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Inhibitory effect of bee venom on blood coagulation via anti-serine protease activity

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ABSTRACT

Maintaining a balance between blood flow and hemostasis is very important because both excessive bleeding and excessive coagulation and aggregation can cause death. In this study, the preventive effect of bee venom (BV) on blood coagulation was investigated. BV prolonged coagulation periods in a dose dependent manner compared with the negative control. It did not target any specific coagulation factor(s) but showed a broad spectrum of action against various coagulation factors. BV did not inhibit fibrinolytic activity. BV inhibited trypsin and chymotrypsin activities dose-dependently. In addition, BV did not induce bleeding disorders. In conclusion, BV plays a role in anticoagulation by inhibiting the activities of coagulation factors, which are serine proteases, not via inhibiting thrombin activity.

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Introduction

Maintaining a balance between blood flow and hemostasis is very important because excessive bleeding can cause death, whereas excessive coagulation and aggregation can cause thrombus formation in blood vessels, blocking blood flow and causing death (Palta et al., 2014). In addition, high blood pressure is an important risk factor for cardiovascular disease that is one of leading causes of death worldwide, especially in developed countries. Blood clotting occurs via the activation of coagulation factors, and platelets are also involved in coagulation. Platelets plays a significant role in hemostasis (Walsh, 2004). They become activated by binding with collagen derived from the damaged endothelium, which induces the release of the contents of granules including adenosine diphosphate (ADP), serotonin, platelet activating factor (PAF), and thromboxane A_2 (TXA_2) into the blood plasma (Rao, 2012). The reactions that follow this lead to the aggregation of more adjacent platelets, resulting in the completion of primary hemostasis. The blood coagulation cascade consists of two pathways: intrinsic and extrinsic blood coagulation pathways that are a slower and process and a very rapid process, respectively. These pathways are the contact activating pathway and the tissue factor pathway, respectively; they lead to fibrin production, resulting in the completion of secondary hemostasis. Coagulation factors are zymogens of serine protease.

Anticoagulants can be taken orally or intravascularly to prevent blood clotting. Many types of anticoagulants have been developed, including aspirin, warfarin, heparin, coagulation factors inhibitors, and antithrombin. Batroxobin, a toxin from snake venom, lyses fibrinogen without affecting platelet function and its activity closely resemble thrombin (Kumar et al., 2015; Vu et al., 2013). Batroxobin plus aspirin may be a more effective treatment in diabetic patients than aspirin alone (Robertson et al., 2012). Prolonged bleeding time is the major risk associated with anticoagulants such as aspirin and warfarin (Hansen et al., 2010). Alternative therapeutic strategies have been studied to improve blood circulation and hypertension using polyphenol-rich diet, herbs, and fiber-rich diet (Stanger et al., 2012). Recent studies show that honeybee venom (BV), a traditional medicine, can be used to treat certain diseases including rheumatoid arthritis, Parkinson's disease, multiple sclerosis, and inflammatory disease (Alvarez-Fischer et al., 2013; Hwang et al., 2015). BV contains many kinds of peptides, proteins, and amines such as melittin, apamin, phospholipase A2,

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hyaluronidase, histamine, and dopamine and its major component is melittin (Son et al., 2007). In this study, we investigated the ability of honeybee (*Apis* spp.) venom to inhibit blood coagulation.

Materials and methods

Chemicals

BV used in this study containing 56.7% melittin was obtained from Kukjeon Pharmacy (Anyang, Korea). β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (β-NADH), pyruvate, heparin, Triton X-100, trypsin, chymotrypsin, human fibrinogen, and aspirin (Lot No. MKBQ8444V; purity, ≥99.5) were purchased from Sigma (St. Louis, MO, USA).

Animals and blood platelet preparation

Eight-week male Sprague Dawley (SD) rats and eight-week old male ICR mice were purchased from Koatech (Pyeongtaek, Korea). The animals were acclimatized for 1 week at 22 ± 2 °C and 50 ± 5% humidity, with 12-h cycles of light and dark. All animal experimental procedures were approved by the Korea University Institutional Animal Care and Use Committee (Approval No. KUIACUC-2013-154) and were performed in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996).

Blood was collected from the abdominal aorta of eight-week male SD rats weighing 200–250 g. After centrifugation at 200*g* for 10 min at room temperature, the supernatant (platelet-rich plasma, PRP) was removed. The remaining material was then centrifuged at 1200*g* for 10 min at room temperature, and the supernatant obtained was platelet-poor plasma (PPP).

Cell cytotoxicity

The cytotoxicity of BV to platelets was determined by measuring the amount of lactate dehydrogenase (LDH) released from platelets (Lee et al., 2016a) The PRP (2×10^8 platelets/ml) was incubated with the vehicle (distilled water, negative control) or with 5 μg/ml, 10 μg/ml, or 20 μg/ml of BV for 5 min and was centrifuged for 1 min at 10,000g. An aliquot of the supernatant (50 μl) was mixed with 1.8 ml of β-NADH solution (0.17 mM reduced form of β-NAD disodium salt in Tris-EDTA buffer, pH 7.4) and incubated for 5 min at 37 °C. 100 μl of a pyruvate solution (14 mM pyruvate in Tris-EDTA buffer, pH 7.4) that had been preincubated at 37 °C was then added, and the absorbance at 340 nm was immediately measured. The decrease in the absorbance at 340 nm of the reaction mixture due to the conversion of NADH to NAD was determined. The positive control group was treated with 1% Triton X-100. The amount of LDH released by the negative control was considered to be 100%.

Anticoagulation assay

The anti-coagulation effect of BV was evaluated by measuring prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) using an automated DCA-2 coagulometer (Dutch Diagnostics, Zutphen, Netherlands). Blood was collected from healthy volunteers (male, ages 26–28) in BD vacuum tubes containing sodium citrate (3.8%) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Plasma was collected after centrifugation of the whole blood for 15 min at 1500g. After citrate-anticoagulated human PPP was obtained as described above, 90 μl of PPP was mixed with 10 μl of BV (0, 0.5, 1, 5, 10, and 20 μg/ml) and incubated at 37 °C for 1 min. Heparin was used as a positive control. 200 μl of PT reagent (Haematologic Technologies Inc., Essex Junction, VT, USA) was then added, and PT was measured. Additionally, in the aPTT assay, 90 μl of PPP was mixed with 10 μl of BV and 100 μl of aPTT reagent (Haematologic Technologies Inc.) and incubated at 37 °C for 2 min. Coagulation was then initiated by adding 100 μl of 0.25 M $CaCl_2$ and aPTT was measured. For the TT assay, 90 μl of citrated coagulated PPP was mixed with 10 μl of BV and incubated at 37 °C for 2 min. Coagulation was then initiated by adding 100 μl of thrombin reagent (Haematologic Technologies Inc.) and TT was measured.

Coagulation factor inhibition assay

To assess the inhibitory effect of BV on each coagulation factor in the coagulation pathway, the aPTT and PT assays were performed with coagulation factor deficient plasmas (Haematologic Technologies Inc.). The aPTT and PT assays were performed using the methods as described above.

Factor Xa (FXa) activity assay

FXa activity assay was measured in 96-well plates with the reaction mixture containing 110 μl of BV (0.001, 0.01, 0.1, 1, 10, and 100 μg/ml) in 50 mM Tris-HCl buffer (pH 7.4) containing 7.5 mM EDTA and 150 mM NaCl and 30 μl of anti-thrombin III (AT III) (200 nM) (Haematologic Technologies Inc.) or buffer. The reaction mixture was incubated at 37 °C for 2 min and 30 μl of FXa (1 U/ml) (Haematologic Technologies Inc.) was then added. After 1 min, 30 μl of substrate S-2222 (1.5 mM) (Chromogenix, Milano, Italy) was added and absorbance was measured at 405 nm for 2 min. Heparin was used as a positive control.

Fibrinolytic cleavage assay

To confirm the effect of BV on fibrinolytic activity, a plasmin inhibition assay was performed by determining the time course of human fibrin degradation. Human fibrinogen (200 μg) that had been clotted with 1 unit of thrombin in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $CaCl_2$ was incubated with plasmin (500 ng) alone or plasmin and BV (1 and 5 μg/ml) at 37 °C. The fibrinolytic activity was analyzed using 12% SDS-PAGE (10 μg/lane).

Serine protease inhibition assay

To investigate the inhibitory effect of BV on serine protease activity, trypsin and chymotrypsin were used. The assay was performed in 96-well plates with 160 μl of BV (0, 0.001, 0.01, 0.1, 1, 10, and 100 μg/ml) in 100 mM Tris-HCl buffer (pH 8.0) containing 20 mM $CaCl_2$ and 0.05% Triton X-100 and 400 ng trypsin or chymotrypsin. The reaction mixture was incubated at 37 °C for 30 min and BApNA or SucAAPF-pNA (0.4 mM) (Sigma) was added; absorbance at 405 nm was then measured for 2 min. The serine protease activity was calculated as the 50% inhibitory concentration (IC_{50}).

Tail bleeding time

Bleeding times were determined using male ICR mice weighing 30–35 g as previously described (Lee et al., 2016a). BV at doses of 0.0625, 0.125, and 0.25 mg/kg, 500 μM of aspirin or distilled water (DW) were injected intravenously into the tail of ICR mice. After 2 h-treatment, the mice were lightly anaesthetized using ether and were maintained at body temperature, 37 °C. The tail was severed 3 mm from the tip using a surgical blade and then immersed in a transparent 15-ml conical tube containing normal saline that had been pre-warmed to 37 °C. The time until bleeding ceased was measured.

Statistical analysis

The data were analyzed using the SPSS statistical analysis program (Chicago, IL, USA). The statistical significance of the difference was

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