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A Novel Fibrinolytic Enzyme Extracted from the Earthworm, *Lumbricus rubellus*

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Abstract A strong fibrinolytic enzyme was readily obtained in saline extracts of the earthworm, *Lumbricus rubellus*. It hydrolyzed not only plasminogen-rich fibrin plates, but also plasminogen-free fibrin plates. The average fibrinolytic activity was about 100 CU (plasmin units) or 250 IU (urokinase units)/g wet weight. The molecular weight and isoelectric point were about 20,000 and 3.4, respectively. The enzyme was heat-stable and displayed a very broad optimal pH range. DFP and SBTI strongly inhibited the enzyme, but the anti-plasmin agent, t-AMCHA, exerted little effect under the same conditions. Purification of the enzyme was performed and three partially purified fractions were obtained. These three fractions were further subdivided. The first fraction (F-I) was divided into three fractions (F-I-0, F-I-1, and F-I-2), which exhibited similar biochemical characteristics. The second fraction (F-II) could not be subdivided. The third fraction (F-III) was divided into two fractions (F-III-1 and F-III-2). Based on results for their enzymatic activities against various substrates, the fraction I enzymes are thought to represent a chymotrypsin-like enzyme and the fraction III enzymes to represent a trypsin-like enzyme. The fraction II enzyme appears to be neither a trypsin- or chymotrypsin-like enzyme nor an elastase. The amino acid compositions of the six enzymes were estimated. Compared with other serine enzymes, these enzymes contained very abundant asparagine or aspartic acid, and there was very little proline or lysine. From the above data, these enzymes are regarded as novel fibrinolytic enzymes, and we name them collectively as Lumbrokinase from the generic name of the earthworm.

Key words: earthworm, fibrinolytic enzyme, serine protease, thrombolysis.

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For several thousand years, the earthworm has been rather widely used in China and the Far East as a drug material for the treatment of various diseases. However, detailed pharmacological studies have not yet been undertaken except on lumbrofebrin as an antifibrile. We recently obtained a novel fibrinolytic enzyme from the earthworm, *Lumbricus rubellus*, which has been abundantly vermicultured for use in the disposal of waste materials in stock raising, pulp companies, and other industrial processes. We report here our investigations on the biological nature and biochemical characteristics of this enzyme.

MATERIALS AND METHODS

Reagents. These included aprotinin (Trasylol; Bayer Pharmaceutical Co., Ltd., Leverkusen, Germany), *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB; United States Biochem., Cleveland, Ohio, U.S.A.), diisopropyl fluorophosphate (DFP; Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.), N^{α}-tosyl-L-lysylchloromethane (TLCK; Sigma Chemical Co., Ltd.), *p*-chloromercuribenzoic acid (PCMB; Sigma Chemical Co., Ltd.), *trans*-4-aminomethyl cyclohexanecarboxylic acid (t-AMCHA; Daiichi Seiyaku Co., Ltd., Tokyo, Japan), soybean trypsin inhibitor (SBTI; Sigma Chemical Co., Ltd.), and bovine fibrinogen (Miles Laboratories Inc., Kankakee, IL, U.S.A.). All other chemicals obtained from commercial sources were of the best grade available.

Earthworm powder. The earthworm, *Lumbricus rubellus*, was vermicultured for the disposal of waste material produced at the Experimental Animal Center, Miyazaki Medical College. One kilogram of earthworms (about 20,000 animals) was washed with tap water to remove attached mud. The earthworms were then left to evacuate casts from their alimentary tract in distilled water overnight under light conditions. Next day, after the castings had been washed out, the living earthworms were homogenized in an ultrahomomixer (Nihon Seiki Co. Ltd., Tokyo, Japan) and lyophilized. The resultant powder was employed as the starting material for subsequent experiments.

Assays of fibrinolysis and protein concentration. Fibrinolytic activity was determined by comparison with the Japanese Standard UK preparation (MM 004), using both the plasminogen-rich fibrin plate method and plasminogen-free fibrin plate method of ASTRUP and MÜLLERTZ (1952). The protein concentration of the sample was determined from absorbance measurements at 280 nm or by the method of LOWRY *et al.* (1951) using bovine serum albumin (Armour Pharmaceutical Co., Phoenix, AZ, U.S.A.) as the reference protein.

Gel exclusion chromatography. Gel exclusion chromatography with Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) was performed on a 2.0×55 cm column equilibrated at 4°C in 0.05 M sodium phosphate buffer containing 0.2 M NaCl and NaN_3, pH 7.4. The earthworm powder enzyme (EPE) sample (0.5 ml total volume) was applied to the column and eluted with the equilibration buffer at a rate of 5 ml/h. Both the protein concentration and fibrinolytic activity were

monitored. A standard curve for estimation of the molecular weight was constructed using bovine serum albumin, ovalbumin, chymotrypsinogen A, and horse heart cytochrome c.

Polyacrylamide gel electrophoresis. Disc electrophoresis of purified EPE was carried out by the method of DAVIS (1964) employing a 0.7 × 10 cm polyacrylamide gel column at 4 mA per tube for 3 h. The acrylamide column concentration in the gel was 7%. Protein was colored with 2% Coomassie brilliant blue solution.

Isoelectric focusing. This was performed by the method of VESTERBERG and SVENSSON (1966) using ampholytes of pH 3.5–10.5. The column (LKB 8101, 110 ml) was kept at 3–4℃ and the potential was maintained at 900 V for 36 h.

Affinity chromatography. Affinity chromatography of the fibrinolytic enzyme using N^{α}-(ε-aminocaproyl)-DL-homoarginine hexylester-Sepharose was performed as described previously by SUMI *et al.* (1978).

RESULTS

When an individual earthworm was cut into pieces and placed on a plasminogen-rich fibrin plate, fibrinolytic activities were observed at the anterior portion of the earthworm body (Fig. 1). The fibinolytic enzyme appeared to be secreted from the pharyngeal region, crop, gizzard, and anterior portion of the intestine.

Figure 2 shows the time course of fibrinolytic activity build-up due to EPE on incubation of the starting material dissolved in 10 times the amount of saline solution containing 0.1% NaN_3 at 37℃. The fibrinolytic activity was measured by the plasminogen-rich fibrin plate method. The activity of EPE rose sharply during the first 10 days, and continued to rise gradually until and after 75 days.

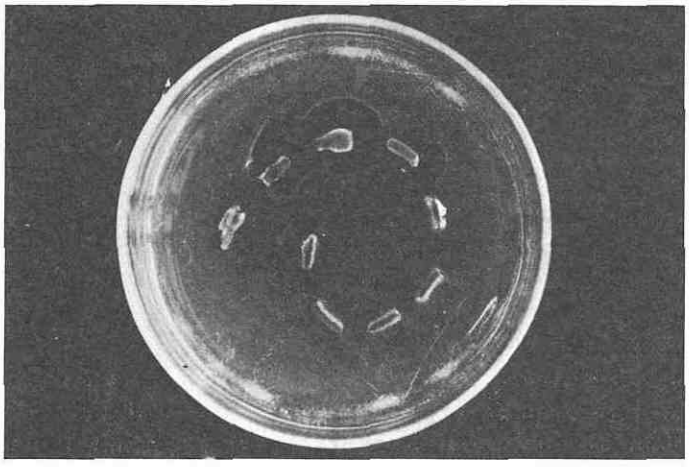

Fig. 1. Demonstration of strong fibrinolysis in the earthworm. A frozen and cut earthworm was applied directly to a plasminogen-rich fibrin plate. The photograph was taken after 18 h at 37℃.

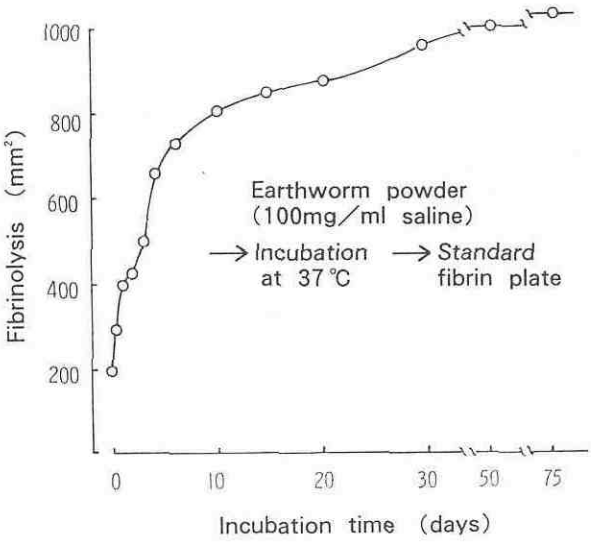

Fig. 2. Incubation effect of earthworm extract on fibrinolysis. Lyophilized earthworm powder (500 g) was dissolved in 5*l* of saline containing 0.1% NaN_3. After incubation at 37℃ for several days, the fibrinolytic activity was assayed in 30 μl samples of the supernatant.

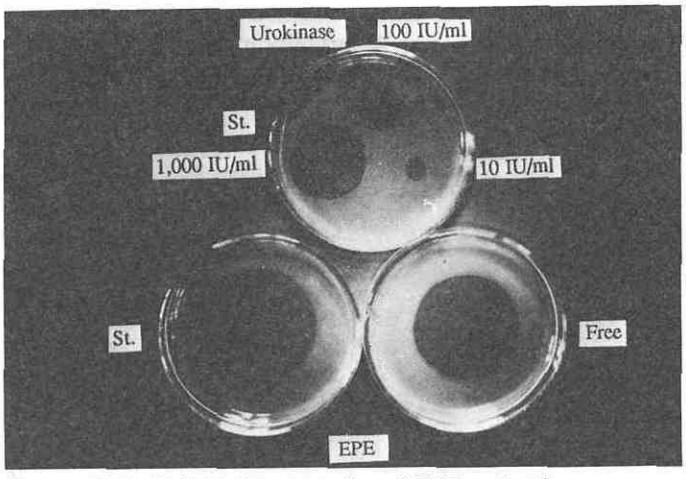

Fig. 3. Comparative fibrinolytic properties of EPE and other enzymes. EPE was extracted from 500 g of lyophilized earthworm powder with 500 ml of saline for 5 h with stirring at 4℃. After centrifugation at 1,500×*g* for 10 min, 30 μl of the supernatant obtained (ca. 14.2 μg protein) was used as the sample. Each sample volume was 30 μl, and incubation was performed at 37℃ for 18 h. St: plasminogen-rich fibrin plate. Free: plasminogen-free fibrin plate.

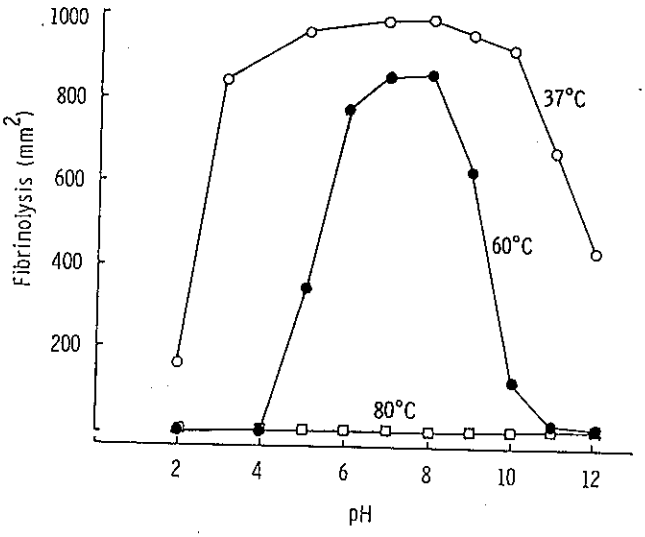

Fig. 4. Heat- and pH-stability of EPE. The EPE extract was incubated in water at several pH values and temperatures for 1 h. It was then neutralized by 30-fold dilution with 0.1 M phosphate buffer, pH 7.4. The residual activities were assayed by measuring the lysis area on plasminogen-rich fibrin plates.

Figure 3 gives a comparison of the activities of the thrombolytic agent, double-chain urokinase, and the earthworm supernatant (i.e. that obtained after 50 days of incubation). The upper fibrin plate shows the activities of 1,000, 100, and 10 international units (IU/ml) of urokinase. The lower fibrin plates show the activities of the earthworm supernatant on a plasminogen-rich plate (left) and a plasminogen-free plate (right). Based on the lysis areas, the activity of the 50-day solution was calculated to be equivalent to about 8,700 IU/ml by comparison with the urokinase activity. Subsequent experiments on the characterization of the enzyme were performed with the 50-day solution.

As shown in Fig. 4, after 30-min heating at 37℃, the enzyme activity was observed in the range between pH 3 and pH 10 on plasminogen-rich fibrin plates. On 30-min heating at 60℃, the activity still remained between pH 6 and pH 9. However, on heating at 80℃ for 30 min, the activity disappeared completely. EPE is thus considered to be rather stable to heat and a broad pH range.

The effects of various inhibitors on the EPE-induced fibrinolytic activity were assessed by the fibrin plate method. Although the fibrinolytic activity was generally measured after 18-h incubation on fibrin plates, the fibrinolytic activity of the 50-day solution with various inhibitors added was observed qualitatively after 2-h incubation, since the fibrinolytic activity of the EPE was too strong and completely dissolved the fibrin plate after 18-h incubation. As shown by the data in Table 1, the fibrinolytic activity of EPE was inhibited by SBTI, aprotinin and DFP, respectively. t-AMCHA had a small inhibitory effect, but PCMB and TLCK

Table 1. Effects of various inhibitors on EPE-induced fibrinolytic activity.

Protease inhibitor	Concentration	Inhibitory activity
SBTI	10 mg/ml	+++
Aprotinin	100 kIU/ml	+++
DFP	1 mM	++
t-AMCHA	10 mg/ml	+
PCMB	5 mM	−
TLCK	5 mM	−

The reaction mixture (1 ml) contained 20 μl of earthworm extract (ca. 500 μg protein), 1–10 mM each inhibitor and 0.1 M phosphate buffer, pH 7.4. After incubation for 10 min at 37°C, the residual fibrinolytic activity was estimated by the plasminogen-rich fibrin plate method. Each value is the mean of 3 determinations.

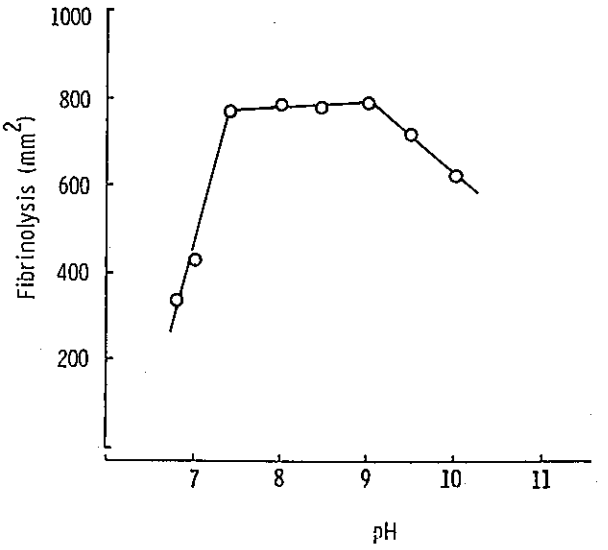

Fig. 5. Optimal pH of EPE activity. The EPE activity was determined with plasminogen-rich fibrin plates at various pH values as indicated on the abscissa.

exerted no effect on the EPE-induced fibrinolytic activity. The enzyme was thus assumed to be a serine protease.

The optimal pH of the enzyme was found to lie between pH 7.4 and pH 9 (Fig. 5), but unlike other enzymes no optimal peak was observed. The enzyme was thus presumed to have a broad pH range.

Polyacrylamide gel electrophoresis of the enzyme was carried out, and the enzyme activity was eluted together with bromophenol blue (BPB; Fig. 6). The enzyme was thought to be very acidic. In fact, as shown in Fig. 7, the enzyme was eluted at pI 3.4 on isoelectric focusing.

On Sephadex G-200 gel filtration, the main peak of fibrinolytic activity was

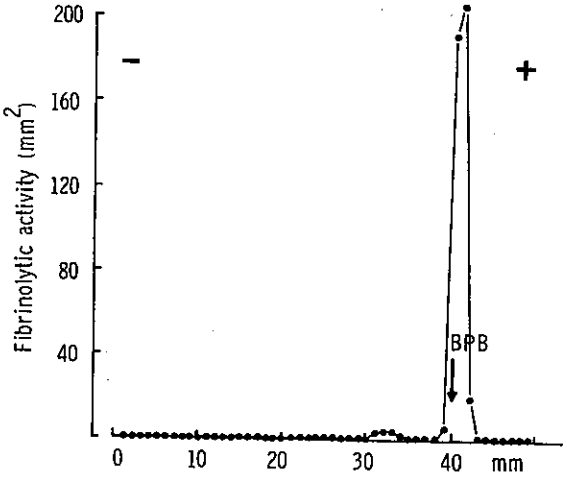

Fig. 6. Polyacrylamide gel electrophoresis of EPE. EPE extract (40 μg) was subjected to electrophoresis at pH 7.4 and 3 mA.

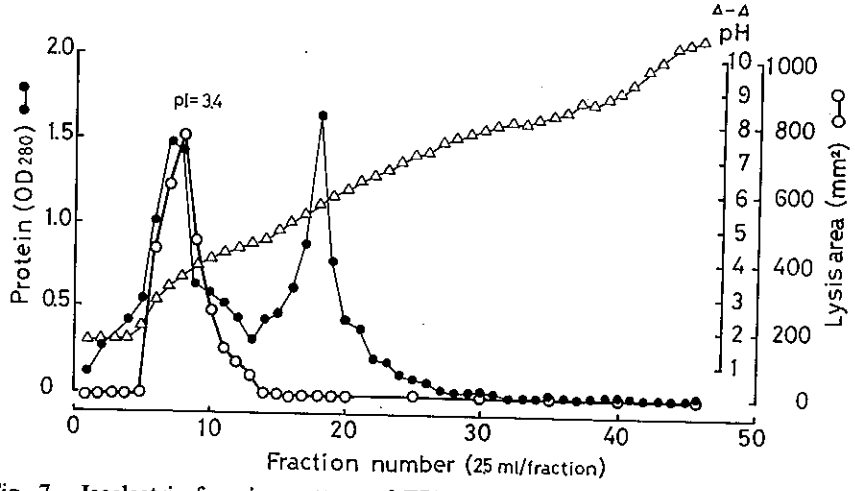

Fig. 7. Isoelectric focusing pattern of EPE. Column (LKB 8101), 110 ml; carrier ampholytes, pH 3.5–10.5; potential, 900 V; fraction volume, 2.5 ml; applied sample, 88 mg EPE; time, 36 h.

observed at tube number 52 (Fig. 8) and a smaller peak of caseinolytic activity corresponded to the main fibrinolytic peak. The main peak of caseinolytic activity occurred at tube number 49 and a smaller peak of fibrinolytic activity corresponded to the main peak of caseinolytic activity. Based on the results for the Sephadex G-200 pattern, the molecular weight of the fibrinolytic enzyme was calculated to be ca. 20,000–30,000.

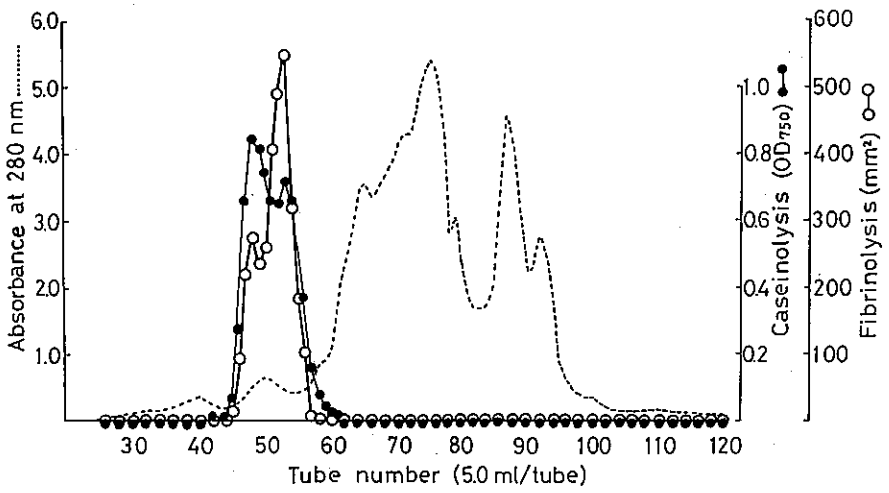

Fig. 8. Gel filtration pattern of EPE on Sephadex G-200. The sample (500 mg EPE/5 ml saline) was applied to a Sephadex G-200 column (2.5×66 cm) equilibrated with 0.1 M phosphate containing 0.2 M NaCl, pH 7.4. Elution was made with the same buffer at a flow rate 40–50 ml/h.

Purification of the enzyme was then performed as follows. One kilogram of earthworm powder was stirred with 10 *l* of saline containing 0.1% NaN_3 for 72 h at 25℃. After centrifugation at 26,500×*g* for 90 min, the supernatant obtained was salted out with a 60% saturation of ammonium sulfate. The resultant precipitate was dissolved in 20 mM phosphate buffer, pH 7.4, and was successively subjected Sephadex G-200 gel filtration. The eluate obtained was further fractionated by column chromatography on DEAE-cellulose, yielding three fractions: F-I, F-II, and F-III. F-I was passed through a DEAE-cellulose column, equilibrated with 10 mM phosphate buffer, pH 8.0, so as to purify the material further. Gradient elution was performed with 0 to 100 mM NaCl in phosphate buffer. After gel filtration on Sephadex G-75, three active fractions designated as F-I-0 (final yield, ca. 20 mg), F-I-1 (final yield, ca. 70 mg), and F-I-2 (Final yield, ca. 60 mg) were obtained.

F-II was passed through a Toyopearl HW-55 column (Toyo Soda Co.), equilibrated with 30% ammonium sulfate, followed by elution with a concentration gradient of 10 to 30% ammonium sulfate. After dialysis against 0.1 M phosphate buffer containing 2 M NaCl, pH 7.4, the eluate was subjected to N^{α}-(ε-amino-caproyl)-DL-homoarginine hexylester-Sepharose affinity chromatography. Gel filtration was performed with Sephadex G-75, and ca. 100 mg of purified F-II was obtained.

F-III was purified by benzamidine-Sepharose affinity chromatography on a column equilibrated with 20 mM phosphate buffer, pH 7.5. Elution was performed with 0.5 M arginine and 1 M urea in phosphate buffer, followed by Toyopearl HW-55 column chromatography. After gel filtration on Sephadex G-75, two active

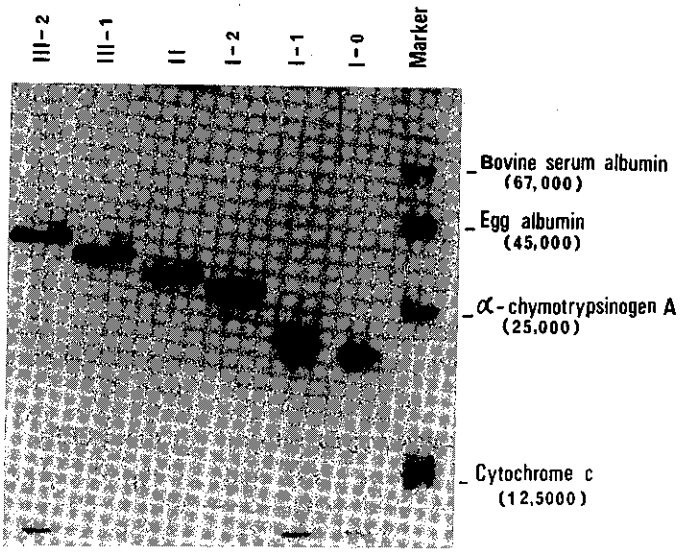

Fig. 9. SDS-polyacrylamide gel electrophoresis of purified EPEs.

Table 2. Physical properties of various EPEs.

	I-0	I-1	I-2	II	III-1	III-2
Molecular weight*	23,500	27,400	27,000	28,500	34,000	34,200
Isoelectric point**	4.12	4.00	3.90	3.80	3.70	3.52
$E^{1\%}$ (280 nm)	12.5	14.2	12.8	16.6	13.1	12.7

*By SDS-polyacrylamide gel electrophoresis. **By isoelectric focusing.

fractions designated as F-III-1 (final yield, ca. 70 mg) and F-III-2 (final yield, ca. 60 mg) were obtained. The enzyme in each of the six fractions revealed a single band, respectively, with or without reduction on SDS-polyacrylamide gel electrophoresis, as shown in Fig. 9.

Based on the results of SDS-PAGE and isoelectric focusing, the molecular weights and isoelectric points of each enzyme were estimated. As shown in Table 2, the molecular weights were found to range between 23,500 and 34,200, and the isoelectric points between pH 4.12 and pH 3.52. The enzymes were thus confirmed to be rather acidic.

The effects of various inhibitors on each individual enzyme were assessed. As shown in Table 3, lima bean trypsin inhibitor (LBTI) and DFP completely inhibited all the enzymes. SBTI completely inhibited the II, III-1, and III-2 fractions, but showed lesser inhibition of I-1 and I-2. From these results, the enzymes were considered to be serine proteases.

A comparison of the enzymatic activities against different substrates showed

Table 3. Effects of various inhibitors on purified EPEs.

Inhibitors (0.8 mg/ml)	Residual activity (%)					
	I-0 (20 μg/ml)	I-1 (10 μg/ml)	I-2 (10 μg/ml)	II (20 μg/ml)	III-1 (2 μg/ml)	III-2 (2 μg/ml)
Control	100	100	100	100	100	100
LBTI	0	0	0	0	0	0
DFP	0	0	0	0	0	0
SBTI	0	16	89	0	0	0
N-Ethylmaleimide	74	0	0	0	100	100
t-AMCHA	30	83	66	23	49	47
ε-ACA	38	100	80	50	68	70

The residual activity was determined by the plasminogen-rich fibrin plate method.

Table 4. Comparative enzyme activities of purified EPEs.

Substrate	I-0	I-1	I-2	II	III-1	III-2
Fibrin (IU)	66.8	102.0	97.0	234.0	87.0	95.0
Casein (CU)	251.2	285.8	266.6	183.2	173.4	146.6
S-2444 (A/min)	0.102	0.268	0.496	0.748	1,236.0	2,675.0
S-2251 (A/min)	1.225	0.370	0.360	0.120	240.0	63.5
TAMe (U)	1.180	7.390	6.600	0.120	151.0	50.7
BAMe (U)	ND	ND	ND	ND	ND	ND
TLMe (U)	ND	0.016	0.014	0.012	61.3	25.6
BTEe (U)	2.430	3.350	2.630	ND	ND	ND
p-NPGB titration (%)	60.3	62.4	58.6	ND	82.7	72.9

ND, not detected.

that each fraction displayed caseinolytic as well as fibrinolytic activity (Table 4). In particular, fractions III-1 and III-2 were found to split arginine and lysine bonds very well. On the other hand, the I fractions split tyrosine bonds. Based on these results, the fraction III enzymes are thought to be trypsin-type enzymes, and the fraction I enzymes to be chymotrypsin-type enzymes. The fraction II enzyme appeared to be neither a trypsin- or chymotrypsin-like enzyme nor an elastase. These enzymes represent novel proteolytic enzymes which are distinct from other well-known proteolytic enzymes. We name them collectively as Lumbrokinase obtained from *Lumbricus rubellus.*

The amino acid compositions of each enzyme were estimated to be as listed in Table 5. The values are given as percentages of residues per molecule. Some interesting results were obtained as compared to other well-known serine proteases. There was very abundant asparagine or aspartic acid, which may be related to the fact that these enzymes were acidic and very stable to heat and pH. On the other hand, there was very little proline or lysine. The present enzymes are thus clearly distinct from other well-known fibrinolytic enzymes, such as plasmin and uro-

Table 5. Amino acid compositions of purified EPEs.

Residue	I-0	I-1	I-2	II	III-1	III-2	Urokinase*	L-Plasmin**	Trypsin***
Asx	12.26	15.65	16.06	14.81	15.25	14.99	19	15	21
Thr	12.22	8.25	8.46	8.40	6.09	6.24	19	14	10
Ser	9.38	11.30	10.85	12.05	10.08	10.39	22	18	24
Glx	3.97	6.05	6.13	6.04	7.04	7.63	28	22	21
Pro	0.40	0.37	0.41	0.44	0.55	0.57	16	15	9
Gly	13.48	15.39	15.41	14.48	12.66	12.89	22	21	20
Ala	12.95	9.49	9.45	10.27	6.90	6.57	10	12	13
Half-Cys	1.44	1.09	1.67	0.80	1.36	1.40	10	9	8
Val	7.03	5.46	5.51	7.77	10.33	10.67	11	22	6
Met	0.55	0.97	0.82	0.98	1.36	1.34	5	2	1
Ile	5.38	6.54	6.64	5.96	7.89	7.62	16	8	12
Leu	7.47	7.95	8.01	7.46	3.73	3.41	21	20	12
Tyr	3.91	3.69	3.79	3.24	4.63	4.83	13	6	7
Phe	2.00	1.11	1.11	0.58	3.09	2.67	9	8	4
Trp	0.64	0.98	ND	1.63	0.83	1.09	5	5	3
Lys	0.04	0.50	0.53	0.45	1.65	1.67	17	14	11
His	2.45	2.75	2.73	2.30	2.37	2.12	10	8	3
Arg	3.98	2.46	2.42	2.34	4.19	3.90	14	14	6

Values are given as % amino acid residues per molecule. ND, not detected. *WHITE *et al.* (1966). **GROSKOPF *et al.* (1969). ***TRAVIS and ROBERTS (1969). *,**,***Values are given in number of residues per molecule.

kinase.

DISCUSSION

The earthworm, *Lumbricus rubellus*, has been abundantly vermicultured for use in the disposal of waste materials produced in stock raising, pulp companies, and other industrial processes. However, there are at present no known ways of putting the growing earthworm to practical use except as fishing bait. On the other hand, the earthworm has been used in the treatment of various diseases. The present study revealed the presence of a very strong and novel fibrinolytic enzyme in this earthworm.

It was reported previously by FRÉDÉRICQ (1878) and by WILLEM and MINNE (1899) that an enzyme which was secreted from the alimentary tract of the earthworm could dissolve fibrin. However, these researchers were not investigating fibrinolysis in particular, but merely employed fibrin as a protein which was most readily obtainable at that time. They found that the proteolytic enzyme was secreted from the pharyngeal region, crop, gizzard, and anterior portion of the intestine. As shown in Fig. 1 of this report, fibrin plates were dissolved by cut pieces of earthworm from the corresponding regions including the above organs.

It was unexpectedly discovered that the fibrinolytic activity increased over long

periods of incubation at 37℃, such as 50 days. This phenomenon lay outside our general understanding of the properties of proteolytic enzymes, and is assumed to reflect the fact that earthworm powder includes abundant zymogen and the enzyme is itself very heat-stable. Comparison of the lysis areas between plasminogen-rich fibrin plates and plasminogen-free fibrin plates showed that the EPE activity was stronger on the former than on the latter. This indicated that EPE had both a plasminogen activator activity and a direct digestive action on fibrin. Although we obtained six enzyme fractions, we consider that all these enzymes represent a novel substance based on the data for their amino acid compositions (Table 5) and interactions with various inhibitors. We therefore decided to refer to these enzymes collectively as Lumbrokinase. Since EPEs also have a high stability and broad pH range, it seems possible that they could be used as an oral thrombolytic agent. In fact, we have heard that the earthworm is eaten as a protein source among natives in Australia. We are planning to carry out trials on earthworm powder as a potential oral thrombolytic agent.

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