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Effects of Insect Crude Drugs on Blood Coagulation and Fibrinolysis System

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Abstract - The *in vitro* anticoagulant and fibrinolytic activities of crude extracts from insects were evaluated in order to find effective therapeutic drugs for the treatment of myocardial and cerebral thrombosis. We prepared three types of extracts (water, methanol and ethylacetate) from 28 insects for use as raw materials for the activity assays. The fibrinolytic activity was tested using the fibrin plate method and the activated partial thromboplastin time and thrombin time were measured for blood clotting activity. With regards to the fibrinolytic system, water extracts of six kinds of insects displayed a remarkable level of activity with a plasmin-like action. The water extracts of [*Catharsius molossus, Eupolyphaga sinensis, Huechys sanguinea, Mantidis oötheca, Mimela splendens*, and *Polistes mandarinus* (Vespae Nidus)] exhibited the activity. On the other hand, the methanol extracts did not display any fibrinolytic activity. In terms of the coagulation system, an aqueous extract of silkworm Tongchunghacho (*Paecilomyces japonica*), *Oxya japonica japonica* and *Buthus martensi* (Scorpion) increased the clotting time significantly longer (181 times) than the control. These results suggest that crude drugs from insects are useful sources for the development of new drugs for use in treatments involving blood coagulation and fibrinolysis.
Keywords – insect crude drugs; inhibition; fibrinolytic and coagulation system

Introduction

Over the last ten years, thrombolytic therapies have played major roles in the early treatment of myocardial infarction. Many kinds of effective thrombolytic agents have been identified (Walker, 1985) and characterized from the vampire bat (Cartwright, 1974), snake venoms (Bajwa *et al.*, 1982), microorganisms (Fujita *et al.*, 1993; Kim *et al.*, 1996), hematophagus and non-hematophagus insects (Hellman and Hawkins, 1964; Ben Hamouda and Ammar, 1984; Amarant *et al.*, 1991; Matsushima *et al.*, 1993), leech (Chudzinsji-Tavassi *et al.*, 1998) and earthworms (Nakajima *et al.*, 1993; H rzenjak *et al.*, 1998), marking a new era in the early treatment of heart attack. However, the biochemical properties of the specific agents have not been successfully investigated.

Peptide bond cleavage is one of the most frequent and important enzymatic modifications of proteins. Recent studies of proteolytic enzymes have focused on their regulatory roles in variety of physiological processes. Among the most thoroughly studied regulatory proteases are those associated with fibrionlysis, coagulation and complement systems.

Wang , *et al.*, studied the effects of 120 kinds of oriental plant drugs and 37 kinds of animal crude drugs on blood coagulation and fibrinolytic activity (Wang, *et al.*, 1989). Very recently, Hahn, *et al.*, demonstrated the presence of a serine protease with fibrinolytic activity in the egg cases of the praying mantis, *Tenodera sinensis* (Hahn, *et al.*, 1999). We have examined insect crude drugs and insects with the aim of discovering effective therapeutic drugs for myocardial and cerebral thrombosis from naturally occurring insects.

Experimental

Materials – The crude insect drugs; *Catharsius molossus, Ciacadae periostracum, Eupolyphaga sinensis, Whitmania Pigra* (Hirudo), *Huechys sanguinea, Mantidis oötheca, Mylabris phaelerata, Formica rufa* (Red ant), *Scolopendra morsitans, Buthus martensi* (Scorpion) and Tabanus were purchased at a local market in Beijing, China. And others were supplied by the Department of Sericulture and Entomology, National Institute of Agricultural Science and Technology, Korea. Fibrinogen (bovine plasma), thrombin, plasmin and aPTT reagent were purchased from Sigma Co.

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(St. Louis, U.S.A.).

Preparation of Test Solution – Each insect crude drug (50 g) was homogenized. The samples were extracted with 50 ml of 40 mM Tris-HCl (pH 7.4) for the water extracts. The other homogenized materials were soaked with methanol and ethyl acetate and were extracted using the respective solvent for the methanol and ethyl acetate extracts. The samples were filtered with Whatman paper and dried using a Hanil vacuum concentration system (Seoul, Korea) or concentrated by evaporating. Each sample (10 mg) was dissolved in 500 μl of PBS buffer (final concentration 0.5% ethanol or 0.5% DMSO) as a test solution.

Biological assays

Fibrin plate assay – The mixture of 10 ml of 0.8% (w/v) of bovine fibrinogen solution and 5 NIH units of human thrombin was poured into petri dishes and left for 30 min at room temperature. Insect fractions were spotted onto the plate and incubated at 37°C overnight. Fibrinolytic activity was assessed on measuring the lysis zone. The procedure was repeated at least three times. The value of fibrinolytic activity (unit) was calculated based on plasmin activity (Thompson, 1983)

Proteolytic activity assay – Proteolytic activity was measured using azocasein as a substrate according to a described method (Beynon and Kay, 1978). The reaction mixture, composed of 1 ml of azocasein (2 mg/ml in 0.2 M sodium borate buffer, pH 7.8) and 1 μg of sample, was incubated at 37°C. After 1 hr, 0.25 ml of the mixture was transferred to a 1.5 ml tube containing 1 ml of 5% (w/v) trichloroacetic acid and mixed. The tubes were then centrifuged at 11,000×g for 5 min and the absorbance of the supernatant was measured at 340 nm.

Effects on coagulation systems – 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

Table 1. Screening of Insect Crude Drugs for Activities on Blood Coagulation and Fibrinolysis System

Insect name (Korean name)	Insect source	Extract[a]	Fibrinolysis (U/mg)[b]	Proteolytic Activity (U/mg)[c]	APTT (sec.)[d]	TT (sec.)[e]
Control (saline)			–		68	55
Agrius convolvuli Larvae (bakaksi-nabang Yuchung)	*Agrius convolvuli* LINNAEUS	W	1.0	2.4	133	49
		M	–		56	66
		E	0.1		58	52
Apriona germari Larvae (Boyngnamu-hanlso Yuchung)	*Apriona germari* HOPE	W	–	8.7	86	62
		M	–		170	75
		E	–		47	67
Bumble bee female(Worker) (Hobakbul Ilbul)	*Bombus ignitus* SMITH	W	0.3	17.7	137	52
		M	-		67	63
		E	-		62	165
Bumble bee Larvae (Hobakbul Yuchung)	*Bombus ignitus* SMITH	W	1.0	55.9	99	61
		M	–		70	66
		E	–		68	95
Bumble bee male(Drone) (Hobakbul Susbul)	*Bombus ignitus* SMITH	W	0.5	8.1	77	65
		M	–		54	58
		E	–		47	86
Catharsius (Kangrang)	*Catharsius molossus* L.	W	2.0	95.7	5200	2000
		M	–		170	412
		E	–		50	62
Cicadae periostracum (Suntae)	*Cryptotympana atrula* FABR	W	0.3	62.0	234	52
		M	–		111	56
		E	–		45	53
Cordyceps (Dongchunghacho)	*Paecilomyces japonica*	W	0.5	143.3	12300	40
		M	–		195	89
		E	–		105	56
Dermestid beetles (Susirungie)	*Trogoderma ternkton*	W	–	32.5	94	56
		M	–		95	74
		E	–		48	83
Eupolyphaga (Jachung)	*Eupolyphaga sinensis* WALKER	W	0.6	22.6	105	59
		M	–		135	151
		E	–		68	57

Table 1. Continued

Insect name (Korean name)	Insect source	Extract[a]	Fibrinolysis (U/mg)[b]	Proteolytic Activity (U/mg)[c]	APTT (sec.)[d]	TT (sec.)[e]
Gryllotalpa *orientalis* (Tangangaji)	*Gryllotalpa orientalis* BURNMEISTER	W	0.2	22.7	78	50
		M	-		43	115
		E	0.1		82	62
Harmonia axyridis (Mudangbulre)	*Harmonia axyridis* PALLAS	W	-	1.6	170	64
		M	–		44	106
		E	–		41	65
Hirudo (Sujil)	*Whitmania Pigra* WHITMAN	W	0.2	7.7	34	51
		M	–		189	162
		E	–		47	76
Huechys (Hongrangja)	*Huechys sanguinea* DE GEER.	W	2.3	119.2	95	2024
		M	–		272	52
		E	0.2		94	120
Larvae of *Scarabaeoidea* (jejo)	*Protaetia brevitarsis seulensis* KOLBE	W	0.6	8.6	90	67
		M	–		48	64
		E	0.1		74	101
Lumbricus (Guin)	*Pheretima aspergillum* E. PERR.	W	0.1	9.7	93	90
		M	–		365	329
		E	–		54	120
Mantidis oötheca (Sangpyocho: little mantis)	*Tenodera sinensis* SAUSSURE	W	1.5	138.0	7647	3316
		M	–		92	156
		E	–		60	70
Mantidis oötheca (Sangpyocho: big mantis)	*Paratenodera sinensis* SAUSSURE	W	0.5	263.0	603	81
		M	-		94	103
		E	0.1		40	97
Mimela splendens (Sodong-pungdaengie)	*Mimela splendens* GYLLENHAL	W	2.0	98.0	73	2000
		M	–		71	156
		W	0.1		41	123
Mylabris (Banmyo)	*Mylabris phaelerata* PALL	M	1.0	21.0	129	41
		E	–		111	75
		W	–		58	79
Oxya japonica japonica (Beymeydugi)	*Oxya japonica japonica* THUNBERG	M	1.8	43.2	15140	65
		E	–		60	86
		W	0.5		73	195
Red Ant (Hongui)	*Formica rufa*	M	–	70.8	30000	2000
		E	–		2000	3000
		W	–		48	86
Scolopendra (Ogong)	*Scolopendra morsitans* L.	M	0.3	17.7	250	49
		E	-		50	70
			0.1		93	58
Scorpion (Jungal)	*Buthus martensi* KARSCH	W	–	5.0	1100	61
		M	–		180	74
		E	–		45	61
Silkworm adult male (Susnabang)	*Bombyx mori*	W	–		101	51
		M	–		53	138
		E	–		61	51
Silkworm (Nue)	*Bombyx mori*	W	1.0	23.2	96	55
		M	–		54	49
		E	–		50	73
Tabanus (Maengchung)	*Tabanus sp.*	W	–	16.1	108	94
		M	–		103	76
		E	–		40	100
Vespae Nidus (Oangbulgyp)	*Polistes mandarinus* SAUSS	W	1.9	75.0	294	54
		M	-		42	65
		E	0.1		61	80

[a]W, buffer extract; M, MeOH extract; E, Ethyl acetate extract, [b]One unit is defined as the amount of plasmin compared in terms of lysed area, [c]One unit of azocaseinolytic activity is defined as the amount of enzyme that causes a net increase of 1.0 of absorbance at 340 nm in 1 hr, [d]aPTT, activated partial thrombopastin time. e) TT, thrombin time.

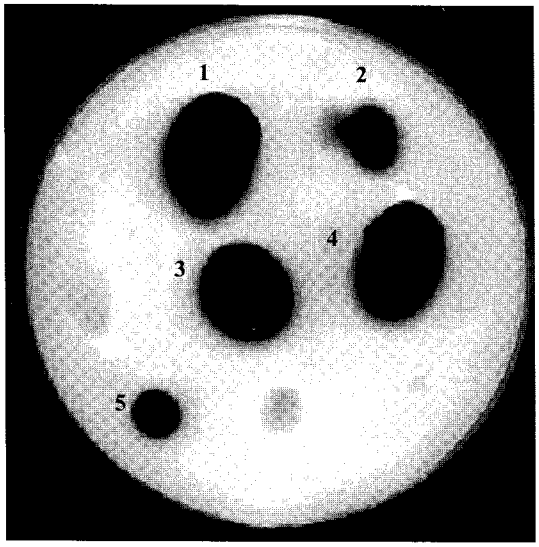

Fig. 1. Fibrinolytic activity of the buffer extract using fibrin plate method.
1. Catharsius; 2. *Tenodera sinensis*; 3. *Oxya japonica japonica*; 4. *Mimela splendens*; 5. Plasmin (1unit).

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).

Results and Discussion

The majority of the insect crude drug buffer extracts displayed markedly increased fibrinolytic activity as shown in Table 1. The six water extracts [*Catharsius molossus*, *Chinese mantidis*, *Eupolyphaga sinensis*, *Mimela splendens*, *Oxya japonica japonica*, *Polistes mandarinus* (Vespae Nidus)] showed strong activity. The water extract of *Catharsius molossus* showed the strongest activity on the fibrinolysis system of these.

The water extract of *Catharsius molossus* was partially purified by a combination of ammonium sulfate fractionation, gel filtration and ion-exchange chromatography. The second peak, Fr II, showed a strong fibrinolytic activity (10 U/mg). Its molecular weight was almost 27,000 Da under the reducing conditions of SDS gel (Fig. 2A and 2B).

On the other hand, the methanol extracts of the insect crude drugs did not exhibit any activity. Indeed, ethyl acetate and methanol extracts inhibited fibrinolytic activity in the fibrin plate (Wang, *et al.*, 1989). Because the fibrin olytic activity was mainly expected to occur by the proteolytic enzymes presenting in the water fraction of the screened insects, we deduced the enzymes might have been denatured in the ethylacetate and methanol extracts. In general, fibrinolysis that dissolves exisiting thrombin through the action of plasmin on fibrin, can be caused by plasminogen

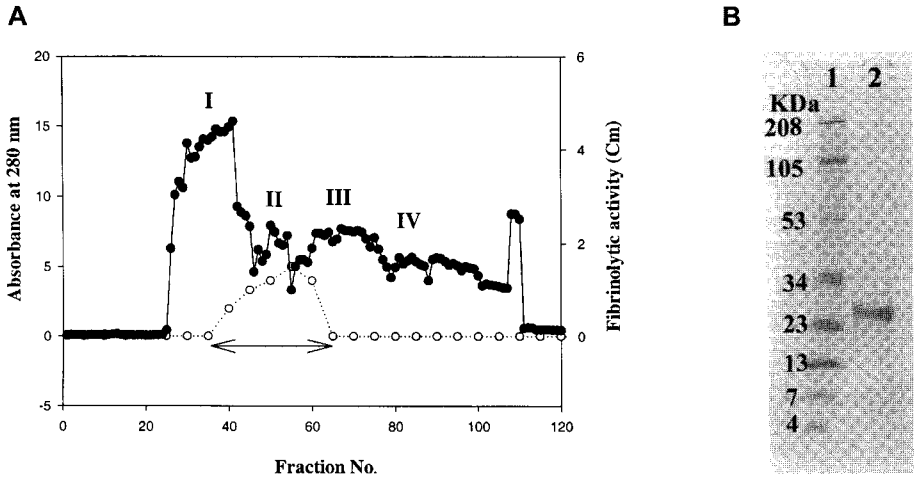

Fig. 2. A) Fractionation of Catharsius extract. Purification of fibrinolytic enzyme (serine protease) was accomplished by using Bio-Gel P-60. The elution was performed with 50 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl at a flow rate of 20 ml/hr. The elution profiles were monitored by reading the absorbance at 280 nm (---). Fibrinolytic activity, based on fibrin plate assay, was shown as dotted line and the active fractions were pooled. B) SDS-PAGE analysis of Catharsius fibrinolytic enzyme under denaturing conditions. *Lane 1*, mixture of marker proteins, myosin (208,000 Da), phosphorylase B (105,000 Da), glyceraldehyde-3-phosphate dehydrogenase (53,000 Da), carbonic anhydrase (34,000 Da), myoglobin-Blue (23,000 Da), lysozyme (13,000 Da), aprotinin (7,000 Da) and insulin B chain (4,000 Da); and *lane 2*, purified enzyme.

activators or plasmin-like proteases.

In our experiment, the low molecular components (i.e., formic acid from red ants) which are also found in other insect species, can influence the degree of coagulation activity. However, the molecular is small enough to be ignored, because we were mainly focused in the rather larger and newer molecules. Animal crude drugs are usually more potent than plant crude drugs as inhibitors on blood coagulation (Wang, *et al.*, 1989). We found that the water extracts of silkworm Tongchunhacho; *Paecilomyces japonica*, *Oxya japonica japonica* and *Buthus martensi,* strongly increased blood coagulation time by more than 100%. *Paecilomyces japonica* is reputed for its biological activities and as a tonic for replenishing vitality according to Chinese traditional medicine (Yan, 1999). *Paecilomyces japonica* is massively produced by inoculating conidia of Cordyceps into live silkworms in Korea.

In conclusion, according to our experiment, insect crude drugs are as potent as animal crude drugs in their effects on blood coagulation and fibrinolytic activity. The present results justify further studies to identify the active constituents of the extracts.

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