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An Anticoagulant/Fibrinolytic Protease from *Lumbricus rubellus*

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Abstract: An anticoagulant/fibrinolytic protease was purified to homogeneity from the earthworm *Lumbricus rubellus*. The protein was a single chain glycoprotein of 32 kDa that exhibited strong proteolytic activity on human thrombin and fibrin clots. Proteolytic degradation of these plasma proteins by the purified enzyme occurred at a neutral pH range. Among several human plasma proteins tested as possible substrates for the protease reaction, the 32 kDa enzyme specifically hydrolyzed both thrombin and fibrin polymers without affecting other proteins, such as serum albumin, immunoglobulin, and hemoglobin. Treatment of the purified enzyme at neutral pH with either phenylmethylsulfonylfluoride or soybean trypsin inhibitor resulted in a loss of catalytic activity. The enzyme hydrolyzed the chromogenic substrate H-D-Phe-L-Pipecolyl-L-Arg-p-nitroanilide with a K_m value of 1.1 μM at a neutral pH. These results suggest that the anticoagulant/fibrinolytic enzyme from *Lumbricus rubellus* is a member of the serine protease family having a trypsin-like active site, and one of the potential clevage sites for the enzyme is the carbonyl side of arginine residues in polypeptide chains.

Key words: anticoagulant, fibrinolytic, protease.

The blood clotting system is composed of intrinsic and extrinsic pathways which produce thrombin by separate initiation processes. Thrombin hydrolyzes fibrinogen to fibrin and factor XIII to XIIIa, forming cross-linked fibrin clots (Jackson, Nemerson, 1980; Lorand *et al.*, 1980). Most of the factors involved in this process have a γ-carboxyglutamyl residue generated by a carboxylase reaction depending on vitamin K, and the residue is thought to have a Ca^{2+} binding site which is required for interaction with membranes and acidic phopholipids.

Dissolution of fibrin in blood clots, called fibrinolysis, usually occurs within a few days after clot formation and is a result of the action of the fibrinolytic system, which is composed of plasminogen, plasmin, plasminogen activator, and α2-antiplasmin (Bachmann, 1987). Because a bleeding risk is raised by excess plamin, the roles of α2-antiplasmin and α2-macroglobulin may be important for physiological homeostasis (Carrel, Boswell, 1986). Activation of plasminogen by the plasminogen activator is regulated within fibrin clots. The plasminogen activator is also regulated by plasminogen activator inhibitor (PAI) (Gurewich, 1988). On the other hand, protein C prevents blood clot formation with thromboplasminogen, or accelerates lysis of fibrin clots by interacting with plasminogen activator inhibitor (PAI) (Anthony, 1991). In spite of regulation, blood clotting and lysis may be disequilibrated by various abnormal factors, leading to serious cardiovascular diseases.

Recently, research progress has been made in developing medicines which prevent thrombus formation, or which dissolve fibrin clots. Plasmin-like enzymes from the snake venom of *Agkistrodon acutus, Crotalus atrox*, and *Agkistrodon halys* have been reported (Ouyang and Huang, 1976; Bajwa *et al.*, 1980, 1981; Chung and Kim, 1991, 1992). Hirudin, an anticoagulant isolated from the leech *Hirudo medicinalis* (Markwardt, 1991), has been fairly well characterized and is now commercially available.

For several thousand years earthworms have been widely used in China and the Far East as a drug material for the treatment of various diseases. It was reported long ago that an enzyme secreted from the alimentary tract of the earthworm could dissolve fibrin (Willem and Minne, 1899). Several kinds of fibrinolytic protease were reported from *Lumbricus rubellus* by Mihara (1991). However, the biochemical characteristics of these enzymes have not yet been reported.

In this work the biochemical and biological properties

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of an anticoagulant/fibrinolytic protease isolated from *Lumbricus rubellus* are reported.

Material and Methods

Materials

The earthworm *Lumbricus rubellus* was obtained from a local farm. Benzamidine-Sepharose 6B, DEAE-Sephacel, Sephacryl S-300, and molecular weight markers for gel filtration were purchased from Phamarcia-LKB (Uppsala, Sweden). Acrylamide and molecular weight markers for SDS-PAGE were bought from BRL (Gaitherburg, USA). Bovine serum albumin (BSA), 2-mercaptoethanol, immunoglobulinG, hemoglobin, phenylmethylsulfonylfluoride (PMSF), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), L-arginine, soybean trypsin inhibitor, and chromogenic substrate were purchased from Sigma Chem. Co. (St. Louis, USA). Fibrinogen and thrombin were obtained from Korea Green Cross Corp. (Seoul, Korea). All other reagents and chemicals used were of analytical quality.

Anticoagulant activity

Anticoagulant activity was estimated by a modification of the clotting time delay method of Baughman (1968). Enzyme samples were incubated with 0.1 ml of a 10 NIH U/ml thrombin solution in 0.05 M sodium phosphate, pH 7.0, containing 0.1 M NaCl for 3 min at 37℃, then 0.5 ml of 0.5% fibrinogen dissolved in the same buffer solution was added. The clotting time delay was then measured. The amount of enzyme required to inhibit 1 NIH unit thrombin per 3 min was defined as 1 U.

Fibrinolytic activity

Fibrinolytic activity was measured by a modification of the fibrin plate method of Astrup and Mullertz (1952) using plasmin as a standard fibrinolytic protease. Five ml of 0.5% human fibrinogen, 0.1 ml of a 100 NIH U/ml thrombin solution, and 2.5 ml of 1.5% agarose were mixed in 0.05 M sodium phosphate, pH 7.0, containing 0.1 M NaCl, and pipetted into a petri dish (100×15 mm). The dish was allowed to stand for 30 min at room temperature to form a fibrin clot layer. Ten μl of each fibrinolytic enzyme sample was dropped onto the fibrin clot layer and incubated at 37℃ for 14 h. The zone clearance areas for standard plasmin at different dilutions were measured, and fibrinolytic activity was expressed in plasmin units.

Zymographic analysis was performed according to a modification of the method of Garneli-Piperno and Reich (1978). After electrophoresis of the enzyme, SDS-PAGE gel was washed in 2.5% Triton X-100 for 30 min under gentle agitation, then extensively rinsed with water . The gel was then put onto the fibrin plate and incubated at 37℃ in a moist atmosphere for an appropriate time until lyzed zones appeared.

Enzyme purification

Enzyme purification was carried out at 4℃. A Teflon-pestle Wheaton Elvehjem homogenizer was used to prepare earthworm homogenate in a solution of 20 mM Tris, pH 7.0. The homogenate was centrifuged at 10,000×g for 30 min, and then the supernatant was treated with $(NH_4)_2SO_4$. A protein precipitate obtained at 20~60% ammonium sulfate saturation was dissolved in a minimal volume of 20 mM Tris, pH 7.0 (buffer A). After dialysis against buffer A the protein solution was then applied to a Benzamidine-Sepharose column (2.2×10 cm) previously equilibrated with the same buffer. Unbound proteins were removed by washing the column with buffer A containing 1 M NaCl, and anticoagulant/fibrinolytic protease activity was eluted with buffer A containing 0.5 M L-arginine. The active fractions were pooled and dialyzed against 10 mM sodium phosphate, pH 8.0 (buffer B). The dialyzed sample was chromatographed on a DEAE-Sephacel column (2.5×10 cm) previously equilibrated with buffer B. After washing, adsorbed proteins were eluted with a linear gradient of 0~0.5 M NaCl in buffer B. Active fractions were collected and concentrated using Centricon. The protein concentration of the purified enzyme was determined by the bicinchonic acid method (Smith, 1985) using bovine serum albumin as a standard protein.

Molecular weight determination

The molecular weight of the denatured protein was determined by 15% SDS-polyacrylamide gel electrophoresis, and the native molecular weight was estimated by gel filtration using a Sephacryl S-300 column (1.5×116.5). Bovine serum albumin (66.2 Kd), ovalbumin (45 Kd), carbonic anhydrase (31 Kd), soybean trypsin inhibitor (21.5 Kd), and lysozyme (14.4 Kd) were used as marker proteins for SDS-PAGE. Size markers for gel filtration were apo-ferritin (480 Kd), γ-globulin (160 Kd), ovalbumin (48 Kd), and ribonuclease A (12.6 Kd).

Carbohydrate staining

Protein glycosylation was identified by the method of Fairbanks *et al.* (1971). After electrophoresis, SDS-PAGE gel was washed in a mixture of 25% isopropanol (v/v) and 10% acetic acid (v/v) for 1 h and left in 0.2% periodic acid for 1 h at 4℃. The gel was then stained with Schiff's reagent for 1 h at 4℃ in the dark and destained with 10% acetic acid.

Inhibition of enzyme activity

Either phenylmethylsulphonylfluoride (PMSF) or 5,5 '-dithiobis(2-nitrobenzoic acid) (DTNB) was dissolved in distilled water at various concentrations. Soybean trypsin inhibitor was dissolved in 20 mM Tris, pH 7.0. After mixing the purified enzyme (6 μg/ml) with each inhibitor, the catalytic activity was measured by the anticoagulant assay, as described above.

Results

Enzyme purification

The anticoagulant/fibrinolytic protease from *Lumbricus rubellus* was purified by a combination of ammonium sulfate fractionation and two chromatographic procedures. The protein precipitate obtained by ammonium sulfate fractionation was redissolved in buffer A and applied to a Benzamidine-Sepharose column. Most of the proteins were removed in the washing step with buffer A. Since benzamidine-Sepharose specifically binds serine protease, application of this affinity procedure at the initial stage of enzyme purification was effective to eliminate other proteins. After additional washing of the adsorbed proteins with buffer A containing 1 M NaCl, the retained anticoagulant/fibrinolytic protease activity was eluted with buffer A containing 0.5 M arginine. The pooled active fractions were dialyzed against buffer B, then the second chromatographic seperation was performed in a DEAE-Sephacel column. The anticoagulant/fibrinolytic activity eluted with a linear gradient of 0~0.5 M NaCl migrated as a single protein band on SDS-PAGE. The purification procedures are summarized in Table 1.

The purified enzyme was found to be homogeneous, not only by SDS-PAGE, but also by gel filtration. The molecular weight of the denatured purified enzyme was 32,000 Da, as shown in Fig. 1. When the SDS-PAGE gel was overlaid on a fibrin-agar plate, a cleared zone on the zymogram appeared at the corresponding position where the protein band was located (Fig. 2). This experimental result clearly indicates that the purified enzyme is able to hydrolyze a fibrin polymer. The enzyme was also identified as a glycoprotein by staining the SDS-PAGE gel with Schiff's reagent and periodic acid (Fig. 2). However, thrombin-mediated conversion of fibrinogen to a fibrin clot was significantly delayed in the presence of the purified enzyme, depending on its concentration. When the purified anticoagulant/fibrinolytic enzyme was passed through a Sephacryl S-300 column, the enzyme activity was eluted as a single, symmetrical peak. At the same time, the native molecular weight was estimated to be 31,600 Da. Therefore, it is surmised that the purified protease from *Lumbricus rubellus* is a monomeric glycoprotein.

Table 1. Purification of 32 KDa anticoagulant/fibrinolytic enzyme from *Lumbricus rubellus*. Enzyme activity was measured by anticoagulant assay method. One unit was defined as the amount of enzyme to inhibit 1 NIH thrombin unit per 3 minute under the experimental condition.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification fold
Homogenate supernatant	500	300	0.6	1
Ammonium sulfate	130	247	1.9	3.2
Benzamidine -Sepharose	9.62	180	18.7	31.2
DEAE-Sephacel	2.5	150	60	100

Biochemical properties

The biochemical nature of the observed anticoagu-

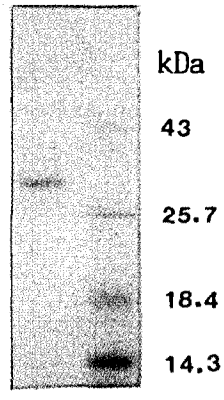

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified anticoagulant/fibrinolytic enzyme from *Lumbricus rubellus*. Purified enzyme (1) was electrophoresed with size markers (2).

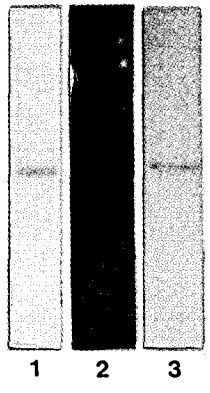

Fig. 2. Zymography and carbohydrate staining of purified enzyme. Purified enzyme on SDS-polyacrylamide gel (1) was zymographed (2) and stained with periodate Schiff's reagent (3).

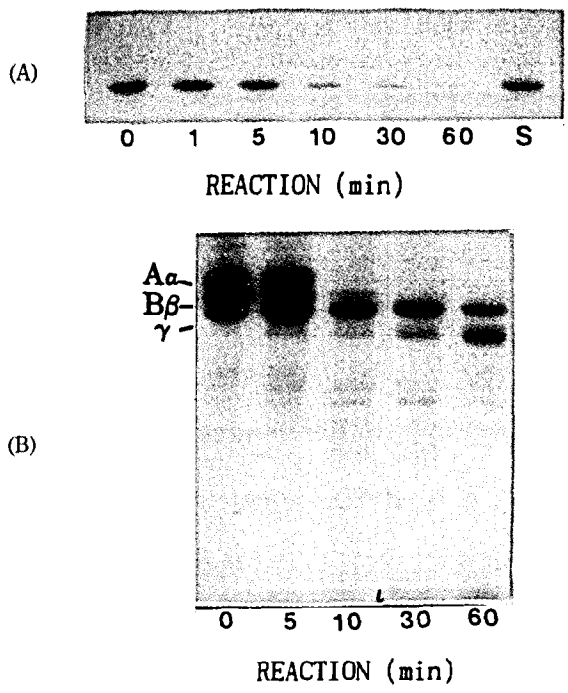

Fig. 3. SDS-PAGE analysis of proteolytic degradation of human thrombin and fibrinogen by the purified enzyme. Thrombin (A) or fibrinogen (B) was reacted with enzyme in 20 mM Tris-HCl,pH 7.0 at 37℃. Thrombin as a standard for autolysis was incubated for 60 min under the same condition(lane S in panel A).

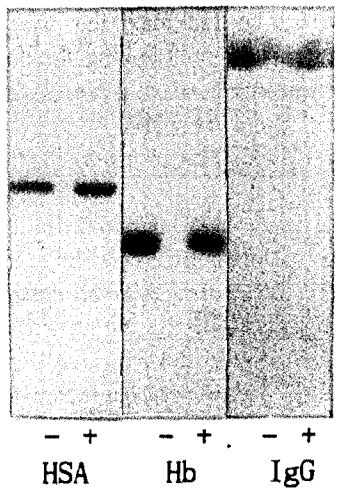

Fig. 4. Non-reduced SDS-polyacrylamide gel electrophoresis of human plasma proteins treated with anticoagulant/fibrinolytic enzyme. Purified enzyme (2.5 μg/ml) was incubated with each plasma protein (2 μg/μl) at 37℃ for 1 h in 20 mM Trix-HCl, pH 7.0. Human serum albumin (HSA), hemoglobin (Hb) or immunoglobulin G (IgG) was reacted with (+) or without (−) enzyme.

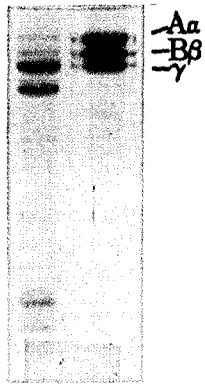

Fig. 5. Inhibition of fibrinogen degradation by anticoagulant/fibrinolytic enzyme treated of phenylmethylsulfonylfluoride (PMSF). Fibrinogen was incubated at 37℃ for 60 min in 20 mM Tris-HCl, pH 7.0 with the purified enzyme; fibrinogen with enzyme (1) and fibrinogen with PMSF-treated enzyme (2).

lant activity of the enzyme was investigated. In order to determine whether thrombin is a proteolytic substrate for the anticoagulant/fibrinolytic enzyme, the reaction product was analyzed by SDS-PAGE. Proteolytic degradation of thrombin by the enzyme was visualized on SDS-PAGE gel as a function of reaction time (Fig. 3A). It was also confirmed that fibrinogen is a member of substrate for the purified enzyme (Fig. 3B). Both the α and β chains of fibrinogen are rather rapidly degraded within 10 min following addition of the anticoagulant/fibrinolytic enzyme. On the other hand, hydrolysis of the γ chain is much slower, but eventually occurs in prolonged incubation with the enzyme for 180 min. The major plasma proteins from a human source, such as serum albumin, immunoglobulin, and hemoglobin, were not affected by the enzyme reaction (Fig. 4). In an attempt to define the potential cleavage site on polypeptide chains, chromogenic peptide substrates were tested with the enzyme. Colorimetric assay results indicated that H-D-Phe-Pipecolyl-Arg-para-Nitro anilide and pyro-Glu-Gly-Arg-para-Nitroanilide are good substrates for the catalytic reaction. Therefore, it seems that cleavage of the carbonyl side of the arginyl residue residue is involved in the proteolytic digestion of thrombin and fibrinogen by the enzyme. Using the chromogenic substrate H-D-Phe-Pipecolyl-Arg-para-Nitroanilide, kinetic constants for amidolytic activity were determined to be K_m=1.1 μM and V_{max}=5.56 μmol/l·min. Both anticoagulant and fibrinolytic reactions of the enzyme proceeded at pH 7.0~8.0. However, the enzymatic activity was strongly inhibited in the presence of divalent metal ions such as Mn^{2+}, Mg^{2+}, and Zn^{2+} while activity was not affected by Na^+ and K^+. The proteolytic activity was also significantly inhibited by the protease inhibitors PMSF (Fig. 5) and soybean trypsin inhibitor. On the other hand, reaction of the enzyme with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), an SH group modification reagant, did not affect its catalytic activity. These results suggest that the anticoagulant/fibrinolytic enzyme belongs to the serine protease family. The enzyme, treated with 2-mercaptoethanol, retained

full fibrinolytic activity, indicating that the disulfide bridge is not responsible for maintaining the functional structure and conformation of the protein molecule.

Discussion

Earthworms have been cultured for use in the disposal of waste materials produced by stock raising, pulp companies, and other industrial processes. Earthworms have also been used in the treatment of various diseases in Asia. In this study, we have demonstrated the purification and biochemical characteristics of an anticoagulant/fibrinolytic enzyme from *Lumbricus rubellus*.

As we have elucidated, it is interesting to note that proteolytic degradation processes for thrombin and fibrinogen shown in Fig. 3. The α and β chains of fibrinogen and thrombin are preferentially degraded by the earthworm enzyme while the γ chain is initially resistant to the proteolytic degradation for 1 h. It is possible that the anticoagulation mechanism of the enzyme can be explained on the basis of proteolytic digestion of both thrombin and fibrinogen. Unlike tissue-type plasminogen activator (tPA), the fibrinolytic mechanism of the enzyme may be attributed to plasmin-like activity which directly hydrolyzes fibrin polymers. Most importantly, the anticoagulant/fibrinolytic enzyme was not able to hydrolyze major plasma proteins, including human serum albumin, immunoglobulin, and hemoglobin, indicating a reasonable substrate specificity which is critical for clinical application. Therefore, the potential value of the enzyme activity may be in preventing thrombus formation and hydrolyzing fibrin clots. On the basis of studies with synthetic peptide substrates to elucidate the possible cleavage site involved in the anticoagulant/fibrinolytic process, it is postulated that the carbonyl side of the arginine residue of the substrate is the hydrolysis target recognized by the enzyme.

Several lines of experimental evidence suggest that the enzyme is a member of the serine protease family, since catalytic activity is rapidly and completely lost by treatment of the enzyme with either PMSF or soybean trypsin inhibitor. It is interesting that many enzymes involved in the blood coagulation cascade belong to the serine protease family (Patty, 1985). Further biochemical information concerning the enzyme will contribute to future therapeutic developments.

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