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Expression and characterization of anticoagulant activity of salivary protein alALP from Asian tiger mosquito *Aedes albopictus*

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Abstract. Several bioactive molecules isolated from the saliva of blood-sucking arthropods, such as mosquitoes, have been shown to exhibit potential anticoagulant function. We have previously identified a 30kDa allergen named Aegyptin-like protein (alALP), which is highly homologous to Aegyptin, from the salivary glands of female *Aedes albopictus* (Asian tiger mosquito). In this study, we identified the conserved functional domain of alALP by using bioinformatic tools, and expressed the His-tagged alALP recombinant protein in sf9 insect cells by generation and transfection of a baculoviral expression plasmid carrying the full-length cDNA of alALP. We purified this recombinant protein and examined its function on the inhibition of blood coagulation. The results showed that the purified His-alALP prolonged the Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) and Thrombin Time (TT) *in vitro* as well as the Bleeding Time (BT) *in vivo*, which suggest that alALP could be a novel anticoagulant.

INTRODUCTION

Mosquito is an important blood-sucking arthropod and transmitting vector for certain pathogens, such as plasmodium and arboviruses (Benelli *et al.*, 2016). Female mosquitoes need to repeatedly probe their mouthparts into the host's skin vessels to search for blood (Fontaine *et al.*, 2011; Chagas *et al.*, 2014). However, this probing activity results in the combination of platelets with collagen, which subsequently activate the host hemostasis at the site of vascular injury (Ribeiro *et al.*, 1984; Mizurini *et al.*, 2013). To successfully take a blood meal, mosquitos inject saliva into the host to interfere with the host hemostasis (Schmid *et al.*, 2016). A number of anti-hemostatic, allergen and immune-modulator compounds from the mosquito saliva have been proposed as markers to be used for surveying mosquito bite exposure (King *et al.*, 2011; Peng *et al.*, 2016), and as a potential anti-hemostatic pharmacological agent (Yoshida *et al.*, 2008; Calvo *et al.*, 2011; Hayashi *et al.*, 2012; Mizurini *et al.*, 2013).

The sialomes, including the transcriptosome and proteasome of salivary glands, in Aedes, Culex and Anopheles mosquitos have been reported (Francischetti *et al.*, 2002; Ribeiro *et al.*, 2004; Ribeiro *et al.*, 2007). This allowed us to search for more functional salivary proteins. For example, an anopheline antiplatelet protein (AAPP) isolated from the saliva of *Anopheles stephensi* exhibits a strong and specific inhibition in collagen-induced platelet aggregation (Yoshida *et al.*, 2008), a factor Xa inhibitor named Alboserpin isolated from the *Aedes albopictus* mosquito binds to heparin and membrane phospholipids to prevent thrombus formation (Calvo *et al.*, 2011). Recently, a family of 30-kDa salivary allergens as a major salivary component have been found in different mosquitoes, including *Aedes* sp. (Valenzuela *et al.*, 2002), *Culex* sp. (Ribeiro *et al.*, 2004) and *Anopheles* sp. (Calvo *et al.*, 2007a).

Aegyptin is a salivary 30-kDa protein of *Aedes Aegypti* mosquito (Calvo *et al.*, 2007b), of which the primary structure displays similarities to the members of 30-kDa salivary allergens, including a region containing 28 negatively-charged amino acid residues with five GEEDA (Gly-Glu-Glu /Asp-Ala) repeats in the N-terminus (Calvo *et al.*, 2007b; Calvo *et al.*, 2010). The recombinant Aegyptin generated from 293-F cells binds to collagens (I-V) and interferes with its interaction with major physiological ligands: GPVI, integrin $\alpha 2\beta 1$ and vWf (Calvo *et al.*, 2010). Notably, Aegyptin inhibits platelet aggregation and adhesion by blocking the interaction of GPVI with collagen, but has no effect on platelet aggregation induced by thrombin, ADP and thromboxane A2 mimetic (U46619) (Mizurini *et al.*, 2013).

We have previously identified a gene encoding 30kDa allergen that specifically expressed in the salivary glands of female *Aedes albopictus*, a mosquito of human Dengue, Zika and Chikungunya virus vector (Li *et al.*, 2014). In this study, the primary and second structures of alALP were analyzed. The gene encoding alALP was cloned into a baculovirus expression vector and the recombinant His-tagged alALP (His-alALP) was generated. The effects of His-alALP on the inhibition of the platelet aggregation and blood coagulation were evaluated *in vitro* and *in vivo*.

MATERIALS & METHODS

Ethical statement

This study was approved by the Animal Ethics Committee of Wenzhou Medical University (Permit number: SYXK (Zhe)-2010-0150). Animal experiments were performed in accordance with National animal ethics guidelines of the Government of P.R. China.

Bioinformatics analysis

The primary structures of 30-kDa allergens in different mosquitoes, including alALP (AAV90693.1, *Aedes albopictus*), Aegyptin (ABF18122.1, *Aedes aegypti*), AAPP (ACE79173.1, *Anopheles stephensi*) and GE-rich protein (AAV90694.1, *Aedes albopictus*) were downloaded from Genebank. The secondary structures of these proteins were analyzed by PRALINE (http://www.ibi.vu.nl/programs/praline www/), using DSSP. The signal peptides were predicted by signal IP 4.1.

Rearing of *Aedes albopictus* mosquitoes

Aedes albopictus mosquitoes (Foshan strain) were kindly provided by Dr. Xiaoguang Chen and reared under optimum temperature at 26±1°C, 72–80% humidity and light 12h per day in an insectary. Adult mosquitoes were maintained in the cages and were fed on water containing 10% glucose. To obtain next generation, 5–6 days old female mosquitoes were fed for two hours on ICR mice as a source of blood meal.

Salivary gland dissection and Isolation of total RNA

Approximately 60 pairs of salivary glands of adult female *A. albopictus* were dissected according to the previous studies (Li *et al.*, 2014), then suspended in 1 mL of Trizol (Invitrogen). The salivary glands were then homogenized by using an electric homogenizer (KIMBLE). Total RNA was then isolated by using the TRIzol® Reagent (Invitrogen), according to the manufacturer's instructions.

Cloning of alALP gene

The synthesis of single stranded cDNA was carried out by reverse transcription (Takara Bio). Then amplification of alALP gene was performed by PCR. The upstream and downstream primers are named as pALP-F (5'-ATCGGGCGCGGATCTGAATTCATGAA ACCCTTGGCAAAATTG-3') and pALP-R (5'-CTAGTACTTCTCGACTCTAGATTAatgatgatg atgatgatgCTGTCCTTTGGAAGATACACAG-3'), respectively. Additionally, the DNA sequence encoding PreScission protease recognition and 6×His tag was added to the 3'terminus of alALP gene.

PCR amplification was performed using 0.1 μg of alALP gene template from preparatory steps, 0.4 μM of each primer, 2 mM $MgCl_2$, 0.2 μM dNTPs, 1 × Taq buffer, 1 U Taq Polymerase (Fermentas) in a final volume of 50 μL. The cycling conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 20 s, 58°C for 30 s, 72°C for 1 min, and final elongation of 72°C for 5 min. The results were analyzed using 1% agarose gel electrophoresis. The PCR products were isolated from the gel by using the Gel-Out kit (Takara Bio) and confirmed by sequencing (BGI, Shenzhen, China).

Construction of PFast-alALP plasmid

Both alALP cDNA and pFastBac™HT Vector (Invitrogen) were digested by EcoR I and Xba I (Fermentas) digestion. Then the DNA fragment of alALP was inserted into pFastBac™HT A vector, using In-Fusion PCR Cloning Kit (Vazyme Biotech, China). The bacterial competent cells (*E. coli* strain DH10Bac™) were transformed with the recombinant vector PFast-alALP. The colonies containing the recombinant bacmid were identified, based on the blue/white colony selection. The recombinant bacmid PFast-alALP was then extracted by Phenol-Chloroform, and confirmed by sequencing.

Protein expression and purification

The expression of recombinant His-tag alALP (His-alALP) protein was carried out, according to the protocol of baculovirus expression system. Briefly, 10 μg recombinant bacmid PFast-alALP was mixed with 8 μL Cellfectin® II in 200 μL Sf-900™ II SFM, and then was transfected into 1 mL Sf9 cells (2×10^6 cells/mL). The mixture was added into a six well plate and incubated for 5 hours at 27°C to generate P0 cells. Then, 500 μL P0 cells were seeded in the 10cm culture dish with Sf9 cells to generate P1, P2 and P3 cells. Finally, 2 mL P3 was added to 100 mL Sf9 (2×10^6 cells/ mL) in culture flask and incubated at 110 rpm, 27°C for 72 hours. Then, the culture medium was centrifuged at 4000 rpm for 15 min at 4°C. 56.1g ammonium sulfate was added to per 100 mL supernatant to deposit the protein overnight at 4°C, then centrifuged at 15000 rpm for 30 min at 4°C.

The purification of aALP was performed, according to the protocol with proper modifications. Briefly, the precipitate was dissolved in 5 mL PBS supplemented with 1mM PMSF, and dialyzed for three times using Buffer A (500mM NaCl, 20mM Tris-HCl, pH 7.9). The His-alALP protein was purified by Ni-NTA affinity chromatography, according to the manufacturer's instructions (Qiagen, Ontario, Canada). Elution was carried out with an imidazole gradient (20–500 mM). The His-alALP containing fractions were further analyzed by SDS-PAGE and Western blot. Then the purified His-alALP protein was dialyzed using PBS plus 20% glycerol, and stored at -80°C.

SDS-PAGE and Western blotting

SDS-PAGE was performed using 12% (w/v) resolution gel (Bio-Rad, USA). In brief, the protein samples were separated by SDS-PAGE at 150 V for 1.5 h, and then stained with Commassie Blue G250. Then, proteins were transferred topolyvinylidene fluoride (PVDF) membranes (Millipore, USA) on a semi-dry blotting devices (Bio-Rad) at 500 mA for 45 min. Subsequently, the membrane was blocked at room temperature for 2 h with 5% (w/v) non-fat milk powder diluted in Tris-buffered saline containing 5% non-fat dry milk and 0.05% Tween 20 (TBST). The blot was then incubated at 4°C overnight in the presence of the Horseradish peroxidase-conjugated polyclonal anti-His antibody (GeneTex, USA) diluted at 1:1000 in TBST. After being washed for three times with TBST, the Western blot band was visualized by using the enhanced chemiluminescence (ECL) reaction (Pierce, USA).

Anticoagulant activity assay *in vitro* and *vivo*

The blood samples from the normal mice were taken for analyzing the biological activities of His-alALP, according the methods described before (Mizurini *et al.*, 2013). Thrombin Time (TT), Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) were measured by TT, PT and

APTT kits (Some Carport, Shanghai, China) according to the manufacturer's instructions. The clotting time was measured by using the coagulometer at ambient temperature. PBS and Heparin Sodium (Meilunbio, Dalian, China) solution were taken as the negative and positive control, respectively.

The mice bleeding time (BT) assay was performed according the methods described before (Hu *et al.*, 2018). The 30 ICR mice, half male and half female, were divided into 6 groups in random, and each group contain 5 mice. The experimental groups were subcutaneously injected with different dose of His-alALP protein (34μg/20g, 136μg/20g and 340μg/20g), respectively. The negative control (Control) group was injected with PBS solution and the positive control group was injected with Heparin Sodium (10μg/20g). The tails of anesthetized mice were transected 3 mm from the tip and vertically immersed in saline solution at 37°C. BT was defined as the time from the moment when incision was made to the point when bleeding stops for over 1 min.

Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was performed by one-sample t-test, followed by pair-sample t-test using the statistical software OringinPro 9.0.

RESULTS

Bioinformatics analysis

The alALP cDNA has 813 bp in length and encodes a protein of 271 amino acid (aa) residues. This protein contains a predicted signal peptide of 19 aa residues, indicating that it is likely a secretory protein (Figure 1). The predicted mature alALP protein has a molecular mass of 28.330 kDa with an isoelectric point of 3.89. The results from an alignment of alALP with Aegyptin show an 60% identity. In addition, similar to the five GEEDA (Gly-Glu-Glu-Asp-Ala) repeats in the N- terminus of Aegyptin, there are five GENA/TD (Gly-Glu-Asn-Ala/Thr – Asp) repeats in alALP (Figure 1). Analyzing the second structure and conservative domains shows that alALP is highly conservative to other 30-kDa allergens in the COOH-terminus, including four conserved cysteines, indicating that alALP may have the similar bioactivity of 30-kDa allergens, such as Aegyptin.

Expression and purification of recombinant His-alALP protein

The total RNA was extracted from the salivary glands of *A. albopictus* Foshan strain female adults. The alALP gene was amplified based on the cDNA temples, then inserted into the multiple clone sites of pFastBac Vector (Figure 2A). By PCR, restriction enzyme identification and sequence analysis, the recombinant plasmid of Fast-alALP was successfully constructed (Figure 2B).

The cell lysates and culture supernatants were collected and analyzed by SDS-PAGE, respectively. As shown in Figure 3A, the recombinant His-tagged alALP protein (His-alALP) with molecular weight of 35 kD was detected in the PFast-alALP transfected cells. The expression of His-alALP protein was confirmed by Western blotting with an anti-His antibody (Figure 3B). The His-alALP showed an anomalously decreased mobility at about 35 kDa position, when analyzed by SDS-PAGE, suggesting that it may be glycosylated. Moreover, the His-alALP was detected in the culture supernatants of PFast-alALP transfected cells (Figure 3C and D).

About 2 mg recombinant His-alALP protein from 100 mL culture supernatants were purified by Ni-NTA affinity chromatography. The purified His-alALP was analyzed SDS-PAGE (Figure 4A), recognized by an anti-His antibody in Western blotting (Figure 4B). Finally, the purified His-alALP protein was concentrated by using centrifugal filter tube and dialyzed against PBS supplemented with 20% glycerol and stored at -80°C. The purified His-alALP protein has more than 90% purity shown by a silver stained SDS-PAGE (Figure 4A). The concentration is 34 μg/μL measured by BCA analysis.

Anticoagulant activity assay

To investigate the potential anticoagulant effect of alALP on inhibiting the extrinsic pathway of coagulation, plasma coagulation

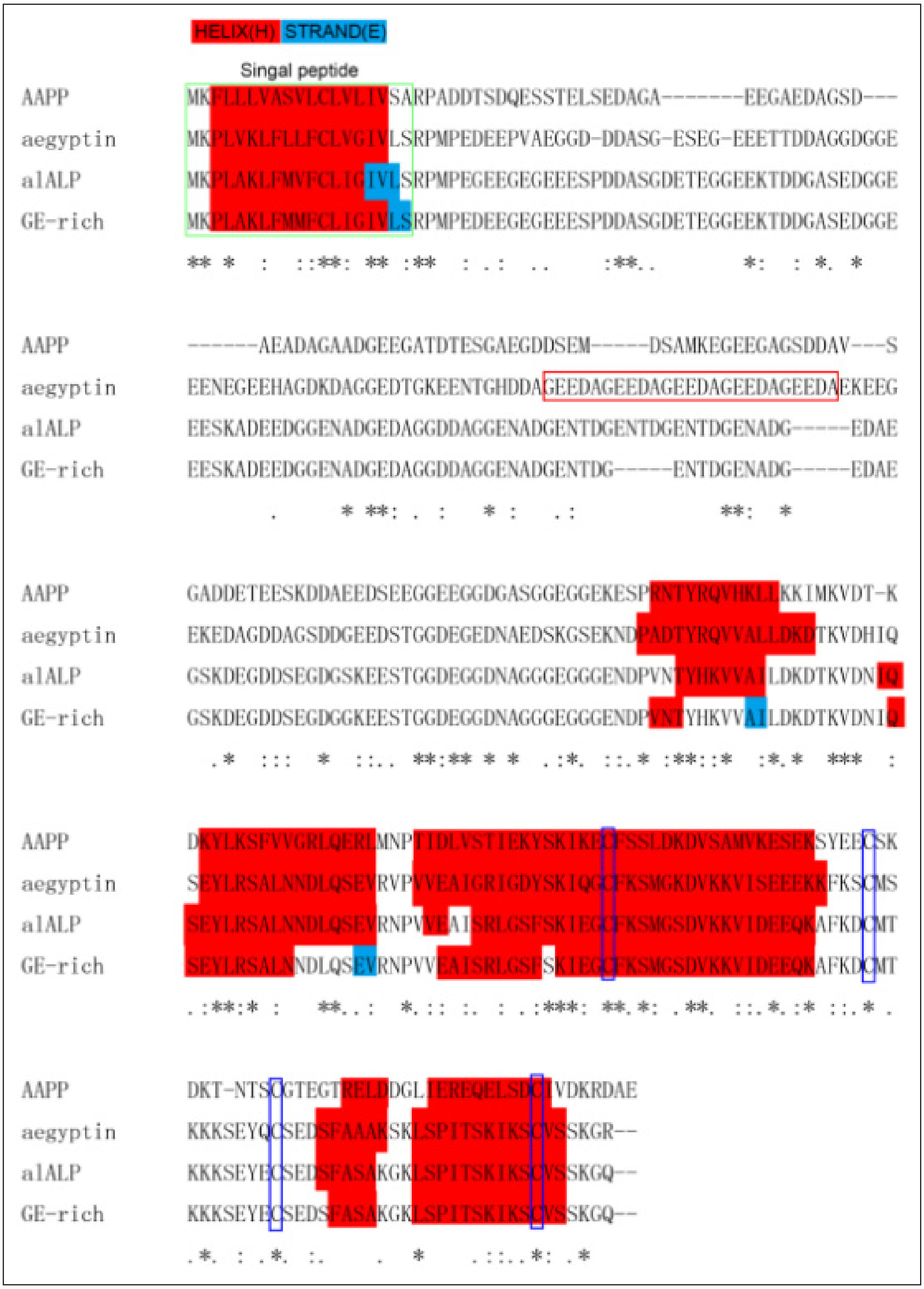

Figure 1. Conservation and second structure analysis of alALP.

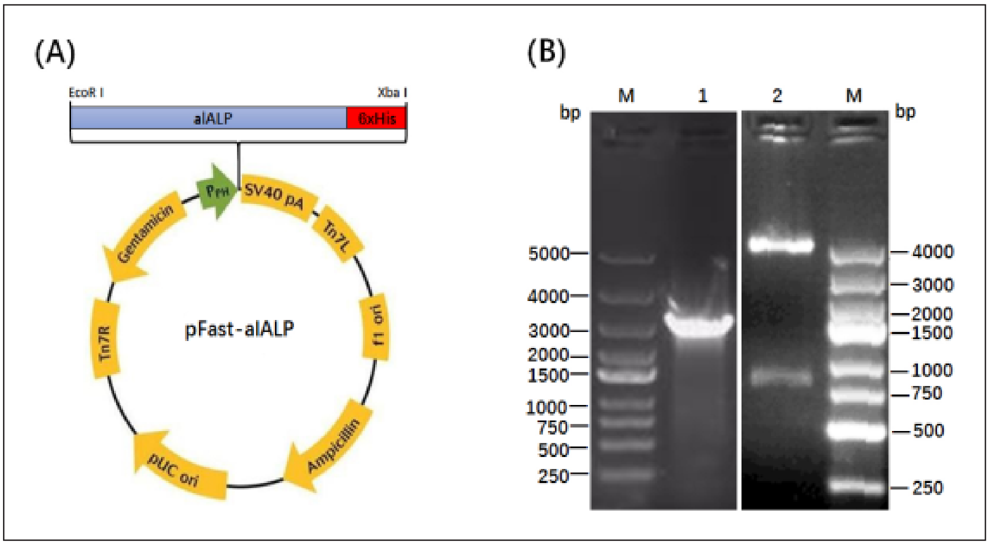

Figure 2. Construction of recombinant PFast-alALP.

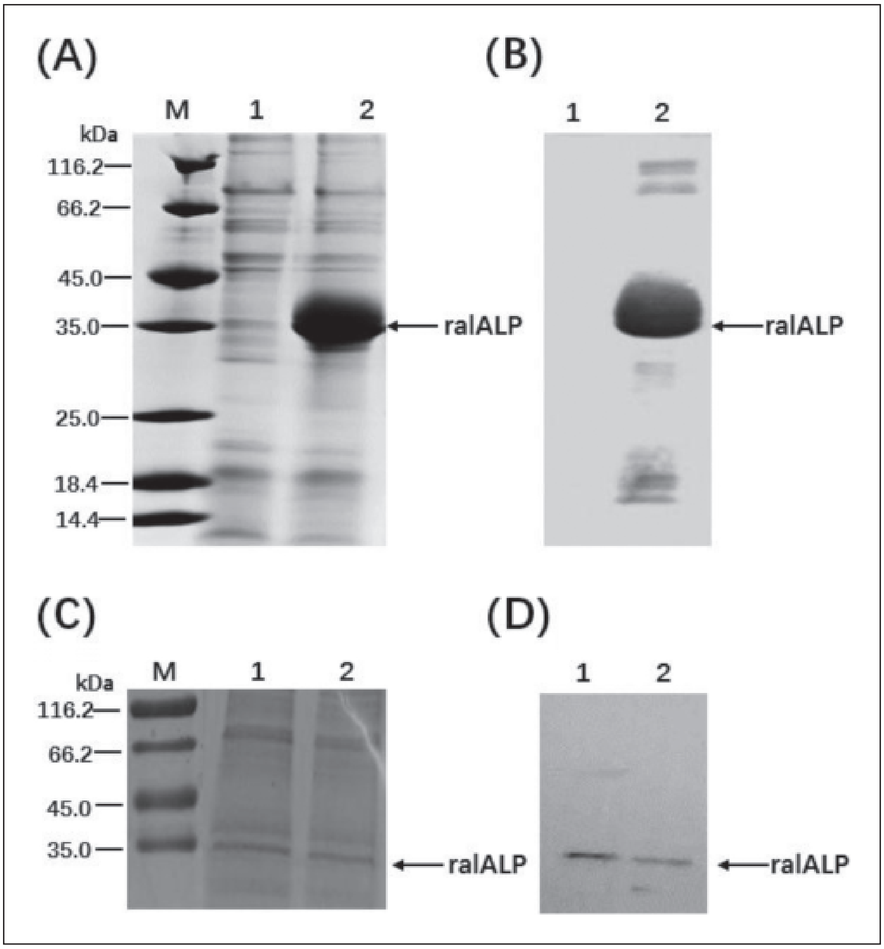

Figure 3. Expression analysis of ralALP by SDS-PAGE and Western blotting.

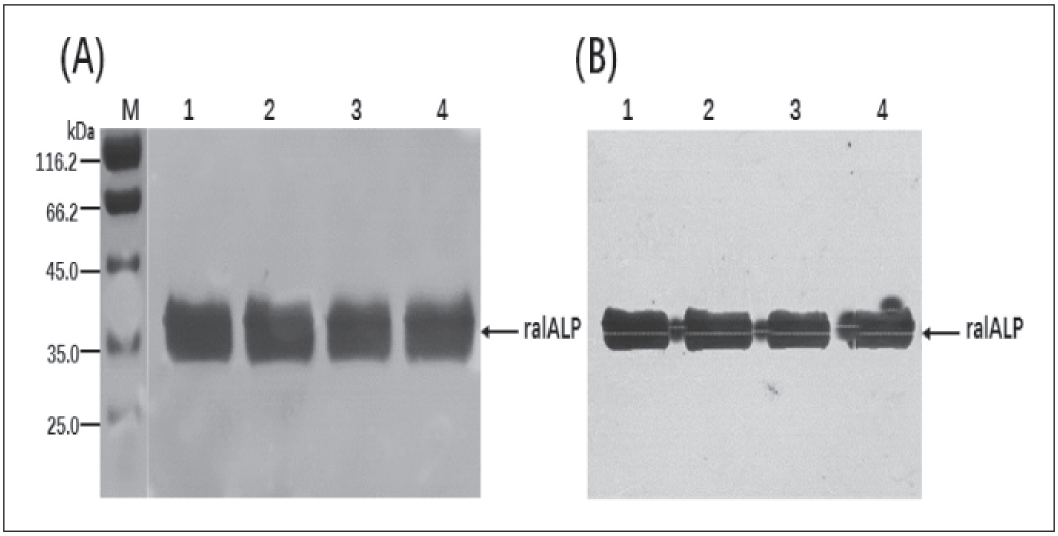

Figure 4. Purification of ralALP by usingNi-NTA affinity chromatography.

experiments were performed to determine the activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT). Comparing to the control group treated with PBS, The His-alALP with a dose of 3.4 μg significantly prolonged APTT (Figure 5A). The similar effect on prolonging plasma coagulation time by using His-ralALP were also detected in both TT and PT assays (Figure 5B and C). However, the higher doses of His-alALP were required in TT (34 μg) and PT (340 μg) assays. The treatment of Heparin (1 μg) was taken as a positive control. There is no significant difference in the APTT, PT and TT between the Heparin and His-ralALP groups ($P > 0.05$). The different doses of His-alALP in anticoagulant activity were carried out in the APTT, TT and PT assays. With the increasing of doses, the effect of His-aALP on anticoagulant activity exhibited more significant in APPT than TT and PT, indicating that His-aALP can prevent the coagulation process through an endogenous way (Figure 5D).

The bleeding time (BT) of His-aALP was further examined with a standard tail-bleeding model, which has been widely used for evaluating the efficacy of novel anticoagulant activity assay of drugs in the preclinical studies. In this assay, the treatment of His-alALP resulted in prolonging BT, when compared to the treatment with PBS (Figure 6A). The enhancement of prolonging BT was observed in the higher doses of His-aALP, indicating that His-alALP has the potent capability of anticoagulant (Figure 6B).

DISCUSSION

To acquire a successful blood meal, mosquitoes and other blood-sucking arthropods have evolved a number of saliva components, including vasodilators and antagonists of platelet aggregation, to suppress the hemostatic system of vertebrate hosts. Some of them have been identified as an effective antithrombotic agent in both *in vitro* and *in vivo* models. In this study, we described the cloning, expression and characterization of anti-thrombotic function of alALP, a 30-kDa allergen identified in the salivary glands of *Aedes albopictus* mosquitoes.

The sialome of an adult female *Ae. albopictus* was reported in 2007. It contains a complex of as many as 34% secreted proteins, such as D7 proteins, antigen 5 family members and 30-kDa allergens (Arca *et al.*, 2007). The 30-kDa allergens are known to be widely distributed in the saliva of female adult mosquitoes, including AAPP from *An. stephensi* and Aegyptin from *Ae. aegypti* (Francischetti *et al.*, 2002; Valenzuela *et al.*,

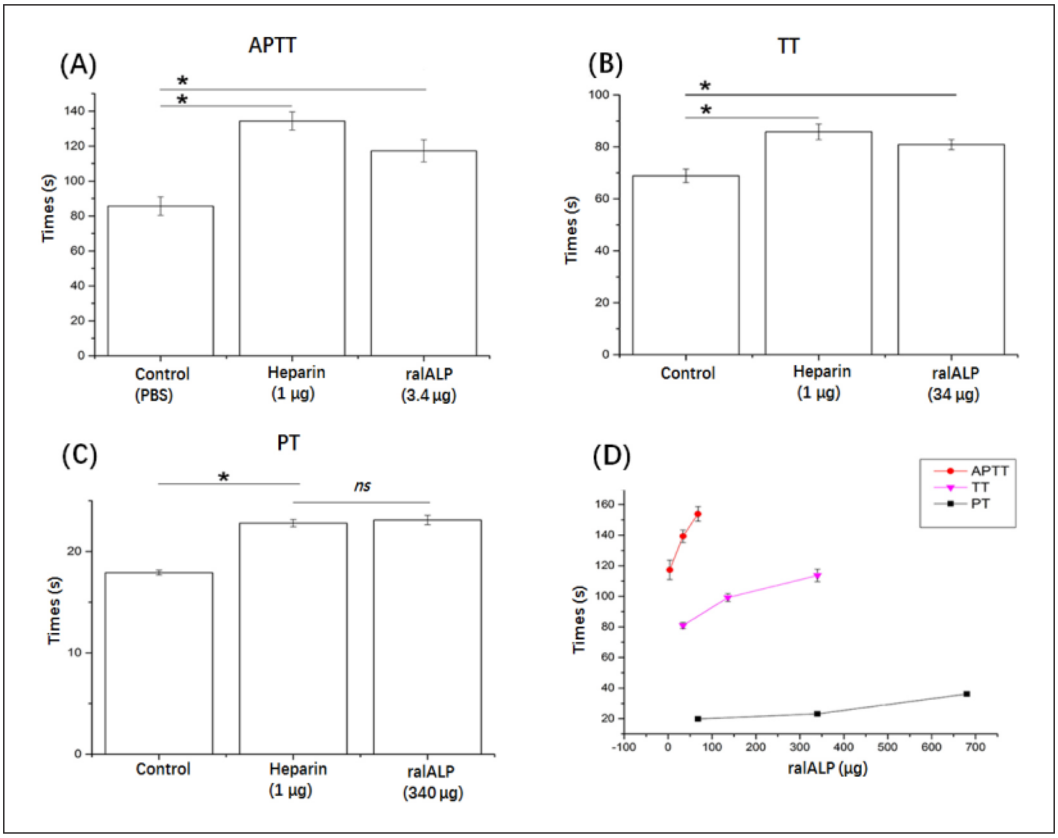

Figure 5. Anticoagulant activity assay of ralALP *in vitro*.

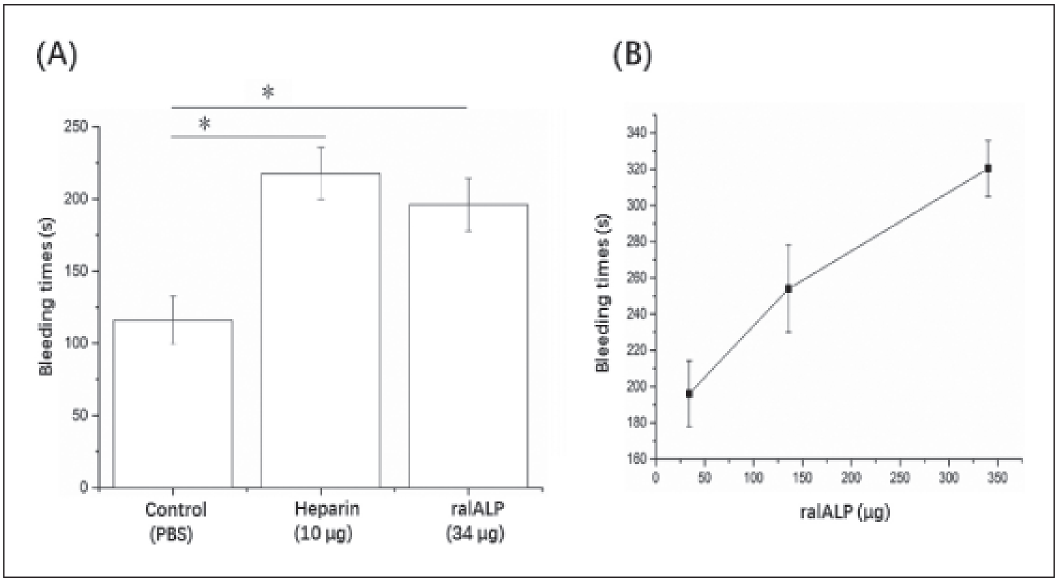

Figure 6. Bleeding time assay of ralALP *in vivo*.

2002). AAPP is a member of the GE-rich proteins, which is highly conserved among anopheline mosquitoes. The recombinant AAPP generated by prokaryotic expression system exhibited the ability of inhibiting collagen-induced platelet aggregation (Yoshida *et al.*, 2008). By crystal structure analysis, AAPP has four cysteine residues from 205 aa to 269 aa, which is a function domain in collagen binding (Sugiyama *et al.*, 2014). We also found the conserved 4 cysteine residues in the COOH terminus of alALP, suggesting that alALP may possess similar anti-coagulation function.

Aegyptin has been also demonstrated as a specific antagonist of collagen-induced platelet aggregation/adhesion (Calvo *et al.*, 2007b). Collagen is a matrix protein and plays an important role in the process of primary hemostasis by inducing platelet activation cascades (Abdel-Naim *et al.*, 2015). Aegyptin specifically blocks collagen-induced human platelet aggregation by high-affinity binding to collagens (Calvo *et al.*, 2007b). The Gly-Glu-X-Asp repeats of Aegyptin mediate specifically blocking collagen-induced human platelet aggregation and granule secretion (Calvo *et al.*, 2007b). Similar to the Aegyptin, alALP was also found to contain Gly-Glu-X- Asp repeats in the N-terminus, indicating that alALP has the potential activity of binding to collagen.

Our previous studies have demonstrated that the recombinant alALP protein was recognized by the serum of mice after blood feeding by *Ae. albopictus*, and induced a high titer of antibodies by injecting into the mice (Li *et al.*, 2014). Since alALP is a potential anticoagulant protein derived from mosquitoes, it is critical to choose a suitable expression system to retain its biological activity. In this study, the Sf9 insect expression system was applied to produce the recombinant alALP protein. The His-alALP was successfully expressed and secreted into culture supernatants in Sf9 cells, indicating that alALP is a secretory protein. By using an *in vitro* anticoagulant activity assay, the recombinant alALP protein prolonged the Thrombin Time (TT), Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT) and Clotting time (CT), suggesting that alALP can inhibit the coagulation process through an endogenous way. Moreover, the enhancement of prolonging BT was observed in the higher doses of recombinant aALP protein.

In conclusion, we have demonstrated that alALP share the conserved functional domain with other 30-kDa allergens from mosquitoes. The recombinant alALP protein generated from insect cells expression system exhibits an anticoagulant activity by both *in vitro* and *vivo* experiments. Thus, it is necessary to investigate the mechanism of anticoagulation by alALP in the further study.

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