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Cloning and characterization of a mannose binding C-type lectin gene from salivary gland of *Aedes albopictus*

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Abstract

Background: The studies on sialomes have shown that hematophagous mosquito saliva consists of a lot of pharmacologically active proteins, in which C-type lectins have been identified and regarded as an important component of saliva. The previous studies showed that C-type lectins play crucial roles not only in innate immunity but also in promoting disease transmission in mammals. However, the function and mechanism of C-type lectins from the mosquito sialome is still elusive.

Methods: A putative C-type lectin gene (*Aalb_CTL1*) was cloned and expressed from *Aedes albopictus* by RT-PCR. The deduced amino acid sequence was analyzed by bioinformatic methods. The gene expression profiles in different tissues and various blood-fed stages of *Ae. albopictus* were examined by Real-Time qRT-PCR and the biological functions of the recombinant mature Aalb_CTL1 were tested by hemagglutination and sugar inhibitory agglutination assays. Moreover, the capabilities of rAalb_CTL1 against microorganisms were measured by microbial-agglutination assay.

Results: The full-length Open reading frame (ORF) of *Aalb_CTL1* consisted of 462 bp, encoding 153 amino acid residues. The deduced amino acid sequence contained a putative signal peptide of 19 amino acids. It also contained a CRD domain with a WND (Trp¹³⁷-Asn¹³⁸-Asp¹³⁹) motif that needed calcium for the hemagglutinating activity and an imperfect EPS (Glu¹²⁸-Pro¹²⁹-Ser¹³⁰) motif that had a predicted ligand binding specificity for mannose. The mRNA level of *Aalb_CTL1* was much higher in female mosquito salivary gland than those in fat body and midgut which was down-regulated in salivary gland after blood feeding. The rAalb_CTL1 contained not only hemagglutinating activity and a high affinity with mannose but also agglutinating activity against yeast *C. albicans* and Gram-positive bacteria *S. aureus* in Ca²⁺ dependent manner.

Conclusion: Aalb_CTL1 was a mannose-binding C-type lectin and constituted one of the important components in saliva of *Ae. albopictus*, which could be involved in the defense against yeast and Gram-positive bacteria infection.

Keywords: C-type lectin, Salivary gland, *Ae. albopictus*, Prokaryotic expression, Agglutinating activity

Background

C-type lectins (CTLs) constitute the largest and most diverse super family of proteins that share a characteristic carbohydrate recognition domain (CRD) with two or three pairs of disulfide bonds [1,2]. Due to their ability to bind to specific carbohydrates on the surface of

microorganisms, CTLs have been regarded as primary candidates for pattern recognition receptors (PRRs) to mediate pathogen recognition, which play an important role in the clearance of pathogens in innate immunity in vertebrates [3,4]. In invertebrates, the previous studies demonstrated that CTLs are also involved in innate immune responses, including the promotion of phagocytosis [5], nodule formation, encapsulation, melanization [6,7] and the activation of prophenoloxidase [8].

Mosquitoes are one of the most important medical arthropods. The harm to human beings caused by mosquitoes

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are not only in its harassment and blood-feeding habits, but also in its transmission of various diseases, such as malaria, dengue fever, Japanese encephalitis, yellow fever, Venezuelan equine encephalitis and Ross River fever [9]. The whole genomics studies demonstrated that there are 25, 39 and 55 CTLs respectively in *Anopheles gambiae* [10], *Aedes aegypti* [11] and *Culex quinquefasciatus* [12], which mostly are soluble proteins containing a single CRD without accessory domains. Several CTLs have exhibited a diverse range of function in mosquito innate immunity. The CTL4 and CTLMA2 from malaria vector *An. gambiae* can inhibit *Plasmodium berghei* ookinete melanization, indicating that they act as agonists of *Plasmodium* development in the vector [13]. In *Ae. aegypti*, a C-type lectin (AegmosGCTL-1) induced by West Nile Virus (WNV) infection, can interact with WNV in a calcium-dependent manner, and facilitate flavivirus invasion *in vivo* and *in vitro* [14]. By *in vivo* reverse genetic analysis, 9 mosGCTL genes from *Ae. aegypti* are identified as important susceptible factors to produce DENV-2 infection, of which mosGCTL-3 exhibits the most significant effect [15]. Based on the evidence above, it can be suggested that mosquito CTLs could be used as ligands by pathogens to promote the infection of a vector. On the other hand, the CTL4 and CTLMA2 have also been found to be required for the clearance of *Escherichia coli*, but not *Staphylococcus aureus*, suggesting that they play a role in fighting against Gram-negative bacteria infection [16]. Therefore, it could be inferred that the CTLs from mosquitoes could serve pleiotropic functions in the interaction of mosquito and pathogen.

Ae. albopictus is an efficient laboratory vector for a large number of arboviruses such as dengue virus, yellow fever virus, West Nile virus and several others. In China, it is also an important vector to transmit dengue fever. In *Ae. albopictus*, two CTLs, AY826070 and AY806069, have been identified by sialotranscriptome analysis [17]. In this study, the open reading frame (ORF) of AY826070 from *Ae. albopictus* Guangzhou strain (named *Aalb_CTL1*) was cloned by RT-PCR and expressed with prokaryotic expression system. The results of Real-Time quantitative RT-PCR (Real-Time qRT-PCR) showed that *Aalb_CTL1* was expressed specifically in female mosquito salivary gland. rAalb_CTL1 demonstrated agglutinating activity against animal erythrocyte, Gram-positive bacteria and yeast. It laid a preliminary foundation to make further study on the role of *Aalb_CTL1* in salivary immunity.

Methods

Mosquitoes and sample collection

Ae. albopictus mosquitoes (Guangzhou strain) were kindly provided by Prof. Zhao Tongyan from Beijing Institute of Microbiology and Epidemiology and routinely reared at $25 \pm 1^\circ\text{C}$ with 75-80% relative humidity

and a 14:10 (light: dark) photoperiod. Mosquitoes were fed on anesthetized mice.

Tissue samples including salivary glands, midgut and fat body from the sugar-fed adult female mosquitoes at 3–5 days old were dissected in the normal saline (NS: 0.9% NaCl, pH 6.5) and immediately soaked in the TRIzol Reagent (Invitrogen Life Technologies) and stored at -80°C until use. Six biological replicates, each consisting of salivary glands from 6 mosquitoes, midgut and fat body from 1 mosquito, were collected for each sample.

Salivary glands were further dissected at various time points: 3–5 days post emergence (PE) and 0 h, 24 h and 72 h post blood-feeding. Mosquitoes, 3–5 days PE, were fed on anesthetized mice for 2 h, and the mosquitoes with engorged abdomen were selected and regarded as 0 h post blood-feeding. Six biological replicates, each consisting of salivary glands from 6 mosquitoes, were collected for each time point.

Full-length ORF cloning of *Aalb_CTL1*

Total RNA was extracted from female mosquito with TRIzol Reagent according to the manufacturer's protocol. The single-stranded cDNA was synthesized using PrimeScript™ RT Kit (TaKaRa). *Aalb_CTL1* specific primers for full length ORF (Table 1) were designed according to the published cDNA sequence of *Ae. albopictus* [GenBank: AY826070]. The desired products were amplified by 35 cycles of PCR using 2 μl synthesized cDNA as template in 50 μl reaction containing 5 μl $10 \times$ Ex Taq buffer (containing Mg^{2+}), 40pmol each primer and 5 U Ex Taq DNA Polymerase (TaKaRa). The PCR conditions were 5 min at 94°C for one cycle, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min. In the last cycle, the PCR product was further incubated at 72°C for 7 min to allow the completion of DNA synthesis.

The amplified products were purified using DNA purification kit (TaKaRa). The purified PCR products were cloned into the pMD18-T cloning vector (TaKaRa) at mole ratio 5:1 at 16°C and then transformed into freshly prepared *E. coli* strain DH5 α competent cells. Recombinant clones were selected using blue/white screening on X-gal (25 $\mu\text{g}/\text{mg}$)/IPTG (25 $\mu\text{g}/\text{ml}$)/Ampicillin (50 $\mu\text{g}/\text{ml}$) LB plates. The clones were confirmed by *Nde I* and *Hind III* restriction enzymes digestion. Three positive clones were picked up for sequencing in both directions.

Sequences analysis

The homology analyses of the nucleotides and deduced amino acid sequences of *Aalb_CTL1* were carried out using the BLAST programs at the NCBI (<http://www.ncbi.nlm.nih.gov/blast>). Translation of the cDNA was performed using the Expert Protein Analysis System

Table 1 Primers used in present study

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
<i>Aalb_CTL1</i> ORF cloning			
<i>Aalb_CTL1</i>	ATGGGGCAGCCATCATCATCATCAT	ACATTCGTTCCGACACAAAAAC	462
Recombinant expression			
<i>Aalb_CTL1</i>	GTCCATATGCAACAGGAATGCGACTGTA AAAATAG	GTGAAGCTTACATTCGTTCCGACACAAAAAC	405
Quantitative real-time PCR (Q-PCR)			
<i>Aalb_CTL1</i>	GCTATCTCTCTCGAGACCTCAC	CCAATCTCCAGCCGTTCTCT	130
Rsp 5	ATTACATCGCCGTC AAGGAG	TCATCATCAGCGAGTTGGTC	126

The restriction endonuclease sites in italics.

(<http://au.expasy.org>). Signal peptide and CRD domain predictions were conducted using SMART software (<http://smart.embl-heidelberg.de/>). The homologous sequences from various species were downloaded from NCBI. A phylogenetic tree was constructed using the Neighbour-joining method with the Molecular Evolutionary Genetics Analysis (MEGA 4) package.

Real-Time quantitative Reverse Transcription Polymerase Chain Reaction (**Real-Time qRT-PCR**) analysis.

Total RNA was extracted for all the tissue samples separately with TRIzol Reagent according to the manufacturer's protocol. Before reverse transcription, the RNA samples were treated with DNAase I (Invitrogen) and the single-stranded cDNAs were synthesized using PrimeScript™ RT Kit (TaKaRa). According to the cDNA full-length sequence, one pair of gene-specific primers for *Aalb_CTL1* (Table 1) was used to amplify product of 130 bp from the cDNA. Two *rsp5* primers (Table 1) were used to amplify a 126 bp fragment as an internal control to verify the successful transcription and to calibrate the cDNA template for corresponding mosquito samples [18].

Real-Time PCR amplification was carried out in an ABI 7300 Real-Time Thermal Cycler according to the manual (TaKaRa). Dissociation curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The two/double standard curve method was used to analyze the expression level of *Aalb_CTL1*. All data were given in terms of mRNA expressed as means ± SE. Significant differences between means were determined using one way ANOVA and $P < 0.05$ was considered statistically significant.

Prokaryotic expression and purification of recombinant *Aalb_CTL1*

A cDNA fragment encoding a mature *Aalb_CTL1* protein (residues 20 to 153) was amplified by PCR using Ex Taq polymerase (TaKaRa) with the specific primers for recombinant expression (Table 1). *Nde I* and *Hind III* restriction sites were added to the 5' ends of *Aalb_CTL1* primers (after the stop codon), respectively. The PCR

fragment was cloned into the pMD18-T vector (TaRaKa) firstly, it was completely digested with *Nde I* and *Hind III* (TaRaKa), and cloned into the *Nde I* and *Hind III* sites of the expression vector pET28a(+) subsequently, then the recombinant plasmid (pET28-*Aalb_CTL1*) was transformed into *E. coli* BL21 (DE3). Positive clones were screened by enzymatic digestion with *Nde I* and *Hind III* and confirmed by nucleotide sequencing. Finally, positive transformants were incubated in 100 ml LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl/1 l distilled water, pH 7.0) at 37°C with shaking at 220 rpm until the culture reached OD600 of 0.5-0.7, and 1 mmol/L isopropyl-β-D-thiogalactosidase (IPTG) was added to the medium under the same conditions for another 5 h. Bacterial cells expressing recombinant proteins were harvested and sonicated, and the inclusion bodies were resuspended in 1 × phosphate-buffered saline (PBS: NaCl 137 mmol/L, KCl 2.7 mmol/L, Na₂HPO₄ 10 mmol/L, KH₂PO₄ 2 mmol/L, pH 7.4) containing 8 M urea. Recombinant *Aalb_CTL1* protein (r*Aalb_CTL1*) was purified by gradient dialysis at 4°C overnight, and further purified with His•Bind Purification Kit (Novagen) according to the manufacturer's instructions. Purified recombinant protein was dialyzed against 1 × PBS buffer at 4°C overnight. The resulting protein was analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie brilliant blue R-250.

Hemagglutination assays

To test whether or not calcium was required for the hemagglutinating activity of r*Aalb_CTL1*, 12.5 μl of serial dilutions of CaCl₂ in NS was mixed with 12.5 μl of r*Aalb_CTL1* (~50 μg/ml). BSA instead of *Aalb_CTL1* was used as a negative control. After 25 μl 2% suspension of mouse erythrocytes was added, the mixture was incubated for 30 min at 25°C. Hemagglutination was observed under a microscope (Nikon TE2000, Japan). Assays were performed in triplicate independently.

The hemagglutinating activity of r*Aalb_CTL1* was tested using 2% (vol/vol) erythrocytes from rabbits, mice, and rats according to the method described by Luo *et al.* [19]. Erythrocytes were washed five times with NS-Ca

buffer (0.9% NaCl, 40 mM CaCl₂, pH 6.5) and then diluted with NS-Ca buffer to 2% (vol/vol) suspension. Two-fold serial dilutions (25 µl) of rAalb_CTL1 in NS-Ca buffer were mixed with 25 µl of the erythrocyte suspension in a microtiter V plate. The plate was incubated for 1 h at room temperature, and the hemagglutination was observed under a microscope (Nikon TE2000, Japan). Erythrocyte suspension mixed with serial dilutions of bovine serum albumin (BSA, Sigma) in NS-Ca buffer were processed in parallel to serve as negative controls.

Sugar binding specificity of rAalb_CTL1

The sugar binding specificity of rAalb_CTL1 was determined by an inhibitory agglutination assay. Serial dilutions (12.5 µl) of various carbohydrates (Sigma) in NS-Ca buffer, including D-mannose, D-galactose, Lactose, D-glucose, Maltose and Sucrose, were mixed with 12.5 µl of rAalb_CTL1 (12 µg/ml) and incubated for 30 min at 37°C. Then, 2% erythrocytes suspension was added, and the mixture was incubated for 30 min at room temperature. An inhibitory effect was observed at the minimum concentration of a carbohydrate required for complete inhibition of the hemagglutinating activity of rAalb_CTL1. This assay was performed in triplicate independently.

Microbial-agglutination assays

Three microorganisms were used for agglutination tests including yeast *C. albicans* (ATCC10231), Gram-negative bacteria *E. coli* (ATCC25922) and Gram-positive bacteria *S. aureus* (ATCC25923). All three microorganisms were obtained from Beijing Institute of Microbiology and Epidemiology and maintained in our laboratory. Bacteria were streaked on LB agar plates at 37°C for 18 h firstly, then a single colony was inoculated into 5 ml LB medium and cultured overnight at 37°C. The bacteria were cultured again in fresh medium at 1:100 dilution for 5 h at 37°C and collected in mid logarithmic phase by centrifugation at 6000 rpm for 5 min.

C. albicans were streaked in Sabouraud's agar plate (10 g tryptone, 40 g glucose/1 l distilled water, pH 5.6; with 20 g agar) at 25°C for 24 h, then a single colony was inoculated into 5 ml Sabouraud's medium and cultured for 36 h at 25°C. The yeast was cultured again in fresh Sabouraud's medium at 1:100 dilution for 24 h at 25°C and collected by centrifugation at 6000 rpm for 5 min.

The yeast or the bacteria prepared as above were stained by DAPI for 20 min, washed twice with TBS buffer (Tris 50 mmol/L, NaCl 150 mmol/L, pH 7.6) and re-suspended in TBS buffer at a concentration of 2×10^8 cells/ml. Then 50 µl TBS including rAalb_CTL1 at final concentration 50 µg/ml was added into 50 µl of bacteria/yeast. After incubation for 1 h at room temperature in the presence or absence of 40 mM CaCl₂, these

samples were observed and photographed under a fluorescence microscope (Nikon TE2000, Japan). BSA instead of rAalb_CTL1 was used as a negative control (BSA-Ca control).

Ethical approval

Ethical approval documents were given by the Experimental Animal Ethics Committee of Guiyang Medical College.

Results and discussion

Saliva of mosquitoes consists of a milieu of pharmacologically active substances to function not only in food uptake but also in disease transmission [20]. Sialome studies have shown that there are at least 100 proteins in mosquito saliva, and more than one CTL has been identified and predicted in mosquito sialomes [17,21,22]. However, little is known about the functions of them. In our study, we detected the agglutinating activity of Aalb_CTL1.

cDNA cloning and sequence analysis of Aalb_CTL1

Based on the cDNA sequence of *Aalb_CTL1*, gene-specific primers were designed and the ORF of *Aalb_CTL1* was cloned and sequenced from the *Ae. albopictus* Guangzhou strain. A total of 462 bp encoding a protein of 153 amino acids was obtained (Figure 1A). Sequence alignment showed there was a base mutation at position 160 from A to G, which in turn caused an amino acid change from I to V in the *Ae. albopictus* Guangzhou strain. Multiple sequence alignment of carbohydrate recognition domain (CRDs) from *Ae. aegypti* and *Cx. quinquefasciatus* demonstrated that V was more conservative than I in this position (Figure 1B). It is well known that both V and I belong to the aliphatic amino acids, but a further study would be performed to test the effect on function of Aalb_CTL1 with such an amino acid change.

According to the ORF of *Aalb_CTL1*, the deduced amino acids sequence contained a putative signal peptide of 19 residues at the N-terminal, suggesting that it should be a secreted protein and would be a component of saliva in mosquito. The calculated molecular mass of the mature Aalb_CTL1 protein (residues 19 to 153) is 15.71 kDa, with an estimated pI of 5.72. Like other C-type lectins, Aalb_CTL1 had a single characteristic CRD stabilized by two highly conserved disulphide bridges from four cysteine residues Cys 45, Cys 122, Cys 142 and Cys 150 [23] (Figure 1A). Multiple sequence alignment of CRDs (Figure 1B) demonstrated that the CRD of Aalb_CTL1 contained 13 of 14 invariant and 14 of 18 highly conserved amino acid residues, which were identified as a C-type CRD by Drickamer *et al.* [23] and Yamamoto-Kihara *et al.* [24]. A WND motif (Trp137-Asp138-Asn139) was identified in the C-terminal of CRD of Aalb_CTL1, indicating

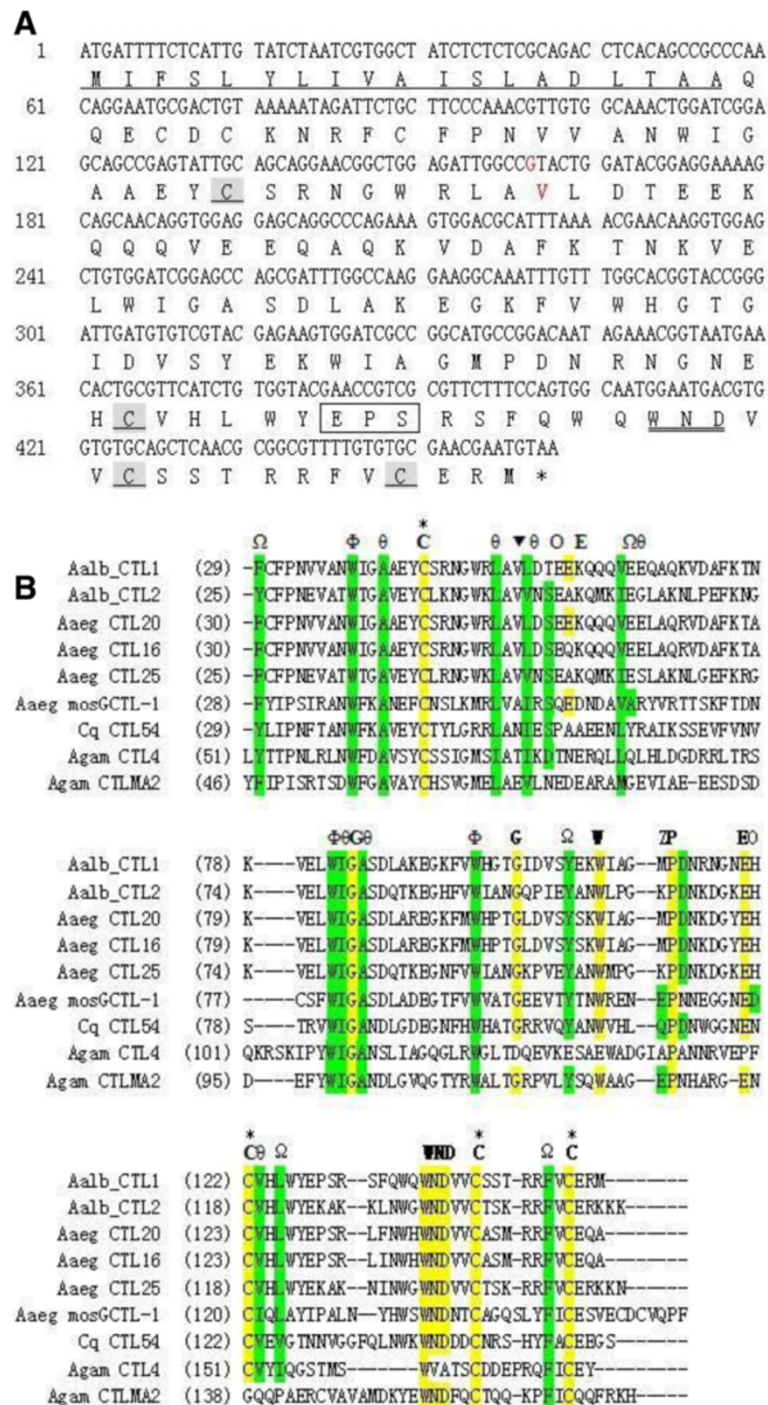


Figure 1 Sequence analysis of Aalb_CTL1. (A) ORF and deduced amino acid sequence of *Aalb_CTL1* from the *Aedes albopictus* Guangzhou strain. The amino acid sequence is represented by a single capital letter below the nucleotide sequence. The putative signal peptide sequence is underlined; four conserved cysteine residues that define the C-type lectin domain are shaded and underlined. The EPS motif for ligand binding specificity is in box. The WND motif is in double lines. The mutation position is in red. **(B)** Comparison of a putative CRD of *Aalb_CTL1* with known and putative mosquito C-type lectins. Alignment of the CRD domain from *Aalb_CTL1*, *Aaeg_CTL16* [VectorBase: AAEL000533-RA], *Aaeg_CTL20* [VectorBase: AAEL011407-RA], *Aaeg_CTL25* [VectorBase: AAEL000556-RA], *Aaeg mosGCTL-1* [VectorBase: AAEL000563-RA], *Aalb_CTL2* [Genbank: AAV90641], *Cq_CTL54* [VectorBase: CPIJ014105], *Agam_CTL4* [VectorBase: ENSANGG00000018677], *Agam_CTLMA2* [VectorBase: ENSANGG00000018421]. Invariant or highly conserved residues within the CRD [Drickamer, 1993] are shown in the upper row: the italic single-letter amino acid codes indicate invariant conserved amino acids. Other abbreviations: Θ, aliphatic; Φ, aromatic; Ω, aliphatic or aromatic; O, oxygen-containing; Z, Glutamine or glutamic acid; triangle: the position for mutation. Cysteine residues forming disulphide bonds are marked with asterisks. Numbers on the left indicate amino acid positions starting from the initial methionine.

that calcium was important for Aalb_CTL1 to possess hemagglutinating activity [25]. In addition to WND motif, an imperfect EPS motif (Glu¹²⁸-Pro¹²⁹-Ser¹³⁰) was also present in the CRD of Aalb_CTL1. For a C-type lectin of uncharacterized function, it can be predicted that CRDs containing a WND motif and an EPN motif might be involved in carbohydrate-binding, whereas, the specificity for either mannose or galactose depends on whether the

motif is EPN or QPD [25]. Therefore, it could be further postulated that Aalb_CTL1 might be mannose-specific though the EPN was substituted by EPS in Aalb_CTL1.

Blast analysis with the deduced amino acid sequence of *Aalb_CTL1* revealed that Aalb_CTL1 shared a high degree of similarity with several known or putative C-type lectin family members from mosquitoes, especially the CTL from *Ae. aegypti* (Aaeg CTL16, similarity 83%).

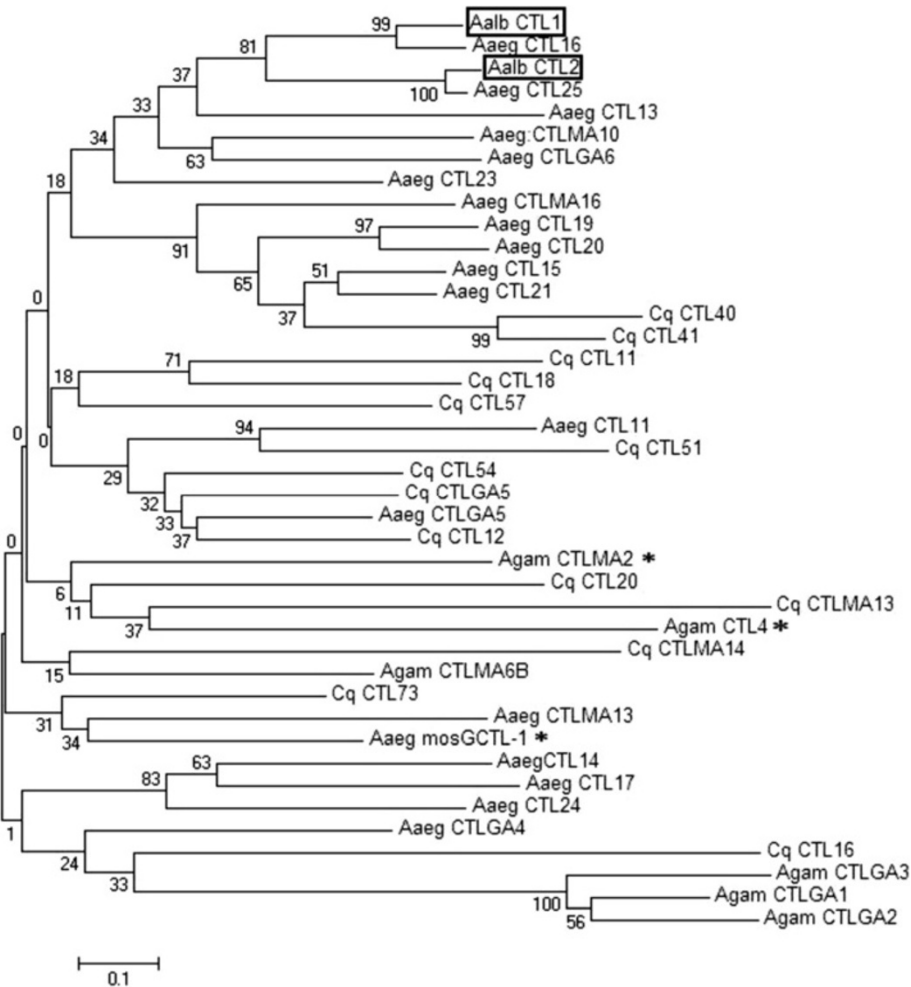


Figure 2 Phylogenetic tree constructed based on full-length amino acids sequence of Aalb_CTL1 and putative or known C-type lectins from mosquitoes. The C-type lectin sequences were from Aaeg CTL11 [VectorBase: AAEL008299], Aaeg CTL13 [VectorBase: AAEL004679], Aaeg CTLGA4 [VectorBase: N41092], Aaeg CTL14 [VectorBase: AAEL011453], Aaeg CTLGA5 [VectorBase: AAEL005641-RA], Aaeg CTLMA10 [VectorBase: AAEL011079-RA], Aaeg CTL15 [VectorBase: AAEL012353-RA], Aaeg CTLGA6 [VectorBase: AAEL009209], Aaeg CTL17 [VectorBase: AAEL011446-RA], Aaeg CTL19 [VectorBase: AAEL011404-RA], Aaeg CTLMA13 [VectorBase: AAEL011621-RA], Aaeg CTL20 [VectorBase: AAEL011407-RA], Aaeg CTL21 [VectorBase: AAEL011408-RA], Aaeg CTL23 [VectorBase: AAEL006456-RA], Aaeg CTL24 [VectorBase: AAEL002524-RA], Aaeg CTL25 [VectorBase: AAEL000556-RA], Aaeg mosGCTL-1 [VectorBase: AAEL000563-RA], Aaeg CTLMA16 [VectorBase: AAEL000283-RA], Aaeg CTL16 [VectorBase: AAEL000533-RA], Cq_CTL11 [VectorBase: CPIJ000449], Cq_CTL12 [VectorBase: CPIJ001323], Cq_CTL16 [VectorBase: CPIJ003650], Cq_CTL18 [VectorBase: CPIJ004339], Cq_CTL20 [VectorBase: CPIJ004916], Cq_CTLMA13 [VectorBase: CPIJ007062], Cq_CTL40 [VectorBase: CPIJ007868], Cq_CTL41 [VectorBase: CPIJ007869], Cq_CTL51 [VectorBase: CPIJ012307], Cq_CTL54 [VectorBase: CPIJ014105], Cq_CTL57 [VectorBase: CPIJ015095], Cq_CTLMA14 [VectorBase: CPIJ015742], Cq_CTL73 [VectorBase: CPIJ016688], Cq_CTLGA5 [VectorBase: CPIJ017075], Agam_CTLMA6B [VectorBase: ENSANGG00000018449], Agam_CTLMA2 [VectorBase: ENSANGG00000018421], Agam_CTL4 [VectorBase: ENSANGG00000018677], Agam_CTLGA1 [VectorBase: ENSANGG00000009790], Agam_CTLGA2 [VectorBase: ENSANGG00000017954], Agam_CTLGA3 [VectorBase: ENSANGG00000009745]. In the Phylogenetic analysis, Aalb_CTL1 and Aalb_CTL2 are in the box from *Ae. albopictus*. Agam CTLMA2, Agam CTL4 and Aaeg mosGCTL-1 whose function has been reported and are marked with*, The bar (0.1) indicates genetic distance.

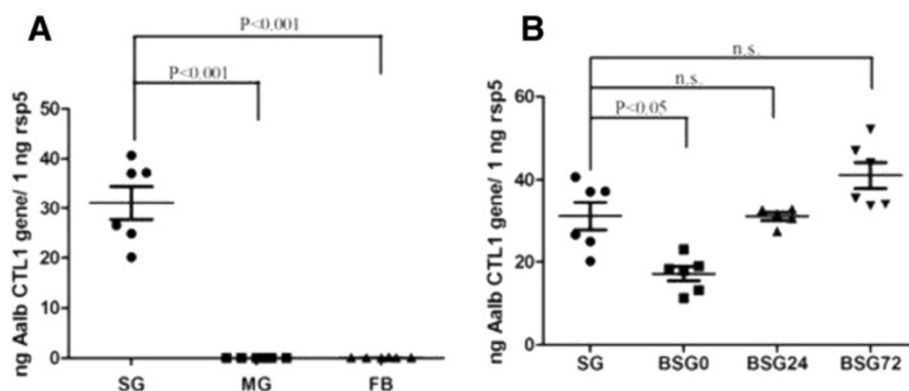


Figure 3 Temporal and spatial expression profiles of *Aalb_CTL1* (n = 6). (A) tissue expression profile of *Aalb_CTL1* in *Ae. albopictus*. SG: salivary gland, MG: midgut, FB: fat body. (B) stage-dependent expression of *Aalb_CTL1* after blood-feeding. BSG_0: 0 time after engorgement; BSG_24: 24 hours after engorgement; BSG_72 hours after engorgement.

These mosquito CTLs contain only one CRD (Figure 1B), which is different from most of the CTLs from the Lepidopteran containing dual CRDs [26]. In order to understand the evolutionary relationship of *Aalb_CTL1* in mosquitoes, a phylogenetic tree was constructed with the CTLs sequences from *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* that was highly similar with *Aalb_CTL1*. In the phylogenetic tree (Figure 2), the *Aalb_CTL1* was clustered with *Aaeg_CTL16* firstly, then clustered with *Aalb_CTL2* and *Aaeg_CTL25*, and they further formed a big branch with some CTLs from *Culex* subfamily. CTLs from *Anopheles* subfamily were clustered together with the other CTLs from *Culex* subfamily. The relationships displayed in the phylogenetic tree showed that *Aalb_CTL1* could belong to *Culex*-specific proteins. The above-mentioned evidences in our data has supported the Batholomay's notion [27] that *Aaeg_CTL16* locates in the upper part of the phylogenetic tree and no CTLs from *An. gambiae* and *Drosophila melanogaster* are clustered together with it.

Tissue-specific expression and stage-dependent expression of *Aalb_CTL1*

The tissue-specific expression and temporal expression pattern of *Aalb_CTL1* in the salivary gland after blood-feeding were determined by Real-Time qRT-PCR. The expression of *Aalb_CTL1* at transcription level was much higher in the salivary gland than those in the midgut and fat body (Figure 3A, $P < 0.001$), indicating that *Aalb_CTL1* would be expressed specifically in salivary gland and could be one of the important components in saliva as a secreted protein. *Aaeg_CTL16* from *Ae. aegypti*, revealing a high degree of sequence identity with *Aalb_CTL1*, has been verified to be salivary gland-specific lectin by semi-quantitative RT-PCR [27]. The expression level of *Aalb_CTL1* was significantly down-regulated at 0 time after blood-feeding (Figure 3B,

$P < 0.05$), suggesting that the expression of *Aalb_CTL1* transcript could be modulated by blood-feeding.

Hemagglutinating activity (HA) and sugar binding specificity of rAalb_CTL1

It is well known that not all proteins containing C-type CRDs can actually bind carbohydrate or even calcium [25]. To test the biological function of *Aalb_CTL1*, a recombinant plasmid pET-28a-*Aalb_CTL1* was transformed

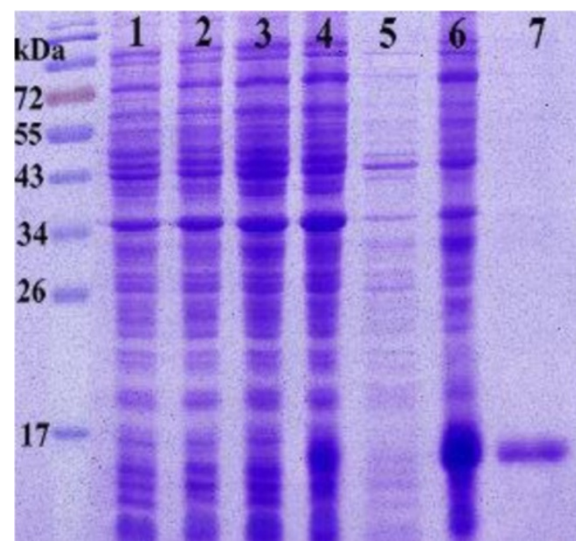


Figure 4 Expression and purification of recombinant mature *Aalb_CTL1* (rAalb_CTL1). Lane M: protein molecular standard. Lane 1: pET-28a(+) in BL21, uninduced. Lane 2: pET-28a(+) in BL21, IPTG-induced for 5 h. Lane 3: pET-28a(+)-*Aalb_CTL1* in BL21, uninduced. Lane 4: pET-28a(+)-*Aalb_CTL1* in BL21, IPTG-induced for 5 h. Lane 5: Supernatants of lysate of BL21 cell containing pET-28a(+)-*Aalb_CTL1* induced with IPTG. Lane 6: Sediments of lysate of BL21 cell containing pET-28a(+)-*Aalb_CTL1* induced with IPTG. Lane 7: pET-28a(+)-*Aalb_CTL1* recombinant proteins purified by the affinity purification method.

Table 2 Sugar binding specificities of rAalb_CTL1

Sugar name	Minimal Inhibitory Concentration (MIC) (mM)
D-Glucose	25
D-Mannose	6.25
D-Galactose	25
Lactose	50
Sucrose	50
Maltose	25

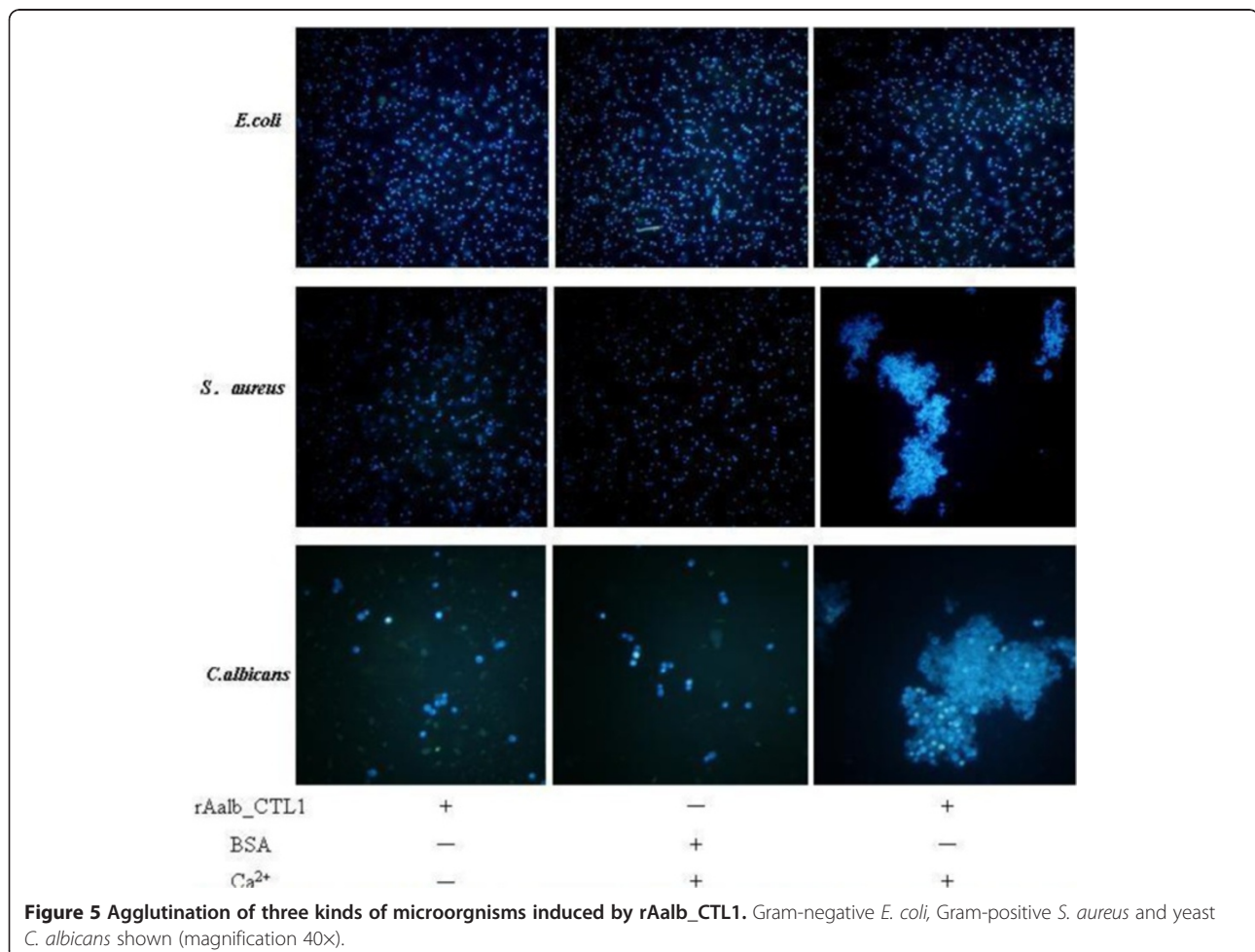
and expressed in *E. coli* BL21 (DE3). One clear band of the rAalb_CTL1 with molecular mass of 17 kDa was detected by SDS-PAGE analysis (Figure 4). The hemagglutination assay showed that rAalb_CTL1 at 40 mM CaCl₂ could induce the hemagglutination of rabbit erythrocytes, rat erythrocytes and mouse erythrocytes, and no agglutination was observed in the rAalb_CTL1 without CaCl₂ group and BSA-Ca negative control under the same conditions. Furthermore, the agglutination was inhibited in the presence of EDTA, indicating that the agglutination of animal erythrocytes by rAalb_CTL1 was Ca²⁺-dependent. The agglutinating activity of rAalb_CTL1 for rabbit

erythrocytes was higher than that for mouse and rat erythrocytes at a minimal agglutination concentration of 6.25 µg/ml, compared with the minimal agglutination concentration of 12.5 µg/ml for mouse erythrocytes and rat erythrocytes.

The carbohydrate-binding inhibitory hemagglutination assay demonstrated that the hemagglutinating activity of rAalb_CTL1 were inhibited by all the six types of sugars at different concentrations in a calcium-dependent way (Table 2), but among the carbohydrates tested, D-mannose inhibited the hemagglutinating activity of rAalb_CTL1 most effectively with a minimal concentration of 6.25 mM. The results suggested that rAalb_CTL1 might have a high ligand affinity to bind mannose, which was associated with their structural features.

Agglutination of rAalb_CTL1 to microbes

CTLs are pattern recognition receptors that can distinguish self from non-self in mammals [28]. Many studies on insects demonstrated that CTLs were involved in the insect innate immunity either *in vivo* or *in vitro*, especially in Lepidopterans [29,30]. In the present study,



to determine whether rAalb_CTL1 could induce the aggregation of microbial pathogens, DAPI-labeled yeast *C. albicans*, Gram-positive bacteria *S. aureus* and Gram-negative bacteria *E. coli* were incubated with the rAalb_CTL1. The rAalb_CTL1 displayed the activity to agglutinate yeast *C. albicans* and Gram-positive bacteria *S. aureus*, but no agglutinating activity toward Gram-negative bacteria *E. coli*. Meanwhile, there was also no agglutinating activity observed in the BSA-Ca control and the rAalb_CTL1 without calcium group, exhibiting that the agglutinating activity against yeast *C. albicans* and Gram-positive bacterium *S. aureus* was in a calcium-dependent fashion (Figure 5). The agglutinating activity of rAalb_CTL1 suggested that Aalb_CTL1 might be involved in eliminating pathogens from food. In the mosquito *Ae. aegypti*, immunity-related proteins expressed in salivary gland, such as lysozymes and C-type lectins, are postulated to have a role in controlling bacterial growth in sugar solutions stored in the crop or in the gut following a blood meal [21,31,32]. In addition, a previous study on CTLs of *An. gambiae* showed that CTL4 and CTLMA2 can recognize the surface molecules of Gram-negative *E. coli* but not Gram-positive *S. aureus*, which is different from that of rAalb_CTL1. Furthermore, the defensive function against Gram-negative bacteria is attributed to the CTL4-CTLMA2 heterodimer *in vivo* [16]. Thus, combined with the reasonable deduction of the result of the phylogenetic tree, it could be inferred that they might belong to the different members of CTLs and be involved in the recognition of different types of microorganisms.

Conclusions

In this study, a putative C-type lectin (*Aalb-CTL1*) was successfully cloned and expressed from *Ae. albopictus* Guangzhou strain. Aalb_CTL1 was only clustered with CTLs from *Culex* subfamily in the phylogenetic tree, suggesting that it would be a *Culex*-specific protein. *Aalb_CTL1* was expressed specifically in salivary gland and its transcript level could be regulated by blood-feeding. rAalb-CTL1 exhibited not only the hemagglutinating activity and a high affinity for mannose but also the agglutinating activity against yeast *C. albicans* and Gram-positive bacteria *S. aureus* in a calcium-dependent manner. Taken together, our results showed that Aalb_CTL1 was a mannose-binding C-type lectin and could act as a PPR involved in the anti-microbial activity. More efforts should be focused on its role on the relationship of mosquito and pathogen in saliva in the future.

Abbreviations

Ae. albopictus: *Aedes albopictus*; *Ae. aegypti*: *Aedes aegypti*; *An. gambiae*: *Anopheles gambiae*; *Cx. quinquefasciatus*: *Culex quinquefasciatus*; *E. coli*: *Escherichia coli*; *S. aureus*: *Staphylococcus aureus*; *C. albicans*: *Candida albicans*; CTLs: C-type lectins; Aalb_CTL1: C-type lectin 1 in salivary gland of *Ae. albopictus*; CRD: Carbohydrate recognition domain; PRRs: Pattern recognition receptors; IPTG: Isopropyl β -D-Thiogalactoside; SDS-PAGE:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA: Bovine serum albumin; Real-Time qRT-PCR: Real-Time quantitative reverse transcription polymerase chain reaction.

Competing interests

All the authors declare that they have no competing interests.

Authors' contributions

CJZ participated in the experiment design, carried out the experiment and drafted the manuscript. WY analyzed the data and drafted the manuscript. LFZ and LJ carried out bioinformatics analysis and prokaryotic expression. SY collected the tissue samples including salivary gland, midgut and fat body. WJH conceived of the study, analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

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