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BIOLOGICAL PROPERTIES OF LECTINS IN MARINE SPONGES FROM CHONBURI PROVINCE, THAILAND.

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ABSTRACT

In some invertebrate marine animals, lectin or agglutinin possesses defense properties against other marine animals. We examined the lectins toward hemagglutinating activity of human and animal erythrocytes, sugar-binding specificities, and effect of heat and divalent cations requirement to elucidate the biochemical properties of the sponge lectins. The preliminary screening for the hemagglutinating activity in 48 marine sponges was carried out by extraction of the fresh sponges using 0.85% NaCl. It was found that the protein extracts from nine sponges' species gave more high agglutinating activity against trypsin or papain-treated human erythrocytes more than normal erythrocytes. However, the protein extracts obtained from three species of sponges, i.e., *Chondrilla australiensis, Hyrtios erecta,* and *Callyspongia (Euplacella) joubini,* contained high activity of hemagglutinin specific to normal human A erythrocytes with titers approximately at 12,049, 1,965, and 1,932/mg protein, respectively.

Results of sugar-binding specificities assays performed with several carbohydrates and glycoproteins showed that the lectins from *C. australiensis, H. erecta, and C. (Euplacella) joubini* were specifically bound to glycoprotein, porcine stomach mucin, and fetuin more than mono- or oligosaccharide. The hemagglutinating activity of lectin derived from *C. australiensis,* and *H. erecta* was stabilized at 25-50°C and of *C. (Euplacella) joubini* was stabilized at 25-35°C. The activity of lectin from *H. erecta* could be enhanced by magnesium ion.

Keywords: Lectin, hemagglutination, marine sponge.

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INTRODUCTION

Lectins are antibody-like molecules and have multivalent by bearing at least two sugar-binding sites but have no enzymatic activity (Goldstein et al., 1980; Kocourek and Horejsi, 1981). The most commonly method for detecting the presence of lectin extract solution is an agglutination of mammalian or animal erythrocytes with untreated or enzyme-treated erythrocytes. The modification of lectins using enzymes glycohydrolases, glycol-oxidases, and the use of trypsin, papain or neuraminidase to uncover hindered residues, have constituted useful methods for preliminary information on the carbohydrate specificity of the lectins (Vasta and Ahmed, 1995). Lectins have been isolated and characterized in various marine invertebrates, including tunicates (Nair et al., 2000), soft coral (Goto et al., 1992), crustaceans (Ravindranath et al., 1985; Vazquez et al., 1993), echinoderms (Giga et al., 1987; Matsui et al., 1994) and clams (Tunkijjanukij and Olafsen, 1998). Lectins have been found in the hemolymph and tissue extracts of marine invertebrate.

Due to the fact that lectins have the ability to bind to carbohydrate and promote the agglutination of different cells, such as bacteria and other invading pathogens, it is reasonable to assume that these molecules may be regarded as having a potential role in invertebrate non-self-recognition reactions (Marques and Barracco, 2000). There are increasing data suggesting the involvement of invertebrate lectins in processes of differentiation and development of organisms as well as in elimination of foreign substances through binding to their carbohydrate on the cell surface, cell-to-cell or cell-to-matrix interaction (Arason, 1996).

Marine sponges represent a rich source of lectins. In searching for agglutinins in marine invertebrates collected near Fiji, the agglutinin was found from the sponge *Dysidea herbacea* (Kamiya et al., 1985). A lectin in the sponge *Phyllospongia foliascens* and *Halichondria panicea* was subsequently isolated and characterized (Kamiya et al., 1986; Kamiya et al., 1990). We have screened nine marine sponges for lectin using a variety of different animals and human erythrocytes and some properties of these lectins in this study.

MATERIALS AND METHODS

Extraction of the sponges

Sponges were collected by scuba diving team at Lan Island and Srichang Island as shown in Table 1. One gram of fresh samples was homogenized in 10 ml of 0.85% NaCl using mortar. The extracts were subsequently centrifuged at 3,000 rpm for 15 min. The supernatant was frozen and kept at -20 °C until use. The identification of sponge specimen was kindly performed by Dr. Sumaitt Putchakarn, Institute of Marine Science, Burapha University, Chonburi, Thailand.

Table 1. A list of sponges species that were collected and used in this study.

Field code	Order	Family	Species
LSAB-02	*Haplosclerida*	*Callyspongiidae*	*Callyspongia (Euplacella) joubini*
LSAB-03	*Verongida*	*Aplysinellidae*	*Suberea praetensa*
LANT-04	*Halisarca*	*Halisarcidae*	*Halisarca ectofibrosa*
LANT-05	*Hadromerida*	*Clionaidae*	*Spheciospongia congenera*
LKRK-19	*Hadromerida*	*Clionaidae*	*Spheciospongia congenera*
LANY-03	*Hadromerida*	*Spirastrellidae*	*Spirastrella solida*
LNOL-07	*Dictyoceratida*	*Thorectidae*	*Hyrtios erecta*
LKRK-14	*Chondrosida*	*Chondrillidae*	*Chondrilla australiensis*
SICA-04	*Chondrosida*	*Chondrillidae*	*Chondrilla australiensis*

Hemagglutination assay

The hemagglutination (HA) assays were performed in U-bottomed micro-titer plate. A serial two-fold dilutions of a 50 µl of sponge extract samples were made with an equal volume of 0.15 M NaCl or 0.05 M Tris-HCl, 0.15 M NaCl pH 7.6 (TBS). A 50 µl of 2% human erythrocytes groups A, B, AB, and O obtained from Burapha Hospital and animal erythrocytes from Mah idol University was added to each well and incubated for 1 h at 25 °C. The hemagglutinating titers were recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of erythrocytes. The control for all assays consisted of the substitution of the sample by buffer. Each experiment was performed in triplicate. Enzyme treated human A erythrocytes were prepared by incubating 2% erythrocytes suspension with 0.2% trypsin or papain in TBS at 37 °C for 1 h. After washing four times, a 2% erythrocytes suspension was prepared in TBS. The results were expressed by the titer value, which is the maximum dilution for positive agglutination.

Protein determination

Protein concentrations of the sponge extracts were determined by the Bio-Rad protein assay according to Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as a standard.

Inhibition of hemagglutination by sugars

Inhibition tests by sugars and glycoproteins were conducted using the dialytic sponge extracts against TBS showing a titer of 1:8. The samples (50 µl) were allowed to react with various concentrations of inhibitors (50µl) for 1 h. Fifty µl of 2% human erythrocytes group A suspension was then added to the mixture and the agglutination was measured after standing for 1 h at room temperature. The results were expressed by the minimum concentration of the inhibitors that completely inhibit the hemagglutination. The following sugars (200 mM or 83 mM) and glycoprotein (0.4167 mg/ml) were used: D-fructose, D-ribose, D-xylose, L-arabinose, D-galactose, D-glucose, D-mannose, D-rhamnose, L-fucose, D-sorbital, D-galactosamine, D-glucosamine, N-acetyl-D- glucosamine, N-acetyl-D-galactosamine, N-acetylneuraminic acid, α methyl-D-mannopyranoside, cellobiose, lactose, maltose, melibiose, sucrose, raffinose, porcine stomach mucin Type II (PSM) and fetuin from fetal calf serum.

Divalent cat ion dependency

To examine the divalent cation requirement of lectin from sponge extracts, the samples were dialyzed against 0.01 M EDTA in 0.1 M Tris-HCl buffer pH 7.6 for 48 h. After that, the samples were dialyzed against 0.15 M NaCl for 24 h to remove EDTA. The samples were tested for hemagglutinating activity against human A erythrocytes in the presence of 10 mM $CaCl_2$, $MgCl_2$ or $MnCl_2$ in 0.05M TBS (Goto et al., 1992).

Temperature stability

Heat stability was examined by incubating the sponge extracts at 4, 25, 35, 40, 60, 70, 80 or 100 °C for 30 min. After cooling in an icebox, the remaining agglutinating activity against 2% human erythrocytes suspension was examined as previously described.

RESULTS

The marine sponges were collected from Chonburi province during 2005-2006 for elucidating the biochemical properties of the sponge lectins. We examined the lectins toward hemagglutinating activity of human and animal erythrocytes. The results are shown in Tables 2 and 3. The sponge extracts of nine species from Lan Island and Sichang Island agglutinated normal human erythrocytes with the reciprocal titers ranging from 8-2,048, while the enzyme treated erythrocytes gave more high hemagglutinating activity than native erythrocytes. Moreover, the lectin from *Callyspongia (Euplacella) joubini, Chondrilla australiensis* and *Hyrtios erecta* agglutinated horse erythrocyte with reciprocal titers at 4,096, 8 and 256, respectively. Comparison of the lectin titer per protein values among the sponge extracts showed *C. australiensis* extract contained the highest lectin, as shown in Table 4.

Table 2. Hemagglutination activity of protein extracts from some marine sponges against human erythrocytes.

Sponges	Hemagglutinating activity (titer)											
	A_N	A_T	A_P	B_N	B_T	B_P	O_N	O_T	O_P	AB_N	AB_T	AB_P
C. (Euplacella) joubini	8	128	256	8	256	1,024	128	256	2,048	16	512	4096
C. australiensis (LKRK-14)	32	128	512	64	512	512	512	1,024	2,408	64	128	4096
C. australiensis (SICA-04)	256	32	256	32	32	64	16	32	64	8	32	64
H. ectofibrosa	512	13,1072	16,384	512	262,144	524,288	16	128	1,024	32	1,024	4,096
H. erecta	8	128	2,048	512	16,384	4,096	64	512	4,096	2048	32,768	66,536
S. congenera (LANT-05)	64	128	128	64	2,048	32,768	16	128	512	16	256	1,024
S. congenera (LKRK-19)	1,024	1,024	4,096	256	4,096	32,768	1,024	1,024	1,024	1,024	64	4,096
S. solida	16	32	64	32	256	256	64	256	64	32	256	256
S. praetensa	32	128	256	32	32	32	32	128	2,048	32	128	8

N = normal; T = trypsin treated erythrocytes; P = papain treated erythrocytes

Table 3. Hemagglutination activity of protein extracts from some marine sponges against animal erythrocytes.

Sponges	Hemagglutinating activity (titer)									
	Rat	Mouse	Guinea pig	Rabbit	Dog	Sheep	Pig	Horse	Chicken	Goose
C. (Euplacella) joubini	0	0	2	16	8	0	32	4,096	0	2
C. australiensis (SICA 04)	8	8	8	16	16	0	0	8	8	8
H. erecta	128	8	0	8	8	2	1,024	256	0	0

Table 4. Hemgglutinating specific activity of some sponges.

Sponges	Hemagglutinating activity		Protein	Specific activity
	Reciprocal titer	Reciprocal titer	(mg/ml)	(mg/ml)
C. (Euplacella) joubini	8	160	0.0828	1,932
C. australiensis (SICA 04)	256	5,120	0.4249	12,049
H. erecta	64	1,280	0.6511	1,965

The sugar-binding specificities and divalent cations requirement are summarized in Tables 5 and 6. Determination of sugar specificity of the sponge lectins showed that lectins from *C. australiensis* highly specifically bound α-lactose but *H. erecta* and *C. (Euplacella) joubini* bound PSM and fetuin more than mono- or oligosaccharide. The divalent cations requirement was observed in hemagglutinating activity after the addition of 10 mM of $CaCl_2$, $MgCl_2$ and $MnCl_2$ to the lectin preparation. The result showed that the lectin from *H. erecta* gave higher activity than the control in the present of $MgCl_2$. The hemagglutinating activity of the serum showed resistance in some degrees to heating. Heating at 80 °C was required to completely destroy the activity of the extracts from *C. (Euplacella) joubini* and *H. erecta* (Table 7).

Table 5. Sugar-binding specificities of marine sponge lectins.

Sugar and glycoprotein	Minimum inhibitory concentration (mM)		
	C. australiensis	*H. erecta*	*C. (Euplacella) joubini*
Sugar			
1.2 M L-Arabinose	0	200	0
1.2 M D-Mannose	0	0	100
1.2 M L-Fucose	100	100	0
1.2 M D-Galactosamine	0	50	0
1.2 M D-Glucosamine	200	50	0
1.2 M α-Methyl-D-mannopyranoside	100	0	0
0.5 M α-Lactose	2.6	0	0
1.2 M D-Maltose	0	100	0
1.2 M D-Melibiose	200	50	0
Glycoprotein (mg/ml)			
0.25% PSM (Mucin type II from porcine stomach)	2.54×10^{-5}	1.02×10^{-4}	4.07×10^{-4}
0.25% Fetuin (from fetal calf serum)	8.14×10^{-4}	0.2083	8.14×10^{-4}

Table 6. Effects of divalent cation on hemagglutinating activity.

Sponges	Hemagglutinating activity (titer)			
	Control	$MnCl_2$	$CaCl_2$	$MgCl_2$
C. (Euplacella) joubini	64	64	64	64
C. australiensis (SICA 04)	256	256	256	256
H. erecta	64	128	128	512

Table 7. Effects of temperature on hemagglutination.

Sponges	Temperature (°C)							
	25	**35**	**50**	**60**	**70**	**80**	**90**	**100**
C. (Euplacella) joubini	256	256	8	2	2	0	0	0
C. australiensis (SICA 04)	256	512	256	128	128	4	2	0
H. erecta	64	64	64	32	8	0	0	0

DISCUSSION

Lectins or hemagglutinins are a class of proteins which form complexes with carbohydrates or substances containing carbohydrates. Some have found wide application as tools for the characterization and isolation of material possessing carbohydrate moieties on cell membranes and cell surfaces. Sponges have been excellent sources for marine bioactive products focusing mainly on small molecular compounds representing a chemical structures and pharmacological properties. Macromolecules, proteins and polypeptide have received little attention. To date, studies on bioactive proteins deal mainly with hemagglutinins and aggregation factors. Aqueous extracts of 48 sponge species from the Red Sea, the Australian Barrier Reef and Florida Keys were screened for hemagglutinating, hemolytic, ichthyologic and lethal activities. Forty two per cent of the sponge species exhibited agglutinating properties to human erythrocytes of ABO groups (Mebs et al., 1985). On the screening for lectins in some sponges from Chonburi province, our results showed that the protein extracts from 33 out of 48 sponge species agglutinated human, animal, and trypsin or papain-treated human erythrocytes group A at the reciprocal titer of 8 to 16,384 (Watanachote et al., 2007). Results suggested that a variety of different human, animal, and enzyme-treated erythrocytes could be used to detect marine sponge lectins. However, none of them reacted specifically against erythrocytes of special blood groups. Similarly, a lectin from the Adriatic sponge, *Haliclona cratera,* which can agglutinate native and trypsinized, papainized, and neuraminidase-treated human erythrocyte groups A, B, AB, and O, and sheep erythrocytes, but the hemagglutinating activity is independent of Ca^{2+}, Mn^{2+} and Mg^{2+} ions (Pajic et al., 2002). A lectin from marine sponge, *H. panacea,* could agglutinate sheep erythrocytes at the lower limit of sensitivity at 0.025 μg of lectin protein per ml and the lectin was independent of the presence of divalent cations (Müller et al., 1981). On the contrary, the lectin from *C. australiensis, H. erecta,* and *C. (Euplacella) joubini* were specifically bound to glycoprotein (PSM) more than mono- or oligosaccharide similarly to the lectin from *Crenomytilus grayanus* (Belogortseva et al., 1998). Several lectins that were found in lower eukaryotic, multi-cellular animals, i.e., soft coral *Lobophytum variatum,* and *Sinularia* species were Ca^{++}-independent (Goto et al., 1992; Goto-Nance et al., 1996). The lectins from *S. lochmodes* (9.1 mg protein/ml) agglutinated rabbit erythrocytes (titer of 8,192) and horse erythrocytes (titer of 512). The agglutinating activity was not changed after the addition of 10 mM of $CaCl_2$, $MgCl_2$ or $MnCl_2$ to the lectin preparation (Jimbo et al., 2000).

Animal lectins are a heterogeneous class of molecules, which exhibit a high structural diversity. The binding of invertebrate lectins to their carbohydrate moieties may require the presence of calcium (C-

type lectins) or other divalent cations such as some lectin from Penaeid shrimp (Watanachote and Tunkijjanukit, 2006) or crab *Scylla serrata* (Kongtawelert, 1998). However, the functions of these proteins have evolved differently in the different animal including adhesion between animal cells within a single organism, targeting of bacterial toxins to animal cells and immune recognition of bacteria and fungi by animals (Dodd and Drickamer, 2001). Because of the growing number of applications in the biochemistry and cell biology, the isolation and characterization of a new lectin requires a detailed study of its carbohydrate binding specificity. However, the lectin in marine sponges is not as widespread as in other marine organisms (mollusks and crustaceans) or in plants (Miarons and Fresno, 2000). Müller et al. (2004) concluded that the *Suberites domuncula* lectin (*Suberites* lectin, tachylectin) acted as an antimicrobial molecule involved in immune defense against bacterial invaders. It is recommended that we should characterize and investigate the role of purified lectin from *C. australiensis, H. erecta,* and *C. (Euplacella) joubini* with antimicrobial activity.

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