THE CYTOTOXIC ACTIVITY OF TWO D-GALACTOSE-BINDING LECTINS PURIFIED FROM MARINE INVERTEBRATES

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Abstract - The present investigation was undertaken in order to evaluate the cytotoxic effect of two D-galactose-binding lectins using the brine shrimp lethality bioassay technique. Both lectins were purified from the marine invertebrates, sea hare *Aplysia kurodai* eggs and polychaete *Perineries nuntia* by conventional affinity chromatography methods. The molecular mass of *Aplysia kurodai* egg lectin (AKL) was determined to be 32 kDa and 56 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing and non-reducing conditions, respectively. On the other hand, polychaete *Perineries nuntia* lectin (PnL) was determined to be 32 kDa in both reducing and non-reducing conditions. AKL and PnL showed strong agglutination activity against trypsinized and glutaraldehyde-fixed human and rabbit erythrocytes. AKL significantly affects the mortality rate of brine shrimp. Experimental results revealed that AKL was found to be more toxic (63.33% mortality) than PnL (33.33% mortality) and the mortality rate of brine shrimp nauplii was increased with the increase in concentration of lectins. These cytotoxic results indicate that future findings of lectin applications obtained from marine invertebrates may be of importance to clinical microbiology, and that they could have application as potent chemotherapeutic agents.

Key words: Invertebrates, brine shrimp, cytotoxic, mortality, lectin.

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INTRODUCTION

Lectins are a group of sugar-binding proteins which recognize specific carbohydrate structures and agglutinate a variety of animal cells by binding to cell-surface glycoproteins and glycolipids. Lectins are found in a wide range of organisms including viruses, bacteria, fungi, plants and animals (Sharon, 2008). Lectins are involved in various biological functions, such as, host defense, cell-cell interaction, folding of glycoproteins and other functions (Sharon, 2007). Based on their binding specificity, lectins have been used as reagents to detect sugar chains in biochemical and histochemical investigations (Valbuena et al., 2010). Invertebrates lack an adaptive immune system and lectins play an important role in their innate immune systems by recognizing invading microbes or pathogens (Kawabata, 2000). Although invertebrate lectins are mainly involved in immune system response (Vasta, 1992), some marine invertebrate lectins show distinct biological functions such as the inhibition of calcium carbonate crystallization in acorn barnacles (Matsubara et al., 2008) and sperm-egg recognition in oysters (Moy et al., 2008). Some lectins act as cytotoxic and mitogenic agents. Such properties make them useful tools for the isolation and characterization of polysaccharides and glycoconjugates (Green et al., 1989) in cancer research, as diagnostic tools for the investigation of the early cell-membrane alterations and carbohydrate changes that accompany neoplastic processes and immunological studies (Sugiura et al., 1988; Sharon, 2007).

Marine invertebrates, which develop in a different environment from terrestrial animals, are the source of a broad range of pharmacological substances. They may contain

special host defense factors because their defense mechanisms differ from the immune system of highly developed vertebrates. Sea hares belong to the opithobranch mollusks of marine gastropods. In phylum Mollusca, some lectins with antibacterial, opsonizing and cytotoxic activities were found from their organs (Banerjee et al., 2004; Melo et al., 2000). 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). A 70 kDa hexameric galacturonic acid-binding lectin has been isolated from eggs of the species, and has demonstrated potent agglutinins in the extracts of *A. kurodai* egg masses which could agglutinate mammalian erythrocytes (Kamiya & Shimizu, 1981). Two D-galactose binding lectins with cell attachment potency against human sarcoma cells were purified from the mantle (Ozeki, 1998). Recently, β-1,3-glucanases were purified from the digestive fluid (Kumagai & Ojima, 2010) of *Aplysia kurodai*. *Aplysia* gonad lectin (AGL) is a galactophylic lectin It has antibacterial activity and is cytotoxicity for carcinoma cells (Zipris et al., 1986).

Polychaeta is a large class in the annelid phylum. A number of lectins which recognize D-galactose, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine are isolated from annelids (Kawsar et al., 2009; Molchanova et al., 2007; Wang et al., 2006; Ozeki et al., 1997). Lectins have attracted the attention of numerous researchers by virtue of the potentially exploitable activities that they manifest, including anti-proliferative, antitumor, immuno-modulatory (Singh et al., 2005; Wang et al., 2006), antifungal and antiviral (Ye et al., 2001) activities. Two polychaeta GlcNAc- and β-galactose-specific lectins from Chaetopteridae and Sabellidae inhibited the syncytium formation of HIV-I infected C8166 cells (Molchanova et al., 2007; Wang, et al., 2006). The study of the properties and functions of marine invertebrate lectins is promising for the discovery of new medical and biological applications and an understanding of carbohydrate-protein interaction.

The brine shrimp lethality bioassay (Mayer et al., 1982; McLaughlin et al., 1991) is a recent development in the assay procedure of bioactive compounds which indicates cytotoxicity as well as a wide range of pharmacological activities (e.g., anticancer, antiviral, insecticidal, pesticidal, AIDS etc.) of the compounds. *Artemia salina*, the brine shrimp, is an invertebrate component of the fauna of saline aquatic and marine ecosystems. It plays an important role in the energy flow of the food chain and it has been used in laboratory bioassay in order to determine toxicity by the estimation of the medium lethal concentration (Meyer et al., 1982). The method is attractive because it is simple, inexpensive and low toxin amounts are sufficient to perform the test on the microwell scale. Lectins play a key role in the control of various normal and pathological processes in living organisms. Research in the field of lectins has been ongoing in many research laboratories. So far more than a hundred lectins have been purified and characterized, but their toxicological study against the mortality of brine shrimp has not yet been carried out extensively. As a continuation of our research, we previously determined the kinetics, glycan-binding properties, cell proliferation, partial primary structure (Kawsar et al., 2009a; 2009b) and antimicrobial activities (Kawsar et al., 2010) of the lectins. In this report, we evaluate the cytotoxicity of two D-galactose-specific lectins purified from marine invertebrates.

MATERIALS AND METHODS

Chemicals

A standard protein marker mixture (Daiichi-III) for SDS-PAGE was purchased from Daiichi Pure Chem. Co. Ltd., Japan. A Sephadex G-75 column was obtained from GE Health Sciences and Sigma, USA. Lactosyl-agarose and protease inhibitor mixture were purchased from Seikagaku Kogyo Co. Ltd., Japan. Mono- and disaccharides were purchased from Wako Pure Chemical Ind. Ltd., Tokyo, Japan and were of the highest purity grade.

Purification of D-galactose-specific lectins

Two D-galactose-binding lectins were purified from the Japanese sea hare, *Aplysia kurodai* (Kawsar et al., 2009a) and marine worm, *Perinereis nuntia* (polychaeta) (Kawsar et al., 2009b). In brief, 200 g (wet weight) of frozen sea hare *A. kurodai* eggs (A) as yellow string noodles was crushed into particles in a mortar and marine worms *P. nuntia* (B) were dispersed to paste with a razor blade and homogenized with 10 volumes (w/v) of Tris-buffered saline (TBS) pH 7.4, containing 0.9% NaCl) containing 10 mM of a protease inhibitor mixture. Homogenates (A) and (B) were centrifuged at 14,720 g in 500 ml centrifuge bottles for 40 min at 4°C with a Suprema-21 centrifuge equipped with an NA-18HS rotor (TOMY Co. Ltd., Japan). Supernatants (A & B) were centrifuged again at 27,500 g for 30 min at 4°C and the precipitate (obtained from B) was homogenated again with 3 volumes (w/v) of TBES (TBS containing 10 mM EDTA) containing 50 mM galactose for 4 h to over night at 4°C. It was centrifuged at 27,500 g for 40 min at 4°C and the supernatant was dialyzed against TBS till free of galactose. Finally, both supernatants (A & B) were applied to the lactosyl-agarose affinity columns (4 ml) being fitted with a Sephadex G-75 pre-column (3 ml), separately. After applying the extracts, the columns were extensively washed with TBS. The lactosyl-agarose columns were eluted with 100 mM galactose/TBS and each 1 ml of elution was collected in tubes with a fraction collector. Each chromatography step during washing and elution was monitored by the measurement of the absorbance at 280 nm using a UV monitor (ATTO Co. Ltd., Japan). 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 galactose.

Agglutinating activity by human and rabbit erythrocytes

The 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 twice-serially-diluted dialyzed lectins with TBS in 96 well V-shape titer plates for 1 h. The hemagglutination activities of the lectins were expressed as a titer defined as the reciprocal of the highest dilution giving positive hemagglutination. To determine the sugar-binding specificity of the lectins, each 20 μl of the sugar (200 mM) and glycoprotein (5 mg/ml) was serially diluted with TBS and added with lectins to previously diluted TBS to adjust the titer to 16, 1% Triton X-100, and erythrocytes in 96 well V-shape titer plates for 1 h. The minimum inhibitory sugar concentrations against the lectins were expressed as negative activity scored.

Polyacrylamide gel electrophoresis

The molecular mass of the polypeptide was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Purified lectins were 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°C for 10 min. Aliquots of 25 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, with 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 gels were 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.

Hatching of brine shrimp

Brine shrimp eggs, *Artemia salina* leach were hatched in artificial seawater prepared by dissolving 38 g of sea salt (Sigma Chemicals Co., U.K) in 1000
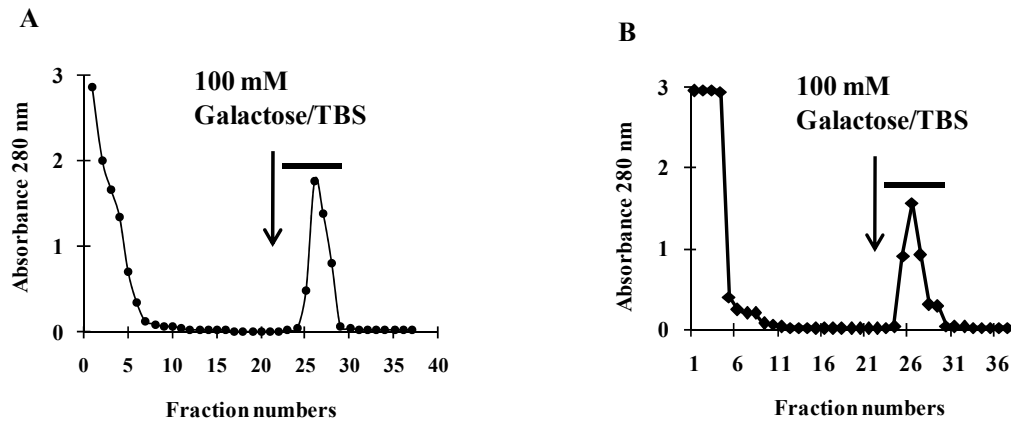
Fig. 1. Affinity purification of lectins. Crude extract of (A) *A. kurodai* and (B) *P. nuntia* were applied to lactosyl-agarose affinity columns and equilibrated with TBS. The columns were extensively washed with TBS and lectins were eluted with 100 mM galactose/TBS (arrow). The column-bound fractions shown by the bar were collected and designated as purified lectins after dialysis against TBS
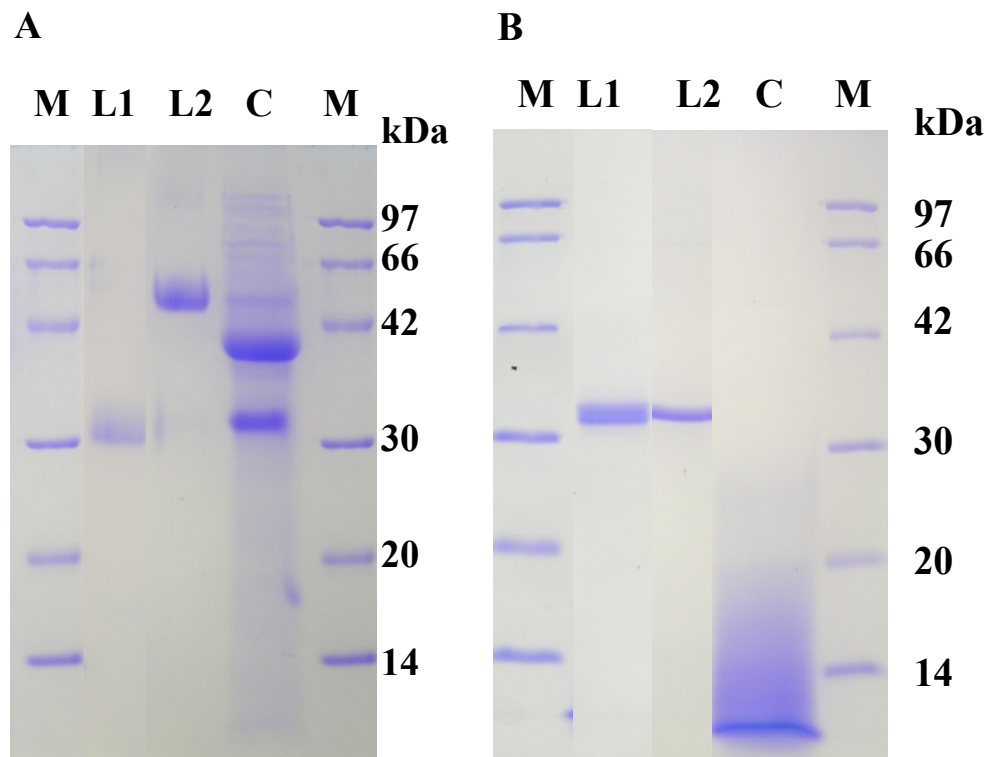

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified lectins. C: crude extract of (A) *A. kurodai* and (B) *P. nuntia* from TBS (pH 7.4); L1: AKL (A) & PnL (B) non-reducing condition; L2: AKL (A) & PnL (B) reducing condition. 12% polyacrylamide was used as separating gel and the gels were stained with Coomassie brilliant blue. M: molecular weight markers (from top to bottom): 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. Effects of AKL purified from *Aplysia kurodai* on brine shrimp lethality bioassay.

Sample code (vial type)	Conc. of sample (µg/ml)	No. of shrimp (each vial)	Number of shrimp died			Average No. of deaths	Mortality* (%)
			Vial 1	Vial 2	Vial 3		
Type-A	2	10	1	1	2	1.333	13.33
Type-B	4	10	3	2	3	2.666	26.66
Type-C	8	10	4	3	4	3.666	36.66
Type-D	16	10	5	4	5	4.666	46.66
Type-E	32	10	6	7	6	6.333	63.33
Control	0	10	0	0	0	0	0

*Values are mean of three replicates

ml of distilled water, and then filtered off. The eggs (Carolina Biological Supply Company, Burlington, NC, USA) were placed in one side of a tank divided by a net containing 3.8% NaCl solution for hatching, while in the other side of the tank a light source was placed in order to maintain the temperature. Two days were allowed for the hatching of the eggs and the sufficient maturation of nauplii for experimental purposes as described by Mayer et al., (1982). The phototropic nauplii that had migrated to the lighted compartment were collected by pipette.

Cytotoxic activity by brine shrimp bioassay

From the stock solutions of the purified lectins (0.9 mg/ml), 10, 20, 40, 80 and 160 µl were placed in different vials and NaCl solution was added to each vial to bring the volume up to 5 ml. The final concentrations of sample in the vials were 2, 4, 8, 16 and 32 µl/ml in vials labeled A, B, C, D and E, respectively. Three sets of experiments were done for each concentration and 10-brine shrimps nauplii were placed in each vial. A control experiment was performed in a vial containing 10 nauplii in 5 ml seawater. After 24 h of incubation at room temperature, the vials were observed using a magnifying glass and the number of survivors in each vial were counted and noted. No deaths were found in the controls. From the data, the mean percentage of mortality of nauplii was calculated for each concentration.

RESULTS AND DISCUSSION

Two D-galactose-specific lectins were purified from the marine invertebrates such as sea hare, *Aplysia kurodai* eggs (AKL) and polychaeta, *Perineries nuntia* (PnL) by lactosyl-agarose affinity columns via elution with 50 mM lactose (Galβ1-4Glc) (Kawsar et al. 2008) containing TBS (Fig. 1A & 1B). AKL was a disulfide bonded dimeric protein consisting of two 32 kDa polypeptides (Fig. 2A). On the other hand, PnL was shown to be a monomeric protein with 32 kDa polypeptide in the presence or absence of 10 mM β-mercaptoethanol by SDS-PAGE (Fig. 2B). Both D-galactose-binding lectins were agglutinated with trypsinized and glutaraldehyde-fixed human and rabbit erythrocytes in the absence of any divalent cations.

The brine shrimp lethality assay is considered a useful tool for the assessment of toxicity (Carballo et al., 2002; Mayer et al., 1982; McLaughlin et al., 1991). It is based on the ability to kill laboratory-cultured *Artemia* nauplii brine shrimp. The assay is considered a useful tool for the preliminary assessment of toxicity (Solts et al., 1993) and it has been used for the detection of fungal toxins (Harwig and Scott, 1971), plant extract toxicity

Table 2. Effects of PnL purified from *Pereneries nuntia* on brine shrimp lethality bioassay.

Sample code (vial type)	Conc. of sample (µg/ml)	No. of shrimp (each vial)	Number of shrimp died			Average No. of deaths	Mortality* (%)
			Vial 1	Vial 2	Vial 3		
Type-A	2	10	1	0	0	0.333	3.33
Type-B	4	10	1	1	0	0.666	6.66
Type-C	8	10	2	1	1	1.333	13.33
Type-D	16	10	3	2	2	2.333	23.33
Type-E	32	10	4	3	3	3.333	33.33
Control	0	10	0	0	0	0	0

*Values are mean of three replicates

(McLauglin et al., 1991), cyanobacteria toxins (Jaki et al., 1999) and the cytotoxicity testing of dental materials (Pelka et al., 2000). Many scientists have reported cytotoxicity of lectins using brine shrimp as a zoological specimen (Santos et al., 2010; Absar et al., 2008, 2005).

The cytotoxic activity of the lectins in the brine shrimp lethality bioassay is presented in Tables 1 and 2 and shows the percentage of mortality of shrimps at 24 h. Mortality of the nauplii was noticed in the experimental groups at the same time the control group remained unchanged. The number of surviving nauplii in each vial was counted and the results were noted. From these data the percent of mortality of the shrimp was calculated for every concentration of each sample. The mortality rate of the brine shrimp nauplii was found to increase with the increase in concentration of the sample, and a graph concentration versus percent mortality on graph paper gave an almost linear correlation (Fig. 3A & 3B). However, there was no mortality in the controls. It is evident from the results of the brine shrimp lethality testing that the AKL had the highest levels of toxicity (63.33% and 46.66% death at 32 and 16 µl/mg, respectively) indicating its higher mortality (Table 1). This cytotoxicity result is very similar to that of glucose/mannose-binding lectins purified from snail (Santos et al., 2010), mannose-specific Potca fish and Mullbery seeds lectins (Absar et al., 2008, 2005). On

the other hand, PnL displayed little toxicity (33.33%) at a concentration of 32 µl/mg (Table 2). It can be mentioned that the lectins purified from the sea hare and polychaeta may contain two saccharide-binding sites for showing its biological action as it gave a strong agglutinating property as well as brine shrimp toxicity (Absar et al., 2005). Though both lectins were recognized by D-galactose, their cytotoxic activity is different. AKL showed two times higher toxicity than PnL. Moreover, we found significant antibacterial and antifungal activities in AKL against human and phyto-pathogens (Kawsar et al., 2010). To our knowledge, this is the first report on the cytotoxic study of purified lectins by brine shrimp lethality. However, the mechanism of the cytotoxic activity of lectins is still unknown and it could reveal some new and interesting facts about the role of lectins in the lifestyle and survival of these sedentary animals. This cytotoxic activity is perhaps suitable as a factor in sea hare self-defense, since some means of self-defense against infectious microorganisms is essential for an animal in any life stage. In general, the adaptive immune system of vertebrates takes a long time. In the case of sea hares, they lay eggs which hatch as immature larvae, thus an adaptive immune system is unsuitable. Instead, the immune systems of these animals possess constitutive multitargeting factors. Thus, sea hare cytotoxic lectins including AKL and PnL are suitable factors for their presumed requirements in a self-defense system. Therefore, the presence of the lectins in the eggs of *A.*
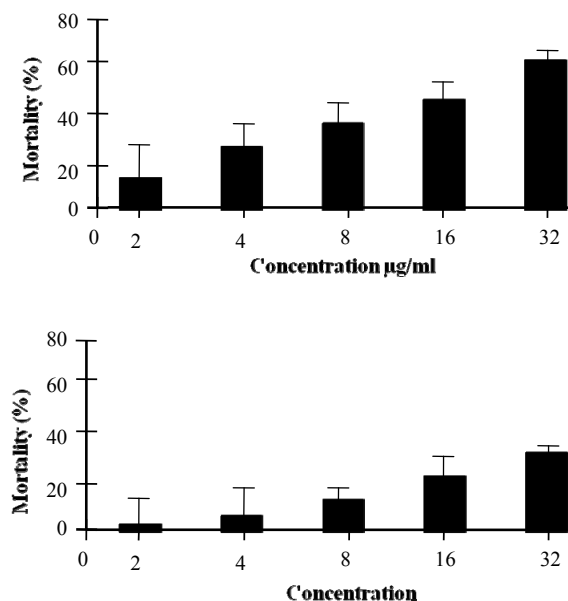
Fig. 3. Graph of concentration (μg/ml) versus percentage of brine shrimp mortality of AKL (A) and PnL (B) purified from sea hare *A. kurodai* eggs and polychaeta, *P. nuntia*, respectively.

kurodai and annelids led us to consider its possible biological involvement in the defense mechanisms of the species. Further studies of the bioactive molecules in the sea hare and polychaeta would facilitate an understanding of the ecological and physiological importance of these proteins in the animal and the discovery of useful molecules in biological sciences.

CONCLUSION

The sea hare eggs lectin (AKL) is more toxic than polychaeta lectin (PnL). AKL may have anticancer and antitumor activities as shown by its higher cytotoxic activity, however, further investigations are needed to confirm their bioactivity which could be explored as a potent chemotherapeutic agent(s) in modern clinical microbiology.

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