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Lactose-Specific Hemagglutinin with *In vitro* Opsonic Effect from the Hemolymph of *Ariadne merione* (Cramer)

Prabhu Sivaprasath[1], Madanagopal Nalini[2]

[1]Department of Biotechnology, Karpagam University, Coimbatore 646 021, Tamil Nadu, India

Abstract: ***The present study was designed to detect the presence of Hemagglutinin (HA) molecule, which recognizes and responds to foreign bodies, in the serum of Ariadne merione. On screening the natural hemagglutinating (HA) activity using various mammalian erythrocytes as indicator cells, highest hemagglutination titer was observed with rat erythrocytes. In cross adsorption tests, this RBC type was able to completely adsorb all the HA activity in serum. Further analysis of the physico-chemical properties revealed that this HA is dependent on Ca^{2+} for its activity, irreversibly sensitive to EDTA, stable only at pH 7 and thermo-labile. Its proteinaceous nature was confirmed by its susceptibility to trypsin and β-mercaptoethanol. HA-inhibition assays with mono-, di-, tri- and polysaccharides revealed that the serum HA is specific for lactose with non-reducing terminal galactose possessing β 1→4 glycosidic linkage. Subsequent role of this HA in mediating cellular immune response revealed that phagocytotic index was enhanced by 6-fold in vitro indicating the opsonic effect of the agglutinin.***

Keywords: *Ariadne merione*, hemagglutinin, lactose, phagocytosis, Opsonin

1. Introduction

Existing in a myriad of environment, insects are susceptible to infection by micro organisms and parasites but they lack adaptive immune system and depend solely on innate immunity [1]. The innate immune response involves variety of humoral molecules namely agglutinins, lysins, antibacterial and antifungal proteins, phenoloxidase system and LPS-, β, 1-3 glucan binding proteins. Due to their functional similarity to that of antibodies in vertebrates, agglutinin in invertebrates are known to mediate defensive function by recognition of non-self and mediate phagocytosis which is the primary mechanism of defense against foreign invaders.

Carbohydrates, structural materials and energy sources, are known to play important role in cellular communication [2], [3], [4]. Agglutinins (=antibodies/lectins in vertebrates) are glycoproteins that can recognize whole sugar, a specific site in a sugar, a sequence of sugars or their glycosidic linkages on the surface of the cells and agglutinate. These agglutinin molecules are ubiquitously distributed in microorganisms, plants, animals [3].

In invertebrates agglutinins are mostly detected in body fluid/ hemolymph and at various levels in other tissues. The hemolymph derived agglutinins are also known to serve as opsonin in mediating/facilitating cellular immune functions like phagocytosis [5], [6], [7], [8]. Phagocytic cells recognize foreign particles by the cell surface receptors and thus further signal the phagocytic cell to ingest the non-self [9].

The current study was aimed for detection of agglutinin like molecule(s) in hemolymph of *Ariadne merione*. Though larvae feed on *Ricinus communis* which is known to possess "ricin", a highly toxic hemagglutinin [10], they are found to be highly resistant to it. Therefore, it was focused to detect and characterize the possible presence of agglutinin-like molecules and its role in cellular immune function in this insect model.

2. Materials and Methods

2.1 *Ariadne merione*

Larvae were collected from the castor plants, *R. communis* distributed around Kurichi Lake, Coimbatore, India (Latitude 10.9600° North, Longitude 77.0100° East) and maintained till fourth instar under laboratory conditions (25±2ºC) in specially modified plastic boxes which provides good ambiance for survival. The bed was changed every day and the larvae were fed *ad libitum* with fresh leaves of *R. communis.*

2.2 Collection of hemolymph and serum preparation

Larvae were surface sterilized by holding gently in between the index finger and thumb. Using fine sterile scissors, the first proleg was cut and the oozing out hemolymph was collected in Eppendrof tube held on ice. The collected hemolymph was centrifuged (400 x *g*, 10 min, 4ºC) and the clear supernatant (=serum) was used for further experiments.

2.3 Reagents

Carbohydrates were purchased from Sigma (St. Louis, MO, USA) and proteases from HiMedia (Mumbai, India). Four types of Tris-buffered saline (TBS) were used in this study: (1) TBS-I: 50 mM Tris-HCl, 120 mM NaCl (300 mOsm), (2) TBS-II: 50 mM Tris-HCl, 100 mM NaCl, 10 mM $CaCl_2$ (300 mOsm), (3) TBS-III: 50 mM Tris-HCl, 5 mM NaCl, 30 mM $CaCl_2$ (135 mOsm), (4) TBS-IV: 50 mM Tris-HCl, 80 mM NaCl, 50 mM EDTA (300 mOsm) and (5) TBS-V: 50 mM Tris-HCl, 120 mM NaCl (300 mOsm). pH of first four buffers were adjusted to 7.2 and the fifth buffer 6.8.

Anticoagulant buffer: cysteine-HCl (5 mg) was dissolved in TBS-I to give a final concentration of 5 mM. The pH was adjusted to 7.2 using 0.1 N NaOH. Saline (0.9 %) with 0.02 % sodium azide (NaN_3) was used for washing erythrocytes.

2.4 Preparation of erythrocytes (RBC) suspension

Human (A, B, O), goat, sheep and rat blood samples were collected in sterile Alsever's solution [11] containing 10 µg/ml streptomycin. Prior to use, the RBC were washed thrice using 0.9 % saline and once with TBS-II by centrifugation (400 x *g*, 5 min, RT). The RBC pellet was re-suspended in TBS-II as 1.5 % suspension (v/v).

2.5 Hemagglutination (HA) and Cross-adsorption assay

HA assays were performed in V-bottomed microtiter plates (Tarsons, India) by serial two-fold dilution of 25 µl of serum sample with equal volume of TBS-II. After dilution, 25 µl of 1.5 % RBC suspension was added to each well and incubated for 45 min at RT. The HA titer was recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of RBC [11]. Cross-adsorption assay was performed by addition of 250 µl serum sample with an equal volume of freshly washed rat RBC pellet and incubated for 1 h at RT with periodical gentle mixing. The suspension was centrifuged (400 x *g*, 5 min, RT) and supernatant was collected and adsorbed for a second and third time under the same conditions. The serum adsorbed thrice was subsequently tested for HA activity against the same as well as other RBC types (Human A, B, O, goat & sheep).

2.6 Cation dependency and EDTA sensitivity

Serum samples were dialyzed (MW exclusion limit <10,000) extensively against TBS-I to test the cation dependency and TBS-IV to examine the EDTA sensitivity. The serum sample dialyzed against TBS-IV was subsequently re-equilibrated by dialysis against TBS-I. The resulting dialysates were centrifuged and the HA activity was determined using rat RBC in the presence of $CaCl_2$, $MgCl_2$ or $MnCl_2$. A serum sample, concurrently dialyzed against TBS-II, was also tested for its HA activity against rat RBC suspended in the same buffer.

2.7 Thermal and pH stability

Thermal stability of the hemagglutinin molecule was determined by holding 100 µl of serum aliquots at different temperatures from 10 to 100ºC for 30 min. The samples were centrifuged (500 x *g*, 5 min, 4ºC) and the supernatants were tested for HA activity using rat RBC. In another experiment, the pH stability was assessed by dialyzing 100 µl of serum aliquots against different buffers at pH ranging from 3 to 12 [12], [13]. 0.2 M acetate buffer (pH 3 to 6), tris-HCl buffer (pH 7 to 9) and 0.1 M glycine-NaOH buffer (pH 10 to12) 12 h at 4ºC. After dialysis, the samples were finally re-equilibrated by dialysis against TBS-II then, the samples were centrifuged (500 x *g*, 5 min, 4ºC) and the residual HA activity was determined using rat RBC.

2.9 HA-inhibition assay

The carbohydrates were tested for their ability to inhibit serum HA activity. For this, the carbohydrates were dissolved in TBS-III. The activity of serum was adjusted to a titer of 4 by diluting the serum using TBS-II. The carbohydrates to be tested were serially diluted with equal volume (25 µl) of serum (HA= 4) in microtiter plates and incubated for 1 h at RT. Finally, 25 µl of 1.5 % rat RBC suspension was added to all the wells, incubated for 1 h at RT and the inhibitory concentration of carbohydrates were determined.

2.8 Trypsin digestion and β-mercaptoethanol (β-ME) effect

Trypsin (Type III from bovine pancreas) was prepared by dissolving 12 mg in 1 ml of TBS-II. The serum was mixed with equal volume of trypsin to a final concentration of 3 mg. ml^{-1} and incubated at 37ºC for 3 h. Controls consisted of serum samples alone and serum samples with heat-inactivated trypsin (100ºC, 10 min). The HA activity of serum samples were checked immediately or after 3 h using rat RBC. The effect of β-ME, a reducing agent was tested for its effect on HA activity. For this, the serum was added with an equal volume of β-ME to give a final concentration of 0.2 M. Then the samples were incubated for 1, 2, 3 and 4 h consecutively at 37ºC, and HA activity was checked at every hour. The remaining samples were dialyzed for 12 h in TBS-II for removal of β-ME, centrifuged (500 x *g*, 10 min, 4ºC) and checked for HA activity using rat RBC.

2.10 *In vitro phagocytosis* assay

By cutting the first proleg of the larvae, hemolymph (25~µl) was collected in 300 µl of anticoagulant buffer in polypropylene tube held on ice. The tube was gently shaken to prevent hemocytes aggregation during hemolymph collection. Five monolayers (50 µl) were made on glass slides and kept in moist chamber for 15 min at 25°C. Then the monolayers were gently washed with TBS-V for rounding up. The first monolayer was overlaid with 50 µl of glutaraldehyde fixed rat RBC suspension (0.5 % v/v) and subsequently the second, third, fourth and fifth monolayers were overlaid with serum, plasma, hemocyte lysate supernatant (HLS) & hemocyte conditioned medium (HCM) pre-treated rat RBC respectively. All the monolayers were left in moist chamber for 30 min at 25°C. Then the monolayers were washed with TBS-I for removal of non-phagocytosed erythrocytes, fixed with 1% glutaraldehyde and observed under phase-contrast microscope (Nikon, Japan) at 20x. The phagocytosed erythrocytes were distinguished from non-phagocytosed by the disappearance of phase brightness and the outer rim not clearly visible. A total of 350-400 hemocytes were checked for engulfment of erythrocytes and scored for percent phagocytosis.

3. Results

3.1 HA and cross-adsorption

The natural hemagglutinin molecule from the hemolymph of larvae of *A. merione* was detected by erythrocyte-binding activity. The agglutinin molecule showed varied hemagglutination (HA) titer with all the mammalian RBC types tested (Table 3.1.1). The highest HA titer of 32 was indicated with rat RBC, hence cross-adsorption test was performed using this sample. On adsorption of serum with rat RBC thrice, it completely removed the HA activity of all the RBC types tested (Table 3.1.2). Therefore, rat RBC was used as suitable indicator cells for further studies.

Table 3.1.1: Hemagglutinating (HA) activity of serum of *Ariadne merione* against various mammalian erythrocytes (RBC).

RBC types tested	HA titer†
Human A	16
Human B	8
Human O	16
Goat	2
Sheep	2
Rat	32

† Values are based on six determinations

Table 3.1.2: Cross adsorption of serum hemagglutinin (HA) of *Ariadne merione* with rat RBC.

Serum adsorbed with	HA titer against RBC types tested†					
	HA	HB	HO	Goat	Sheep	Rat
None	16	8	16	2	2	32
Rat	0	0	0	0	0	0

† Values are based on six determinations

3.2 Effect of cations

The serum samples dialyzed against divalent cation-free TBS (TBS-I) and the HA activity tested in the absence of cations, resulted in complete loss of the activity. But on addition of Ca^{2+} the samples regained its activity, whereas Mg^{2+} and Mn^{2+} were not effective on HA activity regaining. Dialyzing the sample against TBS containing Ca^{2+}, the activity was unaffected. Serum samples dialyzed against TBS containing 50 mM EDTA and tested in absence of cations, the activity was completely lost and it did not resume in the presence of any of the cations tested (Table 3.2.1).

Table 3.2.1: Effect of divalent cations and EDTA on the hemagglutinating (HA) activity of serum of *Ariadne merione*.

Treatment of serum	Cation in sample diluting medium and RBC suspension	HA titer†
Untreated serum (before dialysis)	$CaCl_2$	32
Serum dialyzed against cation-free TBS (Control)	None	0
	$CaCl_2$	32
	$MgCl_2$	0
	$MnCl_2$	4
Serum dialyzed against TBS+10 mM $CaCl_2$	$CaCl_2$	32
Serum dialyzed against TBS +50 mM EDTA followed by dialysis against cation-free TBS	None	0
	$CaCl_2$	16
	$MgCl_2$	0
	$MnCl_2$	0

† Values are based on six determinations

3.3 Thermal stability and pH

The HA activity was stable between 10 and 30°C, it gradually decreased on increase in temperature and was completely lost at 60°C and above. The activity remained stable only at neutral pH 7 and decreased gradually on increase in acidity and alkalinity

3.4 Carbohydrate binding specificity

Among the 13 carbohydrates tested for inhibition, 4 inhibited the serum HA activity of *A. merione* against rat RBC (Table 3.5.1). HA activity was inhibited by simple hexoses (glucose and galactose). Among the disaccharides tested, maltose and lactose with 1→4 linkage bearing glucose or galactose in the non-reducing terminal inhibited unless otherwise stated the activity.

Table 3.4.1 Effect of carbohydrates on serum hemagglutinating (HA) activity of *Ariadne merione*. The starting concentration of each carbohydrate was 200 mM

Carbohydrate tested	Minimum inhibitory concentration (mM)†
MONOSACCHARIDES	
Pentose	
L-Arabinose	
Hexoses	-
D-Fructose	
D-Glucose	-
D-Galactose	**100**
D-Mannose	**100±50**
	-
Deoxy sugar	
2-Deoxy-D-ribose	
	-
Acetylated sugar	
N-acetyl-D-glucosamine (GlcNAc)	
	-
DISACCHARIDES	
Trehalose (glc α 1→1 glc)	
Sucrose (glc α 1→2 glc)	-
Maltose (glc α 1→4 glc)	-
Lactose (gal β 1→4 glc)	**100**
	50
TRISACCHARIDE	-
Raffinose (gal α 1→6 glc α 1→2 fruc)	
POLYSACCHARIDE	-
Laminarin (β 1→3, homopolymer of glucose 0.1%)	

† Values are based on six determinations

3.5 Susceptibility to trypsin and β-ME

Incubation of serum samples with trypsin immediately reduced the HA titer from 32 to 4 and it further decreased to 2 on incubation for 3 h at 37°C. This reduction in activity was attributed by the enzymatic digestion of HA molecules as the heat-inactivated trypsin did not affect the HA activity (data not shown). Treatment of serum with 0.2M β-ME reduced the activity and it did not change after removal of β-ME by dialysis (data not shown).

3.6 Effect of HA on phagocytosis

Cysteine (5 mM) used in this study, enabled the isolation of intact hemocytes with behavioral and functional responses in *A. merione* by preventing plasma gelation and hemocytes aggregation. More than 95% of these intact hemocytes were viable up to 3 h by trypan blue dye-exclusion test. These hemocytes were functionally active, that 5% of them phagocytosed rat RBC (Figure. 1). The phagocytotic activity by hemocytes was significantly (***P*<0.005; **P*<0.05) enhanced to 29, 15, 39 and 29 % on exposure to serum-, plasma-, Hemocyte lysate supernatant- (HLS), or Hemocyte conditioned medium- (HCM) treated rat RBC respectively (Figure. 2).

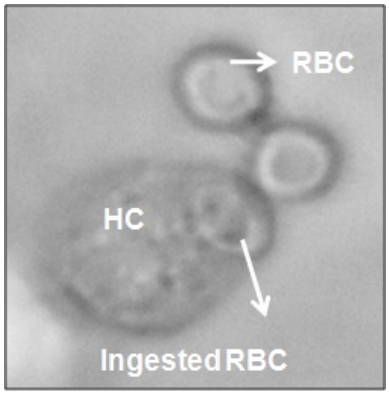

Figure. 1 Phase contrast micrograph of hemocyte (HC) of *A. merione* showing phagocytosis of rat erythrocytes (RBC).

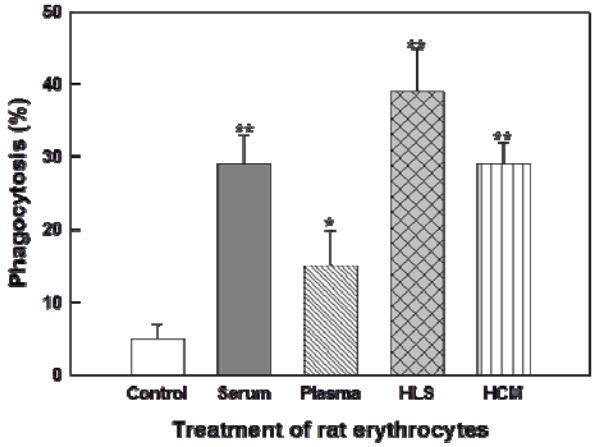

Figure. 2 *In vitro* phagocytic activity of *A. merione* hemocytes. Vertical bars represent mean (±SD) of phagocytotic rates of six independent determinations. The difference in phagocytosis rates between the control and experimental were stastically significant (* $P < 0.05$, ** $P < 0.005$).

4. Discussion

This study demonstrates the presence of naturally occurring hemagglutinin molecules in the serum of the butterfly, common castor, A. *merione*. The HA activity was performed using various mammalian erythrocytes (Human A, B, O, goat, sheep, rat), the highest activity was recorded with rat RBC. Cross-adsorption assays showed that rat RBC completely adsorbed all the HA molecules from the serum, indicating high affinity towards rat RBC. In addition, these results also suggest that the HA binding sites on different RBCs may differ in their quantity.

The HA activity was completely lost in the absence of Ca^{2+} and it completely regained on addition of the same, which is evident by the serum sample that did not lose its HA activity on dialysis against TBS containing Ca^{2+}. C-type lectins are distributed in most biological systems which are known to be dependent on divalent cations, usually Ca^{2+} and are reversible or irreversibly sensitive divalent cation chelators like EDTA or EGTA [14], [15]. Similarly in the present study the serum sample was Ca^{2+} dependent and on dialysis against EDTA, it completely lost its HA activity. This activity did not restore upon addition of any of the cations tested. These results indicate that the HA molecule of *A.*

merione could be a C-type lectin, as observed in *Extatosoma tiaratum* and *Plutella xylostella* [16], [17].

The HA activity in serum samples was destroyed at or above 40°C and the extreme pH above or less than 7 did not facilitate this activity as observed with *P. xylostella* [17]. Additionally, this activity was completely precipitable using ammonium sulphate (at 75% saturation) and appears to possess disulphide bonds which plays significant role in HA activity, wherein the activity was drastically reduced on treating with β-ME as observed in other arthropods [18]. Incubated with trypsin resulted in the reduction of activity and complete loss within 3 hours. All these observations clearly indicate that the serum agglutinin molecule is proteinaceous in nature.

Serum HA activity in arthropods were shown to be specific for galactose and galactose derivatives [19], [20], [21], methyl-α-D-mannopyranoside [17], *N*-acetyllactosamine [23], sucrose [18]; [12] and sulphated polysaccharides [24]. Among the inhibitory sugars, lactose (gal β 1→4 glc) potentially inhibited the HA activity in the serum of *A. merione* suggesting that the HA is specific for lactose. On the other hand, maltose (glc α 1→4 glc) was not as potent as lactose and raffinose possessing galactose at terminal end was not inhibitory, suggesting that galactose at C-1 position in β anomeric form is essential for interaction with the agglutinin molecule. Further, the presence of glucose at subterminal position with α 1→4 glycosidic linkage supports the strength of agglutination, since trehalose and sucrose with α 1→1 or α 1→2 glycosidic linkage were not inhibitory.

Attachment of foreign particle or non-self is a prerequisite for internalization and this process could be mediated by direct attachment of phagocytic cell on non-self or by adherence of humoral molecule on surface resulting in its recognition and thus facilitates phagocytosis. To investigate this possibility, use of serum-pretreated rat erythrocytes as targets which showed enhanced phagocytic response indicating the opsonic role of this hemagglutinin is mediating cellular immune responses which is in accordance to the observations by Wheeler *et al.* (1993) [8] and Rowley & Ratcliffe (1980) [5] in *Melanoplus differentialis*; *Clitumnus extradentatus* and *Periplanata americana* under *in vitro* conditions.

5. Conclusion

Thus, this study demonstrates the presence of a unique agglutinin specific for lactose with β 1→4 glycosidic linkage playing opsonic role in mediating cellular immune function. Further work needs to be undertaken for this novel agglutinin of *A. merione*.

6. Future Scope of this Study

Innate immune system in insects consists of both cellular and humoral molecules and functionally associated with each other during immune response. Further investigations are required to better understand the genomic functions of immune cascade. Future work will be concentrated on developing biocontrol agents for these types of agricultural pests.

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Author Profile

Prabhu Sivaprasath is a research scholar in Department of Biotechnology at Karpagam University. Ph.D research work focus on Invertebrate immunobiology and molecular physiology in the biocontrol aspect. Karpagam University, Eachanari (PO), Coimbatore-21, Tamilnadu, India.

Dr. M. Nalini Padmanabhan is working as Assistant professor at Department of Biotechnology, Karpagam University and guiding students in the field of Invertebrate immunobiology and molecular physiology.