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Effects of Traditional Crude Drugs on Fibrinolysis by Plasmin[1]: Antiplasmin Principles in Eupolyphaga

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Fifteen crude drugs used in traditional Sino-Japanese medicine were investigated to analyze their effects on fibrinolysis by plasmin. Each crude drug was extracted with water (W), boiling water (H) and hot ethanol (E). The extracts were tested for inhibitory effect on fibrinolysis by the fibrin plate method and on plasmin activity by the chromogenic substrate method, and the following results were obtained.

Rhei Rhizoma, Moutan Cortex and Paeoniae Radix: Each extract showed marked inhibitory effects on fibrinolysis and plasmin activity.

Atractylodis Lanceae Rhizoma: H showed an inhibitory effect on fibrinolysis.

Eupolyphaga, Tabanus, Armeniacae Semen, Persicae Semen and Cnidii Rhizoma: E inhibited plasmin activity, while W and H were ineffective.

One of the antiplasmin principles in Eupolyphaga (*Opisthoplatia orientalis* Burmeister) was identified as oleic acid. Some *cis*-unsaturated fatty acids, such as linoleic acid and palmitoleic acid, also inhibited plasmin activity.

Keywords——crude drug; antiplasmin principle; Eupolyphaga; *Opisthoplatia orientalis*; oleic acid; free fatty acid; fibrinolysis; S-2251

Introduction

Crude drugs based on traditional medicines have been widely used in Japan and China, and many investigators have attempted to elucidate their modes of action and active principles. Some crude drugs affect blood coagulation,[2] and their active principles[3] have been investigated. In the previous paper[4] we reported that some crude drugs and prescriptions inhibited fibrinolysis by plasmin and prolonged the activated partial thromboplastin time (aPTT). These observations suggested the presence of inhibitors of plasmin and thrombin in the crude drugs.

In the present study, we examined the enzyme inhibitory activities of crude drugs, and tested the effects of 15 crude drugs, some of which are used as remedies for "oketsu," on fibrinolysis and plasmin activity by using the fibrin plate method and the chromogenic substrate (S-2251) method, respectively.

"Oketsu," blood stasis or stagnant syndrome, is one of the pathological and physiological concepts unique to traditional Sino-Japanese medicine. Although the definition of "oketsu" in terms of Western medicine is obscure because the name may be given to a wide range of diseases, it is clinically recognized as blood stagnation or disorder in the peripheral microcirculation. If the "oketsu" syndrome is considered to be a kind of peripheral circulatory disorder, then crude drugs which are used for "oketsu" may affect blood coagulation and fibrinolysis.

The screening of 15 crude drugs revealed that several drugs potently inhibited plasmin in a dose-dependent manner. Among them, hot ethanol extract (E) of Eupolyphaga (*Opisthoplatia orientalis* BURMEISTER), which is one of remedies for "oketsu," was found to have a strong inhibitory activity. We also report here the identification of the antiplasmin principles in Eupolyphaga.

Results and Discussion

Effects of Crude Drug Extracts on Fibrinolysis and Plasmin

Three kinds of extracts (with water (W), hot water (H) and E) of 15 crude drugs were tested for inhibitory effect on fibrinolysis by the fibrin plate method and on plasmin activity by the chromogenic substrate method. Table I shows the antifibrinolytic activity at a concentration of 5 mg/ml and the antiplasmin activity at a concentration of 60 μg/ml for each extract. As some of E showed unusual lysis which was probably caused by a detergent-like effect of oily materials on the fibrin plate, their inhibitory activities could not be determined.

Every extract of Rhei Rhizoma, Moutan Cortex, Paeoniae Radix strongly inhibited both fibrinolysis and plasmin, and H of Atractylodis Lanceae Rhizoma inhibited fibrinolysis. Among Eupolyphaga, Tabanus, Armeniacae Semen, Persicae Semen and Cnidii Rhizoma, only E inhibited plasmin activity.

H of Rhei Rhizoma, Moutan Cortex and Paeoniae Radix and E of Eupolyphaga and Tabanus inhibited plasmin with dose-dependency (Fig. 1), and the 50% inhibitory concentrations (IC_{50}) with respect to antiplasmin activity were 3.2, 28.2, 63.0, 39.0 and 30.0 μg/ml, respectively. Rhei Rhizoma showed the strongest inhibitory activity. This inhibitory activity of Rhei Rhizoma was mainly due to tannins, which are known to have nonspecific protein-denaturing activity (data not shown).

Okamoto *et al.*[5] reported that Paeoniae Radix, Salviae Miltiorrhizae Radix and Moutan Cortex were typical inhibitory herbs for plasmin. Studies of the active principles in Paeoniae Radix[6] and Moutan Cortex[7] have been reported, but the principles have not been characterized yet. Tannins and related polyphenols[8] in Rhei Rhizoma have antiplasmin activity. The present study supports these findings.

Eupolyphaga and Tabanus, derived from insects, are components of Daiohshachu-gan, used particularly for "oketsu." However, little is known concerning the pharmacological aspects[9] of Eupolyphaga and Tabanus. We therefore investigated the antiplasmin principles of Eupolyphaga.

Characterization of Antiplasmin Principles in Eupolyphaga

Ground Eupolyphaga was extracted with *n*-hexane and the extract was fractionated by silica gel column chromatography to afford six fractions, named frs. I—VI. Fraction III

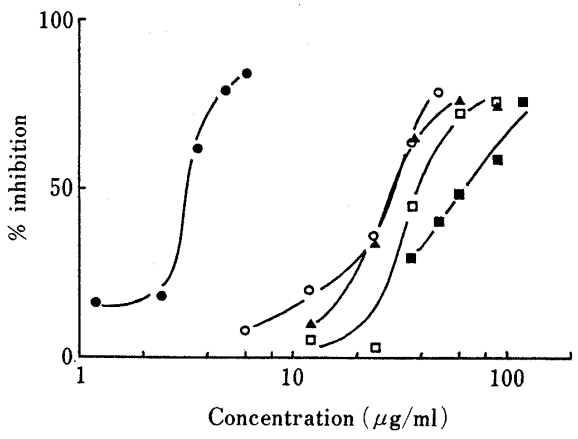

Fig. 1. Effects of Crude Drug Extracts on Plasmin

Antiplasmin activity was determined as described in Experimental. Incubation time was 3 min. H of Rhei Rhizoma (●), Moutan Radicis Cortex (○) and Paeoniae Radix (■). E of Eupolyphaga (□) and Tabanus (▲).

TABLE I. Effects of Crude Drug Extracts on Fibrinolysis and Plasmin

Crude drug	Scientific name		% inhibition of Fibrinolysis[a]	% inhibition of Plasmin[b]
Eupolyphaga	*Opisthoplatia orientalis* BURMEISTER	W	−0.3± 8.4	−2.9± 3.1
		H	−3.9± 1.3	6.9± 1.1
		E	N.D.	68.3± 2.2
Tabanus	*Tabanidae* sp.	W	11.3±13.0	−6.3± 4.1
		H	−11.6±12.2	−1.6± 4.2
		E	N.D.	75.7± 1.3
Hirudo	*Hirudidae* sp.	W	5.7± 7.3	12.0± 3.7
		H	15.8± 6.4	6.4± 9.4
		E	N.D.	17.7± 1.8
Atractylodis Rhizoma	*Atractylodes japonica* KOIZUMI	W	21.1± 1.8	12.3± 1.6
		H	21.1± 7.7	1.4± 2.5
		E	N.D.	0.6± 1.4
Atractylodis Lanceae Rhizoma	*Atractylodes lancea* DC	W	29.9± 6.6	−1.2± 1.9
		H	52.7± 2.8	−2.2± 0.8
		E	N.D.	2.5± 3.9
Moutan Radicis Cortex	*Paeonia moutan* SIMS	W	57.0± 8.9	53.1± 2.4
		H	86.7±23.0	90.3± 1.2
		E	100.0± 0.0	88.7±18.3
Paeoniae Radix	*Paeonia albiflora* PALL	W	41.9± 5.6	22.4± 0.8
		H	73.2± 1.6	55.6± 2.0
		E	67.5±12.8	83.2± 1.1
Rhei Rhizoma	*Rheum* sp.	W	100.0± 0.0	93.2± 1.6
		H	100.0± 0.0	96.4± 0.1
		E	100.0± 0.0	97.4± 0.9
Aconiti Tuber (Shirakawabushi)	*Aconitum japonicum* THUNBERG	W	19.3± 3.5	−1.3± 2.9
		H	14.7± 5.7	−2.2± 1.3
		E	20.9± 6.6	−4.6± 2.5
Aconiti Tuber (Uzu)	*Aconitum japonicum* THUNBERG	W	15.7± 6.8	2.2± 6.6
		H	16.4± 8.5	−3.1± 0.7
		E	31.4± 5.5	−3.7± 2.2
Persicae Semen	*Prunus persica* BATSCH	W	15.4± 5.1	3.4± 2.9
		H	−2.3± 2.0	−2.3± 1.9
		E	N.D.	33.1± 0.5
Armeniacae Semen	*Prunus armeniaca* LINNE	W	8.0± 9.6	2.6± 4.2
		H	7.5± 8.1	−1.3± 6.9
		E	N.D.	54.8± 6.1
Angelicae Radix (Yamatotohki)	*Angelica acutiloba* KITAGAWA	W	−1.4±10.8	−1.7± 2.5
		H	12.5±10.0	1.5± 2.1
		E	N.D.	3.6± 2.0
Angelicae Radix (Karatohki)	*Angelica sinensis* DIELS	W	13.4±12.7	3.6± 1.4
		H	13.0± 9.1	2.6± 0.3
		E	N.D.	17.9± 3.2
Cnidii Rhizoma	*Cnidium officinale* MAKINO	W	11.9± 6.4	−1.8± 9.4
		H	21.7± 5.8	−3.6± 4.1
		E	N.D.	34.3± 0.5

Each value shows the mean±S.D. ($n=3$). *a*) Antifibrinolytic activity was determined by the standard fibrin plate method as described in Experimental. Sample concentration was 5 mg/ml. *b*) Antiplasmin activity was determined by the chromogenic substrate (S-2251) method as described in Experimental. Sample concentration was 60 μg/ml and incubation time was 3 min. W: H_2O extract. H: hot H_2O extract. E: hot EtOH extract. N.D.: not determined.

showed inhibitory activity and was rechromatographed to afford more active fr. III-2. Fraction III-2 was further separated into three fractions (fr. III-2-a, b, c) by reversed-phase thin layer chromatography (TLC) (RP-18). Fraction III-2-c showed a strong inhibitory effect

TABLE II. Effects of Free Fatty Acids (FFA) on Plasmin

FFA[a]	% inhibition of plasmin[b]
Lauric acid (12:0)	11.0± 5.0
Myristic acid (14:0)	−2.4± 7.4
Palmitic acid (16:0)	6.1± 4.8
Margaric acid (17:0)	11.9± 2.7
Stearic acid (18:0)	7.1± 6.5
Nonadecanoic acid (19:0)	13.5± 2.7
Arachidic acid (20:0)	12.0± 5.4
Palmitoleic acid (16:1, *cis*-9)	61.9± 1.9
Petroselinic acid (18:1, *cis*-6)	83.2± 2.4
Oleic acid (18:1, *cis*-9)	85.0± 2.6
Elaidic acid (18:1, *trans*-9)	6.3± 1.1
Linoleic acid (18:2, *cis*-9, *cis*-12)	52.5±21.1
Linolenic acid (18:3, *cis*-9, *cis*-12, *cis*-15)	21.5± 3.9
γ-Linolenic acid (18:3, *cis*-6, *cis*-9, *cis*-12)	45.2± 1.6
Arachidonic acid (20:4, *cis*-5, *cis*-8, *cis*-11, *cis*-14)	46.9± 3.4

Each value shows the mean±S.D. ($n=3$). *a*) Concentration of FFA was 60 μM. *b*) Antiplasmin activity was determined as described in Experimental. Incubation time was 3 min.

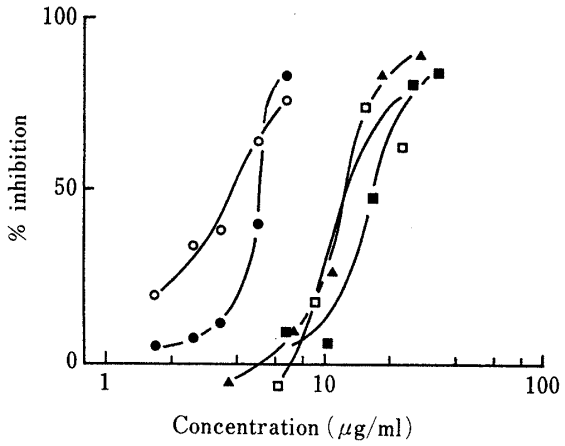

Fig. 2. Effects of *cis*-Unsaturated Fatty Acids on Plasmin

Antiplasmin activity was determined as described in Experimental. Incubation time was 3 min. Oleic acid (●), petroselinic acid (○), linoleic acid (■), palmitoleic acid (□) and arachidonic acid (▲).

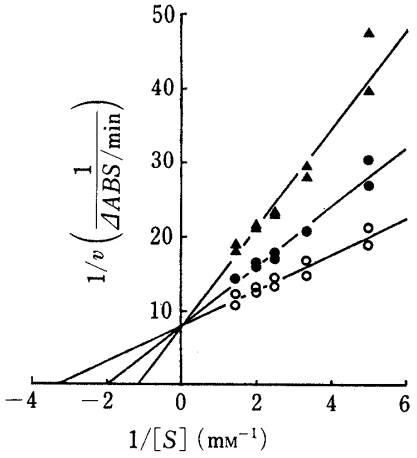

Fig. 3. Lineweaver–Burk Plot for Inhibition of Plasmin by Oleic Acid

Antiplasmin activity was determined as described in Experimental. Incubation time was 5 min. The concentrations of oleic acid were 0 μM (○), 6 μM (●) and 9 μM (▲).

and fr. III-2-b showed a weak one. These fractions were found to contain several free fatty acids by gas chromatography-mass spectrometry (GC-MS): oleic acid (79.1%) and palmitic acid (20.9%) in fr. III-2-c, and linoleic acid (69.1%) and palmitoleic acid (26.1%) in fr. III-2-b. The IC_{50} values of antiplasmin activity of the *n*-hexane extract, and fractions III, III-2, III-2-c and III-2-b were 24.5, 13.1, 10.3, 7.6 and 14.7 μg/ml, respectively. The identification was confirmed by direct comparison of the compounds with authentic samples of free fatty acids.

The IC_{50} values of antiplasmin activity of oleic acid, palmitoleic acid and linoleic acid were 5.4, 13.0 and 17.0 μg/ml, respectively. Palmitic acid at a concentration below 350 μg/ml did not inhibit plasmin. On the other hand, Inagaki *et al.*[10] reported that the fatty acid in Eupolyphaga was mainly oleic acid (*ca.* 70%) and palmitic acid (*ca.* 20%). These results indicate that the inhibitory activity on plasmin of Eupolyphaga is due to oleic acid.

Effects of Free Fatty Acids on Plasmin

Furthermore, we tested a range of free fatty acids for antiplasmin activity (Table II). *cis*-Unsaturated free fatty acids inhibited plasmin activity dose-dependently (Fig. 2), but the *trans*-isomer of oleic acid, elaidic acid, and saturated fatty acids such as stearic acid, were almost ineffective.

The kinetics of inhibition of plasmin by oleic acid was studied. The inhibition was competitive, as determined from a Lineweaver–Burk plot (Fig. 3). The K_i value of oleic acid was 9.0 μM. However, the mode of inhibition changed to complex type, as the concentration of oleic acid was raised (data not shown).

Various effects of fatty acids on the activities of other enzymes have been reported: unsaturated fatty acid inhibits human platelet phospholipase A_2,[11] human granulocyte elastase[12] and chymase[13] (chymotrypsin-like protease). Ashe and Zimmerman[12] reported that human granulocyte elastase is inhibited by *cis*-unsaturated fatty acids, but not by their *trans*-isomers or by saturated fatty acids. We observed similar effects of fatty acids on plasmin.

In this work, we found that several drugs had antiplasmin activity. It might be difficult to correlate antiplasmin activity directly with effectiveness against "oketsu" syndrome. However, considering that "oketsu" syndrome is similar to disseminated intravascular coagulation (DIC), which involves disorders of both blood coagulation and fibrinolysis, it is of interest that antiplasmin principles have been found in these crude drugs. A study of antiplasmin principles of other crude drugs is in progress in our laboratory.

Experimental

Materials——Plasmin (from human serum), thrombin (from human plasma), fibrinogen (from human plasma) were obtained from Green Cross Co., Osaka. Urokinase (from human urinary) was from Mochida Pharmaceuticals Co., Ltd., Tokyo. Chromogenic substrate, H-D-Val-Leu-Lys-*p*-nitroanilide (S-2251), was from Kabi Diagnostica, Stockholm. TLC plates (Silica gel 60 F_{254} and RP-18 F_{254}s) were from Merck, Darmstadt, Germany. Free fatty acids and other chemicals were from Wako Pure Chemical Industries Ltd., Osaka, Nakarai Chemicals Ltd., Kyoto, and Tokyo Chemical Industries Co., Ltd., Tokyo.

Enzymes were dissolved in 50 mM Tris–HCl buffer (pH 7.4) and 100 mM NaCl–50 mM Tris–HCl buffer (pH 7.4) for the fibrin plate method and the chromogenic substrate method, respectively. S-2251 was dissolved in distilled water. Free fatty acids were dissolved in methanol or ethanol and diluted with the buffer to the required concentrations.

Crude Drugs——Crude drugs used in this study were obtained from Tochimoto Tenkaido, Osaka, and Uchida Wakan-yaku, Tokyo. Each crude drug (5 g) was extracted with water (200 ml) at room temperature for 2 d (W), with water (200 ml) at 100 °C for 2 h under reflux (H) or with ethanol (200 ml) at 70—80 °C for 2 h under reflux (E). Each extract was filtered, concentrated, and lyophilized (W, H) or evaporated under nitrogen (E). Each extract was dissolved in the buffer and insoluble materials were removed by centrifugation. The resultant supernatant was diluted to the required concentration with the same buffer.

Assay——Standard Fibrin Plate Method[14]: Fibrin plates were prepared from 0.15% fibrinogen with 3 drops of 10 U/ml thrombin. Aliquots of 30 μl of a mixture of urokinase (100 I.U./ml) with an equal quantity of sample were applied to fibrin plates, and the area of lysis after incubation at 37 °C for 6 h was measured; 30 μl of urokinase (50 I.U./ml) was used as the control. The amount of inhibition was calculated by means of the following equation.

$$\% \text{ inhibition} = (1 - \text{sample value/control value}) \times 100$$

Chromogenic Substrate Method[15]: Plasmin activity was determined by using synthetic chromogenic substrate S-2251 based on the method recommended by the manufacturer. A mixture of 0.6 ml of sample and 0.2 ml of 0.5 CU/ml plasmin was incubated for 3 or 5 min at 37 °C before the addition of 0.2 ml of 3.5 mM S-2251. Buffer (0.6 ml) was used instead of a sample as control. Initial reaction rate was measured by recording the increase of absorbance at 405 nm with a Hitachi 200-20 double-beam spectrophotometer equipped with a thermostated cuvette holder and connected to a Hitachi 200 recorder. The amount of inhibition was calculated in the same way described as above. In the kinetic study, 0.125 CU/ml plasmin was used. The incubation time was 5 min, and the S-2251 concentration was varied from 1 to 3.5 mM.

Isolation of Active Principles from Eupolyphaga (*Opisthoplatia orientalis* BURMEISTER)——Ground Eupolyphaga

(10 g) was successively extracted with *n*-hexane and ethanol. The inhibitory activities of these extracts at a concentration of 24 μg/ml were 62.1% for the former, and -3.2% for the latter.

In a large-scale extraction, ground Eupolyphaga (300 g) was extracted with 10 l (1000 ml × 10) of *n*-hexane under reflux to give 24 g of extract, which was applied to a silica gel column chromatography (Wakogel C-200; 6 cm × 59 cm) and eluted with benzene–acetone (9 : 1) and methanol. Six fractions (frs. I—VI) were obtained. The inhibitory activities of these fractions at a concentration of 12 μg/ml were 5.0% (fr. I), 12.1% (fr. II), 81.7% (fr. III), -10.8% (fr. IV), 3.3% (fr. V) and -3.4% (fr. VI). Fraction III (5.0 g), the main active fraction, was rechromatographed on a silica gel column chromatography (Wakogel C-200; 3 cm × 37 cm) with *n*-hexane–diethyl ether–acetic acid (80 : 30 : 1) and (60 : 40 : 1), and the eluate was checked by TLC (Silica gel 60). Five fractions (frs. III-1—III-5) were obtained. The inhibitory activities of these fractions at a concentration of 12 μg/ml were 15.4% (fr. III-1), 90.1% (fr. III-2), 14.5% (fr. III-3), 16.1% (fr. III-4) and 6.6% (fr. III-5). Fraction III-2 was subjected to reversed-phase TLC (RP-18) developed with acetonitrile–methanol–water–formic acid (95 : 2 : 3 : 0.5) and three spots were visualized by iodine vapor. These spots were scraped off and extracted with chloroform. The inhibitory activities of these three fractions at a concentration of 12 μg/ml were -9.2% (fr. III-2-a), 37.7% (fr. III-2-b) and 83.2% (fr. III-2-c). Fraction III-2-b and fr. III-2-c were methylated with diazomethane and analyzed by GC with FID (Shimadzu GC-7A; 10% EGSS-X 2 mmϕ × 2 m, N_2 45 ml/min, 175 °C) and identified by GC-MS (JEOL JGC-20K, JMS-D200). Methylated fr. III-2-b contained methyl palmitoleate (26.1%; *m/e*, M^+ 268) and methyl linoleate (69.1%; *m/e*, M^+ 294) and methylated fr. III-2-c contained methyl palmitate (20.9%; *m/e*, M^+ 270) and methyl oleate (79.1%; *m/e*, M^+ 296).

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References and Notes

1) A part of this study was presented at the 31st Annual Meeting of the Pharmacognostical Society of Japan, Tokyo, October 1984.
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