Joannsin, a novel Kunitz-type FXa inhibitor from the venom of *Prospirobolus joannsi*

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Summary

The repugnatorial glands of millipedes release various defensive chemical secretions. Although varieties of such defensive secretions have been studied, none of them is protein or peptide. Herein, a novel factor Xa (FXa) inhibitor named joannsin was identified and characterised from repugnatorial glands of *Prospirobolus joannsi*. Joannsin is composed of 72 amino acid residues including six cysteines, which form three intra-molecular disulfide bridges. It is a member of Kunitz-type protease inhibitor family, members of which are also found in the secretory glands of other arthropods. Recombinant joannsin exhibited remarkable inhibitory activity against trypsin and FXa with a Ki of 182.7 ± 14.6 and 29.5 ± 4.7 nM, respectively. Joannsin showed strong anti-thrombosis functions *in vitro* and *in vivo*. Joannsin is the first peptide component in millipede repugnatorial glands to be identified and is a potential candidate and/or template for the development of anti-thrombotic agents. These results also indicated that there is Kunitz-type protease inhibitor toxin in millipede repugnatorial glands as in other arthropods secretory glands.

Keywords

Millipede, repugnatorial gland, Kunitz-type inhibitor, factor Xa, thrombosis

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Introduction

Arthropods, common on land and in water, are rich in biologically active peptides including protease inhibitors (1, 2). Millipedes are widely distributed in the world. Most of them possess defensive/repugnatorial glands in the form of integumental sacs arranged segmentally along the length of the body. These glands provide the best protection from their predators (3). There are various chemical toxins such as 1, 4-benzoquinones, phenols, hydrogen cyanide, quinazolinones, alkaloids, benzaldehyde, benzoyl cyanide, benzoic acid, mandelonitrile, and mandelonitrile benzoate in the defensive glands (3, 4). Most of these compounds might be produced by symbiotic microbes and readily available through food intake or aspiration (5). So far, no peptide or protein has been investigated in the defensive glands (6). Many bioactive peptides and/or proteins with functions related to defense, preying and blood-sucking have been identified from the arthropods' secretory glands, including salivary glands and venom glands. It is hypothesised that the repugnatorial gland, which is a specialized secretory gland in millipedes, contains bioactive peptides and/or proteins. The current work prospects gene-encoded proteins or peptides from the repugnatorial glands of the *Prospirobolus joannsi*, which is widely distributed in China with great abundance.

Materials and methods

Anatomical studies

P. joannsi (individual weight of 5–6 g) were collected from the forest of Jiangsu Province of China. The millipedes were dissected in phosphate-buffered saline (PBS) (150 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM K_2HPO_4, pH 7.2) immediately. The sacs of defensive glands were displayed after dissecting the millipedes and removing the innards carefully according to the described method (3).

Collection of secretions from the millipede repugnatorial glands

The secretions (crude sample) were collected manually by stimulating the repugnatorial glands of the millipedes using a 3 V alternating current according to our previous report (7). The crude

sample was filtrated by the Centrifugal Filter Devices (Millipore, 3000 NMWL), and the components with a molecular weight greater than 3000 Da (crude extracts) were kept for further analysis.

cDNA library construction and sequencing

The defensive glands of *P. joannsi* were dissected as described above and cDNA was prepared as detailed in our previous study (8). Total RNA was extracted from the defensive glands of five millipedes using TRIzol (Life Technologies Ltd., Carlsbad, CA, USA) and used to prepare cDNA using a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA). cDNA library was constructed by using a Creator SMART™ cDNA Library Construction Kit (Clontech) according to manufacturer's instructions. A cDNA library of about 3×10^6 independent colonies was produced. The clones with cDNA > 350 bp were selected and sequenced on an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Expression and purification of recombinant joannsin

The DNA sequence encoding recombinant joannsin was synthesised (Sangon Biotech, Co., Ltd. Shanghai, China). As described in our previous study (9), a chemical cleavage site (-DP-) for formic acid was designed at right upstream to joannsin coding sequence. The joannsin/pET-32a (+) construct was transformed into *Escherichia coli* strain BL-21 (DE3) for recombinant expression. 50 % formic acid (v/v) treatment was used to release recombinant joannsin from the fusion protein. The hydrolysed joannsin was purified by a Sephadex G-50 (Superfine, Amersham Biosciences, Piscataway, NJ, USA; 2.6 cm × 100 cm) gel filtration column eluted by 0.1 M phosphate buffer, pH 6.0 (PBS) and C_4 reverse phase high performance liquid chromatography (RP-HPLC, Unisil C_4 column, 5 µm particle size, 10 mm × 250 mm) eluted with a gradient of increasing acetonitrile concentration containing 0.1 % (v/v) trifluoroacetic acid (TFA). Lyophilised HPLC fractions were dissolved in 0.1 % trifluoroacetic acid/water. 0.5 µL sample was spotted onto a matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) plate with 0.5 µl α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 60 % acetonitrile). Spots were analysed by an UltraFlex I mass spectrometer (Bruker Daltonics, Billerica, MA, USA) in a positive ion mode, with ± 0.06 % accuracy of mass determinations.

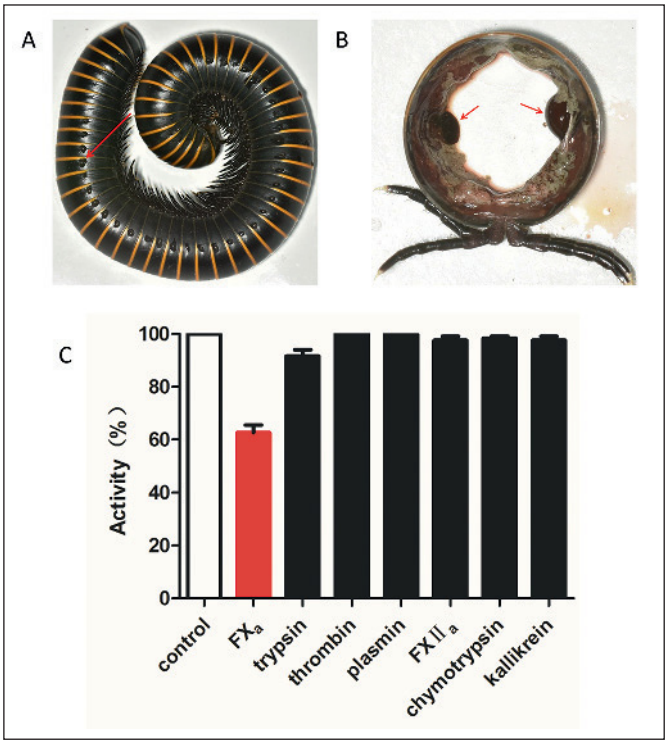

Figure 1: Repugnatorial glands in *P. joannsi* and FXa-inhibiting activity in its secretions. A) Defensive secretions from the millipede repugnatorial glands. B) Pairs of defensive glands in each body somite of the millipede are indicated by the red arrow. C) The components containing FXa-inhibiting activity. At the concentration of ~ 0.1 mg/ml, the mixture components significantly inhibited FXa.

Blood coagulation time assay

Blood recalcification time was measured according to the method described by Gulliani et al. (10). Briefly, 20 µl of platelet-poor plasma (PPP) collected from healthy human subjects was dispensed into round-bottomed 96-well plates, and then the testing sample which was dissolved in 80 µl of HEPES buffer (with 0.15 M NaCl, pH 7.4) was added to the plates. After incubation for 10 minutes (min) at room temperature, 50 µl of 0.025 M calcium chloride ($CaCl_2$) was added to the plates and the clotting time was recorded. The activated partial thromboplastin time (APTT) assay was performed according to the previous method (12). APTT reagent (50 µl) was incubated with the mixture of PPP (40 µl) and testing sample (10 µl) at 37°C for 3 min, and clotting time was recorded after the addition of 50 µl of 0.025 M $CaCl_2$. For prothrombin time (PT) assay, PT reagent (50 µl) was added to the incubated mixture at 37°C for 3 min of PPP (40 µl) and testing sample (10 µl), and clotting time was recorded. All the analyses above were monitored by the Microplate Spectrophotometer (BioTek Instrument, Inc., Winooski, VT, USA) at the absorbance of 650 nm.

Serine protease inhibitory testing

According to our previous report (9), experiments were assayed in buffer (0.05 M Tris–HCl, pH 7.8) at 37°C. The proteases (Factor Xa, 0.1 nM, Enzyme Research Laboratories, South Bend, IN, USA, HFXa 1011; trypsin, 10 nM, Sigma, St. Louis, MO, USA, T4665; thrombin, 10 nM, Sigma, T4393; plasmin, 10 nM, Sigma, P1867; Factor XIIa, 10 nM, Enzyme Research Laboratories, HFXIIa 1212a; chymotrypsin, 400 nM, Sigma, CHY5S; kallikrein, 400 nM, Enzyme Research Laboratories, HPKa 1303) and different amounts of joannsin (final concentrations ranging from 0.0 to 0.262 µM) were pre-incubated for 10 min at 37°C. After incubation of the inhibitor with the enzyme for 30 min, the reaction was initiated by the addition of 0.5 mM substrate F3301

(CH_3OCO-*D*-CHA-Gly-Arg-*p*NA-AcOH, Sigma) for FXa, B3133 (*N*-benzyl-L-arginine-4-nitroanilide-hydrochride-*p*NA, Sigma) for trypsin, *H*-*D*-Phe-Pip-Arg-*p*Na·2HCl (Hyphen Biomed, Neuville-sur-Oise, France) for thrombin, G8148 (Gly-Arg-p-nitroanilide dihydrochloride, Sigma) for plasmin, *H*-*D*-Pro-Phe-Arg-*p*NA·2HCl (Hyphen Biomed, France) for FXIIa , S1899 (*N*-Succinyl-Gly-Gly-Phe-*p*-nitroanilide, Sigma) for chymotrypsin, and C9521 (*Z*-Phe-Arg-7-amido-4-methylcoumarin hydrochloride, Sigma) for kallikrein. The initial rate of product formation in each reaction was monitored continuously at 405 nm for 2 min. The inhibition constant *Ki* was determined according to the Dixon method (11). To further study the inhibition of joannsin on FXa, the physiological substrate human prothrombin (Enzyme Research Laboratory, HP 1002) was used. The reaction was assayed in buffer (0.05 M Tris–HCl, pH 7.8) at 37°C. After incubation of FXa (10 nM) and joannsin (0, 10, 20, 50 nM) for 30 min, human prothrombin (10 nM) was added to the mixture and incubated for 5 min, and then the reaction was stopped with the addition of 5×SDS-loading buffer. After boiling for 5 min, the mixtures were checked by the SDS-PAGE.

Anti-thrombosis in mouse model

Carrageenan-induced mouse tail thrombosis model (13) was used in this experiment. Apixaban and 0.9% saline were used as positive control and blank control, respectively. Testing sample with different concentrations was injected intravenously into male Kunming mice (n=8, 20–25 g). After 30 min, mice were intraperitoneally injected with 100 µl (1%) κ-Carrageenan (type I, Sigma) dissolved in 0.9% NaCl with a dose of 60 mg/kg to induce thrombosis in the tail. Six hours (h) after the injection of κ-Carrageenan, the testing sample was injected into mice by caudal vein again. The length of thrombus in mouse tail was measured at 12 h and 24 h after treatment, respectively. All the experimental protocols using animals in this work were approved by the Animal Care and Use Committee at Kunming Institute of Zoology, Chinese Academy of Sciences (SMKX-2015022).

Statistical analysis

Data were assessed for statistical significance using Student's (unpaired) t-test. Results were reported as mean ± *SE* with significance accepted at $p < 0.05$.

Results

Anatomical studies

Copious secretion was observed in the millipede repugnatorial glands after stimulating by a 3 V alternating current (▶Figure 1A). There was a pair of defensive glands in each body somite of the millipede (▶Figure 1B).

FXa inhibiting activity in the secretions of the millipede defensive glands

Under the assay conditions, at the concentration of ~ 0.1 mg/ml, the crude extracts of the defensive secretions showed significant inhibitory activity on FXa (▶Figure 1C), indicating that there are components that inhibit FXa in the crude extracts. However, there

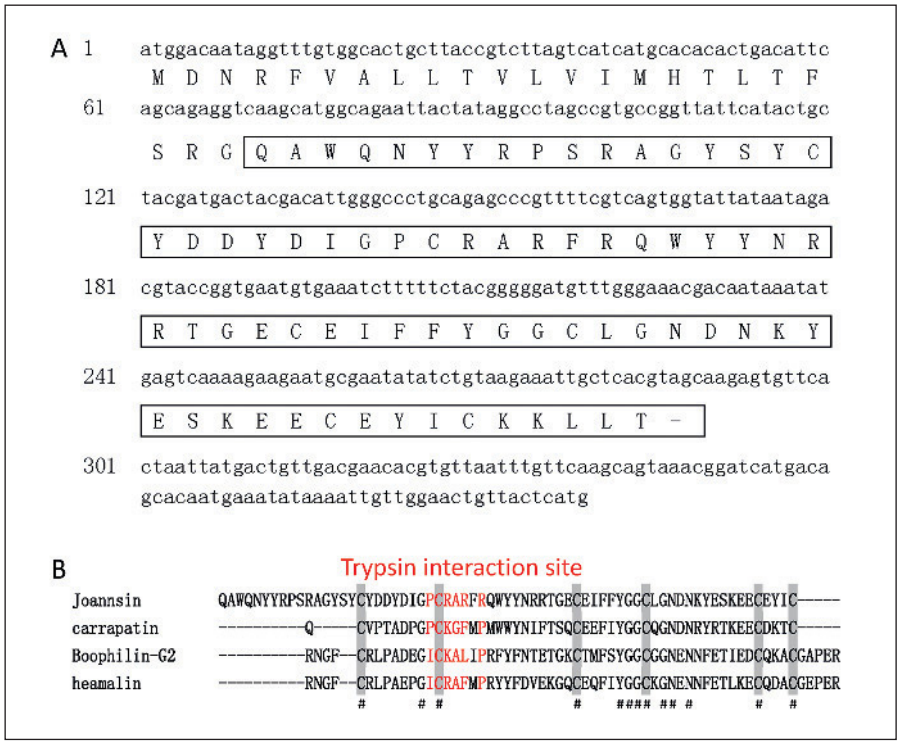

Figure 2: Primary structure of joannsin (A) and sequence alignment with other Kunitz-type serine protease inhibitors (B). Mature joannsin is boxed, and the stop codon is indicated by a bar (-). The identical amino acid residues are indicated by the symbol (#). Cysteine residues are highlighted in grey and the trypsin interaction sites are shown in red.

were only ~ 1–3 % total proteins in the crude extracts and the sample was insufficient for purification of native FXa inhibitors from the secretions. Other approaches including cDNA cloning and recombinant expression were used to identify and characterise potential FXa inhibitors.

Primary structure of joannsin

A cDNA encoding a protein precursor composed of 95 amino acid (aa) residues was found (►Figure 2A). After online analysis to predict cleavage sites and a signal peptide/non-signal peptide by

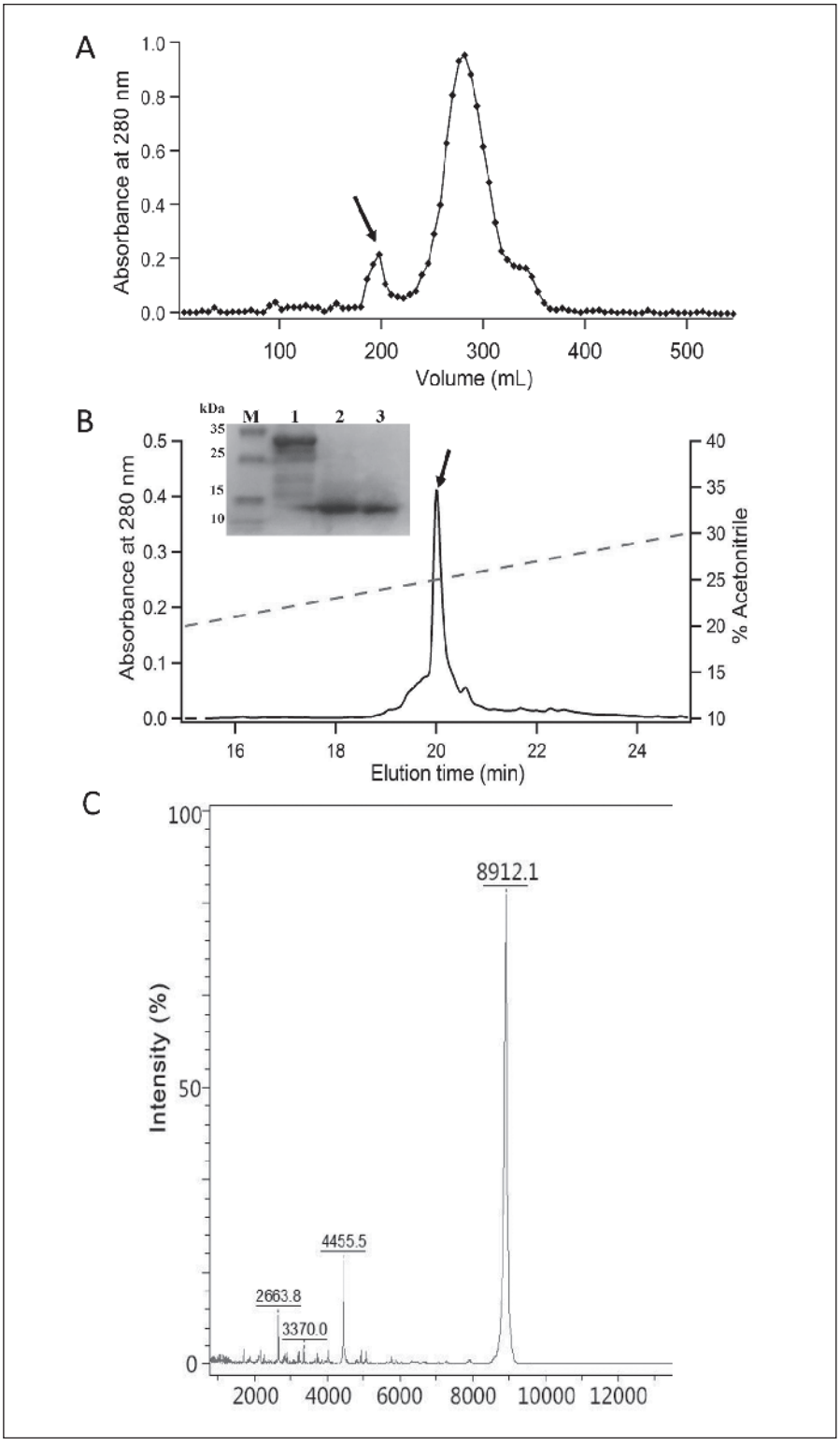

Figure 3: Recombinant expression of joannsin. Recombinant joannsin was purified by Sephadex G-50 gel filtration (A) and C4 RP-HPLC (B), indicated by an arrow. SDS-PAGE of samples after each purification (inserted in B): collected fusion protein purified with affinity (line 1); after chemical cleavage, recombinant joannsin was purified by a Sephadex G-50 (line 2); recombinant joannsin purified by a C4 RP-HPLC (line 3). The purified recombinant joannsin was subjected to MALDI-TOF mass spectrometry analysis (C).

the SignalP 4.1 Server tool (http://www.cbs.dtu.dk/services/SignalP/), a hypothetical 23-aa signal peptide and a hypothetical 72-aa mature peptide named joannsin were obtained. BLAST search indicated that joannsin shared high homology with other known Kunitz-type protease inhibitors, which are thrombin inhibitors from salivary glands of blood-sucking ticks (14–17) (►Figure 2B). Especially, there is a putative trypsin interaction site present in other serine protease inhibitors. The distribution motif of six cysteines in joannsin is also identical to other serine protease inhibitors. Based on the sequence alignment, joannsin may contain inhibitory ability on serine proteases, especially on blood coagulation factors.

Expression and purification of recombinant joannsin

The joannsin/pET-32a (+) construct was transformed into the *E. coli* strain BL-21 (DE3) for recombinant expression. The recombinant joannsin was expressed by the induction of IPTG (1 mM) and purified from the soluble fraction of the *E. coli* lysate. The recombinant protein was isolated on a His-binding resin Ni^{2+} affinity column and hydrolysed by 50% formic acid. The products were first separated by the Sephadex G-50 gel filtration column (►Figure 3A), and then purified further by the C_4 RP-HPLC (►Figure 3B). After each purification, SDS-PAGE was used to identify the quality of collected samples (inserted in the ►Figure 3B). The purified joannsin was subjected to matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis, which gave an observed molecular mass of 8911.1 (►Figure 3C) by using a positive ion and linear mode. The theoretically predicted molecular mass of joannsin is 8916.9 Da. Considering that the sequence contains six cysteine residues, which likely form three intra-molecular disulfide bridges as found in other Kunitz-type serine protease inhibitors, the theoretically predicted molecular mass of joannsin containing three intra-molecular disulfide bridges should be 8910.9 Da, which corresponded with the observed molecular mass.

Joannsin prolonged blood coagulation time

The influence of joannsin to blood coagulation was explored through the recalcification time, APTT and PT assay. Compared with normal recalcification time of ~ 10 min, recalcification time was significantly prolonged by 0.125 mg/ml joannsin, and no coagulation was observed even at 15 min (►Figure 4A). Normal APTT is ~ 30 seconds (s). However, no coagulation appeared even at 120 s after 0.125 mg/ml joannsin treatment (►Figure 4B).

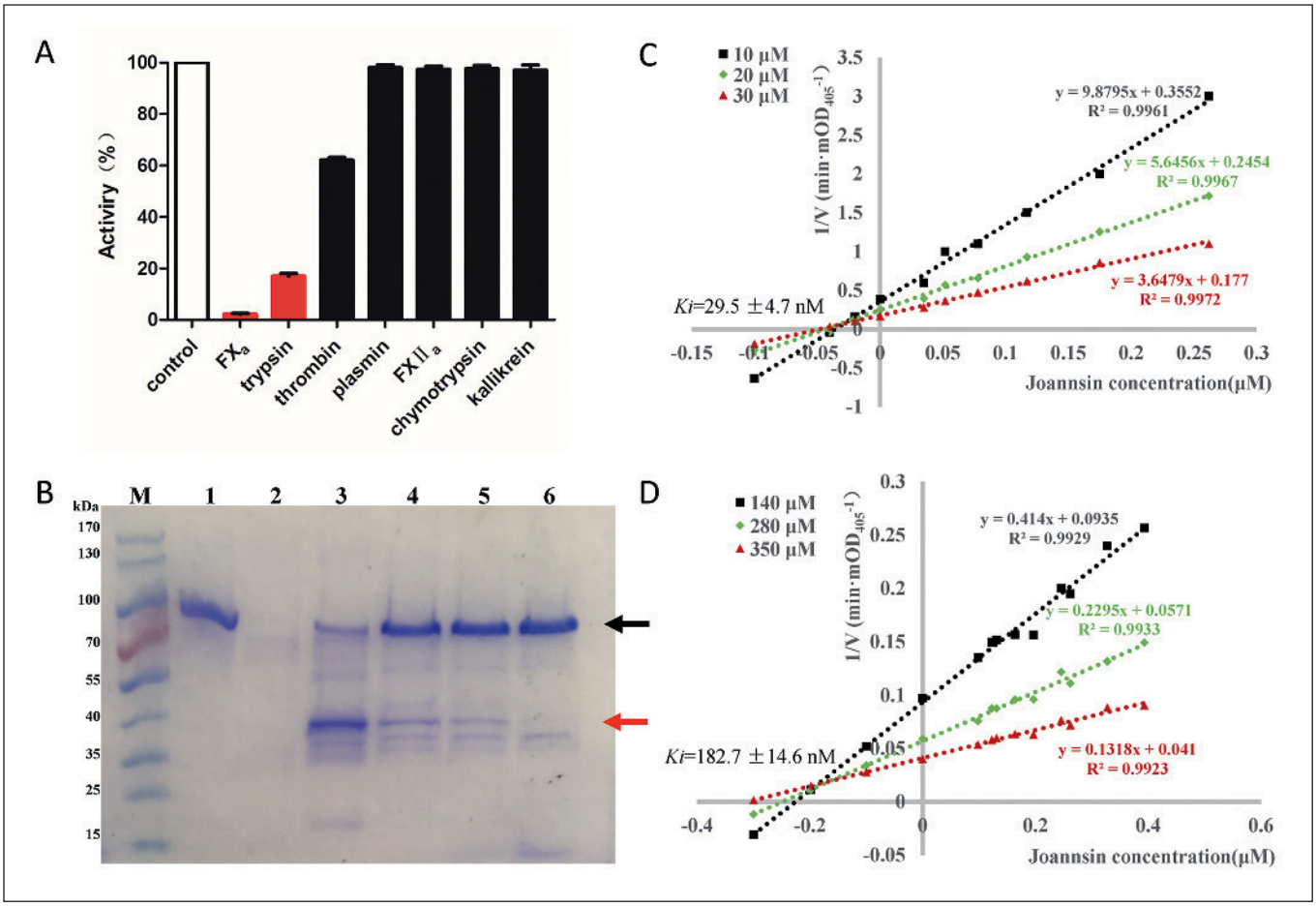

Figure 4: Coagulation effect of joannsin. Compared with the control group, the recalcification time (A), APTT (B) and PT (C) were prolonged.

Compared with the normal PT of ~ 10 s, the PT induced by 0.125 mg/ml joannsin was ~ 60 s (▶Figure 4C).

Protease inhibitory activity of joannsin

The inhibition activity of joannsin to a diversity of proteases was further explored. We confirmed that joannsin strongly inhibited the activity of FXa and trypsin and moderately inhibited the activity of thrombin (▶Figure 5A). It had no effect on plasmin, FXIIa, chymotrypsin, and kallikrein, which are related with blood coagulation, inflammation, or digestion. With the incubation of joannsin and FXa, it was suggested that joannsin inhibited the activity of FXa to its physiological substrate human prothrombin in a dose-dependent manner (▶Figure 5B). Enzymatic kinetic studies showed that joannsin was a non-competitive inhibitor towards FXa and trypsin with *Ki* of 29.5 ± 4.7 and 182.7 ± 14.6 nM, respectively (▶Figure 5C, D).

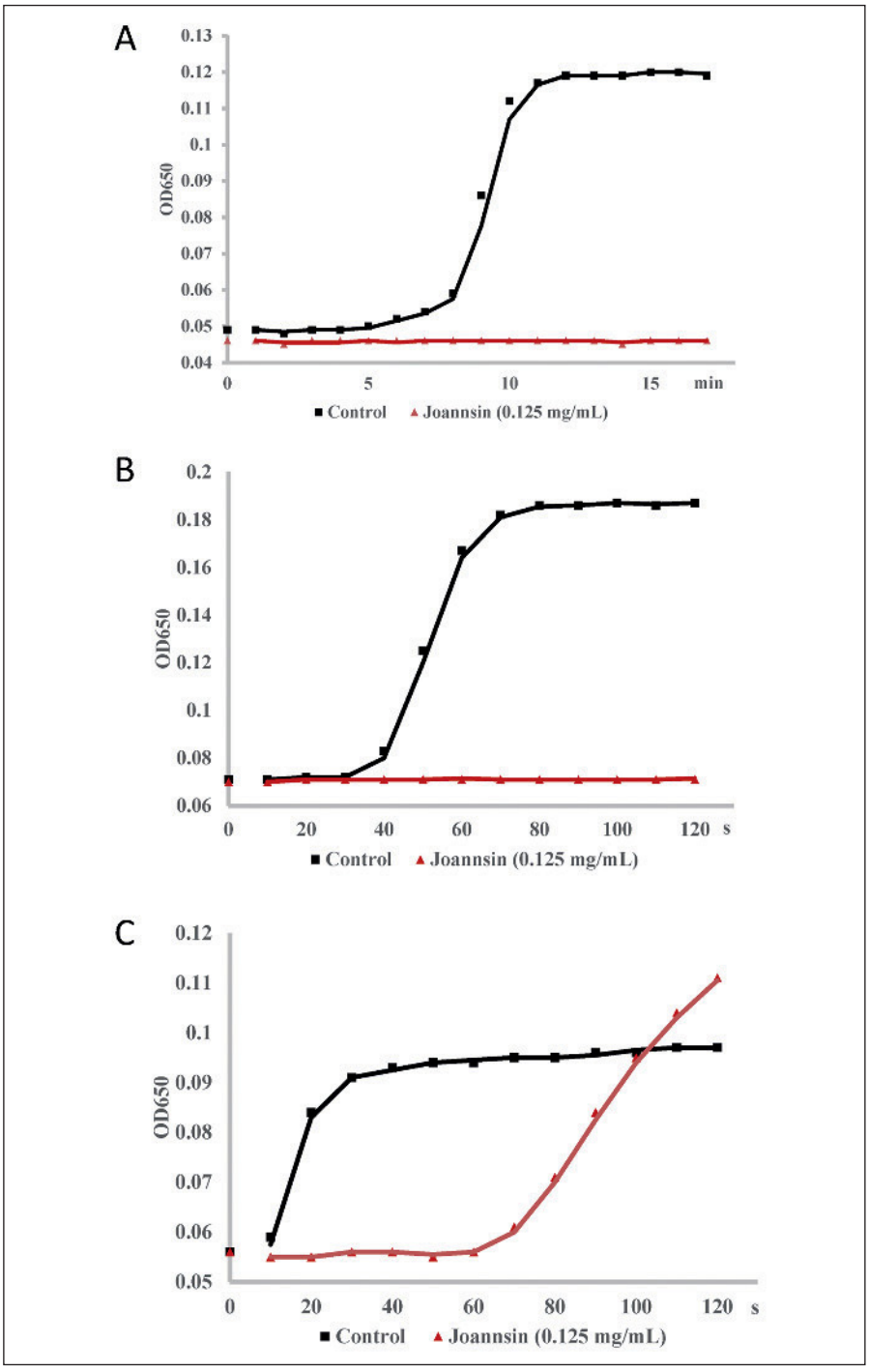

Figure 5: Protease inhibiting activity of joannsin. The effect of joannsin on FXa, trypsin, thrombin, plasmin, FXIIa, chymotrypsin and kallikrein with the chromogenic substrate (A). SDS-PAGE after the reaction of joannsin and FXa (B): human prothrombin (line 1); FXa (line 2); joannsin inhibited the activity of FXa to human prothrombin with different concentrations (0 nM: line 3; 10 nM: line 4; 20 nM: line5; 50 nM: line 6), black arrow and red arrow indicated human prothrombin and thrombin, respectively. According to the Dixon method, the *Ki* of joannsin towards FXa and trypsin is 29.5 ± 4.7 nM (C) and 182.7 ± 14.6 nM (D), respectively.

Joannsin inhibited thrombus formation

As shown in ▶Figure 6, recombinant joannsin inhibited thrombosis in a dose-dependent manner in carrageenan-induced mouse tail thrombosis model. After 12 h injection of joannsin, thrombus formation was inhibited by 28.3%, 50.0% and 70.0% at the dosage of 2 (67.33 μM), 4 (134.66 μM) and 6 mg/kg (201.99 μM), respectively, compared with the 38.3% inhibitory rate induced by 0.2 mg/kg (130.58 μM) apixaban, which is a clinical FXa inhibitor (▶Figure 6A). At the time of 24 h, 26.1%, 54.2% and 61.1% thrombus formation was inhibited by 2, 4 and 6 mg/kg joannsin, respectively. The thrombus formation inhibition by 0.2 mg/kg apixaban is 44.4% (▶Figure 6B). The representative actual thrombus length of each group at 24 h was measured and the photos taken (▶Figure 6C).

Discussion

Many arthropods contain specialised secretory glands, which have been found in groups of chelicerata, hexapoda, myriapoda and insects (18). For instance, horseflies (19, 20) and ticks (21, 22) have salivary glands containing bioactive peptides or/and proteins with functions (i.e. anti-thrombosis, analgesia, and anti-inflammation) to facilitate blood-sucking; wasps (23, 24), centipedes (25–27), scorpions (8, 28), and spiders (29–32) have venomous glands containing peptides or/and proteins to exert defensive and predatory functions. Many components in those glands are small peptides in which disulfide bonds form the scaffolds which are referred to as disulfide-rich domains (21). Particularly, many of these small peptides belong to the group of Kunitz-type serine protease inhibitors which possess diverse functions, they act on voltage-gated ion channels, proteases, coagulation factors, receptors, and so on (33–35). Significantly specialised repugnatorial glands in the body of millipedes are considered as defensive organs. Many chemical compounds exerting defensive functions have been identified from repugnatorial glands of millipedes (3, 4). Most of the compounds are originated from foods. Limited information is available on peptides or proteins from repugnatorial glands of millipedes.

A Kunitz-type serine protease inhibitor (joannsin) was identified and characterized from the defensive glands of the *P. joannsi*. Joannsin is a peptide containing 72 amino acid residues including three intra-molecular disulfide bridges, which form disulfide-rich domains as found in other secretory glands of venomous or blood-sucking arthropods (21). Joannsin shares significant sequence similarity with thrombin inhibitors from salivary glands of ticks, a group of blood-sucking arthropods (14–17). Among the seven tested serine proteases, joannsin showed inhibitory activity on two coagulation factors (thrombin and FXa) and trypsin (▶Figure 5). It showed the strongest ability to inhibit FXa especially. Amblyomin-X, TFPI (tissue factor pathway inhibitor) and joannsin all belong to the Kunitz-type serine protease inhibitor, but they have different *Ki* to FXa: 3.9 μM for Amblyomin-X; 1.24 nM for TFPI; 29.5 ± 4.7 nM for joannsin (36–37). The structural and functional similarity of Kunitz-type serine protease inhibitors in both ticks and millipedes may imply their evolution connection, which opens up a new avenue for further study. FXa plays a key role in blood coagulation and is an important target for the development of anti-thrombosis agents. As illustrated in ▶Figure 6, *in vivo* anti-

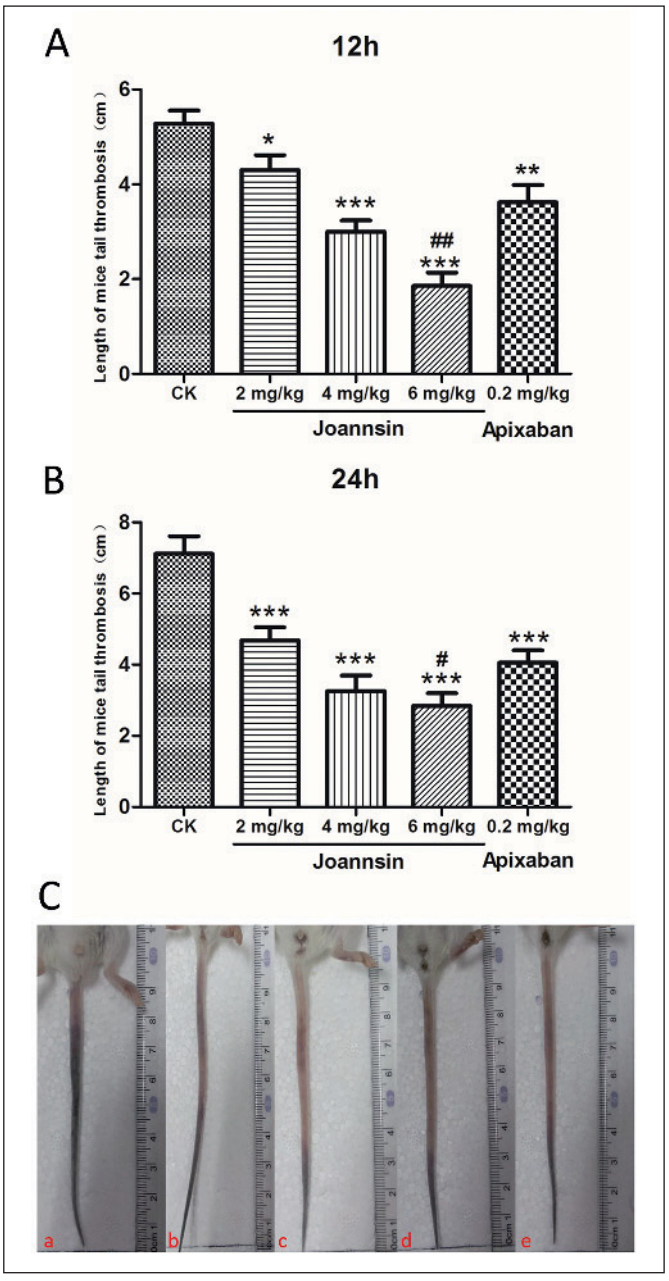

Figure 6: Joannsin inhibited thrombus formation *in vivo*. Joannsin inhibited carrageenan-induced thrombosis in mouse tail at 12 (A) and 24 (B) h after injection in a dose-dependent manner. (C) The representative thrombus in the mouse tail at 24 h. (a) The control group, (b, c, d) the joannsin groups with different concentrations (2 mg/kg, 4 mg/kg and 6 mg/kg, respectively), (e) the group of apixaban (0.2 mg/kg). Data were presented as mean ±SE. * P< 0.05, ** P< 0.01, *** P<0.001 compared with CK, # P< 0.05, ## P< 0.01, compared with Apixaban. Analysed by student's t test for two-sample comparison.

What is known about this topic?

- The repugnatorial glands of millipedes release various defensive chemical secretions, such as 1, 4-benzoquinones, phenols, hydrogen cyanide, quinazolinones, alkaloids, benzaldehyde, benzoyl cyanide, benzoic acid, mandelonitrile, and mandelonitrile benzoate, to provide the best protection from their predators.
- Although varieties of such defensive secretions have been studied, none of them is protein or peptide.
- We hypothesised that the repugnatorial gland, which is a specialised secretory gland in millipedes, contains bioactive peptides and/or proteins.

What does this paper add?

- In this study, we have prospect a novel coagulant factor Xa (FXa) inhibitor named joannsin, which from the protein components of the defensive chemical secretions of *Prospirobolus joannsi*.
- To our knowledge, so far, this is the first peptide investigated in the repugnatorial glands of the millipede. As a Kunitz-type protease inhibitor, joannsin exhibited remarkable inhibitory activity against trypsin and FXa with a Ki of 182.7 ± 14.6 and 29.5 ± 4.7 nM, respectively.
- Besides, joannsin showed strong anti-thrombosis functions *in vitro* and *in vivo*.
- Our findings support that joannsin can be a potential candidate and/or template for the development of anti-thrombotic agents.

thrombosis ability of joannsin is comparable with that of apixaban, a clinical anti-thrombotic agent. Three intra-molecular disulfide bridges in the small peptide of joannsin may render high stability *in vivo*, which combined with the potential anti-FXa and anti-trypsin abilities may make joannsin an excellent candidate and/or template to develop anti-thrombosis drugs.

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Conflicts of Interest

None declared.

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