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	100
D-Galactose	100±50
D-Mannose	-
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)	100
Lactose (gal β 1→4 glc)	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).

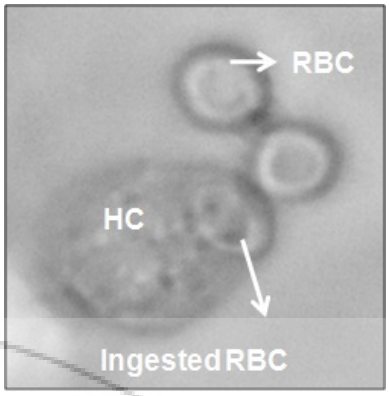

Figure. 1 Phase contrast micrograph of hemocyte (HC) of *A. merione* showing phagocytosis of rat erythrocytes (RBC).

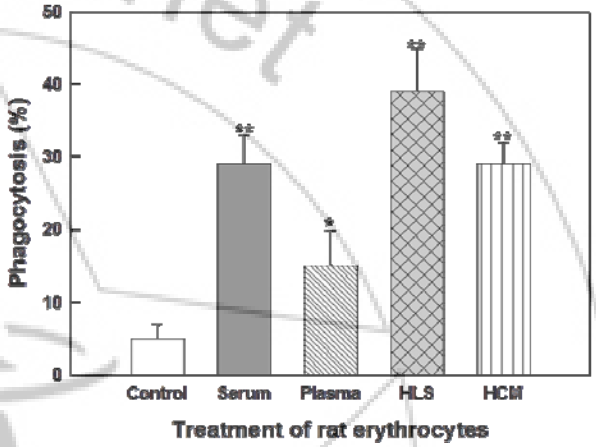

Figure. 2 *In vitro* phagocytic activity of *A. merione* hemocytes. Vertical bars represent mean (±SD) of phagocytotic rates of six independent determinations. The difference in phagocytosis rates between the control and experimental were stastically significant (* P< 0.05, ** P< 0.005).

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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