Arch Pharm Res Vol 29, No 5, 418-423, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Purification and Characterization of Anticoagulant Protein from the Tabanus, *Tabanus bivittatus*

Mi Young Ahn, Bum Soo Hahn[1], Pyeong Jae Lee, Song Ji Wu[2], and Yeong Shik Kim[2]

Department of Agricultural Biology, National Institute of Agricultural Science and Technology, Suwon 441-100, Korea, [1]Department of Molecular Physiology and Biochemistry, National Institute of Agricultural Biotechnology, Suwon 441-707, Korea, and [2]Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 110-460, Korea

(Received December 5, 2005)

Tabanus anticoagulant protein (TAP) was isolated from the whole body of the tabanus, *Tabanus bivittatus*, using three purification steps (ammonium sulfate fractionation, gel filtration on Bio-Gel P-60, and ion exchange chromatography on DEAE Sephadex gel). The purified TAP, with a molecular weight of 65 kDa, was assessed to be homogeneous by SDS-polyacrylamide gel electrophoresis, and an isoelectric point of 7.9 was determined by isoelectric focusing. The internal amino acid sequence of the purified protein was composed of Ser-Leu-Asn-Asn-Gln-Phe-Ala-Ser-Phe-Ile-Asp-Lys-Val-Arg. The protein was activated by Cu^{2+} and Zn^{2+}, and the optimal conditions were found to be at pH 3~6 and 40~70°C. Standard coagulation screen assays were used to determine thrombin time and activated partial thromboplastin time. Chromogenic substrate assays were performed for thrombin and factor Xa activity. TAP considerably prolonged human plasma clotting time, especially activated partial thromboplastin time in a dose-dependent manner; it showed potent and specific antithrombin activity in the chromogenic substrate assay. Specific anti-factor Xa activity in TAP was not detected. Overall, this result suggested that TAP has significant anticoagulant activity on blood coagulation system.

Key words: Anticoagulant activity, Purification, *Tabanus bivittatus*

INTRODUTION

Haematophagous arthropods have a major economic impact on cattle production worldwide. By sucking blood, they produce wounds in the skin; at the site of injury, they inject saliva containing various bioactive substances (Kazimírová *et al.*, 2002). The pharmacologically active substances in their salivary glands include inhibitors of blood coagulation and platelet aggregation, as well as vasodilatory and immunosuppressive substances (Ribeiro *et al.*, 1989).

The incidence of thrombotic disorders, including cerebral stroke, myocardial infarction, and venous thromboembolism, are rapidly increasing throughout the world. During the past decade, treatments for thrombotic and vascular disorders have been developed with the intention of improving therapeutic efficacy and safety (Ouriel, 2002). Potent antithrombin activity in the salivary glands of tabanids was noted during the discovery of tabanin, a thrombin inhibitor from *Tabanus bovinus* (Markwardt & Leberecht 1959; Markwardt & Schulz 1960). A platelet aggregation inhibitor and a potent glycoprotein IIb/IIIa fibrinogen receptor antagonist were recently isolated from the deerfly *Chrysops* species (Grevelink *et al.*, 1993; Reddy *et al.*, 2000).

According to our previous report, the water extract of *Tabanus bivittatus* (Diptera: Tabanidae) was as potent as other crude animal drugs, having strong effects on blood coagulation and showing cytotoxicity against cancer cells (Ahn *et al.*, 2000; Ahn *et al.*, 2002).

Until recently, there has been a lack of research on the anticoagulant proteins of *Tabanus bivittatus* and other related insects. Our study was designed to characterize a new anticoagulant protein of the coagulation system from the whole body of the tabanus through the evaluation of its thrombin inhibitor.

Correspondence to: Mi Young Ahn, Department of Agricultural Biology, National Institute of Agricultural Science and Technology, 61 Sudun-Dong, Kwonsun-gu, Suwon 441-100, Korea
Phone: 82-31-290-8577, Fax: 82-31-290-8543
E-mail: amy@rda.go.kr

MATERIALS AND METHODS

Chemicals

Tabanus bivittatus was purchased at a local market in Beijing, China. Bio-Gel P-60 gel was obtained from Bio-Rad (Hercules, CA). DEAE Sephadex A-25, Human thrombin, factor Xa from bovine plasma and *N*-ρ-tosyl-glycyl-propyl-arginine-ρ-nitroanilide were purchased from Sigma Chemicals (St. Louis, MO). Chromozym® X was purchased from Roche (Mannheim, Germany).

Purification of the anticoagulant protein, TAP

TAP was isolated by a combination of ammonium sulfate fractionation, gel filtration, and anion exchange chromatography. *Tabanus bivittatus* (500 g) was suspended in 500 mL of 40 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, and any insoluble material was removed by centrifugation at 8,000×g for 30 min at 4°C. Up to 60% of the supernatant was added with ammonium sulfate, after which it was centrifuged and decanted away. Then, up to 90% was added with ammonium sulfate, and the supernatant was centrifuged and removed. The precipitate was suspended in 15 mL of 40 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. The resulting solution was applied on a Bio-Gel P-60 column (80×2.5 cm) equilibrated with 40 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.4) at 4°C, and the column was eluted with the same buffer at a flow rate of 20 mL/h. Fractions showing optimal anticoagulant (aPTT/TT) activity were pooled. The sample was concentrated using a DIAFLO UM 10 from Amicon (Beverly, CA) and then loaded onto a DEAE Sephadex A-25 gel chromatography column (38×1.2 cm) equilibrated with the 50 mM phosphate buffer (pH 7.4). The non-interacting solutes were washed from the column with the equilibration buffer. The bound fractions were eluted using a linear sodium chloride gradient from 0 to 2.5 M NaCl in phosphate buffer (pH 7.4) at a flow rate of 20 mL/h. Fractions showing maximal aPTT activity and thrombin time inhibition were pooled and concentrated as previously described. Desalting and molecular sieving were done using an TSK G3000 SW (7.5 mm×30 cm)-gel column (Phenomenex, Torrance, U.S.A.). The concentrated fraction was eluted using an isocratic HPLC equipment consisted of a Thermo Spectra Products (San Jose, U.S.A.) liquid chromatographic system (UV 3000 photodiode UV-Vis detector) in 30 min from 100% water, flow-rate 0.8 ml/min. Fractions were collected by peaks in Fig. IC and freezing dried, and major peak (Fr I) used as TAP (test sample).

Measurement of clotting time and protein determination

Human plasma from the Blood Bank of Seoul National University Hospital was used for measuring clotting time in both activated partial thromboplastin time (aPTT) and thrombin time (TT). The clotting time tests were performed on a Beckton Dickenson BBL Fibrosystem (Cockeysville, U.S.A.). In brief, a mixture containing 40 μL of test solution and 80 μL of prewarmed plasma for one minute was incubated for three minutes under stirring, then dropped into 0.02 M calcium chloride solution at 37°C. When the clot was formed, aPTT was measured. For the measurement of TT, an equal volume of thrombin (10 U/mL) and test insect fractions were mixed and incubated at 37°C for 5 minutes. The 50 μL of reaction mixture was added to 250 μL of pre-warmed fibrinogen and the clotting time was determined (Astrup and Mullertz, 1952). Protein concentration was determined by Bradford method (Bradford, 1976).

Determination of molecular weight and isoelectric point of TAP

SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970) using a 4% stacking and 12% resolving polyacrylamide gel. Isoelectric focusing was performed using a Bio-Rad's Model 111 Mini IEF Cell (Hercules, CA) according to manufacturer's procedure (Ahn *et al.*, 2003). Gels were visualized with either Coomassie Brilliant Blue R250 or silverstain.

Determination of internal amino acid sequence of TAP

The internal amino acid sequence of purified TAP was determined using an Applied Biosystems Precise clc492 protein sequencer at the Korea Basic Science Center in Seoul.

Effects of divalent metal cations and inhibitors

TAP (1 μg) was preincubated with 5 mM of Ca^{2+}, Ba^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}, 5 mM EDTA and 5 mM EGTA at 37°C for 2 h. The residual enzyme activity was determined measuring clotting time, aPTT (Grevelink *et al.*, 1993).

Effects of pH and temperature

The optimal pH for clotting time, aPTT was determined by varying the pH of the reaction mixture between 3 to 11. TAP (1 μg) was dissolved in either citrate buffer (50 mM, pH 3-6), Tris-HCl buffer (50 mM, pH 7-9) or phosphate buffer (50 mM, pH 10-11), and incubated at 37°C for 2 h. Temperature dependency of the purified enzyme was determined under standard conditions at different temperature. The protein (1 μg) was incubated at each temperature for 15 min after which its clotting time was determined using the coagulation assay (aPTT) as described above.

Chromogenic substrate assays for thrombin and factor Xa

The assays were adapted to 96-well microtitre plates. For screening of antithrombin activity, 190 μL of Tris buffer (50 mM Tris, 227 mM NaCl, pH 8.3, containing 0.1% BSA and 0.1% sodium azide), 10 μL of Tris buffer containing 0.056 U thrombin from bovine plasma (Sigma, Steinheim, Germany), 10 μL of 150 mM NaCl (control) or 10 μL of TAP (6 μg protein) were pipetted into a well. After a 10-min incubation at room temperature (RT), 20 μL of 1.9 mM *N*-p-tosyl-glycyl-prolyl-arginine-p-nitroanilide, acetate salt (Sigma) in redistilled water were added to each well. For screening of antifactor Xa activity, 190 μL of Tris buffer (50 mM Tris, 150 mM NaCl, pH 8.3, containing 0.1% BSA and 0.1% sodium azide), 20 μL of Tris buffer containing 0.05 U factor Xa from bovine plasma (Sigma) and 10 μL of 150 mM NaCl (control) or 10 μL of TAP (6 μg protein) were pipetted into a well and incubated at RT for 10 min. Twenty μL of 2 mM Chromozym® X (*N*-methyoxycarbonyl-D-norleucyl-Gly-L-Arg-p-nitroanlilde acetate) (Roche, Mannheim, Germany) in redistilled water were then added. After addition of the substrates, the absorbance was read at 405 nm for 30 min at 2 min intervals using an ELISA reader. Substrate hydrolysis by bovine thrombin or factor Xa was monitored in two parallel samples and expressed as the rate of change in absorbance at 405 nm.

RESULTS

Purification

The isolation of TAP was achieved by a combination of three purification steps. The crude extract containing 976.6 mg of protein showed a specific activity of 81.0 sec/mg. Ammonium sulfate fractionation at between 60 to 90% gave maximum anticoagulant activity. Gel-filtration chromatography of the 60-90% ammonium sulfate fraction on Bio-Gel P-60 could yield a fraction (V, NO. 91-93) with the anticoagulant activity increased approximately four times (Fig. 1A). The second step in the purification was accomplished using DEAE Sephadex A-25 chromatography with a linear sodium chloride gradient from 0 M to 2.5 M.

Three bound fractions were observed (Fig. 1B). A fraction (II, NO. 43-44) had anticoagulant activity in aPTT. The total protein of TAM in the second bound fraction (II) was about 0.97 mg, with 556 sec/μg anticoagulant activity in aPTT and a specific activity of 92.3 sec/μg in TT as a substrate. These results are shown in Table I. TAP was found to be homogeneous by SDS-PAGE with a molecular weight of 65,000 Da (Fig. 2), and the same result was obtained by gel filtration chromatography on TSK-3000 SW using water as an eluant (Fig. 1C), indicating the monomeric structure of the TAP (Fr I). TAP has an isoelectric

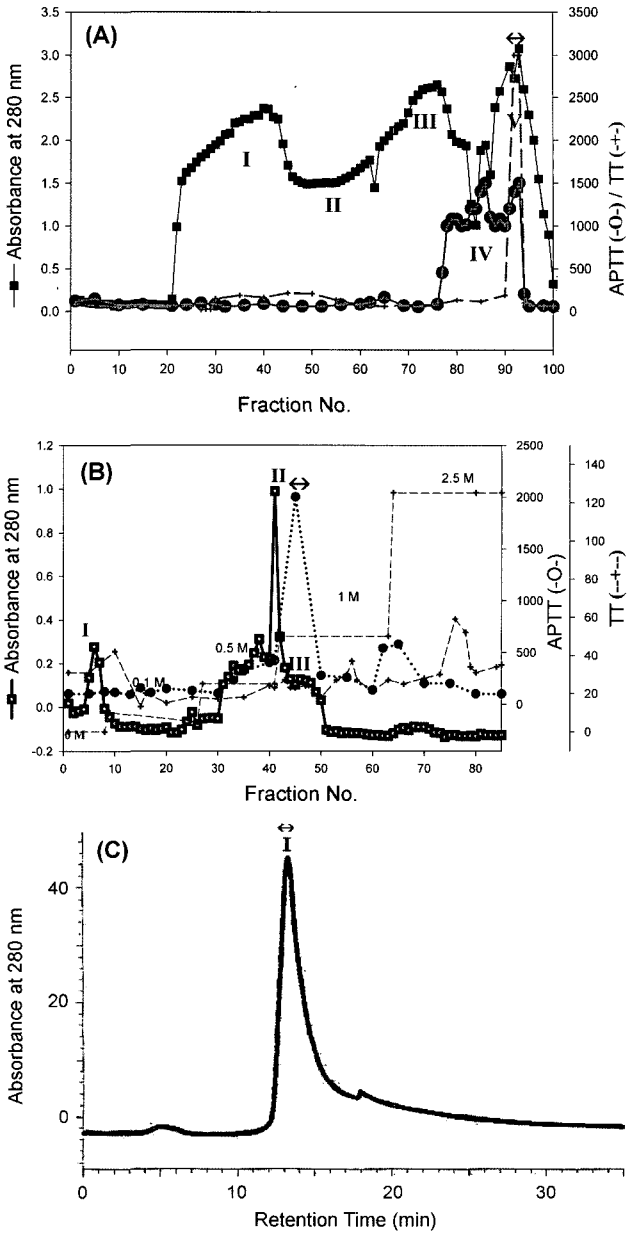

Fig. 1. Fractionation of tabanus extract. Purification of anticoagulant protein by gel chromatography. Fractionation of tabanus (*Tabanus bivittatus*) extract. Purification of TAP was accomplished by using (A) Bio-Gel P-60, (B) DEAE Sephadex A-25 gel. (A), The elution was performed with 50 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl at a flow rate of 20 mL/h. The elution profile was monitored by reading the absorbance at 280 nm (-■-). Anticoagulant activity, based on aPTT/thrombin time assay, was shown as (-●-)/(-+-) dotted line and the active fractions were pooled (↔). (B), About eleven milligrams of material pooled from the previous step was applied to the column. Elution (50 mM phosphate buffer, pH 7.4) was performed with a linear salt gradient from 0 M to 2.5 M at a flow rate of 20 mL/hr. The elution profiles were monitored by spectrophotometry at A_{280} (-■-). Anticoagulant (aPTT/thrombin time) assay was shown as (-●-)/(-+-) dotted line and the active fractions were pooled (↔). (C), Gel filtration chromatography on TSK-3000 SW using water as an eluant. The purified fraction (I: TAP) was pooled.

Table I. Purification of Tabanus anicoagulant protein (TAP) from *Tabanus bivittatus*

Purification step	Protein (mg)	aPTT (sec)[1)]	Yield	Purification fold
Crude extract	976.60	81	100	1
Ammonium sulfate Fractionation (60-90%)	61.81	251	20	3
Bio-Gel P-60	4.74	347	2	4
DEAE Sephadex Gel	0.97	556	1	7

[1)]aPTT, activated parrtial thromboplastin time.

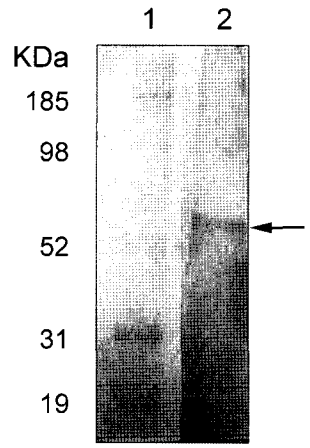

Fig. 2. (A) Silver stained SDS-PAGE analysis of DEAE Sephadex A-25 fractions for TAP under denaturing conditions using a 4% stacking and 12% resolving polyacrylamide gel. *Lane 1*, mixture of marker proteins; *lane 2*, TAP (TSK Fr I).

point of 7.9, as determined by isoelectric focusing (data not shown).

Effects of the anticoagulant activity on pH and temperature

The optimum pH for anticoagulant (aPTT) activity was found to be 5.0, and the protein was stable within the pH range of 3-6 (Fig. 3). The optimum temperature was 40°C, and the enzyme activity was stable between 10°C to 60°C (data not shown).

Effect of divalent metal ions

The effects of various divalent metal ions on the purified TAP are shown in Table II. Cu^{2+} and Zn^{2+} also activated the anticoagulant activity of TAP by 89% and 49%, respectively. As seen in most anticoagulant insect proteins, TAP was unaffected by metallic cofactors, such as calcium ions, magnesium ions, barium ions, and copper ions (Table II).

Chromogenic substrate assay

Incubation of bovine factor Xa with its chromogenic substrate, Chromozym® X, resulted in an increase in absorbance at 405 nm. The reaction was not inhibited by TAP, and reaction curves of substrate hydrolysis were similar to the control (Fig. 4A).

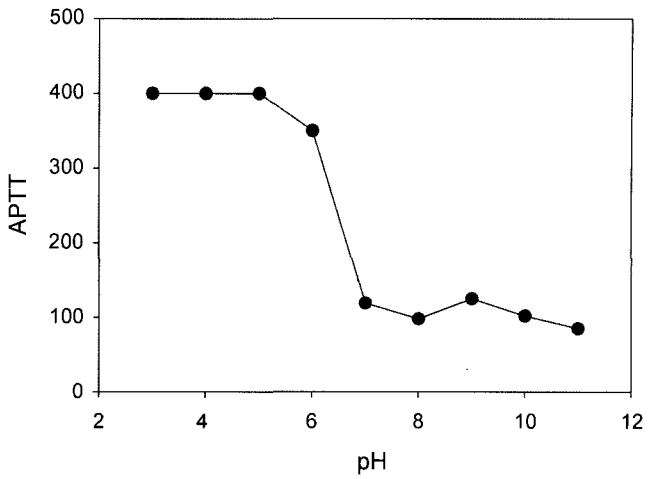

Fig. 3. Optimal pH level of TAP. TAP (1 μg) was incubated at each pH buffer solutions for 15 min after which its clotting time was determined using the coagulation assay (aPTT).

Table II. Effect of the divalent cations on aPTT activity of TAP[1)]

Divalent	Cation concentration (mM)	aPTT (sec.)
None		84.7±19.0
$CaCl_2$	5	90.2± 2.7
$MgCl_2$	5	81.5± 2.8
$BaCl_2$	5	90.2± 2.7
$CuCl_2$	5	600.7±27.3
$ZnCl_2$	5	1050.0± 7.7
$MnCl_2$	5	83.6± 4.1

[1)]A mixture containing 40 μL of TAP (1 μg) solution and 80 μL of prewarmed plasma for one minute was incubated for three minutes under stirring, then dropped into 0.02 M calcium chloride solution at 37°C. When the clot was formed, aPTT was measured. Values represent the mean and standard deviation of triplicate experiment

The chromogenic substrate assay for thrombin confirmed specific antithrombin activity in the investigation of TAP (Fig. 4B), although substrate hydrolysis was reached to acme by TAP for approximately 5 min. Inhibition activity of TSK Fr I (TAP) against thrombin was marked 37.7% and DEAE Sepha Fr II was 27.4% compared to the control.

Determination of the internal amino acid sequence of TAP

The internal amino acid sequence of TAP was determined to be Ser-Leu-Asn-Asn-Gln-Phe-Ala-Ser-Phe-Ile-Asp-Lys-Val-Arg, and was compared with other sequences in the protein databases using the BLAST program (NCBI, Bethesda, MD). The enzyme was not homologous to any

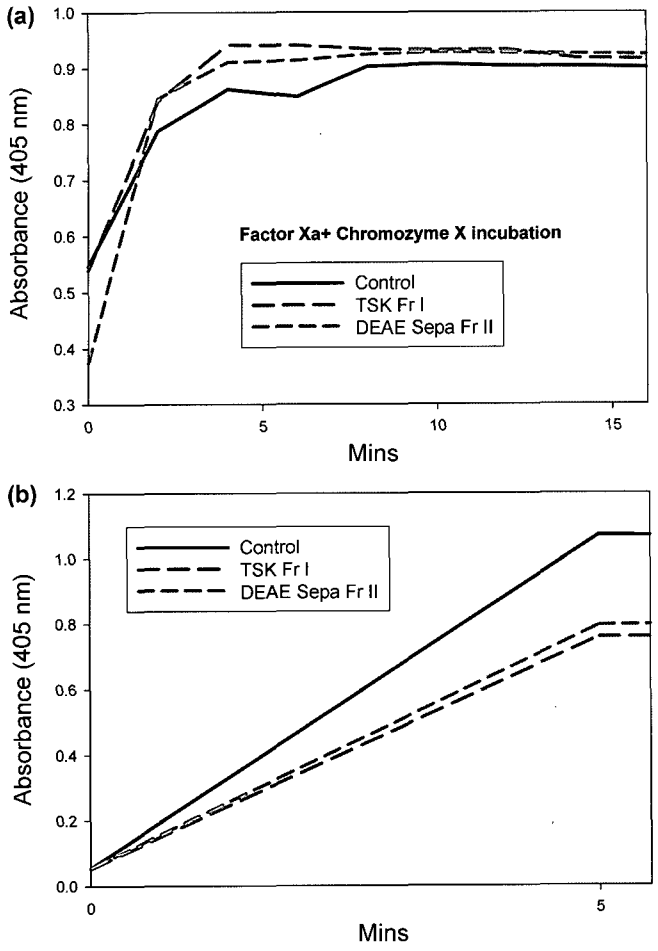

Fig. 4. Reaction curves showing the substrate hydrolysis. (a) by factor Xa (0.05 U/well, substrate 2 mM Chromozym® × 12 μL/well) (b) by human thrombin (0.056 U/well, substrate 1.9 mM *N*-p-tosyl-glycyl-propyl-arginine-p-nitroanilide-acetate 20 μL/well), affected by TAP (TSK Frl) 3 μg protein/well and partial purified TAP (DEAE sepa FrII, 3 μg protein/well). Control = 0.15 M NaCl.

other insect proteins.

DISCUSSION

A tabanus anticoagulant protein (TAP) was purified from *Tabanus bivittatus* in three chromatographic steps (Table I). The final recovery was about 1%, and its purification factor increased by 7-fold. TAP has a molecular weight of about 65 kDa and shows a monomeric enzyme with a pI of 7.9.

TAP was insensitive to most typical serine protease inhibitors, including TLCK, soybean trypsin inhibitor, aprotinin, benzamidine (data not shown), and metalloprotease inhibitors (EDTA and EGTA).

The specific activity of TAP on aPTT (556 sec/μg) was higher than that of control (68 sec/μg). The purity of TAP was demonstrated using SDS-PAGE and TSK-HPLC. There have been comparatively few studies on the anticoagulant enzyme of tabanid, antithrombin, molecular weight of approximately 7-kD peptide named tabanin (Markwardt, 1994) with similarities to the mode of action of the leech anticoagulant, hirudin and partially purified (one step RP-HPLC) horsefly salivary gland protein, molecular weight of 10-60 kDa (Kazimý´rova´ *et al.*, 2001). In this report, we found and purified a new anticoagulant protein with a molecular weight of 65 kDa, 14 internal amino acid sequences that were not matched by the BLAST program (NCBI, Bethesda, MD), and showing a selective anticoagulation activity. Anticoagualnts only revealed strong anticlotting and antithrombin activities in the thrombin time (TT) activities that have been studied in subfamily of Tabaninae using specific chromogenic substrate assay (Kazimírová *et al.*, 2002). We purified the protein showing inhibition of clot formation in aPTT assay and having target factors in the coagulation pathway related thrombin (not related factor Xa). It may be related to inhibition of prothrombin activation to thrombin.

With the above data, TAP was identified as a naturally occurring candidate for anticoagulant therapy. Further studies on the physiological function of TAP, as well as *in vivo* thrombin inhibition, are required.

REFERENCES

Ahn, M. Y., Ryu, K. S., Lee, Y. W., and Kim, Y.S., Cytotoxicity and L-amino acid oxidase activity crude insect drugs. *Arch. Pharm. Res.*, 5, 477-481 (2000).

Ahn, M. Y., Hahn, B. S., Ryu, K. S., and Cho, S. I., Effects of insect crude drugs on blood coagulation and fibrinolysis system. *Natural Product Sciences*, 8, 66-70 (2002).

Ahn, M. Y., Hahn, B. S., Ryu, K. S., Kim, J. W., Kim, I., and Kim, Y. S., Purification and characterization of a serine protease with fibrinolytic activity from dung beetles, *Catharsius molossus*, *Thrombosis Research*, 112, 339-347 (2003).

Astrup, T. and Mullertz, S., The fibrin plate method for estimating of fibrinolytic activity. *Arch Biochem Biophys*, 40, 346-351 (1952).

Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72, 248-254 (1976).

Grevelink, S. A., Youssef, D. E., Loscalzo, J., and Lerner, E. A., Salivary gland extracts from the deerfly contain a potent inhibitor of platelet aggregation. *PNAS*, 90, 9155-9158 (1993).

Kazimírová, M., Sŭlanová, M., Trimnell, A. R., Kozánek, M., Vidlička, L., Labuda M., and Nuttall, P. A., Anticoagulant activities in salivary glands of tabanid flies. *Med. Vet. Entomol.*, 16, 301-309 (2002).

Kazimírová, M., Sţulanová, M., Kozánek, M., Takáč, P.,

Labuda, M., and Nuttall, P. A., Identification of Anticoagulant Activities in Salivary Gland Extracts of Four Horsefly Species (Diptera, Tabanidae). *Haemostasis*, 31, 294-305 (2001)

Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685 (1970).

Markwardt, F. and Leberecht, E., Untersuchungen über den blutgerinnung-shemmenden Wirkstoff der Tabaniden. *Naturwissenschaften*, 46, 17-18 (1959).

Markwardt, F. and Schulz, E., Über den Mechanismus der blutgerinnungshemmenden Wirkung des Tabanins. *Naunyn-Schmiedeberg's Archiv für Experimentelle Pathologie Undpharmakologie*, 238, 320-328 (1960).

Markwardt, F., Coagulation inhibitors from animals feeding on blood. *Rev Iberoamer Tromb Hemost*, 7, 225-231 (1994).

Ouriel, K., Current status of thrombolysis for peripheral arterial occlusive disease. *Ann Vas Surg*, 16, 797-804 (2002).

Reddy, V. B., Kounga, K., Mariano, F., and Lerner, E. A., Chrysoptin is a potent glycoprotein IIb/IIIa fibrinogen receptor antagonist present in salivary gland extracts of the deerfly. *J. Biol. Chem.*, 275, 15861-15867(2000).

Ribeiro, J. M. C., Vachereau, A., Modi, G. B., and Tesh, R. B., A novel vasodilatory peptide from the salivary glands of the sand fly *Lutzomyia longipalpis*. *Science*, 243, 212-214 (1989).