Evidence for Existence of a Water-Extractable Anticoagulant in an Earthworm, *Lumbricus rubellus*

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Abstract: We have isolated a water-extracted novel regulator for blood coagulation from an earthworm, *Lumbricus rubellus*. As a folk remedy, the earthworm has been known to facilitate blood circulation. After complete heat inactivation of endogenous proteases in the earthworm, an anticoagulant(s) was purified through ammonium sulfate fractionation and three consecutive gel permeation chromatography of Sephacryl S-300, Sephadex G-75, and G-150 by measuring activated partial thromboplastin time (APTT). The anticoagulant was further purified to 2,800 fold with a C4 reversed-phase HPLC. This activity was stable under heat (100°C for 30 min) and acidic conditions (0.4 N HCl). The effects of this partially purified anticoagulant on thrombin were observed with various substrates such as Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), H-D-phenylalanyl-L-pipecoyl-L-arginine-p-nitroanilide (S-2238), Nα-p-tosyl-L-arginine methyl ester (TAME), and fibrinogen as a natural substrate. Only TAME hydrolysis, due to an esterase activity of the enzyme, was inhibited among the chromogenic substrates. In addition, the anticoagulant not only inhibited the conversion of fibrinogen to fibrin but also prolonged the fibrin clot formation monitored with the *in vitro* coagulation test. Based on these observations, we suggest the significance of measuring the ability of antithrombotic drugs to inhibit the esterase activity of thrombin. In this report, it was also shown that the earthworm indeed contained a water-extractable, heat- and acid-stable anticoagulant which could be used as a novel antithrombotic agent.

Key words: anticoagulant, blood coagulation, earthworm, esterase activity, thrombin.

The blood coagulation system is a part of a process called hemostasis with simultaneous participation of vascular constriction and platelet aggregation following vessel injury (Mann *et al.*, 1990; Davie *et al.*, 1991). Coagulation which transforms circulating blood into an insoluble gel takes place by either the intrinsic or the extrinsic pathway (Longberry, 1982). While the extrinsic pathway is activated by tissue factors exposed from subendothelium only after vascular laceration, the intrinsic pathway can be activated in normal blood circulation even in the absence of external causes. Activation of either pathway ends up with the conversion of factor X to Xa, which starts a common pathway resulting in fibrin clot formation. This hemostatic mechanism should be well-balanced with the fibrinolytic system and the naturally occurring inhibitors for clot formation. When this careful regulation is disrupted, devastating outcomes such as bleeding disorders or frequent thrombi formations which interfere with optimal blood circulation result. A large amount of research, therfore, has

been carried out to develop effective drugs to control hemostasis.

The earthworm itself has been used as an anti-inflammatory, analgesic, and antipyretic agent in oriental medicine (Nagasawa *et al.*, 1991). In addition, its effects on tumor, epilepsy, and blood coagulation have been also suggested (Wang *et al.*, 1989; Nagasawa *et al.*, 1991; Noda *et al.*, 1992). It was found that proteases from an earthworm, *Lumbricus rubellus*, exhibited high fibrinolytic activity, which have been recognized and patented for their therapeutic applications (Mihara, 1986 Kim *et al.*, 1995). It is, however, still questionable whether proteases can actually dissolve fibrin clots exclusively without affecting other proteins in blood. It is also difficult to imagine that proteases could survive against hostile environments during the course from oral administration to blood circulation. Since the earthworm has been known to facilitate blood circulation, our research has focused on an anticoagulant selectively related to the instrinsic pathway. Facilitation of blood circulation can be achieved by selective fibrinolysis by proteases and the effective retardation of the blood coagulation system since clot formation and dis-

solution are in a continuously dynamic steady state even in the absence of external stimuli. In this report, we present evidence for the existence of a water-extracted anticoagulant from L. rubellus for the first time, which is stable under heat (100°C for 30 min) and acidic (0.4 N HCl) conditions. In addition, its effects on thrombin, a pivotal protease in the blood coagulation system, have been also studied with various chromogenic substrates.

Materials and Methods

Materials

Adult earthworms, L. rubellus, were provided by the Shingal School of Agriculture located at Kihung, Korea. The fresh earthworms were divided into several batches (50 g/batch) after washing with distilled water and kept frozen at −70°C until being used. Thrombin and fibrinogen (type I-S) from bovine plasma, TAME, BApNA, trifluoroacetic acid, Tris, Sephadex G150-120, and a test kit for thrombin clotting time (TCT) were obtained from the Sigma Chemical Co. (St. Louis, USA). S-2238 was from Chromogenix (Mölndal, Sweden). Dade® Thromboplastiin IS, Dade® Actin® Activated Cephaloplastin Reagent, and Dade® Ci-Trol® Coagulation Control (level I) were from Baxter Diagnostic Inc. (Deerfield, USA). Sephacryl S-300 and Sephadex G-75 were purchased from Pharmacia (Uppsala, Sweden). A C4 reversed-phase (RP) column was obtained from Millipore (Bedford, USA). Solvents for RP-HPLC including methanol, acetonitrile, and isopropanol were obtained in Optima grade from Fischer Scientific (Fair Lawn, USA).

Activated partial thromboplastin time (APTT) test

The APTT was monitored manually by the tilt tube method with an Actin® activated cephaloplastin reagent and Dade® Ci-Trol® Coagulation control (level I) as normal human plasma. Actin® (0.1 ml) was preincubated for 1 min at 37°C, then mixed with 0.1 ml of the control plasma and incubated for an additional 3 min. The time required for fibrin web formation was measured after 0.1 ml of prewarmed 20 mM CaCl$_2$ was added to the reagent plasma mixture in the presence and absence of the anticoagulant from L. rubellus. The data was analyzed in % coagulation time acdording to an equation of $t_o/t_o \times 100$, where t_o, an t_o represented APPT in the absence and presence of the anticoagulant, respectively. One unit of the anticoagulant was defined as the amount required to increase 1% of % coagulation time compared with a control. In addition, the thrombin clotting time (TCT) was measured with the control plasma and a thrombin time reagent. The

effect of anticoagulant was examined according to the method provided by the manufacturer.

Purification of anticoagulant from L. rubellus

The anticoagulant purification was started by mixing 50 g (wet weight) of the frozen earthworm with distilled water in a ratio of 1 : 1 (w/v) and followed by grinding with an Omni-Mixer (Model-17105, Omni Co.). After further homogenization at 1,200 rpm with a glass potter homogenizer with a tight-fitting, heat extraction was performed at 100°C for 30 min in a water bath. The supernatant was collected with centrifugation at 50,000 ×g for 20 min. Anticoagulatory activity was fractionated with ammonium sulfate at final concentrations of between 50% to 80% at 4°C. Following the same centrifugation procedure, the resulting pellet was resuspended in a minimum volume of 50 mM Tris-HCl, pH 8.0. The sample was purified with Sephacryl S-300 gel permeation chromatography (1.5 cm×60 cm) with a flow rate of 0.25 ml/min in 50 mM Tris-HCl, pH 8.0. The anticoagulatory fractions were concentrated to 5-fold with Speed-Vac. and subjected to two more consecutive gel permeation chromatographies using Sephadex G-75 (2.8 cm×62.5 cm) at a flowrateof2.5 ml/min) and G 150-120 (1.8 cm×53 cm) at a flow rate of 0.22 ml/min. As a final step, the anticoagulant was further purified with a C4 reversed-phase HPLC. The sample was injected into the column pre-equilibrated with 0.1% trifluoroacetic acid (TFA) as an A-buffer, and any substances attached to the matrix were eluted with a B-buffer composed of 80% acetonitrile in the A-buffer under a linear gradient (0% to 100 % B-buffer within 50 min). All the collected fractions were lyophilized to remove any residual organic solvent and TFA. The pellet was resuspended with 50 mM Tris-HCl, pH 8.0 and the anticoagulatory activity was measured.

Measurements of thrombin activity in the presence or absence of the anticoagulant

The esterase activity was assayed according to the method of Hummel (1959). In brief, a typical reaction was carried out directly in a cuvette which contained 0.5 μM thrombin, 1.0 mM TAME, and various amounts of the anticoagulant in a total volume of 0.6 ml with 50 mM Tris-HCl (pH 8.0). For the hydrolysis of TAME an increase in absorbance at 247 nm was continuously monitored using a Uvikon spectrohotometer 930 (Kontron). Both S-2238 and BApNA were used to examine the amidase activity of thrombin. The hydrolyzed products, p-nitroaniline, from both substrates were continuously monitored by measuring the absorbance at 405 nm. S-2238 (1.0 mM) was added to a reaction

mixture of 0.6 ml containing 50 nM thrombin and 50 mM Tris-HCl (pH 8.0). When BApNA was used as a substrate, 0.5 μM thrombin and 1 mM substrate were added to the same reaction mixture. The initial rates were obtained from slopes of the curves within 1 min. The effects of anticoagulant on thrombin were analyzed by calculating v/v_o, where v and v_o represented the activities in the presence and absence of the anticoagulant, respectively. The conversion of fibrinogen to fibrin by thrombin was spectrophotometrically monitored at 600 nm (Valembois et al., 1988). Thrombin (10 μg) and the sample were preincubated in 50 mM Tris-HCl, pH 8.0 for 3 min at 37°C and the change in transmittance was measured as soon as fibrinogen was added to 0.15% in the final volume of 3 ml. The delays in the fibrin web formations were compared in the presence and absence of anticoagulant.

Other methods

Polyacrylamide gel electrophoresis was done with a pre-casted 10~20% Tricine gradient gel obtained from Novex™ or the method of Laemmli (1970). Proteins on the gel were visualized by Coomassie blue stain. The amounts of proteins collected from each purification step were determined by the method of Bradford (1976).

Results and Discussion

Anticoagulant effects of water extract from earthworms

As from any components of herbal medicine in Chinese medical prescriptions, the most popular way to obtain the naturally occurring drugs from earthworms has been water-extraction with slow and continuous boiling. The heat treatment which was employed in this study was important, because it not only simulated the traditional drug extraction method but also inactivated endogenous proteases which might have interfered with our purpose of isolating an anticoagulant(s) from L. rubellus. As mentioned above, the facilitation of blood circulation by the crude extract of earthworms can be achieved by either anticoagulatory or fibrinolytic activity. It was necessary, therefore, to inactivate completely the endogenous proteases to specifically search for the anticoagulant. When the extract was preincubated at three different temperatures (60°C, 80°C, 100°C) for 30 min the residual trypsin-like and fibrinolytic activities were measured. The endogenous proteolytic activities were completely eliminated at a temperature above 80°C, even though the possible presence of proteases with different substrate specificities should not be ignored (data not shown). On the other hand, the anticoagulatory potency influencing especially the intrin-

Table 1. Anticoagulatory activity of the crude earthworm extract after 30 min of preincubation at various temperatures

Temperature (°C)	% Coagulation time[b,c]
Control[a]	100
60	120.6± 2.0 (5)[d]
80	135.7+ 2.4 (6)
100	138.8± 1.9 (6)

[a]Control represents the activity without the crude earthworm extract.
[b]The fibrin clot formation was measured with the APTT test.
[c]% Coagulation time was obtained with an equation of te/to× 100, where to and te represented APTT in the absence and presence of the anticoagulant, respectively.
[d]The values inside the brackets indicated numbers of experiments performed at each condition.

Table 2. Effect of acidification on the anticoagulatory activity of the heat-treated earthworm extract[a]

Final HCl conc. (N)	% Coagulation time[b]
0	126.7± 2.7 (3)[c]
0.05	130.0± 1.0 (2)
0.1	142.0+ 2.0 (2)
0.2	129.0± 4.6 (3)
0.4	124.0± 1.0 (2)

[a]The anticoagulatory activity was measured with the APTT test after neutralization with 3 M Tris.
[b]The activity in the absence of the extract was considered as control to obtain the % coagulation time.
[c]The numbers of experiments performed for each condition were indicated within the brackets.

sic pathway was gradually increased as the extraction temperatures were raised (Table 1). At a 100°C extracting temperature for 30 min the % coagulation time was increased by 39% as compared with that of the control, which did not contain the extract. The additional incubation at 100°C did not change the % coagulation time. The anticoagulant, therefore, is a heat-insensitive molecule, which could eliminate a possibility that the anticoagulatory factors are related to structure-based functional proteins.

Since the crude water-extract from earthworms exhibits its function on blood circulation following oral administration, a unknown regulator(s) should survive from hostile environments such as the acidic condition inside the stomach and the digestive tract where various hydrolases including proteases are involved. The heat-treated water extract from L. rubellus after removal of precipitate was subjuced to acidification with 2 N HCl. After 20 min of preincuation at room temperature in acidic conditions, the extract was neutralized with 3 M Tris and the remaining activity was measured with

Table 3. Purification of the anticoagulant from *Lumbricus rubellus*

Procedure	Total protein (mg)	Total activity[a] (U)	Specific activity[a] (U/mg)	Yield (%)	Purification (fold)
Crude extract	2244.0	118156	52.7	100	1.0
Heat treatment	61.6	170424	2766.6	144.2	52.5
ASF[b]	33.7	166296	4934.6	140.7	93.6
S-300	24.1	210961	8753.6	178.5	166.1
G-75	3.93	95586	24322.1	80.9	461.5
G-150	1.68	82467	49087.5	69.8	931.5
C-4	0.026	3856	148307.7	3.3	2814.2

[a]One unit was defined as the amount of anticoagulant which increased 1% of % coagulation time from the control.
[b]Ammonium sulfate fractionation. The anticoagulatory activity was measured after dialysis against 50 mM Tris-Cl, pH 8.0.
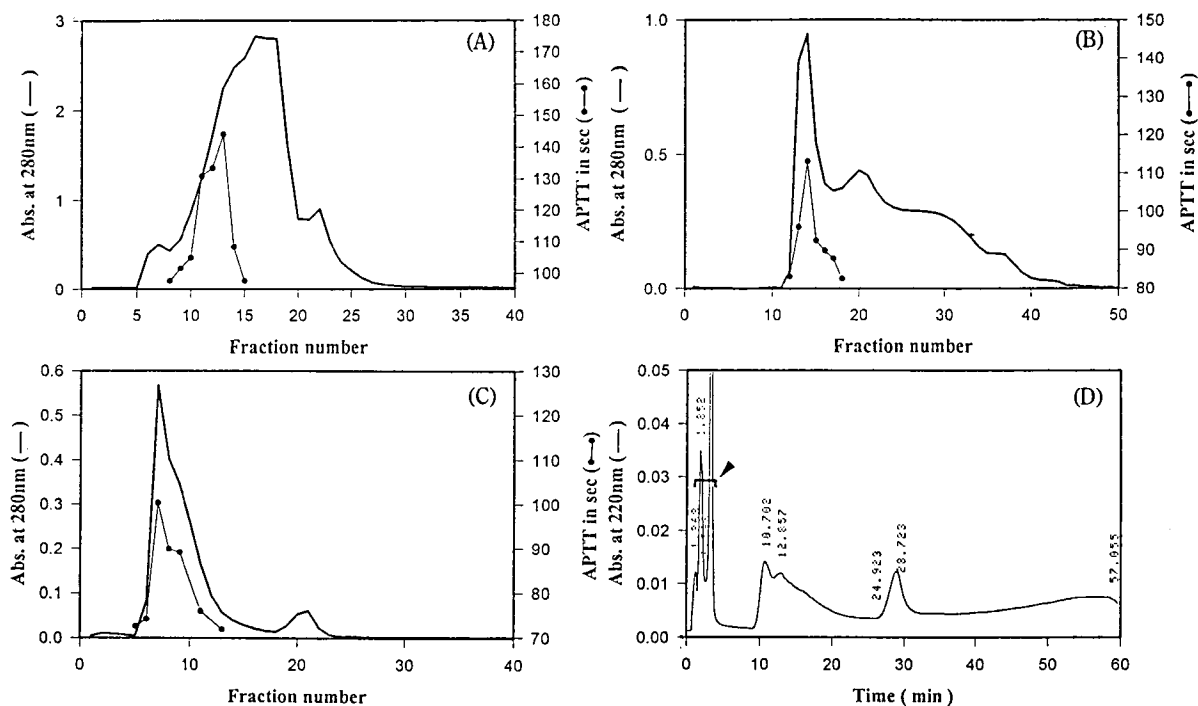

Fig. 1. Elution profiles of the anticoagulant during the purification with various chromatography steps. Proteins and the activity were measured at 280 nm ( ) and with the APTT test (● ●), respectively. A, Sephacryl S-300 gel filtration chromatography; B, Sephadex G-75; C, Sephadex G150-120; D, C4 reversed-phase HPLC where elution was spectrometrically monitored at 220 nm and the active fractions were indicated as an arrow head with a bracket.

the APTT. The anticoagulatory effect did not disappear with these preincubations even at 0.4 N HCl (Table 2). This possibly indicates that the anticoagulant might not be related to glycosaminoglycans such as heparin, a popular anticoagulant, because glycosidic linkages are known to be vulnerable in acidic conditions. When the heparin was treated with 0.4 N HCl, its anticoagulatory activity was indeed decreased in % coagulation time from 133% to 104.3%.

Purification of the anticoagulatory factor from earthworms

An anticoagulant from the water extract of *L. rubellus*

was partially purified by ammonium sulfate fractionation (50~80%), three subsequent gel filtration chromatography steps, and the final reversed-phase HPLC with a C4 column (Table 3). The yield was increased by 70.3% after S-300 gel permeation chromatography, indicating a possible presence of a reversible inhibitor for the anticoagulant in the ammonium sulfate precipitate. The anticoagulant was purified to about 2,800 fold with a yield of 3.3%. The gel filtration chromatography steps were mainly employed for purification because other methods of chromatography using salts could cause a difficulty in estimating the clotting time in the APTT test. The elution profiles of the anticoagulant from the
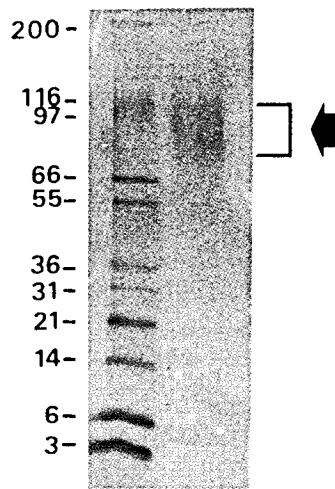
Fig. 2. Gel electrophoresis with a 10~20% Tricine gradient gel from Novax™. The gel was visualized by Coomassie blue stain. A broad band was indicated with an arrow with a square bracket. The protein markers used were; myosin (rabbit muscle), 220 KDa; galactosidase (*E. coli*), 116.3 KDa; phosphorylase b (rabbit muscle), 97.4 KDa; bovine serum albumin, 66.3 KDa; glutamic dehydrogenase (bovine liver), 55.4 KDa; lactate dehydrogenase (porcine muscle), 36.5 KDa; carbonic anhydrase (bovine erythrocyte), 31 KDa; tyrosine inhibitor (soybean), 21.5 KDa; lysozyme (chicken egg white), 14.4 KDa; apotinin (bovine lung), 6 KDa; insulin B chain (bovine pancreas), 3.5 KDa.

chromatography steps are shown in Fig. 1. The substance was eluted at relatively earlier fractions from all of the gel filtration steps (Fig. 1A-C). Interestingly, this molecule did not bind to the hydrophobic matrix of the C4 reversed-phase column (Fig. 1D). Although two major peaks appeared on the chromatogram of HPLC as the active fraction, both peaks were eluted as a flow-through because their elution positions were still before the actual gradient started. This indicated a hydrophilic nature of the anticoagulant. When the final active fraction was analyzed with a 10~20% Tricine-gradient gel and visualized with Coomassie blue stain, a blurry and broad band appeared at an apparent molecular weight of between 80 to 100 kDa (Fig. 2). The exact nature of this anticoagulant, however, is yet to be known as to whether the activity was due to a protein. In addition, when the purified fraction was digested with trypsin, the anticoagulatory activity became slightly more stimulated. This indicates that the anticoagulatory effect might be due to either a peptide core(s) of a heat-denatured protein resulting from proteolysis or a protease-insensitive biomolecule. In fact, we are still exploring the characterization of the biochemical nature of this novel molecule.

Antithrombotic drugs can be divided into three categories; anticoagulatory, fibrinolytic, and antiplatelet types. Although coumarin-type drugs and heparin have been widely used as anticoagulants, these type of drugs

have severe hemorrhagic complications (Kelton and Hirsh, 1980). Drug development related to hemostasis with low toxicity *in vivo*, therefore, has drawn a considerable amount of attention (Wang *et al.*, 1989). The earthworm has been scientifically searched for the presence of natural drugs during the last 40 years. The first substance isolated was 2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate, lombricine (Van Thoai and Robin, 1954), which showed antipyretic and anticancer effects by preventing an excessive uptake of glucose as a nutrient or an energy source (Nagasawa *et al.*, 1991). Besides the proteases whose therapeutic uses have been suggested as fibrinolytic agents, lyso platelet-activating factors such as 1-O-Alkyl-*sn*-glycero-3-phosphocholines have been isolated from the earthworm, *Pheretima asiatica* (Noda *et al.*, 1992). Our preliminary observation indicates that the total lipids of the earthworm extracted with chloroform/methanol (1 : 2, v/v) prolonged the fibrin clot formation, indicating a possible presence of another type of anticoagulant in the lipids. As far as a water-extracted anticoagulant from the earthworm is concerned, however, this study is the first to show evidence of its existence.

Thrombin inhibition by an anticoagulant

In order to characterize this anticoagulant, its function as a protease inhibitor was investigated with thrombin, the multifunctional protease in the blood coagulation system (Callas *et al.*, 1995). Thrombin plays pivotal roles in the blood coagulation cascade as either a procoagulatory or anticoagulatory agent by regulating its own level (Davie *et al.*, 1991). The primary function of the enzyme is to convert fibrinogen into fibrin which provides the basis of a blood clot. The conversion of prothrombin to thrombin is accelerated by coagulation factors such as Va, VIIIa, and VIIa. The level of the enzyme, on the other hand, is down-regulated by itself through activating protein C in the presence of thrombomodulin, which ultimately eliminates the activated factors V and VIII by proteolysis (Esmon, 1989). Thrombin, therefore, has become a main target for drug development influencing hemostasis (Stubbs and Bode, 1995).

Effects of the anticoagulant on thrombin were examined with various substrates such as Nα-benzoyl-DL-arginine-p-nitroanilide (BApNA), H-D-phenylalanyl-L-pipecoyl-L-arginine-p-nitroanilide (S-2238), and Nα-p-tosyl-L-arginine methyl ester (TAME). Thrombin activities with the substrates in the presence of the anticoagulant are shown in Fig. 3. Only the esterase activity of thrombin assayed with TAME was significantly inhibited by 83% at 0.38 μg of the anticoagulant while the amidase activities of thrombin measured with either BApNA or
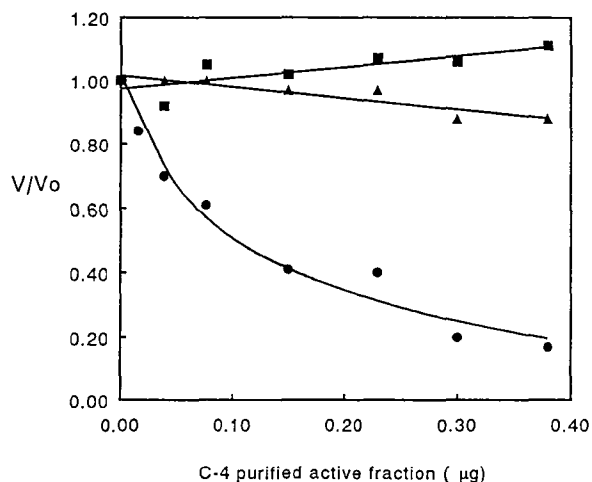
Fig. 3. Effects of the anticoagulant on thrombin activity were analyzed with three different chromogenic substrates such as BA-pNA (■), S-2238 (▲), and TAME (●). Its inhibitory effect was shown as v/vo, where v and vo represented the activities in the presence and absence of the C4 purified active fraction, respectively.
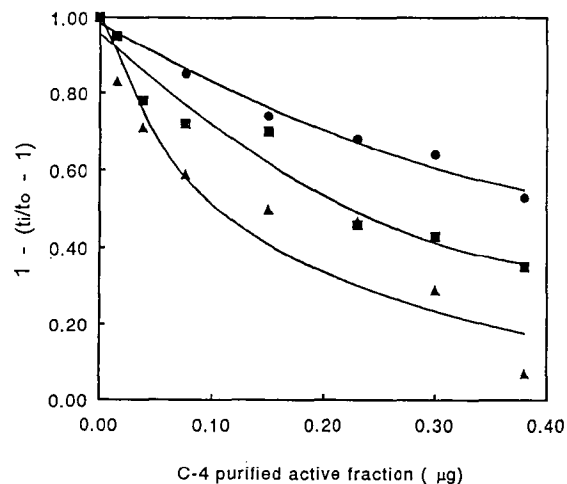
Fig. 4. Effects of the anticoagulant on the APTT (●), the thrombin clotting time (■), and the conversion of fibrinogen (▲). Its effects on increasing the duration of the fibrin clot formations were analyzed by calculating $1-[ti/to-1]$, where ti and to indicated the time required for either the clot formation or the fibrin production in the presence and absence of the C4 purified active fraction, respectively. The conversion of fibrinogen to fibrin was spectrophotometrically monitored at 600 nm.

S-2238 were little affected. It was previously shown that the rate of TAME hydrolysis was influenced by both acylation and deacylation steps, whereas BApNA was catalyzed with a rate-determining step in which deacylation is exclusively involved (Curragh and Elmore, 1964). Our results, therefore, could suggest that the anticoagulant selectively exerts the acylation step of esterase activity, so that the accessibility of TAME to the enzyme has been limited.

In addition, the anticoagulant inhibited the conversion of fibrinogen to fibrin by thrombin and also prolonged the duration required for the fibrin clot formation in both APTT and TCT (Fig. 4). Although there is a possibility that the anticoagulant may interact with other factors besides thrombin in the intrinsic pathway, the parallel correlations between the TAME specific inhibition of the enzyme and the prolongation of APTT and TCT without affecting S-2238 hydrolysis deserve the following considerations. Recently, we have suggested that the esterase activity of thrombin is essential to study any antithombotic drugs especially for those not directed to an active enzyme site (Paik et al., 1996). Although the S-2238 has become the most popular substrate for thrombin research, there have been several examples in which the fibrin clot formations were definitely affected without S-2238 hydrolysis being influenced. The anticoagulant from the earthworm provided another excellent for the case. This result has made us propose that researches into TAME hydrolysis of thrombin would be more appropriate in the study of blood coagulation.

In conclusion, the anticoagulant isolated from the

earthworm was a hydrophilic, heat- and acid-stable macromolecule which not only inhibited the esterase activity of thrombin but also prolonged both the APTT and the TCT. In this report, we have presented evidence that water-extracted anticoagulant indeed exists in L. rubellus. We also suggest the significance of the TAME-hydrolyzing esterase activity of thrombin in the course of drug screening. Although complete purification and characterization of the anticoagulant is not yet finished, we expect that this substance will be very useful, not only as a probe to study the coagulation system, but also for a therapeutic purpose.

Acknowledgement

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