

Isolation and Characterization of a Novel Antithrombotic Peptide from Enzymatic Hydrolysate of *Agkistrodon acutus* Venom

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Abstract Snake venoms contain a large variety of proteins and peptides that affect the hemostasis and thrombosis. Numerous antithrombotic peptides have been found in snake venom. However, few studies have been performed on the proteolysis of snake venom for obtaining bioactive peptides. In this study, the *Agkistrodon acutus* venom was hydrolyzed using four commercial proteases (pepsin, papain, neutrase, and alcalase) and the hydrolysate was tested for antiplatelet aggregation activity. The pepsin hydrolysate, exhibiting the highest activity, was further purified by gel filtration and reversed-phase high performance liquid chromatography. A novel antithrombotic peptide SP-14 was obtained, and its sequence was identified as SHIHGDYSSPSGAP using tandem electrospray mass spectrometry. SP-14 inhibited U46619-induced rabbit platelet aggregation in a dose-dependent manner. It reduced the mortality of mice in acute pulmonary thrombosis model and decreased thrombus weight in rat arteriovenous shunt model, while with lower bleeding risk than aspirin. Therefore, SP-14 may be beneficial for new antithrombotic drug design and development.

Keywords *Agkistrodon acutus* venom · Enzymatic hydrolysis · Platelet aggregation inhibitor · Antithrombotic peptide · Bleeding evaluation

Introduction

It is widely believed that thrombus formation influences the progression of various cardiovascular or cerebrovascular disorders, including myocardial infarction, unstable angina, transient ischemic attack, and atherosclerosis (Fan et al. 2010). In the process of thrombus formation, platelets play a crucial role because they can adhere, become activated, release agonists and finally aggregate at the injured vascular site (Peng et al. 2011). Accordingly, antiplatelet agents have been widely developed as an important tool for preventing thrombotic events. However, these drugs may lead to severe hemorrhagic risk or upper gastrointestinal bleeding (Hao et al. 2015). Therefore, research to find new antiplatelet agents is necessary for the prevention of thrombotic disease.

Natural bioactive peptides are considered to be one of the most important sources for drug development. In recent years, much attention has been paid to unraveling the structural and functional properties of bioactive peptides. Solvent extraction, enzymatic hydrolysis, and microbial fermentation are three methods to generate natural bioactive peptides in drug discovery. Among them, the enzymatic hydrolysis method is the best choice because of little residual organic solvents or toxic chemicals in the products (Kim and Wijesekara 2010). A large number of bioactive peptides have been discovered using enzymatic hydrolysis, such as antioxidant peptide (Kim et al. 2013), anticoagulant peptide (Ren et al. 2014), antithrombotic peptide (Shimizu et al. 2009), antihypertension peptide (Lee et al. 2010), and immunomodulatory peptide (Gauthier et al. 2006).

Snake venoms contain a large variety of proteins and peptides affecting hemostasis and thrombosis (McCleary and Kini 2013). Several studies have reported antithrombotic proteins or peptides derived from snake venom and their potential for use as alternative antithrombotic drugs, such as

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SV-PAD-1 from *Protobothrops elegans* venom (Oyama et al. 2009), Ruviprase from *Russell's viper* venom (Thakur et al. 2014), and AgkTx-II from *Agkistrodon halys* venom (Perumal Samy et al. 2008). However, few studies have evaluated the antithrombotic properties of peptides derived from enzymatic hydrolysates of snake venom. In this study, the antiplatelet aggregation activities of *Agkistrodon acutus* venom hydrolysates prepared using four commercial proteases (pepsin, papain, neutrase and alcalase) were investigated. A novel antithrombotic peptide, named SP-14, was isolated from pepsin hydrolysate by gel filtration chromatography and reversed phase high performance liquid chromatography (RP-HPLC). This peptide was further synthesized to evaluate its antithrombotic activity by acute pulmonary thrombosis model in mice and arteriovenous shunt thrombosis model in rats. Moreover, the bleeding risk of this peptide was examined by tail cutting in mice.

Materials and Methods

Materials and Animals

Lyophilized *Agkistrodon acutus* venom was obtained from YuJiang snake farm (Jiangxi province, China) and stored at -20°C . Adenosine diphosphate (ADP), thrombin, and U46619 (a TxA_2 analog) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Collagen was purchased from Hyphen-Biomed (Neuville Sur Oise, France). All other chemicals used in this study were of analytical grade. ICR mice (18–22 g), Sprague–Dawley rats (180–220 g), and New Zealand white rabbits (2–2.5 kg) were purchased from Jiangsu Province Laboratory Animal Center.

Preparation of *Agkistrodon acutus* Venom Hydrolysates

Each 100 mL of buffer solution was added to each 1 g of snake venom powder and then added different enzymes. These buffer solutions were as follows: 0.1 M sodium phosphate buffer solution, pH 8.0 for neutrase; 0.1 M sodium phosphate buffer solution, pH 8.0 for alcalase; 0.1 M glycine–HCl solution, pH 2.0 for pepsin; 0.1 M sodium phosphate buffer solution, pH 6.0 for papain. The mixtures were hydrolyzed for 4 h separately by neutrase (6.43×10^4 U/g) at 50°C , alcalase (1.51×10^5 U/g) at 50°C , pepsin (8.03×10^4 U/g) at 37°C , and papain (8.91×10^4 U/g) at 37°C . The enzyme/substrate ratios (w/w) were 1, 0.5, 1, and 1 %, respectively. Samples were taken out at 2-, 3-, and 4-h intervals and followed by immediate heating at 100°C for 15 min to inactive the enzyme. The hydrolysates were centrifuged at 10,000 rpm for 15 min and the supernatants were stored at 4°C .

Platelet Aggregation Activity Assay

In vitro platelet aggregation was measured according to the turbidimetric method (Born and Cross 1963) using four-channel aggregometer (Beijing Steellex Science Instrument Company, China). Blood was withdrawn from the carotid artery of New Zealand white rabbits and anti-coagulated with 3.8 % sodium citrate (1:9 citrate/blood, v/v). Platelet-rich plasma (PRP) was obtained by centrifugation at $120 \times g$ for 15 min. Platelet-poor plasma (PPP) was prepared from the precipitated fraction of PRP by centrifugation at $1600 \times g$ for 10 min. PRP was adjusted to 3×10^8 platelets/mL. Aliquots of 270 μL of PRP were preincubated at 37°C for 5 min in the cuvette with 20 μL of sample solution at appropriate concentrations (200, 400, 600, 800 and 1000 $\mu\text{g/mL}$). Saline was vehicle control. After incubation, platelet aggregation was induced by the addition of 10 μL ADP (10 μM), collagen (2.5 $\mu\text{g/mL}$), thrombin (2 U/mL) or U46619 (6 μM). The maximum platelet aggregation rate was recorded within 5 min with continuous stirring at 37°C . The light transmittance was calibrated with PPP. The inhibition of platelet aggregation was measured at the maximum aggregation response.

Antithrombotic Peptide Purification

The samples having antiplatelet aggregation activity was applied to a column (2.6 \times 100 cm) packed with Sephadex G-50 (Pharmacia Biotech, Sweden), equilibrated with distilled water and eluted with distilled water at a flow rate of 0.6 mL/min. The absorbance of the eluates was monitored at 280 nm. The fractions were collected at 6-min intervals, and then tested for their effects on the antiplatelet aggregation activity. The fraction with the highest antiplatelet aggregation activity was collected, lyophilized, and then purified by a semi-preparative RP-HPLC column (Habon C_{18} ; 20 \times 250 mm) in the BioLogic Duoflow system (Bio-Rad, USA) using acetonitrile as the organic modifier and trifluoroacetic acid (TFA) as the volatile buffer. Eluent A consisted of 0.1 % TFA in 10 % acetonitrile/ H_2O (v/v), and eluent B consisted of 0.1 % TFA in 90 % acetonitrile/ H_2O (v/v). The linear gradient elution conditions was conducted using an isocratic step for 0–48 mL with 10 % acetonitrile containing 0.1 % TFA and a subsequent 132 mL linear gradient of 10–80 % acetonitrile containing 0.1 % TFA at a flow rate of 6.0 mL/min. The eluent profile was monitored at 214 nm. The fraction with highest antiplatelet aggregation activity was further separated by analytical RP-HPLC column (Kromasil C_{18} ; 4.6 \times 250 mm) using a linear acetonitrile gradient (20–75 %) at a flow rate of 1.0 mL/min. Eluates was monitored at 214 nm. Each fraction was evaluated for antiplatelet aggregation activity. The fraction displaying strongest antiplatelet aggregation activity was

collected and lyophilized. To evaluate the purity, the sample was checked on the analytical C₁₈ RP-HPLC column using LC-20AT system (Shimadzu, Japan).

Determination of Molecular Mass and Peptide Sequence

The molecular mass and amino acid sequence of the purified peptide was determined by tandem electrospray mass spectrometry (MS) (Agilent 6520 Q-TOF, USA) coupled with an electrospray ionization (ESI) source. The purified peptide was dissolved in H₂O/acetonitrile (85:15, v/v), containing 0.1 % formic acid. Ionization was carried out in positive mode with a capillary voltage of 4000 V. Nitrogen was maintained at 40 psi for nebulization and 8 L/min at 325 °C for evaporation temperature. Data were collected in centroid mode from m/z 100 to 1700. Peaks Viewer 4.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used in combination with manual de novo sequencing to process the MS/MS data and to analyze peptide sequence.

Peptide Synthesis

The platelet aggregation inhibitory peptide identified from the hydrolysate was synthesized by the stepwise manual solid phase peptide synthesis (SPPS) technique according to a previous study (Kong et al. 2013). The synthesized peptide was purified by the preparative reversed-phase HPLC on a C₁₈ column (Habon C₁₈; 20 × 250 mm). Elution was performed using a linear gradient (10–60 % B in 30 min) of 0.1 % TFA in 10 % acetonitrile (A) and 0.1 % TFA in 90 % acetonitrile (B) at a flow rate of 6.0 mL/min. The absorbance was monitored at 214 nm. The main peak was pooled, dried in vacuum, lyophilized, and stored at –20 °C. The purity was analyzed by HPLC and ESI-MS. The purity of this peptide was more than 96.0 %.

Acute Pulmonary Thrombosis Assay

Acute pulmonary thrombosis was induced according to a previously described method (Hsiao et al. 2003). Vehicle control (saline), SP-14 (5, 15, and 45 mg/kg) and aspirin (50 mg/kg) were injected into the tail vein of mice (18–22 g). Fifteen minutes later, ADP (300 mg/kg) was injected into the contralateral vein to induce thrombosis. Mortality of mice in each group after injection was recorded within 15 min.

Arteriovenous Shunt Thrombosis Assay

Male Sprague–Dawley rats (180–220 g) were used for arteriovenous shunt thrombosis model according to the previously described method, with minor modifications

(Sperzel and Huetter 2007). Saline, aspirin (50 mg/kg) or SP-14 (5, 15, and 45 mg/kg) was administered through tail veins. Sixty minutes after administration, rats were anesthetized with chloral hydrate (300 mg/kg, i.p.). The right common carotid artery and left jugular vein were isolated and cannulated with two 40-mm-long, saline-filled catheters (PE-60, Becton–Dickinson, Sparks, MD, USA). A 120-mm-long polyethylene tube (PE-160, Sparks, USA) was used to connect the two PE-60 catheters. The polyethylene shunt containing a cotton thread (100 mm in length and 0.26 mm in diameter) was filled with heparin saline solution (50 U/mL) before installation. The extracorporeal circulation of blood through the shunt tube was opened for 20 min and then the cotton thread was withdrawn from the middle PE-160 catheter. The wet and dry weights of thrombi were measured by subtracting the pre-experiment weight of the wet and dry 10-cm cotton thread.

Bleeding Evaluation

For evaluating the bleeding time, mice tail cutting method was performed according to the previously described method with some modifications (Tucker et al. 2012). Briefly, fifty mice (18–22 g, male and female in half) were divided into 5 groups: saline, SP-14 (5, 15, and 45 mg/kg) and aspirin (50 mg/kg) group. Fifteen minutes after the administration, mice were anesthetized with chloral hydrate (300 mg/kg, i.p.) and the tails were transected at 3 mm from the tip using a disposable surgical blade. The remaining tail was immediately immersed into saline at 37 °C. The bleeding time were recorded for 20 min.

Statistical Analysis

All data were analyzed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Results were expressed as the mean of three replicate determinations and standard deviation (SD). Statistical significance was evaluated using *t* test for two-sample comparison or ANOVA followed by Tukey's test for multiple comparisons. *P* < 0.05 was considered to be statistically significant.

Results

Preparation of *Agkistrodon acutus* Venom Hydrolysates

Enzymes have their specific cleavage positions within proteins. To select a suitable protease, *Agkistrodon acutus* venom was independently hydrolyzed with alcalase,

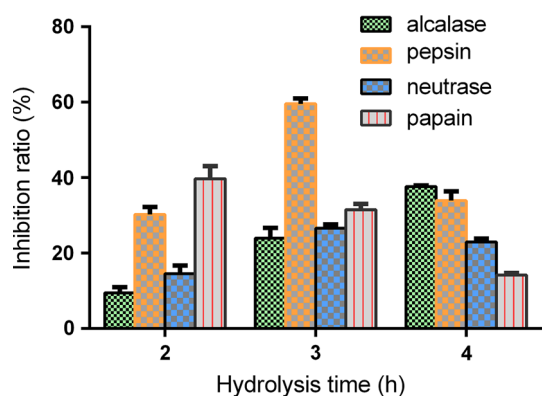


Fig. 1 Platelet aggregation inhibitory activities of *Agkistrodon acutus* venom hydrolysates prepared using four enzymes at different hydrolysis times. All data are expressed as mean \pm SD ($n = 3$)

neutrase, pepsin, and papain followed by antiplatelet activity test. As shown in Fig. 1, the antiplatelet activity of alcalase hydrolysate increased with prolongation of hydrolysis time, while papain acts as opposite trend. Hydrolysates produced by neutrase or pepsin showed an increase in antiplatelet activity at the first 3 h, and then decreased. Amongst the four enzymes examined, pepsin digest for 3 h resulted in the highest antiplatelet activity, from which the maximum inhibitory rate of platelet aggregation by the hydrolysate reached 59.56 ± 1.41 %. Therefore, the *Agkistrodon acutus* venom hydrolysate produced by pepsin treatment for 3 h was selected for further study.

Purification and Determination of an Antithrombotic Peptide

The purification procedure was a combination of gel filtration and reversed-phase chromatography guided by monitoring antiplatelet activity. The pepsin hydrolysate was dissolved and subjected to a gel filtration column (2.6×100 cm) packed with Sephadex G-50, and three fractions designated as A, B, and C were isolated (Fig. 2a). Each fraction was collected and independently tested for antiplatelet activity. The fraction C, which displayed the strongest inhibitory effect on platelet aggregation, was further purified by RP-HPLC on a semi-preparative RP-HPLC column using a linear acetonitrile gradient of 10–80 %. The elution profile obtained contained five fractions, designated as C1–C5 (Fig. 2b). The fraction C3 displayed the highest antiplatelet activity was further purified on an analytical RP-HPLC column using a linear acetonitrile gradient of 20–75 %. The eluted peak indicated by an arrow was found exhibiting antiplatelet activity (Fig. 2c). The peak was subsequently subjected to tandem mass spectrometry for peptide sequence identification, and

its amino acid sequence was determined to be SHIHG-DYSSPSGAP (Fig. 3).

Platelet Aggregation Activity

To study the antiplatelet effects of SP-14 in vitro, rabbit platelet-rich plasma (PRP) aggregation induced with various agonists was used as an in vitro model. The results showed that SP-14 selectively inhibited platelet aggregation induced by different agonists. As shown in Fig. 4, SP-14 inhibited U46619-induced platelet aggregation in a dose-dependent manner, with a maximum inhibition rate of 69.77 ± 5.62 % at $1000 \mu\text{g/mL}$ ($n = 3$, $P < 0.01$). But it did not significantly influence ADP-, thrombin- or collagen-induced platelet aggregation compared to control group.

Effects of SP-14 on ADP-induced Acute Pulmonary Thrombosis Model In Vivo

To examine whether SP-14 exerted antithrombotic effects in vivo, SP-14 was challenged in an acute pulmonary thrombosis model induced by ADP, and aspirin, which is a clinical anti-thrombosis medicine, was used as a positive control. The results summarized in Table 1 showed that SP-14 significantly decreased mice mortality compared to saline-treated group, in a dose-dependent manner. Administration at 15 mg/kg (i.v.), this peptide significantly prevented mice death, with a protection rate of 80 %. Compared to positive control aspirin, SP-14 showed a more potent effect, as aspirin reached 80 % protection rate with an over threefold higher dose of 50 mg/kg .

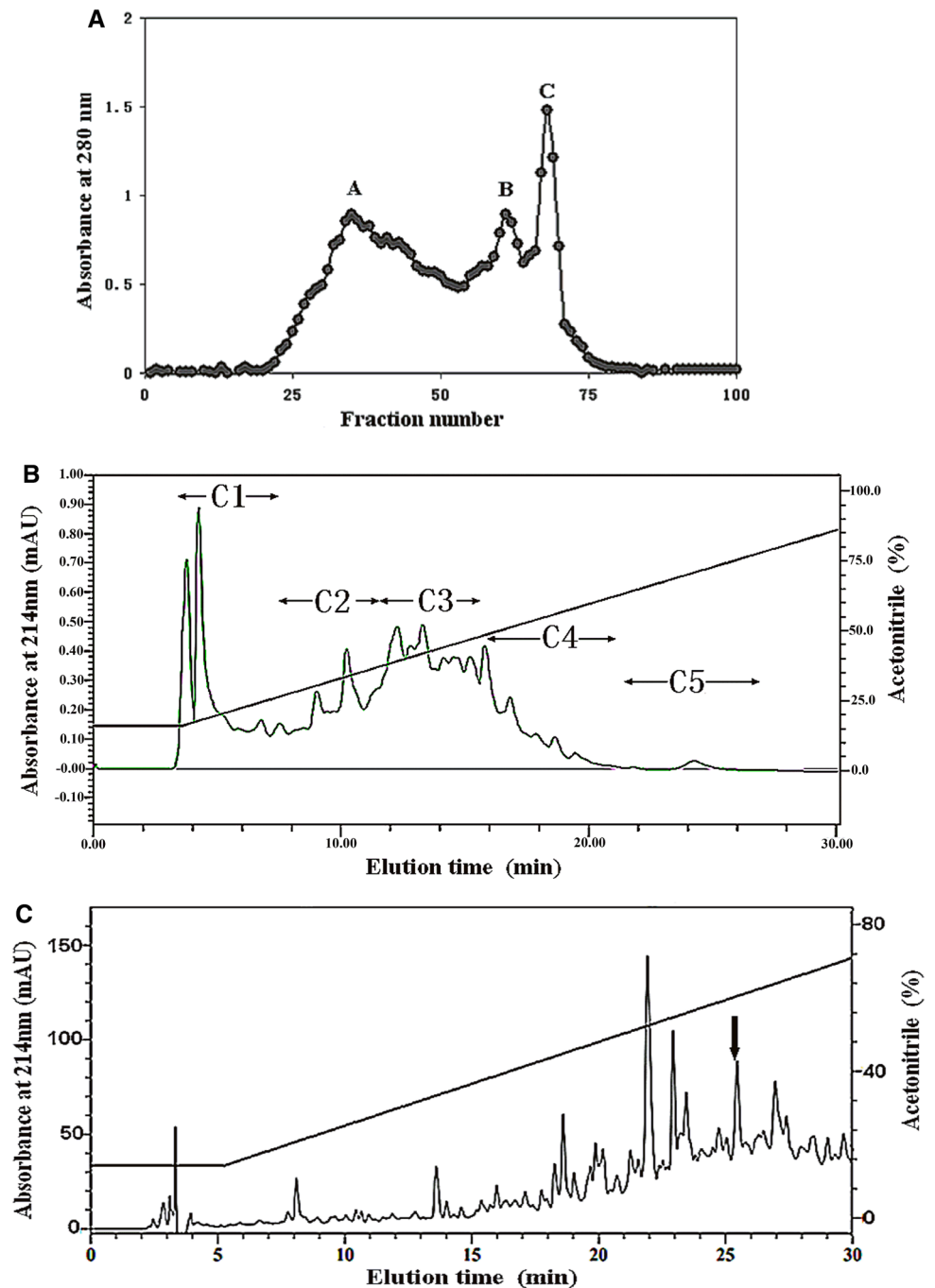
Effects of SP-14 on Arteriovenous Shunt Thrombosis Model In Vivo

To further confirm the antithrombotic activity of SP-14 in vivo, an arteriovenous shunt thrombosis model was used. As shown in Fig. 5, SP-14 inhibited thrombus formation in a dose-dependent manner. After the administration of SP-14 (5, 15, and 45 mg/kg), the thrombus dry weight was reduced by 14.63 ± 4.48 , 29.55 ± 3.69 and 48.36 ± 3.08 % ($n = 6$, $P < 0.01$), respectively, compared with that of saline-treated group. While aspirin reduced thrombus weight by 57.31 ± 5.85 % ($n = 6$, $P < 0.01$) at dose of 50 mg/kg , indicating that SP-14 and aspirin have similar antithrombotic effect in this animal model.

Bleeding Evaluation

To assess the bleeding risk of SP-14, the bleeding time of SP-14-treated mice was measured by a mice tail cutting

Fig. 2 Purification of an antithrombotic peptide.
a Sephadex G-50 gel filtration of snake venom hydrolysate.
b The fraction C after gel filtration chromatography was separated by a semipreparative C₁₈ RP-HPLC column.
c The fraction C3 was further purified by an analytical C₁₈ RP-HPLC column. The final purified peptide was indicated by an arrow



assay at concentrations of 5, 15, and 45 mg/kg. SP-14 increased the bleeding time in a dose-dependent manner. At a dose of 45 mg/kg, it increased the bleeding time to 14.15 ± 2.31 min, while a positive control aspirin increased the bleeding time to 19.13 ± 5.23 min at the similar dose, indicating that the bleeding risk of SP-14 is lower than that of aspirin.

Discussion

Peptides have been widely used in the biotechnological field, especially in the pharmaceutical applications. Generation of bioactive peptides by proteolysis using exogenous proteases, is an interesting approach for identifying novel therapeutic agents (Korhonen and Pihlanto 2006). In

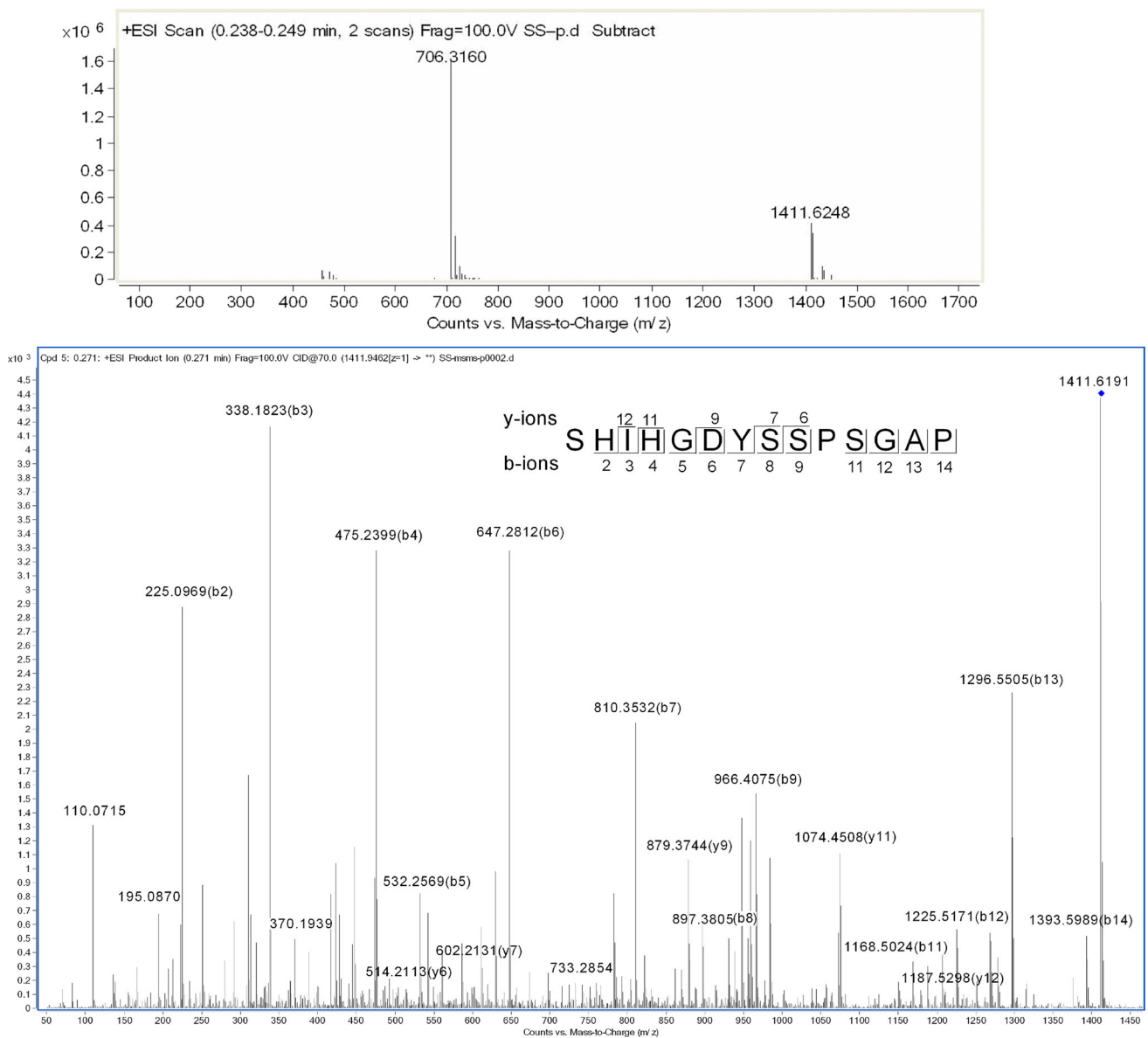


Fig. 3 Mass spectrum of the antithrombotic peptide. **a** The m/z value of the singly-charged ions ($[M+H]^+$) was 1411.62 Da and the doubly-charged ions ($[M+2H]^{2+}$) was 706.32 Da. **b** Respective b-ions and y-ions are annotated in the spectrum

the present study, a novel antithrombotic peptide, named SP-14, was isolated from pepsin hydrolysate of *Agkistrodon acutus* venom. SP-14 inhibited platelet aggregation in vitro, and inhibited thrombus formation in vivo, while with low bleeding risk. To the best of authors' knowledge, this is the first report that snake venom was enzymatically hydrolyzed, leading to a discovery of a novel antithrombotic peptide.

The molecular masses of reported platelet aggregation inhibitors isolated from snake venom was in a range from 10 to 110 kDa, such as the SV-PAD-1 (10 kDa) (Oyama et al. 2009), BmooAi (15 kDa) (de Queiroz et al. 2014), and AgkTx-II (13.87 kDa) (Perumal Samy et al. 2008).

The high molecular mass proteins or peptides tend to stimulate immune response, and may lead to side effects in some people, which limit their clinical application. SP-14, a 14-amino acid peptide with the molecular weight of 1410.62 Da, is much smaller than other antiplatelet aggregation proteins and peptides isolated from snake venom. Thus, this peptide may be able to avoid such problems. What's more, small peptides are relatively easy to be absorbed compared with proteins and macromolecular peptides. Extensive protein homology search results manifested that SP-14 was a novel antithrombotic peptide.

Platelet aggregation is a complex process in thrombus formation. It is mediated primarily through platelet

Fig. 4 Effects of SP-14 on platelet aggregation. PRP was preincubated for 5 min with different concentrations of SP-14 or vehicle control (saline). Platelet aggregation was initiated with ADP (10 μ M), collagen (2.5 μ g/mL), thrombin (2 U/mL) or U46619 (6 μ M), respectively. All data are expressed as mean \pm SD. * P < 0.05, ** P < 0.01 versus vehicle control (n = 3)

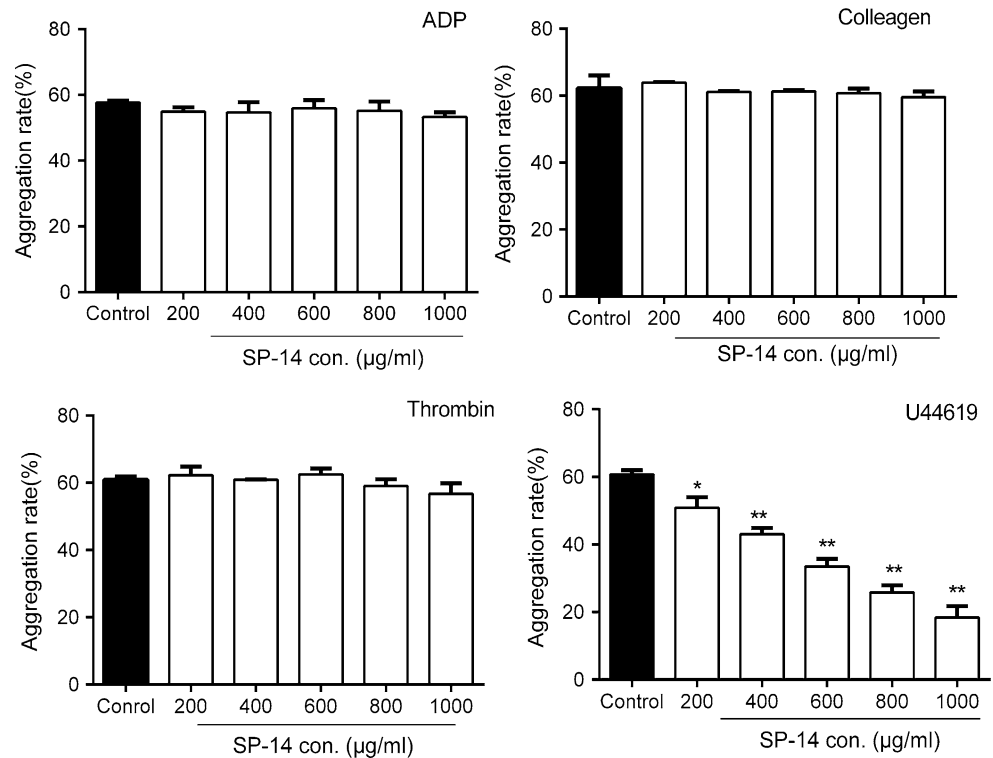


Table 1 Effects of SP-14 on ADP-induced acute pulmonary thrombosis and bleeding time in mice

Treatment	Dose (mg/kg)	Survival rate (%)	Bleeding time (min)
Saline	–	10	9.674 \pm 2.54
SP-14	5	60**	10.02 \pm 2.85
	15	80**	12.75 \pm 1.72*
	45	90**	14.15 \pm 2.31**
Aspirin	50	80**	19.13 \pm 5.23***

Each data was expressed as the mean \pm SD (each group, n = 10)

* P < 0.05; ** P < 0.01; *** P < 0.001; compared with vehicle control (saline)

adhesion at the injured site, and the action of endogenous agonists such as adenosine diphosphate (ADP), thrombin, collagen, and thromboxane (TxA₂). These agonists further promote platelet activation and aggregation via specific receptors to form platelet thrombosis (Sambrano et al. 2001). TxA₂, a strong platelet agonist, induces platelet aggregation and vasoconstriction through binding to thromboxane prostanoid (TP) receptor. TP receptor is expressed not only in platelets, but also in endothelial cells, monocytes, and macrophages, which contributes to the development of thrombotic diseases. Thus, the TxA₂ pathway may be a major target in the treatment of cardiovascular disease (Fontana et al. 2014). SP-14 potently inhibited platelet aggregation induced by U46619 rather than other agonists (ADP, collagen, or thrombin). These

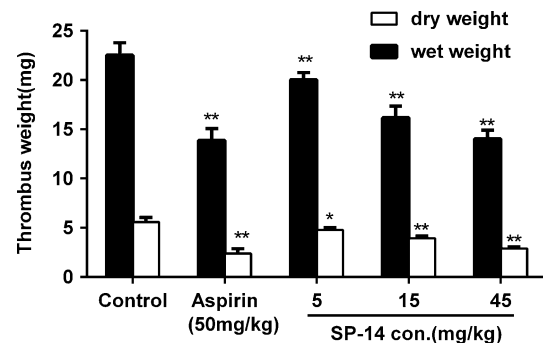


Fig. 5 Effects of SP-14 on arteriovenous shunt thrombosis in rats. The shunt thrombosis model was tested 20 min after injection of saline (vehicle control), aspirin or SP-14 (5, 15 and 45 mg/kg). The thrombus weight (wet and dry) was measured. All data are expressed as mean \pm SD. * P < 0.05, ** P < 0.01 versus vehicle control (n = 6)

findings implied that SP-14 might inhibit platelet aggregation via inhibiting the binding of TxA₂ to TP receptor. Many reported antithrombotic proteins or peptides inhibited platelet aggregation induced by various agonists rather than one specific agonist, such as salvianolic acid A from danshen inhibiting platelet aggregation induced by ADP, thrombin, collagen, and U46619 (Huang et al. 2010), YY-39 from ticks inhibiting platelet aggregation induced by ADP, thrombin, and U46619 (Tang et al. 2014).

Acute pulmonary thrombosis mode is created by injecting platelet activator into mice which cause thrombus formation. This model is characterized by the massive

activation of circulating platelets and the widespread platelet thrombosis in the microcirculation of the lungs leading to disseminated pulmonary microembolism and death in mice (Gresele et al. 1990). In the rats arteriovenous shunt model, thrombosis is initiated by platelet adherence to a cotton thread anchored in the shunt, and both activation of platelets and coagulation contribute to thrombus formation (Vogel et al. 1989). To determine the effect of SP-14 on thrombosis in vivo, these two animal models were chosen to examine antithrombotic effects of this peptide. It was found that SP-14 prevented death in mice and the protective rate (90 %) of SP-14 was similar with aspirin (80 %) at an approximate dose (50 mg/kg) (Table 1). Both the wet and dry weight of thrombus were significantly decreased compared with saline-treated group (Fig. 5). Thus, these results suggested that SP-14 had potent antithrombotic effect in vivo, which was caused by platelet aggregation.

Generally, the more potent an antithrombotic agent is, the more bleeding risks comes, which is the key problem limiting clinical usage of antithrombotic drugs (Tang et al. 2014). Mice tail cutting method was used to evaluate the bleeding risk of SP-14, which is a popular tool to estimate how well platelets interact with blood vessel walls to form blood clots (Morris et al. 1996). SP-14 showed a trend toward to prolong bleeding time in mice tail transection, but its bleeding risk was lower than aspirin (Table 1).

In addition, the coagulation cascade is a complicated and dynamic process triggered by endothelial and/or plaque damage and included the intrinsic and extrinsic pathways. APTT is related to the intrinsic coagulation phase in plasma, while PT is related to the extrinsic phase (Wang et al. 2014). Neither APTT nor PT were affected by SP-14 in vitro, even tested at the concentration of 1 and 3 mg/mL, suggesting that SP-14 apparently has no anticoagulant properties and does not affect the coagulation cascade (data not shown).

In conclusion, a novel antithrombotic peptide SP-14 was isolated from the hydrolysate of snake venom. SP-14 inhibited U46619-induced platelet aggregation in vitro, and exhibited potent antithrombotic activity in vivo. This study proposed a new method for producing bioactive peptides by the enzymatic hydrolysis of snake venom.

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Conflict of interest Xiaohui Ye, Meimei Chen, Yahui Chen, Xingli Su, Ying Wang, Wen Su and Yi Kong declare that they have no conflict of interest.

Informed Consent This manuscript does not contain any studies with human subjects performed by any of authors.

Human and Animal Rights All experiments executed in according to the Guide for the Care and Use of Laboratory Animals and approved by the institutional animal ethical committee.

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