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Hannahpep: A Novel Fibrinolytic Peptide from the Indian King Cobra (*Ophiophagus hannah*) Venom

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A novel fibrinolytic peptide (Hannahpep) was isolated and purified from the venom of the Indian King Cobra (*Ophiophagus hannah*) by thin-layer chromatography followed by reverse-phase high-performance liquid chromatography. The MW of the peptide was found to be 610 Da and the amino acid sequence of Hannahpep was determined to be Arg, His, Ala, Arg, His, Asp. Hannahpep produced defibrinogenating activity in male albino mice. It exhibited significant fibrinolytic and fibrinogenolytic activity *in vitro*. Hannahpep showed plasma-anticlotting activity. However, it lacked hemolytic, hemorrhagic, or phospholipase activity. This peptide may have possible therapeutic application in the management of thrombosis or occlusion of a blood vessel. © 1999 Academic Press

Key Words: **snake venom; *Ophiophagus hannah*; King Cobra; fibrinolytic factor; thrombolytic factor.**

Thrombosis or occlusion of a blood vessel is a major killer throughout the world (1). The classical use of heparin, urokinase, streptokinase in the management of thrombosis is expensive and possess side effects. Antiplatelet drugs (ticlopidine, aspirin), has become an essential part of the treatment of thrombosis (2). Increased understanding of platelet biology has revealed that platelets play a key role in acute arterial thrombosis. The platelet receptor for fibrinogen (glycoprotein receptor, GPIIb/IIIa) is the chief receptor responsible for platelet aggregation by its ability to find soluble fibrinogen, which forms bridges between platelets and leads ultimately to thrombus formation.

Natural peptides derived from snake venoms have demonstrated the ability to block the GPIIb/IIIa receptor and prevent aggregation. These include trigramin from the snake *Trimeresurus graminues,* bitistatin from *Bitis arietans* and kistrin from pit viper *Agkistrodon rhodostoma.* But serious adverse effects of these peptides of disintegrin family have severely limited their potential use as therapeutic agents (2). Recently, several compounds possessing fibrinolytic activity and clot lytic property have been isolated from snake venom (3, 4). This initiated the possible search for snake venom compounds in the therapeutic management of thrombosis. The present study reports the isolation, purification, and characterisation of a novel fibrinolytic peptide from the venom of the Indian King Cobra (*Ophiophagus hannah*), the largest venomous land snake, mainly found in the northeastern hilly regions of India (5).

MATERIALS AND METHODS

Isolation and Purification

Thin-layer chromatography (TLC). Lyophilised venom of Indian King Cobra (*Ophiophagus hannah*) was obtained from Calcutta Snake Park, India. A 10 mg/ml solution was made in distilled water. Thin-layer chromatography of King Cobra venom (KCV) was done in preactivated glass plates (20 × 10 cm) coated with silica gel G (type 60), using solvent system isopropanol:0.1 N HCl (7:3, v/v). Spots were visualised in (a) UV (254 nm) chamber (b) iodine vapour (c) 0.1% ninhydrin in acetone. The R_f value was calculated.

High-performance liquid chromatography (HPLC). The active compound was purified further on reverse-phase HPLC (RP-HPLC) using a Waters 486 system using a Nova-Pak C_{18} column (60 Å, 4 μm, 3.9 × 150 mm) using solvent system methanol:water (60:40, v/v). The TLC purified active compound was passed through a Millipore filter (0.4 μm) and applied to RP-HPLC column.

Mass spectrometry. Electron impact mass spectra (EIMS) of the compound was done in Jeol A_x 500 spectrophotometer using spectral methanol.

Amino acid sequence analysis. The N-terminal sequences of the isolated peak fraction from HPLC column was carried out by automated Edman degradation with a gas phase protein sequencer Model 476A (Applied Biosystem Co) with an online phenylthiohydantoin (PTH)-derivative analyzer. Sample hydrolysis, derivatization, chromatography, and data analysis were carried out by the instrument as mentioned in the instruction manual.

Defibrinogenating Activity

Defibrinogenating activity was assayed according to Theakston and Reid (6) Male albino mice (20 ± 2 g) were injected (iv) with the

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compound/saline for experimental/control groups. After an hour, blood was collected in test tubes (75 × 12 mm) by puncturing retro-orbital plexus and the clotting time was recorded. The minimum defibrinogenating dose (MDD) was defined as the minimum amount of venom which, when injected iv into male albino mice produced incoagulable blood 1 hr later (6).

Fibrinolytic Activity and Fibrinogenolytic Activity

Fibrinolytic activity was measured by using the fibrin plate technique of Astrup and Mullertz (7) 166 mg of fibrinogen in 10 ml 0.07 M ammonium sulphate solution was taken in a petri dish. Then 0.2 ml of bovine thrombin solution (120 U/ml) was added on it and kept at room temperature for 2 hrs 0.02 ml sample was added on it and incubated at 37 ± 1°C for 20 min. Diameter of lysed zones were measured with the help of millimeter scale and photograph was taken.

Fibrinogenolytic activity was estimated according to the method of Ware *et al.* (8). To 2.5 ml of fibrinogen solution in a test tube 0.5 ml of sample was added and kept at 37 ± 1°C for 1 hr. To this bovine thrombin (120 U/ml) was added and allowed to stand at 37 ± 1°C for 30 min. The whole mixture was centrifuged at 2000 rpm for 20 min. The fibrin clot was removed with a glass rod, blotted on filter paper and washed with phosphate buffer saline (pH 7.4). The fibrin clot was poured in a tube containing 1 ml 10% NaOH and boiled for 30 min. Then 2.5 ml distilled water, 1.5 ml 20% Na_2CO_3 and 0.5 ml phenol reagent was added and mixed. It was allowed to stand for 20 min and the OD measured at 470 nm against blank.

Plasma Recalcification

The minimum clotting dose of plasma (MCDP) was determined as described by Theakston and Reid with some modification. Goat plasma (0.2 ml) was incubated in a water bath at 37 ± 1°C. To each tube 0.1 ml of the compound in different concentrations was added (0.1 ml saline in control). Finally 0.1 ml of 25 mM of $CaCl_2$ was added and clotting time recorded. The minimum clotting dose of plasma (MCDP) induced by venom was defined as the least amount of venom that produced clot of standard citrated solution of plasma in 60 sec at 37 ± 1°C [(Theakston and Reid (6)].

Hemorrhagic Activity

Hemorrhagic activity was tested on shaved albino mice (20 ± 2 g) by modified method of Kondo (9). The minimum hemorrhagic dose (MHD) of venom is defined as the least amount of venom which when injected intradermally (id) into mice result in a hemorrhagic lesion of 10 mm diameter 24 hrs later (6).

Hemolytic Activity

Human, goat, mice erythrocytes were used to determine the hemolytic activity. Erythrocytes were washed with saline (0.9%) by centrifugation (2000 rpm) for 10 minutes. After repeated washings with saline a 1% cell suspension was prepared. The compound was mixed with 1% cell suspension in saline and the mixture was incubated at 37 ± 1°C for 60 min. Reaction was stopped by adding 3 ml of chilled phosphate buffer saline (PBS). The tubes were centrifuged at 2000 rpm for 10 min and absorbance of the supernatant was measured at 540 nm. Supernatant of tube treated with 3 ml distilled water was taken as 100% lysis.

Phospholipase Activity

Phospholipase activity was estimated by egg yolk coagulation method according to Habermann and Neumann (10).

All results were expressed as mean ± SEM. The significance of the difference between means was determined by Student's 't' test. P values <0.05 were considered significant.

RESULTS AND DISCUSSION

There have been various reports on the use of snake venom components in the management of thrombosis or occlusion of a blood vessel. Bitistatin (from *Bitis arietans*) and kistrin (from *Agkistrodon rhodostoma*) of the disintegrin family have demonstrated the ability to inhibit platelet aggregation and subsequent thrombosis in canine models when administered with heparin (2). Recently there has also been a number of investigations towards using snake venom fibrinolytic enzymes to combat thrombosis. Some of them are atroxase from *Crotolus atrox* (3), lebetase from *Vipera lebetina* (4) and basilase from *Crotolus basiliscus basiliscus* (11).

Fibrinolytic activity has been detected in a number of snake species within crotolid and viperid families. One α-fibrin(ogen)olytic enzyme has been reputed from elapidae venom; α-fibrin(ogen)ase proteinase F1 from *Naja nigricollis* venom (12).

In this study a novel fibrinolytic peptide has been reported from Indian King Cobra (*Ophiophagus hannah*) venom. Previous studies in our laboratory have revealed that Indian King Cobra (*Ophiophagus hannah*) is a complex mixture of several bioactive compounds (13). Studies with crude venom have indicated the presence of a defibrinogenating factor in the venom.

Isolation and Purification of Peptide from Indian King Cobra (Ophiophagus hannah)

Thin layer chromatography of KCV produced a single spot having yellow fluorescent at 254 nm. In iodine, the spot appeared yellow in colour and in ninhydrin it appeared purple in colour. The R_f of the spot was 0.48. Rechromatography of this spot in the same solvent/detection system produced a single spot (R_f 0.48). This purification process produced 0.04% yield of the active compound.

For further purification of the compound preparative TLC was performed. The peptide isolated by TLC was scraped and eluted in distilled water. It was subsequently passed through a Millipore fitter (0.4 μm). The peptide was further purified on reverse-phase high performance liquid chromatography (RP-HPLC). Figure 1 shows the elution pattern of the peptide on RP-HPLC. The elution pattern shows a single sharp peak, with a retention time of 2.23 min. The HPLC purified compound was provisionally named "Hannahpep" derived from the name, *Ophiophagus hannah.*

Characterization of Hannahpep

Electron impact mass spectra (EIMS) of the peptide showed M^+ at *m/z* 610 (Fig. 2). The molecular weight

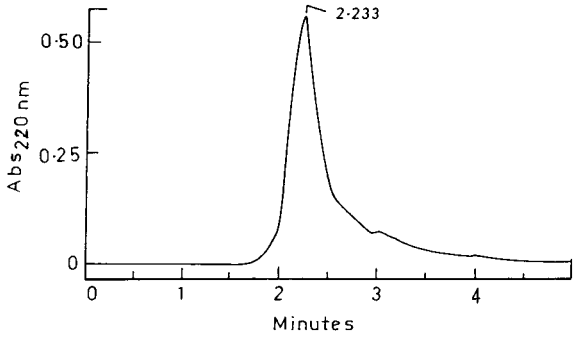

FIG. 1. HPLC purification of Hannahpep on reverse-phase C_{18} column. The column was eluted by an isocratic gradient of methanol: water (60:40, v/v).

revealed that it contained approximately 6 residues taking the molecular weight of one amino acid as 100. The N-terminal amino acid sequence of the purified Hannah peptide, was determined by automatic Edman degradation using an Applied Biosystems pulsed liquid sequencer equipped with an online 120A PTH amino acid analyzer. Hannah peptide was a 6-residue peptide. The N-terminal sequence was as follows: Arg, His, Ala, Arg, His, Asp (Fig. 3). Peptides like mellitin F and Secapin isolated from honey bee have been reported to contain 19 and 24 residues respectively (14). Even Apamin a neurotoxic peptide from bee venom have been reported to contain 18 amino acids (15). Recently, a novel peptide of 11 amino acids with the ability to contract rat stomach fundus was isolated from *Agkistrodon halys blomhoffii* (16). It is for the first time that such a small peptide has been reported from snake venom.

Biological Activity

Hannahpep (2.5 μg) produced defibrinogenation in male albino mice. HP (2.5 μg/20 g) was injected intravenously to a group of 6 mice. For comparison 0.1 ml of 0.9%. NaCl was also injected in control mice (n = 6). After 1 hr blood was collected from retro-orbital plexus

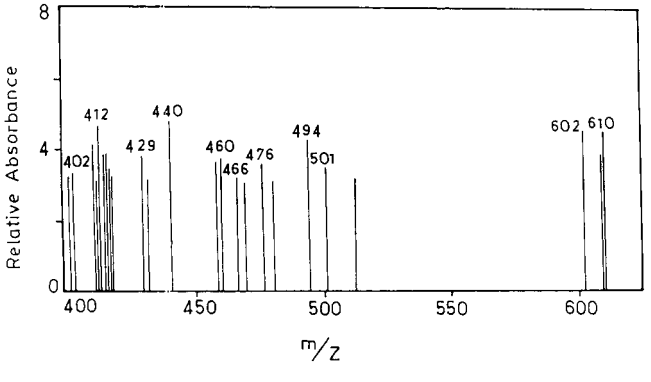

FIG. 2. Mass spectra of Hannahpep.

FIG. 3. Sequence analysis of Hannahpep. N-terminal amino acid sequence was determined by automatic gas-phase Edman degradation (→).

of mice and clotting time was recorded. Compared to control where blood clotted within 57.5 ± 1.78 sec, blood from Hannahpep injected mice produced non-clotted blood (observed up to 6 hr).

Hannahpep (2 μg) exhibited significant fibrinolytic activity (Fig. 4). It produced halos (10.6 ± 0.33 mm, diameter) on fibrin plates as compared to streptokinase (Sigma, U.S.A.), which produced halos (10.8 ± 0.33 mm). Hannahpep (2 μg) also produced 67.14 ± 0.63% fibrinogenolytic activity as compared to 100% streptokinase induced fibrinogenolytic activity.

In plasma recalcification, the minimum clotting dose of plasma for Hannahpep was 2 μg. In the presence of Ca^{2+} Hannahpep increased the plasma recalcification time significantly. However, Hannahpep did not exhibit any hemorrhagic, hemolytic or phospholipase activity.

Such unique property of Hannahpep indicates its possible use as a therapeutic agent in thrombosis or occlusion of a blood vessel. Thrombosis within the coronary arteries involves activation of both platelets and the coagulation system (1). Thrombolytic drugs such as streptokinase, urokinase, staphylokinase are currently used in curing such diseased conditions. However, isolation of these enzymes is expensive and time consum-

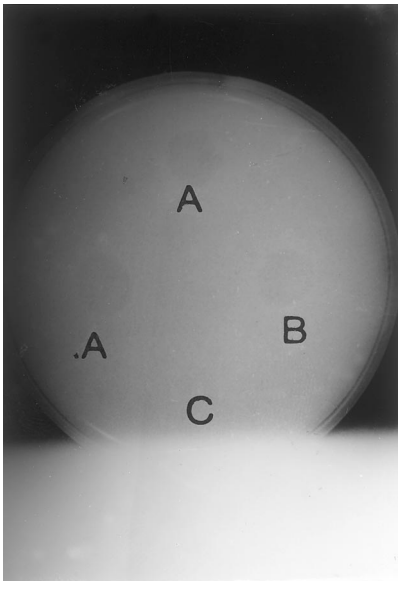

FIG. 4. Fibrinolytic activity of Hannahpep. A, Hannahpep (2 μg). B, Streptokinase. C, Control (0.9% saline).

ing. On the other hand the natural peptides of disintegrin family derived from snake venom that are GP IIb/IIIa antagonist, and inhibit platelet aggregation have serious side effects. They tend to induce thrombocytopenia and are highly antigenic, capable of generating an immune response. These side effects limit the potential use of disintegrins as therapeutic agents. Hannahpep has been found to be a poor antigen. Attempt to raise antiserum against it in rabbits had been a failure, probably because of its low molecular weight (unpublished data). Hannahpep has been found to have no significant effect on platelet aggregation (unpublished data). This indicated that Hannahpep act mainly on the blood coagulation system to induce clot lytic effect. Hannahpep with its simple purification process pose as a promising compound with fibrinolytic activity.

In conclusion, it may be stated that a novel, six-residue peptide with fibrinolytic activity has been isolated from the Indian King Cobra venom. It is the first time probably that such a small peptide with fibrinolytic property has been reported from snake venom. Further, structural analysis of the peptide will help to enlighten its possible use as a therapeutic agent.

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