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Fibrinolytic and Antithrombotic Protease from *Spirodela polyrhiza*

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A fibrinolytic protease was purified from a Chinese herb (*Spirodela polyrhiza*). The protease has a molecular mass of 145 kDa and 70 kDa in gel filtration and SDS-polyacrlamide gel electrophoresis (PAGE), respectively, implying it is a dimer. Its optimum pH was 4.5–5.0. The enzyme was stable below 42°C and after lyophilization. The enzyme activity was inhibited significantly by leupeptin and aprotinin. The protease hydrolyzed not only fibrin but also fibrinogen, cleaving A_α and B_β without affecting the γ chain of fibrinogen. It preferentially cleaved the peptide bond of Arg or Lys of synthetic substrates (P_1 position). The enzyme had an anticoagulating activity measured with activated partial thromboplastin time (APTT), thrombin time (TT), and prothrombin time (PT) tests. It delayed APTT, TT, and PT two times at the concentration of 36, 39, and 128 nM, respectively and this was drastically reduced after heat treatment.

Key words: anticoagulatory; antithrombotic; fibrinolytic protease; *Spirodela polyrhiza*; thrombin time

Blood clots are formed from fibrinogen by thrombin and are lyzed by plasmin, which is activated from plasminogen by a tissue plasminogen activator.[1-3] The blood clotting and lysis systems are tightly regulated. Its disturbance results in serious cardiovascular disease and cerebral infarction. The factors involved in fibrinolysis and thrombolysis are a promising target for chemotherapy.[4-6] Hemorrhagic toxin from the snake venom of *Crotalus atrox*,[7] fibrinolytic and thrombolytic agents from *Lumbricus rubellus*,[8] fibrinolytic enzyme from *Bacillus* sp. in the traditional Korean and Japanese fermented foods, chungkook-jang[9] and natto,[10,11] respectively, an anticoagulant, hirudin from *Hirudo* medicinalis,[12] fibrinolytic protease from *Pleurotus ostreatus*,[13] *Flammulina velutipes*,[14] and *Ganoderma lucidum*[15] have been reported and characterized. *Spirodela polyrhiza* is an ingredient of traditional Oriental medicines. It has been used for lowering blood pressure and detoxification of snake venom. We have purified and characterized a fibrinolytic protease from *Spirodela polyrhiza*. The protease showed anticoagulant activity probably due to its fibrinolytic and antithrombotic activity. This enzyme may be useful in clinical applications for reducing thrombosis.

Materials and Methods

Materials. PM 10 membrane was purchased from Amicon (Bedford, MA). Ammonium sulfate, ethanolamine, *p*-aminobenzamidine-Agarose, bovine fibrinogen, thrombin, subtilisin Carlsberg, human fibrinogen, thiobenzyl benzyloxycarbonyl-L-lysinate (Z-Lys-SBzl), 5,5′-dinitrobis(2-nitrobenzoic acid), heparin (low molecular weight), APTT reagent, and thrombomax reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Phenyl-Sepharose, Polybuffer Exchanger (PBE) 94, Polybuffer 74 and 96 were purchased from Pharmacia (Uppsala, Sweden).

Purification. A dried preparation of *Spirodela polyrhiza* was obtained from a local Oriental medicine supply store. Fifty grams of dry material was resuspended with 300 ml of 10 mM Tris-HCl, pH 8.0 and was disrupted for 5 min on ice with pulses in an ice-chilled bead beater (Biospec Products, U.S.A.). The homogenate was filtered through several sheets of cheesecloth and the filtrate was centrifuged at $10,000 \times g$ for 40 min in a refrigerated centrifuge, T-324 using A-8.24 rotor (Kontron Instruments, Sweden). The supernatant was concentrated by ultrafiltration using PM 10 membrane. Solid ammonium sulfate was added to the clear supernatant to reach the concentration of 1.0 M. The solution was put on a phenyl-Sepharose column (2×7 cm) which was equilibrated with 10 mM Tris-HCl, pH 7.0 containing 1.0 M ammonium sulfate. The column was

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; APTT, activated partial thromboplastin time; TT, thrombin time; PT, prothrombin time; Z-Lys-SBzl, thiobenzyl benzyloxycarbonyl-L-lysinate; PBE, Polybuffer Exchanger; PMSF, phenylmethylsulfonyl fluoride; E-64, trans epoxysuccinyl leucylamide 4-guanidinobutane; SEM, standard error of the mean; TPCK, *N*-tosyl phenylalanine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid

washed with the buffer and the bound protein was eluted with 10 mM Tris, pH 7.0. The enzymatically active fractions were pooled, and concentrated by ultrafiltration with 10 mM Tris-HCl, pH 7.0. A PBE 94 column (1 × 9.4 cm) was equilibrated with 25 mM ethanolamine-HCl, pH 9.4 and the enzyme was eluted at pH ~3.9 with 15 bed volume of Polybuffer 96, pH 7.0 and 15 bed volume of Polybuffer 74, pH 3.0. Desalted preparation was put on a *p*-aminobenzamidine-Agarose column (1.2 × 3 cm) equilibrated with 10 mM Tris-HCl, pH 7.0 and proteins were eluted with 10 mM Tris-HCl and 0.5 M arginine, pH 7.0. Fractions containing fibrinolytic protease activity were pooled and concentrated by ultrafiltration using a PM 10 membrane.

Enzyme activity. The fibrinolytic activity was measured in the fibrin plate by a modification of the method of Jeon *et al.*[8] and also analyzed by SDS-PAGE under reducing conditions. For fibrin plate methods, twelve ml of 0.8% bovine fibrinogen was dissolved with 0.2 M borate buffer, pH 7.8 containing 50 mM NaCl. This was poured into a petri dish and 10 U/ml of thrombin was added, and stirred vigorously. The dish was left for 2 h at room temperature to form a fibrin clot layer. Fifteen μl of each sample was put on the fibrin clot layer and incubated at 30°C for 15 h. The zone of clearance areas in the fibrin plate were measured and the relative activity was compared. One unit was defined as the amount of enzyme which produced a clear zone of 1 mm^2. For SDS-PAGE analysis, human fibrinogen was treated with protease in 50 mM Tris-HCl, pH 8.0 at 37°C. The reaction was stopped by boiling for 3 min. The reaction mixture was combined with SDS sample buffer and boiled for 3 min. Electrophoresis was done on a 10% gel. Gels were stained by Coomassie brilliant blue R-250 and destained by 10% acetic acid and 7% methanol. Proteolytic activities for hemoglobin, immunoglobin, and bovine serum albumin was measured by the methods used for human fibrinogen. The amidolytic activity was measured with several synthetic substrates. Initial rate of hydrolysis were measured using a Kontron spectrophotometer (UVKON 860) equipped with a thermostated cell compartment. Z-Lys-SBzl, a substrate for trypsin-like protease was routinely used for characterization of the enzyme. The reaction mixture was incubated at 30°C with 50 μM Z-Lys-SBzl, 0.7 mM 5,5′-dinitrobis(2-nitrobenzoic acid), and 50 mM Tris-HCl, pH 7.0. The rate of hydrolysis of Z-Lys-SBzl was measured by increase of absorbance at 412 nm due to formation of 3-carboxy-4-nitrophenoxide ion from released benzyl mercaptan and 5,5′-dinitrobis(2-nitrobenzoic acid) ($\varepsilon = 13.6$ mM^{-1} cm^{-1}). Hydrolysis of substrates conjugated to *p*-nitroaniline was measured by monitoring the rate of absorbance at 405 nm from the release of *p*-nitroaniline ($\Delta\varepsilon = 10$ mM^{-1} cm^{-1}). The reaction was monitored for up to 10 min and the rate of reaction was calculated from the straight portion of the activity curve. Five hundred μl of azocasein or azoalbumin (0.6%), 600 μl of 50 mM Tris-HCl, pH 8.0 and an appropriate amount of enzyme were incubated in 1.5-ml microfuge tubes. For each time point, a 200-μl sample was removed, treated with 10% trichloroacetic acid, and neutralized for absorbance at 440 nm.

Anticoagulant activity. Fresh normal human blood (54 ml) was collected in 6 ml of 3.8% sodium citrate. Plasma was prepared with an initial whole blood centrifugation at 400 × *g* for 10 min, and a second centrifugation of the plasma at 800 × *g* for 20 min to eliminate residual platelets. APTT, TT, and PT were measured by the increase of absorbance at 420 nm. A typical sample assay for APTT contained 0.3 ml platelet poor plasma, 0.3 ml APTT reagent, and various concentrations of protease in a final volume of 1 ml. The reaction mixture was incubated at 37°C for 2 min and the reaction was started by adding 8 μl of 1 M $CaCl_2$. That for TT excluded APTT reagent and $CaCl_2$ and the reaction began by adding thrombin. That for PT was started by adding thrombomax reagent. The clotting time was the time when a drastic increase of absorbance occurred (ΔA420 > 0.010). The same method was used to measure reptilase activity from snake venom (*Bothrops atrox*, Sigma) but 4 μg/ml of snake venom was added instead of thrombin.

Results and Discussion

Purification and molecular properties

Protease activity was detected from crude extracts of *Spirodela polyrhiza* and purified using a fibrin plate assay. The enzyme was bound tightly to phenyl-Sepharose and eluted with 10 mM Tris-HCl, pH 7.0. It was eluted at pH ~3.9 on a chromatofocusing gel. Finally purified protease was obtained with elution with arginine through an affinity column of *p*-aminobezamidine-Agarose. The enzyme was purified 14.7-fold with a 6% recovery (Table 1) and elecrophoretically homogeneous. The molecular mass of purified enzyme was 145 kDa on a Sephadex G-150 gel filtration column and 70 kDa on SDS-PAGE (Fig. 1), indicating that the enzyme is a dimer. The effects of pH on the enzyme activity were tested with various buffers. Maximum activity was found in the range of 4.5 to 5.0. Thermal stability of purified protease was measured after treatment at different temperatures for 15 min. Half the activity was lost by incubation at 42°C and totally inactivated at 50°C (Data not shown). Since lyophilization did not affect enzyme activity, the enzyme was stored after lyophilization.

Table 1. Purification of Fibrinolytic Protease in *Spirodela polyrhiza*

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Homogenate	282600	148	1909	100	1.0
Phenyl-Sepharose	84301	27.6	3054	30	1.6
PBE 94	45828	2.4	19095	16	10.0
p-aminobenzamidine-Agrose	16799	0.6	27998	6	14.7

Table 2. Substrate Specificity of Fibrinolytic Protease

Substrate	K_m (M)	V_m (mM sec^{-1} mg^{-1})	Relative V_m/K_m [a] (%)
Z-Lys-SBzl	1.3×10^{-5}	6.1×10^{-2}	326
N-Tosyl-Gly-Pro-Arg *p*NA	1.8×10^{-5}	2.6×10^{-2}	100
N-Tosyl-Gly-Pro-Lys *p*NA	7.6×10^{-5}	2.1×10^{-2}	19
N-Suc-Ala-Ala-Pro-Phe *p*NA	2.1×10^{-4}	1.6×10^{-2}	5.3
N-Suc-Ala-Ala-Pro-Leu *p*NA	3.8×10^{-4}	1.3×10^{-2}	2.4
Lys *p*NA	—	—	—
Arg *p*NA	—	—	—

[a] The value of relative V_m/K_m of each compound was estimated relative to that of N-*p*-Tosyl-Gly-Pro-Arg-*p*NA.

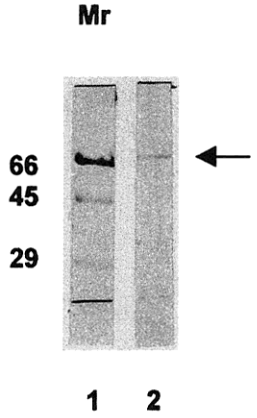

Fig. 1. SDS-PAGE Analysis of Protease.
The M_r standards consisted of bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) in lane 1. The active fraction after mono Q column chromatography was put on a 10% SDS-PAGE in lane 2.

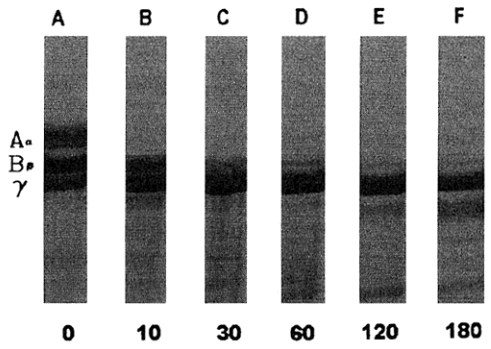

Fig. 2. Protease Activity with Human Fibrinogen as a Substrate.
Degradation of A_α and B_β chain of human fibrinogen was observed as the incubation time (A, 0 min; B, 10 min; C, 30 min; D, 60 min; E, 120 min; F, 180 min) went by at the ratio of substrate/enzyme (10/1, w/w).

Substrate specificity

When the purified enzyme was incubated with human fibrinogen, A_α and B_β chain disappeared without affecting the γ chain as the incubation time progressed (Fig. 2). However, the enzyme did not attack human hemoglobin, immunoglobulin, or bovine serum albumin. No hydrolysis of azocasein and azoalbumin was detected, indicating that it was relatively specific to fibrin or fibrinogen as a protein substrate. As shown in Table 2, the protease most actively hydrolyzed N-Tosyl-Gly-Pro-Arg *p*NA, which is a synthetic substrate for thrombin. A synthetic plasmin substrate, N-Tosyl-Gly-Pro-Lys *p*NA was hydrolyzed in a less degree. Corresponding aminopeptidase substrates, Arg *p*NA and Lys *p*NA were not cleaved at all. Z-Lys-SBzl was also significantly hydrolyzed. The enzyme cleaved the peptide bond which connected basic residues of synthetic substrates at P_1 site. Similar patterns of substrate specificities were observed in other serine proteases like thrombin, trypsin or plasmin.

Effects of group-specific agents on enzyme activity

Fibrinolytic and amidolytic activity of the purified protease in the absence or presence of various reagents is summarized in Table 3. The result of fibrinolytic activity was consistant with that of amidolytic activity with Z-Lys-SBzl as a substrate. The enzyme was strongly inhibited by leupeptin, and aprotinin, but to a lesser degree with phenylmethylsulfonyl fluoride (PMSF) and soybean trypsin inhibitor, indicating it may be a serine protease. The fibrnolytic protease was not sensitive to inhibitors of metalloprotease, such as 1,10-phenanthroline, phosphoramidon, EDTA, and EGTA nor those of cysteine protease including *N*-ethylmaleimide, iodoa-

Table 3. Effects of Inhibitors on Fibrinolytic Protease

Reagent	Concentration (mM)	Enzyme activity (%)[a] fibrin plate	Enzyme activity (%)[a] Z-Lys-SBzl
None		100	100
PMSF	0.1	86	62
	1.0	55	29
Aprotinin	0.015	27	10
Soybean trypsin inhibitor	0.005	55	55
TPCK	1.0	90	85
Leupeptin	0.2	0	6
Pepstatin	0.2	100	94
E-64	0.2	100	100
Bestatin	0.2	103	90
Phosphoamidon	0.01	103	82
Phenanthroline	10	103	86
EDTA	10	100	94
EGTA	10	93	86
N-ethylmaleimide	1.0	100	86
Iodoacetic acid	10	107	93
Dithiothreitol	10	100	96
SDS	0.1%	—	78
Urea	1000	90	77

Each enzyme activity was measured after 1 hr of incubation with various inhibitors at room temperature.
[a] The values are compared with the control (none) = 100%.

cetic acid, and trans epoxysuccinyl leucylamide 4-guanidinobutane (E-64). It was also insensitive to pepstatin, which reacts with aspartic protease and to bestatin, which is known to be an aminopeptidase inhibitor.

Anticoagulant activity

The fibrinolytic protease from *Spirodela polyrhiza* delayed TT, APTT, and PT using human plasma (Fig. 3). Unless specified otherwise, the following coagulation data is presented as the mean ± SEM (n = 6). TT was more sensitive than APTT and PT at the high concentration of the protein. TT was prolonged 2 and 3 times the control value at approximately 39 and 48 nM, respectively. The control TT was 20.0 ± 3.1 seconds. Compared with its effect on TT, the enzyme induced more gradual delay of APTT at high concentrations of the enzyme. APTT required 36 and 89 nM to reach 2 and 3 times the control value (35.4 ± 3.1 seconds). PT was prolonged twice the control value at 128 nM. As a control, heparin prolonged clotting times twice at 1.5 and 3.3 μM for APTT and TT, respectively, in the above system. The anticoagulant activity of *Spirodela* protease was stronger than that of heparin in terms of concentration. At a high concentration of the protease, TT was much more sensitive than PT and APTT. APTT and PT can detect coagulation defects in the intrinsic and extrinsic pathway, respectively. Since degradation or defect of fibrin, fibrinogen, or thrombin could be a cause of prolonged TT, the effect of *Spirodela* protease on thrombin was examined. Clotting of plasma can also be catalyzed by reptilase, an enzyme from the snake venom of *Bothrops atrox*.[16] *Spirodela* fibrinolytic protease did not inhibit this enzyme (Data not shown). The effect on conversion of purified fibrinogen to fibrin by thrombin was also tested. The clotting time induced by thrombin was prolonged, but that by reptilase was not changed. These results demonstrated that *Spirodela* fibrinolytic protease is specific to thrombin. The effect of *Spirodela* fibrinolytic protease on coagulation times for the thrombin catalyzed fibrin formation indicated that the binding of the protease and fibrinogen to thrombin are mutually exclusive. The delay fold of clotting times was calculated as 1/initial velocity. At the lower concentration of fibrinogen the delay of clotting time was more pronounced as shown in Fig. 4. Analysis of

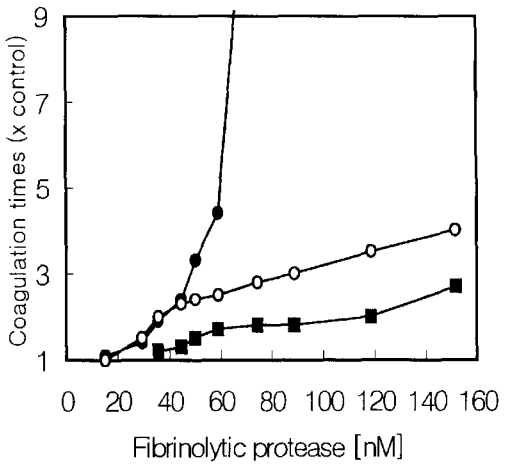

Fig. 3. Anticoagulant Effects of *Spirodela* Protease.
The effect of *Spirodela* protease on TT (—●—), APTT (—○—), and PT (—■—) was measured and data was expressed as the mean (SEM < 5%). Each set of assays was done in a single subject.

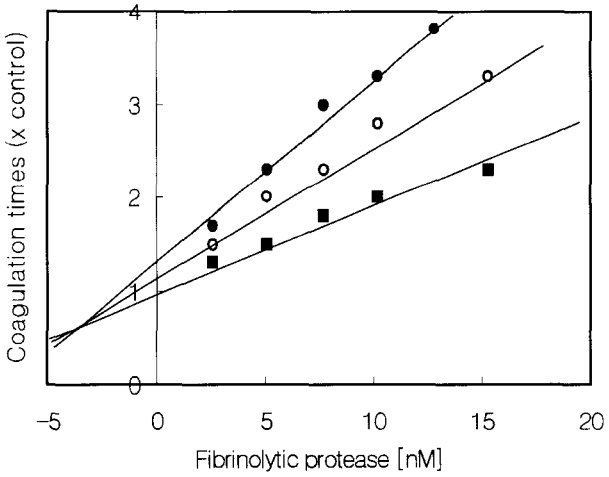

Fig. 4. Inhibition of Clotting by *Spirodela* Protease at Different Fibrinogen Concentrations.

The clotting time was measured by adding thrombin to the indicated concentrations (2.3 μM, —●—; 4.7 μM, —○—; 9.4 μM, —■—) of fibrinogen in 0.2 M borate buffer, pH 7.8 containing 50 mM NaCl at 37°C using the increase in the absorbance at 420 nm.

the effect of *Spirodela* fibrinolytic protease as an inhibitor of thrombin catalyzed fibrin formation yielded an apparent K_i value of 4.5 nM with a competitive inhibition pattern. It indicated that *Spirodela* fibrinolytic protease inhibited thrombin by binding at fibrinogen binding sites which is distinct from the active site. The pI value (3.9) of *Spirodela* protease also supported the idea that the protein bound to a fibrinogen binding site of thrombin which is one of the extended patches of mainly positively charged residues.[17,18] Since *Spirodela* fibrinolytic protease shares synthetic substrates with thrombin, the effect on an active site of thrombin could not be measured using synthetic substrates. The central role of thrombin in the coagulation cascade[19-22] makes thrombin the target of physiological inhibitors in the blood as well as a variety of inhibitors found in blood-sucking animals.[6,12,23-26] The anticoagulation activity of *Spirodela* fibrinolytic protease could be due to not only its fibrinolytic activity, but also antithrombotic activity.

Acknowledgments

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