishes. At the center of each plate, 5 days old fungal mycelial block (4 mm in diameter) was inoculated and incubated at 27^0C. A control set was also maintained in each experiment. Linear mycelial growth of fungus was measured after 3-5 days of incubation in triplicate. The average of two measurements was taken as mycelial colony diameter of the fungus in mm. All the antifungal results were compared with the standard antifungal antibiotic Nystatin (100 µg/mL PDA). Lactose was used as negative control. The percentage inhibition of radial mycelial growth of the test fungus was calculated as follows:

$$\% \text{ Inhibition} = (C\text{-}T/C) \times 100$$

Where, C = diameter of the fungal colony in the control petridish and T = diameter of the fungal colony in the treated petridish.

3. Results and Discussion

Sea hare, *Aplysia kurodai* eggs of crude extraction showed strong hemagglutination activity against human and rabbit red blood cells (Table 1) and the activity were cancelled by the presence of saccharides such as galactose and lactose. *A. kurodai* eggs lectin was purified on a lactosyl-agarose column via elution with 50 mM lactose containing TBS (Figure 1). 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 (Figure 2). On the other hand, crude extracts of eggs contained various proteins by SDS-PAGE (Figure 2), indicated that the AKL was present as a disulfide-bounded dimeric protein consisting of two 32 kDa polypeptide subunits.

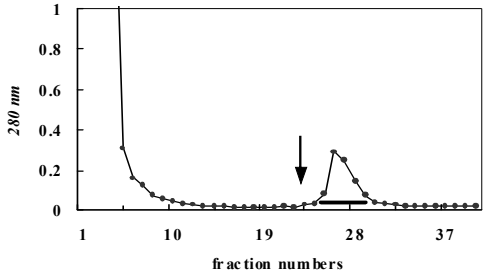

Figure 1. Affinity purification of AKL. Crude extract of *A. kurodai* was applied to a lactosyl-agarose column equilibrated with TBS. The column was washed with TBS and eluted with TBS containing 50 mM lactose (arrow).

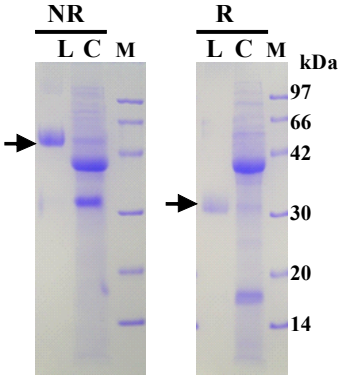

Figure 2. SDS-PAGE pattern of AKL. Purified lectin (L), (10 µg) and crude extract (C), (10 µg) were subjected to SDS-PAGE under non-reducing (NR) and reducing (R) conditions. Standard marker proteins (M) were used as follows, phosphorylase b (97 kDa); bovine serum albumin (66 kDa); ovalbumin (42 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20 kDa); and lysozyme (14 kDa).

Table 1. Hemagglutination of different erythrocytes by AKL

Erythrocytes*	Titer (HU)
Rabbit	2048
Human Type-A	2048
Human Type-B	1024
Human Type-O	1024

Note: *Trypsinized and glutaraldehyde fixed erythrocytes were used

AKL had so similar physicochemical properties with another hexameric 13 kDa lectin (Kamiya & Shimizu, 1981) purified from same species eggs on the affinity to galacturonic acid and the independence from divalent cations for its activity. This result indicated that eggs of *A. kuroai* contain multiple lectins. AKL strongly recognizes both α-galactosides as melibiose (Galα1-6Glc), methyl α-D-galactopyranoside and β-galactosides as lactose (Galβ1-4Glc), *N*-acetyllactosamine (Galβ1-4GlcNAc), methyl β-D-galactopyranoside and fetuin. *N*-acetyl neuraminic acid (Neu5Ac) was not recognized by the lectin since fetuin did not inhibit hemagglutinating activity of AKL and the terminal D-galactose of saccharides was an important for the binding with AKL. From these results, AKL has been characterized as a D-galactose-binding or galactophilic lectin as same as AGL purified from gonad of *Aplysia depilans* (Gilboa-Garber et al., 1985). AKL was suggested to be a glycoprotein containing saccharides as 6-14% molar ratio depending on the sample lots of the lectin detected by the phenol-sulfuric acid method.

Sea hares of *Aplysia* species have been reported to certain some biological active substances, including antibacterial factors (Iijima et al., 2003), toxins (Yamada et al., 2009) and chemical defense substances (Kamiya et al., 2006). Most of these substances are low molecular weight compounds derived from the algae on which the sea hares feed. However, no bioactive high molecular substances except agglutinin have previously been identified in sea hares (Kamiya and Shimizu, 1981). Here our purified lectin was subjected to screening for *in vitro* antibacterial inhibition growth by disc diffusion method (Bauer et al., 1996) against eleven pathogenic bacteria and compared to that of antibacterial antibiotic, ampicilin. AKL (250 µg/disc) exhibited a strong antibacterial activity on the gram-positive bacteria, as *Staphylococcus aureus* and *Bacillus megaterium*. The diameter of zone inhibition by the addition of AKL was significant effective for *Staphylococcus aureus* and *Bacillus megeterium* to be 12 and 11 mm, respectively (Table 2). This result is very similar with antitumor glycoprotein aplysianin E (250 kDa), in the eggs of *A. kurodai* which is inhibited the growth of *Staphylococcus aureus* bacteria (Yamazaki, 1993) and also sea hare, *Dolabella auricularia* (Iijima et al., 2003). However, the lectin has inhibited less effect for *Bacillus cereus* and *Bacillus subtilis.*

Table 2. Antibacterial activity of AKL against gram-positive bacteria

Name of bacteria	Diameter of zone of inhibition in milimeter	
	Lectin (250 µg/disc)	Ampicillin* (20 µg/disc)
Staphylococcus aureus	12±1	21±1
Bacillus megaterium	11±1	20±1
Bacillus cereus	6±1	16±1
Bacillus subtilis	4±1	18±1

Note: *Standard antibacterial antibiotic.

Table 3. Antibacterial activity of AKL against gram-negative bacteria

Name of bacteria	Diameter of zone of inhibition in milimeter	
	Lectin (250 µg/disc)	Ampicillin* (20 µg/disc)
Shigella dysenteriae	9±1	21±1
Salmonella typhi	5±1	19±1
Salmonella paratyphi	5±1	18±1
Shigella sonnei	4±1	20±1
Vibrio cholerae	4±1	15±1
Pseudomonas sp	4±1	14±1
Escherichia coli	0	19±1

Note: *Standard antibacterial antibiotic.

The antibacterial & antineoplastic activity showed against gram-positive bacteria of sea hare, *Aplysia juliana* (Kamiya et al., 1989). However, AKL did not inhibit well against all gram-negative bacteria was tested. *S. dysenteriae* exhibited little sensitivity by the lectin (Table 3), though the control antibiotic, ampicillin inhibited the growth against all gram-negative bacteria. Amongst the gram-positive and gram-negative bacteria, gram-positive bacteria were more effective to the lectin as compared to gram-negative bacteria.

Table 4. Antifungal activity of AKL purified from the sea hare, *A. Kurodai*

Name of fungi	Percentage inhibition of fungal mycelial growth	
	Lectin (100 µg/ml PDA)	Nystatin* (100 µg/ml PDA)
Curvularia lunata	21.53±1	67.86±1
Botryodiplodia theobromae	18.72±1	58.39±1
Macrophomina phaseolina	11.67±1	66.46±1
Alternaria alternata	10.56±1	49.78±1
Colletotrichum corchori	9.63±1	40.57±1
Fusarium equiseti	4.38±1	36.63±1

Note: *Standard antifungal antibiotic.

On the other hand, Aplysianin-A, an antibacterial glycoprotein in the albumen gland of the sea hare *Aplysia kurodai*, inhibited the growth of both gram-positive and gram-negative bacteria (Takamatsu et al., 1995). The growth of inhibition may cause to the presence of H_2O_2 which killed bacteria or halts bacterial growth, for example antibacterial protein aplysianin A (ApA) purified from albumen gland of *A. kurodai* (Jimbo et al., 2003). Recently, a β-galactoside binding pearl shell lectin purified from marine bivalve, *Pteria penguin* (Naganuma et al., 2006) had shown the similar antibacterial activity with AKL, as it effectively inhibited the growth against both gram-positive and gram-negative bacteria. Also rhamnose-binding steelhead trout (*Oncorhynchus mykiss*) eggs lectin inhibited the growth of gram-positive and gram-negative bacteria by recognizing lipopolysaccharide or lipoteichoic acid (Tateno et al., 2002) as same as AKL.

Antifungal activity by AKL was determined against six phytopathogenic fungi with antifungal antibiotic nystatin as positive control. Generally, the lectin had less inhibited the growth of fungal than the case of bacteria. AKL (100 µg/mL in PDA medium) exhibited significant inhibition of mycelial growth against *Curvularia lunata* (21.53%) among all tested fungi. AKL also showed good inhibition of mycelial growth against *Botryodiplodia theobromae* (18.72%) (Table 4). On the other hand, the mycelial growth of *Alternaria alternata*, *Macrophomina phaseolina* and *Colletotrichum corchori* (11.67-9.63%) was moderately inhibited by the lectin. However, the growth of *Fusarium equiseti* was least inhibited by AKL, though the growth of all the six fungi was totally inhibited by antifungal antibiotic Nystatin (100 µg/mL PDA). Although the growth of inhibition effect by AKL against fungi was not strong, some other galactose-, mannose- or fucose-binding lectins have reported (Broekaert et al., 1998) and also sea hare showed antifungal activity (Iijima et al., 1995).

Aplysia kurodai eggs contain a large quantity of AKL but its physiological function is unknown yet. But the egg mass of a sea hare showed antibacterial activity and that the catalytic activity of the eggs decreases during hatching (Kamiya et al., 1984). The presence of the lectins in the eggs of *A. kurodai* led us to consider its possible biological involvement in the defense mechanisms of the species. The wide distribution of lectin with antimicrobial property in the animal kingdom indicates that this lectin has been well conserved during evolution, which is understandable because animals cannot survive unless they can eliminate invading bacteria.

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