Original Article

Lectin Activity in Gut Extract of *Culex pipiens*

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Abstract

Background: The role of lectins is important in interaction between pathogens and mosquito vectors. This study was performed to identify agglutinin activities of protein molecules on the midgut of *Culex pipiens*.

Methods: *Culex pipiens* was reared in insectray condition and the midguts of males and females (blood fed and unfed) were dissected separately in Tris-HCl buffer. The extracts of midguts were applied for hemagglutinin assay against red blood cells of rabbit, mouse, rat, dog, horse, sheep, guinea pig, cow, human (A, B, AB, O groups). Then, the RBCs with relatively high agglutinin activity were chosen for carbohydrate inhibition assay. D (+) glucose, D (+) galactose, D (+) mannose, D (-) fructose, D (-) arabinose, L (-) fucose, lactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, sialic acid were used to specify carbohydrate binding lectin.

Results: The highest agglutinin activities were found against sheep and rabbits RBCs. Sexual diversity of agglutinin activities was observed among midgut extraction of males and females. In addition, variation in agglutinin activity of blood fed and unfed female mosquitoes were detected. The lectin activity was inhibited highly with glucose, galactose, fucose and fructose but less inhibitor activities was observed by arabinose, N-acetyl-D-galactosamine, n-acetyl-d-glucosamine, lactose and mannose.

Conclusion: The secretion of hemagglutinins (lectins or lectin-like molecules) in the digestive system depends on the type of food in the gut. This suggests that emptying of the gut in preparation for protein rich food probably starts the secretion of hemagglutinins.

Keywords: Mosquitoes, *Culex pipiens*, Lectin, Hemagglutination activity, Midgut

Introduction

Lectins are defined as carbohydrate-binding proteins or glycoproteins of non immune origin which agglutinate cells and/or precipitate glyco-conjugates (Goldstein and Poretz 1986). Lectins in insects with distinct sugar specificities involve in recognition and protective roles in immune defense against microbial pathogens. The agglutinins against vertebrate erythrocytes have been reported in various insects (Basseri 2002). Insect hemagglutinins are lectin or lectin-like molecules that are ubiquitous, non-enzymatic carbohydrate binding proteins or glycoproteins and once bound to erythrocytes or other cells, usually cause their agglutination, and may also precipitate glycol conjugates (Stebbins and Hapner1986, Ingram 1997).

Most studies have concentrated on the hemagglutination activity (HA) in the gut of hematophagous insects especially Dipteran (Rudin and Hecker 1989, Volf 1993, Grubhoffer et al. 1994, Volf et al. 1994, 1995, 1998). The role of lectins are important in the life cycle of some parasitic protozoa carried by mosquitoes, sand flies, tsetse flies, and many other bloodsucking insects (Ingram

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and Molyneux 1991, Basseri 2002, Basseri et al. 2008). These molecules in the gut and hemolymph cause the establishment of infection and parasitic development (Ibrahim et al. 1984, Mello et al. 1999).

In addition, anti-parasite agglutinins have been detected in midgut extracts of trypanosome vectors (Ibrahim et al. 1984, Wallbanks et al. 1986). Unfortunately there is not enough information about the nature of lectin- carbohydrate in insect-vector–parasite interaction. In spite of the fact that there are specific mechanisms between lectin receptors on surface of parasites of vector tissue which can ensure the success or failure of infection (Rudin 1991, Billingsley 1997, Basseri et al. 2008), some investigators suggest that interactions between parasites and vector gut walls may be mediated by the carbohydrates on the surface of parasites. Besides the lectins in the vector gut, as characteristic carbohydrate markers have been identified on the surface of parasites such as *Trypanosoma* and *Leishmania* (Schottelius 1982a, 1982b, 1982c). The midgut lectins have been identified from such vectors as *Rhodnius prolixus* (Pereira et al. 1980) *Glossina austain* (Ibrahim et al. 1984) *Phlebotomus papatasi* (Wallbanks et al. 1986) and *Anopheles gambiae* (Mohamed and Ingram 1994).

Culex pipiens is usually the most common pest mosquito in urban as well as rural areas of Iran (Azari-Hamidian 2007, Dehghan et al. 2010). The purpose of present study was to detect the hemagglutinin activity in the midgut extraction of males and females of *Cu. pipiens*. Furthermore, to find changes in hemagglutination activity in blood fed and unfed female mosquitoes. Moreover, specific carbohydrates of lectins were surveyed to characterize the lectins in the midgut. The mosquito lectin has been partially characterized for further study and their functional roles.

Materials and Methods

Insect rearing and sample preparation

The laboratory strain of the *Cx. pipiens* was used. The mosquitoes were reared for more than 48 years in the insectray. During the present study they were occasionally allowed to feed on laboratory guinea pigs and reared under a photoperiod of 12:12 day/night at 28±2 ºC and 50–60% relative humidity. The adult females including fed and unfed females as well as males were then applied separately for midgut dissections.

Preparation of gut

Mosquitoes gut were dissected separately in TN buffer (20mM Tris - HCl, 0.15M NaCl, pH=7, 5mM $CaCl_2$). The guts were collected and washed with the buffer and homogenate using mechanical homogenizer in cold condition. Then, the homogenate samples were centrifuged at 10,000g for 15min, three times. The supernatant were kept in -80 ˚C until use.

Protein Assay

The concentration of midgut proteins was estimated as discussed by Bradford (1976) and in order to obtain standard curve, serial dilution of different concentrations of bovine serum albumin (BSA) was used.

Preparation of erythrocyte

Blood from rabbit, mouse, rat, dog, horse, sheep, guinea pig, cow, human (A, B, AB, O groups) were prepared in 3.8% (w/v) trisodium citrate. In order to prepare red blood cells, whole bloods were washed three times in TN buffer at 1500g for 5min each to remove serum and gain RBCs.

Finally a 2% (v/v) suspension of RBC was prepared and kept at +4˚C until use for hemagglutination assay and also hemagglutination inhibition assay.

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Hemagglutination assay

Five microlitre of each midgut extract was serially diluted in TN buffer (as suggested by Uhlir et al. 1996) in the v-bottom wells of micro titration plates. Then 5 microlitre of 2% mentioned erythrocytes suspension was added to each well. The titer of hemagglutination activity was determined under stereomicroscope after 60 min incubation at room temperature. Unagglutination described as RBCs with clear dot on the bottom of the well, and agglutinatinated targets formed a diffuse mat.

All experiments were repeated three times. The controls contained TN buffer and 2% BSA. Finally, the erythrocyte which had the highest dilution activities visually was chosen for next experiments. The reciprocal value of the highest dilution with positive reaction was scored as the titer.

Hemagglutination inhibition assay (HIA)

The HIA was performed to determine the inhibitory activities of different carbohydrates against the midgut lectin activities as follows: D (+) glucose, D (+) galactose, D (+) mannose, D (-) fructose, D (-) arabinose, L (-) fucose, lactose, N-acetyl-D-glucosamine, N- acetyl –D-galactosamine, sialic acid (all form sigma). The stock solutions of carbohydrates were prepared in NaCl/Tris/Ca 2+ buffer at 0.2M stored at -20 ºC until use. For each inhibition (5µl) aliquots of buffer contain carbohydrate was added in each microlitre wells, followed by 5 microlitre of gut extracted adjusted subsequently to titer of 1: 4096. These were mixed gently by shaking and incubated for 60 min at room temperature. Finally, 5 microlitres of 2% RBCs of sheep or rabbit suspension was added into each well and left the microplates for 1 h at room temperature to incubate the mixtures. These tests were done three times. Well without lectin or inhibitors were considered as control.

Enzyme treatment of RBCs

In order to expose more receptors of RBCs to extracted lectins, all RBCs were treated with trypsin. Equal volume of 2% above RBCs, trypsin (2 mg/ml) solution prepared in tris-HCl, were mixed together and incubated at 37 ºC for 25 min.

The RBCs were then washed three times with buffer and adjusted to 2% suspensions. Treated or untreated RBCs were incubated with gut extract at room temperature for 30 min. subsequently, hemagglutination titers were assessed.

Results

Hemagglutination assay

The hemagglutination patterns of whole proteins from the midguts of *Cx. pipiens* against a range of erythrocytes were determined (Fig. 1). The highest activates occurred against sheep erythrocytes (titer of 256) followed by rabbits (titer of 64). The less activity was observed against Guinea pig erythrocytes (titer≤ 16). Thus, sheep erythrocyte was used for inhibition assay.

Trypsinization assay

Modification of rabbit's erythrocyte membranes by trypsin treatment increased the agglutinin activities more than eight times indicating more lectin molecules present in the extracted proteins.

Appearance of hemagglutinating activity at sex and fed condition

The hemagglutination activity of midgut extracts of male and female mosquitoes was different (Table 1) while the proteins of males' midgut showed less agglutinin activities rather than females. In addition, fed females of *Cx. pipiens* demonstrated two times more agglutinin activity than unfed females (Table 1).

Carbohydrate inhibition assay

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As shown in Table 2, the hemagglutination inhibition of sugars for extracted proteins of midgut was dissimilar. The lectin activity was inhibited highly by glucose followed by galactose, fucose and fructose but less inhibitor activities were observed by arabinose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, lactose and mannose.

Table 1. Effect of induction and blood meal feeding and compartment with mosquito sex on Lectin Hemagglutination Activity (HA)

	Protein Concentration (mg/ml)	HA (with candidate RBCs) (Titer)
Fed	0.17	32
Unfed	0.06	16
Male	0.15	8

The number in last column represents endpoint titers expressed as the reciprocals of the dilutions (1/n). All samples were prepared three times and the assay repeated

Table 2. Effects of inhibitory sugars on agglutinin activities of protein on midgut extract of *Culex pipiens*

Inhibitor	Titer
D(+) glucose	>256
D(+) galactose	>128
D(+) mannose	>64
L(+) fucose	>128
Lactose	>64
N-acetyl-D-glucosamine	>64
N-acetyl-D-galactoseamine	>64
L(+) Arabinose	>64
D(+) Fructose	>128

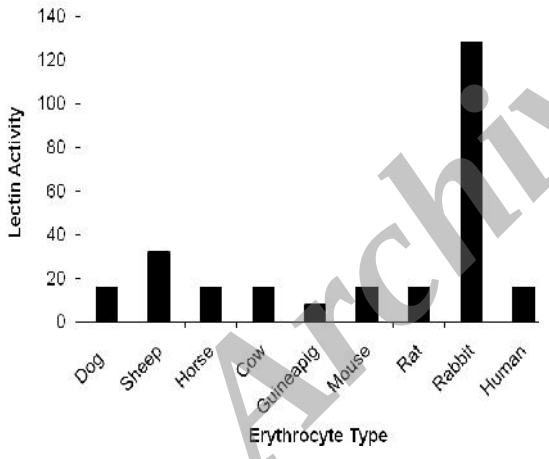

Fig. 1. Agglutination activities of midgut extract of *Culex pipiens* against different erythrocytes

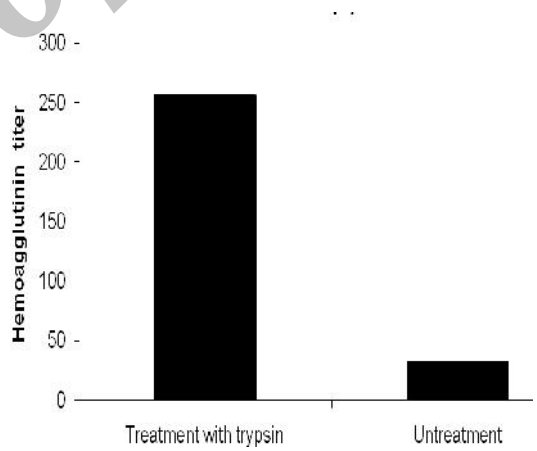

Fig. 2. Comparison between Trypsin enzyme treated and untreated RBC agglutinin activities against midgut extract of *Culex pipiens*

Discussion

The present study shows that hemagglutination activity exists in midgut of adults of both sexes. In addition, this activity was higher among fed females. The hemagglutination activities were described in digestive systems of six species of phlebotomine sand flies (Volf and Killick-Kendrick 1996). They showed that higher hemagglutination activity in the gut of females and characterized the lectins in the

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females' midgut. The results of their study showed that, the agglutinin activity was more than 50 times higher in unfed females than in males. Similarity, blood meal increased agglutinin levels in the midgut extraction of *Cx. quinquefasciatus* against *Escherichia coli* as well as rabbit RBCs (Ayaad 2009).

Presence of variation in lectin activity between males and females is typical only for hematophagous Nematocera (suborder of dipterous insects), presumably because only the females of these insects take blood meals. This presumption is supported by findings in Glossinae, in which both sexes take blood and where there are no significant differences between lectin activities of males and females (Ingram and Molyneux 1988, 1991).

However, our results indicated that the secretion of hemagglutinins (lectins or lectin-like molecules) in the digestive system depends on the type of food in the gut. Interestingly, trypsinization increased binding of agglutinin of midgut extracted proteins with rabbit erythrocytes indicating there are more hidden lectins molecule receptors.

Though, fasting adults have very high hemagglutination activity, which may be due to the degradation of gut cells and presence of innate lectin molecules in the epithelial cells. On the other hand, the high activity in the rest of abdomen may be correlated with the presence of symbionts.

Generally, in adults of mosquitoes, symbionts might use 'host' reserves for their own growth and start to act like pathogens inside the body. In this case, haemocytes, which presumably produce the lectins or hemagglutinins, act to eliminate infection. This concept is supported by the observation the levels of hemagglutinins in females taking blood meals are uncharged.

However, our results is different with those presented by Gelbic and Olejnicek (2004) though they stated that hemagglutination activity in the midgut of *Cx. pipiens* complex is not dependent on a blood meal or the uptake of protein food. Apparently, the variation in results may be due to different population used. We showed the effect of geographical populations of *Anopheles stephensi* on agglutinin activities of the mosquitoes' midgut (Basseri et al. 2004). Similarly, Grubhoffer and Noriega (1995) and also Grubhoffer et al. (1997) reported significant increases in hemagglutination activity in female *Aedes aegypti* after the ingestion of protein food and Volf and Palanova (1996) obtained similar results in phlebotomids. Mohamed et al. (1992) did not find differences either between sexes or after blood fed of *An. gambiae*. According to the present results, hemagglutination activity correlates with some digestive processes and sex of the mosquitoes.

It has been shown that haemagglutinins in the gut of *Cx. pipiens* may separate food from liquid of blood meal or nectar and retard the passage food through the gut (Gelbic and Olejnicek 2004).

Interestingly, significant increase of hemagglutination titer was observed in females after blood meal. This suggests that the empty gut probably starts the secretion of hemagglutinins to prepare for protein-rich food. Male mosquitoes do not suck blood, and therefore the filling of the gut does not start the reaction in the same way as in the females.

Hemagglutination assay on the midgut of *Ae. aegypti* showed that the agglutinin might not be protein (lectin) but a glycan (Olejnıcek et al. 2000). However, further species of Culicinae need to be investigated for induction of gut hemagglutination activity because different responses in different mosquito genera or even species can be expected (Nayar and Knight 1997).

The mosquito midgut represents one of the most challenging environments for many microorganism born diseases such as *Plasmodium*, arboviruses and fungus (Tajedin et al. 2009, Chugh et al. 2011, Cox et al. 2011). The ability of a panel of lectins to potentiate the uptake of a variety of microorganisms is especially important in terms of nonself recognition

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in invertebrate immune system (Rattclife and Whitten 2004, Yoshida et al. 2007).

However, agglutinin activity may be important for the elimination of infections, as well as for the processing of food and the utilization and transportation of nutrients. In conclusion, the secretion of hemagglutinins (lectins or lectin-like molecules) in the digestive system of *Cx. pipiens* depends on the type of food as well sex.

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