



Purification and Characterization of a Novel Anticoagulant Peptide from Protein Hydrolysate of *Eupolyphaga sinensis* Walker

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Abstract

Eupolyphaga sinensis Walker (ESW), an animal drug in traditional Chinese medicine, has been used clinically for thousands of years for cardiovascular disease and osteoarthritis. Many studies of ESW have reported that it had anti-inflammatory and anti-tumor activities due to the small-molecule ingredients. However, large-molecule compounds of ESW representing significant pharmacological effects such as anti-thrombotic, anti-cancer, and anti-inflammatory have not been revealed yet. Here, a novel anticoagulant peptide (P9) containing 9 amino acids was isolated from the hydrolysate of aqueous extracts of ESW. Further, P9 synthesized by solid-phase synthesis showed 70% inhibition of thrombin, prolonging thrombin time to 17 s and activated partial thromboplastin time to 33 s. Molecular docking and spectroscopy demonstrated that P9 could inhibit thrombin activity by binding to the active site of thrombin and altering the secondary structure of thrombin. In an in vivo study, P9 enhanced the intensity of Phenylhydrazine-induced cardiac staining in thrombosed zebrafish with antithrombotic activity. The results suggest that peptides originating from ESW hydrolysates could exert an anticoagulant effect, which is likely to be a potential source of bioactive peptides with anticoagulant activity.

Keywords *Eupolyphaga sinensis* Walker · Animal drug · A novel Peptide · Anticoagulant activity

Introduction

Animal traditional Chinese medicine, including drugs derived from insects, has been used in the clinic to cure thrombosis, arthritis, stroke, etc. for thousands of years (Li et al. 2023; Wang et al. 2023; Zhang et al. 2022). In the past, a lot of small-molecule compounds, such as polysaccharides, alkaloids, and aliphatic, have been isolated and demonstrated anticancer, anti-inflammatory, and antibacterial

activities (Fu et al. 2022; Wang et al. 2017; Wu et al. 2021). In recent years, some pharmacologically active peptides derived from insect drugs, such as earthworms and centipedes, have been reported (Kong et al. 2013; Liu et al. 2016, 2019), which revealed the need to study more on animal traditional Chinese medicines.

Eupolyphaga sinensis Walker (ESW) was first recorded in the Shennong Bencao Jing of the Han Dynasty (Wang et al. 2019) and had the effects of breaking blood and stasis and promoting the reunion of fractured bones (Committee 2020). It is mainly used to treat diseases such as cardiovascular and cerebrovascular diseases and osteoarthritis. ESW is one of the main ingredients of Gubi Fang, which is used clinically to treat osteoarthritis (Jiang et al. 2017; Yuan et al. 2018; Zhou et al. 2018a) to help regulate the balance of catabolism to improve inflammation (Liu et al. 2015; Zhou et al. 2016) and anti-thrombotic (Guan et al. 2021). In addition, Chinese patent medicines such as Tongxinluo capsules and Huoxuezhitong capsules, whose ingredients include ESW, have been widely used clinically because they can improve blood flow and reduce fibrinogen (Huang et al. 2018; Liu et al. 2014) or improve inflammation (Ju et al. 2020). Although the efficacy of ESW has been clinically

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proven, little is known about the mechanism of action and the specific active ingredients of ESW. Hence, research on the active ingredients of ESW and the targets of action must be continued.

Bioactive peptides are a group of compounds consisting of 2–20 amino acids that are beneficial to human health or biological activity (Xu et al. 2020). They are attracting attention due to their efficacy in anti-thrombosis with low side effects (Rengasamy et al. 2019). In the past ten years, many peptides from animal drugs, including leeches (Zhang et al. 2022) and fleas (Lu et al. 2021), were found to have antithrombin and thrombolytic activities. ESW, a Chinese medicine commonly used as an antithrombotic, is a better source of active peptides for the treatment of thrombosis. Our previous study found that the aqueous extract of ESW has antithrombin activity (Li et al. 2006). Thus, we hypothesize that the substances in ESW that exert antithrombin activity are proteins or peptides.

Therefore, this study aimed to isolate, identify, and characterize a peptide with anticoagulant activity from the enzymatic product of ESW aqueous extract with anticoagulant activity using ultrafiltration and nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) technique. The interaction and molecular effects of P9 with thrombin were investigated in vitro using molecular docking, spectral analysis and experimental validation. In addition, a thrombotic model of zebrafish larvae was used to determine the antithrombotic efficacy of P9 in vivo.

Materials and Methods

Materials

Eupolyphaga sinensis Walker was collected from Sciendan Co., Ltd., Shanxi, China. Platelet-poor plasma (PPP) from rabbits was purchased from Senbeijia, Nanjing, China. APTT, PT and TT kits were purchased from Sunbio, Shanghai, China. Fibrinogen (from bovine), thrombin (from bovine, 2000 u/mg), aspirin (ASP) and Heparin (Hep.) were purchased from Shanghai Yuanye Biotechnology Co., Ltd, China. O-dianisidine and Phenylhydrazine (PHZ) were from Shanghai Aladdin Biochemical Technology Co., Ltd, China. All reagents were of analytical grade.

The ESW was pulverized into fine powder. The power (1000 g) was decocted twice with 8 quantities of water for 2 h. The extract was filtered, and the solvent was evaporated using a rotary evaporator at 65 °C. Then the crude extracts were centrifuged at 5000 rpm for 10 min at 4 °C. The resulting supernatants were freeze-dried and stored at -20 °C.

AB strains of zebrafish embryos treated with 1-phenyl 2-thiourea (PTU) were obtained from Nanjing YSY Biotech

Co, Ltd, China. The embryos were maintained in an incubator at 28 °C for 4 days. The experimental schemes involving conscious animals were approved by the Institutional Animal Ethical Committee of Nanjing University of Chinese Medicine (No. 202304A058) and were conducted according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (Publication no. 80–23, revised 1996).

Isolation and Purification of Anticoagulation Peptides from *Eupolyphaga Sinensis* Walker

Preparation of Protein Hydrolysate of ESW and Ultrafiltration

The lyophilized powder of crude extracts of ESW was added into distilled water on the solid-to-liquid ratio of 1:10 (w/v) and centrifuged at 5000 g for 10 min. Afterward, the mixed solution was hydrolyzed separately using two different proteases, including pepsin at pH 2.0, 37.5 °C, and trypsin at pH 7.8, 37.5 °C, with an enzyme substrate ratio of 10%. After 4 h, the enzyme-treated solutions were kept in a 95 °C water bath for 10 min, and centrifuged at 5,000 g for 10 min. All hydrolysates were lyophilized and kept at -20 °C for plasma clotting time (plasma recalcification) test and thrombin time assays. Trypsin hydrolysates exhibited the highest anticoagulant activity in both protein hydrolyzing species and were selected for the preparation of anticoagulant peptides. To obtain peptides of different molecular weight (> 50, 50–30, 30–10, 10–3, < 3 kDa) from trypsin-hydrolysates, cut-off membranes with various size molecular weights, 50 kDa, 30 kDa, 10 kDa and 3 kDa (Amicon ® Ultra, Millipore, Carrigtwohill, Cork, Ireland) centrifugal ultrafiltration tubes were used for ultrafiltration. Briefly, samples were placed in centrifuge tubes and centrifuged at 3000 g for 45 min at 4 °C. The fractions were collected as follows: >50 kDa; peptides retained without passing through the 50 kDa membrane, 50–30 kDa; peptides permeating through the 50 kDa membrane but not the 30 kDa membrane, 30–10 kDa; peptides permeating through the 30 kDa membrane but not the 10 kDa membrane 3–10 kDa; peptides permeating through the 10 kDa membrane but not the 3 kDa membrane, <3 kDa; peptides permeating through the 3 kDa membrane. All the collected fractions were lyophilized and kept at -20 °C for plasma clotting time tests and thrombin time assays.

Peptide Identification by Nano-LC-MS/MS

The fraction with the highest anticoagulant activity was analyzed using an EASY-nLC 1000 system (Thermo Fisher Scientific, MA, USA) coupled with an Orbitrap Fusion Lumos

mass spectrometer (Thermo Fisher Scientific, MA, USA). 8 μL of peptide solution in 0.1% formic acid and 5% ACN was loaded onto a trap column (Thermo Scientific Acclaim PepMap C18, 100 mm \times 2 μm) coupled with an analytical column of Acclaim PepMap C18 (75 μm \times 15 cm). Peptides were eluted with formic acid/CAN (0.1/80, v/v) at a gradient from 3 to 99% in 60 min. The fusion mass spectrometer was operated in a data-dependent acquisition mode to MS scan (m/z 350–1550). Peptide identification was carried out with PEAKS Studio (Version 8.0, bioinformatics Solutions Inc., Waterloo, ON, Canada) and searched using the Uniprot Proteome database.

PRT Assay

The plasma recalcification time (PRT) assay was conducted using a previously reported method with a slight modification (Zhang et al. 2022). Rabbit plasma samples (100 μL) were mixed with each fraction in a 96-well plate, which was incubated in 37 °C water baths for 1 min; to this, 100 μL 0.025 M calcium chloride was added, and the timer was started simultaneously. The rabbit plasma and fractions were gently swirled by a glass capillary at approximately 15 s intervals to observe the appearance of the clot. The time between the addition of calcium chloride and the appearance of the earliest clot was recorded as the PRT. The experiment was repeated three times.

APTT, PT, TT Assay

Activated partial thromboplastin time (APTT), plasma prothrombin time (PT), and thrombin time (TT) were determined by diagnostic kits (Sunbio, Shanghai, China), and based on the method of Shuzhen Cheng et al. with some slight modifications (Cheng et al. 2018). For the APTT assay, 50 μL APTT reagent was incubated with 50 μL platelet-poor plasma from rabbit (Senbeijia, Nanjing, China) and 50 μL of each sample. After incubation for 5 min, 50 μL CaCl_2 (25 mM) preheated at 37 °C was added, and the clotting time was timed by an SC40 semi-automatic coagulometer (Steellex, Beijing, China). To test PT, another 50 μL plasma and 50 μL of the sample were incubated at 37 °C, after incubated for 3 min, 50 μL PT was added, and the reagent was the clotting time was timed by SC40 semi-automatic coagulometer. For the TT assay, 50 μL PPP was first incubated at 37 °C. Then 50 μL of each sample was incubated with 50 μL of PPP for 60 s at 37 °C, and coagulation steel beads were added, followed by pressing the timer while 50 μL of TT reagent was added, and the coagulometer automatically recorded by SC40 semi-automatic coagulometer. The experiment was repeated three times.

In Silico Screening of Thrombin-Inhibitory Peptides

The peptides obtained from nano-LC-MS/MS were subjected to *in silico* analysis to predict the physiochemical properties of peptides. In order to determine the pharmaceutical properties of the peptides, NovoPro was used to assess the molecular weight, hydrophobicity, net charge of the peptides, the isoelectric point (pI), and water solubility of the peptides; Peptide Ranker was used to predict the potential biological activity, moreover, the toxicity of the identified peptides was assessed using Toxin Pred (Lafarga et al. 2016). Peptide Ranker scores above 0.8 were considered biologically active for further study.

Molecular Docking

To screen anticoagulant peptides from a small number of biologically active peptides for further studies to investigate the interactions of small molecule peptides with thrombin, HPEPDOCK was used for molecular docking (Yang et al. 2020; Zhou et al. 2018b). For the docking study, the X-ray crystal structure of thrombin-fibrinogen was obtained from the Protein Data Bank (PDB code: 1UCY) (Martin et al. 1996). The receptor was uploaded in PDB format after removing fibrinogen and water, adding hydrogen, and adding missing amino acid residues. Docking scores and rankings were calculated for all active peptides. The high score reflected the strength of the peptide and thrombin binding. The highest-scoring peptides were selected for further analyses. The docking results of the peptides and thrombin were analyzed visually using Discovery Studio Visualizer 2019.

Peptide Synthesis

The peptide identified by Nano-LC-MS/MS and screened for activity was selected and chemically synthesized using the solid-phase peptide synthesis methods. Synthetic peptide P9 was provided by Nanjing TGpeptide Biotechnology Co., Ltd (Nanjing, China). The purity of these synthetic peptides was determined to be >98%, and the molecular masses of these peptides were confirmed by HPLC and LC-MS (Fig. S1b and c).

Determination of the Antithrombotic Activity

The antithrombotic activity *in vitro* was determined according to previous reports with some slight modifications (Chen et al. 2020). Firstly, P9, thrombin and fibrinogen were dissolved respectively in Tris-HCl buffer (0.05 M Tris-HCl and 0.154 M NaCl, pH 7.4) and stored at 4 °C. The absorbance was measured at 405 nm using the Synergy2 microplate reader (Biotek, Vermont, USA). In brief, 10 μL of

thrombin (12 U/mL) and 40 μ L of different concentrations of P9 solution were mixed and incubated in a 37 °C water bath for 10 min. Immediately afterward, 140 μ L of 1 mg/mL fibrinogen was added to each well of the 96 microplate well and the absorbance at 405 nm was measured and recorded as C_0 . The mixture was then added to a 96 microplate well and then incubated in a 37 °C water bath for 10 min, and its absorbance at 405 nm was immediately measured and recorded as C_2 , while the group with distilled water added was recorded as C_1 . The following equation represents the antithrombotic activity. Each sample was measured at least three times. The experiment was repeated three times.

$$A = \frac{(C_1 - C_2)}{(C_1 - C_0)} \times 100\% \quad (\text{A.1})$$

Enzymatic Kinetics of Thrombin Inhibition

The measurement inhibition kinetic modes and the corresponding inhibition constant (K_i) value of P9 with thrombin were slightly modified from the previous reports (Chen et al. 2020). P9, thrombin and fibrinogen were dissolved respectively in Tris-HCl buffer (0.05 M Tris-HCl and 0.154 M NaCl, pH 7.4) and stored at 4 °C. 10 μ L of thrombin solution (12 U/mL) was preincubated with 40 μ L of P9 (15, 30, 60 μ M) or distilled water at 37 °C for 10 min. Different concentrations (0.735 mM, 1.471 mM, 2.941 mM, 4.412 mM, 5.882 mM, 7.353 mM) of fibrinogen were then added to the mixture at 37 °C for 10 min, the absorbance of 405 nm was then measured. Lineweaver-Burk plots were used to determine the type of inhibition and inhibition constants K_i . The inhibition mechanism can be analyzed using the following equation. The experiment was repeated three times.

$$\frac{1}{V} = \left(1 + \frac{[I]}{K_{is}}\right) \times \frac{K_m}{V_m} \times \frac{1}{[S]} + \left(1 + \frac{[I]}{K_{ii}}\right) \times \frac{1}{V_m} \quad (\text{A.2})$$

Where V is the velocity of the reaction; V_{\max} is the maximum velocity; $[S]$ and $[I]$ are the substrate and inhibitor concentrations, respectively; K_m is the Michaelis constant; K_{is} is the inhibitor constant describing the affinity of the inhibitor towards the target enzyme (Bovine thrombin); K_{ii} is the inhibitor constant describing the affinity of the inhibitor towards the target enzyme (Bovine thrombin)-substrate complexes.

UV-vis Spectroscopy Study

UV-vis absorption section spectroscopy was determined by a UV-2401PC spectrophotometer (Shimadzu, Japan). P9 and thrombin were first dissolved separately in Tris-HCl

buffer (0.05 M Tris-HCl and 0.154 M NaCl, pH 7.4). 1 mL of P9 solution (concentration) and 2 mL of thrombin (5 μ M) solution were mixed and incubated at 37 °C for 10 min. The mixture was then placed in a UV spectrophotometer to measure the spectra at 180–400 nm. All spectra were collected in a 1 cm light-range quartz cell (Chen et al. 2019).

Circular Dichroism (CD) Spectroscopy

A J-1500 spectrometer (JASCO, Japan) was used in the measurement of Circular dichroism spectra, according to Xu et al. with some modifications (Xu et al. 2020). All reagents were dissolved in Tris-HCl buffer (0.05 M Tris-HCl and 0.154 M NaCl, pH 7.4). The concentration of thrombin was 5 μ M, and the concentration of peptide was 11.2 μ M and 22.4 μ M. The mixture was scanned in the far UV range (190–260 nm, with 50 nm/min) using a CD spectrum with a bandwidth of 2 nm, using a 10 mm quartz cuvette. First, use the measurement of Tris buffer as a baseline, and 3 mL of thrombin (5 μ M) was measured. The incubated peptide-thrombin mixture was then measured. To estimate the content of different secondary structures, the SELCON3 algorithm from the Dichroweb website (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>) was used (Miles et al. 2022).

In vivo Study

To further verify whether the synthetic peptide P9 that was active in vitro had an equivalent in vivo effect on preventing or treating thrombus (Yin et al. 2020), we used Phenylhydrazine (PHZ, 1 μ g/mL) to construct zebrafish thrombus models in the antithrombotic test. A random selection of 360 zebrafish was placed in 12-well plates containing 3 mL of embryo culture water, with 10 fish per well. Then, we divided them into six groups - control group, model group, positive control group, P9 low-dose group, P9 medium-dose group, and P9 high-dose group, which were treated with 0.1% DMSO, aspirin (ASP, 25 μ g/mL), P9 (1 μ g/mL), P9 (5 μ g/mL), and P9 (10 μ g/mL). Then the 12-well plates were placed at 28 °C for 6 h. Immediately afterward, each group was given 3 mL of PHZ dissolved in 0.1% DMSO solution per well; the control group was given 3 mL of 0.1% DMSO solution. The 12-well plates were placed at 28 °C for 1.5 h, and O-dianisidine staining solution was added for 10 min to avoid light staining. 10 zebrafish were randomly selected from each group, and the thrombus in the tail veins and hearts of zebrafish larvae were observed and photographed under a fluorescence microscope (M205FCA, Leica, Germany) to evaluate the intensity of cardiac thrombus staining. The images were processed and calculated using Image Pro Plus 6.0 software.

Statistical Analysis

Each experiment was performed in triplicates. Experimental data are means \pm standard deviations (S.D.). Statistical Product and Service Solutions (SPSS) software, version 19.0 (SPSS, Inc., Chicago, IL, USA), or GraphPad Prism 8.3.0 (GraphPad Software, San Diego, CA, USA) were used for statistical analysis. Two-tailed Student's test and one-way ANOVA analysis of variance were used for quantitative data analysis. For multiple comparisons, Dunnett's post-test was used. Differences with $p < 0.05$ or < 0.01 were considered statistically. Origin 8 (OriginLab Corporation, USA) was used for graphing.

Results

Isolation and Purification of Aqueous ESW Extracts with Anticoagulant Activity

The anticoagulant activity of protein hydrolysates of aqueous extracts of ESW is shown in Fig. 1. At the concentration of 5 mg/mL, trypsin hydrolysate was able to prolong PRT to 227.3 ± 7.5 s, which was significantly higher than hydrolysate prepared from crude extract (185.7 ± 5.1 s) and pepsin (191.3 ± 6.7 s) ($p < 0.01$). Similarly, the ability of trypsin hydrolysate to prolong TT (17.00 ± 1.0) was markedly higher than that of crude extract (12.17 ± 0.1) and pepsin hydrolysates (13.33 ± 0.2) ($p < 0.01$). Therefore, trypsin hydrolysate with the highest activity among the two hydrolysates was selected for the preparation of anticoagulant peptides. Trypsin hydrolysate was fractionated by ultrafiltration membranes with MWCO of 50 kDa, 30 kDa, 10 kDa, and 3 kDa, respectively, and five peptide fractions were prepared. As shown in Fig. 2a, among the five components at a concentration of 10 mg/mL, all four

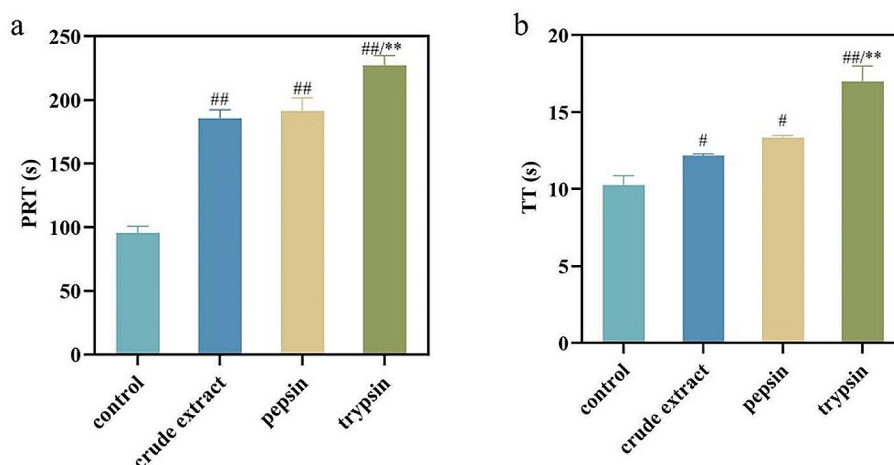
groups were able to significantly prolong the PRT, except for the component of 30–10 kDa, which was not statistically significant compared with the control group ($p < 0.01$). Notably, the component with a molecular weight of less than 3 kDa was the one that prolonged the PRT the longest among the four components, at 253.7 ± 6.1 s. Figure 2b, TT time increased with the decreasing molecular weight and all were able to significantly prolong the TT ($p < 0.1$). The fraction < 3 kDa was the fraction that prolonged the TT the longest at 54.03 ± 3.6 s. In summary, < 3 kDa had the strongest anticoagulant activity and was selected for subsequent analysis.

A total of 4701 peptides were identified from < 3 kDa molecular weight hydrolysate by Nano LC-MS/MS, these sequences have not been mentioned previously in the literature. The amino acid composition of all identified peptides ranged from 4 to 20. These peptides had molecular masses between 598 and 2913 Da, with more than 84% less than 1500 Da.

In silico Screening of Antithrombin Peptides

With the development of artificial intelligence, molecular docking, a more mature computational simulation method, has become a powerful tool for screening drugs and interpreting their mechanism of action with their targets (Vidal-Limon et al. 2022). The < 3 kDa fraction was composed of a multitude of peptides and thus needed to screen peptides with the highest antithrombin activity. First, we screened the peptides with PEAKS studio ALC $> 80\%$ for further studies (Table S1). Then, Peptideranker was used to predict the biological activity of peptides that had been screened, where 29 peptides with a score above 0.8 were considered most likely to be biologically active. They are non-toxic peptides consisting of less than or equal to 9 amino acids and were submitted to the HPEPDOCK website and

Fig. 1 Anticoagulant activity of two protein hydrolysates of aqueous extracts of ESW using different proteases. **a** Analysis of the biochemical properties of each hydrolysate at a concentration of 5 mg/mL by PRT assay. **b** Analysis of the biochemical properties of each hydrolysate at a concentration of 5 mg/mL by TT assay. Plasma without treatment for drug treatment was used as the control group. Data were expressed as mean \pm SD ($n = 3$). PRT: ## Compared with the crude extract and $p < 0.01$; # $p < 0.05$; TT: ** Compared with the crude extract and $p < 0.01$; * $p < 0.05$



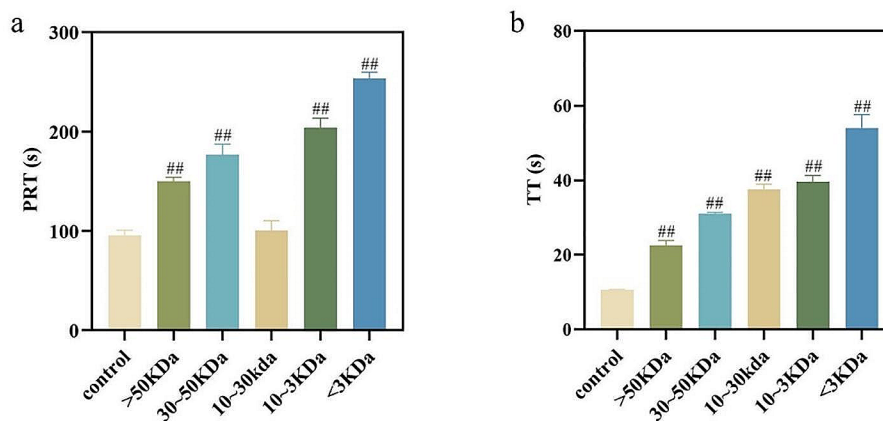


Fig. 2 Anticoagulant activity of five ultrafiltered fractions of trypsin hydrolysate from aqueous extracts of ESW. **a** Analysis of the biochemical properties of each ultrafiltered fraction at a concentration of 10 mg/mL by PRT assay. **b** Analysis of the biochemical properties of each ultrafiltered fraction at a concentration of 10 mg/mL by TT assay.

scored against thrombin; a higher absolute negative score in HPEPDOCK indicates a higher binding affinity between the peptide and thrombin and the higher potential for thrombin inhibitory activity (Table 1). The highest score of -261.326 was obtained for HTWNLHWWR (His-Thr-Trp-Asn-Leu-His-Trp-Trp-Arg), consisting of 9 amino acids (Fig. S1a), while the lowest score of 180.565 was obtained for WPFK consisting of 4 amino acids. Therefore, HTWNLHWWR, which is considered to have the strongest binding affinity and higher thrombin inhibitory activity among the 29 peptides, was selected as the best candidate for chemical syntheses (Fig. 3).

Molecular Docking Simulations

As previously described, molecular docking screening of potentially active peptides was performed using the HPEPDOCK website and the docking behavior of the control (fibrinopeptide A-alpha from *Bos taurus*) with thrombin was studied. Here, P9, which had a binding site for thrombin that was very similar to fibrinogen, showed a higher score (-261.326) than the control fibrinopeptide A-alpha (-193.158) for 1UCY (Fig. 4a and Fig. S2a), suggesting that good interactions between P9 and proteins are established. In this study, Hydrogen bonding is key in the stability of ligand-protein binding, where a hydrogen bond distance of less than 3.5 Å is a more favorable distance (Atef Hatamleh et al. 2020). Both P9 and fibrinopeptide A- α form hydrogen bonds with thrombin at distances of approximately 3.5 Å, suggesting that these bonds are very strong (Fig. 4b and Fig. S2b). From the 2D diagram, it was seen that P9 formed six hydrogen bonds with the receptor 1UCY, in which five amino acid residues in the receptor, including Thr 149, Thr

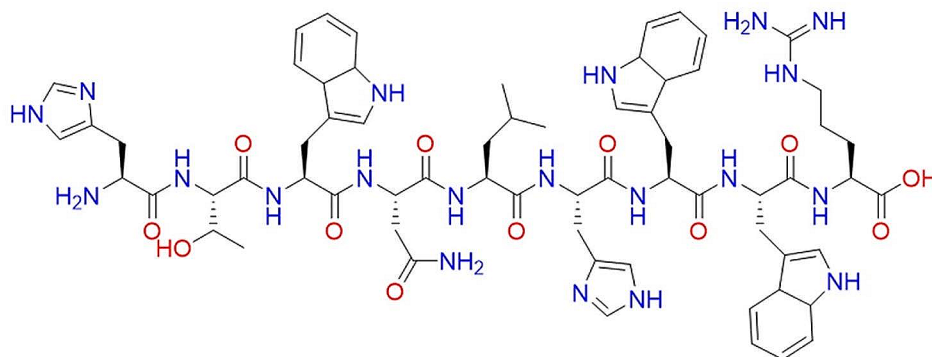
149 A, Arg 221 A, Glu 217, and Glu 97 A, were involved in the formation of the hydrogen bonds. Furthermore, P9 formed alkyl and pi-alkyl bonds with thrombin at thrombin residues Leu 99 and Cys 220, and Van der Waals force interactions involving Gly 219, Lys 97, and Asn 98 were also coordinated with the active site, all of which force facilitates the binding of P9 to thrombin and thus inhibit thrombin activity (Fig. 4c and Fig. S2c). In addition, the hydrophobic amino acids in P9 promote the binding of P9 to the hydrophobic amino acids in the active site of thrombin, forming a hydrophobic surface that facilitates the stability of the complex, which is similar to the contact surface of fibrinopeptide A-alpha binding to thrombin (Fig. 4d and Fig. S2d).

The Anticoagulant Activity of P9

The anticoagulant activity of synthesized P9 was tested using APTT, PT, and TT assays with heparin as a positive control (Fig. 5a). P9 markedly prolonged TT to 17 s ($p < 0.05$) in a dose-dependent manner and significantly extended APTT to 33 s ($p < 0.01$) but did not show a significant difference for PT. Heparin significantly prolonged APTT and TT ($p < 0.01$), but did not affect PT. The above results suggest a similarity between P9 and heparin intervention in the coagulation cascade. Furthermore, to demonstrate the inhibitory effect of P9 on thrombin activity, we used different concentrations of P9 to verify the inhibitory effect of P9 on thrombin and used sodium heparin as a positive control (Fig. 5b). The results showed that the inhibitory effect of P9 on prothrombin activity was proportional to the dose. With increasing concentrations of P9, its inhibitory effect on

Table 1 Molecular docking of potential inhibitor of thrombin peptides

Peptides	Molecular weight	pI	PeptideRanker score	Toxicity	Hydrophilicity	HPEPDOCK score
HTWNLHWWR	1335.47	11.05	0.90	Non-Toxin	-1.13	-261.326
CEFAWWR	997.13	6.13	0.97	Non-Toxin	-0.69	-259.905
HMVWRWWR	1256.48	12.40	0.95	Non-Toxin	-0.94	-257.823
QTWLCWR	992.15	9.02	0.92	Non-Toxin	-0.97	-249.894
WCHHMLK	954.17	8.97	0.89	Non-Toxin	-0.79	-246.571
ELWWFK	908.05	7.00	0.93	Non-Toxin	-0.85	-245.162
CRWWTR	907.05	10.88	0.94	Non-Toxin	-0.37	-243.542
WSGLAYWR	1038.15	9.84	0.92	Non-Toxin	-1.01	-237.348
FCFHFR	856.00	9.02	0.99	Non-Toxin	-1	-237.089
KMVWCWNR	1122.36	10.12	0.87	Non-Toxin	-0.55	-236.411
WCWSTVR	937.07	9.02	0.83	Non-Toxin	-0.91	-235.492
WEWMCWHR	1233.42	7.16	0.97	Non-Toxin	-0.88	-234.138
KLWHWK	897.07	10.60	0.81	Non-Toxin	-0.52	-231.769
WDWFYMK	1075.23	7.00	0.98	Non-Toxin	-0.99	-229.657
CMWPYER	984.15	6.13	0.88	Non-Toxin	-0.29	-227.73
WMHGWNGK	1015.14	10.09	0.83	Non-Toxin	-0.68	-226.81
MWLWEMCK	1126.41	6.13	0.89	Non-Toxin	-0.78	-225.862
WQAHYPR	957.04	9.84	0.81	Non-Toxin	-0.5	-225.036
HVCWLWK	971.18	8.97	0.91	Non-Toxin	-1.23	-221.037
FAPNHWK	899.00	10.09	0.86	Non-Toxin	-0.53	-218.693
WVEFWK	894.02	7.00	0.83	Non-Toxin	-0.8	-216.158
KGMHMLWMK	1161.50	10.60	0.89	Non-Toxin	-0.41	-214.832
CWRCFVR	969.18	9.19	0.94	Non-Toxin	-0.49	-214.264
WFMAR	709.86	11.05	0.97	Non-Toxin	-0.94	-209.376
ECPWLR	802.94	6.13	0.90	Non-Toxin	-0.03	-200.969
LWCCR	679.85	8.30	0.96	Non-Toxin	-0.84	-191.389
WFEDLMRKR	1280.49	10.09	0.82	Non-Toxin	0.67	-189.891
KGFAVCCK	919.12	9.02	0.84	Non-Toxin	-0.16	-185.239
WPFK	576.68	10.09	0.98	Non-Toxin	-0.73	-180.565

Fig. 3 The chemical structure of the synthesized peptide P9 (HTWNLHWWR). The image obtained by ChemDraw 17

prothrombin also enhanced, reaching a peak at a concentration of 0.08 mg/mL. Compared with sodium heparin, P9 at the same concentration showed a weaker inhibition of thrombin, suggesting that P9 has a milder inhibitory effect on thrombin. The data were analyzed using the probit analysis program in GraphPad Prism 8.3.0, resulting in an $IC_{50} = 0.045$ mg/mL.

Determination of Kinetics Parameters

The pattern of inhibition was determined using a Lineweaver–Burk double inverse plot of P9 inhibition of thrombin kinetics (Chen et al. 2020). As shown in Fig. 6, the fitted curves for different concentrations of P9 intersected in the third quadrant and the slope increased with increasing P9

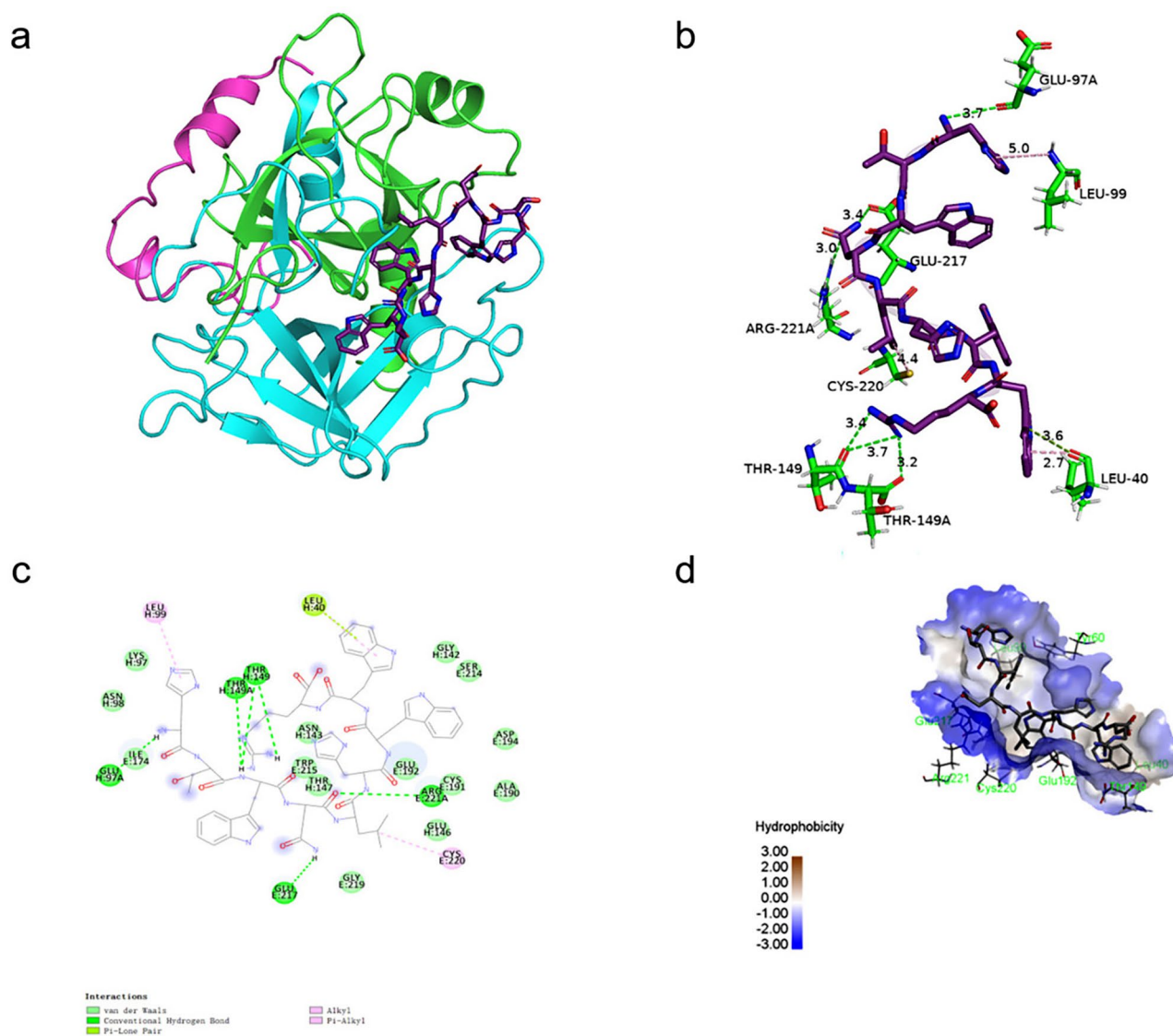


Fig. 4 Visualization of molecular docking of thrombin (from *Bos taurus*, PDB code: 1UCY) with anticoagulant peptide P9. **a** Docked complex. **b** 3D details of the interaction of P9 and thrombin. **c** 2D details

concentration, suggesting a mixed mode of inhibition of anticompetitive and noncompetitive.

According to Eq. A.2, K_m , V_{max} , and V_{max}/K_m decreased with increasing P9 concentration (Table 2), indicating that the catalytic efficiency of thrombin decreased with increasing P9 concentration at constant enzyme concentration. In addition, the dissociation constant K_{is} (0.1291 mM) for thrombin-inhibitor (P9) was greater than the thrombin-substrate-inhibitor compliant dissociation constant K_{ii} (0.0232 mM), indicating that the enzyme-substrate-inhibitor complex was more stable than the thrombin-inhibitor complex, suggesting that P9 possibly influencing the structure of thrombin with the thrombin-substrate-inhibitor complex

of the interaction of P9 and thrombin. **d** The hydrophobic interaction of P9 and thrombin. The darker the blue color, the more hydrophilic

and thus affecting the catalytic efficiency (Forghani et al. 2016).

Interaction Assay between Thrombin and Peptide

UV-Vis absorption spectroscopy is a simple but effective method for investigating structural changes in proteins and the formation of protein-ligand complexes (Yang et al. 2009). In this study, the UV-Vis spectra of P9 and thrombin in the presence and absence of P9 were obtained between 190 and 400 nm (Fig. 7a). Thrombin had two absorption peaks at 215 and 275 nm. When the P9 solution was added to the thrombin solution, the peak at 215 nm was slightly

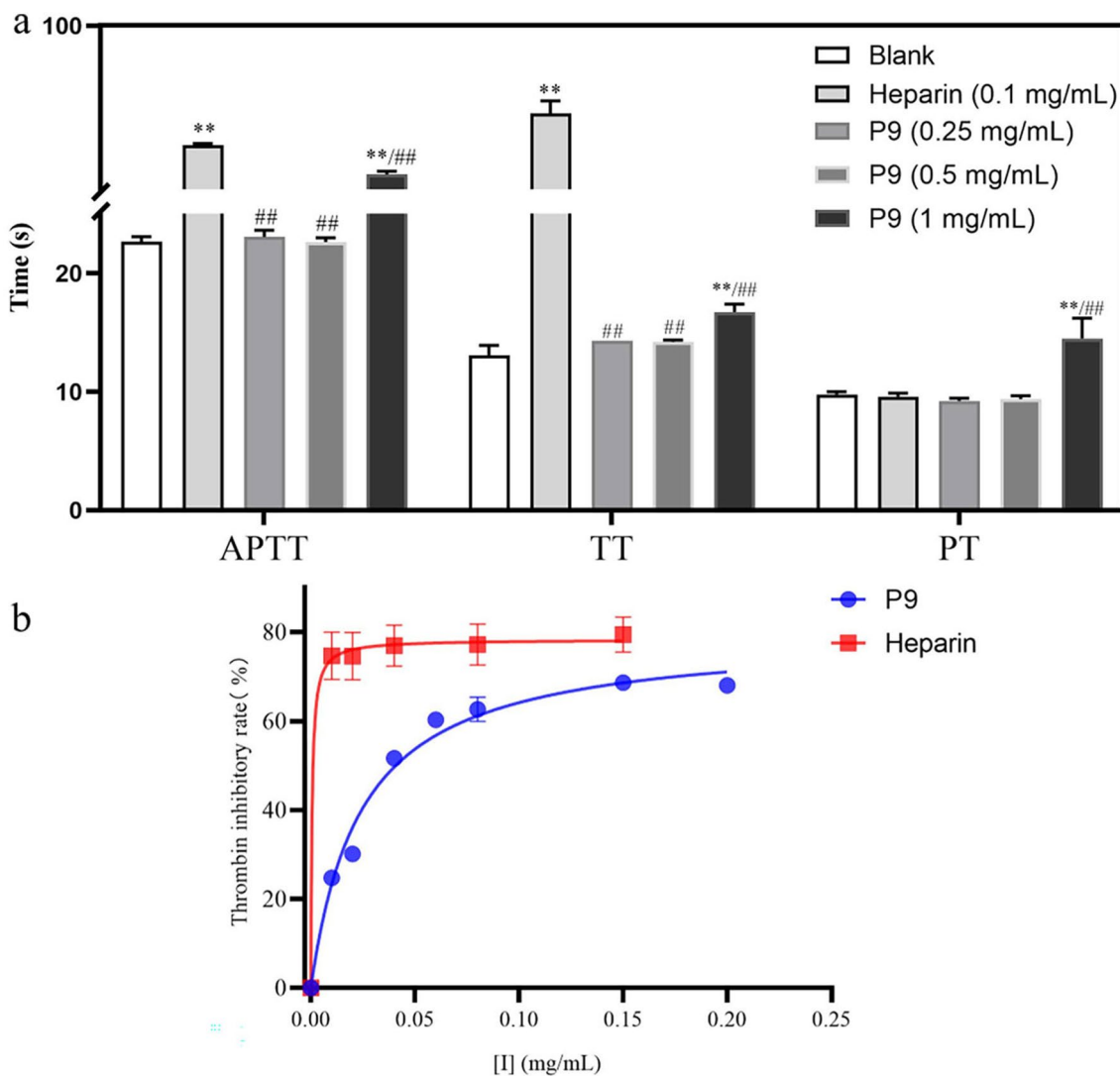


Fig. 5 In vitro anticoagulant activity of P9. **a** Effects of P9 on APTT, PT, and TT. **b** The dose-inhibition curves of [I] (P9 and heparin concentration) against thrombin. The plasma without treatment for administration treatment was used as a blank group. Heparin was used as a

control group. Values are represented as means \pm SD ($n=3$). *Compared with the blank group and $p < 0.05$; ** $p < 0.01$. ##Compared with the Heparin group and $p < 0.01$; # $p < 0.05$

blue-shifted (2 nm) and decreased in intensity, probably due to the interaction of P9 with thrombin, which resulted in the disorganization of the surroundings of amide bonds and the contraction of the C=O bonds in thrombin (Chen et al. 2019; Xu et al. 2012). The slight decrease of the absorption peak at 275 nm may be due to the binding of P9 to thrombin, which altered the structure of thrombin and resulted in the masking of the hydrophobic moiety of aromatic amino acids in thrombin, thus leading to the weakening of the absorption peak (Chen et al. 2011). These results suggested that there was an interaction between P9 and thrombin and that a complex was formed.

CD Spectroscopy is able to measure protein secondary structures, such as α -helix, β -sheet, β -turn, and random coil,

which can cause changes in protein secondary structures when ligands bind to proteins. As shown in Fig. 7b, further evidence of conformational changes in thrombin was obtained by CD spectroscopy at different concentrations of P9 (0, 11.2, 22.4 μ M). Natural thrombin had two negative peaks at 208 and 222 nm, as described by the previous study (Zhang et al. 2011). As the P9 concentration increased, the α -helix and β -turn content of the thrombin decreased while the β -sheet and random coil content increased (Fig. 7c), suggesting that the interaction between P9 and thrombin leads to changes in the secondary structure of thrombin. These results P9 may inhibit thrombin activity by altering the secondary structure of thrombin and thereby inhibiting thrombin activity.

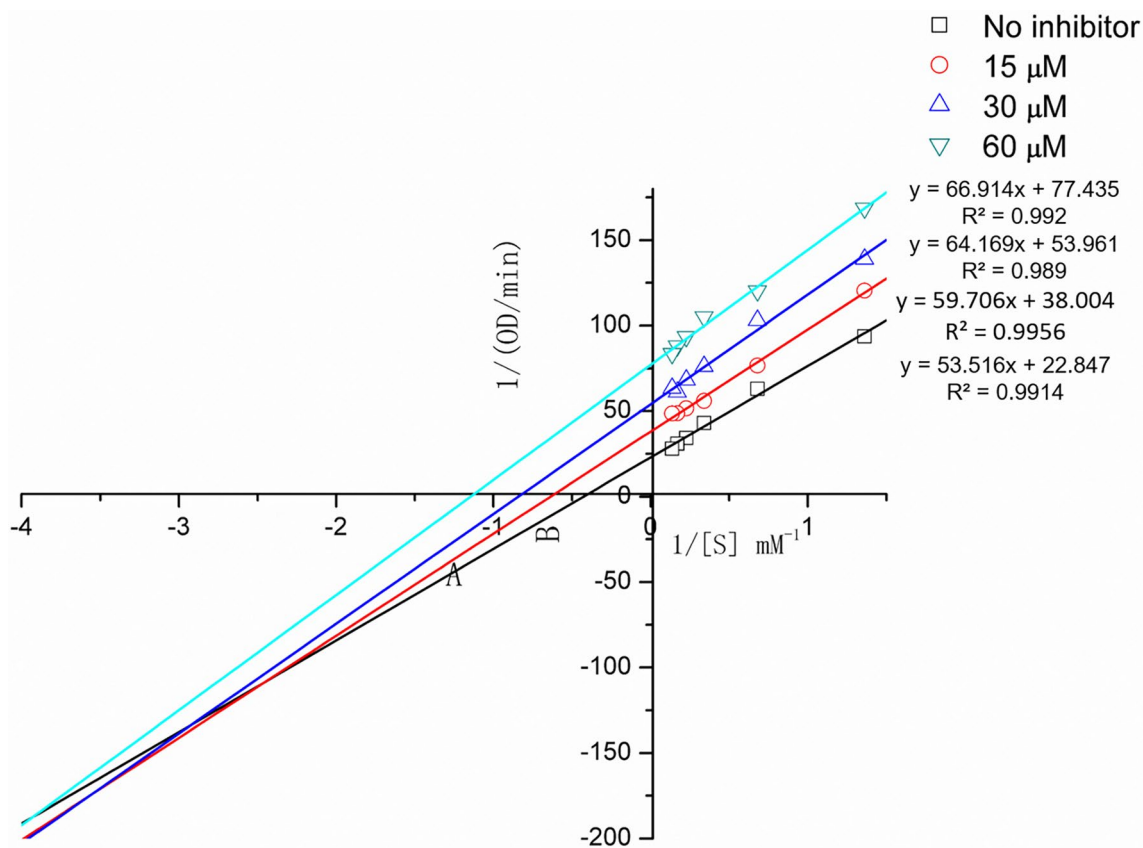


Fig. 6 Lineweaver–Burk plot of P9 inhibition of thrombin-catalyzed fibrinogen (from *Bos taurus*). The [S] represented fibrinogen concentration. 1/(OD/min) represents the rate of conversion of fibrinogen to

fibrin catalyzed by thrombin with time. 15 μM and 30 μM P9 intersected the blank group in the third quadrant, indicating a mixed mode of binding of P9 to thrombin

Table 2 Kinetics parameters of thrombin inhibited by P9

P9 concentration (μM)	K_m/V_{max} ((min $\cdot\mu\text{M}$)/Abs)	K_m (μM)	V_{max} (Abs/min)	K_{is} (mM)	K_{ii} (mM)
0	55.13	2.326	0.04217	0.1291	0.0232
15	59.71	1.571	0.02631		
30	64.17	1.189	0.01853		
60	66.91	0.8644	0.01291		

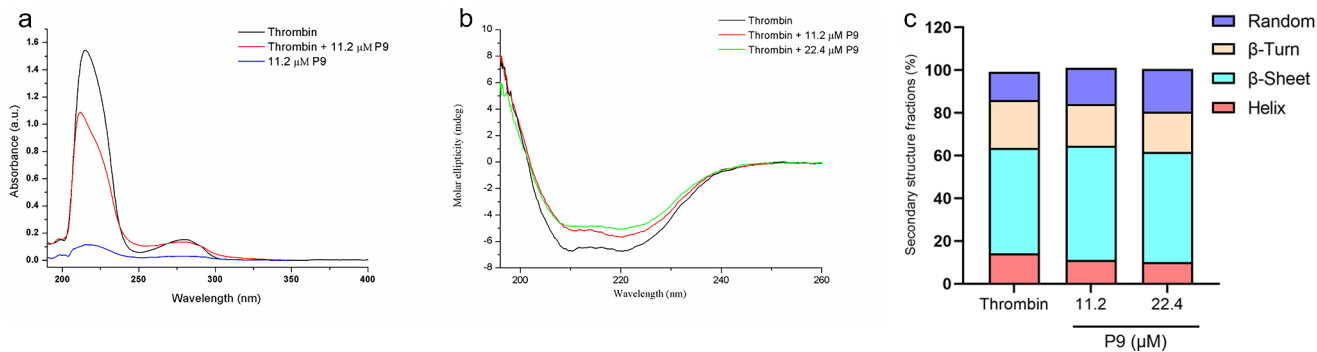


Fig. 7 The interaction analysis of thrombin-induced by the P9 via spectroscopy. **a** UV-Vis spectra of thrombin plus P9 and thrombin. **b** CD spectra of thrombin plus different concentrations of P9 and throm-

bin. **c** Estimated secondary structural fractions of thrombin. Thrombin, thrombin with buffer. The concentration of thrombin was 5 μM

In vivo study

Phenylhydrazine (PHZ) is a common agent for inducing thrombosis, which can lead to oxidative damage of erythrocyte lipid membranes, increase the rate of thrombin generation, induces the formation of thrombus, and the zebrafish thrombus model induced by PHZ has been applied in the study of pharmacological antithrombotic effects (Xu et al. 2022a; Yin et al. 2020). Based on the results of in vitro studies, we chose the phenylhydrazine-induced thrombosis model in zebrafish to further investigate the anticoagulant activity of P9 in vivo. Before the experiment, we evaluated the toxicity of different concentrations of P9 in juvenile zebrafish (3dpf) exposed for 24 h. When the P9 concentration was 10 $\mu\text{g/mL}$, the survival rate of juvenile zebrafish was 100%. Therefore, three gradient concentrations were used for the dosing group based on the maximum tolerated concentration (1, 5, 10 $\mu\text{g/mL}$). Compared with the control group, zebrafish tail vein erythrocytes aggregated in the model group, while the staining intensity (SI) of heart erythrocytes was significantly lower ($P < 0.0001$), indicating the success of the zebrafish thrombotic model induced by PHZ (Fig. 8a, b).

Compared with the model group, the staining intensity of cardiac erythrocytes in the administered and positive groups increased significantly, and the degree of erythrocyte aggregation in the tail vein decreased, showing antithrombotic activity. This indicated that peptide P9 had in vivo activity and could reduce the risk of peripheral circulatory obstruction induced by PHZ. In addition, with the increase in drug

concentration, the staining intensity of cardiac erythrocytes increased, showing a more obvious dose-dependent relationship. Interestingly, P9 had better efficacy than ASP for zebrafish with thrombus when the concentration of P9 was 5 $\mu\text{g/mL}$ (Fig. 8c), whereas low-dose aspirin (ASP) is a well-known anticoagulant drug, which inhibits the formation of thromboxane 2 and platelet COX-1 thereby slowing down the formation of thrombus (Ma et al. 2021), suggesting that P9 has an antithrombotic effect similar to aspirin (25 $\mu\text{g/mL}$) in vivo.

Discussion

Thrombus is one of the most common clinical symptoms in cardiovascular and cerebrovascular diseases, and there are various factors inducing thrombus formation, among which thrombin, as a major enzyme in the coagulation cascade reaction, which converts fibrinogen to fibrin, promotes the formation of cross-linked fibrin, and activates coagulation factors (IX, V, and VIII), is an important cause of thrombus formation (Al-Amer 2022). Therefore, inhibition of thrombin activity helps to reduce the incidence of thrombus formation. Anticoagulant peptides derived from natural sources, such as plants and animal species (Akram et al. 2017; Syed et al. 2018), have generated a great interest for their potential as drug candidates because they exhibit safer and more specific anticoagulant activity than chemical agents such as argatroban and bivalirudin (Jackson et al. 2019; Rengasamy et al. 2019).

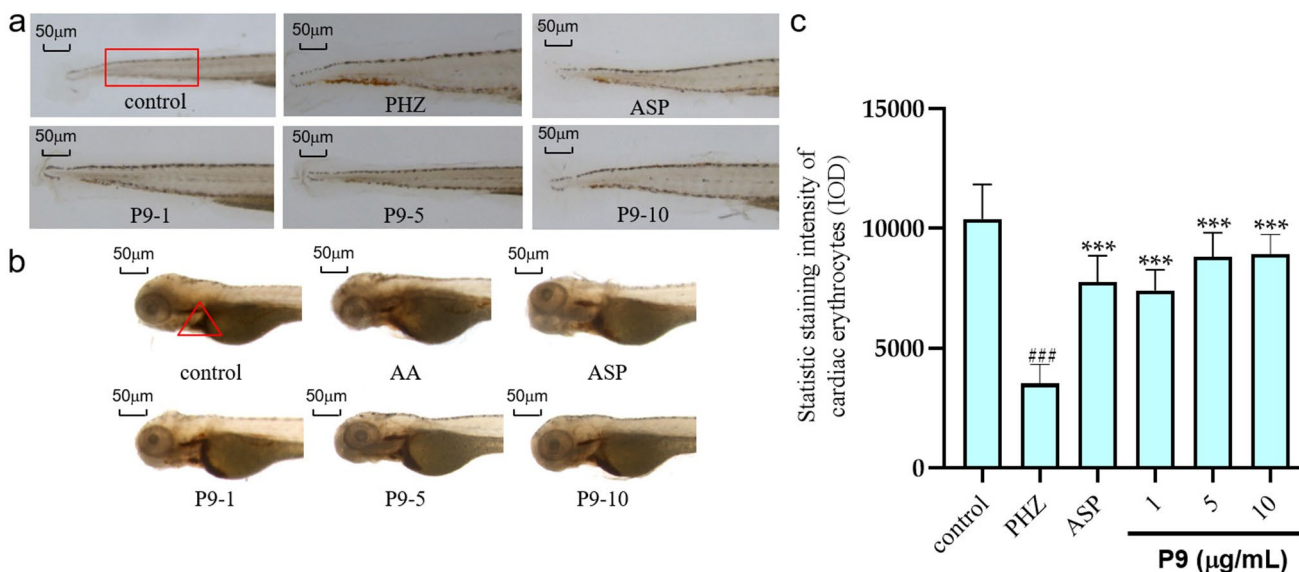


Fig. 8 Antithrombotic activity of P9 in zebrafish. Erythrocytes aggregation in the caudal vein **a** and the thrombus staining area in the heart **b** of zebrafish larvae of the control group (0.1% DMSO), model group (phenylhydrazine group (PHZ, 1 $\mu\text{g/mL}$), positive control group (aspirin (ASP), 25 $\mu\text{g/mL}$), P9 low-dose group (1 $\mu\text{g/mL}$), P9 medium dose

group (5 $\mu\text{g/mL}$) and P9 high dose group (10 $\mu\text{g/mL}$). **c** In vivo visualization of the erythrocytes of zebrafish hearts and statistic staining intensity of the erythrocytes in zebrafish hearts of all the groups. Data are presented as mean \pm SD ($n \geq 8$). *** $p < 0.0001$ compared to the PHZ group. #### $p < 0.0001$ compared to the control group

In this study, the anticoagulant peptide P9 was purified and characterized for the first time from ESW and its anticoagulant effect was demonstrated in *in vitro* and *in vivo* experiments (Fu et al. 2022). Our isolation process showed that fractions with smaller molecular weights had better anticoagulant activity, which could be attributed to the fact that low molecular weight peptides are more likely to bind to target molecules as compared to high molecular weight peptides, and hence they exhibit higher anticoagulant activity (Bhadkaria et al. 2023). According to sequence analysis, *in silico* analysis and molecular docking, a P9 (HTWNLHWWR) with a mass of 1335.5 Da binds to the catalytic luminal active site of thrombin via van der Waals forces, hydrogen bonding, alkyl bonding, and pi-alkyl bonding (Fig. 4), which is similar to the fibrinopeptide A-alpha binding site (Scheraga 2004), indicating that P9 may inhibit thrombin activity by spatial site blocking or by altering the spatial structure of thrombin (Chen et al. 2020), which is similar to the inhibition of thrombin by novel anticoagulant peptides isolated from plants or animals, including QPLPPI, GNWGPLV, and FFPDIPKIK from soybean (Xu et al. 2022b), ELEDSDLSE, RGILTLK, RGMVAGDSK, RGVNDELVY, AGFAGDDAPR from *Tenebrio molitor* (Qiao et al. 2018), NAESLRK, and TARNEANVNIY from *Crassostrea gigas* (Cheng et al. 2018, 2021). In addition, the active site on the surface of thrombin contains a large number of charged amino acids and hydrophobic amino acids (Feng et al. 2017; Tapparelli et al. 1993), and hence peptides containing hydrophobic amino acid residues at the C-terminal and N-terminal ends are more likely to bind to the active site of thrombin (Wei et al. 2019). The hydrophobic amino acids and charged amino acids at the C-terminus of P9 (Fig. 3) may be a factor affecting its binding to thrombin, but the exact effect has not yet been clarified and needs to be further investigated.

APTT, PT and TT are important indicators for evaluating different stages of the coagulation pathway, the ability of P9 to prolong APTT and TT suggests that the synthetic peptide P9 is able to affect factors IX, X, XI and II, but does not affect extrinsic pathways (Fig. 5) (Zhang et al. 2022). Prolongation of thrombin time implied that P9 interacts negatively with thrombin (factor II) in the coagulation pathway, altering the secondary structure of thrombin and thus inhibiting the activity of thrombin (Fig. 7) (Cheng et al. 2021). The IC_{50} of P9 on thrombin was 0.045 mg/mL, which is similar to the results of a previous study, such as an active peptide, XC-43, obtained from the flea *Xenopsylla cheopis*, with an IC_{50} value of 0.032 mg/mL for thrombin, which rapidly and tightly binds to thrombin and inhibits thrombin consequently (Cheng et al. 2021), and Ren et al. identified peptides from *Buthus martensii* Karsch protein hydrolysate that inhibited thrombin activity with an IC_{50} of 0.012 mg/

mL (Ren et al. 2014), suggesting that P9 may be an ideal thrombin inhibitor (Fig. 5).

Enzymatic kinetics supports the *in silico* prediction that P9 is essentially a mixed-mode thrombin inhibitor, and chemically synthesized P9 can bind non-competitively and anti-competitively to thrombin (Fig. 6), meaning that P9 may not only occupy the substrate-thrombin binding site, but may also bind to thrombin to form an enzyme-substrate-P9 complex affecting influence on V_{max} , thereby inhibiting enzyme activity. Although most thrombin-inhibiting peptides derived from plant and animal proteins are usually competitive (Chen and Huang 2020; Cheng et al. 2021; Zhang et al. 2022), certain non-competitive thrombin-inhibiting peptides can also exhibit satisfactory effects, such as peptides from soybean, and Cassiae semen (Xu et al. 2022b; Yu et al. 2019). It is noteworthy that, this binding mechanism was verified by molecular docking and spectroscopy, P9 has a very similar binding site to fibrinogen but differs in the site where the force is formed, which may hinder the binding of fibrinogen to thrombin in terms of spatial structure, while the ability of P9 to form a more stable complex with thrombin renders the original ordered spatial structure of thrombin disordered, leading to a reduction of the binding rate of fibrinogen to thrombin.

Recently, zebrafish has been widely used as a model animal for life science research in toxicology, genetics, and human diseases because of its advantages of rapid reproduction and high genetic homology with humans (Choi et al. 2021; Qiu et al. 2019). In fact, the transparency of zebrafish larvae makes it possible to observe thrombosis in zebrafish without using invasive methods, making zebrafish particularly suitable for the study of thrombosis (Raghunath et al. 2022; Zhu et al. 2016). In this study, we showed that the anticoagulant activity of P9 at a safe and effective dose was favorable *in vivo*, and the anticoagulant effect was dose-dependent (Fig. 8), which was consistent with the results of *in vitro* anticoagulation experiments and may be related to the fact that P9 has anticoagulant activity. Based on the above results, in our further study, we need to further identify the mechanism of P9 action *in vivo*.

Conclusion

In conclusion, a novel anticoagulant peptide named P9 (HTWNLHWWR) was isolated and identified from trypsin hydrolysates of aqueous extracts of *Eupolyphaga sinensis* Walker. P9 prolonged APTT and TT times *in vitro* and was able to treat phenylhydrazine-induced thrombosis in zebrafish *in vivo*. The anticoagulant mechanism of P9 may be that P9 binds to thrombin in a mixed mode and alters the secondary conformation of thrombin. The results of this study

provide an experimental basis for the preparation of ESW anticoagulant peptides. However, ESW exerts its antithrombotic effects through the coregulation of multiple components and multi-targets, and these effective components and potential molecular mechanisms need to be further investigated by in vivo and in vitro experiments.

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Author Contributions X.L.: Undertook the experiments and wrote the manuscript. X.Z.: In vivo study supervision. H.L.: In vivo study supervision. L.P.: Methodology, review & editing. R.L.: Methodology, Software. H.Z.: Supervision, Writing - review & editing. Z.T.: Supervision, Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethical Approval No human sample is used in the study.

Competing Interest The authors declare that they have no known competing financial interests or personal relationships that appeared to influence the work reported in this paper.

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