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Physico-Chemical Characterization of a Naturally Occurring Agglutinin in the Hemolymph of the Larva of Red Palm Weevil, *Rhynchophorus ferrugineus*

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Abstract

The hemolymph of most invertebrates contains naturally occurring hemagglutinins with diverse specificities for erythrocytes. A natural agglutinin with high affinity for rabbit erythrocytes was detected in the hemolymph of the larva of the red palm weevil, *Rhynchophorus ferrugineus.* The HA titer can be ranked as rabbit > guinea pig = dog = horse > human B > human O = buffalo > goat = pig > human A = rat = mice = cow erythrocytes. Maximum hemagglutinin activity with rabbit erythrocytes was observed at pH 7.5 and temperature 30˚C to 40˚C. The hemagglutinability was calcium dependent and reversibly sensitive to EDTA. The HA activity of the larval hemagglutinin was inhibited by the glycoproteins, Porcine stomach mucin = Fetuin > Bovine thyroglobulin > Transferrin = Apotransferrin > Bovine submaxillary mucin and sugars, N-acetyl mannosamine = melibiose = α-lactose > D-galactosamine > N-acetyl-D-galactosamine = N-acetyl neuraminic acid. Reduction in HA titer with the sialidase treated rabbit erythrocytes revealed the sialic acid specificity of the agglutinin. Presence of a single agglutinin and its proteinaceous nature were unveiled by cross adsorption studies and treatment with denaturing agents respectively. Therefore, the preliminary description of the hemolymph agglutinin would offer ways to perform lectin purification from *Rhynchophorus ferrugineus*, the red palm weevil.

Keywords: EDTA, erythrocytes, hemolymph, Rhynchophorus ferrugineus and sialic acid.

INTRODUCTION

An agglutinin with specificity for diverse species of mammalian erythrocytes is identified in the larval hemolymph of the red palm weevil, *Rhynchophorus ferrugineus* by hemagglutination (HA) assays. pH, temperature and ionic conditions are the most common factors that influence lectin activity. (Naeem et al. 2007). Most insect agglutinins are thermolabile inspite of the occurrence of heat stable molecules in some species (Minnick et al. 1986; Basil Rose et al. 2014). Factors including denaturing agents, variation of temperature and nature of medium affect the tertiary structure and henceforth hemagglutination activity of

lectins (Kabir et al. 2012). In this paper, an attempt has been made to investigate the physico-chemical characteristics and sugar specificity of the hemagglutinin in the hemolymph of the larva of the red palm weevil *Rhynchophorus ferrugineus* so as to develop strategies for the purification of this lectin.

MATERIALS AND METHODS

Animal collection and maintenance

The larvae of *Rhynchophorus ferrugineus* were collected from dead coconut trees found in the coconut groves of Azhagappapuram, Kanniyakumari District, Tamil Nadu, India. The collected larvae were maintained in plastic containers containing cut petiole or stem tissues from coconut tree as recommended by Rananavare et al. (1975).

Collection of hemolymph

The larvae of the red palm weevil were anaesthetized using chloroform vapour for 5 minutes. Hemolymph was collected by cutting off the posterior tip of the larva, after wiping with 70% ethanol. The exuded hemolymph was collected in prechilled centrifuge tubes with phenyl thiourea placed on ice. The collected hemolymph samples were centrifuged and stored at -20˚C for further analysis.

Erythrocyte collection and preparation

Mammalian erythrocytes for hemagglutination assays were collected from different sources: Human blood samples (A, B and O) were collected from Kanya Blood Bank, Nagercoil. Goat and Pig blood samples were collected from slaughter houses and blood of other animals were collected from the veterinary hospital by veinpuncture of the neck (Cow, Buffalo and Horse), ear (Rabbit), forearm (Dog), and heart puncture (Rat, Mice and Guinea pig).

Hemagglutination assay (HA)

Hemagglutination assays were performed in 96-well, 'U'- bottomed microtitre plates as described by Ravindranath and Paulson (1987). Hemolymph samples (25 µl) were serially diluted with 25 µl of TBS in microtiter plates and mixed with 25 µl of 1.5% erythrocyte suspension. The plates were incubated for one hour at room temperature and examined for hemagglutination. The hemagglutination titer or HA titer (the unit of agglutinin activity) was the reciprocal of the highest dilution of the sample that still agglutinated erythrocytes.

pH and thermal stability

To study the effect of pH on hemagglutination, 25 µl of the hemolymph was serially diluted with equal volume of Tris Buffered Saline (TBS) at varying pH (5-10) in microtiter plates and incubated at room temperature (30 ± 2˚C) for 1 hour and mixed with 25 µl of 1.5% suspension of rabbit erythrocytes and the HA titer was determined after 1 hour at room temperature (30˚C). To assess the thermal stability of the hemagglutinin, 300 µl of larval hemolymph was incubated in a water bath for 1 hour, at different temperatures ranging from 10˚C to 80˚C and the samples were tested for HA activity against rabbit erythrocytes.

Effect of divalent metal ions and EDTA

To study the effect of divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) and chelators on HA activity, 25 µl of the larval hemolymph was serially diluted with equal volume of Tris Buffered Saline (TBS) with different concentrations (0-50 mM) of divalent cations and chelators (EDTA and trisodium citrate). After incubation for 1 hour, the HA activity of each sample was studied against rabbit erythrocytes.

Hemagglutination inhibition (HAI) assay

Known concentration (sugar: 100 mM; glycoprotein: 5 mg/ml) of inhibitors (25 µl) were serially diluted with 25 µl of TBS in microtiter plates. Then 25 µl of hemolymph diluted to subagglutination concentration in TBS (to give a HA titer of 2) was added to each well and incubated for 1 hour. After incubation, 25 µl of 1.5% rabbit erythrocyte suspension was added, mixed and incubated. Hemagglutination inhibition titer was reported as the reciprocal of the highest dilution of inhibitor giving complete inhibition of agglutination after 1 hour.

Enzyme treatment of erythrocytes

Protease treatment

Following the procedure of Pereira et al. (1981), rabbit erythrocytes were washed five times with TBS (pH 7.5) by centrifugation at 4000 x g for 5 minutes at room temperature (30 ± 2˚C) and resuspended in the same buffer. Equal volume of 1 mg/ml proteases (trypsin, pepsin and neutral protease) in TBS (pH 7.5) was added to the washed erythrocytes, mixed and incubated at room temperature (30 ± 2˚C) for 1 hour. The enzyme treated erythrocytes were then washed five times in TBS and used for hemagglutination assay.

Neuraminidase treatment

Asialoerythrocytes were prepared following the method of Mercy and Ravindranath (1993). A reaction mixture (total 5 ml) containing 10% washed rabbit erythrocytes in PBS-BSA (pH 7.5) and 140 mU (milliunits) of neuraminidase of *Clostridium perfringens* (Type X: Sigma) was incubated at 37˚C for 4 hours. The treated cells were then washed with PBA-BSA three times and pelleted by low-speed centrifugation followed by the final wash in TBS-BSA (pH 7.5) and tested for HA activity.

Chemical stability

To assess the chemical stability of the agglutinin, the larval hemolymph was mixed with chloroform in the ratio 1:3 and incubated for 5 minutes. After discarding the precipitate, the supernatant was dialyzed against distilled water for 24 hours at 4˚C and then assayed for HA with rabbit erythrocytes. To analyse the effect of denaturing agents, 0.1 ml aliquot of larval hemolymph was mixed with 0.1 ml of denaturing agents, HCl (0.1 N) and NaOH (0.01 N) and allowed to react for two hours at room temperature (30 ± 2˚C) and then checked for hemagglutination activity.

Cross adsorption assay

The cross adsorption assays were performed following the method of Hall and Rowlands (1974) and Mercy and Ravindranath (1992). Hemolymph sample (1 ml) was mixed with an equal volume of washed and packed human B, O, horse, dog, pig, rabbit, goat, guinea pig and buffalo erythrocytes. This erythrocyte-hemolymph mixture was incubated at 10˚C overnight (18 hours) with gentle occasional shaking. After centrifugation, the supernatant was tested against the selected erythrocytes for hemagglutination assay. The supernatant that gave hemagglutination activity was readsorbed with equal volume of their respective washed and packed erythrocytes till it showed no agglutination activity/no change in agglutinability with the tested erythrocytes.

RESULTS

HA assay

The larval hemolymph of *Rhynchophorus ferrugineus* agglutinated a wide variety of mammalian erythrocytes. The agglutination profile of the larval hemolymph of *Rhynchophorus ferrugineus* was rabbit > guinea pig = dog = horse > human B > O = buffalo > goat = pig > human A = rat = mice = cow (Figure 1).

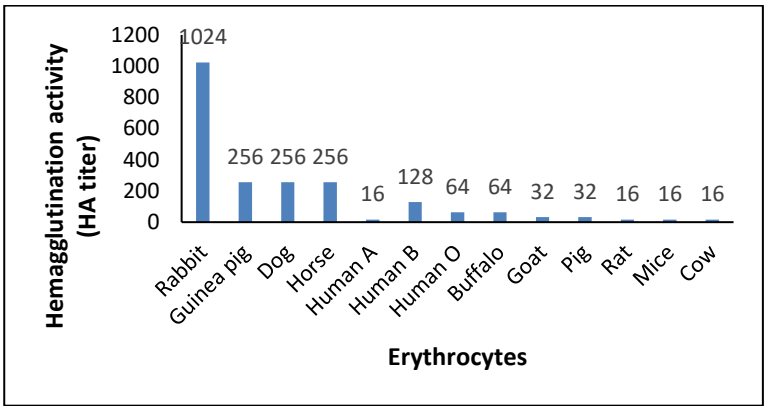

Figure 1. Hemagglutination titer of the hemolymph agglutinin of the larva of the red palm weevil, *Rhynchophorus ferrugineus* against different mammalian erythrocytes.

pH and thermal stability of the hemagglutinin

Optimum hemagglutination of the hemolymph agglutinin was observed at pH 7.5. A gradual decline in HA titer was observed consistently towards increase in alkalinity (Figure 2).

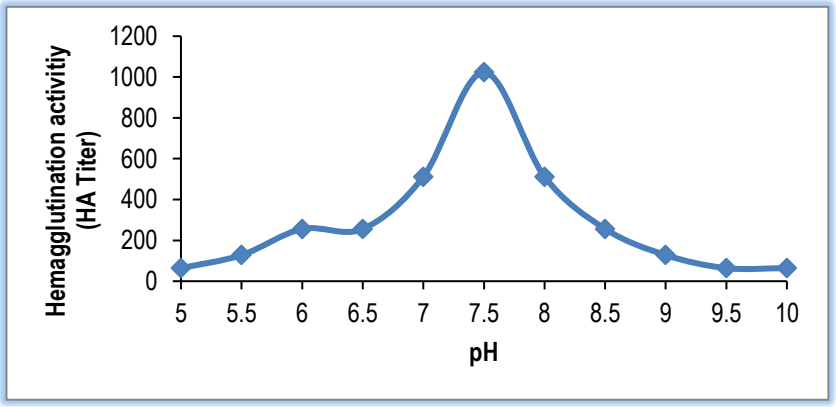

Figure 2. Hemagglutination titer of the hemolymph agglutinin of the larva of the red palm weevil, *Rhynchophorus ferrugineus* in relation to pH

Temperature changes affected the hemagglutination titer of the larval hemolymph. HA titer was very low (HA titer = 16) at low temperature (10˚C to 20˚C). A sudden peak in agglutination activity (HA titer = 1024) was observed between 30˚C to 40˚C. The HA titer began to decrease at 50˚C and disappeared totally at 80˚C (Figure 3).

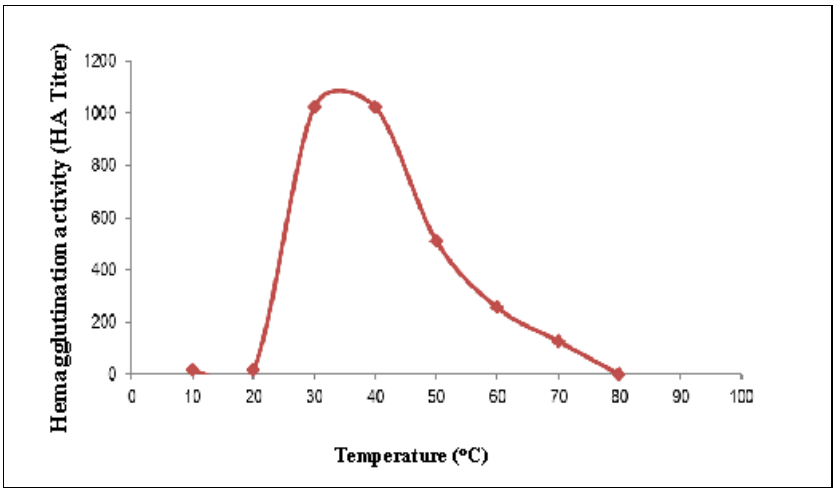

Figure 3. Hemagglutination titer of the hemolymph agglutinin of the larva of the red palm weevil, *Rhynchophorus ferrugineus* in relation to temperature

Effect of the divalent cations and calcium chelator

Maximum HA titer was observed when HA assay was carried out in TBS with 10 to 30 mM Ca^{2+}. HA titer began to decrease on further increase in concentration of Ca^{2+} in the buffer. Agglutination activity seemed to be unaffected by the addition of Mg^{2+} and Mn^{2+} ions in the buffer (Table 1).

Table 1. Effect of divalent cations on the hemagglutinating activity of the hemolymph agglutinin of the larva of the red palm weevil, *Rhynchophorus ferrugineus*

Concentration (mM) (n = 5)	HA titer		
	$CaCl_2$	**$MgCl_2$**	**$MnCl_2$**
0	256	256	256
0.01	256	256	256
0.1	256	256	256
1	256	256	256
5	512	256	256
10	1024	256	256
20	1024	256	256
30	1024	256	256
40	512	256	256
50	128	256	256

n = number of animals tested

Impact of Calcium chelator

Addition of low concentrations (0.01-5 mM) of di- and tetra sodium EDTA, revealed either no change or just two fold decrease in HA titer. But when the concentration of EDTA was increased to 10 mM or higher, a tremendous fall in HA titer was observed. Addition of low concentration (0.01 to 5 mM) of trisodium citrate exhibited a twofold reduction in HA activity which was revived to normalcy with further increase in the concentration (10-50 mM) of trisodium citrate (Table 2).

Table 2. Effect of EDTA and Trisodium citrate on the hemagglutination titer of the hemolymph agglutinin of the larva of the red palm weevil, *Rhynchophorus ferrugineus*

Concentration (mM) (n = 5)	**HA titer**		
	Di sodium EDTA	**Tetra sodium EDTA**	**Tri sodium citrate**
0	256	256	256
0.01	128	256	128
0.1	128	256	128
1	128	256	128
5	128	128	128
10	16	32	256
20	8	4	256
30	4	2	256
40	2	0	256
50	2	0	256

n = number of animals tested

Hemagglutination inhibition assay (HAI)

Glycoproteins

Among the glycoproteins tested, Porcine stomach mucin and Fetuin showed maximum inhibitory potency. The order of inhibition is: Porcine stomach mucin = Fetuin > Bovine thyroglobulin > Transferrin = Apotransferrin > Bovine submaxillary mucin (Table 3).

Table 3. Hemagglutination inhibition (HAI) titer of the hemolymph agglutinin of the larva of the red palm weevil, *Rhynchophorus ferrugineus* by different glycoproteins

Glycoproteins (n = 5)	HAI titer	Minimum concentration for inhibition (µg)	Relative inhibitory potency (%)
Porcine stomach mucin	512	9.76	100
Fetuin	512	9.76	100
Bovine thyroglobulin	256	19.53	50
Transferrin	32	156.25	6.25
Apotransferrin	32	156.25	6.25
Bovine submaxillary mucin	8	625	1.56

n = number of animals tested

Sugars

Of the various sugars tested for inhibition, N-acetyl mannosamine, melibiose and α- lactose inhibited the HA titer with the same potency whereas D-galactosamine, N-acetyl-D-galactosamine and N-acetyl neuraminic acid inhibited HA relatively with less potency (Table 4).

Table 4. Hemagglutination inhibition (HAI) titer of the hemolymph agglutinin of the larva of the red palm weevil, *Rhynchophorus ferrugineus* by different sugars

Sugars (n = 5)	HAI titer	Minimum concentration for inhibition (mM)	Relative inhibitory potency (%)
N-acetyl mannosamine	128	39.06	100
Melibiose	128	39.06	100
α-Lactose	128	39.06	100
D-galactosamine	64	78.12	50
N-acetyl-D-galactosamine	32	156.25	25
N-acetyl neuraminic acid	32	156.25	25

n = number of animals tested

Enzyme treatment of erythrocytes

HA titer remained unchanged when HA assay was carried out with pepsin treated rabbit erythrocytes. However, two fold increase in HA titer was observed when analysed with Trypsin and Neutral protease treated rabbit erythrocytes, and 16 fold decrease in HA titer was observed when checked with neuraminidase treated rabbit erythrocytes (Table 5).

Table 5. Effect of enzyme treatment of erythrocytes on HA titer of the hemolymph agglutinin of the larva of the red palm weevil, *Rhynchophorus ferrugineus*

Enzymes (n = 5)	Site of enzyme activity	HA titer
None	-	1024
Trysin (1 mg/ ml)	Arg-Lys-	2048
Pepsin (1 mg/ ml)	C-terminal of Phe, Leu & Glu	1024
Neutral protease (1 mg/ ml)	-	2048
Neuraminidase	NeuAc-D-Gal, NeuAc-D-GalNac	64

n = number of animals tested

Effect of denaturing agents

Treatment of the larval hemolymph with denaturing chemicals like chloroform, HCl and NaOH resulted in a tremendous reduction of the HA titer (Figure 4).

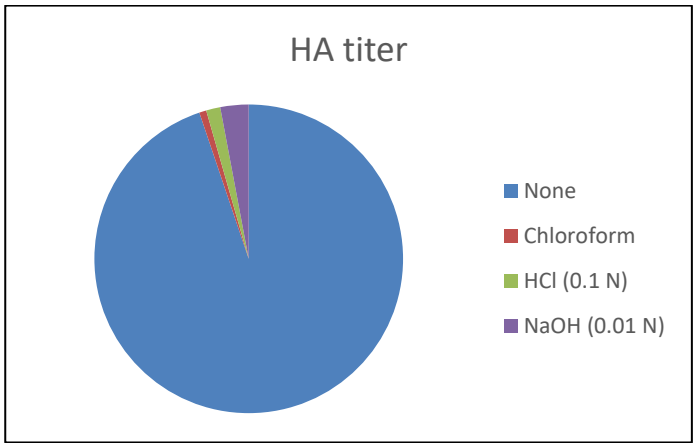

Figure 4. Effect of denaturing agents on HA titer of the hemolymph agglutinin of the larva of the red palm weevil, *Rhynchophorus ferrugineus*

Cross adsorption test

The hemolymph after adsorption to a particular species of erythrocyte, failed to agglutinate the erythrocytes of the same or other species (after first or second or third adsorption in certain cases) revealing the presence of a single agglutinin in the hemolymph (Table 6).

Table 6. Hemagglutination (HA) titer of the hemolymph of the larva of *Rhynchophorus ferrugineus* after adsorption with different erythrocytes

Erythrocytes adsorbed (n = 10)	Human B	Human O	Horse	Dog	Goat/ Pig	Rabbit	Guinea pig	Buffalo
None	128	64	256	256	32	1024	256	64
Human A	0	0	0	2 (0)	0	128 (16) (0)	0	8 (0)
Human B	0	0	0	0	0	8 (0)	0	0
Human O	0	0	0	2 (0)	2 (0)	64 (8) (0)	0	8 (0)
Horse	0	0	0	0	0	4 (0)	0	0
Dog	0	0	0	0	0	8 (0)	0	0
Pig	0	0	0	0	0	8 (0)	0	0
Rabbit	0	0	0	0	0	0	0	0
Cow	0	0	0	0	0	0	0	0
Goat	0	0	0	0	0	16 (8) (0)	0	0
Guinea pig	0	0	0	0	0	16 (4) 0	0	0
Rat	0	0	0	0	0	4 (0)	0	0
Baffalo	0	0	0	0	0	16 (8) (0)	0	0

n = number of animals tested
Note: Values in parenthesis refer to HA titer after successive adsorption.

DISCUSSION

The present study reveals the presence of an agglutinin capable of agglutinating a wide variety of mammalian erythrocytes in the hemolymph of the larva of red palm weevil, *Rhynchophorus ferrugineus*. The apparent lack of specificity of anti-RBC agglutinins is common among insects (Fries, 1984) and numerous reports discussed the presence of naturally occurring agglutinins towards a wide range of vertebrate erythrocytes in various members of insect species (Nalini and Kim, 2007; Tian et al. 2009; Learnal-Sudhakar, 2014; Tamilarasan et al. 2021) and the occurrence of non-specific hemolymph agglutinins against the human ABO (H) blood group or Rhesus system as a common feature amongst insects (Molyneux et al. 1986). The observed similarity and difference in hemagglutination activity in the larval hemolymph with different blood groups may be due to either similarity or differences in the number of sugar haptens exhibited on the cell surfaces of the different blood groups.

Among the various erythrocytes tested for HA, the hemolymph gives high HA titer with rabbit erythrocytes suggesting that the receptor determinants preferentially recognized by the hemolymph agglutinin are either abundant or more accessible on rabbit erythrocytes rather than on other tested erythrocytes. Hakomori (1973) has reported that lectins recognize sugar constituting the glycocalyx of eukaryotic cells which serve as receptors to ligands. As observed in the hemolymph of the larva of red palm weevil *Rhynchophorus*

ferrugineus, hemolymph agglutinin with great affinity for rabbit red blood cells is also reported in the hemolymph of *Balberus craniifer* (Anderson et al. 1972), *Leucophaea maderae* (Amirante, 1976) and *Oryctes rhinoceros* (Jayalakshmi, 2005).

Lectins have different optimum pH to maintain their stability (Utarabhand and Akkayanont, 1995). Optimum hemolymph hemagglutination occurred at pH 7.5 in the larva of the red palm weevil. Above and below pH 7.5, hemagglutination titers gradually declined. These results demonstrate that the lectin activity was pH dependent. It has been reported that, more acidic or basic pH decreases both the stability and activity of lectins (Utarabhand and Akkayanont, 1995). Different pH conditions were found to have profound effects on the tertiary and ternary structure of proteins and can perturb protein conformational stability Ugwu and Apte, 2004).

The activity of the hemagglutinin was found to be optimum between a temperature range of 30˚C to 40˚C. Agglutination was very low at temperature between 10˚C to 20˚C and was totally lost when the hemolymph was incubated at 80˚C. The loss of hemagglutinating activity with increasing temperature is evidently due to heat induced denaturation of the lectin. This denaturation may expectedly weaken the interaction between lectin and the carbohydrate ligand leading consequently to attenuated agglutinating activity (Qadir et al. 2013). These observations indicate that hemagglutinin of the larva of *Rhynchophorus ferrugineus* is heat-labile in nature. Thermolabile nature is characteristic of lectins of a few Lepidopteran insects like *T. commodus* (Hapner and Jermyn, 1981), *Melanoplus sanguinipes* (Stebbins and Hapner, 1985), the Phasmid, *Extatosoma tiaratum* (Richards et al. 1988) and the Dipteran, *Glossina fuscipes* (Ingram and Molyneux, 1990).

The bonding process between lectins and carbohydrates is generally dependent on the presence of metallic ions. The results of the present study also indicate the requirement of divalent cations like calcium for HA activity of the larval hemolymph. However, the hemagglutinin activity of the larval hemolymph remained unchanged when tested with rabbit erythrocytes in TBS with either Mg^{2+} or Mn^{2+} suggesting that the hemagglutinin activity is independent of these metal ions but dependent on Ca^{2+} ions. There are reports suggesting that a few lectins are metallo proteins (Goldstein and Hayes, 1978) and a part of metal is essential for the activities of lectins (Paulova et al. 1971).

In relation to immune reactions, C-type lectins are implicated in most biological systems, which are known to be dependent upon divalent cations, usually calcium ions and are reversibily or irreversibily sensitive to divalent cation chelators such as EDTA or EGTA (Vasta et al. 1999). Such cation-protein linkages have been previously reported for lectins of Arthropods (Grubhoffer amd Matha, 1991). In this investigation, EDTA had no significant effect upto 5 mM concentration on the HA activity of the hemolymph of the larva of *Rhynchophorus ferrugineus*. However, further increase in concentration reduced the HA activity drastically suggesting that the hemagglutinin of *Rhynchophorus ferrugineus* is a calcium dependent one. The Ca^{2+} dependent lectins are also reported in insects such as *Teleogryllus commodus* (Hapner and Jermyn, 1981), *Spodoptera exigua* (Pendland and Boucias, 1986), *Periplaneta americana* (Kubo and Natori, 1987), *Schistocera gregaria* (Ayaad, 2004), *Helicoverpa armigera* (Chai et al. 2008), *Odoiporus longicollis* (Tamilarasan et al. 2021).

In the present study, when Trypsin and Neutral protease treated rabbit erythrocytes were used for HA, elevation in HA titer was observed, but the HA titer remained unchanged when analyzed with pepsin treated rabbit erythrocytes. This must be due to the site of action of the tested enzymes. Enzymes may alter or modify the chemical nature of RBC surface structures (receptors) by removing the glycopeptide fragments and their accessibility (Uhlenbruck and Rothe, 1974). In addition, stereochemical hindrance by glycoproteins may limit the expression of hidden (cryptic) antigenic determinants (receptors) which are unmasked following enzymatic treatment and now mediate lectin binding to RBC agglutination which can be used to ascertain the nature of carbohydrate involved in binding with the relevant agglutinin (Mohamad et al. 1992).

The agglutinability of the hemolymph agglutinin of the larva of *Rhynchophorus ferrugineus* was inhibited by the sialo glycoproteins, Porcine stomach mucin = Fetuin > Bovine thyroglobulin > Transferrin = Apotransferrin > Bovine submaxillary mucin and sugars N-acetyl mannosamine = melibiose = α-lactose > D-galactosamine > N-acetyl-D-galactosamine = N-acetyl neuraminic acid. Affinity for a broad spectrum of carbohydrates is also reported among the lectins purified from a number of Orthopteran insects (Drif and Brehelin, 1989; Tamilarasan et al. 2021). HA assay of the hemolymph of the larvae of *R. ferrugineus* with sialidase treated rabbit erythrocytes showed a great reduction in HA titer. HAI with sialoglycoproteins and N-acetyl neuraminic acid and reduction in HA titer when assayed with asialo rabbit erythrocytes suggest that the agglutinin found in the hemolymph of the larva of *R. ferrugineus* could be a sialic acid specific one which can be confirmed only after purification.

The cross adsorption tests performed with the larval hemolymph of *Rhynchophorus ferrugineus* showed that, all erythrocyte types tested adsorbed various amount of agglutinating activity from the hemolymph and subsequent readsorptions completely adsorbed the agglutinating activity of the erythrocyte types tested. This reveals the presence of a single hemagglutinin in the hemolymph of the larva of *R. ferrugineus* and the agglutinin probably share a common surface receptor but with quantitative difference in its agglutinin binding sites. The physico-chemical characteristics and sugar binding specificity of the agglutinin studied would help in the affinity purification of this hemagglutinin.

CONCLUSION

A Ca^{2+} dependent rabbit erythrocyte specific agglutinin with maximum activity at pH 7.5 and temperature 30 - 40°C is identified in the hemolymph of the larva of red palm weevil *R. ferrugineus*. Hemagglutinability is inhibited by the sugars N-acetyl mannosamine, melibiose, and α-Lactose, the glycoprotein fetuin, and PSM. Treatment with denaturing enzymes reveals the proteinaceous composition of the agglutinin. Cross adsorption assay revealed the presence of a single agglutinin. This work provided all the details necessary for the purification of the agglutinin by affinity chromatography. Sialic acid specificity suggest the possibility of the agglutinin to be included in the biomedically significant lectin database if purified.

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