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# Antithrombotic Effects by Oral Administration of Novel Proteinase Fraction from Earthworm *Eisenia andrei* on Venous Thrombosis Model in Rats

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A novel proteinase fraction, SPP-501, was purified from the earthworm, *Eisenia andrei*, and its antithrombotic effects compared with those of urokinase and t-PA (tissue type-plasminogen activator) in a thrombosis model, induced by the insertion of a stainless wire coil into the inferior vena cava. SPP-501, urokinase and t-PA were administrated once a day for 14 days. On the oral administration of SPP-501, as well as urokinase and t-PA, the thrombus weight was dramatically decreased. The euglobulin lysis time (ELT) was also shortened by SPP-501, but urokinase and t-PA failed to dissolve the euglobulin clot. Conversely, urokinase and t-PA produced detectable fibrinogen/fibrin degradation products (FDP), but SPP-501 did not. Thrombin induced platelet aggregation was desensitized in the SPP-501 treatment groups. With a high dose of SPP-501 (45 mg/kg), the APTT (activated partial thromboplastin time) was prolonged. These results suggest that SPP-501 shows both antithrombotic and fibrinolytic activities when orally administered.

**Key words** Antithrombosis, Fibrinolysis, Lumbrokinases, Venous thrombosis model

## INTRODUCTION

Earthworms have been used as anticoagulant and fibrinolytic medicines in East Asia for several thousands of years. Therefore; in this study, earthworms were investigated to determine the active components of the traditional prescription as starting materials for the development of new antithrombotic and fibrinolytic agents. Mihara *et al.* (1991) reported the extraction of strong and novel fibrinolytic enzymes from the earthworm, *Lumbricus rubellus*. These enzymes were purified into six groups of fibrinolytic enzymes, collectively named lumbrokinases (LKs). The LKs were heat-stable, and showed a broad range of optimal pHs. LKs are fibrin specific proteases, which show high activity in the presence or absence of plasminogen (Nakajima *et al.*, 1993), with molecular weights between 23,500 and 34,200. The enzyme of LKs has been reported to possibly possess therapeutic benefit the treatment of thromboembolic symptoms, due to the potent activity and stability of the enzyme. In this study, the fibrinolytic enzymes (we give a name to SPP-501) were partially purified from the earthworm, *Eisenia andrei*, and the antithrombotic and fibrinolytic effects investigated using a venous thrombosis rat model.

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## MATERIALS AND METHODS

### Materials

The t-PA (Actylase®) was purchased from Boehringer Ingelheim Pharma (Ingelheim am Rheim, Germany), and the Urokinase from Shinpoong Pharm Co. (Ansan, Korea). The thrombin and bicinchoninic acid were purchased from Sigma Chem Co (St. Louis, Mo, U.S.A.). Standard marker proteins were purchased from BIO-RAD (Hercules, CA, U.S.A.). All other chemicals were of analytical grade.

### Animals

Male Sprague-Dawley rats, weighing 250 to 300 g, were purchased from SLC Inc. (Shizuoka, Japan). The rats were housed under standard conditions; temperature 23 ± 2°C, humidity of 50 ± 10%, 12 h light/dark cycles. Food and water

were available *ad libitum*. To ensure adaptation to their new environment, the rats were housed in the departmental holding room for 1 week prior to the experiments.

### Extraction of SPP-501

The sample (SPP-501) used in these experiments was extracted using a modification of the method of Cho *et al.* (2004). The earthworm, *Eisenia andrei,* was vermicultured at a breeding center. Briefly, the live earthworms were washed with distilled water to remove attached mud, homogenized in a homogenizer and then centrifuged at 4,500 g and 4°C for 60 min. The supernatant was filtered with Celite, with 95% EtOH added to the filtered solution to make a 40~80% EtOH solution, which was then centrifuged at 4,500 g and 4°C for 60 min. The precipitate was resuspended in distilled water, filtered with a membrane filter (0.45 μm) followed by an ultra filter (NMWC 10,000). The ultra filtered solution was lyophilized, with the resultant powder designated as SPP-501.

### Measurement of proteolytic activity and purity

The proteolytic activity of SPP-501 was determined as the casein lysis activity, using the method developed by Robbins & Summaria (1976). Briefly, 2 mL of 4% α-casein solution (pH 7.4), 1.6 mL of 67 mM sodium phosphate buffer (pH 7.4) and 0.4 mL of SPP-501 solution (1 mg/mL) were pipetted into a plastic tube at 4°C, with 4 mL of the solution incubated for 30 min at 37°C. The incubation was stopped the addition of 6.0 mL of 15% trichloroacetic acid. The precipitate was removed by filtration through a Whatman, No. 1 filter paper. The absorbance of the solution was read at 280 nm using a spectrophotometer (Hewlett-Packard 8453, Germany). One unit of activity was defined as the quantity of the enzyme able to release 450 μg of TCA-soluble tyrosin in 1 h.

The purity of SPP-501 was determined using by 10% polyacrylamide gel. Protein samples were subjected to electrophoresis in a buffer solution containing 10% glycerol, 1% SDS, 2% β-mercaptoethanol and 0.0025% bromophenol blue.

### Venous thrombosis and treatment of drugs

A venous thrombosis model was developed according to the system performed by Kumada *et al.* (1980). Briefly, the animals were anaesthetized with ketamine (100 mg/kg, i.p.). After making a midline incision, the inferior vena cava was exposed and a stainless steel wire coil (a dental paste carrier, 15 mm long, Dentaurum®, Germany) inserted into the lumen of the inferior vena cava, at the site just below the junction, by puncturing the vein wall with the sharpened end of the wire coil, which was gently twisted toward the iliac vein. The inserted part of the wire coil was 13 to 14 mm long. After the wire insertion, the incision was sutured and disinfected with povidone solution. SPP-501 (5, 15 and 45 mg/mL/kg) was orally administered once a day for 14 days. Urokinse ($3\times10^5$ unit/kg/30 min) and t-PA (1.5 mg/kg/30 min) were intravenously injected into the tail vein once a day for 14 days, using an infusion pump, under ketamine anesthesia. After the last drug administration, the rats were laparotomized under ketamine anesthesia. For coagulation and fibrinolysis assays, 9 mL of blood samples were taken from abdominal aorta into a syringe containing 1 mL of 3.2% sodium citrate. The blood was centrifuged at 4°C and 1800 g for 10 min to obtain platelet-poor plasma (PPP). After clamping the vena cava at both the distal and proximal sites to the inserted wire, the vein wall was longitudinally cut open. The wire was carefully removed, together with the thrombus. The weight of the thrombus on the steel wire was measured, and the total protein content obtained using the bicinchoninic acid method.

### Euglobulin clot lysis time

This assay was performed using a modification to the method described by Copley *et al.* (1959). The euglobulin fraction was prepared by the addition of 0.5 mL of PPP to 6.1 mL of acetic acid solution (0.1 mL of 1% acetic acid in 6 mL of distilled water), placed in a refrigerator for 10 min and centrifuged at 1600 g for 3 min at 4°C. The supernatant was discarded, and the precipitate resuspended in 0.388 mL of a DPBS solution, with 0.012 mL thrombin reagent (100 unit/mL) added, with gentle shaking. The reactants were placed in a water bath at 37°C, and the complete euglobulin clot lysis time (ELT) checked every 5 min.

### Determination of fibrin/fibrinogen degradation products

A fibrin/fibrinogen degradation products (FDP) plasma kit (Diagnostica Stago, France) was used for the detection and semi-quantification of the FDP in the plasma using latex particles coated with monoclonal antibodies to FDP. The assay was performed according to the manufacturer's procedure. A latex suspension was mixed with PPP, with the degree of agglutination compared with positive (5-10 μg/mL) or negative control (less than 2 μg of FDP/mL) sera.

### Coagulation assays

The activated partial thromboplastin time (APTT) and prothrombin time (PT) were determined using a coagulation laboratory device (KukJe Pharm. Co. Japan), with Dade® Actin® Activated cephaloplastin reagent and Dade® Thromboplastin C plus.

### Preparation of washed platelet and aggregation of platelet

Washed rat platelets were obtained using the method described by Radomski and Moncada (1983). Briefly, prostaglandin $E_1$ was added to the blood ($PGE_1$, 2 μg/mL whole blood), centrifuged at 250 g and 15°C for 15 min. The platelet-rich plasma (PRP) was removed, supplemented with 0.3 μg/mL $PGE_1$, and centrifuged at 900 g and 15°C for a further 7 min. The supernatant was discarded, with the platelets resuspended in either 10 mL of washing buffer, containing (mM): NaCl 130, KCl 4.74, glucose 11.5, bovine serum albumin (0.2%), HEPES 10 and EGTA 0.02, along with 0.3 μg/mL $PGE_1$, or in suspension buffer, containing (mM): NaCl 130, KCl 4.8, $NaHCO_3$ 4, $MgCl_2$ 1.2, $KH_2PO_4$ 1.2, $CaCl_2$ 1.8, glucose 11.5, bovine serum albumin (0.2%), HEPES 10 and EGTA 0.02, without $PGE_1$, to a final concentration of $3\times10^8$ platelets/mL. The platelet suspensions were stored at 4°C. 0.5 mL of the platelet suspension was warmed to 37°C in a flat glass cuvette, with stirring at 1000 rpm. Platelet aggregation was induced by thrombin (2 units/mL), and the degree of aggregation measured in a dual-channel aggregometer (model 560-VS, Chrono-Log Corp, U.S.A.) within 1 h of finally being resuspended.

### Statistical analysis

Statistical analyses were performed using either the Student's *t*-test or ANOVA. $P<0.05$ and $P<0.01$ were taken to indicate statistical significance, as shown in the appropriate figure legends.

## RESULT

### Extraction of SPP-501

The fibrinolytic enzyme fraction, SPP-501, from the earthworm was extracted by ethanol precipitation and ultra filtration. The proteolytic activity of SPP-501 was 17,300 unit/g, with a recovery of 86% (Table I). The molecular weight of SPP-501 was estimated to be between 10,000 and 70,000 from SDS-PAGE (Fig. 1). SPP-501 contained not only the six fractions identified by Mihara *et al.* (1991), but one higher molecular weight and four lower molecular weight fractions, and one fraction with a molecular weight between those of LKF4 and LKF5.

**Table I.** Comparison of the yields and proteolytic activities of the materials obtained from *Eisenia andrei*

| Materials | Total Protein (g) | Total Activity (Unit) | Specific Activity (Unit/g) | Recovery (%) | Purification (Fold) |
|---|---|---|---|---|---|
| Homogenate | 12.0 | 10440 | 870 | 100 | 1.0 |
| EtOH extract | 4.0 | 9440 | 2360 | 90 | 2.7 |
| Ultra filtrate, SPP-501 | 0.7 | 9017 | 17300 | 86 | 19.8 |

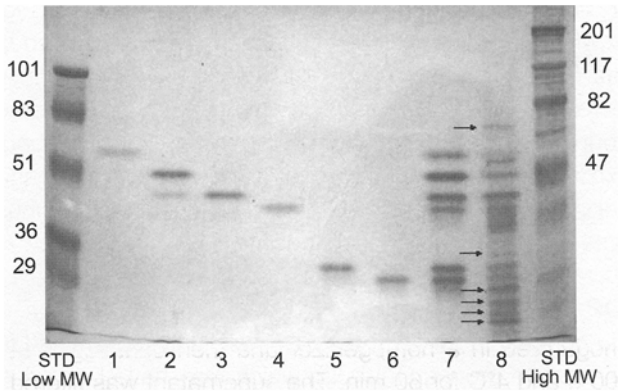


**Fig. 1.** The polyacrylamide gel electrophoresis patterns of the purified antithrombotic enzyme, SPP-501. Electrophoresis was undertaken on 10% polyacrylamide gel, using the procedure of Laemmli, with either 20 or 40 μg of purified enzymes. The standard low molecular weight markers (STD Low MW) consisted of phosphorylase (101,000), bovine serum albumin (83,000), ovalbumin (50,600), carbonic anhydrase (35,500) and soybean trypsin inhibitor (29,100). The standard high molecular weight markers (STD High MW) consisted of myosin (201,000), β-galactosidase (117,000), bovine serum albumin (82,000) and ovalbumin (47,000). The MW of SPP-501 was estimated to be between 10,000 and 75,000 (lane 1, LKF1; lane 2, LKF2; lane 3, LKF3; lane 4, LKF4; lane 5, LKF5; lane 6, LKF6; lane 7, mixture of the six LK fractions; lane 8, SPP-501). Arrows (→) indicate other new bands other than the six for lumbrokinases.

### Antithrombotic effect

In the model used in this study, thrombosis was induced by the insertion of a wire coil into the inferior vena cava. When SPP-501, t-PA, and urokinase were administered

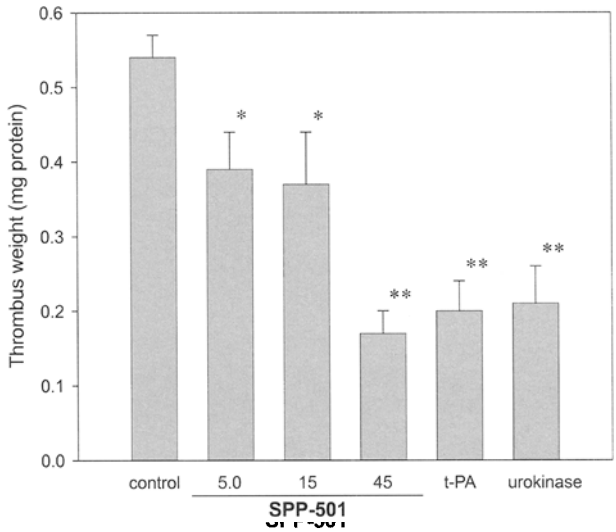


**Fig. 2.** The Effects of SPP-501, t-PA and Urokinase on the Thrombus Formation in Rats. Thrombus weight changes were determined after the administration of the drugs into a venous thrombosis rat model. SPP-501 was orally administered once a day for 14 days. Urokinase and t-PA were intravenously injected into the tail venous once a day for 14 days under ketamine anesthesia. Data are expressed as the mean ± S.E. *p<0.05, **p<0.01; significantly different from the vehicle control group.

for 14 days, the thrombus weights were decreased (Fig. 2). The thrombus weight of the negative control group was 0.54 mg. The treatment with SPP-501 significantly decreased the thrombus weights. In the high SPP-501 dose group (45 mg/kg), the thrombus was decreased about 68.5% that of the negative control group. Also, t-PA and urokinase, used as positive drugs, decreased the thrombus sizes by 63.0 and 61.1%, respectively.

### FDP levels

In contrast to the ELT, the FDP was only determined in the positive groups (Table II), which may have been due to the short half-life time of FDP.

### Fibrinolytic effect

The ELT was used in a screening procedure for measuring the fibrinolytic activity (Table III). In all the SPP-501 treatment groups, the ELT became shorter. However, in the t-PA or urokinase treatment groups, the ELT was not determined. It might be suspected that fibrinolytic enzymes or relative zymogens, for instance plasmin, were affected by the administration of strong proteases administered, i.e. t-PA or urokinase.

### Clotting time

**Table II.** The effects of SPP-501, t-PA and urokinase on the serum FDP in the venous thrombosis rat model

| Groups | Dose (mg/kg/day) | FDP | |
|---|---|---|---|
| | | – | + (2.5 μg/mL) |
| Control | – | 10/10 | 0/10 |
| t-PA | 1.5† | 3/9 | 6/9 |
| Urokinase | $3\times10^5$ †† | 4/10 | 6/10 |
| | 5 | 10/10 | 0/10 |
| SPP-501 | 15 | 10/10 | 0/10 |
| | 45 | 10/10 | 0/10 |

†, mg/kg/30min/day (i.v.); ††, unit/kg/30 min/day (i.v.).

**Table III.** Activated partial thromboplastin time (APTT), prothrombin time (PT) and euglobulin clot lysis time (ELT) in the venous thrombosis rat model

| Groups | Dose (mg/kg/day) | N | APTT (sec) | PT (sec) | ELT (min) |
|---|---|---|---|---|---|
| Normal Control | – | 30 | 24.3 ± 0.4 | 18.4 ± 0.1 | 46.4 ± 2.0 |
| Negative Control | – | 30 | 22.8 ± 0.2** | 17.7 ± 0.1** | 50.2 ± 2.1 |
| t-PA | 1.5† | 9 | 22.0 ± 0.4 | 17.8 ± 0.1 | N.D.$ |
| Urokinase | $3\times10^5$ †† | 29 | 29.7 ± 4.7 | 19.0 ± 0.5* | N.D.$ |
| | 5.0 | 26 | 22.9 ± 0.3 | 17.4 ± 0.1 | 47.5 ± 1.9 |
| SPP-501 | 15 | 20 | 23.3 ± 0.3 | 17.7 ± 0.1 | 44.8 ± 2.1* |
| | 45 | 20 | 23.6 ± 0.2** | 17.6 ± 0.1 | 41.5 ± 2.6* |

†, mg/kg/30 min/day (i.v.); ††, unit/kg/30 min/day (i.v.). Data are expressed as the mean ± S.E. $, N.D.: not determined (until 120 min after clotting). *, $p<0.05$ vs. negative control; **, $p<0.05$ vs. normal control.

**Table IV.** Antiplatelet effects of SPP-501 in the venous thrombosis rat model

| Groups | Dose (mg/kg/day) | N | Platelet ($\times10^8$/mL) | Aggregation (%) |
|---|---|---|---|---|
| Normal control | - | 30 | 18.9 ± 0.8 | 89.6 ± 1.8 |
| Negative control | - | 30 | 17.0 ± 1.0 | 89.1 ± 2.1 |
| t-PA | 1.5† | 9 | 19.7 ± 3.6 | 94.8 ± 1.6 |
| Urokinase | $3\times10^5$ †† | 29 | 19.9 ± 5.0 | 87.8 ± 2.4 |
| | 5.0 | 26 | 18.6 ± 0.7 | 84.0 ± 3.4 |
| SPP-501 | 15 | 20 | 17.2 ± 0.9 | 79.2 ± 3.7* |
| | 45 | 20 | 17.8 ± 0.9 | 75.6 ± 4.4* |

†, mg/kg/30min/day; ††, unit/kg/30 min/day. Data are expressed as the mean±S.E. *$p<0.05$ vs. negative control.

Due to the induction of a thrombosis, the clotting times of APTT and PT in the negative (thrombosis-bore and not treated to any drugs) control group were significantly shorter than that of the normal control group (Table III). However, in the high SPP-501 dose group (45 mg/kg), the clotting time recovered to normal values. In contrast, in the t-PA group, no recovery of the clotting time was observed. The venous administration of urokinase influenced both the PT and APTT.

### Inhibition of platelet aggregation

The effect of antithrombosis by SPP-501 was investigated with respect to platelet aggregation (Table IV). SPP-501 was found to dose-dependently inhibit the aggregation of washed rat platelets induced by thrombin. However, neither t-PA nor urokinase inhibited the aggregation.

## DISCUSSION

Hemostasis, the arrest of bleeding from an injured blood vessel, requires the combined activities of vascular, platelet and plasma factors, counterbalanced by mechanisms to limit the accumulation of platelets and fibrin to the area of the injury. Fibrinolytic enzymes dissolve fibrin, the main protein component of blood clots, and medications containing these enzymes are the most effective methods used for the treatment of thrombosis. A variety of therapeutically important fibrinolytic enzymes, such as urokinase, streptokinase, tissue-type plasminogen activator (t-PA), staphylokinase and recombinant prourokinase, have been extensively investigated and used clinically as thrombolytic agents. However, these enzymes, which are applied *via* intravenous administration, are expensive and suffer from a number of significant limitations, including fast clearance during circulation in blood prior to showing their therapeutic effects and lack of resistance to reocclusion, as well as bleeding complications. In addition, various side effects, such as anaphylaxis and immunoreactions, are often caused by the administration of foreign proteins.

The earthworm has traditionally been used as a medicine for thrombotic disorders in oriental countries. However, despite several reports on the application of earthworm powder for thrombotic disorders, there have been few scientific studies to find the mechanism or to characterize the active components (Chang *et al.*, 1995, Hahn *et al.*, 1997, Kim *et al.*, 1998). Since earthworm powder is usually administered orally, it should be clarified whether the LKs in the powder are absorbed from the intestinal tract into the systemic blood for their activities to be displayed.

The fibrinolytic system primarily consists of the precursor enzyme, plasminogen, which circulates in the plasma as glu-plasminogen (Becker, 1991). Two plasminogen activators; t-PA and urokinase, mediate the critical conversion of plasminogen to plasmin. Natural inhibitors of fibrinolysis block either plasmin or the two activators of plasminogen. Plasmin is rapidly inactivated by the interaction, *via* its lysine site, with its inhibitor, $\alpha$2-antiplasmin. Plasminogen activator inhibitors (PAI) inhibit the plasminogen activators, of which, PAI-1 in the plasma is known as the main inhibitor of t-PA and urokinase (Vassalli *et al.* 1991). Its expression and production are under complex control, and the level of PAI-1 in the plasma has a significant relationship with thrombosis formation (Sawdey and Loskutoff, 1991). Taking these facts into consideration, it can be postulated that some earthworm components, e.g. proteases, may act on fibrin, and/or modulate the levels of $\alpha$2-antiplasmin or PAI-1.

The intestinal absorption of proteins (e.g. fibrinolytic enzymes) remains a controversial, as the passage of high molecular weight molecules through the gastric and intestinal mucosa, in the intact state, coupled with their charge density, is difficult to accept. There have been similar reports showing enhanced fibrinolytic activity after the oral administration of either urokinase or nattokinase (Sumi *et al.*, 1980; Sumi *et al.*, 1990). However, the possibilities that small molecular components of the enzymes can enter the blood stream and act as inducers of the plasminogen activator bound to the endothelium can not be over ruled. Recently, LK III-1 was demonstrated to be absorbed, intact, into the blood through the intestinal epithelium, with the maximum activity in blood found after around 60 min (Fan *et al.*, 2001; Wu *et al.*, 2002).

In the present study, the antithrombotic and fibrinolytic enzymes, SPP-501, were purified from the earthworm, and found to contain six LK fractions (Fig. 1). Of the SPP-501 fractions, LKF3 was found to have the highest concentration. SPP-501 was also found to contain a few new proteolytic enzymes, other than the six lumbrokinase fractions.

Previous observations have presented the potential antithrombotic potential of LKs (Ryu *et al.*, 1993, 1994, 1995; Jeon *et al.*, 1995; Hrzenjak *et al.*, 1998; Park *et al.*, 1999; Jin *et al.*, 2000; Hwang *et al.*, 2002; Cho *et al.*, 2004). Ryu *et al.* (1995) immobilized LK on a polyurethane surface, *via* a maleic anhydride/methyl vinyl ether copolymer, and demonstrated that LK-immobilized polyurethane had specificity for fibrinogen and fibrin, resulting in a non-thrombogenic surface. When LK was used in a patient cerebral infarction, the kaolin partial thromboplastin time (KPTT) was prolonged, but the t-PA activity and D-dimer level were increased, while the fibrinogen content was significantly decreased (Jin *et al.*, 2000). Consequently, they suggested that LK might be used for the prevention of cerebral infarction and a second cerebral infarction in patients with a previous cerebrovascular ischemic event.

In our experimental venous thrombosis rat model, SPP-501 (45 mg/kg, p.o.) dramatically decreased the thrombus, and the effect was similar to those obtained with the intravenous injection of either t-PA or urokinase. The antithrombotic effect of SPP-501 treatment is considered to be due to the proteolytic enzymes absorbed into the blood steam prolonging the PT and APTT, with the inhibition of the aggregation of washed rat platelets induced by thrombin. Conversely, SPP-501 enhanced the fibrinolytic activity and ELT. The results showed that SPP-501 evoked, in a complex way, the coagulation and fibrinolytic system in blood, *via* an antithrombotic effect in our venous thrombosis rat model.

## REFERENCE

Becker, R. C., Seminars in thrombosis, thrombolysis and vascular biology. 4. Fibrinolysis. *Cardiology*, 79, 188-210 (1991).

Chang, C. S., Lee, C. K., Shin, J. S., Cho, I. H., and Suh, J. J., Fibrinolytic and anticoagulant effects of earthworm, *Lumbricus rubellus,* extracts. *Yakhak Hoeji,* 39, 666-670 (1995).

Cho, I. H., Choi, E. S., Lim, H. G., and Lee, H. H., Purification and characterization of six fibrinolytic serine-proteases from earthworm *Lumbricus rubellus*. *J. Biochem. Mol. Biol.*, 37, 199-205 (2004).

Cho, I. H., Lee, C. K., Lim, H. G., and Lee, H. H., Pharmaceutical characteristics of korean *Lumbricus rubellus* lumbrokinase. *Korean J. Biotechnol. Bioeng.*, 19, 274-283 (2004).

Copley A. L., Niewiarowski, S., and Marechal, J., A micromethod of euglobulin fibrinolysis in plasma of human subjects and small laboratory animals. *J. Lab. Clin. Med.*, 53, 468-473 (1959).

Fan, Q., Wu, C., Li, L., Fan, R., Wu, C., Hou, Q., and He, R., Some features of intestinal absorption of intact fibrinolytic enzyme III-1 from *Lumbricus rubellus*. *Biochim. Biophys. Acta.,* 1526, 286-292 (2001).

Hahn, B. S., Jo, Y. Y., Yang, K. Y., Wu, S. J., Pyo, M. K., Yun-Choi, H. S., and Kim, Y. S., Evaluation of the *in vivo* antithrombotic,

anticoagulant and fibrinolitic activities of *Lumbricus rubellus* earthworm powder. *Arch. Pharm. Res.,* 20, 1-23 (1997).

Hrzenjak, T., Popovic, M., Bozic, T., Grdisa, M., Kobrehel, D., and Tiska-Rudman, L., Fibrinolytic and anticoagulative activities from the earthworm *Eisenia foetida*. *Comp. Biochem. Physiol.,* 119, 825-832 (1998).

Hwang, C. M., Kim, D. I., Kim, J. E., Huh, S. H., Min, B. G., Park, J. H., Han, J. S., Lee, B. B., Kim, Y. I., Ryu, E. S., and Kim, J. W., *In vivo* evaluation of lumbrokinase, a fibrinolytic enzyme extracted from *Lumbricus rubellus*, in a prosthetic vascular graft. *J. Cardiovasc. Surg.*, 43, 891-894 (2002).

Jin, L., Jin, H., Zhang, G., and Xu, G., Changes in coagulation and tissue plasminogen activator after the treatment of cerebral infarction with lumbrokinase. *Clin. Hemorheol. Microcirc.,* 23, 213-218 (2000).

Kim, Y. S., Pyo, M. K., Park, K. M., Hahn, B. S., Yang, K. Y., and Yun-Choi, H. S., Dose dependency of earthworm powder on antithrombotic and fibrinolitic effects. *Arch. Pharm. Res.,* 21, 374-377 (1998).

Kumada, T., Ishihara, M., Ogawa, H., Abiko, Y., Experimental model of venous thrombosis in rats and effect of some agents. *Thrombosis Res.*, 18, 189-203 (1980).

Mihara, H., Sumi, H., Akazawa, K., Yoneds, T., Mizumodo, H., and Maruyama, M., A novel fibrinolytic enzyme extracted from the earthworm *Lumbricus rubellus*. *Jap. J. Physiol.,* 41, 461-472 (1991).

Nakajima, N., Mihara, H., and Sumi, H., Characterization of potent fibrinolytic enzymes in earthworm: *Lumbricus rubellus*. *Biotech. Biochem.,* 57, 1726-1730 (1993).

Park, Y. D., Ryu, E. S., Kim, H. J., Jeong, J. S., Kim, J. H., Jeon, S.M., Jo, Y. H., Kim, W. K., and Min, B. G., Characterization of antithrombotic activity of Lumbrikinase-immobilized polyurethane valves in the total artificial heart. *Artif. Organs,* 23, 210-214 (1999).

Radomski, M. and Moncada, S., An improved method for washing of human platelets with prostacyclin. *Thromb. Res.,* 30, 383-389 (1983).

Robbins, K. C. and Summaria, L., Plasminogen and plasmin. *Methods Enzymol.*, 45, 257-273 (1976).

Ryu, G. H., Park, S. Y., Han, D. K., Kim, Y. H., and Min, B. G., Antithrombotic Activity of a Lumbrikinase immobilized polyurethane surface. *ASAIO J.,* 39, M314-M318 (1993)

Ryu, G. H., Park, S. Y., Kim, M.R., Han, D. K., Kim, Y. H., and Min, B. G., Antithrombogenicity of Lumbrikinase-immobilized polyurethane. *J. Biomedi. Mater. Res.,* 28, 1069-1077 (1994).

Ryu, G. H., Han, D. K., Park, S. Y., Kim, M. R., Kim, Y. H., and Min, B. G., Surface characteristics and properties of Lumbrikinase-immobilized polyurethane. *J. Biomedi. Mater. Res.,* 29, 403-409 (1995).

Sawdey, M. S. and Loskutoff, D. J., Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. Tissue specificity and induction by lipopolysaccharide, tumor necrosis factor-alpha, and transforming growth factor-beta. *J. Clin. Invest.,* 88, 1346-1353 (1991).

Sumi, H., Sasaki, K., Toki, N., and Robbins, K. C., Oral administration of urokinase. *Thromb. Res.,* 20, 711-714 (1980).

Sumi, H., Hamada, H., Nakanishi, K., and Hiratani, H., Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. *Acta Haematol.,* 84, 139-143 (1990).

Vassalli, J. D., Sappino, A. P., and Belin, D., The plasminogen activator/plasmin system. *J. Clin. Invest.*, 88, 1067-1072 (1991).

Wu, C., Li, L., Zhao, J., Fan, Q., Tian, W. X. and He, R. Q., Effect of $\alpha$2-M on earthworm fibrinolytic enzyme III-1 from *Lumbricus rubellus*. *Int. J. Biol. Macromol.*, 31, 71-77 (2002).