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Purification and characterization of a novel antithrombotic peptide from *Scolopendra subspinipes mutilans*

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ABSTRACT

Ethnopharmacological relevance: The centipede has been prescribed for the treatment of cardiovascular diseases in Korea, China and other Far Eastern Asian countries for several hundred years.
Materials and methods: A novel antithrombotic peptide was isolated from *Scolopendra subspinipes mutilans* using a combination of ultrafiltration, Sephadex G-50 column, Source 15Q anion exchange column and RP-HPLC C18 column.
Results: The molecular mass of the purified peptide is 346 Da measured by Electrospray Ionization Mass Spectrometry (ESI-MS). The primary structure of the peptide is Ser-Gln-Leu (SQL) determined by Edman degradation. SQL potently prolonged the activated partial thromboplastin time (aPTT), and inhibited platelet aggregation.
Conclusions: These results help to clarify the mechanism of the antithrombotic activity of the centipede for effective treatment of cardiovascular and cerebrovascular diseases.

1. Introduction

Centipedes (*Scolopendra subspinipes mutilans* L. Koch) are predatory, elongated and dorsoventrally flattened arthropods, which belong to the Chilopod class (Negrea and Minelli, 1995). In China, the centipede has been used for thousands of years as a traditional Chinese medicine to treat disorders, such as stroke-induced hemiplegia, apoplexy, epilepsy, tetanus, whooping cough, tuberculosis, scald burns, and pyocutaneous disease. In addition, the centipede has been described for the treatment of cardiovascular diseases in Korea, China and other Far Eastern Asian countries for several hundred years (Pemberton, 1999).

Thrombosis is caused by two main pathways. One is blood clotting, which is processed by various coagulation factors at sites of vascular injury. Because endogenous or exogenous anticoagulants interfere with coagulation factors, blood coagulation can be prolonged or stopped (Jung et al., 2007). The other thrombotic pathway is platelet aggregation, which plays an important role in hemostasis. The process of platelet aggregation includes the adhesion and activation of platelets, secretion of the granular contents, and aggregation of platelets again (Kong et al., 2009). There are few reports about antithrombotic agents identified from the centipede. You et al. (2004) isolated a 25 kDa serine protease from *Scolopendra subspinipes mutilans* that demonstrated fibrinolytic activity by converting human Glu-plasminogen to activated plasmin. Wu et al. (2009) found that centipede acidic protein (CAP) significantly suppressed the development of atherosclerosis, and improved the hemorrheological disturbances and histopathological changes in the atherogenic-diet rat model. These effects may be attributed in part to reversal of dyslipidemia, inhibition of lipid peroxidation, and regulation of nitric oxide (NO) and endothelin-1 (ET-1) systems.

Centipedes consist of many kinds of protein, amino acid, lipids and enzymes. Many peptides were purified or identified from Centipedes (Wang et al., 1997; Peng et al., 2010; Undheim et al., 2011; Yang et al., 2012). In the present study, we report the purification and characterization of a novel peptide (Ser-Gln-Leu) with antithrombotic activity, which may contribute to the traditional usefulness of centipede as antithrombotic medicine.

2. Materials and methods

2.1. Materials

Centipedes were obtained from Xiansheng Pharmacy (Nanjing, Jiangsu province, China). The ultrafiltration tube was purchased from Millipore (Billerica, Massachusetts, USA). The BioLogic Duoflow

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system was obtained from Bio-Rad (Richmond, CA, USA). The Lichrospher C18 reverse-phase HPLC column was from Jiangsu Hanbon Science & Technology Co. Ltd (Huai'an, China). The ESI-MS System was from Waters Company (Milford, Massachusetts. USA).

2.2. Extraction of peptides from Scolopendra subspinipes mutilans

Chinese Pharmacopoeia (I) provides a centipede medicinal method: centipede was extract by ethanol (50%) and the dried powder can take orally. So, *Scolopendra subspinipes mutilans* ($n=100$) were ground to a powder in a mortar and then extracted by stirring for 24 h in 1000 mL of 50% ethanol solution. The homogenate was centrifuged at $8000\times g$ for 15 min at 4 °C. The supernatant was collected. The ethanol was removed from the supernatant by rotary evaporation.

2.3. Purification of an anticoagulant peptide from Scolopendra subspinipes mutilans

The supernatant was filtered through a Millipore membrane filter (0.45 μm) and then applied to an ultrafiltration tube of 10,000 Da, and centrifuged at 10,000 rpm for 5 min. The low molecular weight fraction after ultrafiltration was collected and then applied to a Sephadex G-50 column (26 mm × 100 cm), equilibrated with distilled water and eluted with the same buffer at a flow rate of 0.8 mL/min. The fractions were collected at 15-min intervals. The activated partial thromboplastin time (aPTT) activity was evaluated for each fraction. The fraction with the highest aPTT activity (Faction C) was collected, lyophilized, dialyzed with 50 mM Tris–HCl buffer (pH 7.4), and further separated by Source 15Q anion exchange column (12 mm × 150 mm) preliminarily equilibrated with 50 mM Tris–HCl buffer (pH 7.4) in a high performance liquid chromatographic (HPLC) system (Bio-Rad, Richmond, CA, USA). A linear gradient of NaCl (0–2 M) in the same buffer was maintained at a flow rate of 3 mL/min. Absorbance was monitored at 214 nm. Each fraction was evaluated for aPTT activity. The fraction with aPTT activity (Faction C1) was separated by RP-HPLC on a C18 column (10 × 250 mm; Kromasil, China) under linear gradient elution conditions using methanol as the organic modifier and trifluoroacetic acid (TFA) as the volatile buffer. Eluent A consisted of 0.1% TFA in 10% methanol (v/v), eluent B of 0.1% TFA in 90% methanol (v/v). The chromatographic column was conditioned with 100% of eluent A, after which 1 mL of the peptide solution was applied on the C18 column and eluted by the remaining eluent A for 10 min and with the following increasing eluent B concentrations: 0–10 min, 0%; 10–45 min, 0–75%. The flow rate was 3 mL/min. The UV absorbance of the eluent was monitored at 214 nm. Each fraction was evaluated for aPTT activity. The active fraction (C-3) was concentrated by lyophilization and further purified by another C18 column (4.6 × 250 mm; Hanbon, China) to produce the final purified peptides. Eluent A consisted of 0.1% TFA in 10% methanol (v/v), eluent B of 0.1% TFA in 90% methanol (v/v). The chromatographic column was conditioned with 80% of eluent A. The second and final re-chromatography involved solvent system A and B at the gradient of 20% B to 40% B in 40 mL at the flow rate of 0.8 mL/min. Elution was monitored at 214 nm. Each fraction was evaluated for aPTT activity. The faction C-2-2 showed aPTT activity.

2.4. Determination of molecular weight and peptide sequence

The molecular mass of the purified peptide was determined using ESI-MS. Complete peptide sequencing was undertaken by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491 (Carlsbad, CA, USA).

2.5. Prothrombin time (PT) and aPTT clotting assays

Nine parts of blood was drawn by venipuncture into one part of 3.2% sodium citrate from healthy volunteers and pooled. The blood was separately centrifuged at $2500\times g$ for 10 min to obtain platelet poor plasma (PPP). Briefly, normal citrated PPP (80 μL) was incubated with sample solution (20 μL) for 3 min at 37 °C. For the aPTT clotting assay, 100 μL of aPTT reagent was added to the mixture (PPP and sample) and incubated at 37 °C for 10 min. Clotting time was immediately recorded after the addition of 100 μL of 20 mM $CaCl_2$. For the PT clotting assay, PT reagent was added to the incubated mixture of PPP (80 μL) and sample (20 μL), and clotting time (s) was recorded. All coagulation assays were performed with three individual replicates (Majdoub et al., 2009). Heparin, a commercial anticoagulant, was used as positive control.

2.6. Assay of anti-platelet aggregation activity

Inhibition of platelet aggregation induced by ADP (10 μM) was determined according to the reported method (An et al., 2011). Human platelet-rich plasma (PRP) provided by Jiangsu Blood Center, Jiangsu, China, was centrifuged at 500 g, for 10 min, at room temperature (RT). The pellets were suspended in Tyrode's buffer A (137 mM NaCl, 2 mM KCl, 0.3 mM NaH_2PO4, 12 mM $NaHCO_3$, 5.5 mM glucose, 0.35% bovine serum albumin (BSA), 1 mM $MgCl_2$, and 0.2 mM EGTA, pH 6.5). After successive washes and centrifugation, the pelleted platelets were resuspended in Tyrode's buffer B (137 mM NaCl, 2 mM KCl, 0.3 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.5 mM glucose, 0.35% BSA, and 2 mM $CaCl_2$, pH 7.4) at the concentration of 5×10^8 platelets/mL, counted with a thrombocounter. Aliquots of 270 μL of PRP plus saline, acetylsalicylic acid (ASA, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) or sample (0.2, 0.4, 0.6, 0.8 and 1 mg/mL) were placed in translucent tubes containing magnetic stir-bar and were preincubated at 37 °C for 1 min. Then the aggregating agent ADP (10 μM) was added in 30-μL amounts and aggregation (the change in light transmittance) was monitored for 300 s on a chart recorder connected to the aggregometer (Wei et al., 2006a, 2006b).

3. Results

3.1. Effect of peptides extracts

The extracts of *Scolopendra subspinipes mutilans* prolonged aPTT but not PT in blood clotting time assays (data not shown).

3.2. Purification of an anticoagulant peptide from Scolopendra subspinipes mutilans

The extract of *Scolopendra subspinipes mutilans* was separated into low weight fraction and high weight fraction by an ultrafiltration tube of 10,000 Da. The low molecular weight fraction showed aPTT activity, and then was separated into three factions by Sephadex G-50 column (Fig. 1A). The faction C showed aPTT activity, and then was separated into four fractions by Source 15Q anion exchange column (Fig. 1B). The faction C1 showed aPTT activity, and then was separated into four factions by RP-HPLC on a C18 column (Fig. 1C). The fraction C-3 showed aPTT activity, and then was separated into two fractions on another C18 column. The fraction C-2-2 showed aPTT activity and represented the final purified peptide (Fig. 1D).