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Toxicity assessment of a β-galactoside-binding lectin (galectin-1) from bullfrog *Rana catesbeiana* unfertilized eggs

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Abstract

The present study was undertaken to investigate the toxicity of β-galactoside-binding lectin (galectin-1) using the brine shrimp lethality bioassay technique. Galectin-1 was purified from the American bullfrog *Rana catesbeiana* unfertilized eggs by conventional affinity chromatography method. The molecular mass of the galectin-1 was determined to be 15 kDa by SDS-PAGE under non-reducing and reducing conditions, respectively. Galectin-1 affects significantly the mortality rate (56.66%) of brine shrimp (*Artemia salina* L.) lethality bioassay. The results exhibited that galectin-1 was found to be moderate toxic and the mortality rate of brine shrimp nauplii was increased with the increase in concentration of galectin-1. These findings suggest that the galectin-1 obtained from amphibian may have anticancer and antitumor activity and would be useful to clinical microbiology to play a significant role in human therapy.

Keywords: galectin-1, *Rana catesbeiana*, brine shrimp, toxicity, mortality

Introduction

Lectins are proteins that can bind to sugar groups of various kinds. Galectins are a family of lectins that bind galactose and share sequence homology in their carbohydrate recognition domains (CRD) (Barondes et al. 1994). Galectins, formerly known as S-type lectins, are β-galactoside-binding proteins that have been proposed to participate in a variety of biological processes such as early development, tissue organization, immune functions, host parasite interactions, tumor evasion and various cancer metastasis (Leffler et al. 2004, Liu and Rabinovich 2005, Rabinovich et al. 2007, Ahmed et al. 2009). Galectins are present in vertebrates and invertebrates and do not require any divalent cations for their binding activity (Vasta 2009). Based on their primary structure and subunit architecture, galectins have been

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classified as "proto" (subunit molecular mass 14.5-16 kDa), "chimera" (29-35 kDa) and "tandem repeat" (32-36 kDa) types (Hirabayashi and Kasai 1993) or galectins 1-12 (Barondes et al. 1994). Although all members of the galectin family bind lactose (Galβ1-4Glc)/*N*-acetyllactosamine (Galβ1-4GlcNAc), a limited diversity exists in the carbohydrate specificity (Ahmed et al. 1990). Based on the differences in specificity and the conservation of amino acid residues that interact with the carbohydrate ligans, galectins are classified into two type: conserved (type-1) and variable (type-II) (Ahmed and Vasta, 1994). In amphibians, galectins have been purified from the genus *Rana* (Ozeki et al. 1991a), *Bufo* (Ahmed et al. 1996) and *Xenopus* (Shoji et al. 2003) and proto type galectin (galectin-1) was isolated in abundance from oocytes of the American bullfrog *Rana catesbeiana* living in aqua (Ozeki et al. 1991a) and found to exist free from the endogenous ligand and abundant in oocytes. American bullfrog oocytes galectin-1 is located in the yolk platelets and is distributed in the extracellular matrices of these organs after development into adults (Uchiyama et al. 1997). It was also shown to have potential cell adhesive activity to human rhabdomyosarcoma cells (Ozeki et al. 1991b) and tissue fibronectin was determined to be a putative endogenous ligand (Ozeki et al. 1995).

Galectin-1 plays an important role in CD8+ T cells mediated cytotoxicity by inducing immunological tolerance (Xu et al. 2010). It induces apoptosis of activated CD8 T cells and transition of Th2 cytokine (Perillo et al. 1995). In addition, many other effects of galectin-1 have been reported on cell adhesion, T cell proliferation, monocytes and neutrophils (Lowe 2001). Galectin-1 also plays a number of major roles in cancer biology in that it interacts with major signaling pathways involved in cancer biology (Camby et al. 2006). Galectin-1 is thus a promising molecular target for the development of new and original therapeutic tools. An investigation of galectins from amphibian will allow deeper understanding of their biological role.

Artemia salina, the brine shrimp, is an invertebrate component of the fauna of saline aquatic and marine ecosystems. *Artemia*'s high sensitivity to a broad range of compounds, allied to the fact that its eggs can be stored for years at room temperature and larvae obtained in 24-48 h, have promoted its use as a test organism for bioassay. It used in laboratory bioassay in order to determine toxicity by the estimation of the medium lethal concentration (Meyer et al. 1982). Brine shrimp lethality bioassay (Meyer 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, AIDS etc.) of the compounds. The method is attractive because it is simple, inexpensive and low toxin amounts are sufficient to perform the test on the microwell scale.

Galectins play a key role in the control of various normal and pathological processes in living organisms. Research in the field of galectins has been going on in many research laboratories of the world. So far few galectins have been purified and characterized but their toxicological study against mortality of brine shrimp have not yet been carried out extensively. We previously determined the glycan-binding properties of the lectin by using frontal affinity chromatography technology (FACT) (Kawsar et al. 2009) and growth inhibitory effects against human and

phytopathogens (Kawsar et al. 2010). In this paper, we evaluate the cytotoxicity of the galectin-1 by brine shrimp bioassay as first time.

Materials and Methods

Reagents

Brine shrimp eggs, *Artemia salina* were purchased from Carolina Biological Supply Company, Burlington, NC, USA and sea salt from Sigma Chemicals Co., U.K. Lactosyl-agarose was purchased from Seikagaku Kogyo Co. Ltd., Japan. Standard protein marker mixture (Daiichi-III) for SDS-PAGE was purchased from Daiichi Pure Chem. Co. Ltd., Japan. Mono- and disaccharides were purchased from Wako Pure Chemical Ind. Ltd., Tokyo, Japan. Sephadex G-75 column was obtained from GE Health Sciences and Sigma, USA.

Affinity purification of galectin-1

Galectin-1 was purified from the oocytes of the bullfrog (*Rana catesbeiana*) by conventional affinity column chromatography (Ozeki et al. 1991a). In brief, Ten grams of eggs collected by laparotomy of *Rana catesbeiana* were immediately homogenized with 100 ml physiological saline and 800 mL acetone. The precipitate was centrifuged and suspended with acetone, followed by filtration on Buchner's funnel. The powder of the eggs thus obtained was homogenized with 20 times (w/v) TBS (10 mM Tris(hydroxymethyl)aminomethane-HCl, 150 mM NaCl, pH 7.4) and centrifuged at 14,720 *g* for 40 min at 4°C and the supernatant was applied to an affinity column of lactosyl-agarose (5 mL) that was fitted with a Sephadex G-75 pre-column (3 mL). The affinity column was washed with TBS to remove the unbound proteins and the lectin was obtained by elution with 50 mM lactose containing TBS using an automated fraction collector. The chromatography profile was monitored during washing and elution with a UV spectrophotometer at 280 nm (ATTO Co. Ltd., Japan). The eluted fractions were collected together and dialyzed against 1000 vol. of TBS to remove any remaining lactose in the solution.

Hemagglutination assay by erythrocytes

Lectin activity was monitored by the hemagglutination using trypsinized and glutaraldehyde-fixed rabbit erythrocytes as described previously (Matsui 1984). Galectin-1 was serially diluted with TBS on a V-bottom microtiter plate and hemagglutination was performed in the presence of 1% BSA (Bovine serum albumin), 0.05% Triton X-100 and 0.25% erythrocytes at 25°C for 1 h. The titer was expressed as the reciprocal of the highest dilution giving positive hemagglutination. For the specific sugar analysis, galectin-1 (the titer was previously adjusted to 16) was mixed with serially diluted sugar solution (200 mM and the glycoprotein 5 mg/mL) in the presence of 1% BSA, 0.05% Triton X-100 and 0.25% erythrocytes. The most effective minimum sugar concentration to inhibit lectin-induced hemagglutination was calculated.

Molecular weight by SDS-PAGE

The purified lectin was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for molecular mass determination in accordance with the procedure of Laemmli 1970. Electrophoresis was performed in reducing and non-reducing conditions (with and without 2-mercaptoethanol) using 15% polyacrylamide gel. 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°C for 10 min. The molecular mass of purified lectin was estimated by comparing its mobility with that of the following 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). The gel

was stained with 0.1% (w/v) Coomassie Brilliant Blue (CBB) R-250 in (4:1:5 v/v) methanol: acetic acid: water for 30 min at room temperature followed by destaining the gel for 1-2 h in destaining solution methanol (40%) and acetic acid (10%) until proteins bands were clearly defined.

Hatching of brine shrimp

Artemia salina, the brine shrimp eggs were hatched in sea water and used after 48 h. The eggs were placed in a tank with two compartments. The compartment containing the eggs was covered to keep the eggs in a dark environment. The other compartment was lighted in order to attract shrimp through holes in the dividing wall panel. After 24 h, the phototropic nauplii that had migrated to the lighted compartment were collected by pipette and incubated under 24 h illuminations at ambient temperature.

Toxicity assessment by brine shrimp lethality

The purified galectin-1 (0.8 mg/mL), 10, 20, 40, 80 and 160 µL were placed in different vials and NaCl solution was added to each vial make the volume up to 5 mL. The final concentration of the sample in the vials became 1.8, 3.6, 7.2, 14.4 and 28.8 µL/mL in vial type-A, B, C, D and E, respectively. Three sets of experiment were done for each concentration and 10-brine shrimps nauplii were placed in each vial. To check the shrimp susceptibility, a blank containing only sea water as control experiment was performed in a vial containing 10 nauplii. 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 (McLaughlin et al., 1991; Meyer et al., 1982).

Results and Discussion

Galectin-1 was purified from the bullfrog *Rana catesbeiana* unfertilized eggs by lactosyl-agarose column chromatography via elution with 50 mM lactose containing TBS (Fig. 1).

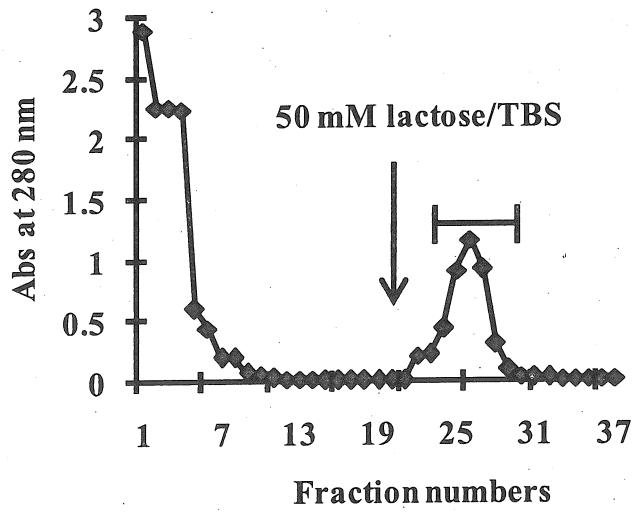

Figure 1. Affinity chromatography purification of galectin-1. Crude extract of *R. catesbeiana* unfertilized eggs were applied to a lactosyl-agarose affinity column and equilibrated with TBS. The column were extensively washed with TBS and column bound galectin-1 was eluted with 50 mM lactose/TBS (arrow). The column bound fractions shown by the bar were collected and designated as purified galectin-1 after dialysis against TBS.

The purified galectin-1 showed strong hemagglutinating activity against trypsinized and glutaraldehyde-fixed human and rabbit erythrocytes. Galectin-1 was purified as a single 15 kDa polypeptide by SDS-PAGE under both reducing and non-reducing conditions, respectively (Fig. 2). A partial primary structure analysis of the lectin showed that the amino acid sequence of the protein belonged to a superfamily of galectins in addition to the carbohydrate binding specificity against β-galactoside (Ozeki et al. 1991c).

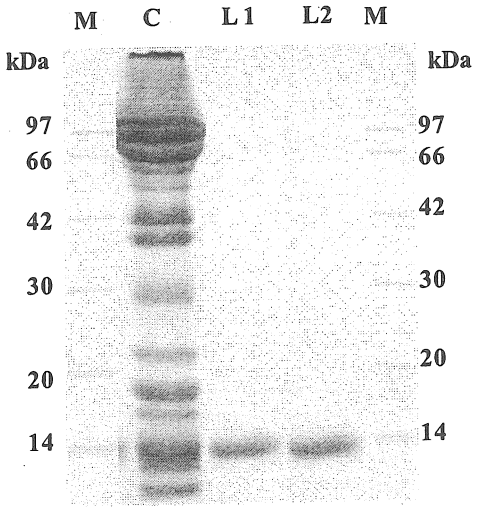

Figure 2. SDS-polyacrylamide gel electrophoresis of purified galectin-1. C: crude extract of *R. catesbeiana* unfertilized eggs from acetone powder. L1 and L2: purified galectin-1 non-reducing and reducing conditions, respectively. 15% polyacrylamide was used as separating gel and the gels were stained with Coomassie brilliant blue. M: standard molecular marker proteins (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).

The brine shrimp lethality assay is based on the ability to kill laboratory-cultured *Artemia* nauplii brine shrimp. The assay is considered a useful tool for preliminary assessment of toxicity (Solts et al. 1993, Carballo et al. 2002) and it has been used for the detection of fungal toxins (Harwig and Scott, 1971), plant extract toxicity (McLauglin et al. 1991), cyanobacteria toxins (Jaki et al. 1999) and cytotoxicity testing of dental materials (Pelka et al. 2000). Many scientists have reported cytotoxicity of lectins using brine shrimp as a zoological specimen (Yeasmin et al. 2001, Absar et al. 2008, Santos et al. 2010). The cytotoxic activity of the galectin-1 in the brine shrimp lethality bioassay is presented in Table 1 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 survived 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 brine shrimp nauplii was found to increase with the increase in concentration of sample and a plot concentration versus percent mortality on graph paper gave in Fig. 3.

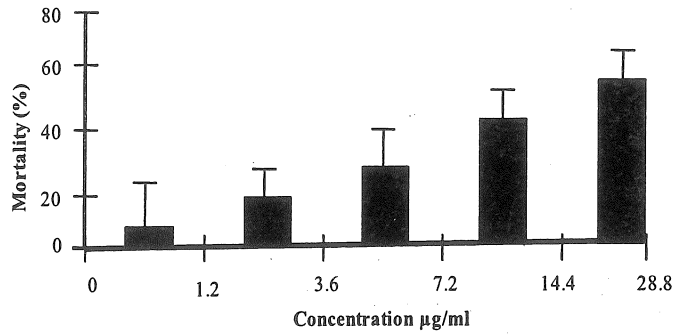

Figure 3. Percentage mortality *vs.* concentration of the galectin-1 on brine shrimp bioassay. Results represent the mean of duplicate analysis and bar indicates ± standard deviation.

However, there was no mortality in the controls. It is evident from the results of brine shrimp lethality testing that the galectin-1 was the highest levels of toxicity (56.66%) at a concentration of the galectin-1 28.8 µg/mL indicating its higher mortality (Table 1).

Table 1. Toxicity effects of galectin-1 from *Rana catesbeiana* unfertilized eggs on brine shrimp bioassay

Sample code (vial type)	Conc. of sample (µg/ml)	No. of shrimp (each vial)	Number of shrimp died			Average no. of death	Mortality* (%)
			Vial 1	Vial 2	Vial 3		
Type-A	1.8	10	1	1	0	0.666	6.66
Type-B	3.6	10	2	2	1	1.666	16.66
Type-C	7.2	10	3	3	2	2.666	26.66
Type-D	14.4	10	5	4	4	4.333	43.33
Type-E	28.8	10	6	6	5	5.666	56.66
Control	0	10	0	0	0	0	0

*Values are mean of three replicates

Till yet, cytotoxic activity has been reported in many lectins (Absar et al. 2008, Santos et al. 2010). Our results suggested that galectin-1 has cytotoxic activity similarly to glucose/mannose-binding lectins from snail (Santos et al. 2010) and mannose-specific Potca fish lectin (Absar et al. 2008). Galactose specific lectins purified from Mulberry seeds (Yeasmin et al. 2001) also showed cytotoxic activity in a similar fashion to galectin-1 by brine shrimp lethality assay. It might be mentioned that the lectins purified from bullfrog *R. catesbeiana* may contain two saccharide binding sites for showing its biological action as it gave strong hemagglutinating property as well as brine shrimp toxicity (Absar et al. 2005). Galectin-1 showed a number of important roles as in acute and allergic inflammation (Rabinovich et al. 2002). Galectin-1 inhibits proliferation of mitogen-activated T cells and reduces clonal expansion of antigen-primed CD8+ T cells and human leukemia T cells in a carbohydrate-dependent manner (Novelli et al. 1999). Galectin-1 induces apoptosis of human and murine T cells during development in

the thymus (Hernandez and Baum 2002). However, the mechanism of the cytotoxic activity of *R. catesbeiana* is still unknown and it could reveal some new and interesting facts about the role of galectins in the lifestyle and survival of these sedentary animals. Since galectin-1 can be purified in large amount from bullfrog unfertilized eggs, it may be a potential drug discovery target for both anticancer and antitumor agents which may useful in modern clinical biochemistry.

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