SENRA Academic Publishers, Burnaby, British Columbia
Vol. 4, No. 2, pp. 1141-1149, June 2010
ISSN: 1715-9997

Canadian Journal of pure&applied sciences

INHIBITORY EFFECTS OF RCG1, A β-GALACTOSIDE-BINDING LECTIN FROM *RANA CATESBEIANA* (AMERICAN BULLFROG) OOCYTES AGAINST HUMAN AND PHYTOPATHOGENS

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ABSTRACT

A β-galactoside-binding galectin-1 (RCG1) was purified from the oocytes of the American bullfrog, *Rana catesbeiana* by affinity column chromatography and was evaluated for its growth of inhibition effects on bacteria and fungi. Through SDS-PAGE and gel permeation chromatography, RCG1 was found to be a non-covalently-bonded dimeric protein that consisted of two 15 kDa polypeptide subunits. This lectin showed significant hemagglutinting activity against trypsinized human and rabbit erythrocytes and it was inhibited by asialofetuin, thiodigalactoside and lectose. RCG1 was screened for *in vitro* antibacterial activity against eleven human pathogenic bacteria and significantly inhibited the growth of gram-positive bacteria than the gram-negative bacteria. *Bacillus subtilis* (10±1 mm) and *Bacillus cereus* (8±1 mm) were exhibited the highest growth of inhibition by the lectin (250 µg/disc). At the same time, RCG1 showed good growth inhibition against the gram-negative bacterium, *Salmonella typhi* but not others such as, *Pseudomans* sp., *Escherichia coli* and *Vibrio cholerae*. Antifungal activity also was investigated against six phytopathogenic fungi based on a food poisoning technique. Among the test fungi, the maximum inhibition of mycelial growth were observed in *Fusarium equiseti* (18.4±1%) followed by *Colletotrichum corchori* (13.3±1%) and *Curvularia lunata* (8.4±1%) at a concentration of the RCG1 100 µg/ml. These results suggest that future findings of lectin applications obtained from bullfrog oocytes may be of importance to clinical microbiology and have potential drug-resistant agents.

Keywords: *Rana catesbeiana*, antibacterial, antifungal, inhibition zone, mycelial growth, lectin.

INTRODUCTION

Galectins, comprise S-type β-galactosyl binding proteins that are present in vertebrates and invertebrates and do not require divalent cations for their binding activity (Vasta, 2009). The galectin family has been subdivided in several subgroups; 'proto', 'tandem' and 'chimera' (Rabinovich *et al.*, 2007) and they are bind to lactose/*N*-acetyllactosamine (Ahmed and Vasta, 1994). Galectin has been found in many organs of many organisms including liver, placenta, lung, heart, spleen, testis, and even also electric organ of electric eel - however the physiological function of galectin is still unknown. Galectins have activity about apoptosis (Koh *et al.*, 2008), cell adhesion (Stowell *et al.*, 2008), immune response and cancer metastasis (Hernandez and Baum, 2002) *in vitr*o, all suggesting that the protein has highly potency for creatures. In amphibians, galectins have been purified from the genus *Rana* (Ozeki *et al.*, 1991[a]), *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.*, 1991[a]) 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.*, 1991[b]) and tissue fibronectin was determined to be a putative endogenous ligand (Ozeki *et al.*, 1995).

Gram-positive bacteria are those that are stained blue or violet by gram staining. This is in contrast to gram-negative bacteria, which cannot retain the crystal violet stain, instead taking up the counterstain (safranin) and appearing red or pink. Gram-positive organisms are able to retain the crystal violet stain because of the high amount of peptidoglycan in the cell wall. Gram-positive cell walls typically lack the outer membrane found in gram-negative bacteria. The cell wall of virtually all bacteria consists of a rigid peptidoglycan layer that is either overlaid by an outer lipopolysaccharide (LPS) layer in gram-negative bacteria or remains exposed on the

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surface of gram-positive bacteria. Peptidoglycan is a polymer of alternating *N*-acetylglucosamine (GlcNAc or NAG) and *N*-acetylmuramic acid (NAM) units connected by short pentapeptides. The β-1,4-glycosidic bond of the *N*-acetylglucosamine-*N*-acetylmuramic acid peptide-glycan backbone can be hydrolyzed by lysozyme (muramidase; mucopeptide *N*-acetylmuramoylhydrolase), a ubiquitous enzyme involved in innate immune reaction of numerous animal species (Ito *et al.*, 1999). Galectins can interact directly with bacterial surface glycans. Furthermore, bacterial infection can modulate galectin expression, which in turn regulates leukocyte function and inflammatory responses. Both gram-positive bacteria, such as *Streptococcus pneumoniae* and gram-negative bacteria, such as *Klebsiella pneumoniae* display surface carbohydrate galectin ligands (Mey *et al.*, 1996; Mandrell *et al.*, 1994). Galectins can binds to bacterial lipopolysaccharides (LPSs) in a dual manner: the C terminus CRD binds to lactosyl moieties of *K. pneumoniae* LPS, whereas the non-carbohydrate-binding *N*-terminal domain of galectin-3 binds to the lipid A moiety of *Salmonella enterica* subsp *enterica* serovar Minnesota LPS (Mey *et al.*, 1996). Galectins also binds mycobacterial phosphatidylinositol mannosides, glycolipids that accumulate on the membrane of *Mycobacterium*-containing phagosomes during infections (Barboni *et al.*, 2005). The galectin BbtGal-L from the cephalochordate amphioxus (*Branchiostoma belcheri*) specifically recognizes *Vibrio vulnificus* but not *Vibrio parahaemolyticus* or *Staphylococcus aureus* and its expression is upregulated by bacterial challenge (Yu *et al.*, 2007). Galectins also can recognize surface glycans on the surface of saprophytic or pathogenic fungi and could function as a macrophage receptor for fungal pathogens (Fradin *et al.*, 2000). Interestingly, the selective binding of galectin-3 to the *Candida albicans* cell wall glycans is fungicidal (Kohatsu *et al.*, 2006) as function in innate immunity. It has been reported that many lectins from marine invertebrates show antibacterial activity. The lectin from the horse mussel *M. modiolus* exhibited strong antibacterial activity against tested *vibrio* strains (Tunkijjanukij and Olafsen, 1998). T-antigen binding lectin purified from sea cucumber showed strong broad spectrum antibacterial activity against both gram-positive and gram-negative bacteria (Gowda *et al.*, 2008).

We previously evaluated the cell adhesion activity by human rhabdomyosarcoma cells and determined the glycan-binding properties of the lectin by using frontal affinity chromatography technology (FACT) (Kawsar *et al.*, 2009). In this paper, we evaluate the antibacterial and antifungal activities by the determination of growth inhibitory effects of the purified lectin, RCG1 from the American bullfrog *Rana catesbeiana* oocytes against human and phytopathogens.

MATERIALS AND METHODS

Drugs and reagents

A lactosyl-agarose column was purchased from Seikagaku Kogyo Co. Ltd., Japan and standard protein marker mixture (Daiichi-III) for SDS-PAGE was purchased from Daiichi Pure Chem. Co. Ltd., Japan. A bicinchoninic acid (BCA) kit was purchased from Pierce Co. Ltd., USA. Superdex 75 and Sephadex G-75 were obtained from GE Healthcare, USA. Agar, dextrose, peptone, beef extract were purchased from Merck Ltd., India and BDH Ltd., Bangladesh and were of the highest purity grade. Female American bullfrogs (*Rana catesbeiana*) were purchased from an experimental animal company, Nippon Seibutsu Zairyo Co. Ltd., Tokyo, Japan. Eggs were stored at -80°C or used after collection according to the situation.

Purification of the lectin (RCG1)

RCG1, β-galactoside-binding lectin was purified from the oocytes of the American bullfrog (*Rana catesbeiana*) by affinity column chromatography (Ozeki *et al*., 1991[a]). Briefly, unfertilized oocytes (100 g w/v) were obtained from the abdominal cavity of a female by laparotomy, homogenized in a cooled pestle and frozen in 500 ml of acetone. During homogenization lipids located in the upper layer were discarded. After removal of lipids several times via frozen acetone, the precipitate was dried completely into powdered form by vacuum drying. Acetone-extracted oocyte powder (10 g) was homogenized with 20 times (w/v) TBS (10mM Tris(hydroxymethyl)aminomethane-HCl, pH 7.4, containing 150 mM NaCl) and centrifuged at 27,500 *g* for 1 h 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 (5 ml). After application of extracts, the lactosyl-agarose column was extensively washed with TBS. The lectin was eluted with 100 mM lactose in TBS and each 1 ml of elution was collected in tubes with a fraction collector. The eluted fractions were analyzed by spectrophotometer at 280 nm. The fractions were dialyzed against 1,000 times volumes of TBS to remove free lactose.

Hemagglutinating activity

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 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.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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°C for 15 min. Aliquots of 30 µl were applied to the well of a mini-slab gel (gel size: 80mm × 100 mm with 1 mm thickness; 12% and 5% polyacrylamide were used in separation and upper gels, respectively, constant current at 30 mA for 1h) 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.

Gel permeation chromatography

The purified lectin was dissolved in 2.5% glycerol and subjected to gel permeation chromatography (GPC) utilizing a Superdex 75 column (1.0 × 65 cm) connected to an FPLC system (GE Healthcare, USA) in the presence of 50 mM lactose containing TBS. The elution time of the lectin from the column was detected by UV at an absorbance of 280 nm. Bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), myoglobin (17 kDa) ribonuclease (14 kDa) and cytochrome *c* (6; 12 kDa) were used as standard molecular marker.

Protein determination

Protein concentrations were determined using BCA protein assay kit (Smith *et al.*, 1985; 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).

Tested pathogens

The bacterial and fungal pathogens 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. 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.

Culture and media

Standard NA (Nutrient Agar) medium was used for growing bacterial strains throughout the work whereby 20 g of agar powder, 5 g of peptone, 3 g of beef extract and 0.5 g of NaCl were added per liter of water. The medium was autoclaved for 15 minutes at 121°C with 15 psi. Older cultures were transferred to freshly prepared NA slants separately for each species via sterilized bacterial loop. In such a way, four tubes were freshly prepared for each bacterial pathogen. These tubes of inoculated slants were incubated at 35±2°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°C.

Antibacterial assay

The *in vitro* growth inhibition assay against bacteria by RCG1 was carried out by the disc diffusion method (Bauer *et al.,* 1966). 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°C, 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°C 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 20 µl (250 µg/disc) from 5% phosphate buffered saline (PBS, pH 7.4) solution of RCG1 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 RCG1 diffused from disc to the surrounding medium by this time. The plates were then incubated at 35±2°C 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. Galactose was used as negative control. All the results were compared with the standard antibacterial antibiotic ampicillin (20 µg/disc), (BEXIMCO Pharm., Bangladesh Ltd.).

Antifungal activity

The *in vitro* antifungal activity of the bullfrog oocytes lectin was determined by the poisoned food technique (Grover and Moore, 1962) with some modification (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°C, 15 psi) for 15 minutes. Purified lectin (in PBS solution) was mixed with sterilized melted PDA medium to have 100 µg/ml in 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°C. 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 milimeter. All the antifungal results were compared with the standard antifungal antibiotic nystatin (100 µg/ml) in PDA. Galactose 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.

RESULTS AND DISCUSSION

The supernatant extract from acetone-powdered bullfrog oocytes showed strong hemagglutinating activity and the activity was cancelled by the co-presence of β-galactosides such as lactose but not by the α-galactose or melibiose and therefore it was applied to a lactosyl-agarose column. After column washing with TBS, the column bound lectin was specifically purified by the addition of 100 mM lactose in TBS (Fig. 1). Four milligrams of RCG1 was purified from 100g (wet weight) of unfertilized oocytes and it was concentrated approximately 410 fold by affinity chromatography (Table 1). The purified *Rana catesbeiana* oocyte galectin-1 (RCG1) showed strong hemagglutinating activity against trypsinized and glutaraldehyde-fixed human erythrocytes and was slightly more sensitive to human erythrocytes when compared to those from rabbit (Table

Table 1. Purification of galactose-binding lectin from bullfrog *Rana catesbeiana* oocytes.

Purification Steps	Titer (HU)	Volume (ml)	Total activity	Protein conc. (mg/ml)	Specific activity	Purification fold	Recovery of activity (%)
Crude extract obtained by acetone	512	100	51200	2.1	2.4	1	100
Purified lectin	4096	5	20480	0.83	987	411	40

Total activity is shown by Titer × volume
Specific activity was shown by titer/mg of protein.

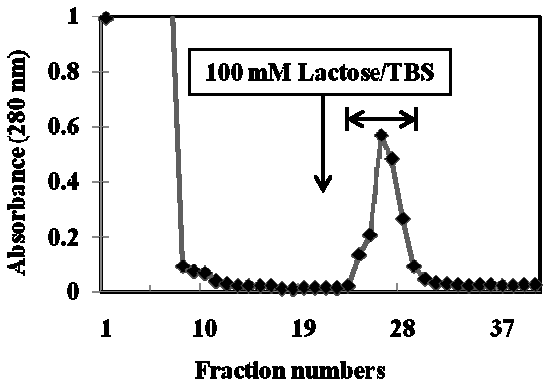

Fig. 1. Lactosyl-agarose affinity chromatography. Crude extract of *R. catesbeiana* oocytes were applied to an affinity column equilibrated with TBS. The column was extensively washed with TBS and the lectin was eluted with 100 mM lactose in TBS (arrow). The column bound fractions shown by the bar were collected and designated as purified lectin after dialysis against TBS.

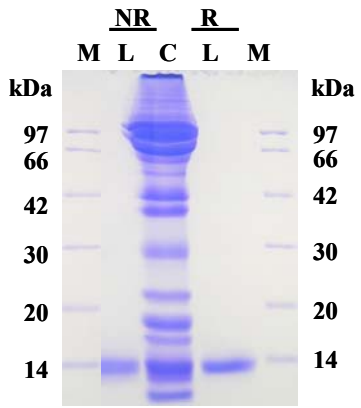

Fig. 2. SDS-polyacrylamide gel electrophoresis. C: crude extract of *R. catesbeiana* oocyte from acetone powdered; L: purified RCG1; NR: non-reducing and R: reducing conditions. 12% polyacrylamide was used as separating gel and the gels were stained with Coomassie brilliant blue. M: molecular weight 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).

2). RCG1 was purified as a single 15 kDa polypeptide under both reducing and non-reducing conditions by SDS-PAGE (Fig. 2, lane L). However, crude extract of the acetone powdered oocytes contained various proteins by SDS-PAGE (Fig. 2, lane C). On the other hand, RCG1 appeared to have a molecular mass at 30 kDa in GPC (Fig. 3A & B), indicating that the lectin was a non-covalent bound dimeric protein consisting of two 15 kDa polypeptide subunits. A partial primary structure analysis of the lectin showed that the amino acid sequence of the protein belonged to a superfamily of galectins (Ozeki *et al.*, 1991[c]) in addition to the carbohydrate binding specificity against β-galactoside.

Table 2. Hemagglutinating activity of RCG1 by human and rabbit erythrocytes.

Blood type*	Titer (HU)
Human (Type O)	2048
Human (Type A)	2048
Human (Type B)	1024
Rabbit	1024

*Trypsinzed and glutaraldehyde fixed erythrocytes were used.

Table 3. Antibacterial activity of RCG1 against gram-positive bacteria.

Name of bacteria	Diameter of zone of inhibition in milimeter	
	Lectin (250 µg/disc)	Ampicillin* (20 µg/disc)
Bacillus subtilis	10±1	20±1
Bacillus cereus	8±1	19±1
Bacillus megaterium	4±1	18±1
Staphylococcus aureus	0	21±1

Note: *Standard antibacterial antibiotic, Statistical analysis (RBD) at 1% level, organisms significant (F value 183.5), replica significant (F value 6.95).

The inhibitory effects of eleven human pathogenic bacteria to RCG1 was tested and compared to that of the antibacterial antibiotic, ampicilin. The results of the sensitivity test are presented in Tables 3 and 4. It was found that the RCG1 (250 µg/disc) gave promising inhibitory effects against gram-positive bacteria such as *Bacillus subtilis*. The diameter zone of inhibition by the

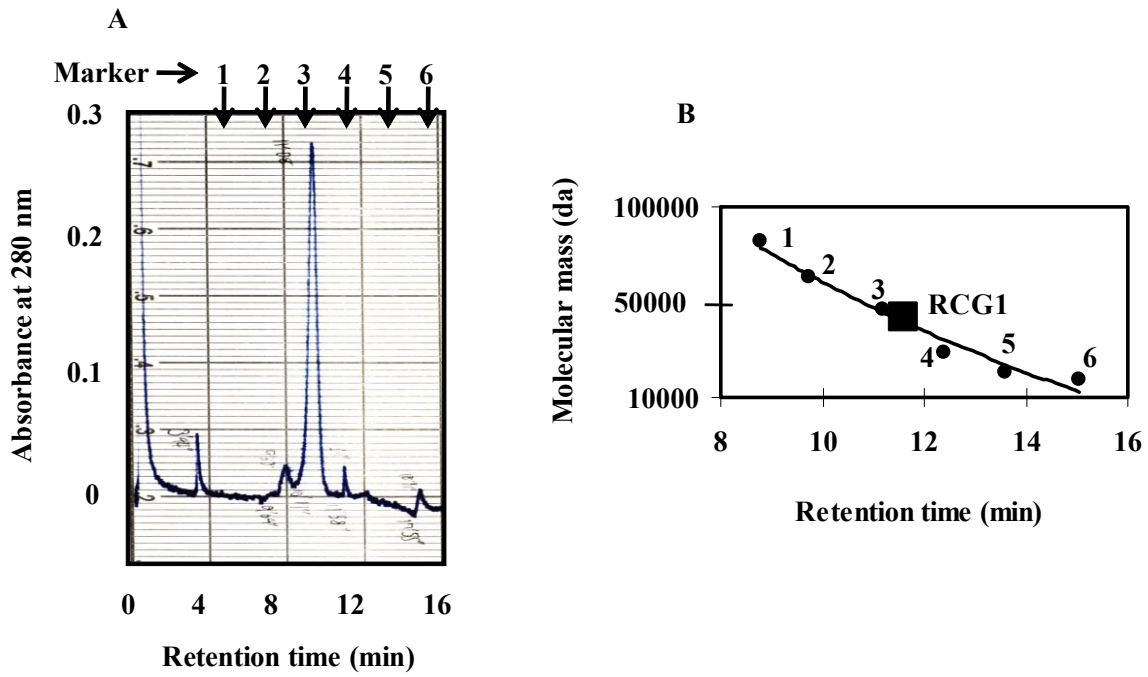

Fig. 3. Determination of the molecular weight by GPC. A: RCG1 (20 µg) was separated on a Superdex 75 column using FPLC system at a flow of 0.5 ml/min (chart speed is 0.5 cm/ml). B: Calibration line for the determination of molecular weight of RCG1 was determined using standard molecular mass marker proteins as; bovine serum albumin (1; 66 kDa), ovalbumin (2; 43 kDa), carbonic anhydrase (3; 30 kDa), myoglobin (4; 17 kDa), ribonuclease (5; 14 kDa) and cytochrome *c* (6; 12 kDa).

addition of RCG1 was significantly effective for *Bacillus subtilis* and *Bacillus cereus*; 10 and 8 mm, respectively (Table 3) although the growth inhibition of *Bacillus megaterium* was less effective. On the other hand, RCG1 showed good antibacterial activity against gram-negative bacterium *Salmonella typhi* but it did not inhibit the growth of other gram-negative bacteria such as *Pseudomonas* sp., *Escherichia coli* and *Vibrio cholerae* (Table 4), though the control antibiotic, ampicillin (20 µg/disc) inhibited the growth against all gram-negative bacteria. Amongst all bacterial strains, the gram-positive bacteria were more susceptible to the lectin as compared to gram-negative bacteria. This result suggested that the structure of surface-exposed carbohydrates on gram-positive bacteria were different even if they belong to the same genus within *Bacillus*.

Table 4. Antibacterial activity of RCG1 against gram-negative bacteria.

Name of bacteria	Diameter of zone of inhibition in milimeter	
	Lectin (250 µg/disc)	Ampicillin* (20 µg/disc)
Salmonella typhi	8±1	22±1
Salmonella paratyphi	6±1	19±1
Shigella sonnei	4±1	18±1
Shigella dysenteriae	3±1	20±1
Pseudomonas sp	0	19±1
Escherichia coli	0	17±1
Vibrio cholerae	0	18±1

Note: *Standard antibacterial antibiotic, Statistical analysis (RBD) at 1% level, organisms significant (F value 183.5), replica significant (F value 6.95).

A glycomics approach to determine the structure of surface glycans of bacteria may provide more useful clinical information to prevent disease using lectins. Indeed, galectins may be interesting candidates for interacting with bacteria owing to their specificity for β-galactosides in that they might be able to interact with some of the bacterial carbohydrates on the cell surface (Mandrell *et al.*, 1994). The glycan binding profile of RCG1 was analyzed by frontal affinity chromatography (Kawsar *et al.*, 2009) and it specifically recognizes branched complex type *N*-linked oligosaccharides having a lactosamine (Galβ1-4GlcNAc) structure. It seems reasonable that RCG1 may catch bacteria containing a lipopolysaccharide layer because the β-galactoside structure (Galβ1-4Glc) is contained in most of the major lipopolysaccharides at the non reducing terminal. In many galectins, a specific affinity moiety was reported against the *N*-acetyllactosamine and poly *N*-acetyllactosamine (Sharma *et al.*, 1992), indicating that galectin family is available as a candidate molecule to trap gram-positive bacteria by binding to lipopolysaccharides that express the "asialo-lactoneo-series" structure. Recently, a lipopolysaccharide-binding lectin purified from the seeds of *Eugenia uniflora* showed antibacterial activity similar to RCG1, as it effectively inhibited growth against gram-positive bacteria such as *Bacillus subtilis* (Oliveira *et al.*, 2008) and a β-galactoside binding pearl shell lectin purified from the marine bivalve, *Pteria penguin* showed antibacterial activity similar to RCG1 as well (Naganuma *et al.*, 2006). Rhamnose-binding steelhead trout (*Oncorhynchus mykiss*) egg lectin inhibited the growth of gram-positive and gram-negative bacteria by recognizing lipopolysaccharide or lipoteichoic acid (Tateno *et al.*, 2002) and in addition, galectin interactions with lipopolysaccharides in the gram-negative bacteria as *Salmonella minnesota* inhibited the growth of same genus *Salmonella* in a similar fashion to RCG1 (Mey *et al.*, 1996).

It has been reported (Yu *et al.*, 2007) that host galectins can bind directly to glycoconjugates on the surface of bacteria, either facilitating or inhibiting pathogen entry, followed by positive and negative regulation of host innate and adaptive immunity. The presence of the lectin in the oocytes of *R. catesbeiana* led us to consider its possible biological involvement in the defense mechanisms of the species. RCG1 is the first lectin from frog reported to exhibit microbial growth inhibition. This activity was previously postulated for invertebrates (Inamori *et al.*, 1999), which expressed soluble and bound membrane lectin forms that appeared to be one of the groups of molecules for recognition and defense. An instance of the above is provided by several types of hemocyte-derived lectins which may play a role of functional in the innate immunity. The same fact was suggested for mammalian lectins, such as mannose-binding lectins as collectin family, which play an important role in host-pathogen interactions by specific recognition of the cell surface substances of bacteria (Rabinovich and Gruppi, 2005). Some lectins seem to be useful for identification of pathogenic bacteria based on the specific binding moieties of lectins to the characteristic glycans on cell wall of such types of bacteria (Munoz-Crego *et al.*, 1999). The characterization of glycans that presented the cell walls of gram-positive and gram-negative bacteria was characterized by lectins, finding to their peptidoglycan and lipopolysaccharides (Doyle, 1994).

Contrastively, the interactions between lectins and glycans in fungi are not well known. In this study, we have found that RCG1 reduced the growth rate of some strains of fungi against six phytopathogenic strains by comparing with the antifungal antibiotic nystatin as positive control. The inhibition of fungal mycelial growth by RCG1 and nystatin are given in Table 5. From these results we observed that the mycelial growth of *Fusarium equiseti* was found to be inhibited (18.4±1%) by RCG1

(100 µg/ml), which showed highest inhibition towards the mycelial growth of all tested fungal strains, although the inhibition effect by the lectin was less than the case of bacteria. The growth of *Colletotrichum corchori* and *Curvularia lunata* (13.3-8.4±1%) was moderately inhibited but *Botryodiplodia theobromae* was least inhibited by RCG1. On the other hand, *Alternaria alternata* and *Macrophomina phaseolina* were never inhibited by the lectin, though the growth of all six fungi was totally inhibited by standard antibiotic, nystatin (100 µg/ml). By now, antifungal activity has been reported in many plant lectins (Broekaert *et al.*, 1989). Our results suggested that RCG1 has antifungal activity similarly to mannose-binding lectins from red cluster pepper (*Capsicum frutescens*) and *Pisum sativum* seeds that inhibited the growth of fungi *Fusarium moniliforme* and *Fusarium oxysporum, Aspergillus flavus* and *Trichoderma viride* (Nagi and Ng, 2007; Sitohy *et al.*, 2007).

Table 5. Antifungal activity of RCG1 from the bullfrog *R. catesbeiana* oocytes.

Name of fungi	Percentage inhibition of fungal mycelial growth	
	Lectin (100 µg/ml PDA)	Nystatin* (100 µg/ml PDA)
Fusarium equiseti	18.4±1	47.4±1
Colletotrichum corchori	13.3±1	50.3±1
Curvularia lunata	8.4±1	46.5±1
Botryodiplodia theobromae	4.6±1	40.4±1
Alternaria alternate	0	39.4±1
Macrophomina phaseolina	0	52.2±1

Note: *Standard antifungal antibiotic, Growth measured- radial growth in cm.

RCG1 has belongs to the galectin family. Many galectins were reported to have multivalent functions such as in cell adhesion and apoptosis through the carbohydrate-binding activity. In this study, we have found that the lectin possess antimicrobial activity in addition to cell adhesive activity (Kawsar *et al.*, 2009). Because the lectin is located on the outer membrane of frog eggs, it may play an integral role in defense against bacterial pathogens. Recently, a number of galactose-binding lectin were shown to act as host receptors for bacteria and fungi (Vasta, 2009; Ideo *et al.*, 2009) by apparent direct or indirect galectin-dependent specific host-pathogen interactions. Since RCG1 can be purified in large amounts from bullfrog eggs, it may be a potential drug discovery target for both anti-cancer and anti-inflammatory agents.

CONCLUSION

The present study showed that RCG1 displayed significant growth inhibition effects against selected human and phytopathogens. American bullfrog *Rana catesbeiana* oocytes galectin-1 (RCG1) was screened for the first time for antibacterial and antifungal activities and these antimicrobial activities may provide an effective defense capability against invading microbes in the amphibian *Rana catesbeiana*.

ACKNOWLEDGEMENTS

We are grateful to the Chairman of the Department of Microbiology, University of Chittagong, Bangladesh to give us the opportunity to carry out the microbiology work. We are also thankful to Dr. Robert A. Kanaly (USA & YCU) for improving our manuscript. This work was supported by Grants-in Aid for Scientific Research from JSPS (Japan Society for the Promotion of Science).

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Received: March 1, 2010; Revised: May 22, 2010; Accepted: May 24, 2010