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Review

Earthworm-serine protease: characterization, molecular cloning, and application of the catalytic functions

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

An earthworm, *Lumbricus rubellus*, produces alkaline serine proteases that are greater than trypsins in their activity and stability. The proteases which were purified from the earthworm were composed of six isozyme proteins. Each isozyme consisted of a single polypeptide chain which was derived from the different genes. The enzymes had activity and were stable at below 60 °C over a wide range of pH 2–11 and were strongly resistant to organic solvents and detergents. Moreover, they retain full activity for long years at room temperature. They acted on various proteins, such as elastin as well as fibrin, and some peptides, such as β-amyloid 1–40 and solubilized actual fibrin clots of whole blood in a rat's vena cava. They also catalyzed the hydrolysis of various esters. The cDNAs encoding the proteases were cloned and sequenced. They showed similarity to mammalian serine proteases and conserved the catalytic amino acid residues, however, neither arginine nor lysine residues were present in the autolysis region. The gene encoding the native form of an isozyme protein was expressed in *Pichia pastoris* to produce the active protease in the culture medium. The proteases contributed to the production of the "earthworm autolysate". The extracts of the autolysate could be used as a "peptone substitute" in media for the efficient growth of microorganisms.

Keywords: Serine protease; Fibrinolytic enzyme; Structure and function; Application of the catalytic functions; Earthworm

1. Introduction

Earthworms have long been used as a drug for antipyretic and diuretic purposes in Chinese medicine under the name of "*Jiryu*" [1–3]. Earthworms secrete the fibrinolytic enzymes and the enzymes purified from an earthworm, *Lumbricus rubellus*, were alkaline serine proteases with a potent fibrinolytic activity [4,5]. Fibrinolytic enzymes dissolve fibrin, the main component of blood clots. To date, therapeutically important fibrinolytic enzymes, such as urokinase and tissue plasminogen activator have been used clinically as chemotherapeutic agents [6–10].

Recently, we have characterized and cloned the serine proteases with fibrinolytic activity from the earthworm, *L. rubellus* (Fig. 1) and showed that the enzymes are composed of six isozymes (isozymes A, B, C, D, E, and F), derived from different genes.

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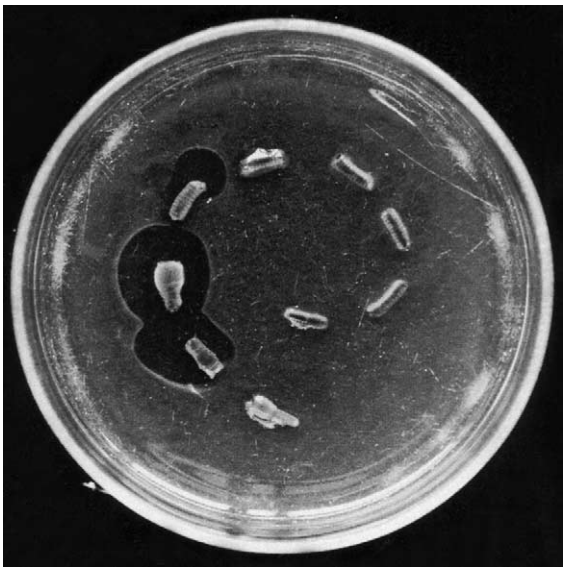

Fig. 1. Fibrinolytic activity of the earthworm proteases. Fibrinolytic activity was detected on a fibrin plate.

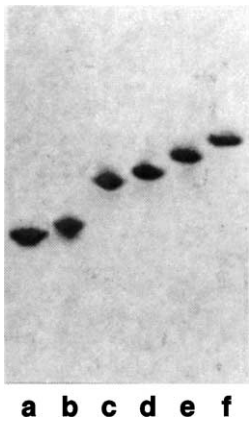

Fig. 2. Polyacrylamide gel electrophoresis of the purified enzymes. Electrophoresis was done on 7.5% polyacrylamide gel with 20 μg of the purified enzymes: (a) isozyme A; (b) isozyme B; (c) isozyme C; (d) isozyme D; (e) isozyme E; (f) isozyme F.

The proteases have a wide catalyzing ability with high stability and strong tolerance towards organic solvents and detergents. On the basis of results of their cleavage specificity against peptide substrates, isozymes A, B, D, E, and F had both trypsin- and chymotrypsin-like activities, but isozyme C also acted like an elastase [11–16].

In this review, we describe the purification, characterization, gene cloning, sequencing, and expression of the earthworm proteases to clarify the protein structure and function with emphasis on application of the catalytic functions of the enzymes for therapeutics, synthesis of useful compounds, and degradation of organic waste products.

2. Isoforms of the proteases

Earthworm fibrinolytic enzymes were purified from *L. rubellus* by chromatographic procedures including some anion exchange and gel filtration chromatographies [11,14]. Each enzyme protein was homogeneous on polyacrylamide gel (Fig. 2) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). About 50–100 mg each of the six homogenous enzymes (isozymes A, B, C, D, E, and F, formerly named F-III-2, F-III-1, F-II, F-I-2, F-I-1, and F-I-0, respectively [11]) were obtained from 1 kg of dry earthworm powder. The enzymes are monomer proteins consisting of single polypeptide chains and are not glycoproteins. The molecular masses of the six isozymes are approximately 23–30 kDa, respectively, on ionspray-MS analysis and their p*I* values are from 3.4 to 4.9 [11].

To date, other earthworm fibrinolytic enzymes with the enzymatic properties similar to those of the enzymes from *L. rubellus* have been reported in different earthworm, such as *Eisenia* species [17–19].

3. Properties of the proteases

3.1. Effects of temperature and pH on the enzyme activity and stability

When the enzyme activity was measured for a chromogenic substrate, H-D-Phe-Pip-Arg-*p*NA, all the isozymes had amidolytic activity and were stable at below 60 °C over a wide range of pH 2–11. The enzymes were most active at 55 °C but lost activity substantially upon incubation at more than 60 °C. On heating at 80 °C for 30 min, the activity completely disappeared [11,16].

We determined the remaining activities of the isozymes (1 mg each) in 1 ml of 0.1 mM Tris–HCl

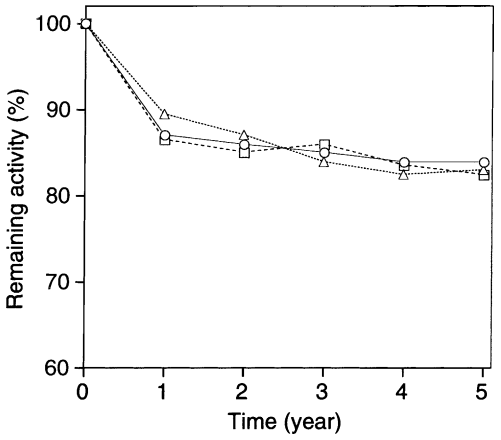

Fig. 3. Stability of the earthworm proteases in buffer at room temperature. One milligram of the earthworm-protease isozymes dissolved in 1 ml of 100 mM Tris–HCl buffer (pH 8.0) in sealed vials were left to stand at room temperature for several years. The remaining fibrinolytic activities were assayed using a fibrin plate as described in the text. (○) Isozyme A; (△) isozyme C; (□) isozyme D.

buffer (pH 8.0) containing 0.1% NaN_3. As shown in Fig. 3, the isozymes exhibited fibrinolytic activity (>80% residual activity) for at least 5 years at room temperature [14]. Thus, the enzymes were considered to be rather heat- and pH-resistant proteases.

3.2. Tolerance of the enzymes to organic solvents

The stability of the proteases towards organic solvents was tested with isozymes A and B at room temperature in 1 ml of 100 mM Tris–HCl buffer (pH 8.0) containing 25% (v/v) acetone, 2-propanol, toluene, and *n*-hexane, respectively [14].

As shown in Fig. 4, the isozymes remained fully active against all of these organic solvents for at least 100 days. However, trypsin lost its activity within 1–10 days. The stability of the proteases against water-soluble and -insoluble organic solvents, even toluene and *n*-hexane, was highest among the various proteases reported [20]. The enzymes showed also strong tolerance to detergents and NaCl as described further. From these results, the proteases are considered to be very useful biocatalysts for synthetic reactions, such as peptide synthesis [21,22] in organic media because of their high stability toward organic solvents and detergents.

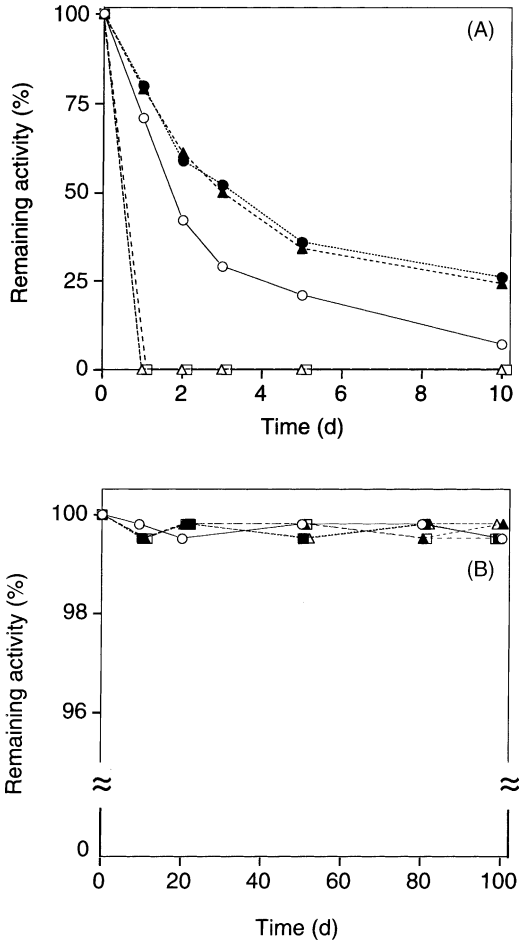

Fig. 4. Tolerance of the earthworm protease to organic solvents. One milligram of porcine trypsin (A) and the earthworm protease (isozyme B) (B) were dissolved in 1 ml of the buffer and were incubated at 25 °C with shaking in the absence or presence of 25% (v/v) of various organic solvents: (○) none; (●) 2-propanol; (▲) acetone; (△) toluene; (□) *n*-hexane. The relative residual activities were assayed with H-D-Phe-Pip-Arg-*p*NA for the earthworm proteases and Bz-L-Arg-*p*NA for trypsin.

3.3. Substrate specificity of the enzymes

The isozymes, especially A and B among the six isozymes, have much higher strong caseinolytic [23] and fibrinolytic activities [24] than plasmin [25] as shown in Fig. 5. The enzymes were plasmin-like enzymes, however, they do not serve as a plasminogen activator, because there was no difference in the fibrinolytic activities on plasminogen-free and

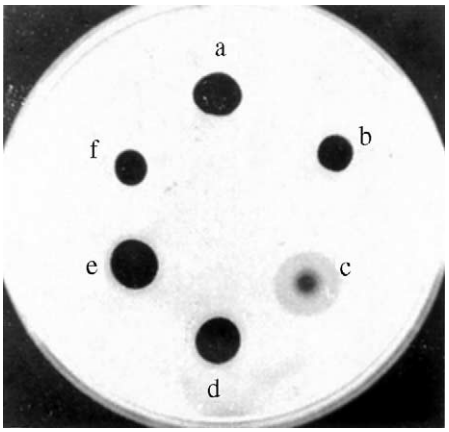

Fig. 5. Fibrinolytic activity of the purified enzymes. The purified enzymes were separately placed on a fibrin plate: (a) 0.1 μg of isozyme A; (b) 0.1 μg of isozyme B; (c) 1 μg of isozyme C; (d) 1 μg of isozyme C; (e) 1 μg of isozyme D; (f) 1 μg of isozyme F. The fibrinolytic activity was measured after incubation for 2 h at 37 °C using human plasmin as a standard.

-containing fibrin plates [26]. The fibrinolytic activity of each isozyme (A, B, C, D, E, and F) was 204, 223, 22, 5.6, 6.1, and 3 casein units (CU)/mg, respectively, with human plasmin as a standard [11,25]. The enzymes had also strong amidolytic activity [27] for various chromogenic substrates as summarized in Table 1. The amidolytic activity (pH 7.2) of each isozyme for H-D-Phe-Pip-Arg-*p*NA was 150, 72, 0.1, 0.4, 0.2, and 0.1 units/mg, respectively [11]. Isozymes A and B were most reactive toward the various substrates and had almost the same spectrum of substrate specificity. The substrate specificities of isozymes D and E resembled each other, and they were most reactive to H-D-Ile-Pro-Arg-*p*NA (2.8 units/mg). Isozymes C and F were reactive to MeO-Suc-Ala-Ala-Pro-Val-*p*NA (0.3 units/mg) and H-D-Val-Leu-Arg-*p*NA (0.4 units/mg), respectively.

3.4. Effects of inhibitors on the enzymes

The effects of various inhibitors on the enzyme activity were investigated using H-D-Phe-Pip-Arg-*p*NA as a substrate. Although the enzymes were rather stable toward various compounds at room temperature for 30 min, diisopropyl fluorophosphate completely inhibited the activity of all the isozymes. The activity of isozymes A, B, and C was inhibited strongly by soybean trypsin inhibitor and aprotinin, but the enzyme activity of D, E, and F was partially inhibited by these inhibitors. Tosyl-phenylalanyl-chloromethylketose, tosyl-lysyl-chloromethylketose, ε-aminocaproic acid, elastatinal, various metal ions (such as Fe^{2+}, Cu^{2+}, and Mn^{2+}), and EDTA did not affect the enzyme activity at all. From these results including substrate specificity, the enzymes were considered to be trypsin-like serine proteases [11].

3.5. Amino acid composition of the enzymes

The amino acid composition of each enzyme was analyzed. Similar amino acid compositions were found in the six enzymes, especially, in isozymes A

Table 1
Substrate specificity of the earthworm proteases

Substrate	Isozyme A	Isozyme B	Isozyme C	Isozyme D	Isozyme E	Isozyme F
Fibrin[a]	204	243	22	5.6	6.1	3
H-D-Phe-Pip-Arg-*p*NA[b]	100	100	33	14	10	25
Pyr-Glu-Gly-Arg-*p*NA[b]	93	75	67	0	0	0
H-D-Ile-Pro-Arg-*p*NA[b]	20	40	0	100	100	25
H-D-Val-Leu-Arg-*p*NA[b]	27	58	0	21	23	100
H-D-Val-Leu-Lys-*p*NA[b]	14	39	0	0	0	0
Bz-L-Arg-*p*NA[b]	2	3	0	0	0	0
Bz-L-Tyr-*p*NA[b]	0	0	0	0	0	0
MeO-Suc-Ala-Ala-Pro-Val-*p*NA[b]	0	0	100	0	0	0
Suc-Ala-Ala-Ala-*p*NA[b]	0	0	0	0	0	0

[a] The enzyme activity was expressed as CU/mg of protein, with human plasmin as a standard.
[b] The relative substrate specificity (%) for the chromogenic substrates was determined spectrophotometrically.

and B, and in isozymes D and E. These enzymes had very abundant asparagine and aspartic acid, compared with other well-known serine proteases [11]. On the other hand, these enzymes had very low lysine contents. It was found that these enzymes were clearly distinct from other fibrinolytic enzymes, such as plasmin [25] and urokinase [28] in amino acid composition.

4. Further characterization of the proteases

4.1. Cleavage specificity of the enzymes

Based on the results of their enzymatic activities for various chromogenic and peptide substrates, isozymes A, B, D, E, and F represent both trypsin- and chymotrypsin-like activities, but isozyme C also serves as an elastase-like enzyme [11,13].

As shown in Fig. 6, nine peptides were recovered by HPLC and six sites of β-amyloid 1–40 as a peptide substrate were cleaved by isozyme A in 12 h hydrolysis, and eight peptides were recovered and six sites were cleaved by isozyme C. Only one bond of Arg5–His6 was cleaved commonly by both isozymes. Parallel to the experiment on the earthworm proteases, the digestion of the peptide by other serine proteases had been done with trypsin and chymotrypsin [29–31]. Under the same reaction conditions (12 h), only the bond of Lys16–Leu17 of the peptide was cleaved by trypsin and only the bond of Phe4–Arg5 by chymotrypsin. These results showed that isozyme A cleaved potentially not only the bonds which were digested by both trypsin and chymotrypsin, but also all other bonds of Phe–X, Arg–X, and Lys–X, including the site of Leu34–Met35. Isozyme C digested preferentially the bonds consisting of neutral amino acids, such as Val–X and X–Gly, including the site of Arg5–His6 which was digested also by isozyme A.

The cleavage of the oxidized insulin B-chain by the two isozymes was investigated in the same manner, and these cleavage sites were compared with those of other proteases [29–33]. As shown in Fig. 7, 8 peptides were recovered by HPLC and 5 sites of the peptide were cleaved by isozyme A in 12 h hydrolysis, and 14 peptides were recovered and 10 sites were cleaved by isozyme C. Two sites of Phe25–Tyr26 and Lys29–Ala30 were cleaved commonly by both isozymes. The digestion of Leu15–Tyr16, Tyr16–Leu17, Phe25–Tyr26, and Lys29–Ala30, which were cleaved by isozyme A, was also demonstrated with other serine proteases [29–31]. Isozyme C showed broader specificity than isozyme A and hydrolyzed the bonds consisting of neutral or hydrophobic amino acids, especially in the hydrophobic region, as well as the digestion of the sites of aromatic amino acid–X and basic amino acid–X. The cleavage specificity of isozyme C, which preferred valine, alanine, glycine, serine, and histidine, resembled those of elastase [29,32], a cysteine protease, and papain [30,31,33].

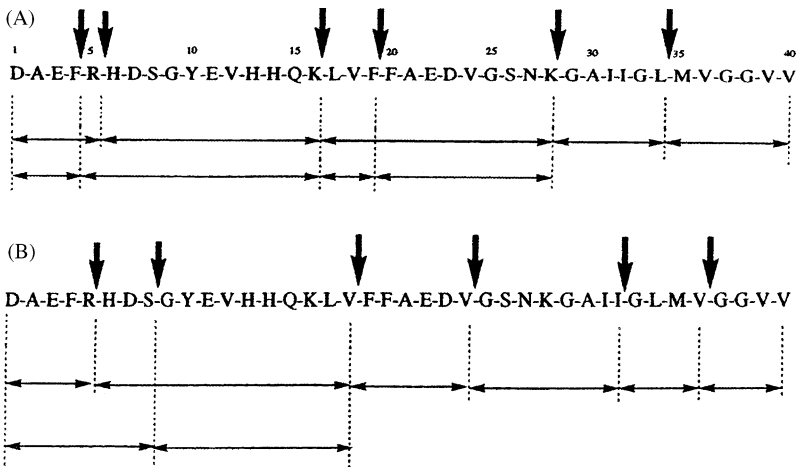

Fig. 6. Cleavage patterns of the earthworm proteases on β-amyloid 1–40. Horizontal arrows show cleaved peptide fragments and vertical arrows show cleavage sites. The reaction conditions are described in the text. (A) Isozyme A; (B) isozyme C.

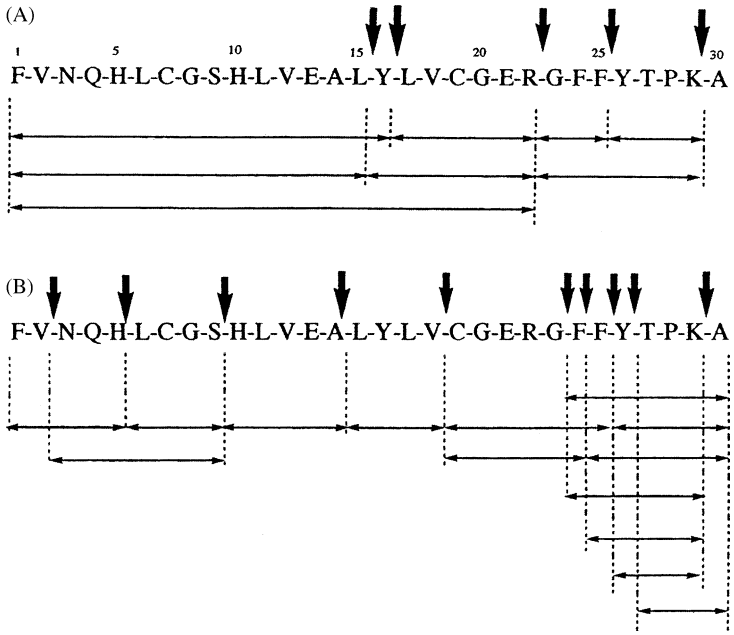

Fig. 7. Cleavage patterns of the earthworm proteases on oxidized insulin B-chain. Horizontal arrows show cleaved peptide fragments and vertical arrows show cleavage sites. The reaction conditions are described in the text. (A) Isozyme A; (B) isozyme C.

Thus, among the earthworm-serine proteases, isozyme A had a cleavage specificity essentially the same as trypsin and chymotrypsin and potentially cleaved β-amyloid 1–40 and insulin B-chain more than the proteases [20,29–31]. Isozyme C showed broader specificity than isozyme A and preferentially digested the bonds consisting of neutral or hydrophobic amino acids in these peptides.

4.2. Autolytic cleavage site of the enzyme

The heat-induced autolysis site of the enzyme was studied using isozyme A (Fig. 8A). When the enzyme (10 μg) was digested at 60 °C for 30 min in a reaction mixture (1 ml) containing 10 mM Tris–HCl buffer (pH 9.0) and 0.1% SDS, the two bands of smaller peptide fragments with molecular masses of 15 and 14 kDa and the non-digested native enzyme (29 kDa) appeared regularly on SDS-PAGE (Fig. 8B) [13,16]. The N-terminal amino acid sequences of the two fragments produced were Ile-Val-Gly-Gly-Ile-Glu-Ala-Arg-Pro-Tyr-Glu-Phe- (the sequence of the native polypeptide after autolysis) and Tyr-Val-Thr-Leu-Asn-Ile-Thr-Thr-Asn-Ala- (the sequence of the polypeptide digested). This result indicated that isozyme A itself was autolyzed initially on the site of Arg144–Tyr145 of the enzyme protein [15]. The temperature (60 °C), at which the autolysis arose on heating, coincided with that on the heat-inactivation curve of the enzyme (Fig. 8A). Heating for longer than 30 min at higher temperature caused the appearance of many smaller peptides from the native polypeptide [13,16]. The enzyme has no Arg or Lys residues in the sequence comprising Asn131 to Asp141 corresponding to the autolysis loop (Asn143 to Asp153) of bovine trypsin [34] but has Arg144, which triggers the autolysis of the enzyme as described further [13,15]. These results provide information concerning the higher stability of the enzyme.

4.3. Hydrolysis pattern by the enzyme for fibrinogen and fibrin

To elucidate the reaction mode of the earthworm protease for decomposing fibrinogen and fibrin, analyses of the degraded products were performed on SDS-PAGE with the same units of human plasmin as a control. In the hydrolysis of fibrinogen with isozyme

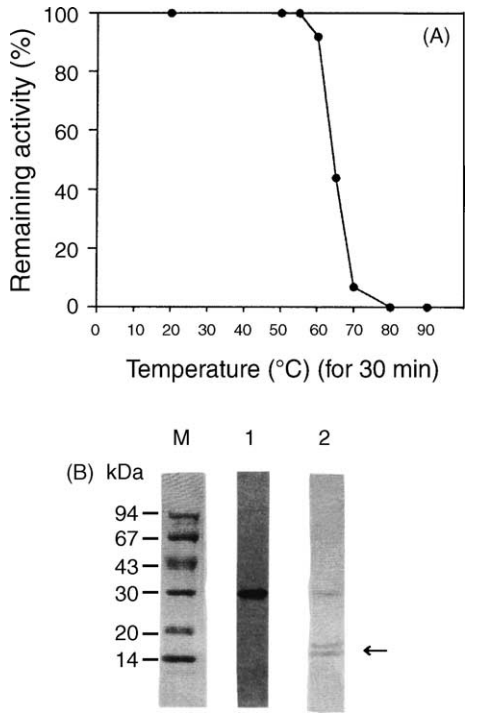

Fig. 8. Inactivation curve of the enzyme by heating and the degradation pattern of the enzyme protein into the peptide fragments by autolysis. (A) Isozyme A (1 mg) was incubated in 1 ml of 50 mM Tris–HCl buffer (pH 9.0) at various temperatures for 30 min. The remaining activity was assayed in the buffer as described in the text. (B) The degradation pattern of the enzyme protein was analyzed by SDS-PAGE (12.5%) before (1) and after (2) heat treatment for 30 min at 60 °C in the buffer; (M) standard protein markers (Pharmacia Biotech). Arrow shows two peptide fragments produced by autolysis.

A, Aα- and Bβ-chains were cleaved first, followed by slower degradation of the γ-chain, producing many more products with lower molecular masses than those of plasmin within the time tested. The enzyme had a very strong activity for decomposing Aα- and Bβ-chains and also the γ-chain [12].

When fibrin was incubated with isozyme A, all of the α-, β-, and γ-chains were readily hydrolyzed faster than plasmin within the time tested. Although in the fibrin hydrolysis by plasmin, some of the degraded polypeptides of fibrin remained unaffected after 6 h incubations, the hydrolysis by the enzyme resulted in several fibrinopeptides with molecular masses of below 10 kDa. These results indicated that the earthworm proteases were proteolytic enzymes different from the typical blood clotting enzyme, thrombin, because fibrinogen was hydrolyzed, but there was no fibrin clot observed, and the earthworm proteases were the fibrinolytic enzyme having much higher activity than the typical fibrinolytic enzyme, plasmin [12].

5. Cloning and DNA sequences of the proteases

5.1. Sequence analysis of the enzyme cDNAs

To obtain the cDNA fragments of isozymes A and B, the primers for RT-PCR were designed on the basis of the N-terminal and partial amino acid sequences of isozyme A. After subcloning the fragments and analyzing the nucleotide sequences of the clones, two kinds of cDNA fragments were obtained. Both cDNA fragments comprised 491 bp and encoded 164 amino acid residues. There were 434 nucleotides and 146 amino acid residues conserved between the nucleotide sequences and the deduced amino acid sequences of the two cDNA fragments, respectively. To obtain the complete nucleotide sequences of the two cDNAs, the primers for RACE PCR were designed based on the nucleotide sequences that show a difference between the two cDNA fragments.

The nucleotide sequences, except for the poly(A) sequence, and the deduced amino acid sequences of the two full-length cDNAs are shown in Fig. 9. In the sequence of the 1011 nucleotides (Fig. 9A), an open reading frame from a start codon, ATG, at position 107 to a stop codon, TAA, at position 842 encodes a polypeptide of 245 amino acid residues. A putative polyadenylation signal, AATAAA, at positions 991–996 was found. The amino acid sequences from Ile1 to Thr71 and Thr97 to Lys123 correspond to those of the N-terminal region and a peptide derived by Lys C endopeptidase digestion of isozyme A, respectively. In the sequence of the 973 nucleotides (Fig. 9B), an open reading frame from a start codon, ATG, at position 107 to a stop codon, TAA, at position 845 encodes a polypeptide of 246 amino acid residues. A putative polyadenylation signal, AATAAA, at positions 932–937 is found. The amino acid sequence from Ile1 to Asp24 on the deduced polypeptide corresponds to that of the N-terminal region of isozymes A and B. However, Ser49 and Ser62 of the N-terminal region and Ala98 and Ile99 of the peptide derived by

(A)

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GTTACTTCTCGCTCTTGCATCGCTGGTAGCGGTGGGCTTTGCCCAACCACCTGTCTGGTACCCCGGTGGTCAATGCGGTGTCAGCCAGTA    90

TTCAGATGCTGGCGACATGGAACTTCCTCCCGGAAAGATTGTCGGAGGAATTGAAGCCAGACCATACGAGTTCCCATGGCAGGTGTCCGT   180
                M  E  L  P  P  G  K  I  V  G  G  I  E  A  R  P  Y  E  F  P  W  Q  V  S  V     18
                           (-1)  (+1)
CCGAAGGAAGTCTTCTGATTCCCATTTCTGCGGAGGTAGCATCATCAACGATCGTTGGGTTGTCTGCGCTGCTCACTGCATGCAGGGAGA   270
 R  R  K  S  S  D  S  H  F  C  G  G  S  I  I  N  D  R  W  V  V  C  A  A  H  C  M  Q  G  E     48

GAGCCCTGCCCTGGTCTCATTGGTCGTCGGCGAGCACGATAGCAGCGCTGCGAGTACAGTACGTCAGACTCATGATGTTGATAGCATCTT   360
 S  P  A  L  V  S  L  V  V  G  E  H  D  S  S  A  A  S  T  V  R  Q  T  H  D  V  D  S  I  F     78

CGTCAACGAAAACTACGATCCCCGTACACTAGAAAACGACGTTTCTGTCATCAAGACAGCTATCGCTATCACCTTCGACATCAACGTTGG   450
 V  N  E  N  Y  D  P  R  T  L  E  N  D  V  S  V  I  K  T  A  I  A  I  T  F  D  I  N  V  G    108

ACCAATCTGTGCTCCAGATCCGGCTAACGACTACGTCTACCGTAAGAGCCAGTGTTCCGGATGGGGAACTATCAATTCAGGTGGAATCTG   540
 P  I  C  A  P  D  P  A  N  D  Y  V  Y  R  K  S  Q  C  S  G  W  G  T  I  N  S  G  G  I  C    138

CTGTCCCGCAGTTTTGCGATATGTTACACTGAACATCACGACCAACGCCTTCTGCGACGCCCGTCTACACATCGGACACTATCTACGACGA   630
 C  P  A  V  L  R  Y  V  T  L  N  I  T  T  N  A  F  C  D  A  V  Y  T  S  D  T  I  Y  D  D    168

TATGATCTGCGCCACAGACAACACTGGGATGACCGACAGAGACTCCTGCCAGGGTGACTCCGGCGGCCCTCTGAGCGTCAAGGATGGCAG   720
 M  I  C  A  T  D  N  T  G  M  T  D  R  D  S  C  Q  G  D  S  G  G  P  L  S  V  K  D  G  S    198

CGGAATCTTCAGTCTGGGTGGCATTGTGTCTTGGGGAATTGGTTGCGCCTCTGGCTATCCAGGAGTTTACTCCCGCGTCGGATTTCATGC   810
 G  I  F  S  L  G  G  I  V  S  W  G  I  G  C  A  S  G  Y  P  G  V  Y  S  R  V  G  F  H  A    228

TGGATCGATCACCGACACGATCACCAACAACTAAACCGACGATGGCCAGTCAACTATAACGGACTCTTATTACCTGCAGTTAACGTTGCT   900
 G  W  I  T  D  T  I  T  N  N  *                                                              238

CATGCAGAACGAACATGCACTTTCATCGCCTATAGGTCCACTAAACACAGCATGGATGACATATGAGTTAAAAGTATCGTGGTGACACGA   990
AATAAAAATCTATCTACTGGG                                                                        1011
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(B)

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GTTACTTCTCGCTCTTGCATCGCTCGTAGCGGTGGGCTTTGCCCAACCACCAGTCTGGTACCCCGGTGGTCAATGCGGTGTCAGCCAGTA    90

CTCAGATGCTGGTGACATGGAACTTCCTCCCGGAACAAAAATTGTCGGAGGAATTGAAGCCAGACCATACGAGTTCCCATGGCAGGTGTC   180
                M  E  L  P  P  G  T  K  I  V  G  G  I  E  A  R  P  Y  E  F  P  W  Q  V  S     17
                           (-1)  (+1)
CGTCCGAAGGAAGTCTTCCGATTCCCATTTCTGCGGAGGTAGCATCATCAACGATCGTTGGGTTGTCTGCGCTGCTCACTGCATGCAGGG   270
 V  R  R  K  S  S  D  S  H  F  C  G  G  S  I  I  N  D  R  W  V  V  C  A  A  H  C  M  Q  G     47

AGAGGCCCCCGCTCTGGTTTCATTGGTCGTGGGTGAGCACGACAGGAGTGCAGCGAGTACAGTACGTCAGACTCATGACGTTGATAGCAT   360
 E  A  P  A  L  V  S  L  V  V  G  E  H  D  R  S  A  A  S  T  V  R  Q  T  H  D  V  D  S  I     77

CTTCGTCCACGAGGACTACAACGCAAATACCCTAGAGAACGACGTTTCTGTCATCAAGACATCTGTTGCCATCACTTTCGACATCAACGT   450
 F  V  H  E  D  Y  N  A  N  T  L  E  N  D  V  S  V  I  K  T  S  V  A  I  T  F  D  I  N  V    107

TGGTCCAATCTGCGCCCCAGATCCGGCTAACGACTACGTCTACCGTAAGAGCCAGTGTTCCGGATGGGGAACTATCAATTCAGGTGGAAT   540
 G  P  I  C  A  P  D  P  A  N  D  Y  V  Y  R  K  S  Q  C  S  G  W  G  T  I  N  S  G  G  I    137

CTGCTGTCCCAACGTTCTGCGATACGTGACGCTGAATGTCACAACCAACCAATTCTGCGAAGATGTATACCCACTAAATTCAATCTACGA   630
 C  C  P  N  V  L  R  Y  V  T  L  N  V  T  T  N  Q  F  C  E  D  V  Y  P  L  N  S  I  Y  D    167

CGATATGATTTGCGCGTCGGACAACACTGGGGGTAACGACAGAGACTCCTGCCAGGGTGACTCCGGCGGCCCTCTGAGCGTCAAGGATGG   720
 D  M  I  C  A  S  D  N  T  G  G  N  D  R  D  S  C  Q  G  D  S  G  G  P  L  S  V  K  D  G    197

CAGTGGAATCTTCAGCCTGATTGGTATTGTGTCTTGGGGAATTGGTTGCGCTTCTGGCTATCCAGGAGTCTACTCCCGCGTCGGATTCCA   810
 S  G  I  F  S  L  I  G  I  V  S  W  G  I  G  C  A  S  G  Y  P  G  V  Y  S  R  V  G  F  H    227

TGCTGCATGGATCACCGACATCATCACCAACAACTAAACCGGAGTTATCCAGTCGACTATAACTGGAACGACTTTACCATCACGAACGAC   900
 A  A  W  I  T  D  I  I  T  N  N  *                                                           238

ACATTAATTAAAATTATTGTATTAAACATAAAATAAAATCTCTCTACTCACAGATCGTTTGTAGAACAATTGT                    973
```

Fig. 9. Nucleotide and deduced amino acid sequences of cDNAs encoding earthworm-serine proteases: (A) isozyme A; (B) isozyme B. The nucleotides and the amino acids are numbered from the 5′ end of the cDNA and from the N-terminal Ile of the native enzyme, respectively. A stop codon is indicated by an asterisk and a putative polyadenylation signal is underlined. The cleavage site for the activation peptide is marked by an arrow.

Lys C endopeptidase digestion of isozyme A were replaced by Ala49, Arg62, Ser98, and Val99 on the deduced polypeptide, respectively. We have shown that the amino acid sequences of the N-terminal 24 amino acid regions of isozymes A and B were identical, while those of isozymes C, D, and E had 12, 13, and 13 amino acid residues identical with isozymes A and B, respectively [11]. These results show that the cDNAs of 1011 and 973 nucleotides encode the earthworm proteases, isozymes A and B, respectively.

5.2. Amino acid sequence similarity

When the deduced amino acid sequences were compared with the N-terminal sequences of the native enzymes [11], extensions of seven and eight amino acid residues were found in the N-termini of the deduced isozymes A and B, respectively. Trypsin-like proteases are synthesized as preproenzymes that contained an N-terminal signal peptide (11–15 amino acid residues) followed by an activation peptide (6–8 amino acid residues) and have a highly conserved N-terminal amino acid sequence (Ile-Val-Gly-Gly-) in the active form [35]. These data show that the proteases, isozymes A and B, are translated as proenzymes that lack the signal peptide and are changed to the active forms by post-translational modification. The amino acid sequences, except for the N-terminal extensions, of the two enzymes comprise 238 amino acid residues, and 215 amino acid residues are conserved between isozymes A and B. This implies that both isozymes A and B are almost the same in substrate specificity, N-terminal amino acid sequence, and immunochemical properties [11].

The deduced amino acid sequences of the earthworm proteases were compared with those of proteins in the SWISS-PROT and PIR data banks. They showed high similarity with mammalian serine proteases. Bovine trypsin [36], bovine chymotrypsin [37], canine factor IX [38], sheep plasmin [39], and human thrombin [40], respectively, showed 38, 37, 37, 36, and 33% similarity to both the earthworm proteases, isozymes A and B.

Fig. 10 illustrates the alignment of the active forms of isozymes A and B, and bovine trypsin. The catalytic triad, His57*, Asp102*, and Ser195* (asterisks

```
      1        10        20        30        40        50        60
A     IVGGIEARPYEFPWQVSVRRKSSDSHFCGGSIINDRWVVCAAHCMQGESPALVSLVVGEH
B     IVGGIEARPYEFPWQVSVRRKSSDSHFCGGSIINDRWVVCAAHCMQGEAPALVSLVVGEH
BT    IVGGYTCGANTVPYQVSL---NSGYHFCGGSLINSQWVVSAAHCYKSGIQVRL----GED
         20        30        40        50        60        70

              70        80        90       100       110       120
A     DSSAASTVRQTHDVDSIFVNENYDPRTLENDVSVIKTAIAITFDINVGPICAPDPANDYV
B     DRSAASTVRQTHDVDSIFVHEDYNANTLENDVSVIKTSVAITFDINVGPICAPDPANDYV
BT    NINVVEGNEQFISASKSIVHPSYNSNTLNNDIMLIKLKSAASLNSRVASISLP-TSCASA
              80        90       100       110       120       130

              130       140       150       160       170       180
A     YRKSQCSGWGTINSGGICCPAVLRYVTLNITTNAFCDAVYTSDTIYDDMICATDNTGMTD
B     YRKSQCSGWGTINSGGICCPNVLRYVTLNVTTNQFCEDVYPLNSIYDDMICASDNTGGND
BT    GTQCLISGWGNTKSSGTSYPDVLKCLKAPILSDSSCKSAYPGQ-ITSNMFCAGYLEG--G
              140       150       160       170       180

              190       200       210       220       230       238
A     RDSCQGDSGGPLSVKDGSGIFSLGGIVSWGIGCASGY-PGVYSRVGFHAGWITDTITNN
B     RDSCQGDSGGPLSVKDGSGIFSLIGIVSWGIGCASGY-PGVYSRVGFHAAWITDIITNN
BT    KDSCQGDSGGPVVCSG-----KLQGIVSWGSGCAQKNKPGVYTKVCNYVSWIKQTIASN
      190       200       210   ***220       230       240   245
```

Fig. 10. Sequence alignment of the earthworm proteases, isozyme A (A), isozyme B (B), and bovine trypsin (BT). The numberings shown above and below the sequence are based on those of the active earthworm proteases and the chymotrypsin A, respectively. Gaps, indicated by a dash, are introduced in the sequences to maximize the homology. The amino acid residues of the catalytic triad and the primary substrate specificity determinant are boxed. The subsites, S1, S2, and S3, are indicated by an asterisk. An arrowhead denotes the peptide bond cleaved by autolysis.

show chymotrypsinogen numbering [41]), and the primary substrate specificity determinant, Asp189*, of the trypsin [42] correspond to His43, Asp91, Ser188, and Asp182 of both isozymes A and B, respectively. Furthermore, subsites, S1, S2, and S3 (Ser214*, Trp215*, and Gly216*) of the trypsin [42] correspond to Ser208, Trp209, and Gly210 of both isozymes A and B, respectively. These results show that the amino acid residues constituting the active site of the trypsins are conserved in the earthworm proteases, isozymes A and B.

5.3. Expression of the enzyme activity

The 803 bp cDNA fragment amplified by PCR encodes the native form of isozyme A at position Ile8 to Asn245 (Fig. 9A). The fragment inserted into the *Pichia* expression vector pPICZαB was fused with a gene encoding the *Saccharomyces cerevisiae* α-factor secretory peptide, which is cleaved by proteases and placed under control of the *Pichia* AOX1 promoter [43]. After induction of the gene by methanol, the fibrinolytic activity of the culture medium was analyzed using an artificial fibrin plate (Fig. 11). The lytic activities on the fibrin plate of isozyme A was around 200 CU/mg protein [11]. Therefore, the protease activities in the concentrated medium and the earthworm powder corresponded to 110 and 60 CU/mg protein, respectively. These results show that the cDNA encodes the earthworm protease and that the specific activity of the culture medium is higher than that of the earthworm crude extract. The culture medium of *Pichia pastoris* cells harboring pPICZαB showed no fibrinolytic activity. The recombinant protease from the medium was purified easily by chromatographic procedures basically according to the our procedures described previously [11,14]. No differences with respect to the fibrinolytic activity of the enzyme were observed when compared with those of the wild-type one (isozyme A).

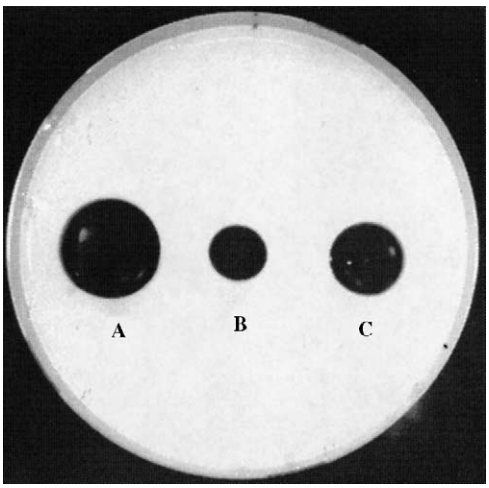

Fig. 11. Secretion of the earthworm protease, isozyme A, from the transformed *Pichia* cells. *P. pastoris* cells harboring pPICMMZ-1 were grown for 1 day in BMMY medium containing methanol. The medium (20 μl) was spotted onto the fibrin plate and incubated at 37 °C for 10 h. (A) The purified isozyme A (1 mg/ml); (B) crude extracts of the earthworm-lyophilized powder (1 mg protein/ml); (C) culture medium of *P. pastoris* cells harboring pPICMMZ-1 (1 mg protein/ml).

6. Structural aspects of the proteases

The primary structure of the isozymes A (EMBL, AB045719, by N. Nakajima and M. Sugimoto) and B (isozyme B, AB0457200, by N. Nakajima and M. Sugimoto) has been deduced from the nucleotide sequence of the cDNAs [15] and that of the isozyme C (SWISS-PROT, P83298, by N. Nakajima and M. Sugimoto) has been determined by the amino acid sequence analysis.

Compared with bovine trypsin, isozymes A and B (Fig. 10) have no Arg or Lys residue in the sequence comprising Asn131 to Asp141 corresponding to the autolysis loop (Asn143* to Asp153*) of bovine trypsin [42] but have Arg144, which triggers the autolysis of isozyme A. These results provide interesting information to clarify the relationship between the protein stability and the protein structure [13,15]. The isozymes A and B are translated as proenzymes which lack the signal peptide and have an N-terminal extension of seven and eight amino acids, respectively. The amino acid sequences of the two active enzymes comprise 238 amino acid residues, and 215 amino acid residues are conserved between these enzymes, implying that both the isozymes A and B are almost the same in substrate specificity, N-terminal amino acid sequence and immunological property as described earlier.

Immunochemical studies showed that both isozymes A and B, and isozymes D and E possessed the same antigenecity, respectively, although these isozymes

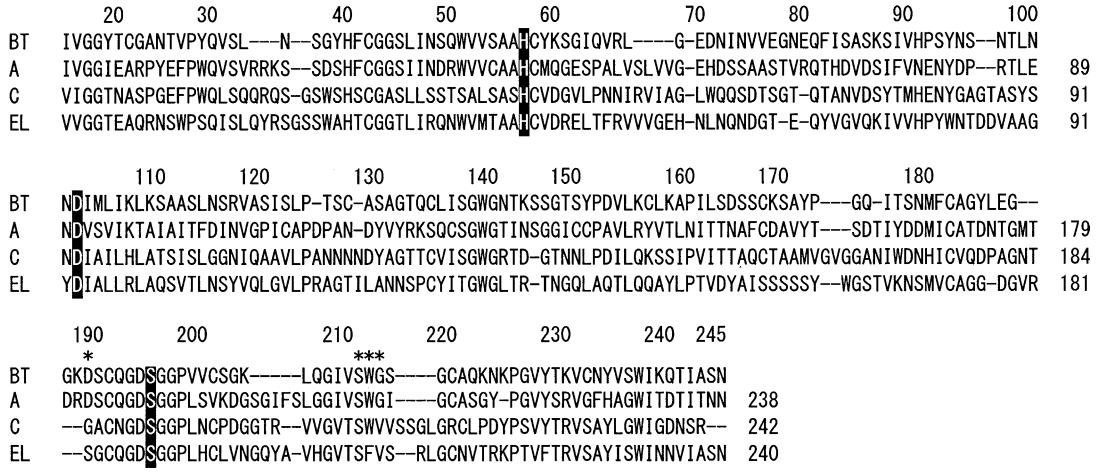

Fig. 12. Sequence alignment of the earthworm proteases, isozyme A (A), isozyme C (C), bovine trypsin (BT), and porcine elastase (EL). The numberings shown above and across the sequence are based on those of the chymotrypsinogen A and the active earthworm proteases, respectively. The amino acid residues of the catalytic triad are represented by reversal letters. The primary substrate specificity determinant and the subsites, S1, S2, and S3, are indicated by asterisks.

were derived from different genes. Two isozymes, C and F, reacted specifically with anti-C-serum and -F-serum, respectively [11].

The isozyme A shows 36% identity with the isozyme C, and both enzymes have high identity (30–38%) with mammalian serine proteases. The catalytic triad of His43, Asp91, and Ser188 on isozyme A, and His44, Asp93, and Ser191 on isozyme C are identified. The primary substrate specificity determinant and subsites conserved in trypsins have been identified as Asp182, Ser214, Trp215, and Gly216 on isozyme A, whereas the primary substrate specificity determinant was lacking and subsite 2 was substituted by Ser214 on isozyme C as the same as elastase (Fig. 12) [15].

7. Bioavailability of the protease functions

Earthworm-lyophilized powder has long been used for antipyretic and diuretic purposes in Chinese medicine as described earlier. Thrombolytic therapy by oral administration with the earthworm powder/enzyme, which could induce plasma fibrinolysis, has been investigated as a therapeutic agent and a health insurance drug for the prevention of life-style disease [44,45].

It was reported that the proteases from *L. rubellus* were non-hemorrhagic proteins and did not induce platelet aggregation and that the chemical modification with human serum albumin fragments was useful for the enzyme to mask undesirable properties of the therapeutic enzyme, such as anaphylaxis and immunoreactions [12]. Further clinical effects of the enzymes as the therapeutic enzymes have been reported [46–48].

7.1. Thrombolysis by the enzyme for actual fibrin clots in rat's whole blood

The fibrinolytic activity of the earthworm protease (isozyme A) was around 10 times that of human plasmin [11]. To investigate the ability of the enzyme to solubilize actual fibrin clots, we have tested thrombolytic activity of the enzyme using fibrin clots induced by thrombin in rat's whole blood. As shown in Fig. 13, 100 μl of fibrin clots formed in a thin plastic tube by the addition of 5 units of thrombin in 5 μl of saline was easily solubilized within 1 h by 50 μg of the enzyme in 100 μl of saline placed on the clots.

The ability of the enzyme to solubilize actual fibrin clots was also demonstrated with a thrombi of whole blood isolated in a rat's vena cava as shown in Fig. 14. The induction of actual fibrin clots (0.5 mm

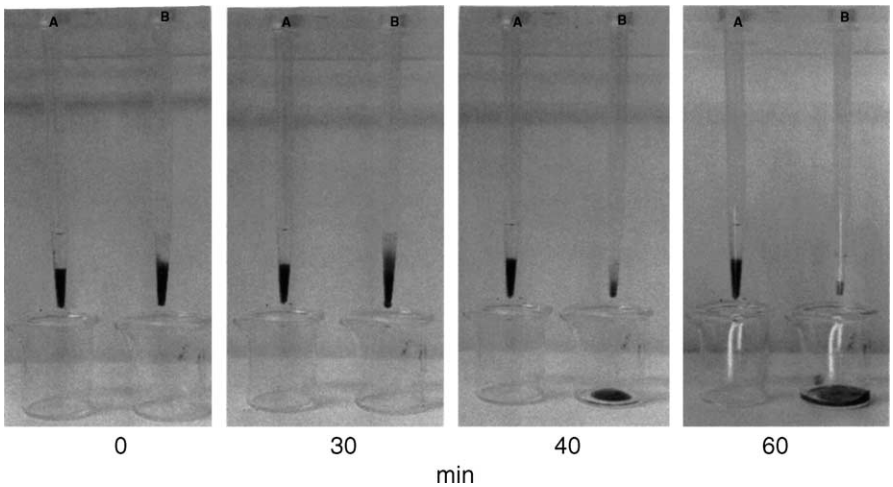

Fig. 13. Thrombolysis by the enzyme of fibrin clots of rat's whole blood. (A) One hundred microliters of saline was placed on the fibrin clots of rat's whole blood in the plastic tubes as a control. (B) Fifty micrograms of the enzyme in 100 μl of saline was added to the fibrin clots. The induction of the fibrin clots was done as described in the text.

i.d. × 10 mm) in the vena cava was formed by the injection of thrombin. Almost all of the fibrin clots (thrombi) isolated from the venous wall of the vena cava were also solubilized within 1.5 h by the addition of 50 μg of the enzyme in 500 μl of saline. These results showed that the enzyme had a potent thrombolytic potential as an anticoagulant protease.

7.2. Modification of the enzyme with human serum albumin fragments

Various serine proteases are known to be inactivated rapidly by protease inhibitors present in sera [49]. To prepare a clinically favorable therapeutic enzyme with a longer in vivo life, but without newly gained specific immunogenicity, we have modified the enzyme chemically with fragmented human serum albumin [12].

Human serum albumin was cleaved with cyanogen bromide into peptide fragments with molecular masses of 10–30 kDa, which may not have a strong inhibitory effect by steric hindrance on the enzyme [50].

Because the enzyme protein had a few amino groups of lysine on the enzyme molecule [11], the carboxyl groups of the acidic amino acids were used for bonding with the amino groups of diaminobutane as a spacer to produce free amino groups on the enzyme molecule using a coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (EDC)

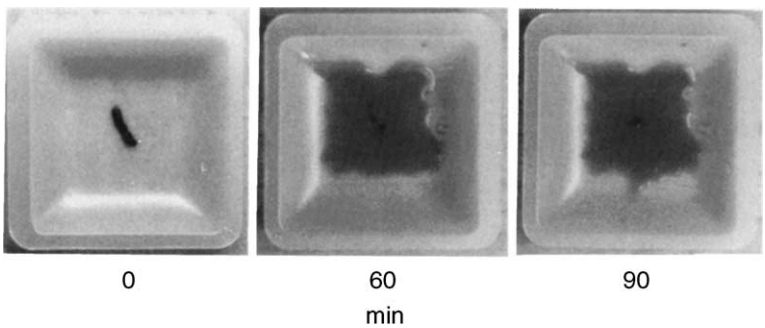

Fig. 14. Thrombolysis of the enzyme for thrombi in rat's vena cava. Fifty micrograms of the enzyme in 500 μl of saline was placed on the thrombi isolated from the rat's vena cava. Saline (500 ml) without of the enzyme was used as a control (data not shown). The thrombus induction was done as described in the text.

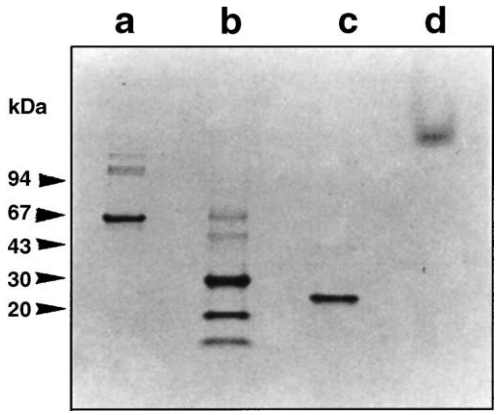

Fig. 15. SDS-polyacrylamide gel electrophoresis of the human serum albumin fragments and the modified enzyme conjugated with the albumin fragments. The electrophoresis was done on 10–20% gel with 20 μg of the samples: (a) human serum albumin; (b) the albumin fragment; (c) the native enzyme (isozyme A); (d) the modified enzyme.

[51]. The diamine-bound enzyme was then covalently attached to the human serum albumin fragments with glutaraldehyde as a coupling reagent [50]. After the coupling reaction of isozyme A with the human serum albumin fragments, the remaining fibrinolytic activity was about 30% of the initial enzyme activity. As shown in Fig. 15, by SDS-PAGE, the modified enzyme migrated as a single band of stained protein with a molecular mass of approximately 150 kDa, and the cleaved products of human serum albumin with cyanogen bromide were composed of three polypeptide bands containing a main protein band with a molecular mass of 30 kDa. On the basis of the molecular mass of the modified enzyme and the native enzyme, at least four to five molecules of the human serum albumin fragments with an average molecular mass of 20–25 kDa were probably attached to one native enzyme molecule with a molecular mass of 29,662 kDa [11].

7.3. Immunological properties of the modified enzyme

The antigenicity (reactivity with an antibody) of the synthesized conjugate was examined by Ouchterlony double-immunodiffusion analyses [12]. In Fig. 16A, where the native enzyme protein (isozyme A) was added to the center well, all of the antisera against human serum albumin, the albumin fragments, and the modified enzyme did not form precipitation lines with the native protein; it reacted only with the antiserum against the native enzyme to form a single precipitation line.

While, in Fig. 16B, where the modified enzyme was added to the center well, as well as the antiserum against the modified enzyme, both the antisera against human serum albumin and the albumin fragments reacted with the native enzyme, and these precipitin lines fused with each other. However, the conjugate protein did not react with the antiserum against the native enzyme. From these results, we suggested that the

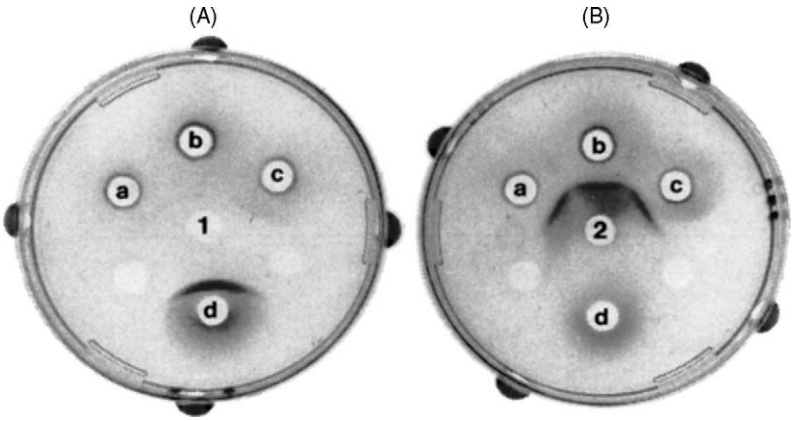

Fig. 16. Ouchterlony double-immunodiffusion analysis of the enzymes. Immunodiffusion was done at room temperature for 15 h. Clear wells contained: (A) 10 μg of the native enzyme (isozyme A) (1); (B) the modified enzyme (2). Peripheral wells containing: (a) 10 μl of anti-human serum; (b) albumin antiserum; (c) 10 μl of anti-modified enzyme antiserum; (d) 10 μl of anti-native enzyme antiserum.

antigenicity of the modified enzyme against the native enzyme protein had disappeared and that the conjugate of the native enzyme with the albumin fragments possessed the antigenicities of human serum albumin, the albumin fragments, and the conjugate itself. The antigenicity of the conjugate seemed to be identical with those of human serum albumin and the albumin fragments.

7.4. Stability of the modified enzyme by protease inhibitors in rat's plasma

The earthworm enzymes were serine proteases similar to plasmin [11] and were known to be inactivated significantly by protease inhibitors present in sera as described earlier. We investigated the inhibitory effects of rat's plasma on the enzyme activity of the modified enzyme by the procedures described previously [50]. As shown in Fig. 17, the conjugate was significantly more resistant to protease inhibitors than the native one: about 40% of the enzyme activity of the conjugate remained after incubation for 20–60 min with rat's plasma, while the native enzyme lost about 95% of its initial activity. From these results, the human serum albumin fragments protected the earthworm protease, though partially, from inactivation by protease inhibitors in rat's plasma, presumably because of steric hindrance in binding of the inhibitors with the enzyme.

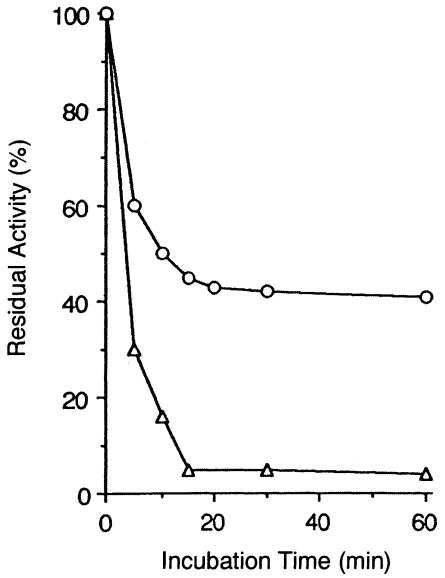

Fig. 17. Inactivation of the enzymes by protease inhibitor in rat's plasma. The modified enzyme (2 units/30 μg) (○) and the native enzyme (isozyme A, 2 units/10 μg) (△) were incubated with rat's plasma (1 ml each) at 37 °C. Samples of the reaction mixtures (10 μl) were taken at various indicated incubation times and the remaining activity in the plasma was measured as described in the text.

8. Further stabilization of the proteases

Isozymes A and B were more stable than trypsin and chymotrypsin. The isozymes had a strong tolerance to organic solvents (25% (v/v)) for at least more than 100 days at room temperature, even against toluene and *n*-hexane as described earlier [14], and retained activity for at least 20 days in 10 mM Tris–HCl buffer (pH 8.0) separately containing SDS (1%), Triton X-100 (1%), and NaCl (20%), compared with the complete inactivation of trypsin as a control. The enzyme retained at least 80% of its activity for at least 5 years at room temperature [14]. Moreover, it maintained almost all of its activity for more than 10 days up to 55 °C under alkaline conditions (pH 8.0) (Fig. 18), although the stability of the enzyme was rather low at pH 6.0, like other alkaline serine proteases [52]. Such

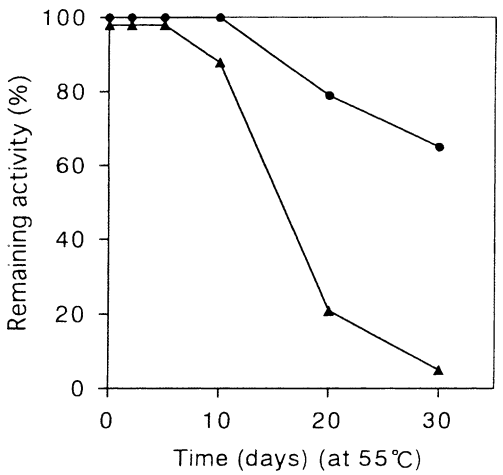

Fig. 18. Stability of the earthworm protease (isozyme A) under both alkaline and acidic conditions. Isozyme A (1 mg) was incubated in 10 mM Tris–HCl buffer (pH 8.0) (●) or 10 mM potassium phosphate buffer (pH 6.0) (▲) for various times at 55 °C. The remaining activity was assayed in 1 ml of 100 mM Tris–HCl buffer (pH 9.0) as described in the text.

stability and tolerance like those were found in other isozymes, isozymes B and C.

On the other hand, a rapid decrease in the activity of isozyme A occurred at more than 60 °C upon heating for 30 min under alkaline conditions (pH 9.0) as described earlier [13,16]. This inactivation was accompanied by autolysis. The autolytic cleavage site of isozyme A was Arg144–Tyr145 of the polypeptide as described earlier [13,15].

8.1. Further stabilization of the protease with chemical modification

We tried further stabilization of isozyme A [16] by chemical modification (cross-linkage) with EDC [53]. Compared with the native enzyme, the EDC-modified enzyme maintained its activity against heating at 65 °C for 30 min and for around 10 h at 60 °C (Fig. 19). The original specific activity of the enzyme for the chromogenic substrate was 150 units/mg protein [11], but the activity of the enzyme decreased by two-thirds after the modification [16]. Similar to the results of the EDC modification, further stabilization of the enzyme was also possible by chemical modification with phenylglyoxal (PGO) [54] These stabilizations against heating (up to 65 °C) via the chemical modification with EDC and PGO seemed to be due to blocking of the Arg residues containing the Arg144 site by chemical modification for the protection of the activity from autolytic inactivation [9,11].

8.2. Further stabilization of the protease with immobilization

As shown in Fig. 20, it was possible to stabilize isozyme A at 30 °C by immobilization with a folded sheet mesoporous material (FSM) 16/52 under acidic conditions (10 mM sodium acetate buffer, pH 5.0), in which the original stability of the enzyme was rather low (see Fig. 18), although the specific activity (150 units/mg) of the enzyme decreased by four-fifths due to the immobilization [16].

Thus, we further stabilized the earthworm protease by chemical modification with EDC and PGO under alkaline conditions, probably by protecting the activity from autolytic inactivation, and also stabilization by immobilization with FSM under acidic conditions, in which the original stability of the enzyme was rather low.

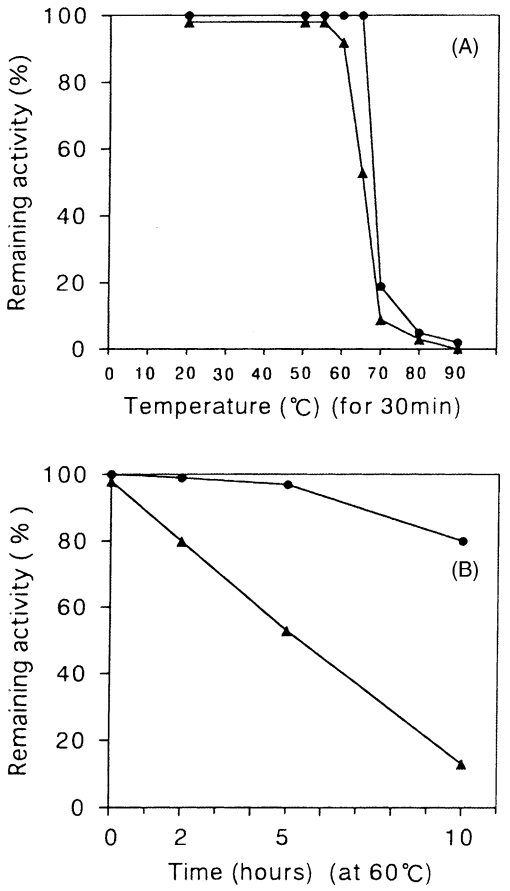

Fig. 19. Heat stability of the EDC-modified enzyme and the native enzyme. Isozyme A (1 mg) was modified in 1 ml of 50 mM sodium acetate buffer (pH 4.0) containing 10 mM EDC and 2 mM *N*-hydroxysuccinimide for 2 h at room temperature by the procedures described in the text. The remaining activities of the modified (3 mg) (●) and the native (1 mg) (▲) enzymes were assayed in 1 ml of 50 mM Tris–HCl buffer (pH 9.0) after heat treatment at various temperatures for 30 min in the buffer (A) and for various times at 60 °C (B).

9. Application of the catalytic functions of the proteases

9.1. Degradation of proteins by the enzymes

The proteolytic activity, except for the fibrinolytic activity, of the earthworm proteases was

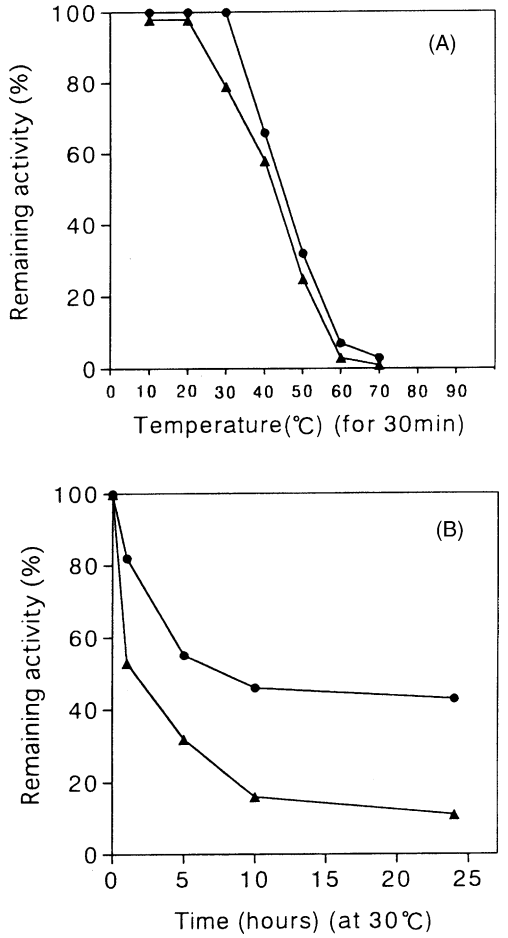

Fig. 20. Heat stability of the FSM-immobilized enzyme and the native enzyme. The immobilization of isozyme A (0.5 mg) with 2.5 mg of FSM 16/52 in 1 ml of H_2O containing 1 mM $CaCl_2$ was done for 2 days at 4 °C by the methods described in the text. The remaining activities of the immobilized (2 mg) (●) and the native (1 mg) (▲) enzymes were assayed in 1 ml of 100 mM Tris–HCl buffer (pH 9.0) after heat treatment at various temperatures for 30 min in 10 mM sodium acetate buffer (pH 5.0) (A) and for various times at 30 °C (B).

studied using various protein substrates. As shown in Table 2, both isozymes A and C were more effective than trypsin in the production of amino acids from elastin, hemoglobin, casein, and collagen, and also in the formation of peptides from albumin, elastin, and casein. Thus, the proteases are useful in the field of waste treatment of non-degradable proteins [55,56].

9.2. Continuous fibrinolysis by the enzyme

We have developed a continuous enzyme reactor system using the immobilized protease with oxirane-activated acrylic beads (Sigma) to investigate whether the enzyme could react for long periods while retaining its high fibrinolytic activity. A fibrin suspension (optical density of 1.0 at 370 nm) was prepared as described previously [20]. The reactor system with a 20 μm glass filter in the bottom of a slurry reactor as shown in Fig. 21 was operated with the immobilized isozyme A (10 mg) with the beads (1 g) by circulating 20 ml of the fibrin suspension in 50 mM borate saline buffer (pH 7.8) at room temperature using a peristaltic pump (flow rate 5 ml/h). The optical density was changed from 1 to 0.1 for the fibrin suspension within 24 h after the substrate solution was added to the reactor. After every 24 h of the substrate being hydrolyzed, the substrate solution was replaced with a new one, and the fibrinolytic reaction was continued for more than 10 cycles in the same manner. The continuous fibrinolysis was possible at least for more than 1 month without adding new immobilized enzyme as shown in Fig. 22. From these results, it was found that the immobilized isozyme A had retained the fibrinolytic activity without any inactivation of the activity after repeated use.

9.3. Hydrolysis of various esters by the enzymes

The esterase activities of several microorganisms have been reported with respect to the hydrolysis of the ester bonds in various esters [57–59]. To further investigate the catalytic functions of the earthworm proteases, the ester-bond hydrolyzing activity of isozymes A and B was determined by measuring the acetic acid and *p*-nitrophenol [14]. As shown in Table 3, hydrolytic activities for ethyl acetate and glucose acetate and for *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate were detected, while trypsin did not act on these substrates. Tosyl-L-Lys-OMe was completely hydrolyzed by isozymes A and B as well as trypsin. The formation of acetic acid from poly(vinyl alcohol) 500 by both the enzymes was also detected, but the amount formed was low.

Recently, the decomposition of bioplastics by microbial esterases was reported [60,61]. When isozyme B (1 mg), dissolved in 1 ml of 0.1 mM

Table 2
Degradation of proteins by the earthworm proteases

Protein	Amino acids released (mg)[a]			Peptides produced (mg)[b]		
	Isozyme B	Isozyme C	Trypsin	Isozyme B	Isozyme C	Trypsin
Elastin	2.8	3.1	0.2	7.2	12.0	2.0
Hemoglobin	31.8	29.4	10.6	112.0	128.0	98.0
Collagen	9.8	7.6	3.1	19.8	20.8	16.8
Casein	33.0	27.6	11.4	164.0	150.0	106.0
Albumin	1.2	1.0	0.6	6.0	5.2	1.2

[a] TCA-soluble amino acids, which were released from 200 mg of the protein substrates upon degradation at 10 CU of the earthworm proteases and porcine trypsin with shaking (150 × *g*) for 10 h at 37 °C, were determined after centrifugation for 20 min at 6000 × *g* as described in the text.

[b] TCA-soluble peptides were measured as described in the text.

Tris–HCl buffer (pH 8.0), was placed onto a poly[(*R*)-3-hydroxybutyrate] film (Showa Denko, Japan) under wet conditions at 25 °C, decomposition of the film occurred after approximately 1 month (Fig. 23), although trypsin did not induce degradation at all. Poly(lactate) film (Shimadzu, Japan) was decomposed to some extent by the enzyme. Furthermore, the earthworm proteases could be used as a biocatalyst for unmasking of the unnecessary acetyl moiety from the building blocks in organic synthesis. For example, the preparation of vinyl *p*-coumarate from the acetyl *p*-coumarate vinyl ester in ethanol was enabled using isozymes A, B, and C [62,63]. Therefore, the earthworm proteases exhibited ester-hydrolyzing ability as well as the proteolytic activity.

9.4. Preparation of "earthworm autolysate"

The homogenate of the earthworm (*L. rubellus*) autolyzed to give a deep black liquid (soy sauce-like liquid) following standing at room temperature [64]. To confirm whether microbial action is involved in

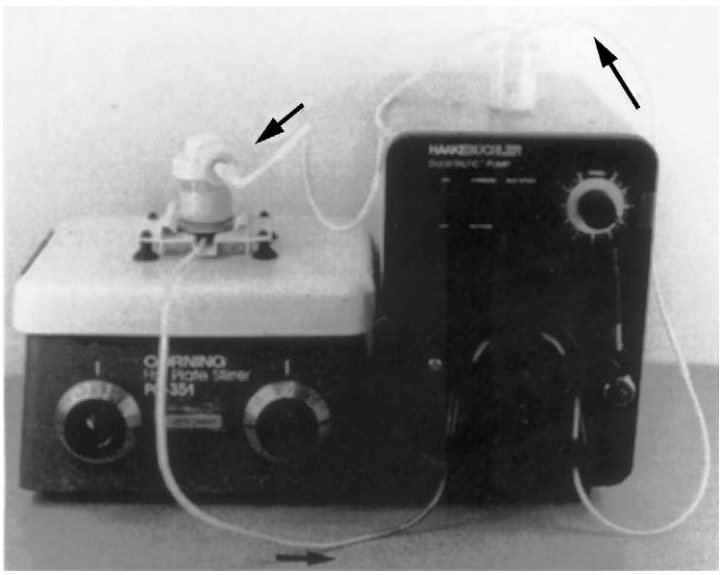

Fig. 21. An immobilized enzyme reactor system for fibrin suspension. The enzyme reactor system had a 20 μm glass filter in the bottom of the slurry reactor (left) with the enzyme immobilized to oxirane-activated acrylic beads for the continuous fibrinolysis. The arrows indicated the direction of flow of the fibrin suspension using a peristaltic pump (flow rate 5 ml/h).

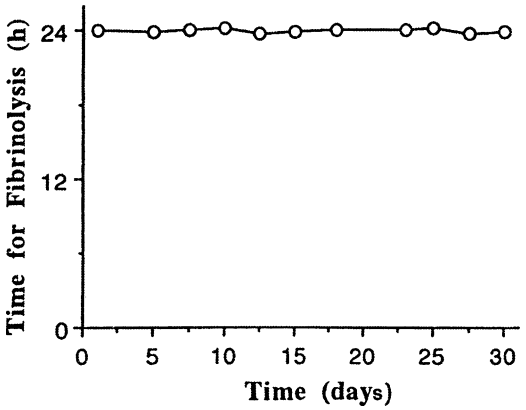

Fig. 22. Continuous fibrinolysis in an immobilized enzyme reactor. Twenty milliliters of fibrin suspension in borate saline buffer (pH 7.8) was fed continuously with the immobilized enzyme (1 g) in the slurry reactor. The continuous fibrinolysis was measured spectrophotometrically at 370 nm after every 24 h.

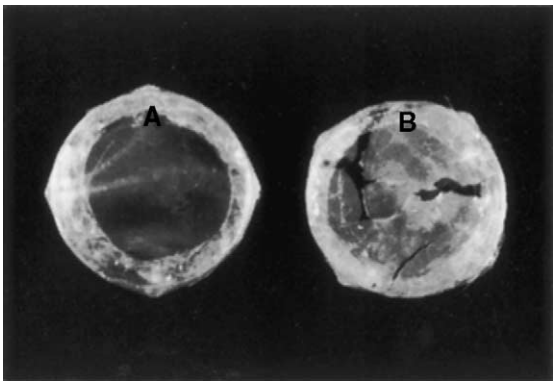

Fig. 23. Degradation of poly[(*R*)-3-hydroxybutyrate] by the earthworm protease. One milligram of the earthworm protease (isozyme B) dissolved in 1 ml of 100 mM Tris–HCl buffer (pH 8.0) was placed onto a film of poly[(*R*)-3-hydroxybutyrate] and left to stand under wet conditions for 1 month at 25 °C: (A) with the buffer solution in the absence of the enzyme; (B) in the presence of the enzyme.

degradation of the earthworm cells, we prepared an autolysate from both the fresh earthworm and earthworm-lyophilized powder in the presence and absence of 0.1% NaN_3. From both the homogenates of the fresh earthworm (twice the wet weight (w/v) of water added) and the earthworm powder (one-tenth the wet weight of water added), the black autolyzed aromatic liquids were formed only following standing in sealed containers at room temperature for over 1 year. Thus, the production of the autolysate was considered to be caused mainly by the action of the earthworm's own proteases without the involvement of microbial degradation [14].

The autolysate still retained strong fibrinolytic activity [14]. To identify the protease isozymes present in the autolysate (10-year-old lysate), the Ouchterlony double-immunodiffusion test of the lysate (10 μl in the center well) was done using antisera against each purified isozyme [11]. Sufficient single precipitin lines were detected between the antisera against the three isozymes B, C, and D. It seems that the protease activity retained in the 10-year-old lysate was due to the three isozyme proteins which possessed antigenic epitopes in the lysate for 10 years.

Table 3
Hydrolysis of esters by the earthworm protease

Ester compound	Product (mmol)
Ethyl acetate[a]	1.0
Glucose acetate[a]	3.0
Poly(vinyl alcohol) 500[a]	0.2
p-Nitrophenyl acetate[b]	0.2
p-Nitrophenyl butyrate[b]	0.1
Tosyl-L-Lys-OMe[b]	0.5

[a] Acetic acid, which was produced in the reaction mixtures (1 ml) containing 10 mM substrate, 10 CU of the earthworm protease (isozyme B), and 100 mM Tris–HCl buffer (pH 8.0) with shaking (150 × *g*) for 10 h at 37 °C was measured as described in the text.

[b] The products, which were formed in the reaction mixtures (1 ml) containing 1 mM substrate, 10 CU of the earthworm protease, and 100 mM Tris–HCl buffer (pH 8.0) for 1 min at 37 °C, were determined spectrophotometrically.

9.5. Enzyme activities in the "earthworm autolysate"

To investigate the enzymatic properties of the protease in the autolysate (10-year-old lysate), we purified the protein which exhibited the highest fibrinolytic and amidolytic activities. The extracts of the autolysate (50 ml) after centrifugation contained 125 mg of protein with a total of 53 units for H-D-Phe-Pip-Arg-*p*NA. The molecular mass of the purified enzyme was calculated to be 36 kDa on SDS-PAGE [14]. These results showed that the enzyme purified from the autolysate

corresponded to isozyme A or B, both of which were purified from the fresh earthworm [11].

Apart from the protease activity, the residual enzyme activities were also detected in the 10-year-old autolysate [14]; lipase (270 units/(ml min) at 30 °C) and amylase (0.25 units/(ml min) at 37 °C), using "Lipase kit S" (Dainippon Pharmaceutical Co. Ltd., Japan) and "Amylase test Wako" (Wako, Japan), respectively.

9.6. Analysis of the components of the "earthworm autolysate"

"Fish sauce" from fish meals, as well as "soy sauce", is produced widely in southeast Asian countries [65]. The chemical components in the supernatant of the "earthworm autolysate" (10-year-old lysate) were analyzed and compared with those in a commercially available "soy sauce". As shown in Table 4, the autolysate and soy sauce had similar amounts of total nitrogen, formol-titrated nitrogen, and L-lactic acid. However, the concentrations of NaCl, alcohol, and reducing sugars were very low in the "earthworm autolysate", despite its strong salty taste like "soy sauce". The extracts of the autolysate included 17.1% (w/v) solid substance, and almost 50% of this solid substance consisted of free amino acids. The amino acid composition of the autolysate was analyzed as shown in Table 5 and compared with that of Polypepton (Daigo Eiyou Co., Japan). The autolysate was rich in Lys, Ala, Leu, and Asx.

Table 4
Analysis of the components in the extract of the earthworm autolysate (10-year-old lysate) and soy sauce[a]

Component[a]	Earthworm autolysate	Soy sauce
Common salt (g/dl)	1.54	16.40
Total nitrogen (TN; g/dl)	1.63	1.59
Formol-titrated nitrogen (FN; g/dl)	0.96	0.95
FN/TN	0.59	0.59
Direct reducing sugars (g/dl)	0.31	4.00
Alcohol (g/dl)	0.12	2.25
L-Lactic acid (g/dl)	0.58	0.70
pH	5.90	4.85
Brix degree (at 20 °C)	17.10	34.00
Standard color	2.50	9.50

[a] These values of the components were measured according to a protocol described in "Analytical methods of Shoyu", Japan Shoyu Lab., Tokyo, Japan, 1985.

Table 5
Amino acid compositions of the extract from the earthworm autolysate and Polypepton

Amino acid	Number of residues (%)	
	Earthworm autolysate[a]	Polypepton[b]
Asx	11.39	6.61
Glx	2.93	20.08
Ser	7.30	4.82
Gly	9.20	1.97
His	1.31	2.17
Arg	1.02	3.76
Thr	5.57	4.74
Ala	12.70	3.01
Pro	5.49	8.57
Tyr	0.56	2.09
Val	8.61	4.06
Met	0.52	1.71
Ile	7.44	4.16
Leu	10.84	8.74
Phe	1.38	4.02
Lys	13.74	13.62
Cys	–	0.02
Trp	–	0.14

[a] One milligram of the lyophilized powder from the extract of the earthworm autolysate (10-year-old lysate) was analyzed according to the Waters Pico-tag amino acid analysis method described in the text. The values (%) are expressed as the number of amino acids, except for Cys and Trp.

[b] Typical amino acid composition of the peptone corresponding to Polypepton (Daigo, Japan) used in the experiments ("Peptone and hydrolysates selection guide in the culture media data sheets", Difco).

Based on a report that melanoidins produced in soy sauce during fermentation exhibit antioxidant ability, we determined the total antioxidant status (TAS; mmol/l) of the "earthworm autolysate" using a "total antioxidant status kit" (Randox Laboratories Ltd., CA, USA) based on the manufacturing instructions [66]: 20 μl of the samples were added in the reaction mixtures (1 ml) containing a radical cation (ferrylmyoglobin) derived from metmyoglobin and H_2O_2. The value of the antioxidant status of the autolysate was about 3.0 mmol/l.

9.7. Effect of replacing Polypepton with the earthworm in microbial media

To investigate the effect of replacing with the "earthworm autolysate", some microorganisms were grown in a medium containing the lyophilized extracts of the

30-day-old autolysate (one-tenth the volume of water) of commercially available earthworm powder [14]. Growth of the microorganisms in the medium with the autolysate in place of Polypepton, and in the original medium (without changes in the other ingredients) was compared [14]. The growth of bakers' yeast (Oriental Yeast, Japan) (Fig. 24A) in the medium containing the same amount of earthworm autolysate as would have been used for Polypepton was substantially better than that in the medium containing Polypepton. *Escherichia coli* XL1-blue (Fig. 24B), as well as *Bacillus coagulans* IFO 12583 and *B. stearothermophilus* DSM 297, could grow in the media containing the autolysate as well as in those containing Polypepton [14].

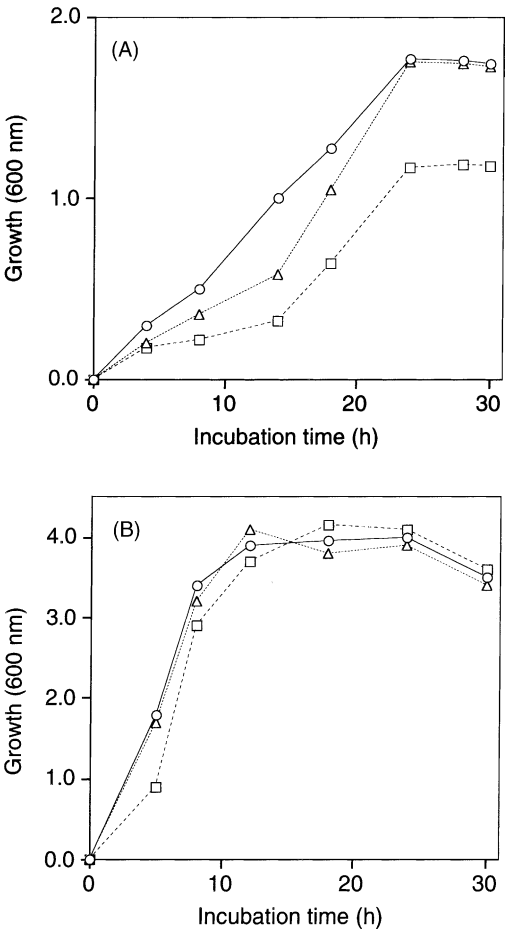

Fig. 24. Growth of microorganisms in media containing the earthworm autolysate in place of Polypepton. Bakers' yeast (Oriental Yeast, Japan) (A) and *E. coli* XL1-blue (B) were grown in media with different compositions at 30 and 37 °C, respectively. (○) The lyophilized extract of the earthworm autolysate (30-day-old lysate) in place of Polypepton; (△) half the amount of Polypepton used was replaced by the earthworm lysate; (□) Polypepton added without changing the other ingredients as described in the text.

Although the addition of the commercially available earthworm-lyophilized powder to media had the same effect on the growth of the microorganisms as did the lyophilized powder from the autolysate, the use of the earthworm powder in the absence of autolysis caused precipitation in the media after autoclaving because of its low solubility. Thus, the use of the autolyzed powder made it easier to prepare the media than did the direct addition of the commercially available earthworm powder. These results indicated that both the fresh earthworm powder and its autolysate could be a "peptone substitute" for the efficient growth of microorganisms.

10. Conclusions

In this review, we reported the wide-ranging catalytic functions of the earthworm-serine proteases which were composed of the six isozyme proteins. The proteases exhibited high stability and strong tolerance towards organic solvents and detergents. The enzymes are expected to be used in therapeutics, the synthesis of useful substances, such as optically active compounds, and the degradation of organic waste products from the livestock industry, the food industry, and so on.

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