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Original article

HSC70 from *Haemaphysalis flava* (Acari: Ixodidae) exerts anticoagulation activity *in vitro*

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ABSTRACT

Ticks and tick-borne diseases are major global health threats. During blood feeding, ticks insert their hypostomes into hosts and inject an array of anticoagulant molecules to maintain fluidity of the blood-meal. These anticoagulant molecules may provide insights into understanding the feeding biology of ticks and to develop vaccines against infestations. In *Haemaphysalis flava*, the heat shock cognate 70 (HSC70), a member of the heat shock protein (HSP) family, is differentially expressed in salivary glands at different levels of engorgement during blood feeding. However, its function in ticks is largely not known. The present study was designed to explore the possible effects of HSC70 on the plasma. The open reading frame (ORF) of *HSC70* was expressed in a prokaryotic system, and recombinant HSC70 (rHSC70) was purified and characterized. The anticoagulation activity of rHSC70 was estimated by measuring prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen (FIB) with/without its inhibitor, VER155008. The results demonstrated that rHSC70 from *H. flava* extended TT ($P < 0.001$) and FIB clotting times (> 300 s), but showed little effect on PT and APTT. Adding an inhibitor reversed anticlotting effects of rHSC70 on TT and FIB. These data indicate that rHSC70 is an anticoagulant agent, and the anticlotting activity likely attributes to the inhibition of thrombin and the transformation of fibrinogen into fibrin.

1. Introduction

As hematophagous arthropods, hard ticks (family: Ixodidae) acquire blood meals during larva, nymph and adult stages, and engorgement on blood is also one of the prerequisites to mate and reproduce. During feeding, ixodid ticks implant themselves with barbed hypostomes, produce a large amount of cements from salivary glands, and form a tightly attachment to the host skin for several days or even weeks. For completion of such a long-term feeding, ticks have to maintain blood in a flow state, and prevent it from coagulation. Salivary glands of ticks' secret a myriad of proteins into saliva to exert anti-hemostatic activities by targeting different stages of blood clotting. Ixolaris, a saliva protein initially characterized in *Ixodes scapularis*, acted as a tissue factor pathway inhibitor (Francischetti et al., 2002). Haemaphysalin, isolated from *Haemaphysalis longicornis*, interfered with the activation of F XII, thus blocked the intrinsic pathway of coagulation (Kato et al., 2005). Americanin, a saliva protein of *Amblyomma americanum*, was proven to be a tight-binding competitive thrombin inhibitor (Zhu et al., 1997). Platelet aggregation is also the acting site of proteins with anti-hemostatic activities. IxscS-1E1, a serpin member in *I. scapularis*, disrupted adenosine diphosphate- (ADP) and thrombin-activated platelet aggregation (Ibelli et al., 2014). Variabilin, an antagonist of the fibrinogen receptor isolated from American dog tick *Dermacentor variabilis*, was verified as an inhibitor of platelet aggregation (Wang et al., 1996). Thrombin is the enzyme responsible for the cleavage of fibrinogen to form fibrin. Four types of thrombin inhibitors have been recognized in the saliva of *Rhipicephalus microplus*, namely, boophilin (Macedo-Ribeiro et al., 2008), BmAP (Horn et al., 2000), microphilin (Ciprandi

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Abbreviations: APTT, activated partial thromboplastin time; BHK, baby hamster syrian kidney; BCA, bicinchoninic acid; BSA, Bovine serum albumin; VIII (F VIII), blood coagulation factor; cDNA, complementary DNA; ER, endoplasmic reticulum; ECL, enhanced chemiluminescence; ELISA, Enzyme Linked Immunosorbent Assay; FIB, fibrinogen; GSH, glutathione; LB, lysogeny broth; LC/MS-MS, liquid chromatography tandem-mass spectrometry; GST, glutathione S-transferase; HSC, heat shock cognate; HSP, heat shock protein; HRP, horseradish peroxide; IPTG, isopropyl-β-D-thiogalactoside; KO, knock out; ORF, open reading frame; OD, optical density; PBS, phosphate buffered solution; PAI-1, plasminogen activator inhibitor-1; PVDF, Polyvinylidene fluoride; PT, prothrombin time; rHLHSP70, recombinantHaemaphysalis longicornis heat shock protein 70; rHSC70, recombinant HSC70; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TT, thrombin time

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et al., 2006), and BmGTI (Ricci et al., 2007). Madanin and chimadanin, saliva proteins from salivary glands of *H. longicornis*, possessed antithrombin activities by binding to thrombin exosite-I and inhibition of enzyme active site, respectively (Iwanaga et al., 2003; Nakajima et al., 2006). On one hand, proteins with anticoagulation activities are regarded as potential antigens for anti-tick vaccine development, which have considered to be the optimal alternative for ticks control compared with applying pesticides. On the other, those proteins will also help to broaden our understanding of tick feeding biology.

Heat shock proteins (HSPs) are expressed both during normal physiological and stress conditions to enable protein folding and transport, and cellular protection among other functions (Boorstein et al., 1994). HSPs represent attractive vaccine candidates for preventions of tick infestations, pathogen infection and transmission (Espinosa et al., 2017). HSPs have been classified into HSP110, HSP90, HSP70, HSP60 and small HSP (sHSP) based on their molecular weights and homology (Shrestha and Young, 2016). Among these, HSP70 is one of the members that have been researched intensively. It exists in both prokaryotes and eukaryotes, is expressed ubiquitously in different tissues in abundance (approximately 1–2% of total cellular protein), and plays a significant role in cell protection (Patury et al., 2009).

Evidence suggests that HSP70 may play a role in anticoagulation (Polanowska-Grabowska et al., 1997; Ishaque et al., 2007; Uchiyama et al., 2007; Rigg et al., 2016). HSP70 has been shown to promote the yield and activity of F VIII by suppression apoptosis and facilitate conformational folding of F VIII (Ishaque et al., 2007); HSP70 inhibited the activity of plasminogen activator inhibitor-1 (PAI-1) (Uchiyama et al., 2007), regulated the activity of integrin $\alpha_{IIb}\beta_3$, as well as the degranulation and aggregation of platelets (Rigg et al., 2016). A recent study indicated that HSP70 obstructed thrombus formation without bleeding risk (Allende et al., 2016).

Heat Shock Cognate 70 (HSC70), a HSP70 of structural type, expressed on the membrane of platelets has been shown to be involved in platelet adhesion (Polanowska-Grabowska et al., 1997). In *Rhodnius prolixus*, HSC70 was shown to play an essential role in the blood meal processing (Paim et al., 2016).

Previously, we have detected HSC70 in midgut contents of *Haemaphysalis flava* in a proteomic study (Liu et al., 2018a, b), and identified unigenes encoding HSC70 by searching the transcriptome library of *H. flava* salivary glands (Xu et al., 2015). In a follow-up proteomic study, at least 6 unique peptides that were functionally annotated as HSC70 were detected in saliva of *H. flava* by LC–MS/MS. These observations indicated that HSC70 was secreted into the saliva and gut lumen. Based on the sequence of contig2727 in the salivary gland transcriptome of *H. flava*, the cDNA of *HSC70* was cloned (Liu et al., 2017). We found that *HSC70* was expressed robustly in the salivary glands and midguts of *H. flava*, and that the mRNA levels in these organs were higher in semi-engorged females than in engorged females (Liu et al., 2017). Thus far, reports on the effects and mechanisms of HSC70 and other members of HSP70 family in ticks are scant. Based on existing evidence, we hypothesized that HSC70 in ticks could participate in the anticoagulation and digestion of blood-meals. In the current study, we expressed recombinant HSC70 from *H. flava* (rHSC70) and characterized its anticoagulation activity *in vitro* to better understand the anticlotting mechanisms.

2. Materials and methods

2.1. Cloning the opening reading frame of HSC70

Total RNA was extracted from engorged *H. flava* females using an *EasyPure* RNA Kit (TransGen Biotech, Beijing, China). cDNA was synthesized using TransScript All-in-One First-Strand cDNA Synthesis SuperMix for PCR (TransGen Biotech, Beijing, China). Primers were designed by Sangon (Shanghai, China) based on the full length sequence of *H. flava* HSC70 (KM111606.1) previously deposited in GenBank by our group. Two round of PCR amplifications were performed to clone the ORF of HSC70. In the first round of amplification, the primers are: *HSC70*-1F: 5′-ACGGACACAGGTTGGTAGTT-3′, *HSC70*-1R: 5′-GTCTAATCCACTTCTTCAATTGTG-3′. PCR cycling programs were: 94 °C for 5 min; 32 cycles of 94 °C for 30 s, 53 °C for 30 s, 2 min at 72 °C; and 72 °C for 10 min.

The products from the first round of amplification were used as templates for the second PCR amplification. The primer sequences for the second PCR amplification are *HSC70*-2F: 5′-tatGAATTCGCGAAGGTGCCCGCAATTGG-3′, *HSC70*-2R: 5′-ggcCTCGAGATCCACTTCTTCAATTGTGGG-3′. The underlined sequences are cleavage sites of restriction enzymes *Eco*R I and *Xho* I. PCR cycling conditions were similar to these of the first round of amplification except 32 cycles of annealing at 65 °C for 30 s. The final PCR products were confirmed using DNA sequencing with the primers *HSC70*-2R and *HSC70*-2 F.

2.2. Construction of recombinant HSC70 expression plasmid

The amplified HSC70 ORF was cloned into *pEASY*-T1, transformed to *Escherichia coli* DH5α (Tiangen Biotech, Beijing, China), and sequenced using the primers *HSC70*-2R and *HSC70*-2 F. Then, the *HSC70*-ORF purified from the positive clones was ligated to pGEX-4T-1 (Dingguo Biotech, Beijing, China) using T4 DNA ligase (Takara, Dalian, China), and then transformed to DH5α. Plasmids extracted from positive clones were subject to the restriction enzyme digestion of *Eco*R I and *Xho* I (Takara, Dalian, China), and also sequenced. Positive plasmids were selected and named as pGEX-4T-1-*HSC70*, and stored at −20 °C for futher use.

2.3. Expression and affinity purification of rHSC70

pGEX-4T-1-*HSC70* was transformed into *E. coli* BL21 (DE3) using standard procedures. A single clone was used to inoculate LB medium containing ampicillin (100 μg/mL), and cultured at 37 °C with shaking at 180 rpm until 0.6 OD_{600} was reached. Isopropyl-β-D-thiogalactoside (IPTG) at 1 mM final concentration was used to induce recombinant protein expression at 16 °C with shaking at 150 rpm. Samples were collected at 2 h, 4 h and 6 h after inducing protein expression; samples from non-induced (without IPTG) media were also collected at the same time points. Proteins were extracted from bacterial cells by ultrasonic lysis, and were subject to SDS-PAGE analysis.

For western blotting, proteins extracted from bacterial cells were transferred to a PVDF membrane, and blocked with 3% BSA at room temperature for 1 h. Mouse Anti-GST monoclonal antibody (Bioworld Technology, MN, USA) was used as the primary antibody (1:5000 dilution) and incubated at room temperature for 1 h. Goat anti-Mouse IgG labelled with horseradish peroxide (HRP) (1: 5000 dillution) (Bioworld Technology, MN, USA) was used as the secondary antibody. HSC70 were then visualized using ECL as described previously (Song et al., 2016). Digital images were obtained by the ChemiDoc XRS + system (Bio-Rad, Hercules, CA, USA).

Total proteins were also collected after 6 h from the IPTG-induced medium and purified using a GST-Resin column (7sea Biotech, Shanghai, China). The target recombinant protein was eluted with 20 mM Tris − HCl containing 6 mM glutathione (GSH) and 250 mM NaCl, and further ultrifiltered to remove GSH using an Amicon Ultra-15 centrifugal filter units (30 kDa) (Merck, Darmstadt, Germany). Purified proteins were resuspended in 1x phosphate buffered solution (PBS), and analyzed by SDS-PAGE. Conventional BCA method was used to measure the concentration of rHSC70 in the samples (UniGeneDx, Ningbo, China).

2.4. Measuring the effects of rHSC70 against blood clotting

All animal experiments in the study were approved and overseen by the Institutional Animal Care and Use Committee at Hunan Agricultural

University (HUNAU). Ten healthy adult Wistar rats (Colloge of Veterinary Medicine, HUNAU), were narcotized with 10% chloral hydrate (3.0 mL/kg) via intraperitoneal injection. Blood samples were drawn from hearts, placed in a clean test tube precoated with 3.8% sodium citrate, and centrifuged at 3000 rpm at 4 °C for 15 min. Plasma was harvested, and stored at 4 °C for further use within 2 h.

To evaluate the dose-response of rHSC70 on the anticlotting activity of rat plasma, plasma was mixed with 3.4 μM rHSC70 solution at ratios of 1:0, 1:1, 1:3 and 1:7 (v:v) to obtain plasma volume fractions at 100%, 50%, 25% and 12.5%, which meant the final concentraction of rHSC70 was 0, 1.75, 2.57 and 3 μM, respectively. Thrombin time (TT), fibrinogen (FIB), prothrombin time (PT) and activated partial thromboplastin time (APTT) of samples were measured with commercial kits (TECO, Niederbayern, Germany) using a MC-1000 coagulometer (TECO Medical Instruments, Niederbayern, Germany). Bovine Serum Albumin (BSA) (Sigma-Aldrich, MO, USA), a protein with no anti-clotting activity was used as the negative control; rSerpin-2 from *H. flava* (recombinant proteins produced in our lab), a protein with known anticloting activity was used as the positive control. All samples and reagents were pre-warmed at 37 °C before the assays. In a TT test, 200 μL testing sample was added to a cuvette, kept at 37 °C for 2 min, and then incubated with 100 μL TT reagents (porcine thrombin, 10 NIH units). The time for clot formation was recorded, and reported as TT. For FIB test, first a standard curve was prepared using a reference plasma of known fibrinogen content. Then, bovine thrombin was added and the FIB was calculated based on the observation that the clotting time is inversely proportional to the fibrinogen content. APTT was tested using APTT reagents (magnesium-aluminum-silicate) and 0.025 M $CaCl_2$. PT was measured by adding PT reagents (containing extracts of Rabbit brain with buffer, stabilizers and $CaCl_2$).

VER155008 (ApexBio Technology, Houston, TX, USA), an inhibitor of HSP70 family including HSC70, Grp78 and Hsp70 ect., at concentrations of 0.01, 0.02, 0.04, 0.08 and 0.1 mM, was incubated with rHSC70, and TT assays were conducted to plot the dose-dependent relation of the inhibitor on blood clotting. Then, to invesigate whether blocking rHSC70 would reverse its effects on APTT, PT, TT and FIB, rHSC70 (3.4 μM), normal saline, and rHSC70 (3.4 μM) with VER155008 (0.1 mM), was mixed with rat plasma at 1:1 dilution (v:v), and assigned as rHSC70 group, controls and inhibitor group, respectively. APTT, PT, TT and FIB were tested as decribed above.

Eight biological replicates were used in all the above blood-clotting tests. Data were analyzed by SPSS 17.0 (Chicago, IL, USA), and are presented as mean ± SD. Statistical significance of the differences among groups was estimated by Student's t test, and $P < 0.05$ was considered to be statistically different.

3. Results

3.1. Cloning the Heat Shock Cognate 70 (HSC70) opening reading frame

Restriction endonuclease digestion of the recombinant plasmid, pGEX-4T-1-HSC70, yielded a product of size 2000 bp, consistent with the length of the target fragment (1959 bp), suggesting that the ORF of *HSC70* was successfully inserted to pGEX-4T-1.

3.2. Expression and affinity purification of rHSC70

A robust protein at about 100 kDa molecular weight was evident in the cell lysate obtained from cultures induced with IPTG, but not in the non-induced group (without IPTG) (Fig. 1A). Western blotting showed a single protein band at 100 kDa in the protein extract from IPTG-induced culture medium (Fig. 1B, Lane 1), but no band was found in the non-induced group (Fig. 1B, Lane 2). The molecular weight of the protein was close to the target recombinant protein whose size was theoretically estimated to be 97.11 kDa. The increase in size of the recombinant protein compared to the putative protein could be contributed to the presence of the GST tag that was fused to itself.

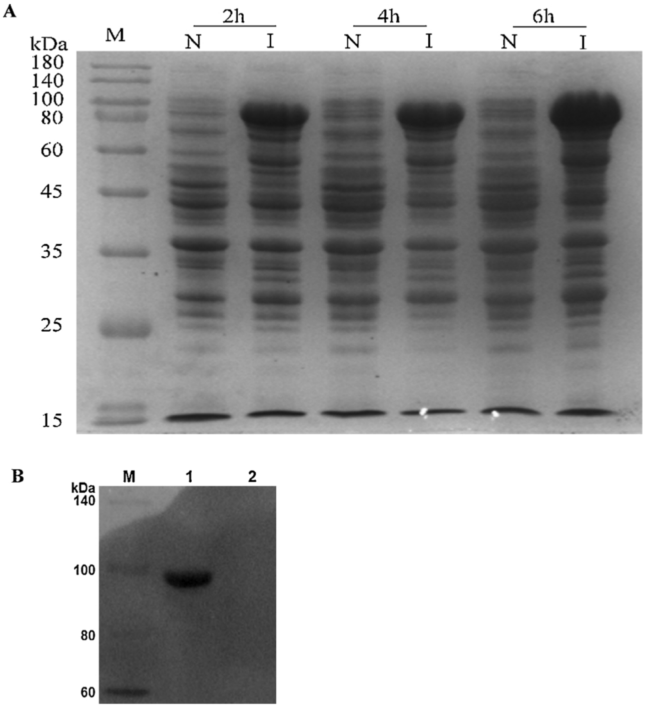

Fig. 1. Expression of rHSC70 in *E. coli.* (A) IPTG was supplied to the culture medium, and the expression was induced for 2 h to 6 h. The protein extracted from bacterial cells was electrophoresed in SDS-PAGE gels (5% stacking gel and 12% separating gel). M, Protein marker; 2 N, Non-induced expression for 2 h; 2 I. Induced expression for 2 h; 4 N, Non-induced expression for 4 h; 4 I, Induced expression for 4 h; 6 N, Non-induced expression for 6 h; 6 I, Induced expression for 6 h. (B) Identification of rHSC70 by western blotting. Proteins extracted from bacterial cells were electrophoresed on SDS-PAGE (6%), and transferred to a PVDF membrane. Mouse anti-GST monoclonal antibody (1: 5000 dilution) was used as the primary antibody, and Goat anti-Mouse IgG labelled with horseradish peroxide (HRP) (1: 5000 dilution) was used as the secondary antibody. M, Protein marker; 1, Induced expression for 6 h; 2, Non-induced expression for 6 h.

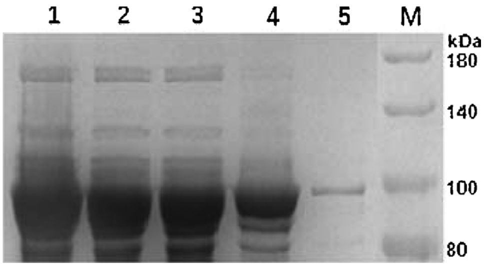

Fig. 2. Purification of rHSC70-GST in *E. coli.* Recombinant proteins were purified by affinity chromatography using a ReGST-Resin column (7sea Biotech, Shanghai, China). Target recombinant protein was eluted using Tris-HCl containing GSH and NaCl, and further ultrafiltered to remove GSH. Proteins were analyzed by SDS-PAGE (6%). 1, Whole cell lysates; 2, Supernatants of cell lysates; 3, Effluents; 4, Precipitates of cell lysates; 5, Purified rHSC70; and M, Protein molecular weight marker.

SDS-PAGE of whole cell lysates (Fig. 2, Lane 1), supernatants (Lane 2) and precipitates (Lane 3) of lysates, and effluents (Lane 4) revealed several protein bands, with the most robust protein at 100 kDa (Fig. 2). This was the only protein (100 kDa) in the purified protein samples (Fig. 2, Lane 5), indicating that the purification of expressed products was successful. As the rHSC70 was purified in its native form by affinity chromatography, no renaturation was necessary for its functional analysis. The presence of a robust protein at 100 kDa indicated that

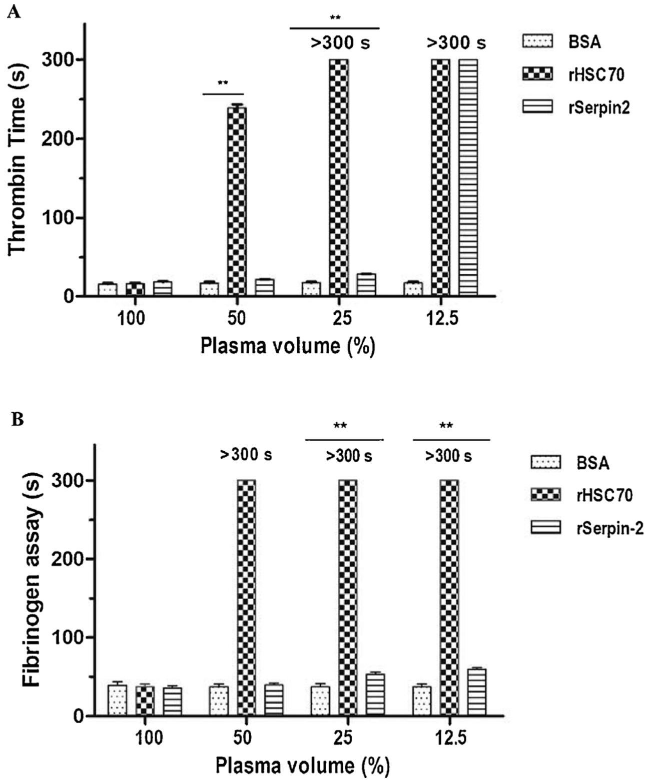

Fig. 3. Effects of rHSC70 on thrombin time (TT) (A) and fibrinogen (FIB) level (B). An initiate concertation of 3.4 μM of rHSC70 was diluted with plasma from rats, and TT and FIB were measured with commercial kits (TECO, Niederbayern, Germany) using a MC-1000 coagulometer (TECO Medical Instruments, Niederbayern, Germany). A 100% of plasma indicates no protein is added to rat plasma, while a 50% of plasma indicates the plasma is mixed with a protein solution at 1:1 (v: v). BSA with concentrations comparable to those of rHSC70 is used as negative controls; rSerpin-2 with concentrations comparable to rHSC70 is used as positive controls. ** indicates a significant difference between the rHSC70/rSerpin-2 group and negative controls ($P < 0.01$). TT of 25% and 12.5% plasma in rHSC70 group and all FIB coagulation time in rHSC70 group exceed 300 s, beyond the scale of the coagulometer. Eight biological replicates are used in total ($n = 8$).

rHSC70 was expressed in both soluble forms and inclusion bodies. Only the soluble form of rHSC70 was collected for further use. The concentration of purified rHSC70 was 0.33 mg/mL or 3.4 μM as measured by the BCA method.

3.3. Bacteria expressed rHSC70 has anticoagulant activities

Mixing 3.4 μM rHSC70 with rat plasma at 1:1 (50% plasma volume) significantly lengthened TT ($P < 0.001$) (Fig. 3A). rHSC70 groups of 25% and 12.5% plasma showed a clotting time > 300 s, a time beyond the scale of the coagulometer (Fig. 3A). In contrast, addition of BSA to plasma (negative controls) did not change TT, and rSerpin-2 group in 12.5% plasma also had a TT > 300 s (positive controls); those data indicated that the kit for measuring TT was reliable (Fig. 3A). Adding rHSC70 to plasma resulted in an FIB coagulation time > 300 s (exceeding the scale of the coagulometer), much longer than the controls (Fig. 3B), suggesting that adding rHSC70 significantly lowered the level of FIB of plasma. As expected, rSerpin-2 groups of 25% and 12.5% plasma significantly extended FIB coagulation time compared with negative controls ($P < 0.01$) (Fig. 3B). However, adding rHSC70 did not significantly change PT and APTT (data not shown).

VER155088 at concentrations of 0.01, 0.02, 0.04, 0.08 and 0.1 mM, was incubated with rHSC70, and TT assays were conducted to plot the dose-dependent relation of the inhibitor on blood clotting. A concentration of 0.01 or 0.02 mM of VER155088 + rHSC70 did not reveal a diffrence in TT compared with rHSC70 alone, however, 0.04 to 0.1 mM of VER155088 dose-dependently extended TT ($P < 0.001$) (Fig. 4A). Then, we tested the hypothesis that blocking rHSC70 would reverse its effects on FIB also. Dilution of plasma with 3.4 μM of rHSC70 + 0.1 mM of VER155008 at 1:1 (v: v) lead to a significantly decrease in FIB clotting time than with rHSC70 alone ($P < 0.001$) (Fig. 4B). These data proved that inhibition of rHSC70 could abolish its anticlotting effects.

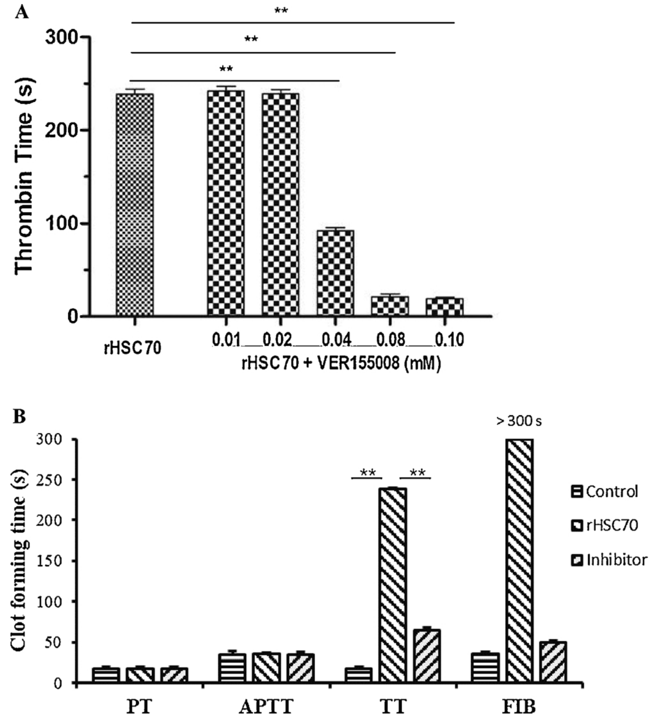

Fig. 4. Effects of inhibition of rHSC70 on PT, APTT, TT and FIB. (A) VER155008 (ApexBio Technology, Houston, TX, US.), an inhibitor of rHSC70, at different concentrations (from 0.01 mM to 0.1 mM) were incubated with rHSC70, and TT was measured as previously described. (B) rHSC70 (3.4 μM), normal saline, and rHSC70 with VER155008 (3.4 μM + 0.1 mM), was mixed with rat plasma at 1:1 dilution. PT, APTT, TT and FIB were measured as described in Fig. 3. ** indicates a significant difference between groups ($P < 0.001$). FIB coagulation time in rHSC70 group exceeds 300 s, beyond the scale of the coagulometer. Eight biological replicates are used in total ($n = 8$).

4. Discussion

The tick, *H. flava*, parasitizes many terrestrial animals, harbors pathogenic microorganisms like new bunyavirus, tick-borne encephalitis virus, etc., and is widely distributed around central and eastern China (He and Cheng, 2017). Thus, it is imperative that viable antigens are screened for developing vaccines against their infestations. Our previous studies confirmed that *HSC70* gene was expressed in *H. flava* and that the HSC70 protein was secreted into the midgut lumen by the tick (Liu et al., 2018a, b). In a saliva proteomic study of the same species, we detected at least 6 unique peptides that was functionally annotated as heat shock cognate 70 of *I. scapularis* or *R. pulchellus*, namely, DAGTIAGLNVLR, FEELNADLFR, WLDTNQLADKEEYEHR, VEIIANDQGNR, TTPSYVAFTDTER and ILNEPTAAALAYGLDK. This data suggests HSC70 is secreted into tick saliva, and ticks do this on purpose. Based on these observations and other reports, we designed the present study to investigate the role of HSC70 on anticoagulation and its possible mechanisms.

Confirmation of the anticlotting effects of HSC70 may be of significance in practical applications to control ticks. Ticks are bloodsucking ectoparasites. Except eggs, the larva, nymphs and adults ticks all need adequate blood meals to grow and enter the next stage of development. Blood-meal in ticks is processed via intracellular digestion in two steps (Coons et al., 1986); first, the blood meal is stored in the digestive tracts in a liquid form for a long time; second, digestive cells take in and break down haemoglobins to meet nutritional requirements. Blood coagulation will hamper the digestion of the blood meal; therefore, anticoagulation is essential for extracting nutrients in ticks (Araman, 1979).

During engorgement, salivary glands secrete large amounts of saliva (Oliveira et al., 2013). In the past 3 decades, dozens of proteins with anticoagulation activity were identified in the saliva (Šimo et al., 2017), but the effect of HSP70 on anticlotting was not clearly understood. Blood clotting is a complicated biochemical process that includes three phases: activation of F X, synthesis of thrombin, and formation of fibrin. It is generally believed that the activation of F X can be triggered by both endogenous pathway and exogenous pathway. PT can be employed as an indicator of the effectiveness of exogenous pathway, while APTT is used as an indicator for the endogenous pathway (Davie et al., 1991). TT indicates the ability to transform fibrinogen into fibrin, and FIB represents the level of fibrinogen in plasma (Davie et al., 1991). Here, we showed that rHSC70 could significantly prolong TT, and decrease the plasma concentration of FIB. Pharmacological blocking of rHSC70 abolished these effects. These results suggested that HSC70 inhibited the activity of thrombin, and prevented FIB from transforming into fibrin, but the anticoagulation of HSC70 was not via activation of F X. We then investigated whether rHSC70 would directly degrade fibrinogen *in vitro*. However, FIB level in the rHSC70 treatment group was not different from that in controls (data not shown), indicating that rHSC70 was not capable of degrading FIB and other indirect mechanisms might account for the inhibition of FIB. However, It was verified that homogenates of salivary glands in *Ixodes scapularis* ticks degraded fibrinogen, and that HSP70 was shown to be responsible for the activity (Vora et al., 2017). rHSC70 used in the present study had a high similarity with HSP70/HSC70 in *R. prolixus* (T1HJT8, E-value 0.0, Score 3 007, identity 90.0%). These data demonstrated that HSC70 was an indispensable molecule for blood meal anticoagulation.

The anticlotting effects of HSC70 may be of high development value (Allende et al., 2016; Paim et al., 2016). Allende et al. (2016) confirmed that HSP70 hampered thrombosis without bleeding risk in a HSPA1 A/B KO mice model. Tian et al. (2011) used rHLHSP70 as an antigen to vaccinate rabbits and then inoculated rabbits with these ticks. Bloodsucking, weights of fully engorged females, egg numbers, etc. were not statistically different from controls. We aligned the aa sequence of rHLHSP70, and found that it belonged to an ER type anchoring to the endoplasmic reticulum. As a comparison, the HSP70 reported in the present study was located in the cytoplasm, and thus could exert its function intracellularly.

To conclude, we confirmed that rHSC70 in *H. flava* exerted an anticlotting effect via the inhibition of thrombin and the transformation of fibrinogen into fibrin. Further studies are needed to elaborate the detailed molecular mechanism of its anticoagulant activity to testify its feasibility as an antigen candidate for the development of vaccines against ticks.

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