Deoxyribonucleic Acid Was Responsible for the Anticoagulatory Effect of an Earthworm, *Lumbricus rubellus*

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Abstract : Earthworm extracts are known for anti-inflammatory, analgesic, antipyretic, and anticancer effects but can also influence blood circulation. It was previously shown that an earthworm, *Lumbricus rubellus*, contained a water-extractable anticoagulant which was a heat- and acid-stable molecule with hydrophilic property. In order to uncover the biochemical nature of this molecule, the anticoagulant was processed with various hydrolases such as trypsin, DNase, RNase, and lysozome. When the digested samples were analyzed with an *in vitro* coagulation test measuring activated partial thromboplastin time (APTT) and agarose gel electrophoresis, the anticoagulant proved to be a relatively homogeneous DNA fragment with relative molecular size around 72 base pairs. Interestingly, the activity was further stimulated with a trypsin digestion. RNA, on the other hand, did not prolong the APTT. It was also demonstrated that the DNA accelerated the antithrombin III (AT-III) inhibition of thrombin from IC_{50} of 0.34 to 0.16 unit determined with S-2238 as a substrate, whereas heparin, a popular anticoagulant, shifted the value to 0.05. Therefore, it is suggested that the DNA could be considered as an alternative antithrombotic agent to heparin, which would exhibits bleeding side effects.

Key words : anticoagulant, antithrombotic agent, DNA, earthworm.

Earthworm has been recognized in oriental medicine as anti-inflammatory, analgesic, and antipyretic agent (Noda *et al.*, 1992). It shows anticancer effect by preventing excess glucose uptake (Nagasawa *et al.*, 1991). It is also implicated in hemostasis by acting either as a fibrinolytic or anticoagulatory agent, which results in the facilitation of blood circulation (Wang *et al.*, 1989). The earthworm, therefore, has been suspected to contain either proteases which specifically dissolve the fibrin clots or anticoagulant(s) which selectively interfere with the intrinsic pathway of the blood coagulation cascade (Mann *et al.*, 1990; Davie *et al.*, 1991; Leipner *et al.*, 1993; Kim *et al.*, 1995; Woo *et al.*, 1996). It was previously shown that an earthworm, *Lumbricus rubellus*, was an excellent source for proteases with high fibrinolytic activities, whose therapeutic significance was also recognized (Kim *et al.*, 1995). On the other hand, we recently demonstrated that the earthworm contained a water-extractable, acid- and heat-stable anticoagulant which could regulate the pre-existing blood coagulation cascade in vivo (Woo *et al.*, 1996). In this report, we identified the nature of the anticoagulant as deoxyribonucleic acid of a relative molecular size around 72 base pairs. In addition, a possibility of this novel anticoagulant as an alternative antithrombotic agent to heparin was examined.

Materials and Methods

Materials

Adult earthworm, *L. rubellus*, had been provided by Shingal School of Agriculture located at Kihung, Korea. Thrombin (cat. # T6634), antithrombin III (cat. # A 9141), TAME, BApNA, trifluoroacetic acid, Tris, and Sephadex G150-120 were obtained from Sigma Chemical Co. (St. Louis, USA). RNase, lysozyme, heparin, and TPCK-treated trypsin were also purchased from the Sigma Chemical Co. DNase was from Promega (Madison, USA). S-2238 was from Chromogenix (MIndal, Sweden). Dade® Actin® Activated Cephaloplastin Reagent, and Dade® Ci-Trol® Coagulation Control (level I) were from Baxter Diagnostic Inc. (Deerfield, USA). Agarose, Sephacryl S-300 and Sephadex G-75 were provided from Pharmacia (Uppsala, Sweden). A C4 reversed-phase (RP) column was obtained from Mil-

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lipore (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

An *in vitro* coagulation test of APTT was performed according to the manufacturer's instruction. The data was analyzed in % coagulation time with an equation of $[(t_e - t_o)/t_o] \times 100$, where t_o and t_e represented APPT in the absence and presence of the anticoagulant, respectively.

Purification of anticoagulant from *L. rubellus*

A detailed purification procedure for the anti-coagulant can be found elsewhere (Woo *et al.*, 1996). In brief, the earthworm was homogenized in distilled water at a ratio of 1:1 (w/v) and followed by heat ex-traction at 100°C for 30 min. After centrifugation, the supernatant was subjected to ammonium sulfate frac-tionation at final concentration of between 50% and 80%. With the precipitate resuspended in a minimum volume of 50 mM Tris-Cl, pH 8.0, the anticoagulant was purified by three consecutive gel permeation chro-matographies with Sephacryl S-300, Sephadex G-75, and Sephadex G 150-120. The final purification em-ployed was a C4 reversed-phase (RP) HPLC using 80% acetonitrile in 0.1% trifluoroacetic acid (TFA) as an elu-tion buffer. After lyophilization and resuspension in 50 mM Tris-Cl, pH 8.0, the anticoagulatory activity was measured with the APTT test. About 2,800-fold pu-rification was achieved when specific activities were cal-culated with respect to their protein contents. The amount of protein was determined with the method of Bradford (1976).

Treatment of the anticoagulant with various hy-drolases

The concentrated sample of 50 μl (0.92 mg/ml) was separately incubated with trypsin (2 μg), DNase (5 U) in the presence of 2 mM $MgCl_2$, RNase (5 μg), and lysozyme (20 μg) at 37°C overnight in a total volume of 150 μl adjusted with 50 mM Tris-Cl, pH 8.0. After heat inactivation of the enzymes at 100°C for 20 min, the anticoagulatory activities and the contents were analyzed with the APTT test and 1% agarose gel elec-trophoresis, respectively.

Effects of the anticoagulant on antithrombin III in-hibition of thrombin

Antithrombin III (AT-III) inhibition of thrombin was examined in the presence and absence of the an-

ticoagulant with the following chromogenic substrates: N-p-tosyl-L-arginine methyl ester (TAME), N-benzoyl-DL-arginine-p-nitroanilide (BApNA), and H-D-phen-ylalanyl-L-pipecoyl-L-arginine-p-nitroanilide (S-2238). An esterase activity of thrombin was measured directly in a cuvette which contained 0.5 μM thrombin and 1.0 mM TAME in a total volume of 0.6 ml with 50 mM Tris-Cl (pH 8.0). The TAME hydrolysis was con-tinuously monitored at 247 nm by measuring the in-crease in absorbance. On the other hand, the gen-eration of a hydrolyzed product, p-nitroaniline, from either S-2238 or BApNA were continuously measured at 405 nm. S-2238 (1.0 mM) and BApNA (1.0 mM) were reacted with 50 nM and 0.5 μM thrombin, respec-tively. The initial rates were obtained from slopes of the curves within 1 min after the reactions started. Throm-bin inhibitions were analyzed with vi/vo. where vi and vo represented the activities in the presence and ab-sence of AT-III. respectively. From these inhibition curves, IC_{50} were obtained in terms of AT-III units which inhibited the thrombin activity by 50%.

Results and Discussion

Earthworm has been scientifically searched for the presence of natural drugs during the last 40 years (Rosenberg and Ennor. 1959). Although several bioac-tive substances such as lombricine exhibiting antipyretic and anticancer effects (Nagasawa *et al.*, 1991). 1-O-alkyl-sn-glycero-3-phosphocholines as lyso platelet-ac-tivating factors (Noda *et al.*, 1992). and proteases responsible for fibrinolysis have been isolated. a water-extractable anticoagulant from *L. rubellus* has never been recognized until our recent demonstration (Woo *et al.*, 1996). Since a water extract of earthworm ob-tained through a continuous boiling used to be orally administered in oriental medicine. an anticoagulant(s) should overcome hostile environments such as heat treatment. acidic conditions inside stomach. and various hydrolases in the digestive tract for their ultimate des-tinations of the blood circulation. The anticoagulant from the earthworm was not affected by either heat (100°C for 30 min) or acid (0.4 N HCl) treatment. in-dicating that the molecule may not be related to either a heat sensitive structure-based functional protein or polysaccharide with acid-vulnerable glycosidic linkages (Woo *et al.*, 1996).

The anticoagulant was not only hydrophilic but also resistant to a trypsin digestion. At the final purification step employing a C4 reversed-phase HPLC. the an-ticoagulatory activity was eluted in a flow-through frac-tion (Woo *et al.*, 1996). Even after an exhaustive di-

alysis against water. 84% of the initial activty was still retained. which excluded a possiblity that the activity was due to salt effect. Interestingly. an overnight trypsin digestion of the C4 active fraction further stimulated % coagulation time by 14.6±4.3% (Fig. 1A). When the digested sample was analyzed with the same reversed-phase HPLC. the activity was still obtained in the same flow-through fraction. This fact indicated that the activity might not be due to proteins or peptides.

When all the evidences were taken into consideration. the anticoagulant was suspected to be a nucleic acid because protease resistance. hydrophilicity. and acid stability eliminated possibilities of the substance as protein. lipid. and carbohydrate. respectively. In order to verify our assumption. the anticoagulant was digested with various hydrolases such as trypsin. DNase. RNase. and lysozyme and analyzed with the APTT test and 1% agarose gel electrophoresis (Fig. 1). As expected. only the DNase-treated sample markedly shortened the duration required for the fibrin clot formation. The % coagulation time became even negative to −24.8±14.2 %. indicating loss of the anticoagulatory activity (Fig.

(A)

	Trypsin	DNase	RNase	Lysozyme
APTT of control (sec)	62±1.5	45.2±3.6	65.8±1.3	61.1±2.2
APTT of sample (sec)[a]	7.1±1.0	33.7±4.5	63.8±3.7	61.5±0.6
% Coagulation time	14.6±4.3	−24.8±14.2	−3.1±4.4	0.8±2.8

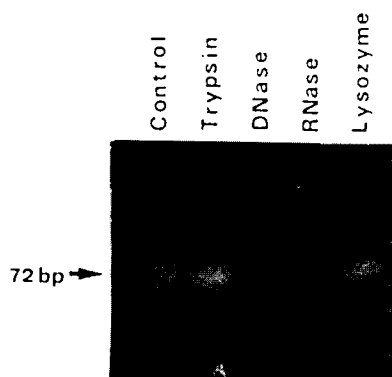[a]$p < 0.05$ vs. control by T-test.

(B)

Fig. 1. Effects of various hydrolases on the anticoagulant. (A) The durations required for the fibrin web formations in the APTT tests are shown in seconds. The differences of APTT with and without the hydrolysis are compared with % coagulation times. The % coagulation time was obtained with an equation of $(t_s / t_c - 1) \times 100$, where t_s and t_c represented APTT with and without digestions by the enzymes, respectively. (B) Agarose gel stained with 1% ethidium bromide after the various hydrolytic reactions. ϕX174 DNA/HaeIII marker was used for size estimation.

1A). The actual APTT after the DNase-treatment became 33.7 sec which was comparable to a control level of 34.6 sec measured in the absence of the anticoagulant. It was also found that Mg^{2+} required for the DNase activity facilitated the coagulation to 45.2 sec from an average of 63.0 sec of other digestion controls. Surprisingly. the anticoagulant was visualized as a relatively discrete band on the agarose gel stained with ethidium bromide. The band was disappeared only after the DNase treatment (Fig. 1B). Taken together, these facts clearly demonstrated that the anticoagulant from L. rubellus was deoxyribonucleic acid with a relative molecular size around 72 base pairs. Another interesting observation was an inverse relationship in % coagulation time between the DNase and the trypsin treatments. which could indicate a possible interaction between the anticoagulatory DNA and proteins. For example, proteolysis of a DNA-protein complex could liberate the DNA as a free anticoagulant. It is also possible that the complex might be necessary for the DNA to reach the blood circulation safely. In other words. the specific complex formation might provide a protection mechanism for the anticoagulant.

Our next question was whether this effect of the DNA was unique for the earthworm, L. rubellus. When salmon sperm DNA was used to measure its effect on the coagulation. it prolonged the APTT (data not shown). The effect was also eliminated by a DNase treatment. Although the salmon DNA was not as homogeneous as the DNA from the earthworm, it could be suggested that DNA with universal anticoagulatory function might be of practical importance. It has been described that a pool of randomly synthesized single-stranded DNA. so-called aptamers, can act as novel structural inhibitors for thrombin and the blood coagulation (Bock, et al., 1992; Griffin et al., 1993). In ad-
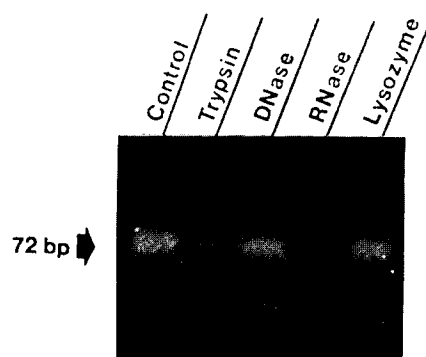

Fig. 2. Agarose gel electrophoresis of the C4-fraction derived from rat liver after the various hydrolase treatments. The 1% gel was visualized with ethidium bromide.

Table 1. IC$_{50}$ of antithrombin III for thrombin in the presence and absence of the anticoagulants

	S-2238	TAME
Control	0.34^a	0.64
DNA^b	0.16	0.33
Heparin^c	0.05	0.38

^{a} Figures are expressed in units of antithrombin III.
^{b,c} The experiments were performed in the presence of 0.2 μg of each anticoagulant.

dition, the aptamers potency as an anticoagulant has been augmented by substituting thymidine with a modified nucleotide, 5-(1-pentynyl)-2-deoxyuridine (Latham *et al.*, 1994). As far as a natural nucleic acid has been concerned, however, this DNA from *L. rubellus* was the first molecule isolated with anticoagulatory activity.

As another member of nucleic acid, a possible function of RNA as an anticoagulant was completely denied by the following control experiment. In order to answer whether the earthworm was the only source for the particular DNA, rat liver was used as a control to follow the same purification procedure employed for the anticoagulatory DNA. Although there was no anticoagulatory activity observed during this purification, the final fraction contained a band on an 1% agarose gel stained with ethidium bromide (Fig. 2). This band disappeared only after a RNase-treatment, indicating RNA did not have the anticoagulatory effect.

The most intriguing phenomenon was that the anticoagulatory activity was exhibited only by DNA. A difference between DNA and RNA would be their extensibilities because single-stranded RNA could be more compact than double-stranded DNA. We assumed, therefore, that the effect was due to negatively charged matrix provided by the extended DNA. If this assumption is valid, the DNA could be compared with heparin in terms of their anticoagulatory mechanisms. It has been already shown that thrombin inhibition by AT-III was accelerated in the presence of heparin since it provides a negatively charged matrix as a template (Nesheim, 1983; Ehrlich *et al.*, 1991). The effect of the earthworms DNA on the AT-III inhibition of thrombin was examined and compared with the result of heparin (Table 1). When the acitivity of thrombin was assayed with TAME, 0.2 μg of the DNA decreased IC$_{50}$ to 0.33 from 0.64 unit of AT-III, while BApNA-hydrolyzing thrombin activity was not affected by the inhibitor. The same amount of heparin (0.2 μg) accelerated the AT-III inhibition to IC$_{50}$ of 0.38, indicating that the DNA

was as effective as heparin on the acceleration of AT-III inhibition for the esterase activity of thrombin. When S-2238 was used as a substrate, however, IC$_{50}$ in the presence of the DNA was 0.16 unit located between the values of 0.34 and 0.05 which were the IC$_{50}$ in the absence and presence of heparin, respectively. The salmon sperm DNA, on the other hand, did not affect the AT-III inhibition (data not shown). Therefore, this moderate acceleration of the AT III inhibition by the DNA led us to cautiously suggest that the DNA could be considered as an alternative antithrombotic agent to heparin which would exhibit bleeding side effects.

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