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Antithrombotic effects of a newly purified fibrinolytic protease from *Urechis unicinctus*

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ABSTRACT

Introduction: The prevalence of thromboembolic disease, one of the top 3 leading causes of mortality worldwide, is being reported continually. More effective and safer antithrombotic drugs may overcome the underlying problems in antithrombotic therapy. In the present work, antithrombotic effects of UFEIII, a newly purified fibrinolytic protease from *Urechis unicinctus* were evaluated.
Materials and methods: UFEIII was purified from the marine invertebrate, *Urechis unicinctus*, using anion exchange and gel filtration chromatography. Molecular weight, fibrinolytic activity and fibrinogenolysis pattern of UFEIII were determined. Furthermore, antithrombotic effects of UFEIII in vivo were investigated through electrical induced carotid arterial thrombosis in rats, $FeCl_3$ induced carotid arterial thrombus model in rabbits and stasis induced vena caval thrombus model in rats.
Results: SDS-PAGE of the purified enzyme showed a single polypeptide chain with molecular weight of 20.8 kDa. In fibrin plate assays, UFEIII could not only directly degrade fibrin but also activate plasminogen. The fibrinogenolysis pattern of UFEIII was Aα-chains > Bβ-chains > γ-chain. Moreover, UFEIII could effectively prolong the time to occlusion in electrical induced carotid arterial thrombosis. Besides, both in rabbits and rats, the administration of UFEIII not only prolonged the activated partial thromboplastin time (APTT) and thrombin time (TT) also decreased the fibrinogen (FIB) content. Further, the thrombus lysis was observed after administration of UFEIII both in rabbits and rats.
Conclusion: UFEIII can possibly be a new potential source of fibrinolytic agent.

Introduction

Thromboembolic diseases, involving cardiovascular disease, cerebrovascular disease and venous thromboembolism, are within the top three leading causes of death as exposited in the global mortality projections to 2030 by WHO [1]. Thromboembolism often occurs when part or all of a thrombus, the archcriminal of the whole complex disorders, breaks away from the vessel wall, travels to other organs with blood circulation, resulting in a myriad of pathological changes wherever they arrive. Thrombus is generated when the physiological equilibrium between coagulation system and fibrinolytic system is disturbed. Regarding the treatment, administration of thrombolytic drugs to dissolve the blood clot is the only alternative of surgical interventions to remove or by pass the blockage [2]. However, the thrombolytic drugs still exhibit many undesired side effects such as hemorrhage, low fibrin specificity and short half-lives after three generations developments. Consequently, searching for ideal thrombolytic drugs has never been stopped.

Marine organisms, which constitute approximately one half of the total global biodiversity, are rich reservoirs of natural biofunctional components [3], and numerous antithrombotic compounds have already been discovered in the ocean [4–6]. *Urechis unicinctus*, which belongs to *Echiuroidea*, *Xenopneusta*, *Urechidae*, mostly inhabits in marine intertidal and subtidal zones of North China, Korea and Japan. In our lab, a cluster of fibrinolytic proteases were discovered from this marine worm and one 10.38 kDa protein [7] was purified and reported before.

In this present study, UFEIII, a newly purified fibrinolytc protease from *Urechis unicinctus* was investigated from multiple perspectives including fibrinolytic activity, fibrinogenolysis pattern and antithrombotic effects in vivo.

Materials and Methods

Reagents and animals

Ehiuroid worms (*U. unicinctus*) were obtained from a local aquatic market in Qingdao, China. Sephacryl S-100 HR, Q-Sepharose FF and Sephadex G-50 were purchased from GE Healthcare Bioscience AB (Uppsala, Sweden). Thrombin, fibrinogen and plasminogen, azocasein and Folin-Ciocalteu's phenol reagent were all purchased from Sigma-Aldrich (St.Louis, USA). Other reagents and chemicals used were of analytical grade and commercially available.

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Wistar rats (male and female half, weights 280 ± 20 g) and male New Zealand white rabbits (weights 3.5-4.0 kg) were purchased from Lukang Pharmaceutical Co. (Shandong, China). Kunming mice (male and female half, weights 20 ± 1 g) were obtained from Qingdao Experimental Animal Center. All animals were accommodated to the new environment for at least 1 week with conditions of moderate temperature and good ventilation. Standard diet and tap water were provided freely. The animal experimental protocol was approved by the Ethics Committee at Ocean University of China (Qingdao, China).

Purification of UFEIII

All fractionation steps were carried out at 4 °C. Washed ehiuroid worms (*U. unicinctus* 2000 g) were laparotomized to remove body walls and viscera, the rest coelomic fluid was centrifuged at 8000 × g for 40 min. Then, the supernatant was pooled and filtered to remove insoluble lipid. After the soluble extracts were dialyzed by membranes with a MWCO of 3500-5000 Da (Spectrum Chemical Mfg. Corp., USA), the semifinished product was obtained by a freeze dry system (Labconco corp., USA). The crude enzyme solution (7.5%) was applied to a Sephacryl S-100 HR column (3.5 cm × 80 cm) equilibrated with triple-distilled water at a flow rate of 1 ml/min. The major active fractions were pooled and introduced into a Q-sepharose FF column (2.5 cm × 20 cm) previously equilibrated with 20 mM Tris–HCl buffer (pH 8.0) and the bound proteins were eluted with a linear gradient of 0-0.5 M NaCl in the same buffer at a flow rate of 5 ml/min. Fractions containing the highest fibrinolytic activity were concentrated by lyophilization after desalting. The concentrated sample was dissolved in a small volume of 20 mM Tris–HCl buffer (pH 8.0) and loaded onto a Sephadex G-50 column (1.6 cm × 80 cm) equilibrated with the same sample dissolving buffer at a flow rate of 1 ml/min. Finally, the active fraction was desalted, lyophilized and used as the purified enzyme preparation.

Protease activity was measured by azocasein assay [8] and chromogenic assay [9] using S-2444 (pyro-Glu-Gly-Arg-p-nitroanilide) as a substrate at 405 nm. Protein concentration was estimated by the method of Bradford [10]. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm.

Determination of purity and molecular weight

High-performance liquid chromatography (Shimadzu LC system, Japan) was employed to analyze the purity of UFEIII using a Shodex OHPak SB-806 HQ (8 mm × 300 mm) column (Shodex, Japan) at 30 °C, with an elution of 0.1 M Na_2SO_4, a flow rate of 1 ml/min and 20 μl sample loaded.

The molecular weight of the purified enzyme was determined by SDS-PAGE using 5% (w/v) stacking and 12% (w/v) resolving polyacrylamide gels. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 for 3 h and destained with a solution containing methanol: glacial acetic acid: distilled water (1:1:8 by volume). A LMW standard protein marker (Takara, Japan) was used for calibration.

Determination of amidolytic activity

Amidolytic activity of UFEIII was measured using chromogenic substrates according to previous report [11] with slight modifications. The reaction mixture (1 ml) contained 20 μl of 0.5 mg/ml enzyme solution, 1 mM substrate solution and 0.1 M Tris-HCl buffer (pH7.4). After incubation for 15 min at 37 °C, absorbance of released p-nitroaniline at 405 nm was determined with a TU-1810 spectrophotometer (Purkinje General, China). Then, the amount of liberated p-nitroaniline was calculated from the change in absorbance. One unit of amidolytic activity (AU) was defined as micromoles of substrate hydrolyzed per min/ml by UFEIII.

Fibrinolytic and fibrinogenolytic activities

Fibrinolytic activity was determined using the fibrin plate method of Astrup and Mullertz [12] with minor modifications. The plasminogen-rich fibrin plate was prepared by pouring the solution composed of 0.68 mg/ml human fibrinogen in 50 mM Tris–HCl buffer (pH 7.4) containing 0.15 M NaCl, 1% agarose, 1.25 U/ml thrombin and 0.25 U/ml human plasminogen into a sterile petri dish with a diameter of 120 mm. The solution in the plate was left for 1 h to form fibrin clot and then 3.5 mm diameter wells were made in the plate for sample application. The plasminogen-free fibrin plate contained no plaminogen and was heated at 85 °C for 30 min in addition. To observe the fibrinolytic activity, 100 μl of sample solution was carefully dropped into each well and incubated for 18 h at 37 °C.

Analysis of fibrinogenolytic activity was performed following the reported method [13] with slight modifications. 200 μl fibrinogen (1 μg/μl) in 10 mM Tris–HCl buffer (pH 7.4) containing 0.15 M NaCl was incubated at 37 °C with 10 μg UFEIII for various time durations. A portion of the incubated sample (20 μl) was withdrawn at each considered time interval, boiled for 3 min to terminate the reaction, and then analyzed by SDS-PAGE.

Hemorrhagic test of UFEIII

Hemorrhagic evaluation was carried out according to previous method [14] with slight modifications. Different doses of the purified UFEIII were subcutaneously injected into the back and belly region of mice. As a positive control, urokinase was also injected into mice. 24 h later, the mice were sacrificed and inspected for the emergence of hemorrhagic halos in the back and belly skins.

Thrombolysis activity of UFEIII in vitro

Blood was collected from the central ear artery of New Zealand white rabbit to a sterilized petri plate. After spontaneous coagulation, the blood clot was blotted up and cut into small pieces. 0.9% saline, lumbrokinase solution (500 U/ml) and UFEIII solution (500 U/ml) were used as the negative control, the positive control and the testing group, respectively. Every group included three parallel tubes, each of which contained 2 g blood clot with 1 ml 0.9% saline, lumbrokinase solution and UFEIII solution. Then, the tubes were incubated in a 37 °C thermostat shaker (60 r/min) for 1 h, 2 h and 3 h. At each considered time interval, a little supernatant fluid was withdrawn to determine the amount of the released red cells microscopically. At last, the residual clot was weighed to calculate the dissolve rate of blood clot. Dissolve rate (%) = $(Mass_{\text{before dissolve}} - Mass_{\text{after dissolve}})/Mass_{\text{before dissolve}} \times 100\%$.

Antithrombotic effects of UFEIII in vivo

Electrical induced carotid arterial thrombosis in rats

This model was a clot prevention model and UFEIII was introduced before thrombogenesis. Wistar rats were anesthetized with intraperitoneal injection of 3% pentobarbital sodium (1 ml/kg) after overnight fasting. Through a midline cervical incision, approximately 15 mm of the right carotid artery was surgically exposed via blunt dissection, and a silkiness membrane was placed under the vessel to isolate it from the vagus nerve and connective tissue. Thrombogenesis was achieved using a BT87-3 Animal Thrombosis Generator (Baotou Medical College, China) following the method previously described with minor modification [15]. Primarily, 0.9% saline, heparin (300 U/kg), high (14 mg/kg), medium (7 mg/kg) and low dosage (3.5 mg/kg) of UFEIII were administrated intravascularly