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A Study of the Anticoagulatory DNA from the Earthworm, *Lumbricus rubellus*, and its Regulatory DNA-Binding Protein

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We have previously shown that a DNA fragment is responsible for the anticoagulatory effect of an earthworm, *Lumbricus rubellus*. The anticoagulant increased the activated partial thromboplastin time (APTT) and also inhibited the thrombin activity observed with either N-α-*p*-tosyl-L-arginine methyl ester (TAME) or H-D-phenyl-alanyl-L-pipecoil-L-arginine-*p*-nitroanilide (S-2238). Since trypsin digestion of the anticoagulant further increased the APTT, the possible presence of a regulatory protein for the anticoagulatory DNA was investigated by digesting the anticoagulant with trypsin and isolating the DNA fragment with C4-reversed phase HPLC. The DNA fragment lacking a regulatory protein was eluted in the flow-through fraction, and analyzed with thrombin and activated factor X. Activated factor X activity was more strongly inhibited than thrombin activity. For DNA digestion, we treated the anticoagulant with DNase and purified the DNA-binding protein with a FPLC Resource-S cation exchange column. This regulatory protein, with an M_r of 55.0 kDa, reduced the anticoagulatory effect of the DNA fragment.

Keywords: Activated factor X, Anticoagulatory DNA, DNA-binding protein, Earthworm, Thrombin.

Introduction

Blood coagulation involves a series of enzyme-catalyzed reactions that lead to a burst of thrombin formation, which ultimately results in the generation of a stable fibrin-based clot. While the extrinsic pathway is activated by tissue factors exposed from the subendothelium only after vascular laceration, the intrinsic pathway can be activated in normal blood circulation even in the absence of external causes. Activated factor X (fXa) plays an essential role in the coagulation cascade since it catalyzes the formation of thrombin, which is common in both pathways. This factor is a vitamin-K-dependent glycosylated serine protease that plays a critical role in maintaining hemostasis (Davie *et al.*, 1991; Mann *et al.*, 1992). With its obvious importances, fXa has become a target for not only research interest but also therapeutic purposes.

Activated factor X is regulated by inhibitors *in vivo* through two different plasma proteases. Antithrombin III (ATIII) is a serpin that, in the presence of heparin or other glycosaminoglycans, results in a rapid and irreversible inhibition of fXa, thrombin, and other plasma proteases (Björk and Danielsson, 1986). The mechanism of heparin-mediated ATIII inhibition is thought to involve either a conformational change in ATIII or the formation of a ternary complex. Another protease inhibitor, tissue factor pathway inhibitor (TFPI), is a protein containing three tandem Kunitz domains and is a slow tight-binding inhibitor of fXa; it also inhibits the tissue factor and factor VIIa (TF·FVIIa) complex in an fXa-dependent manner (Broze *et al.*, 1990). On the basis of its *in vitro* properties, TFPI is thought to regulate the tissue factor inducing the (extrinsic) coagulation pathway by a feedback mechanism (Broze, 1992).

There are several exogenous inhibitors against fXa reported from organisms such as the black fly *Simulium vittatum* (John *et al.*, 1990), lone star tick *Amblyomma americanum* (Kuichun *et al.*, 1997), leech *Haementeria officinalis* (Nutt *et al.*, 1991), and hookworm *Ancylostoma caninum* (Michel *et al.*, 1995). All of these anticoagulants have been identified as either heat-unstable proteins or peptides. However, we reported recently that an earthworm, *Lumbricus rubellus*, contains a water-

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extractable, heat- and acid-stable anticoagulant which turned out to be deoxyribonucleic acid. Interestingly, this anticoagulatory activity was further stimulated upon protease treatment, suggesting that the anticoagulatory DNA could be associated with a possible protein. In this report, we tried to uncover the exact nature of the anticoagulant from the earthworm in respect to its fXa inhibition and possible regulatory protein.

Materials and Methods

Materials Adult earthworm, *L. rubellus*, was provided by the Shingal School of Agriculture located in Kihung, Korea. Factor Xa, thrombin, trifluoroacetic acid, MES, Tris, bovine serum albumin, TPCK-treated trypsin, and Sephadex G150-120 were obtained from Sigma Chemicals Co. (St. Louis, USA). DNase was from Promega (Madison, USA). S-2238 and S-2222 were from Chromogenix (Mölndal, Sweden). Dade Actin Activated Cephaloplastin Reagent and Dade Ci-Trol Coagulation control (level I) were from Baxter Diagnostic Inc. (Deerfield, USA). Sephacryl S-300, Sephadex G-75, and resource-S-cation-exchange FPLC columns 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 An *in vitro* coagulation test of APTT was performed according to the manufacturer's instruction. 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 the absence of the anticoagulant from *L. rubellus*. 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 represent APTT in the absence and presence of the anticoagulant, respectively.

Preparation of the anticoagulatory DNA For this study, the anticoagulant purification procedure by Woo *et al.* (1996) was used. In brief, the earthworm was homogenized in distilled water at a ratio of 1:1 (w/v) followed by heat extraction at 100°C for 30 min. After centrifugation, the supernatant was subjected to ammonium sulfate fractionation at a final concentration of between 50% and 80%. With the precipitate resuspended in a minimum volume of 50 mM Tris-Cl, pH 8.0, the sample was purified by three consecutive gel permeation chromatographies with Sephacryl S-300, Sephadex G-75, and Sephadex G 150-120. The final purification employed a C4 reversed-phase (RP) HPLC using 80% acetonitrile in 0.1% trifluoroacetic acid (TFA) as an elution buffer to give the active fraction named as the anticoagulant. Then, 500 μl of the anticoagulant (1 mg/ml in 50 mM Tris-Cl, pH 8.0) was incubated with trypsin (20 μg) at 37°C overnight. For the purpose of trypsin inactivation and removal, the sample was incubated at 100°C for 20 min and injected into the column pre-equilibrated with buffer A composed of 0.1% trifluoroacetic acid (TFA). Any substances attached to the matrix were eluted with buffer B composed of 80% acetonitrile in buffer A under a linear gradient (0 to 100% buffer B within 50 min). Active fractions were collected, concentrated and then dialyzed with distilled water, yielding the anticoagulatory DNA.

Inhibition test of the anticoagulatory DNA with fXa and thrombin Enzyme activity was observed directly in a cuvette by placing 0.5 μM thrombin, 1.0 mM S-2238 or 0.25 U fXa, 0.4 mM S-2222, and various amounts of the anticoagulant in a total volume of 0.5 ml containing 0.1% BSA and 50 mM Tris-Cl at pH 8.0. The hydrolyzed product, *p*-nitroanilin, from both substrates was continuously monitored by measuring the absorbance at 405 nm. The initial rates of enzymatic activity were obtained from calculating the slopes of the curves within 1 min. Effects of the anticoagulant on thrombin and fXa were analyzed by calculating V/V_o, where V and V_o represent activity in the presence and absence of the anticoagulatory DNA, respectively.

Purification of the regulatory DNA-binding protein The anticoagulant (500 μl, 1 mg/ml in 50 mM Tris-Cl, pH 8.0) was incubated with DNase (5 U) in the presence of 2 mM $MgCl_2$ at 37°C overnight. After heat inactivation of the enzyme, the sample was purified with a FPLC Resource-S-cation-exchange column pre-equilibrated with 20 mM MES, pH 6.5. Any substances attached to the matrix were eluted with a 2.0 M NaCl linear gradient in the equilibrium buffer, from 0 to 100% of the salt solution within 50 min. Each fraction was collected, lyophilized, and dialyzed against distilled water.

Effects of the DNA-binding protein on the anticoagulatory DNA The 380 ng DNA-binding protein was pre-incubated with 0, 100, 200, 300, or 400 ng anticoagulatory DNA at room temperature for 60 min. Each preparation was incubated for 5 min at 37°C with 0.25 U of fXa, and the reactions were carried out in a total volume of 0.5 ml with 0.4 mM S-2222, 0.1% BSA, and 50 mM Tris-Cl, pH 8.0.

Other methods Polyacrylamide gel electrophoresis was performed with a pre-casted 10~20% Tricine gradient gel obtained from Novex™ or the method of Laemmli (1970). The amounts of regulatory DNA-binding protein were determined by the method of Bradford (1976).

Results and Discussion

Preparation of the anticoagulatory DNA Previously, we reported that an earthworm, *Lumbricus rubellus*, contained a hydrophilic, water-extractable anticoagulant that was heat- and acid-stable. The anticoagulant from earthworm inhibited the conversion of fibrinogen to fibrin by thrombin and also prolonged the duration time required for fibrin clot formation in both APTT (activated partial thromboplastin time) and TCT (thrombin clotting time). In addition, the anticoagulant inhibited the esterase activity of thrombin assayed with TAME, but the amidase activities of thrombin measured with L-BapNA or S-2238 were only slightly inhibited (Paik *et al.*, 1996; Woo *et al.*, 1996). In order to uncover the biochemical nature of this molecule, the anticoagulant was processed with various hydrolases

such as trypsin, DNase, RNase, and lysozyme. When the digested samples were analyzed with an *in vitro* coagulation test measuring APTT and agarose gel electrophoresis, the anticoagulant was identified as a DNA fragment. Interestingly, the activity was further stimulated with trypsin digestion but the activity disappeared with DNase digestion. This indicated that the anticoagulatory activity was due to a DNA fragment with a possible involvement of a regulatory DNA binding protein (Paik *et al.*, 1997). We further investigated the properties of the trypsin-digested anticoagulant and the possible existence of a regulatory protein.

Trypsin-digested anticoagulant was separated with a C4 reversed-phase HPLC column, and the active fraction was collected (Fig. 1). The anticoagulatory activity eluted from the flow-through fraction was dialyzed against distilled water, isolating the anticoagulatory DNA fragment. Effects of the anticoagulatory DNA fragment on APTT were examined and compared with the anticoagulant obtained without trypsin digestion (Fig. 2). The activity of APTT was inhibited by 74% at 400 ng of the anticoagulatory DNA fragment, but only by 45% at the same concentration of trypsin-untreated anticoagulant.

Effects of the anticoagulatory DNA fragment on activated factor X (fXa) and thrombin In order to characterize the anticoagulatory DNA fragment, its function as a protease inhibitor was investigated with the serine protease, fXa, and thrombin. Since fXa and thrombin are crucial enzymes for both the intrinsic and the extrinsic coagulation pathways and in the common coagulation pathway, most anticoagulants target fXa or thrombin or both.

The effects of the anticoagulatory DNA were examined with chromogenic substrates such as benzoyl-CO-Ile-Glu(γ-OR)-Gly-Arg-*p*NA·HCl (S-2222) and H-D-Phe-Pip-Arg-*p*NA·2HCl (S-2238), which are specific for fXa and thrombin, respectively. The enzyme activities in the presence of the anticoagulatory DNA are shown in Fig. 3. The inhibition of thrombin and fXa were compared using the same amount of anticoagulatory DNA. It was found that 100 ng of the anticoagulatory DNA inhibited 10% of the thrombin activity whereas it inhibited 90% of the fXa activity. This result shows that the anticoagulatory DNA

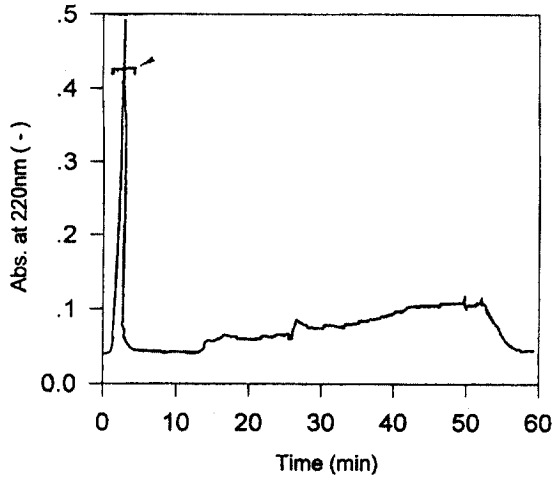

Fig. 1. Elution profiles of anticoagulatory DNA with C4 reversed-phase HPLC. Elution was monitored spectrometrically at 220 nm with the active fraction indicated as an arrowhead with a bracket in the future.

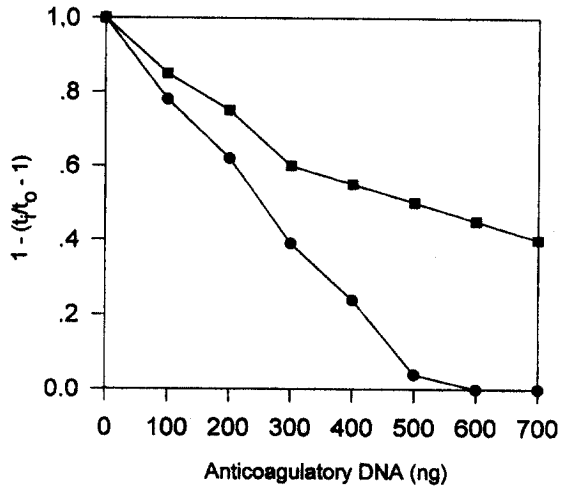

Fig. 2. Effects of the anticoagulatory DNA(●) and the anticoagulant (■) on the APTT. Effects on increasing the duration of fibrin clot formation were analyzed by calculating $1 - [t_i/t_o - 1]$, where t_i and t_o represent the time required for clot formation in the presence and absence of the anticoagulant, respectively.

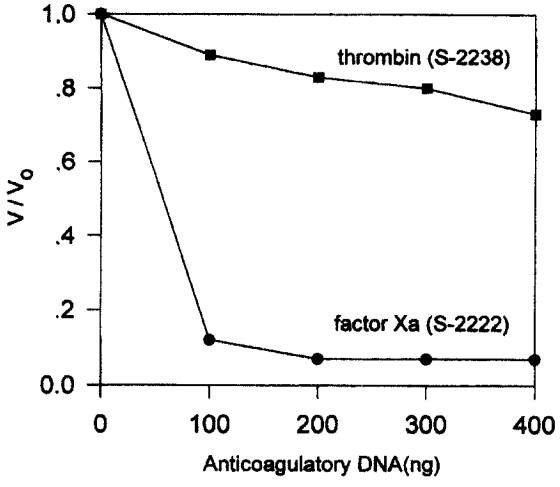

Fig. 3. Effects of the anticoagulatory DNA on thrombin and fXa activity were analyzed with chromogenic substrates S-2238(■) and S-2222(●). Inhibitory effects are shown as V/V_o, where V and V_o represent activities in the presence and absence of the anticoagulatory DNA, respectively.

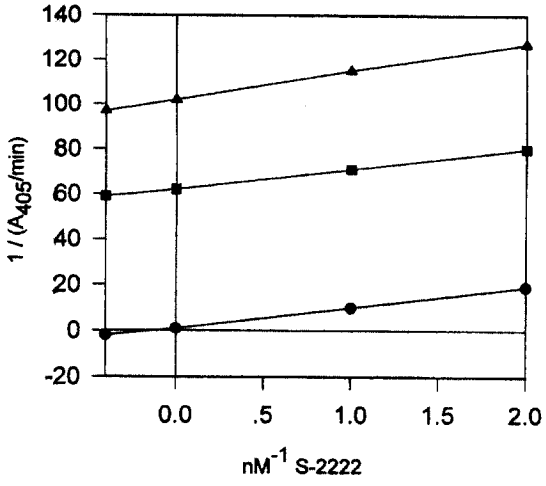

Fig. 4. Double-reciprocal plot of fXa inhibition pattern by anticoagulatory DNA at concentrations of 0 μM (●), 1.8 μM (■), and 2.8 μM (▲), respectively.

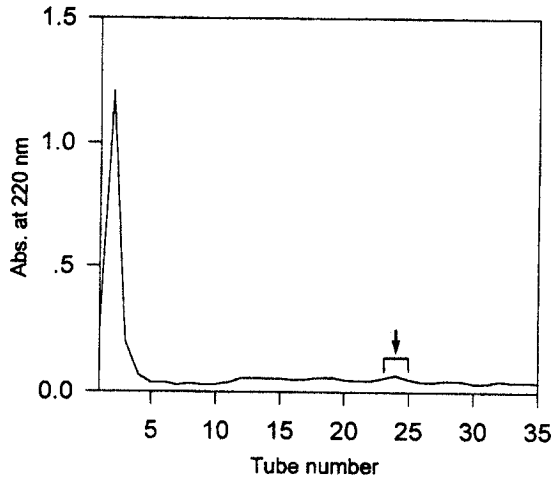

Fig. 5. Elution profiles of regulatory protein with a Resource S FPLC column. Elution was spectrometrically monitored at 220 nm, with the active fraction indicated as an arrowhead.

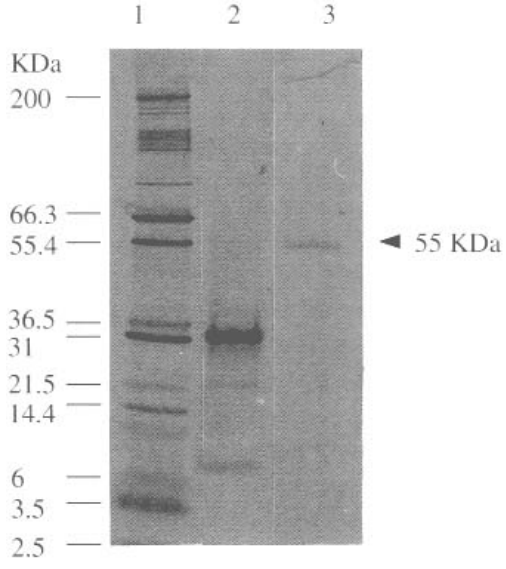

Fig. 6. Gel electrophoresis with a 10–20% Tricine gradient gel from NOVEX™, visualized by silver staining. Lane 1, Marker; Lane 2, DNase; Lane 3, regulatory protein. The protein markers used were: myosin (rabbit muscle), 200 kDa; bovine serum albumin, 66.3 kDa; glutamic dehydrogenase (bovine liver), 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; apotinine (bovine lung), 6 kDa; insulin B chain (bovine pancreas), 3.5 kDa; insulin A chain (bovine pancreas), 2.5 kDa.

was very specific for fXa. Its inhibition pattern was shown to be uncompetitive, as seen with a double-reciprocal plot (Fig. 4). The K_i value obtained with an equation developed for the analysis of uncompetitive inhibitors, $V_i = \{1 + ([I]/K_{ESI})\}/V_{max}$, $K_{ESI} = K_i$, [I] = concentration of substarate (S-2222), was 0.048 μM. Because the uncompetitive inhibitors bind only to the ES complex, we supposed that the anticoagulatory DNA may have bound to the fXa-prothrombin complex.

Purification of the DNA-binding protein Previously, we found that the anticoagulatory activity of the anticoagulant was further increased with the trypsin treatment. Thus, we suspected that the anticoagulant would consist of DNA and its binding protein, which would suppress the DNA's anticoagulatory activity. We isolated the regulatory DNA-binding protein and tested its effects on the activity of anticoagulatory DNA. First, we treated DNase to the anticoagulant at 37°C, overnight, and heat-inactivated. The sample was dialyzed against 20 mM MES, pH 6.5, seperated on a Resource-S-cation-exchange column, and eluted with a 0 to 2 M NaCl gradient (Fig. 5). Each fraction was concentrated, dialyzed against distilled water, and analyzed with a 10~20% Tricine-gradient gel electrophoresis and visualized with silver staining. We obtained a single band with a molecular weight of 55 kDa at fraction #24 and the protein was not related to the treated DNase (Fig. 6).

Interactions between the anticoagulatory DNA and its regulatory protein In order to characterize the purified protein, the anticoagulatory DNA and purified protein were incubated at 25°C for 60 min, and the effects on the fXa were investigated. In the absence of the regulatory protein, anticoagulatory DNA inhibited fXa activity to 80% of control (no anticoagulatory DNA), but this inhibition was completely removed in the presence of the regulatory protein. Since DNA is acidic due to the phosphate group, the binding protein was supposed to be a basic protein. We suspected that the inhibition of the fXa might be due to the negatively-charged DNA, which could be protected by the basic regulatory protein (Fig. 7).

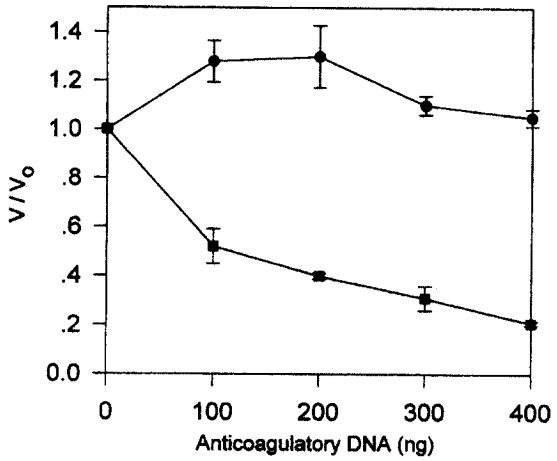

Fig. 7. Effects of the regulatory protein on the anticoagulatory DNA. Activity was detected with fXa and chromogenic substrate S-2222. Effect are shown as V/V_o, where V and V_o represent activities in the presence and absence of the anticoagulatory DNA fraction, respectively. (■), Absence of regulatory protein; (●), presence of 380 ng regulatory protein.

Recent evidences indicated that fXa inhibitors exhibited the increased t-PA induced thrombolysis and prevention of reocculusion in a canine model of arterial thrombosis and suppression of venous thrombosis in a rabbit model (Vlasuk *et al.*, 1991). Furthermore, the fXa has been implicated in determining the procoagulant activity of whole-blood clot (Eisenberg *et al.*, 1993). Therefore, the inhibition of the fXa by natural agents represents an attractive approach for clinical intervention in various thrombotic disorders. In this report, we found that the anticoagulant from *L. rubellus* was heat-stable and its active portion was deoxyribonucleic acid, unlike other fXa inhibitors which were mostly heat-sensitive proteins or peptides. It has been described that a pool of randomly synthesized single-stranded DNA, so-called aptamers, can act as novel structural inhibitors for thrombin (Bock *et al.*, 1992; Griffin *et al.*, 1993). In addition, the potency of an aptamer as an anticoagulant has been augmented by substituting thymidine with a modified nucleotide, 5-(1-pentynyl)-2-deoxyuridine and G quartet form structure (Latham *et al.*, 1994). Further studies are underway to determine whether the anticoagulatory activity is a sequence effect or a conformational effect.

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