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Short communication

Gal/GalNAc specific multiple lectins in marine bivalve *Anadara granosa*

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ABSTRACT

Complete lectin mapping of molluscs with their diversified recognition pattern and possible role in lectin-carbohydrate interaction based immune response triggering need much attention. In this communication, Gal/GalNAc specific three lectins AGL-IA (*Anadara granosa* lectin-IA), AGL-IB (*A. granosa* lectin-IB) and AGL-IV (*A. granosa* lectin-IV) and a lectin having hemolytic activity AGL-III (*A. granosa* lectin-III) were purified from the plasma of *A. granosa* bivalve by a combination of gel filtration and affinity chromatography. AGL-IA and IB were oligomeric lectins whereas, AGL-III and IV were monomeric. The molecular weight of AGL-IA, IB, III and IV were 375, 260, 45 and 33 kDa respectively. AGL-IA and IV agglutinated both rabbit and pronase treated human erythrocytes, whereas AGL-IB agglutinated only rabbit erythrocytes. AGL-III was found to agglutinate rabbit erythrocytes, however, it caused hemolysis of pronase treated human erythrocytes. The activity of all four lectins was calcium dependent and maximum at a pH range 7–8. Apart from Gal/GalNAc specific, the four lectins showed substantial differences in their carbohydrate recognition pattern. Moreover, there was a difference in the carbohydrate specificity between AGL-III and other three lectins (AGL-IA, AGL-IB and AGL-IV) towards polyvalent glycotope. On the one hand, 'cluster glycoside effect' *i.e.*, an enhancement of the activity of a multivalent ligand, was observed for carbohydrate specificities of AGL-IA, AGL-IB, AGL-IV. On the other hand, the effect of multivalent ligands on the carbohydrate specificity of AGL-III was opposite of cluster glycoside effect. The affinity of AGL-IA, AGL-IB and AGL-IV for ligands can be ranked as follows: glycoproteins >> polysaccharide > oligosaccharides and monosaccharides. However, Gal related monosaccharides were the best inhibitors of AGL-III and the inhibitory activity decreased gradually in the following order: monosaccharide > disaccharide > polysaccharide. Thus, the diverse specificity of multiple lectins in *A. granosa* plasma possibly enables to recognize a wide range of microorganisms.

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Marine molluscs live in aquatic environment which is rich in microorganisms. Due to lack of antibody-based immunity, host defense of molluscs and other invertebrates against pathogen infection solely depends on different pattern recognition proteins (PRPs). Different PRPs such as lectins, cytokines, nitric oxide synthases and antimicrobial peptides comprise innate immune systems of molluscs [1,2]. Among them, lectins play a crucial role in innate immunity by recognizing different patterns of carbohydrate exposed on microorganisms and subsequently trigger a series of protective immune responses [3,4]. Lectins from several marine bivalves were purified and characterized for their role as a crucial player in host defense against foreign substances [4–18]. However, detailed carbohydrate recognition patterns of very few of them like codakine [16], *Macoma birmanica* agglutinin (MBA) [19] and *Aplysia* gonad lectin [20] were studied. MBA was found to be β-GlcNAc/α-Man specific and polyvalent glycoside clusters of GlcNAcβ1–2Manα1– was the most potent binding motif [19]. Codakine was a Fuc and Man-specific lectin and had a very fine specificity for N-linked biantennary complex type glycan [16]. *Aplysia* gonad lectin had unique specificity for D-galacturonic acid and its polysaccharides [20].

Again, presence of multiple lectins with their role in innate immunity was well documented for many invertebrates like horseshoe crab [21], cockroach [22] and Japanese eel [23,24]. Because of diverse carbohydrate specificity of these lectins, recognition of vast array of microorganisms becomes possible. Therefore, it is relevant to believe that multiple lectins of diverse carbohydrate specificities present in the circulation of marine molluscs can also play important roles in their defense by recognizing a vast array of

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invading microorganisms [4]. *Anadara granosa*, a widely distributed and economically important marine clam, which belongs to the family arcidae, possesses blood corpuscles (hemocytes) containing hemoglobin that rarely occurred in invertebrates. Two lectins, Anadarine P and Anadarine MS had been reported from the plasma of *A. granosa* [25,26]. Earlier, we have mentioned the presence of four new lectins AGL-IA, AGL-IB, AGL-III and AGL-IV in the plasma of *A. granosa* [4]. In this communication purification, characterization and carbohydrate recognition profile of these four new lectins were elaborately described.

A. granosa clams were collected from mangrove mudflats of deltaic Sundarban of West Bengal, India. Blood was withdrawn by dissecting the adductor muscles. The clear plasma was collected and stored at −20 °C. Primarily, plasma (2 ml) was loaded on a Sephadex G-100 column (68 cm × 1.6 cm) pre-equilibrated with 20 mM Tris buffer saline containing 10 mM $CaCl_2$, pH 7.5 (TBS-Ca), and eluted with the same buffer at a flow rate of 5.4 ml/h. The elution of protein was monitored spectrophotometrically at 280 nm. The hemagglutinating activity of eluted fractions was checked with 2% (v/v) pronase treated human erythrocytes and normal rabbit erythrocytes as described by Adhya et al. [7]. The plasma after gel filtration resolved into four fractions (Supplementary Fig. 1) of which fraction I (Fr 1) and fraction IV (Fr IV) showed hemagglutinating activity against pronase treated human erythrocytes and normal rabbit erythrocyte, whereas fraction III (Fr III) showed hemagglutination activity against rabbit erythrocytes and hemolytic activity against pronase treated human erythrocytes. Fr I and Fr IV were separately loaded on arabinogalactan matrices which was pre-equilibrated with TBS-Ca. Bound proteins of both fractions were desorbed from the columns with 40 mM EDTA in 20 mM TBS, pH 7.5 (TBS-EDTA) and were collected in mixture of 1 M Tris and 1 M $CaCl_2$ (50:1 v/v). The collected bound proteins from Fr I and Fr IV were separately dialyzed in TBS-Ca buffer and concentrated on centricon 10 (Amersham). They were designated as AGL-IA and AGL-IV respectively. The arabinogalactan unbound protein from Fr I showing hemagglutinating activity with rabbit erythrocytes, was concentrated and subsequently loaded on fetuin-agarose matrix previously equilibrated with TBS-Ca. The bound protein AGL-IB was eluted and concentrated as described in purification of AGL-IA and AGL-IV. AGI-III was purified from Fr III on melibiose–Sepharose 4B matrix using similar purification method like AGL-IA, AGL-IB and AGL-IV. Melibiose was immobilized on Sepharose 4B according to the procedure of Porath and Ersson adopted by Teichberg et al. [27] by divinyl sulphone activation method. Protein was estimated following the method of Lowry [28] using bovine serum albumin as the standard. The yields of AGL-IA, AGL-IB, AGL-III and AGL-IV were 0.39%, 0.45%, 0.96% and 0.18%, respectively (Table 1).

The homogeneity of all the lectins was ascertained by non-denaturing polyacrylamide gel electrophoresis (PAGE), which produced single band for each lectin. The molecular masses of AGL-IA and AGL-IB were determined on 6% non-denaturing PAGE by comparing the relative mobility of the lectins with standard proteins (HMW calibration kit, Amersham) and the estimated molecular weight of AGL-IA and AGL-IB were 375 and 260 kDa, respectively (Supplementary Figs. 2 and 3). The four purified lectins AGL-IA, AGL- IB, AGL-III and AGL-IV were separately denatured with 8 M urea containing 2% SDS in absence and presence of 100 mM dithiothreitol for 24 h at 37 °C. Therefore non-reduced and reduced lectins were subjected to 10% SDS-PAGE [29]. The molecular masses of subunits were determined by comparing the relative mobility of subunits with the Precision plus Protein Standards (Bio-Rad). Denaturing of AGL-IA showed three bands in non-reducing (260, 74 and 33 kDa) and reducing (60, 47 and 33 kDa) conditions (Supplementary Fig. 2). Whereas, AGL-IB produced two bands in non-reducing (52 and 105 kDa) and reducing (33 and 65 kDa) condition (Supplementary Fig. 3). From these results, it was concluded that both AGL-IA and AGL-IB were oligomeric lectins of different subunits. AGL-III and AGL-IV were monomeric lectins with molecular weight 45 and 33 kDa respectively, as they showed single band in both non-reducing and reducing conditions (Supplementary Fig. 4). Molecular complexity and self-aggregation were very common in lectins that were isolated from hemolymph of marine clams. This particular trait of these molecules was believed to be caused by either post-translation modifications or effect of multiple genes [4]. Tridacnin, purified from the hemolymph of *Tridacna maxima* clam, has been reported to have molecular weight 470 kDa with subunits of 10, 20 and 40 kDa [9]. Gigalin H and gigalin E from Pacific oyster, *Crassostrea gigas* which showed self-aggregating complexes of molecular weight ranging from 500 to 1600 kDa, were also dissociated in presence of 8 M urea into 21.0, 22.5 and 33.0 kD subunits [30]. Another hemolymph lectin chiletin of 12 kDa monomer, isolated from *Ostrea chilensis* oyster, was found to aggregate and remained in five different isoforms in native condition, and the largest one was 640 kDa. Some aggregates were similarly resolved with 8 M urea into different covalently and non-covalently attached subunits [6]. Similar phenomenon was also reported in hemolymph lectins of horse mussel, *Modiolus modiolus* [8] and pearl oyster, *Pinctada fucata martensii* [14].

There was a similarity between AGL-IA and AGL-IV in hemagglutinating property. AGL-IA and AGL-IV did not agglutinate normal human erythrocytes upto 50 μg/ml. However, they strongly agglutinated pronase treated erythrocytes irrespective of human blood group, and the minimum amounts of AGL-IA and IV required were 0.25 μg/ml and 1.25 μg/ml, respectively. They also showed strong agglutination towards rabbit erythrocytes with a minimum amount 0.25 μg/ml and 5 μg/ml, respectively. Pronase treatment on rabbit erythrocytes, the agglutinating abilities of AGL-IA and AGL-IV, were enhanced by 4 and 8 fold respectively. AGL-IB did not agglutinate human erythrocytes even after pronase treatment upto its concentration of 80 μg/ml but it agglutinated normal rabbit

Table 1
Purification profile of *A. granosa* lectins.[a]

Purification steps	Protein (mg)	Total activity (HU[b])	Specific activity (HU/mg)	Yield (%)
Plasma	46.80	12283.0	262.5	–
Fraction I	23.69	12874.0	543.43	50.62
AGL-IA	0.184	736.0	4000.0	0.39
AGL-IB	0.21	42.0	200.0	0.45
Fraction III	3.0	5.6	1.87	6.4
AGL-III	0.45	90.0	200.0	0.96
Fraction IV	0.595	28.0	47.0	1.27
AGL-IV	0.085	17.0	200.0	0.18

[a] Data shown were mean of five experiments.
[b] HU means the minimum amount of protein (μg/ml) showing visible hemagglutination of rabbit erythrocytes after 30 min of incubation at room temperature.

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