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Biochemical and Hemostatic Mechanism of A Novel Thrombin-Like Enzyme

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ABSTRACT

Thrombin-like enzyme (TLE) plays a significant role in vessel injury hemostasis. A novel snake venom TLE (Agacutin) was purified from *Agkistrodon Acutus* snake venom. Structural analysis indicated that Agacutin is a heterodimer that has a MW of 29,402 Da, a pI value of 5.39, and optimum activity at 35 °C and pH 7.5. The N-terminal 15 amino acid sequences of Agacutin are DSSGWSSYEGHEYYV (small subunit) and DCSSGWSSYEEHQYY (large subunit). *In vitro* studies indicated that the coagulation activity of Agacutin was activated by Ca^{+2} or inhibited by phenylmethanesulfonyl fluoride, but not influenced by heparin or hirudin. The arginine esterase activity and fibrinogen hydrolysis result showed that Agacutin only cleaves α-subunit and releases fibrinopeptide A. *In vivo* studies indicated that Agacutin *iv* (0.01-0.05 U/kg) shortened 30.2-49% of the rabbit blood clotting time, or *ip* (0.5-2.0 U/kg) shortened 29.7-73.1% of the mouse tail bleeding time. Agacutin does not influence APTT, platelet or euglobulin clotting time, and activate Factor II or XIII. It converts fibrinogen into the soluble fibrin that accelerates hemostasis at wound.

Introduction

Snake venom contains various components that affect the mammalian hemostatic system as either procoagulants or anticoagulants [1,2]. These components interact with diverse proteins of the blood coagulation cascade and the fibrinolytic pathway. Generally, according to the results of *in vivo* and / or *vitro* analysis, these proteins are classified into the following seven groups: (i) fibrinogen clotting enzymes; (ii) fibrino (geno)lytic enzymes; (iii) plasminogen activators; (iv) prothrombin activators; (v) factor V, X activators; (vi) hemorrhagins; and (vii) platelet aggregation inhibitors [3]. Thrombin-like enzyme (TLE) is protease that belongs to the first group (i). TLE can accelerate the clotting of plasma or fibrinogen solution *in vitro*, but *in vivo* they have different effects, for example hemostasis and anticoagulation. By exploiting some of the effects, these proteins have been developed into clinical drugs. Reptilase from *Bothrops atrox* snake venom serves as an example of the successful clinical drugs. Reptilase shortens the blood clotting and bleeding time *in vivo* and has been used widely in the treatment of wound hemorrhages and in the surgery to prevent bleeding. In 2004, Reptilase was successfully recombined into *Pichia pastoris* [4]. In 2005, a 35.5 kDa TLE with hemostatic function was purified from *Agkistrodon blomhoffii* ussurensis snake venom [5]. Interestingly, its N-terminal 15 amino acid residue sequence was the same as that of Reptilase and its hemostatic effect was similar to that of Reptilase. In 2006, Wang YN et al [6] purified a novel Factor X activator from *Viper russellii* snake venom. This activator showed better hemostatic activity in mice and rats at a dose of 0.000313 U/kg.

Since 1995, a number of enzymes with structural and functional diversity have been purified from *Agkistrodon acutus* snake venom. In 1995, Chen YL et al [7] purified *Agkicetin* (a heterodimer with 14 and 15 kDa subunits), a *GP1b* antagonist and platelet inhibitor. In 1998, Chia-Hsin Yeh et al [8] purified *Accutin* (5.241 kDa), a new member of disintegrin family, which potently inhibits human platelet aggregation. In 1999, Huang QQ et al [9] reported two TLEs, *Acuthrombin-A* (28 kDa) & *Acuthrombin*-C (69 kDa). Cheng X et al [10] purified the fibrinogenolytic enzyme *Agkisacutacin* (a heterodimer with 14 and 15 kDa subunits). Pan H et al [11] successfully cloned *Acutin* (38 kDa) which caused defibrination. In 2000, Xu XL et al [12] reported two TLEs, *Anticlotting Factor*-I (ACF-I) and *Anticlotting Factor-II* (ACF-II) (a heterodimer with 14.6 and 14.7 kDa subunits). In 2001, Yeh CH

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et al [13] reported a platelet glycoprotein *Ib* antagonist *Agkistin* (a heterodimer with 16.5 and 15.5 kDa subunits) and Liang XX et al [14] reported a fibrinolytic enzyme *F II (a)* (26 kDa). In 2004, Li T et al [15] purified *Acoagulatin* (a heterodimer with 14.4 and 17 kDa subunits), an anticoagulant. The publications on all these enzymes enrich our understanding about the components of the venom.

Since 1993, we have tried to purify the coagulant from *Agkistrodon acutus* snake venom. Fortunately, five novel enzymes which use fibrinogen as substrate have been obtained. Agacutin is the most comprehensively studied one. The biochemical properties and the *in vitro* and *in vivo* hemostatic mechanisms of Agacutin are reported in this paper.

Materials and methods

Preparation of Agacutin

1.0 g of the crude snake venom powder (purchased from the Snake Venom Institute, Guangxi Medical University, China) was dissolved in 8 ml of 0.01 M phosphate buffer (pH 6.8) and centrifuged at 6000 rpm for 20 min. The supernatant was applied to a gel filtration column of Sephadex G-75 and eluted with 0.01 M phosphate buffer (pH 6.8). After the relevant fraction had been collected, it was applied to a DEAE-Sepharose Fast Flow (FF) ion exchange column and eluted with a linear gradient of 0-0.065 M NaCI in 0.01 M phosphate buffer (pH 6.8). After the effective fraction had been dialyzed against 0.01 M phosphate buffer (pH 6.8) for 24 h, the sample was re-applied to the regenerated DEAE-Sepharose FF column to repeat the linear gradient elution of 0-0.065 M NaCI. The effective fraction was subsequently applied to a Sephadex G-25 column for desalting. The preparation procedure was finished after the purified Agacutin was freeze-dried. One vial for the animal experiments contained 1 unit of Agacutin and 1% micro-molecular dextran (purchased from the Sin Food Drug Administration, SFDA). The Econo-system preparative liquid chromatographer (Bio-Rad Co, USA) was used in the preparation.

HPLC analysis of Agacutin

High performance liquid chromatography (HPLC) (Waters 6000E, USA) was performed to analyze the purity of Agacutin. For gel-filtration HPLC column (BIOSEP SEC-2000, Pharmacia-LKB Co), an elution of 0.2 M phosphate buffer (pH 6.8) and a flow rate of 1 ml/min were used. For reverse-phase HPLC C4-column (Hypersil-C4, 300 Å, ODS-BP, 2.6×40 mm), Solution A (0.1% trifluoroacetic acid water solution containing 0.1% n-butylamine), Solution B (acetonitrile solution containing 0.1% trifluoroacetic acid and 0.1% n-butylamine) and a flow rate of 1 ml/min were used. The elution gradients of 0 min/ 40% to 10 min/ 64% to 10.1 min/ 64% to 15 min/ 40% (retention time / Solution B ratio) were utilized during the analysis.

SDS-PAGE analysis

A polyacrylamide gel (T: C = 12: 3.9) was prepared for SDS-PAGE.

MALDI-TOF Mass Spectrographic analysis

0.75 μl of Agacutin (200 ng/μl) was mixed with an equal volume of 10 mg/ml mustard acid. The mixture was applied to a mass spectrometer (ABI Co, USA).

N-terminal amino acid sequencing of Agacutin

Agacutin is a heterodimer consisting of 15 and 16 kDa subunits. In order to sequence the N-terminal 15 amino acid residues, SDS-PAGE (T: C = 15:3.9) and the PVDF membrane electrical transfer technique [16] (Mini Trans-Blot & Power Pac HC, Bio-Rad Co, USA) were used to separate the two subunits of the heterodimer. The Edman Degradation method (Procise^R cLC Protein Sequencer, Applied Biosystems, USA) was used to determine the sequence of each subunit.

Determination of the iso-electric point (pI)

According to the method of Righetti P et al [17], iso-electric focusing (IEF) electrophoresis was performed by using PAG gel matrix (T:C = 7:3) and Ampholine (pH 3.5-9.5, Pharmacia Co. Sweden) to form an appropriate pH gradient. A Sanhen electrophoresis apparatus (ECP3000, Beijing Medical Apparatus Co.) was used in the experiment. The pH values which were measured at each 0.5 cm region of the gel were plotted against the corresponding gel lengths and the resulting pI of Agacutin was calculated.

Study of the biochemical properties

The optimum temperature or pH value of Agacutin-induced clotting time was obtained from the plot of Agacutin-induced clotting time. A 0.2 ml Agacutin water solution (0.2 U) was mixed with 0.2 ml of standard human plasma (Dade Behring Co., Germany) containing 20-500 U sodium heparin, 2.5-25 mM phenylmethanesulfonyl fluoride (PMSF), or 5-100 AT-U hirudin to measure plasma clotting time at 37 °C. To determine the influence of Ca^{2+} on ox fibrinogen clotting time, 0.45-9.0 mM $CaCl_2$ was used. Porcine thrombin (Sigma Co.) was required as a control in some experiments.

Urea dissolving assay

In order to observe whether the plasma clot was re-dissolved, 1 ml of 3 M urea was added to a human plasma clot which was previously prepared by mixing 0.2 ml of human plasma with 0.2 U of Agacutin at 37 °C.

Determination of enzyme activity

Coagulation activity assay

The enzyme activity of Agacutin was estimated according to the Reptilase Unit (KU) method [18]. Here, 0.2 ml of Agacutin-water solution (1 KU/ml) should coagulate both 0.2 ml of standard human plasma (Dade Behring Co., Germany) at 37 °C in 60 ± 20 seconds and 1 ml of ox fibrinogen solution (0.4%) (purchased from SFDA) in 232 ± 56 seconds. The plasma, ox fibrinogen and Agacutin water solution were pre-incubated individually for 3 min at 37C in a glass incubator. When the 0.2 ml of plasma or the 1 ml of ox fibrinogen solution was mixed with 0.2 ml of Agacutin at 37 °C, the cloudiness in the mixture occurred. The plasma or ox fibrinogen clotting time was obtained by counting the time from the mixture to the initial cloudiness. KU was used *in vivo* and *vitro* experiments below. In the coagulation activity assay, because the activation method has a larger eye observation error, each sample was assayed activity three times. The first two activities were preliminary results. The 3rd result which was recorded in the paper was the most accurate and reliable.

Arginine esterase activity assay

Arginine esterase activity was determined according to the BAEE method [19].

Hydrolysis analysis of Agacutin to ox fibrinogen subunit

1 μg / μl of ox fibrinogen and 10 U / 100 μl of Agacutin were prepared with 50 mM Tris-HCl (pH 7.5), respectively. 100 μl of the ox fibrinogen and 100 μl of the Agacutin solution were mixed and incubated at 37 °C for 1, 2, 3, 4 h, respectively. The incubation samples were loaded on SDS-PAGE (T: C = 8:3.9). In the experiment porcine thrombin (1 U / 100 μl) was used as control.