Partial Characterization and purification of a midgut lectin of an orange blister beetle, *Mylabris pustulata* (Linn.)

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ABSTRACT

A natural agglutinin was partially purified from the midgut extract of Mylabris pustulata. Preliminary studies on the hemagglutination of tissue extracts-foregut, midgut, hindgut, fat body, testis and ovary of M. pustulata revealed the preferential agglutinating towards rabbit and rat erythrocytes. Studies on the physico-chemical characterization of the agglutinin revealed that it was sensitive to pH and maximum HA was in the range of 8.5 to 9.5. The agglutinin activity was temperature sensitive with maximum agglutination from 10^{o}C to 30^{o}C. Calcium chelator, EDTA reduced the HA activity of the agglutinin suggesting the role of intrinsic calcium in the agglutination. Hemagglutination inhibition analysis documented glycoprotein - lactoferrin and sugar- N-acetyl galactosamine as the effective inhibitors of the agglutinin. The agglutinin was purified by biospecific adsorption using formalinized rabbit erythrocytes. The lectin agglutinated rabbit erythrocytes with higher avidity like that of the agglutinin. On SDS-PAGE the lectin showed a band of 27 kDa confirming as a single lectin. Since the lectin is sialic acid specific it can be applied in cancer diagnosis and therapy.

Keywords: *Agglutinin, Lectin, Mylabris pustulata, midgut, glycoproteins, sugars*

1. Introduction

Immune response is the basic physiologic function of all living organisms and a series of defense mechanism has been evolved to protect cellular integrity, homeostasis and survival of the host [1]. Cellular immunity is induced by non-self motifs present on the surface of the pathogens which in turn are recognized by cell derived Pattern Recognition Receptors (PRR) with assorted binding specificity [2]. Insects have an innate immune system that includes a wide range of specific and nonspecific reactions triggered by the presence of foreign substances [3].

Agglutinins/ lectins are a family of proteins, mono/ polyvalent in nature that contain characteristic modules of carbohydrate-recognition domains (CRDs) and can bind and agglutinate to the carbohydrate moieties on the surface of erythrocytes, without altering the properties of the carbohydrates in a calcium-dependent manner. It plays an important role in animal immune response, and in insects, they are involved in opsonisation, nodule formation, agglutination, encapsulation, melanisation, and prophenol oxidase activation and maintaining gut micro biome homeostasis [4]. Lectins are involved in various biological functions such as

host defense, cell-cell interaction and folding of glycoproteins, detection of sugar chains in biochemical and histochemical investigations [5] and discrimination of malignant cells from non-malignant cells [6]. The binding of lectins to sugar moieties in cell walls or membranes can alter the physiology of the membrane, resulting in agglutination, mitosis, or other biochemical changes in the cell [7]. Lectins demonstrate antifungal, immunomodulatory, anti HIV and anti-insect properties [8] suggesting lectins as potential biomolecules for the diagnosis of the disease and targeted therapy. Binding of lectins to nanoparticles helps in targeted therapy thus finding immense applications in biomedical fields [9].

Class Insecta has been investigated for agglutinins and among Coleopterans *Allomyrina dichotoma* [10], *Leptinotarsa decemilineata* [11], *Mylabris indica* [12], *Phyllophaga* sp. [13] and *Odoiporus longicollis* [14] have been screened for agglutinins. Antibacterial and anticancerous properties of lectin was documented in the hide beetle, *Dermestes frischii* [15]. Hence, the present study was carried out to identify and characterize an agglutinin and purify a lectin from the midgut of a coleopteran *Mylabris pustulata*.

2. Materials and Methods

2.1 Experimental Animal collection

The orange blister beetle, *Mylabris pustulata* (Plate 1), a pest of leguminous plant was collected from the cotton fields and the places rich in *Ipomoea cornea* plants in and around Kanyakumari District, Tamilnadu, India.

Plate 1: Orange blister beetle, *Mylabris pustulata*

2.2 Preparation of tissue extract

Healthy orange blister beetles were dissected and the tissues were cleansed well in 0.7% saline to remove the haemolymph. The tissue extracts were prepared by homogenizing 100 mg of foregut, midgut, hindgut, fat body, testis and ovary in 1 ml of cold TBS (Tris Buffered Saline: Tris HCl 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM, pH 7.5) using a homogenizer. The

extracts were centrifuged at 4000 rpm for 10 minutes at 30°C and the supernatant was assessed for hemagglutination activity.

2.3 Erythrocyte Preparation

Rat, rabbit, dog, Human, A, B, O, buffalo, cow and goat erythrocytes were prepared following the standard method of Ravindranath and Paulson [16].

2.4 Hemagglutination (HA) Assay

Hemagglutination assay were performed in 96 well, 'U` bottomed microtiter plates (Tarson) as described by Ravindranath and Paulson [16].

2.5 Physico-chemical characterization

pH and thermal stability: pH and temperature dependence of agglutinin was measured by pre-incubating the midgut extract at specific pH (5 - 10) and temperature (10ºC - 60°C) for 1 hour before adding erythrocyte suspension for hemagglutination assay.

Cations and EDTA treatment: To study cations (Ca^{2+} and Mg^{2+}) dependence on hemagglutination, HA assays were performed in TBS (pH 7.5) with and without these ions at varying concentrations (0 to 100 mM). To study the effect of calcium chelators (EDTA) on the agglutinin, the midgut extract was pre-incubated at different concentrations (0.01 to 50 mM) of EDTA and trisodium citrate for 1 hour before adding erythrocyte suspension for HA assay.

Hemagglutination inhibition (HAI) Assay

Hemagglutination inhibition assay were performed in 96 well, 'U` bottomed microtiter plates (Tarson) as described by Ravindranath and Paulson [16].

Purification of *Mylabris pustulata* lectin

Purification of lectin by biospecific adsorption using formalinized rabbit erythrocytes were prepared as described by Nowak and Barondes [17] with slight modification.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide 12.5% slab gel electrophoresis was performed according to Laemmli [18].

3. Results

3.1 Hemagglutination assay (HA)

Hemagglutinins were detected in the tissue extracts of foregut, midgut, hindgut, fat body, testis and ovary of the orange blister beetle *Mylabris pustulata*. Maximum HA was witnessed in the midgut extract against rabbit erythrocytes. The tissues of *M. pustulata* agglutinated rabbit and rat erythrocytes but failed to agglutinate human, buffalo, cow, goat and dog except for midgut which agglutinated cow and dog erythrocytes (Table 1).

Table 1: Survey of hemagglutinins in various tissue extracts of *Mylabris pustulata*

Tissue extracts n = 5	Hemagglutination titre with erythrocytes						
	Rabbit	Human A, B, O	Rat	Buffalo	Cow	Goat	Dog
Foregut	32	0	16	0	0	0	0
Midgut	64	2	16	0	2	0	4
Hindgut	32	0	4	0	0	0	0
Fat body	8	2-4	2	0	0	0	0
Testis	4	0	2	0	0	0	0
Ovary	2	0	2	0	0	0	0

n = number of *Mylabris pustulata* tested

3.2 Effect of pH and temperature

The HA of the extracts of midgut was sensitive to pH. The HA activity was greater at an alkaline pH ranging from 8.5 to 9.5 while acidic pH decreased the HA activity. The HA titer of the extracts of the midgut in *Mylabris pustulata* was also sensitive to change in temperature. Maximum agglutinability was observed at 10°C - 30°C and a reduction in HA was observed at high temperature (Table 2).

Table 2: Hemagglutination titer of midgut extract of *Mylabris pustulata* in relation to pH and temperature

pH	HA titer	Temperature (°C) At pH-8	HA titer
5.0	32	10	128
5.5	32	20	128
6.0	32	30	128
6.5	64	40	64
7.0	64	50	32
7.5	64	60	16
8.0	128	70	16
8.5	128	80	8
9.0	128		
9.5	64		
10	64		

3.3 Effect of cations and calcium chelators

Addition of cations at varying concentration does not have much impact on the HA titer of the midgut extract of *M. pustulata*, Higher concentration of magnesium slightly reduced the HA titer. Addition of calcium chelators Di and tetra sodium EDTA reduced the HA activity at 0.1mM concentration (Table 3).

Table 3: Impact of cations on the hemagglutination titer of the midgut extract of *Mylabris pustulata* at pH 8

Concentration (mM)	Calcium	Magnesium	EDTA	
			Di Sodium	Tetra sodium
0.0	128	128	128	128
0.1	128	128	64	64
1	128	64	64	32
10	128	64	64	32
20	64	32	32	32
30	32	32	32	32
40	32	32	32	32
50	32	32	32	32

3.4 Hemagglutination inhibition test

Sugar specificity of the midgut extract of *Mylabris pustulata* was examined by using various glycoproteins and sugars. Of the various glycoproteins tested, lactoferrin served as the potent inhibitor. Fetuin, porcine thyroglobulin and BSM (bovine submaxillary mucin) showed weak inhibitory potency. PSM (porcine stomach mucin), transferrin, apo transferrin and α-acid glycoprotein lacked inhibitory potency. The HAI of the glycoproteins can be ranked as follows lactoferrin > fetuin > porcine thyroglobulin > BSM. Of the sugars tested N-acetyl galactosamine exhibited inhibition, and other sugars such as, galactosamine and lactose showed very weak inhibitory potency (Table 4).

Table 4: Hemagglutination inhibition of the midgut extract of *Mylabris pustulata* by glycoproteins and sugars

Inhibitors		HAI	Minimum conc. Required (µg/ml)	Relative inhibitory potency (%)
Glycoprotein **(µg/ml)**	Lactoferrin	32	3.12	50
	Fetuin	16	6.25	25
	Porcine thyroglobulin	16	6.25	25
	BSM	4	25	6.25
Sugars **(mM)**	N-acetyl galactosamine	16	312.5	124.8
	Galactosamine	8	625	62.4
	Lactose	8	625	62.4

Glycoproteins PSM, Transferrin, α-acid glycoprotein, Bovine thyroglobulin and sugars

3.5 Characteristics of the purified lectin

The agglutinin purified by biospecific adsorption with formalinised rabbit erythrocytes showed increase in the specific activity (Table 5). Further the purified fraction recognized rabbit erythrocytes with high efficiency similar to that of the crude extract (Table

6). On SDS PAGE the lectin displayed a single band of 27 kDa confirming the homogeneity of lectin (Plate 2).

Table 5: Purification of midgut lectin from *Mylabris pustulata*

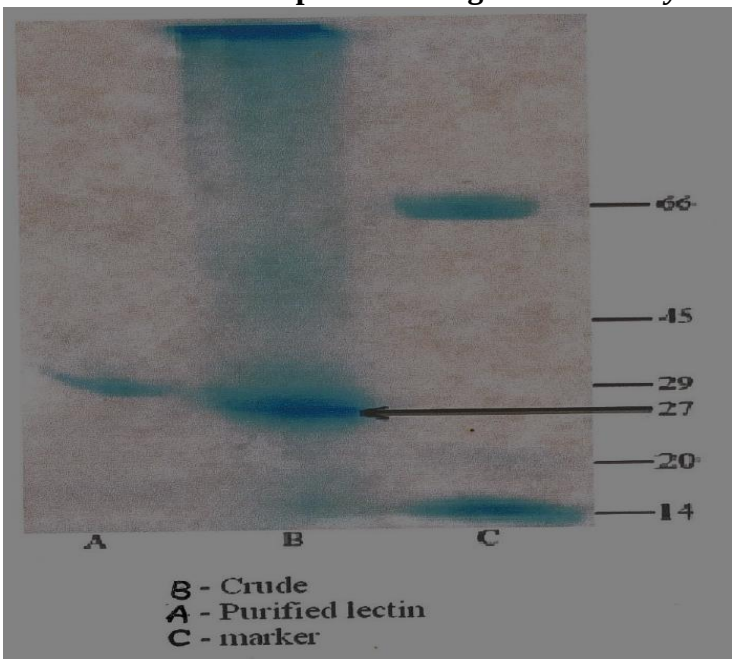
Sample	Volume (ml)	Protein (mg)	Total activity (HA units)	Specific activity (HA units/mg)
Crude extract	10	107.5	25600	238.13
Purified using formalinized rabbit RBC	1	27.3	10240	375.09

Table 6: HA titer of the purified lectin from the midgut extract of *Mylabris pustulata*

Erythrocytes	Sialic acid	HA titer
Rabbit	9-O-AcSia/NeuGc	64
Rat	NeuGc/ NeuAc/O-AcSia	32

Cow, goat and buffalo failed to agglutinate the tested erythrocytes.

Plate 2: SDS-PAGE of the purified midgut lectin of *Mylabris pustulata*



4. Discussion

Blister beetles are phytophagous in nature and feed on flowers of many crops such as red gram, hibiscus, peas, beans, etc. resulting in loss of economic products [19]. Broad studies have shown that lectins from plants and animals can be used for the treatment of cancer and against microbes. An attempt was made to identify a lectin from *Mylabris pustulata* that might have significant biomedical importance.

A careful search of agglutinins in tissues led to a serendipitous discovery of an organ rich in agglutinins. The tissues tested for HA activity are foregut, midgut, hindgut, fat body, testis and ovary of *M. pustulata*. Of all the tissue tested, maximum HA was noted in the midgut extract towards rabbit and rat erythrocytes but failed to agglutinate other mammalian erythrocytes. High HA titer with rabbit erythrocyte, suggests that the receptor determinants recognized by the midgut agglutinin are more accessible on rabbit RBC when compared to other mammalian erythrocyte tested. Lectins have the intrinsic capacity to bind to specific sugars NeuAc, 9-O-acetyl NeuAc, NeuGc and 9-O-acetyl NeuGc that are expressed on the glycocalyx of rabbit erythrocytes [20] [21]. Affinity towards rabbit erythrocytes were observed in *Oxya hyla hyla* [22] and *Phyllophaga* sp. [13]

Mylabris pustulata lectin was stable at pH ranging from 8.5 to 9.5, while acidic pH reduced the HA activity. It has been observed that acidic and neutral pH reduced the HA activity when compared to alkaline pH. Sensitivity of insect lectins to pH have also been demonstrated in *Musca domestica* which was stable from pH 4 to 8 [23]. Study on pH helps to understand the structure of the lectin because when lectins are subjected to different pH there is a structural transition from monomeric to dimeric or tetrameric forms [24]. This in turn increases the specificity of lectin thus suggesting its potential in medical application. Maximum agglutinability of *Mylabris pustulata* agglutinin was observed at temperature from 10-30°C and decrease in HA titer at higher temperatures suggesting the role of temperature in protein denaturation. Temperature intolerance of lectin was also observed in *M. domestica* lectins which was stable at temperatures upto 65°C [23], Orthopteran *T. commodus* [25], dipteran *Glossina fuscipes* [26]. *Mylabris indica* hemolymph lectin was unaffected at a temperature ranging from 5-100°C [12]. *Mylabris pustulata* agglutinin was unique in that its agglutinability was unaffected by metal ions, calcium chloride and magnesium chloride and moderately affected by chelators EDTA as reported in *M. sexta* [27] and *Mylabris indica* [12]. However, in *Mylabris indica* calcium ions at 0.01mM concentration enhanced the HA titer [12] which is similar to our finding. Sensitivity of the lectin to pH is correlated to the amount of calcium and it has been reported that low amount of calcium results in higher sensitivity [28].

Lectin carbohydrate interaction occurs because of the covalent bonds, where the water molecules are displaced around the polar groups of the protein and carbohydrate portion of the protein. This modification forms hydrogen bonding and van der waals force thus stabilizing the interaction [29]. The HA activity with rabbit RBC was inhibited by the glycoproteins lactoferrin, fetuin, porcine thyroglobulin, BSM and sugars N-acetyl galactosamine and lactose. The sialic acid residue of lactoferrin is NeuAc [30] and that of fetuin is NeuGc [31]. Inhibition

by lactoferrin containing NeuAc and fetuin containing NeuGc, agglutination with rabbit erythrocytes containing NeuGc and rat erythrocytes containing Neu Ge/ Neu Ac/ O-Ac-Sia suggest its affinity to sialic acid. The ability of the agglutinin to agglutinate rabbit and rat erythrocytes and the ability of the sialic containing glycoprotein, lactoferrin to inhibit agglutination with great avidity argues for the sialic acid specificity of the lectin. Similar results are reported in the hemolymph and midgut gland of a millipede *Thyropygus descriptus* [32], centipede *Rhysida nuda nuda* [33], Red Palm Weevil, *Rhynchophorus ferrugineus* [34].

The hemagglutinin purified showed an increase in specificity with increase in HA titer. As the HA specificity remains unaltered the agglutinin can be called as lectin. On SDS Page the lectin showed a single band of molecular weight of 27 kDa suggesting it as a single lectin as reported with a 30 KDa lectin of *P. americana* [35] and 20 kDa lectin of *S. peregrina* [36]. Thus, lectins which are immune molecules unique to the insect immune system can be tapped as potential biomolecule for disease diagnosis and treatment.

5. Conclusion

The findings of the study clearly illustrates that the sialic acid specific lectin purified from *Mylabris pustulata* would be of immense value in discriminating cancer cells and pathogenic microbes. The lectin can be used in targeted drug delivery and treatment of malignant cells.

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