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Research Article

IDENTIFICATION AND CHARACTERIZATION OF A NATURALLY OCCURRING AGGLUTININ OF THE MAY BEETLE *PHYLLOPHAGA* SP.

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ABSTRACT

A naturally occurring hemagglutinin with high affinity for rabbit erythrocytes was identified in the whole body extract of the May beetle *Phyllophaga* sp. The extract of the whole body also agglutinated pig, human A, human AB, rat, human B and O erythrocytes with diverse specificities. However, it failed to agglutinate cow, goat and buffalo erythrocytes. Physico-chemical analysis of the agglutinin using rabbit erythrocytes as indicator cells revealed that the agglutinin was at its highest activity between pH 7.5 to 8.5, temperature 35^{o}C and in the presence of 10 mM Ca^{2+} in the buffer. Agglutinability of the whole body extract of the May beetle was slightly enhanced by low concentrations (0.01 to 5 mM) of disodium EDTA, unaffected by trisodium citrate (0.01 to 50 mM) and tetrasodium EDTA (0.01 to 1 mM). However, higher concentration of disodium EDTA (> 10 mM) greatly reduced the agglutinability than tetrasodium EDTA and trisodium citrate. Hemagglutinability inhibition assay recognized lactoferrin (HAI titer = 512) as the potent inhibitor of the agglutinin found in the whole body extract of the May beetle *Phyllophaga* sp. Presence of Ca^{2+} dependent natural agglutinin in the whole body extracts of the May beetle, *Phyllophaga* sp. may contribute to its defense mechanism.

Keywords: Agglutinins, Erythrocytes, Hemagglutination, Hemagglutination inhibition, Lectin, *Phyllophaga* sp.

INTRODUCTION

Immune response against invading pathogens are the basic physiologic functions of all living organisms and a series of defense mechanism has evolved to protected cellular integrity, homeostasis and survival of the host (Buchmann, 2014). The immune systems of vertebrates are complex in terms of the mechanisms employed in immune defense, the abundance of immune related molecules and the diversity of effector cell types (Boehm, 2012). Invertebrates ranging from protozoans to metazoans possess cellular receptors which bind to foreign elements and differentiate from non-self (Dzik, 2010). Smith (2016) reported cellular immunity is induced by non-self motifs on the surface of pathogens recognized by cell derived Pattern Recognition receptors with diverse binding specificity. The innate immunity is the first line of inducible host defense against bacterial, fungal and viral pathogens (Hoebe *et al*., 2004). Important innate effector molecules are agglutinins, antimicrobial peptides, fibrinogen related peptides, hemolysins, lysozymes, pentraxins and complement related proteins (Chettri *et al*., 2011).

Agglutinins/lectins are polyvalent in nature and can bind to the carbohydrates moieties on the surface of erythrocytes and agglutinate the erythrocytes, without altering the properties of the carbohydrates (Lam and Ng, 2011). Lectins are ubiquitous in nature and they are found in a wide range of organisms including viruses, bacteria, fungi, plants and animals (Sharon, 2008). They may bind to the cell surface glycoproteins and glycolipids and 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 (Valbuena *et al*., 2010), discrimination of cancer cells from normal cells (Dan *et al*., 2015). They can bind to sugar moieties in cell walls or membranes thereby change the physiology of the membrane to cause agglutination, mitosis or other biochemical changes in the cell (Hamid *et al*., 2013; Sullivan, 2017). Although a

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number of investigations on lectins are carried out in various insects, there is paucity of information regarding the availability of lectins among the May beetle of the family Scarabaeidae of the class insects. Here an effort is taken in this investigation to identify and characterize agglutinins from May beetle Scarabaeidae sp.

MATERIAL AND METHODS

Collection of animal

Live specimen of insects *Phyllophaga* (Figure 1) species were collected from the village area of Elavuvilai and Nattalam near Marthandam, Vilavancode Taluk, Kanyakumari District, Tamil Nadu, India. The collected animals were transported to the laboratory.

Kingdom	:	Animalia
Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Coleoptera
Family	:	Scarabaeidae
Subfamily	:	Melolonthinae
Genus	:	*Phyllophaga*

Figure 1. Image of *Phyllophaga* sp. and their taxonomic position.

Preparation of whole body extract

The healthy anaesthetized beetles were cleaned with distilled water and then rinsed in cold Tris buffered saline (TBS) to remove the dust. The whole body extracts were prepared following the modified method of Volf *et al.* (2002). The extract was prepared at 1:10 ratio ie. 1 gm beetle was ground in 10 ml cold TBS and centrifuged at 4000 g for 10 min. at 4^{o}C and the supernatant was assessed for hemagglutination activity.

Preparation of erythrocyte suspension

Blood samples were collected from different mammals (human A, B, AB, O, pig, rabbit, rat, buffalo, cow, goat) directly in modified Alseivier's medium (pH 6.1) containing sodium citrate (30 mM), sodium chloride (77 mM), glucose (114 mM), neomycin sulfate (100 µg/ml) and chloramphenicol (330 µg/ml). Erythrocytes were suspended and washed thrice by centrifugation at 4000g with ten volumes of physiological saline and with Tris-Buffered Saline (TBS) pH 7.5 (Tris-HCl: 50 mM, NaCl: 100 mM; $CaCl_2$: 10 mM) and resuspended in TBS as 1.5% suspension (Mercy and Ravindranath, 1993).

Hemagglutination assay

The whole body extract of the May beetle, *Phyllophaga* sp. was assayed for the presence of agglutinins using TARSON 96 well U-bottom microtitre plates described by (Ravindranath and Paulson, 1987). The sample (25 µl) was serially diluted in TBS (25 µl) and incubated with 1.5% suspension of RBCs (25 µl) at room temperature (30±2^{o}C) for an hour or until the negative control showed a red button formation. Agglutination activity was detected based on the RBCs appearance on the well: a positive result appears as a red-carpet layer, while negative results appear as a red button in the bottom of the well.

Effect of pH on hemagglutinating activity

To study the effect of pH on agglutinability of the whole body extract, HA assay was carried out in TBS of different pH (5.0 to 9.5).

Effect of temperature on agglutinating activity

To study the effect of temperature on agglutinability of the whole body extract of the beetle, *Phyllophaga* sp., the extract was aliquoted as 500 µl and incubated at specific temperature (0-85^{o}C) for an hour and used for HA assay.

Effect of divalent cations and chelators on hemagglutination activity

To assess the effect of cations on HA activity of the whole body extract, the extract was serially diluted with 25 µl of TBS with different concentration of cations (Ca^{2+}, Mg^{2+}, Mn^{2+}) and chelators (EDTA and trisodium citrate) and was incubated at room temperature (30±2^{o}C) for an hour prior to the addition of rabbit erythrocytes and the hemagglutination titer was determined.

Hemagglutination Inhibition assay

To a known concentration of serially diluted inhibitor (sugars/glycoproteins) solution (25 µl), 25 µl of the extract of the whole body diluted to sub agglutination concentration was added, mixed and the plate was incubated for 1 hour at room temperature. Finally 25 µl of 1.5% rabbit erythrocytes suspension was added and

incubated for 1 hour at room temperature (30±2°C). The minimum concentration of the inhibitors required to completely block the agglutination after 1 hour of incubation at room temperature (30±2°C) was reported as the HAI titer.

RESULTS

Hemagglutinability of the extract

The agglutinin in the whole body extract of *Phyllophaga* sp. agglutinated rabbit, pig, rat and all the human erythrocytes (rabbit > pig > human A > human AB > rat > human B > human O), but it failed to agglutinate cow, goat and buffalo erythrocytes. Among the various erythrocytes tested, maximum agglutinability was observed with rabbit erythrocytes (Table 1).

Influence of pH on HA

The optimum pH of the agglutinin in the whole body extract of *Phyllophaga* species was observed from pH 7.5 to 8.5. The agglutinability was low at acidic and alkaline range (Table 2).

Impact of temperature on HA

The maximum hemagglutination activity of the extract of the whole body of *Phyllophaga* was observed at 35°C, which got gradually reduced above and below 35°C (Table 2).

Effect of cations and chelators

Maximum hemagglutination was observed in the presence of 10 mM Ca^{2+} but not Mg^{2+} and Mn^{2+}. However, addition of higher concentration of all the three cations (Ca^{2+}, Mg^{2+}, Mn^{2+}) decreased the HA titer (Table 3). A decrease in HA titer was observed with the addition of increasing concentration of EDTA and trisodium citrate.

HAI assay

Among the inhibitors tested, the agglutinability of the agglutinin was highly inhibited by lactoferrin (HAI titre=512) followed by apotransferrin (HAI titre=32), thyroglobulin (HAI titre=16), transferrin (HAI titre = 8), sucrose (HAI titre = 8), D-mannose (HAI titre = 4), D-galactosamine (HAI titre = 2), dextrose (HAI titre = 2) and D-glucuronic acid (HAI titre = 2) (Table 4).

Table 1. HA titer of whole body extract of *Phyllophaga* sp. with different mammalian erythrocytes.

Erythrocytes (n=25)	HA titer
Rabbit	2048
Pig	1024
Human A	512
Human AB	128
Rat	64
Human B	8
Human O	2
Cow	0
Goat	0
Buffalo	0

Table 2. HA titer of the agglutinin in the whole body extract of *Phyllophaga* sp. in relation to pH and temperature.

pH (n=5)	HA titer	Temperature (°C) (n=25)	HA titer
5.0	64-128	0	1024
5.5	256	5	1024
6.0	256	15	1024
6.5	256	25	1024
7.0	512	35	2048
7.5	2048	45	1024
8.0	2048	55	512
8.5	2048	65	512
9.0	1024	75	512
9.5	512-1024	85	256

Table 3. Effect of cations and chelators on HA activity of the agglutinin in the whole body extract of *Phyllophaga* sp.

Concentration (mM) (n=5)	HA titer					
	Ca^{2+}	Mg^{2+}	Mn^{2+}	EDTA		Trisodium citrate
				Disodium	Tetra sodium	
0	128	128	128	128	128	128
0.01	128	128	128	256	128	128
0.1	128	128	128	256	128	128
1	128-256	128	64	256	128	128
5	1024	128	64	256	64	128
10	2048	128	64	32	64	128
20	512	128	64	32	64	128
30	64	128	16	8	64	128
40	32	128	16	0	64	128
50	8	32	16	0	4	128
100	2	16	8	0	0	32

Table 4. HAI titer of the agglutinin in the whole body extract of *Phyllophaga* sp. by various glycoproteins and sugars.

Inhibitors (Glycoproteins/Sugars) (n=5)		HAI titer	Minimum Conc. Required (mg/ml) / (mM)	Relative inhibitory potency (%)
Glycoproteins	Lactoferrin	512	19.53	100
	Apotransferrin	32	156.25	6.25
	Thyroglobulin	16	312.5	3.125
	Transferin	8	625	1.562
Sugars	Sucrose	8	12.5	100
	D-Mannose	4	25	50
	Dextrose	2	50	25
	D-Glucuronic acid	2	50	25
	D-Galactosamine	2	50	25

DISCUSSION

In general lectins interact with their target through multiple binding sites, which increased affinity and specificity. In this investigation, whole body extract of the May beetle, *Phyllophaga* sp. showed the highest specificity with rabbit erythrocytes (rabbit>pig> human A>human AB> rat>human B>human O). Agglutinin / lectin may recognize a whole sugar or a part of sugar or a sequence of sugar or their glycosidic linkages (Brettin and Kobat, 1976; Shimizu *et al*., 1977; Kobiler and Mirelman, 1980; Koch *et al*., 1982). The agglutinin in the whole body extract binds to the particular sugar moiety/receptors on the surfaces of the erythrocytes. The erythrocyte specificity of the agglutinin of the whole body extract argues for the specific recognition of the sugars constituting the glycocalyx of these erythrocytes, which serve as receptors to ligands as in the eukaryotic cells (Hakomori, 1973). Probably the agglutinin may bind to sialic acid of the glycocalyx of these erythrocytes (Yasue *et al*., 1978) because all the erythrocytes recognized by the agglutinin possess sialic acid as the terminal sugar in the glycocalyx. Agglutination is facilitated by two or more combining sites on agglutinating molecules enabling the agglutinin to adhere to more than one erythrocyte species (Goldstein *et al*., 1980). Among the various erythrocytes tested, the agglutinin in the whole body extracts of the beetle gave a higher hemagglutination titre with rabbit erythrocytes. The agglutinin of the beetle, *Oryctes rhinoceros* also showed highest affinity for rabbit erythrocytes (Jayalakshmi, 2005).

In the beetle, *Phyllophaga* sp. maximum HA titer was observed from pH 7.5 to 8.5 and temperature 35^{o}C. Conformational changes occur due to the change/dissociation of the binding sites of the agglutinin when there is decrease/increase in pH and temperature which may suppress/accelerate the hemagglutination activity. The loss of agglutinating activity of the agglutinin in the whole body extract may be due to destabilization of sporadic weak interactions of tertiary structure responsible for binding of native agglutinin. pH and temperature sensitivity was also reported in the beetle, *Oryctes rhinoceros* (Jayalakshmi, 2005), millipede, *Thyropygus descriptus* (Basil-Rose *et al*., 2014) and *Arthrosphaera disticta* (Arul Gandhi, 2013).

Divalent cations are involved in stabilizing the primary structure of hemagglutinins. A calcium ion acts as a bridge between the protein and the carbohydrate through direct interactions with sugar hydroxyl groups. Probably, the divalent cations may trigger/suppress the hemagglutination activity depending on their concentration. Our results imply that the agglutinin in the whole body extract is rich in endogenous calcium and does not respond to the exogenous cations for its activity. C-type lectins are also reported earlier in the beetle *Oryctes rhinoceros* (Jayalakshmi, 2005), crabs, *Cancer antennarius* (Ravindranath and Cooper, 1984), *Scylla serrata* (Mercy and Ravindranath, 1992), *Paratelphusa jacquemontii* (Denis *et al*., 2003), *Episesarma tetragonum* (Devi, 2007), *Lamella lamellifrons* (Mettilda, 2012) and millipede, *Arthrosphaera disticta* (Arul-Gandhi, 2013), *Trigoniulus corallinus* (Anitha and Basil-Rose, 2018).

EDTA is known to be a metal-chelating agent. Addition of 0.01 to 5mM disodium EDTA may cleave the excess calcium resulting in an increase in HA titer. Similar activity was also reported in *Paratelphusa jacquemontii* (Denis *et al*., 2003), *Emerita emeritus* (Jayasuriya, 2002), *Lamella lamellifrons* (Mettilda, 2012), *Arthrosphaera disticta* (Arul-Gandhi, 2013).

Among the sugars tested sucrose, D-mannose, D-galactosamine, dextrose and D-glucuronic acid were inhibited agglutinations at varying capacities. Among the glycoprotein inhibitors tested, lactoferrin (lactoferin> apotransferrin> thyroglobulin> transferrin) was identified as the potent inhibitor with high HAI titer. The glycol-proteins differ not only in their sialic acid content but also with respect to the distribution of the carbohydrate chains and their linkages to protein. Sialyl residue (NeuAc) was abundant in lactoferrin and it consists of a single polypeptide chain with two glycans attached through N-glycosidic linkages. Hydroxyl groups (OH) of carbohydrates may participate in the binding to CRDs of the agglutinin (Seufi *et al*., 2012). Lactoferrin specificity is also reported in arthropods (Arul-Gandhi, 2013; Basil-Rose *et al*., 2014; Anitha and Basil-Rose, 2018).

CONCLUSION

The present study revealed the presence of agglutinin in the whole body extract of the May beetle *Phyllophaga* sp. The agglutinin recognized rabbit erythrocytes with great avidity in the presence of Ca^{2+} ions at pH 7.5 to 8.5 and temperature upto 35^{o}C. The agglutinability was specifically inhibited by the glycoproteins lactoferin>apotransferrin> thyroglobulin> transferrin and sugars, sucrose, D-mannose, D-galactosamine, dextrose and D-glucuronic acid. This study provides the physico-chemical characteristics necessary to purify the agglutinin.

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