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Isolation of midgut agglutinin of *Culex quinquefasciatus* (Diptera , Culicidae)

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ABSTRACT

A Lectin that agglutinates *Eschrechia coli* (ATCC 25922) , *Staphylococcus aureus* (ATCC 6538) live bacteria and various mammalian red blood cells (RBCs) was identified in *Culex quinquefasciatus* midgut extract (Cqlec) by using human (three groups: A, B, and O, RH+)mouse, rat, guinea-pig, rabbit and goat erythrocytes. With the use of $(NH_4)_2 SO_4$ fractionation, anion - exchange and GluNA - CBr-Sepharose 6B affinity chromatography, *C. quinquefasciatus* mid gut lectin has been purified to homogeneity. IEF and reducing SDS/PAGE revealed that the isolated mid gut lectin have isoelectric point (PI) of 6.30, and a subunit approximate molecular weight of 34.5 KDa .The highest agglutination activity of crude and isolated Cqlec were detected against both *Eschrechia coli* cells and rabbit RBCs. Significant differences in hemagglutinin titers and carbohydrate inhibition were detected between sugar fed and blood fed adult female mosquitoes.Overall agglutinin levels were increased following a blood meal feeding and *E. coli* induction using a hypodermic needle. This study presents the first report on the occurrence of heterogeneous anti rabbit RBC agglutinins in the midgut extracts of *C. quinquefasciatus* from Al kharj *area* in Saudi Arabia. The HA of lectins are Ca^{2+}independent, heat-resistant, and are strongly inhibited by D(+)-mannose and D(+) glucose followed by N-acetyl-D-glucosamine. Raffinose and N-acetyl-D-manosamine were found to be moderate inhibitors. Non of the lectins were inhibited by galactose , lactose , trehalose or fetuin (1%) but the glycosubstances mucin and laminarin showed strong inhibitition using low concentrations . 2D-NMR spectroscopy revealed a component of the corresponding residue in structure having group regions of resolution spectrum of Man_9- $GlcNAc_2Asn$.

Keywords: Lectin, hemagglutination, midgut , *Culex quinquefasciatus* , mosquitoes, Culicidae, NMR.

INTRODUCTION

Lectins are defined as carbohydrate-binding proteins or glycoproteins of non-immune origin or as carbohydrate-binding proteins other than antibodies or enzymes. By definition, they are polyvalent, oligomeric, nonimmunoglobulin that bind carbohydrate, agglutinate cells (e.g., RBC, bacteria and viruses or precipitate polysaccharides, glycoproteins or other glycoconjugates (Sharon and Lis, 1989; Ingram ,1997 ; Lis and Sharon, 1998).

Lectins in different insects are important for both the establishment of infection and parasitic development in the gut and hemolymph (Ibrahim *et al.,* 1984 and Mello *et al.,*1999).

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 glycoconjugates (Ingram, 1997). Many hematophagous diptera are vectors of pathogenic parasites of man and animals. Due to social and economic importance of

the diseases caused by these organisms, some investigations have been focused on insect hemolymph or gut extract. Hemagglutinins are involved in carbohydrate binding specificities, because it is considered that they could exert an influence on host-parasite interactions in the appropriate vectors (Hernandez *et al.,* 1986 ; Chen *et al.,* 1993 and Chen & Billingsley, 1999).It is suggested that interactions between parasites and vector gut walls may be mediated by the carbohydrates present on the parasites surface and the lectins in the vector gut. Characteristic carbohydrate markers have been identified on the surface of parasites including T*rypanosome* and *Leishmania* (Lis and Sharon , 1998), and the midgut lectins have been identified from such vectors as *Rhodnius prolixus* (Mello *et al.,*1999) *Anopheles* and *Phlebotomus papatasi* (Volf *et al.,* 2002). Due to the lack of acquired immune response, the host defense against microbial infection in invertebrates solely depends on innate immune systems (Ingram,1997).This has been proposed in invertebrates by the pattern recognition proteins (PRPs) that bind conserved pathogen associated molecular patterns (PAMPs) molecules. The latter is present in the array of carbohydrate components on the surface of microorganisms. Binding of PRPs to PAMPs triggers a series of protective immune responses. Known soluble PRPs include lectins, lipopolysaccharide (LPS)-binding proteins, anti-LPS factors (ALF), peptidoglycan-binding proteins, β-1,3-glucan-binding proteins (Janeway and Medzhitov , 2002). Dodd and Drickamer (2001) have also reported that lectins are often complex, multidomain proteins, but sugar binding activity can usually be ascribed to a single protein module within the lectin polypeptide . Such a module is designated as carbohydrate recognition domain (CRDs) and these CRDs are classified into two categories, the first group is located intracellular, in luminal compartment and has important function in trafficking, sorting and targeting of glycoprotein in the secretary and other pathways. The other CRDs are found in lectins that found largely outside the cell and are either secreted or localized to the plasma membrane. In the present study *C.* mid gut *quinquefasciatus* lectin with various sugar specificity and distinct antigenicity towards mammalian erythrocytes and microorganisms surface determinants was isolated and purified. In addition, a preliminary characterization of the purified lectin was performed.

MATERIAL AND METHODS

Insect rearing and sample preparation:

Culex quinquefasciatus were collected as larvae from a particular rural location in Alkharj area in Saudi Arabia and kept in the insectaries till the adult emergence. Insects were reared at 28 ± 2°C, 75% humidity, with a 12-h light: dark cycle for ten generations to confirm strain purity. Adults were maintained on 10% sucrose solution and females were blood-fed on anesthetized mouse three times a week. Preliminary experiments were conducted to evaluate the HA of the naturally occurring crude mid gut lectins upon *E. coli* induction as well as blood meal feeding of adult female *C.* towards the *quinquefasciatus* various mammalian erythrocytes and both the G (+) and the G (-) bacteria under investigation. Humoral immunity was induced in adult females by experimentally pricking the wing with a needle dipped in a bacterial suspension of 1.1 x $l0^6$ ml^{-1} viable cells Gram negative *Escherichia coli* (ATCC 25922) , equivalent to 1 McFarland tube suspended in 1µl phosphate-buffered saline(PBS, 137mM NaCl, 2.7mM KCl, 8.1 mM Na_2HPO_4/ 1.3mM KH_2PO_4, pH 7.2) and allowed to recover for 24 h in separate laboratory cages. Sugar and semi engorged blood fed females were prechilled at-20°C for 10 min, and dissected in cold

phosphate buffered saline. The technique of tissue isolation was similar to that reported by Russell *et al.* (1991). The midguts were washed three times with PBS,pH7.2 and homogenized with a mechanical homogenizer and then spun down at 10000g for 10 minutes. The supernatants were kept at-20°C until used.

Erythrocyte suspension preparation:

Formalinized rabbit, cow, guinea-pig, rat and mouse blood was purchased from the Egyptian Organization for Biological Products and Vaccines (VACSERA), Cairo, Egypt.& Fresh peripheral human blood (types A, B, and O, RH^+) in citrate/dextrose was obtained from normal human volunteers. All blood samples were prepared for hemagglutination activity (HA) and hemagglutination inhibition assays (HIA) as described by Ayaad *et al.* (2009) with some modifications. RBCs were washed in 10 ml PBS (pH 7.2) The concentration of the original suspension was adjusted to 2% (v/v) in PBS and kept at +4° C until used in HA and HIA. Tested sugars and glycosubstances were purchased from (Sigma Chemical Co. Switzerland).

Hemagglutination and hemagglutination inhibition assays:

Crude mid gut extract and isolated lectins were prepared for HA and HIA .The determination of HA was performed in 96-well U-shaped microtitration plates (Corning) by serial two-fold dilution of 25 µl lectin samples in 25 µl TBS/HCl Ca^{2+}-Mg^{2+}free pH 7.4 (50 mM Tris /HCl , 100mM NaCl,) and 25 µl of 2% (v/v) suspension of the various mammalian RBCs in 0.15 M NaCl . The titer of HA was evaluated after 2 h incubation at room temperature. Unagglutinated RBCs formed a clear dot on the bottom of the well, whereas agglutinated targets formed a diffuse mat. The agglutination titers were determined microscopically and expressed as the reciprocal value of the last sample dilution causing agglutination. The HA in the last test well with positive hemagglutination was defined as 1 HA unit. To determine whether the hemagglutination required calcium, portions of about 100 µl of purified lectin preparation was dialyzed against 200ml of TBS/ Ca^{2+}- Mg^{2+} pH 7.4 (50 mM Tris /HCl , 100mM NaCl, 2mM $CaCl_2$ and 1mM $MgCl_2$) either with or without 500 mM EDTA for 24 h with two changes of buffer. HA assays were then performed using rabbit RBCs washed in the corresponding dialyzing buffer.

The HIA was performed to determine the inhibitor concentration that caused a 50% decrease of HA (HIA 50%). The tested monosaccharides include D-(+)-galactose, D-(+)-glucose, D-(+)-mannose; the oligosaccharides lactose, trehalose and D-(+)-raffinose ; and the sugar derivatives N-acetyl-D-galactosamine, N-acetyl-D-mannosamine and N-acetyl-D-glucosamine . Other glycosubstances as laminarin , fetuin and mucin were also used. All sugars and glycosubstances are purchased from (Sigma, Switzerland) Stock solutions of sugars and glycosubstances were prepared in TBS/HCl Ca^{2+}- Mg^2 free pH7.4 subsequently, 25 µL of mid gut homogenate or isolated lectin preparations were added to each well, mixed gently by shaking, and incubated for 1 h at RT. Then 50 µL of 2% rabbit RBCs suspension was added, and the concentration of these sugars and glycosubstances required to cause 50% inhibition (IC_{50}) of HA were determined. For each inhibition test, serial two-fold dilutions of 25 µL of the inhibitors in TBS/HCl pH 7.4 in microtiter plates were used. Subsequently, 25 µL of lectins were added to each well, mixed gently, and incubated for 1 h at RT. Then 50 µL of 2% RRBCs suspension were added. The minimum concentration of these sugars and glycoconjugates required to cause 50% inhibition (IC50) of HA was identified. All determination were done in triplicate. Wells without lectin or inhibitors were used as control (Goldstein and Hayes,1978).

Bacterial agglutination assay:

For the bacterial agglutination assay, the standard strains Gram negative *Escherichia coli* (ATCC 25922) and Gram positive *Staphylococcus aureus* (ATCC 6538) live cells were resuspended in TBS pH 7.4 at a concentration of 1.1×10^6cells ml^{-1}(suspension adjusted to 1 Macfarland turbidity standard) and agglutinating activities of both crude midgut and purified lectins were assessed by using the same method described for the hemagglutination assay .

Isolation and characterization of lectin

***$(NH_4)_2SO_4$ fractionation*:**

C. quinquefasciatus female whole midgut extract proteins were precipitated with stepwise increasing saturation percentages of ammonium sulfate (30-80% saturation fractions). Precipitates showing positive hemagglutination using rabbit RBCs as indicator were pooled, and subsequently dialyzed against three changes of 50 mL TBS/ HCl pH 7.4 at 4°C.

***Ion-exchange chromatography*:**

The dialyzed samples were centrifuged, and the supernatants were subjected to ion-exchange chromatography using a DEAE-cellulose (Bio-Rad) column (1.0 x 30 cm) equilibrated with TBS and eluted by NaCl of stepwise increased concentration (0.02-0.25M) at a flow rate of 1.0 mL min^{-1}. The hemagglutination activity of the eluted protein fractions were estimated against rabbit RBCs. Fractions corresponding to each peak showing positive agglutination activity were pooled and dialyzed overnight against TBS/HCl (containing 100 mM NaCl) pH 7.4 at +4°C. Active peaks were pooled and used for affinity chromatography.

***Affinity chromatography*:**

A Sepharose 6B affinity column was prepared as follows: 75 mg GluNAc (Sigma) was coupled to 3 g of CNBr-activated Sepharose 6B (Pharmacia, Uppsala, Sweden) according to the method described by Komano *et al.* (1980) with slight modification. The affinity column (20 × 1.5 cm) was pre-equilibrated with TBS and run at a flow rate of 0.33 ml/min. Crude blood fed female mosquito mid gut extract (100 ml) diluted in TBS pH 7.2 buffer to 5 ml (protein concentration about 1 mg/ml) was loaded on the column and incubated for 12 h with the affinity sorbent at +4°C. The column was washed with100ml of TBS/HCl pH 7.4 buffer until the absorbance at 280 nm of the effluent was stable at zero, and specifically eluted with 25ml 0.3 M D-(+) glucose in TBS buffer. Fractions (2.5 ml) were collected, dialyzed against distilled water overnight, and then tested separately , for HA against rabbit RBCs . The active fractions were pooled, lyophilized and stored at -70°C for further characterization.

***NMR spectroscopy*:**

Lyophilized lectin fractions isolated from the GluNAc-CNBr-Sepharose-6B affinity chromatography column were transferred into 5-mm NMR tubes. Spectra were acquired at an ^1H observation frequency of 600.13 MHz and a spectral width of 6, 410 Hz with a relaxation delay of 3 s and an acquisition time of 3 s. A line–broadening factor of 1.0 Hz was applied to all free induction decays prior to fourier transformation .Chemical shifts were referenced to the α – glucose anomeric proton resonance (85.233). Spectra were phase and baseline corrected prior to analysis . Signal assignment was facilitated by acquisition of a suit of two- dimensional heteronuclear NMR spectra, ^1H-^{13}C heteronuclear single –quantum coherence and ^1H-^{13}C heteronuclear multiband correlation experiments (Nicholson *et al.*,1995).

Protein determination:

The protein concentrations of the crude mid gut extract and the purified lectin were determined by the method of Bradford (1976) and bovine serum albumin (BSA) was used as standard.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE):

SDS/PAGE was carried out by the method of Laemmli (1970).Proteins of either crude midgut extract or purified lectins were denatured with 1% SDS and 2% 2-mercaptoethanol by heating at 100°C for 10 min and loaded on SDS slab gels containing 12% acrylamide. After electrophoresis, gels were stained in a freshly made ammoniacal silver nitrate solution for 15 minutes using the SilverQuest Silver stain kit(Invitrogen) according to the method described by (Rabilloud,1990) . After staining, the gel was washed 4 times in deionized water for 4 min. The images were developed in a solution containing 200 ml of water, 1 ml of 1% citric acid and100 µl of 37% formaldehyde for 2 to 15 min. When a slight background stain appeared, development was stopped by rinsing gel with 3 changes of ~200 ml water.

Isoelectric focusing (IEF):

IEF of the purified lectin was performed with an LKB electrophoresis apparatus , using polyacrylamide IEF (Biorad , USA) plate gels (pH 3.6-9.3, Sigma) , according to the method described by O'Farrell (1975). Proteins (15 µg in 2µl distilled H_2O) were applied to the gel plate and focused at a constant power of 20 W for 2h .After the completion of the run the gel was fixed in sulphosalicylic acid containing 10% trichloroacetic acid for 20 min and stained with 0.2% Coomassie Blue R250 in the destaining solution (30% ethanol and 10% acetic acid). Protein bands were visualized after destaining. Focusing protein marker kit (pH 3.6 -9.3, Sigma) containing the following pI reference proteins, amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.6), β-lactoglobulin (pI 5.1), carbonic anhydrase II (bovine, pI 5.9), carbonic anhydrase I (human, pI 6.6), myoglobin (pI 6.8, 7.2), L-lactate dehydrogenase (pI 8.3, 8.4, 8.6), and trypsinogen (pI 9.3) was used for calibration.

Effect of temperature

The effect of temperature on crude and isolated midgut lectins activity was also investigated, using aliquots of 25µl lectin solution in TBS, were kept at 4°C or heated, in a water bath for 1 h at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C. The agglutinating activities of the different lectins were thereafter assessed at room temperature against rabbit RBCs.

RESULTS AND DISCUSSION

Relying on the principal evidence that the majority of vector-borne pathogens are acquired when the arthropod vector ingests an infective blood meal and the arthropod midgut serves as both barrier and gateway to pathogen invasion. The present study attempted at the isolation of the culicid *C. quinquefasciatus* midgut lectin using a combination of $(NH_4)_2SO_4$ fractionation, ion-exchange and affinity chromatographies . Enhancement of agglutinating activities upon infection using *E. coli* live cells (as tested with rabbit RBCs as indicators), suggesting that this lectin is a major component of *C . quinquefasciatus* that may have a role in the defense reaction of insects.

Effects of induction and blood meal feeding on lectin production:

Hemagglutination titers was varied regarding to the feeding status of female mosquitoes as well as to the effect of induction using $1.1\times 10^{6} ml^{-1}$ *E.coli* live cells. The presented data in Table (1) show that great increase of HA of crude midgut homogenate lectin towards various mammalian erythrocyte surface moieties ranged from1:2 to 1:128 and this indicated the enhancement effects of the humoral

induction using *E. coli* cell suspension as an elicitor. Table, 1 also reveal overall lectins HA blood meal enhancement and the hemagglutination titers were higher in blood-fed females than in sugar- fed towards the various RBCs . Therefore, all the following investigations were conducted on induced blood fed females. This could be supported through considering the work of Ratcliffe and Rowley (1983), Pendland and Boucias (1985) and Saad *et al.* (1995) have recorded more or less similar data in different insect species together with the crustacean *Penaeus japonicas* (Yang *et al.*,2007). Moreover, blood meal feeding also significantly elevated the isolated *C. quinquefasciatus* mid gut lectin production. Similar observations were demonstrated by Basseri *et al.* (2004) in the mid gut lectins of different geographical populations of *Anopheles stephensi*.

Table: 1. Effects of induction and blood meal feeding on lectin hemagglutination activity

Mammalian RBCs	**Natural crude midgut lectin**		**Induced crude midgut lectin**	
	Hemagglutination titer		Hemagglutination titer	
	Blood fed	**Sugar fed**	**Blood fed**	**Sugar fed**
Human A+	8	2	16	4
Human B+	16	4	32	8
Human O+	4	2	16	4
Goat	2	2	16	8
Rabbit	32	8	128	64
Guinea pig	-	-	-	-
Rat	-	-	-	-
Mouse	4	2	8	4

Bacterial agglutination:

To test whether Cqlec can interact with the surface moieties of microorganisms, we performed an agglutination assay using *S. aureus* as an example of G(+) bacteria and *E. coli* as an example of a G(-) bacteria (Table 2). Both bacterial strains were comparably agglutinated by Cqlec with the highest agglutinating titer of 256 depicted for the isolated lectin towards *E. coli* cells, however comparable moderate titer of 64 was obtained against *S. aureus* surface moiety. These results indicated that Cqlec recognized the surface molecules on both Gram (-) and Gram (+) bacteria with variable specificities. Yu and Kanost (2000) have detected that *M. sexta* immulectin-2 (IML-2) binds to LPS from *Escherichia coli*. Promising results were also reported by Yu *et al.* (1999) for *M. sexta* immulectin that bind to bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA), as well as to fungal β-1,3-glucan). Yu *et al.* (2006)further recorded the binding specificity of IML-2 of *M. sexta* to bacterial lipid A, several smooth and rough mutants of LPS and peptidoglycan,as well as to fungal mannan and β -1, 3-glucans (laminarin and curdlan).

Table 2. Agglutination of *C. quinquefasciatus* crude midgut extract and isolated lectins against bacteria

Bacteria	**lectin activity**	
	crude lectin	**isolated lectin**
	Agglutination titer	
E. coli **G (-)**	64	256
S. aureus **G (+)**	32	64

Hemagglutination and carbohydrate specificities:

Regarding the screening of the HA and HIA of the crude and isolated lectins towards various erythrocytes surface determinants, table (3) demonstrate high affinity against rabbit RBCs with comparable potancies between crude and isolated lectins followed by human RBCs with the variable types tested . However, no HA appeared directed towards rat or guinea pig , consequently mouse and goat RBCs were moderately agglutinated for both lectins. Rabbit RBCs specificity was also detected for the midgut lectins of *An stephensi* (Basseri , 2004) . Other insects show variable preferences to RBCs of different vertebrates (Komano *et al.*, 1980; Ingram and Molyneux, 1990; Ayaad,2008 and Ayaad *et al.*,2009).

Table: 3. Hemagglutination of crude midgut extracts and isolated lectins of *C. quinquefasciatus* against various erythrocytes

Mammalian RBCs	**lectin activity**	
	crude lectin	**isolated lectin**
	HA titer	
Human:		
A+	16	64
B+	32	64
O+	16	128
Goat	16	16
Rabbit	128	512
Guinea pig	-	-
Rat	-	-
Mouse	8	32

The HA inhibition of sugars & glycosubstances towards rabbit RBCs were used in the carbohydrate specificity study (Table 4). Variable carbohydrate specificities were detected in a comparable pattern between crude and Cqlec, presenting high HIA by D(+) mannose followed by D (+) glucose and N-acetyl-D-glucosamine . Raffinose followed by N-acetyl-D-manosamine were found to be moderate inhibitors. Non of the lectins were inhibited by galactose, lactose, trehalose or fetuin (1%) but the glycosubstances mucin and laminarin showed strong inhibitition using low concentration of (0.003%). The mannose /glucose binding specificity of Cqlec seem to be important in recognition of these sugars on cell and pathogen surfaces. In this respect McGreal *et al.* (2004) reported that carbohydrate binding specificity is crucial in recognition of carbohydrates on cell surfaces, circulating proteins, as well as those on pathogen surfaces. Heterologous patterns were also detected between different geographical populations of *An. stephensi* mid gut lectins (Basseri ,2004) suggesting great carbohydrate specificity . According to Mäkelä, (1957) monosaccharides classification , the isolated Cqlec fall into group III which reacts with glucose / mannose , whereas the isolated ACL of the comparable culicid *Ae. caspius* would be classified as group II , an N- acetyl-D- galactosamine /galactose as defined by (Ayaad, 2008) . It is extremely interesting, and significant that the culicid species are not just confined to one carbohydrate binding group. Therefore the diverse specificities of lectins within culicidae may be related to the physiological functions of these molecule components based on lectin – carbohydrate interactions . Similar mannose binding lectin was detected among the multiple lectins defined by Chen *et a.l* (1993) in the cockroach *Blaberus discoidalis* . For lectin-carbohydrate interactions, there is growing evidence that they can mediate the infection of parasites to their insect vectors, and their disruption can affect subsequent

establishment of the parasite within the vector. The role of lectins and carbohydrate in mosquito-pathogens interactions remains undefined.

Table: 4. Inhibition of HA of *C. quinquefasciatus* mid gut lectins

Sugar or glycosubstance	Minimum concentration (mM/or %) required for 50 % inhibition (IC_{50}) to HA	
	Crude midgut lectin	Isolated lectin
D-(+)-galactose	>100	100
D-(+)-glucose	50	12.5
D-(+)-mannose	25	3.13
raffinose	50	25
lactose	200	>200
Trehalose	200	200
N-acetyl-D-galactosamine	>100	>200
N-acetyl-D-glucosamine	>50	6.25
N-acetyl-D-mannosamine	50	50
Laminarin	0.3%	0.003%
Fetuin	>1%	0.1%
Mucin	0.003%	0.003%

Values > 200 mM sugars or >1 % glycosubstance indicate that no inhibition of agglutination was recorded.Data were obtained from a single experiment repeated four times.Rabbit RBCs are used as indicator.

Physicochemical properties of crude and purified lectins:

Heat stability. HA was reduced to 70% when *C. quinquefasciatus* crude midgut extract was incubated at 100°C for 1h. However, the isolated lectin Cqlec displays the same titration after heating at 100°C for the same period of time suggesting heat resistant type lectin. Therefore, Cqlec appears to be heat resistant. The heat resistance lectins were also evaluated in the coleopteran *L. decemlineata* (Minnick *et al.,* 1986) the orthopteran *L. migratoria* (Drif and Brehelin, 1994) and the culicid *Ae. caspius* (Ayaad,2008) where the lectin activity have been reported to be heat resistant when subjected to 100 °C. However, heat lability is characteristic for lectins of some other insects, e.g. the orthopterans *T. commodus*, (Hapner and Jermyn 1981), and *M. sanguinipes* (Stebbins and Hapner 1985), the dipteran *Glossina fuscipes* (Ingram and Molyneux, 1990) and the orthopteran *S. gregaria* (Ayaad,2004 and Dorrah *et al* .,2009).

Divalent cation requirement:

The isolated Cqlec as well as its crude form do not require Ca^{2+} for expression of activity. When the final concentration of EDTA in the reaction mixture reached 50 mM, HA was unaffected. These results may suggest that Cqlec is a calcium-independent lectin. Coincided results were also obtained by us in the comparable culicid *Ae. caspius* (Ayaad,2008) and the other insect lectins, e.g. *Allomyrina dichotoma* (Umetsu *et al.,* 1984), *Glossina fuscipes* (Ingram and Molyneux, 1990), *Oecophylla smaragdina* (Hassan and Absar, 1995), *Phlebotomus duboscqi* (Volf *et al.,* 2002) and the crustacean invertebrate *p. japonicas* (Yang, *et al.*,2007) .Yu *et al* (2006) have also reported that Ca^{2+} independent lectins are involved in innate immune responses, such as prophenoloxidase activation. However , the present results comes in contrast with the general characteristic of most C-type - lectins of *T. commodus, M. sanguinipes, S. exigua, E. tiaratum,* and nymph & adult *S. gregaria* (Hapner & Jermyn ,1981 ;Pendland & Boucias 1986 ;Stebbins & Hapner, 1985;

Richards *et al.*, 1988, Ayaad, 2004 and Dorrah *et. al.*,2009) that revealed a complete loss of HA in the presence of the strong chelator EDTA .

Isolation of Cqlec

Preliminary tests showed that the supernatant of crude midgut extract of induced blood fed *C. quinquefasciatus* females is able to agglutinate rabbit RBCs, in the absence of Ca^{2+} , at a titer of 64 (Table 1) . This HA was significantly inhibited by D-(+)-mannose (IC_{50}=25 mM) and to a certain extent (IC_{50}= 50 mM) D- (+) - glucose, raffinose followed by N-acetyl-D-glucosamine (Table 4). Therefore, the presence of lectins in the midgut extract was indicated.

Salting out of proteins of the midgut extract by $(NH_4)_2SO_4$ showed that the maximum HA was recovered from the precipitated proteins at 30-80% saturation level. When these proteins were subjected to ion exchange chromatography on CM-cellulose and eluted with a linear gradient of 0.02-0.25 M NaCl, 2 separate peaks, indicated as I and II of protein fractions were obtained (Fig. 1). Among them, only fractions of peak I revealed HA. When these active fractions were pooled and further isolated by affinity chromatography on GluNAc -CBr-Sepharose 6B column and eluted using 3M D (+) Mannose, HA was detected in fractions of the peak I of figure (2), at an agglutination titer of 512 using rabbit RBCs as indicator. Therefore, these fractions seem to contain the putative lectin (Cqlec). Similarly, Chen *et. al* (1993) have purified a mannose binding lectin among the multiple hemolymph lectins of *Blaberus discoidalis* cockroach. They proposed that the occurrence of complex carbohydrate-protein interaction that arise from different tissue lectins within the same individual are possibly dependent on other binding sites, such as those that interact with the prophenoloxidase cascade. Therefore, the biological importance of lectins diversity still need great investigations in order to be well understood.

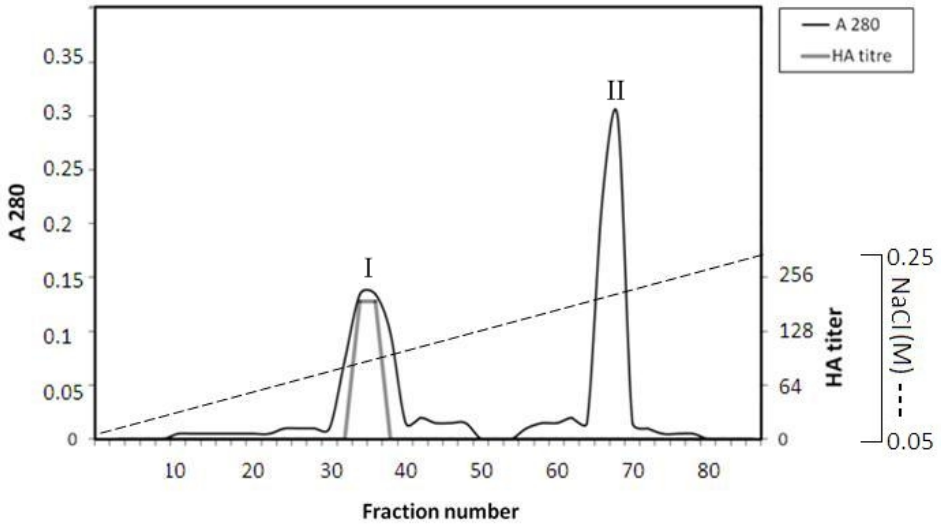

Fig.1. Ion exchange chromatography of 30-80% cut from$(NH_4)_2SO_4$ fractionated on CM-cellulose column equilibrated with TBS. Samples applied at a flow rate of 1.0 ml min^{-1}. Elution of the bound proteins using a linear gradient of 0-0.5 M NaCI. Fractions of peak I were pooled and have HA against rabbit RBCs; but those of peak II have none.

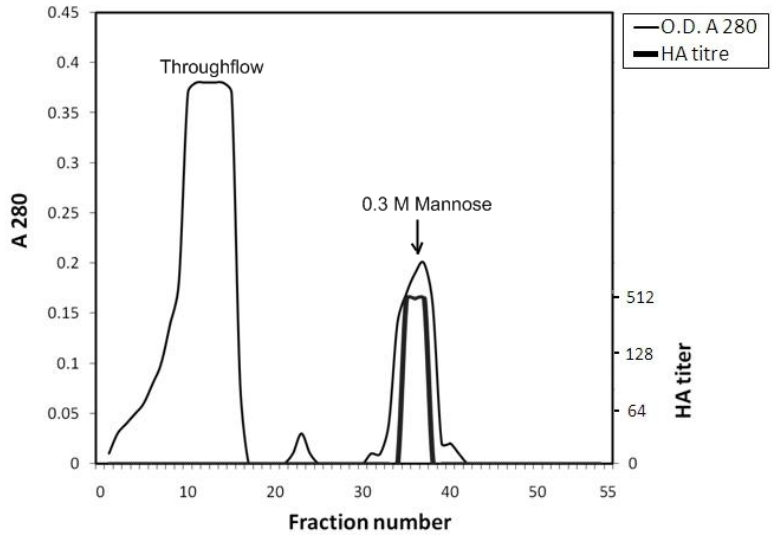

Fig. 2. Typical chromatograph showing GluNAc-CNBr- Sepharose 6B affinity chromatography purified Cqlec lectin preparation. Arrow indicates , 3M D -(+)Mannose elution in TBS.

Molecular characterization of Cqlec:

Molecular weight analysis of Cqlec under reducing conditions revealed that the obtained lectin appeared nearly homogenous on SDS/PAGE, that estimated a single subunit of about 34.5 KDa (Fig.3 A) . This component was further confirmed through pI (determination by IEF) to lie in the acidic range of 6.3 (Fig. 3B) and similar to most of that of other insect lectins where the pI values ranges from 4.7 to 6.4, e.g. the dipterans *G. fuscipes* (Ingram and Molyneux, 1990) and *Ae. caspius* (Ayaad, 2008) , the lepidopteran *M. sexta* (Minnick *et al.,* 1986) and the orthopterans *L. migratoria* (Drif and Berhelin, 1994) and *S. gregaria* (Dorrah *et al.*,2009).

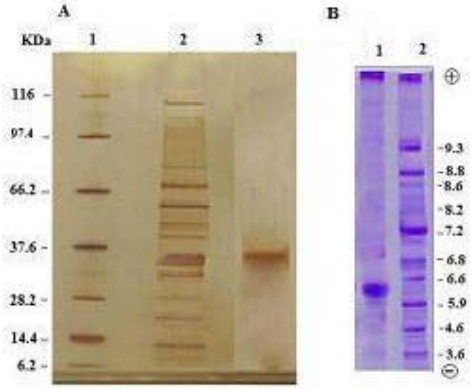

Fig. 3. SDS/PAGE and IEF of *C. quinquefasciatus* crude and purified midgut lectins.

A) Lectins were loaded on 12% polyacrylamide gel under reducing conditions.as indicated in materials and methods. Gels were stained with sliver nitrate , lanes 2 and 3 showed crude and purified Cqlec, respectively and the molecular weight of the standards lane ,1(in kilodaltons) are indicated on the left.

B) IEF of purified Cqlec (lane , 1) using polyacrylamide gel (Sigma) and (lane, 2) standards calibration Kits of Sigma. COBB staining.

The separation of a single subunit lectin was also detected by Saad *et al.* (1995) for the hemolymph lectin of *Parasarcophaga dux* using Sepharose4B affinity chromatography .Moreover we have isolated another homogeneous lectin from the

culicid *Ae caspius* whole body homogenate using anion exchange and gel filtration chromatographies (Ayaad,2008)and it was estimated to be represented by two subunits with two different molecular weights of approximately 32 and 34 under the reducing conditions by 2-mercaptoethanol. The presence of the 34.5 KDa subunit in the isolated mid gut lectin of the present culicid candidate *C. quinquefasciatus* and the corresponding of *Ae. caspius* obtained from whole body homogenate may indicate conserved lectin epitopes of culicidae from different geographical regions. Most of the insect lectin isolated to date presents two different subunits with disulfide linkage, e.g., the mosquito *Ae. stephensi* (Chen and Billingsley, 1999), lectins of the orthopterans *T. commodus* (Hapner and Jermyn, 1981), *M. sanguinipes* (Stebbins and Hapner, 1985) and *S. gregaria* (Dorrah *et. al.,* 2009) and the dictyopteran *B. discoidalis* (Chen *et al.,* 1993).

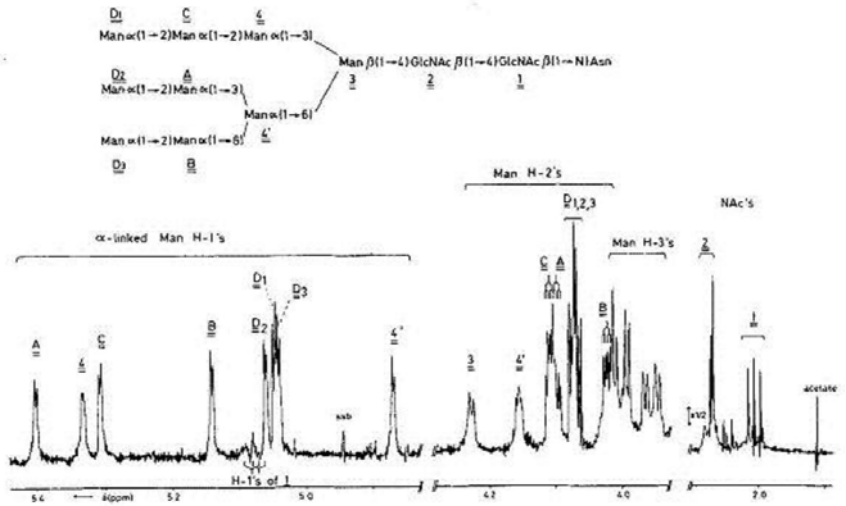

Fig. 4. NMR pattern of carbohydrate component in the isolated Cqlec.

Group regions of resolution- enhanced 500-MHz ^{1}H NMR spectrum of Man $_{9}$-GlcNAc $_{2}$Asn component in D_2 O at 300 K . The numbers and the letters in the spectrum refer to the corresponding carbohydrate residues in the structure.

NMR pattern of carbohydrate component in the isolated Cqlec molecule

Lyophilized lectin fraction isolated from the GluNAcCNBr - Sepharose - 6B affinity chromatography column were transferred into 5-mm NMR tubes. Group regions of the resolution – enhanced 500-MHz ^{1}H NMR spectrum of Man $_{9}$-GlcNAc $_{2}$Asn component in D_2 O at 300 K were obtained. As indicated in Fig. (4) the numbers and letters in the spectrum refer to the corresponding residues in the structure. The presented results on 2D - NMR may refer to the carbohydrate component of the isolated Cqlec to be a predicted glycoprotein component of mannaose , N-acetylglucosamine in a molar ratio of 9 :2 , respectively,attached to the Asn amino acid residue. It would be promising to fully understand the Cqlec's binding properties in further investigatetion of this lectin at the nucleic acid level, such as its molecular cloning , gene expression control and structural information from crystallography or solution 3 NMR.

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ARABIC SUMMARY

عزل عامل تلزن لبعوضة كيولكس كوينكيفاسكياتس (ثنائيات الأجنحة ، كيوليسيدى)

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تم التعرف على عامل للتلزن (ليكتن) فى مستخلص المعى الاوسط للبعوض كيوليكس بيبينس كوينكيفاسكياتوس عن طريق الكشف على مدى قدرة تفاعله التلزنى تجاه بعض سلالات البكتريا النشطة مثال *اشريشيا كولاى* و *ستافيلوكوكوس اوريوس*، وايضا تجاه بعض انواع كرات الدم الحمراء للفقاريات. وباستخدام تقنيات التفتيت الملحى و المبادلات الأيونية وعمود الصلة الكروماتوجرافى تم فصل هذا الليكتن بدرجة نقاء عالية. أعطى تحليل نقاط التعادل الكهربى قيمة 6.30 كما بين الفصل الكهربائى تحت ظروف الإختزال انه كبروتين مكون من وحدات بقيمة 34,5 كيلو دالتون. كانت أعلى معدلات التلزن تجاه كرات الدم الحمراء للأرانب و*لأشريشيا كولاى* . وقد أظهرت التجارب تواجد فروق معنوية فى الإرتباط (التخصصية) بالسكريات والكربوهيدرات بمقارنة الإناث المغتذية بوجبة من الدم و الأخرى التى تغذت على السكريات فقط ، كما يتزايد معدل التلزن فى حال الإستحثاث. وتعد الدراسة كأول تسجيل لتواجد اللكتينات فى المعى الأوسط لهذا النوع من البعوض. ولاتعتمد قدرة التلزن لهذا اللكتين على وجود أيونات الكالسيوم فى الوسط، كما أن لا يتأثر بالحرارة. ويتم تثبيط المقدرة التلزنية لهذا اللكتين ببعض السكريات الأحادية مثال المانوز والجلوكوز.و أظهر تحليل الرنين النووى المغناطيسى ثنائى البعد لهذا الليكتن ك كبروتين مرتبط بسكريات (جليكوبروتين) هذا ويتواجد مناطق إرتباط للأحماض الأمينية بالمانوز.