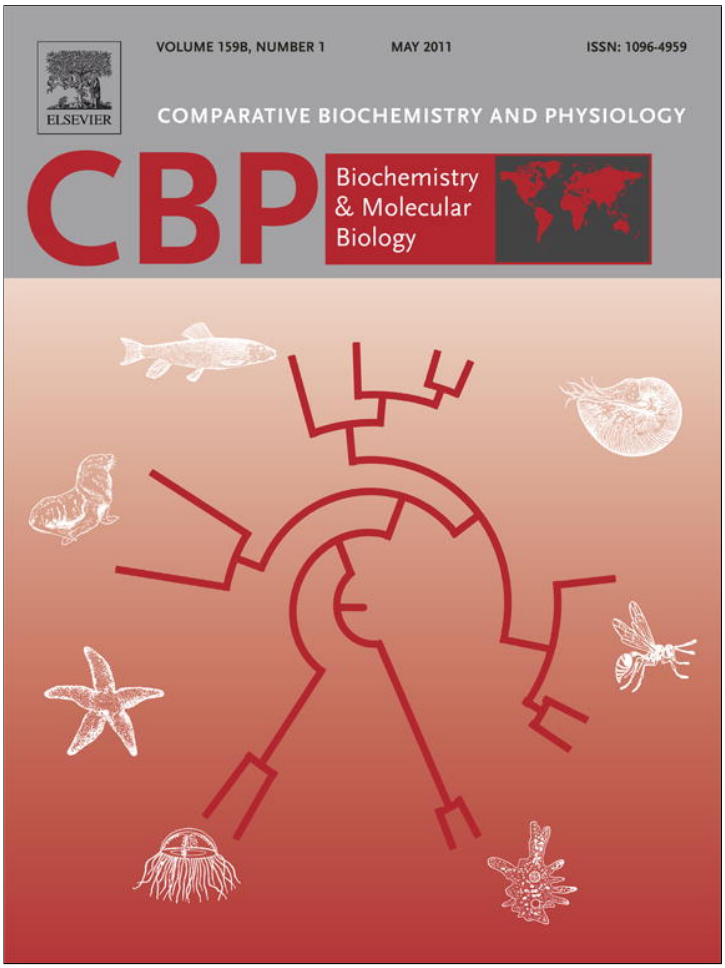

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A novel alkaline serine protease with fibrinolytic activity from the polychaete, *Neanthes japonica*

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ABSTRACT

A new protease named NJP with fibrinolytic activity was isolated from *Neanthes japonica* (Izuka), by a combination of ammonium sulfate fractionation, hydrophobic chromatography, ion-exchange chromatography and gel filtration. The molecular mass of NJP was approximately 28.6–33.5 kDa as estimated by MALDI-TOF mass spectrometry and SDS-PAGE, which revealed a monomeric form of the protease. The isoelectric point of NJP determined by 2-DE was 9.2. NJP was stable in the range of pH 7.0–11.0 with a maximum enzymatic activity at 40 °C and pH 9.0. The hydrolyzing activity of NJP on fibrinogen started from the Aα-chain, followed by the Bβ-chain, and the γ-chain at last. NJP had also a higher specificity for the chromogenic substrate S-2238 for thrombin. NJP activity was completely inhibited by PMSF. Analysis of partial amino acid sequences showed that NJP had very low homology with other known fibrinolytic enzymes. These results indicate that NJP is a novel alkaline thrombin-like serine protease. Thus NJP may have potential applications in the prevention and treatment of thrombosis.

1. Introduction

The incidence of thrombotic disorders including cerebral stroke, myocardial infarction, and venous thromboembolism is rapidly increasing throughout the world, so does the mortality of these disorders (Mine et al., 2005). During the past decade, fibrinolytic agents such as tissue-type plasminogen activator (t-PA) and urokinase (UK) have been widely used in the treatment of thrombosis. However, all these enzymes have undesired side effects, including need of large therapeutic doses, limited fibrin-specificity, re-occlusion and bleeding tendency (Murray et al., 2010). Therefore, the search for other fibrinolytic enzymes from various sources is being continued. The isolation of relatively inexpensive fibrinolytic enzyme with high specificity for fibrin from nature extracts was investigated (Lee et al., 2005). In addition, several fibrinolytic enzymes have been discovered from a variety of sources, such as lumbrokinase from earthworms (Mihara et al., 1991; Nakajima et al., 1993; Wang et al., 2003; Cho et al., 2004), scolonase from the Korean centipede, *Scolopendra subspinipes mutilans* (You et al., 2004), lonofibrase from *Lonomia obliqua* caterpillars (Pinto et al., 2004), nattokinase from vegetable cheese natto (Fujita et al., 1993; Deepak et al., 2009), snake venoms (Bortoleto et al., 2002; Leonardi et al., 2002; De-Simone et al., 2005) and fibrinolytic enzymes from mantis (Hahn et al., 1999; Hahn et al., 2001), making them potent naturally occurring candidates for fibrinolytic therapy.

Neanthes japonica (Izuka) belongs to Annelida/Polychaeta/Nereididae Johnston/Neanthes kinberg as identified by the Institute of Oceanology, Chinese Academy of Sciences. It is a euryhaline and eurythermal nereid polychaete that exists mainly in China and Japan, usually inhabiting the intertidal zone, shallow, sandy-mud sediments and estuaries. A fibrinolytic enzyme from *N. japonica* (Izuka), named NJF (EC 3.4.21.-), has been purified and characterized in our previous research (Deng et al., 2010). During the purification of NJF, in the procedure of ion-exchange chromatography, NJF with high fibrinolytic activity was isolated from the bound fraction after the non-interacting fraction was washed from the column. Yet later on a significant fibrinolytic activity with high pH value was also detected in the non-interacting fraction. So the specific approach was followed for the identification of a new protease. In this study, we report the purification and characterization of a new different serine protease from *N. japonica* (NJP, EC 3.4.21.-).

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; 2-DE, two-dimensional electrophoresis; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MALDI-TOF-TOF, matrix-assisted laser desorption ionization-time of flight; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol tetraacetic acid; SBTI, soybean trypsin inhibitor; BSA, bovine serum albumin.

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2. Materials and methods

2.1. Materials

Specimens of *N. japonica* were collected from the coast of Qinghuangdao Prefecture, China. *N. japonica* was maintained in small plastic boxes at −20 °C. Phenyl Sepharose 6 Fast Flow, DEAE Sepharose Fast Flow, Sephacryl S-100 High Resolution, Sephadex G-25 (medium grade), low molecular weight (LMW) protein standard markers, and immobilized pH 3–10 nonlinear gradient strips were products of Amersham Bioscience (Sweden). Bovine thrombin and human plasminogen were purchased from the National Institute for The Control of Pharmaceutical and Biological Products (Beijing, China). Bovine fibrinogen, BSA, PMSF, aprotinin, EDTA, EGTA, pepstatin A, SBTI, benzamidine, Coomassie Brilliant Blue R250 and iodoacetate were obtained from Sigma-Aldrich (St. Louis, MO, USA). H-D-Phe-Pip-Arg-*p*NA (S-2238) and H-D-Val-Leu-Lys-pNA (S-2251) were procured from Chromogenix (Milano, Italy) and Harbin High-Tech Group (White Swan Pharmaceutical Co., Ltd., UK), respectively. Other reagents were of special grade and commercially available.

2.2. Purification of NJP

Unless otherwise stated, all fractionation steps were performed at 4 °C. Five kg of washed clamworm samples were homogenized in 2 L of 20 mM phosphate buffer (pH 7.0, buffer A) and the solution was centrifuged at 8,000 *g* for 30 min. Solid ammonium sulfate was slowly added to the supernatant up to 20% saturation. The mixture was stored at 4 °C for 4 h or overnight, and centrifuged at 10,000 *g* for 20 min to remove the particulates and pellets. The supernatant was adjusted to 55% ammonium sulfate saturation by adding solid ammonium sulfate and stored for 4 h. The precipitate was collected by centrifugation at 10,000 *g* for 15 min and resuspended in 1 L of buffer A with 1 M ammonium sulfate. The crude enzyme solution was applied to a 7.5×20 cm Phenyl Sepharose 6 FF column (Amersham Bioscience) equilibrated with buffer A containing 1 M ammonium sulfate. The bound fractions were eluted using a linear decreasing gradient of ammonium sulfate from 1.0 M to 0 M in buffer A at a flow rate of 35 mL/min. The major active fraction showing the maximal fibrinolytic activity was collected and loaded onto a 3.5×50 cm G-25 column (Amersham) equilibrated with 20 mM Tris–HCl buffer (pH 7.4, buffer B) and then eluted with the same buffer at a flow rate of 14 mL/min for desalting. The desalted active fraction was applied to a 5.5×20 cm DEAE Sepharose FF column (Amersham), equilibrated with buffer B, and eluted with linear gradient of 0–1 M NaCl in the same buffer at a flow rate of 10 mL/min. The non-interacting fraction was washed from the column with buffer B, and the fraction showing high fibrinolytic activity was collected and concentrated by lyophilization for further purification. The bound fraction was eluted and collected for the purification of NJF as previously described (Deng et al., 2010). The concentrated non-interacting fraction having fibrinolytic activity was dissolved in a small volume of buffer B and loaded onto a 1.6×100 cm Sephacryl S-100 High Resolution column (Amersham) equilibrated with buffer B containing 0.15 M NaCl, and then eluted with the same buffer at a flow rate of 0.85 mL/min. The maximum enzymatic active fraction were collected, desalted and concentrated by lyophilization, and used as the purified enzyme preparation. For all purification steps, the eluates were monitored by spectrophotometry at 280 nm. The activity of the enzyme was estimated with azocasein as a substrate and by the fibrin plate assay as described later. Protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.3. Proteolytic activity

Proteolytic activity was measured using azocasein as a substrate following previously described methods (Beynon and Kay, 1978; Hahn et al., 1999). The reaction mixture, composed of 1 mL of azocasein (2 mg/mL in 0.2 M Tris–HCl buffer, pH 7.8) and 20 μL of purified NJP/column fraction (0.05 μg/μL), was incubated at 37 °C. After 1 h, 0.25 mL of the mixture was transferred to a 1.5 mL tube containing 1 mL of 5% (w/v) trichloroacetic acid and mixed well. The tubes were then centrifuged at 11,000 *g* for 5 min and the absorbance of the supernatant was measured at 340 nm.

2.4. Fibrinogenolytic and fibrinolytic activity

The fibrinogenolytic activity was tested by incubating 500 μL of fibrinogen (2 μg/μL) with 1 μg of NJP in 20 mM Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl at 37 °C. At various time intervals (0, 1, 10, 30, 60, 120, 180, 300 and 420 min), aliquots were taken from the reaction mixture and mixed with an equal volume of sample buffer containing β-mercaptoethanol and were then boiled at 100 °C and analyzed by SDS-PAGE.

The fibrinolytic activity was determined by the method using both plasminogen-rich and plasminogen-free fibrin plates with minor modifications (Astrup and Mullertz, 1952). The plasminogen-free fibrin plate was made up of 3 mL fibrinogen solution (1.5% bovine fibrinogen in 20 mM Tris–HCl buffer, pH 7.4), 1 mL of thrombin solution (1 U/mL in the same Tris–HCl buffer according to British Pharmacopeia) and 3 mL of 1% agarose in Petri dishes (5.5 cm in diameter), this fibrin plate was heated at 80 °C for 30 min to eliminate other fibrinolytic factors, while the plasminogen-rich fibrin plate contained 5 units of plasminogen in addition to the above components and was not heated. The clot was allowed to stand for 1 h at room temperature, and then holes (3 mm in diameter) were punched on the fibrin plate for sample application. To observe the fibrinolytic activity, 10 μL of the sample solution was carefully dropped into each hole and incubated at 37 °C for 18 h. The activity of the fibrinolytic enzyme was estimated by measuring the dimension of the clear zone on the fibrin plate and using UK as a control.

2.5. Determination of molecular mass and isoelectric point

The molecular mass of NJP was determined by SDS-PAGE and MALDI-TOF mass spectrometry (MS). SDS-PAGE was performed according to the method of Laemmli (1970) using 5% stacking and 12% resolving polyacrylamide gel. The gels were stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich). Molecular weight was measured according to the LMW standard protein markers which composed of rabbit phosphorylase B (97.0 kDa), BSA (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and bovine milk α-Lactalbumin (14.4 kDa). The molecular mass of NJP was also analyzed by MALDI-TOF MS (Autoflex III, Bruker) at the National Center of Biomedical Analysis.

The isoelectric point (pI) of NJP was determined by two-dimensional electrophoresis (2-DE) according to the manufacturer's procedure. Isoelectic focusing (IEF) was carried out using 13-cm Immobiline DryStrip gels containing a preformed pH gradient immobilized in homogeneous polyacrylamide gels with a pH range of 3–10 (Amersham). Purified enzyme was loaded by in-gel rehydration with a reswelling solution containing 8 M of urea, 0.3% DTT (w/v) and 0.2% (v/v) pH 3–10 IPG buffer. IEF was carried out at 20 °C in a Multiphor II Electrophoresis System (Amersham), wherein the voltage was linearly increased from 300 to 3500 V at 4 V/min and kept constant for a further 3 h. After IEF, the strip was equilibrated for 15 min in buffer containing 8 M urea, 20% glycerol, 2% SDS, 2% DTT and then for 15 min in the same buffer containing 2.5% iodoacetamide instead of DTT. After equilibration, the strip was loaded on a 12.5% SDS-PAGE gel for second-dimensional separation. The gel was then stained with Coomassie Brilliant Blue R250.

2.6. Determination of amino acid sequence by MALDI-TOF/TOF MS

NJP was separated by 12% SDS-PAGE and the gel was stained with Coomassie Brilliant Blue R250. The purified enzyme in the gel was cleaved by trypsin. The digested peptides were extracted from the gel matrix and analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF/TOF) Autoflex IIITM (Bruker) at Hong Kong Baptist University. The obtained amino acid sequences were submitted to the UniProtKB/Swiss-Prot database. The homology with the amino acid sequences of NJP was searched through the non-redundant protein sequences (nr) and Swiss-Prot databases by using NCBI blastp program.

2.7. Effects of temperature and pH on fibrinolytic activity

The optimal temperature for this enzyme against azocasein was determined by incubating NJP (20 μL of 0.1 μg/μL) in 50 mM Tris–HCl (pH 7.4) at different temperatures (10–80 °C) for 1 h. After incubation, the residual protease activity (%) was measured with 2 mg/mL azocasein as described above.

The optimal pH for NJP was determined in various buffers with pH ranges from 3 to 11. NJP of 10 μL (0.1 μg/μL) was added to 90 μL of 50 mM citrate buffer for pH 3–6, 50 mM Tris–HCl buffer for pH 7–9 and 50 mM glycine-NaOH buffer for pH 10–11, respectively. After incubating for 1 h at 37 °C, the remaining protease activity (%) was measured using azocasein as a substrate.

2.8. Effects of protease inhibitors on enzyme activity

The effects of nine protease inhibitors (PMSF, EDTA, EGTA, β-mercaptoethanol, iodoacetate, benzamidine, aprotinin, pepstatin A and SBTI) were examined. The enzyme sample (2 μg) was pre-incubated in 50 mM Tris–HCl buffer, pH 9.0 with the addition of a protease inhibitor at 37 °C for 1 h. After incubation, the residual enzyme activity was determined using the azocasein assay.

2.9. Amidolytic activities on chromogenic substrates

Amidolytic activities were measured spectrophotometrically using the chromogenic protease substrates, S-2238 (H-D-Phe-Pip-Arg-*p*NA for thrombin) and S-2251 (H-D-Val-Leu-Lys-*p*NA for plasmin and streptokinase-activated plasminogen). Activities were tested by mixing NJP (1 μg/300 μL of 50 mM Tris–HCl (pH 7.4)) with 450 μL of a 0.5 mM substrate. After continuous measurement for 5 min at 37 °C using a spectrophotometer, the amount of *p*-nitroaniline released was determined by measuring the change in absorbance at 405 nm ($\varepsilon = 9.65 \times 10^3\ M^{-1}\ cm^{-1}$, Tris–HCl, pH 7.4).

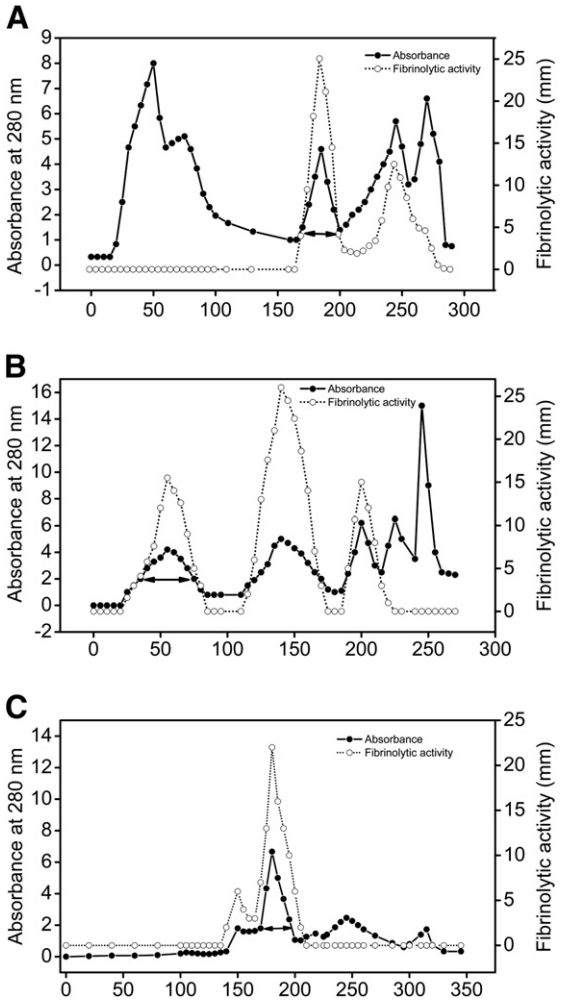

Fig. 1. Chromatography patterns of the purification procedures. (A) Phenyl sepharose 6 FF. (B) DEAE sepharose FF. (C) Sephacryl S-100 HR. The elution profiles were monitored by reading the absorbance at 280 nm (—●—). Fibrinolytic activity, based on fibrin plate assay, was shown as a dotted line (○) and the active fraction was collected (↔).

3. Results and discussion

3.1. Purification of NJP

Purification of this fibrinolytic enzyme was carried out by a combination of various column chromatographic purification steps. The results were listed in Table 1 and shown in Fig. 1. Fibrinolytic activity was mainly used as the index of purification. The crude extract containing 66,000 mg of protein was obtained from the

Table 1
Purification steps of NJP from *Neanthes japonica* (Izuka).

Purification step	Protein (mg)	Azocaseinolytic activity (U/mg)[a]	Fibrinolytic activity (U/mg)[b]	Total activity (units)	Yield (%)[c]	Purification fold
Crude extract	66,000	8.4	6.5	429,000.0	100	1
Ammonium sulfate	19,367	25.4	20.6	398,960.2	93.0	3.2
Fractionation						
Phenyl sepharose 6FF	540	121.0	407.2	219,888.0	51.3	62.6
DEAE sepharose FF	15.6	162.2	5434.1	84,772.0	19.8	836.0
Sephacryl S-100 HR	5.5	306.1	10,113.9	55,626.5	13.0	1556.0

[a] One unit of azocaseinolytic activity is defined as the amount of enzyme which causes a net increase of 1.0 in absorbance at 340 nm in 1 h.
[b] One unit is defined as the amount of enzyme that will hydrolyze 1.0 μmol of S-2238 per minute at 37 °C.
[c] Yield was calculated based on the total fibrinolytic activity.

homogenate of *N. japonica*. Ammonium sulfate fractionation between 20% and 55% produced maximal fibrinolytic activities. In hydrophobic interaction chromatography using a Phenyl Sepharose 6 FF, the fibrinolytic activity was eluted in two fractions (Fig. 1A). The major active fraction was further purified by anion-exchange chromatography using a DEAE Sepharose FF, and the fibrinolytic activity was eluted as three peaks (Fig. 1B). Anion-exchange chromatography was particularly a critical step to the success of NJP purification. NJF was purified from the second active fraction as previously described (Deng et al., 2010). The first active fraction (the non-interacting fraction) was further purified by gel filtration chromatography using a Sephacryl S-100 HR column. The fibrinolytic activity was eluted as two peaks (Fig. 1C), the higher peak with the maximum fibrinolytic activity was collected, desalted and concentrated by lyophilization, and used as the purified enzyme preparation, named as *N. japonica* protease (NJP). The purified NJP was shown as a single band in SDS-PAGE (Fig. 2A) and one protein spot in 2-DE (Fig. 2C). The fibrinolytic activity of NJP was increased by 1556-fold after purification, and the final yield was 13%.

3.2. Molecular mass and pI

NJP was found to be homogeneous by SDS-PAGE and the apparent molecular mass was estimated to be 33.5 kDa (Fig. 2A and B), which was consistent with that of the single spot identified by 2-DE (Fig. 2C) indicating that the enzyme was a monomeric protease. The molecular mass of NJP estimated by MALDI-TOF MS was 28.6 kDa (Fig. 3A). The molecular mass of lumbrokinase isolated from *Lumbricus rubellus* was disparate. It varies from 23.5 to 34.2 kDa as reported by Mihara et al. (1991), and from 24 to 43 kDa as reported by Nakajima et al. (1993). Wu and colleagues isolated eight fibrinolytic enzymes from *Eisenia fetida*, the molecular masses were ranged from 22.5 to 35 kDa (Wu et al., 2007). The molecular mass of NJP was similar to those of the fibrinolytic enzymes from *N. japonica* (Izuka) (NJF, 28–32 kDa) (Deng et al., 2010), *L. rubellus* (F5 (33.1 kDa), F6 (33 kDa)) (Cho et al., 2004) and *E. fetida* (EFE-b (29.5 kDa), EFE-c (29.7 kDa), EFE-g (29.6 kDa)) (Wang et al., 2003). Scolonase, a centipede serine protease, having a molecular mass of 25 kDa was also reported in the invertebrate: *S. subspinipes mutilans* (You et al., 2004). These results suggest that the molecular masses of these proteases with fibrinolytic activities from invertebrates are in a relatively narrow range (22–43 kDa).

To determine the pI of NJP, electronic images of 2-DE gel were analyzed using ImageMaster™ 2D Platinum software (Amersham Bioscience). Only one protein spot was detected and its pI was about 9.2 (Fig. 2C). The pI of NJP was lower than subtilisin DJ-4 (10.0) (Kim and Choi, 2000), but higher than that of NJF (4.4) (Deng et al., 2010), EFEa–g (3.46–3.94) (Wang et al., 2003), lumbrokinase (F-I-0, F-I-1, F-I-2, F-II, F-III-1, and F-III-2) (4.85, 4.30, 4.00, 4.20, 3.60, and 3.40, respectively) (Nakajima et al., 1993) and scolonase (4.8) (You et al., 2004). As the pI of most fibrinolytic enzymes was below pH 7.0, the basic pI of NJP suggests that NJP is a novel enzyme different from other known serine protease with fibrinolytic activity.

3.3. Amino acid sequence analysis of NJP

The trypsin-digested peptides from the band on SDS-PAGE were analyzed using MALDI-TOF/TOF MS. Partial amino acid sequences of three peptides were obtained (Fig. 3B–D). The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession number(s) P86834. The homology was searched through the non-redundant protein sequences (nr) and Swiss-Prot databases, no significant similarities were found between NJP and any other known proteins. Alignment of the obtained sequences of NJP with other proteases sequences by ClustalW software indicated that there was no significant homology between NJP and the selected proteases, hirudin (*Hirudo medicinalis*, GenBank: CAA01205.1) and the recombinant protease of *Perinereis aibuhitensis* Grube (PPA) (Li et al., 2006) (Fig. 4). These results suggest that NJP is a novel protein. Recently a kind of invertebrate fibrinolytic enzyme named lumbrokinase has been expressed by recombinant DNA techniques in yeasts, *Escherichia coli* and milk, respectively (Ge et al., 2007; Xu et al., 2010; Hu et al., 2004). And it has reported that a recombinant cysteine protease inhibitor (CPI)

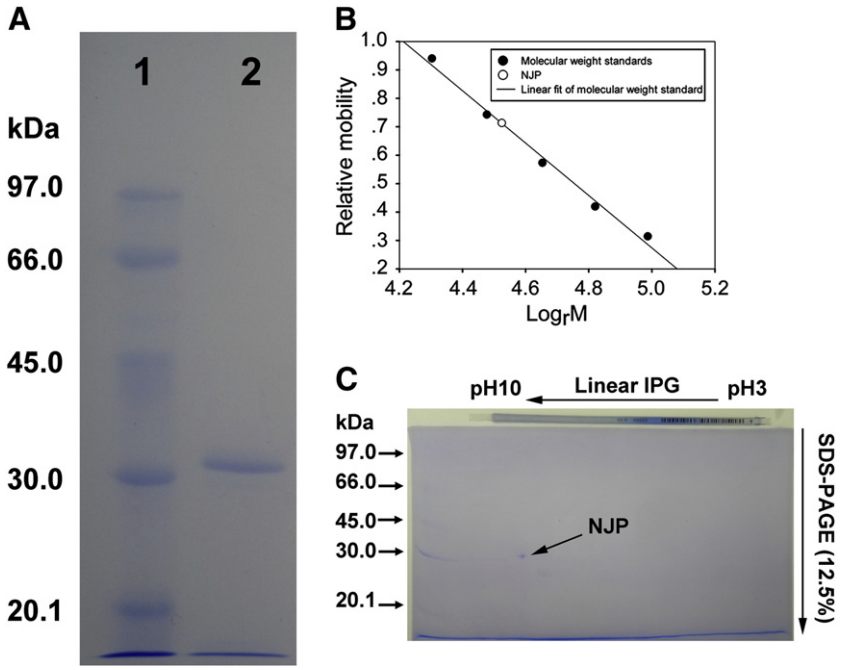

Fig. 2. SDS-PAGE (A), logarithmic plot (B) and 2-DE (C) of the purified NJP. (A) Lane 1, a standard LMW marker; Lane 2, purified NJP. (B) Logarithmic plot for the SDS-PAGE of purified NJP. (C) The first dimension was completed on immobilized pH 3–10 linear gradient strips; the second dimension was analyzed by 12.5% SDS-PAGE. LMW standard protein marker run on the left was indicated. In this image, only the stained spot of NJP was highlighted.

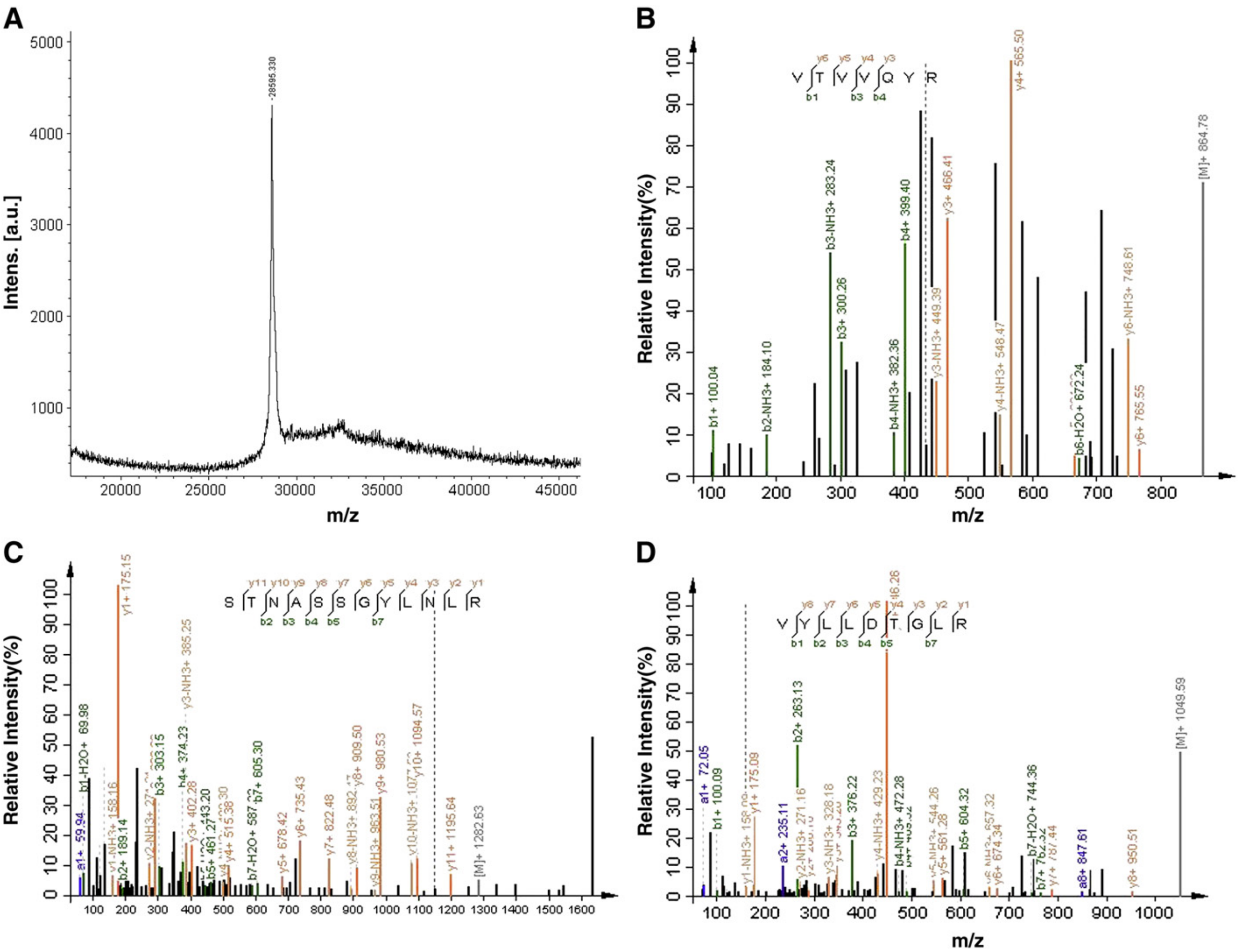

Fig. 3. MALDI-TOF mass spectrum (A) and MALDI-TOF/TOF MS (B–D) of NJP. The molecular mass was analyzed by MALDI-TOF MS (A). Results of MALDI-TOF/TOF MS for NJP. The partial amino acid sequences of the three peptides were (B) V-T-V-V-Q-Y-R, (C) S-T-N-A-S-S-G-Y-L-N-L-R and (D) V-Y-L-L-D-T-G-L-R.

(Li et al., 2010) with strong insecticidal activity and a recombinant perinerin (Zhou et al., 2009) with antimicrobial effects have been cloned and expressed from clamworm *P. aibuhitensis* Grube. Thus, our further research interest will continue with working on the cloning of full-length cDNA and expression of the recombinant NJP protein.

3.4. Fibrinogenolytic and fibrinolytic activity

To elucidate the action mode of NJP, degradation products of fibrinogen digested by NJP were separated by SDS-PAGE. The results showed that the purified NJP hydrolyzed the Aα-chain of fibrinogen completely within 1 min, followed by the Bβ-chain after 30 min, and then the γ-chain after 300 min (Fig. 5A). This hydrolytic pattern was similar to that of CPM-2 (Ahn et al., 2005) and MEF (Hahn et al., 1999), which preferentially hydrolyzed the Aα-chain of fibrinogen rather than the Bβ and γ-chains. However, it was clearly different from that of the subtilisin FS33 (Wang et al., 2006), which preferentially hydrolyzed the Bβ-chain. It was reported that *Ef* P-III-1 could cleave the α, β and γ chains of fibrinogen, showing a strong α-fibrinogenase, moderate β-fibrinogenase, and weak γ-fibrinogenase activities (Zhao

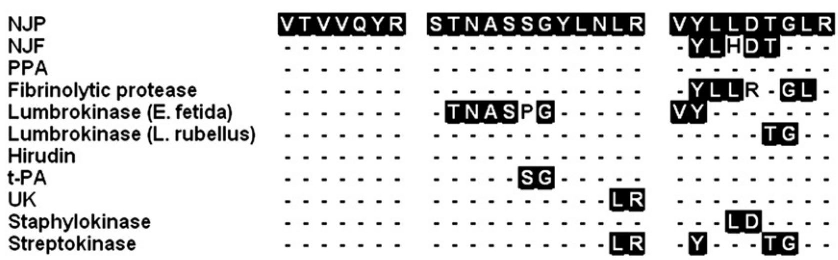

Fig. 4. Comparison of the amino acid sequences. The obtained partial amino acid sequences of NJP was compared with other proteases with fibrinolytic activities such as NJF (*Neanthes japonica*, Swiss-Prot: P86330.1), PPA (the recombinant protease of *Perinereis aibuhitensis* Grube, (Li et al., 2006)), fibrinolytic protease (*Perinereis aibuhitensis*, ACL12061.1), lumbrokinase (*Eisenia fetida*, AAW27919.1), lumbrokinase (*Lumbricus rubellus*, AAN28692.1), hirudin (*Hirudo medicinalis*, CAA01205.1), t-PA (*Homo sapiens*, CAA00299.1), UK (synthetic construct, CAA00829.1), staphylokinase (*Staphylococcus aureus*, CAA24957.1) and streptokinase (*Streptococcus pyogenes*, CAP17326.1). As aligned by the ClustalW program, identical amino acids were boxed in black.

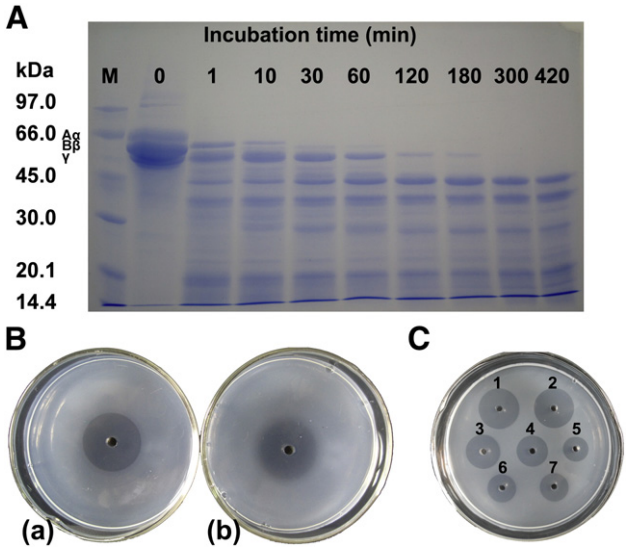

Fig. 5. Hydrolysis of fibrinogen by NJP (A), the mode of fibrinolytic action by NJP (B) and comparison of the fibrinolytic activity of NJP and UK (C). (A) Fibrinogen (1 mg) was incubated with NJP (1 μg) at 37 °C for the various time intervals, and analyzed by 12% SDS-PAGE. (B) Analysis of fibrinolysis by NJP (1 μg) on plasminogen-rich fibrin plate (a) and plasminogen-free fibrin plate (b), respectively. (C) The fibrinolytic activity of NJP was examined by fibrin plate assay. 1: UK (2500 U/mL), 2: UK (1250 U/mL), 3: UK (625 U/mL), 4: UK (312.5 U/mL), 5: UK (156.25 U/mL), 6: NJP (0.02 mg/mL), and 7: NJP (0.02 mg/mL). The calculated fibrinolytic activity of NJP was about 12,000 U (urokinase units)/mg protein versus UK according to the fibrin plate data.

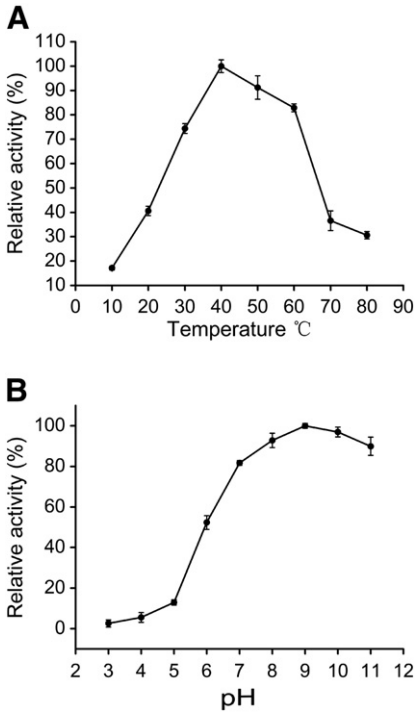

Fig. 6. Effects of temperature (A) and pH (B) on the activity of the fibrinolytic enzyme from *N. japonica*. The remaining proteolytic activity was measured by azocasein assay at 340 nm. (A) Purified NJP was incubated at temperatures from 10 °C to 80 °C. (B) Proteolytic activity was measured by incubating the enzyme at 37 °C for 1 h in the pH range of 3–11. Data were expressed as mean ± SD (n = 3).

et al., 2007), the hydrolysis pattern of NJP was almost identical to that of *Ef* P-III-1. Fibrinogen is the final molecule in the coagulant cascade before the deposition of fibrin; it is involved in primary haemostasis, platelet aggregation and is a major determinant of plasma viscosity (Howard et al., 2008). Therefore, reduction in fibrinogen level may have a benefit in reducing the incidence of thrombosis.

The fibrinolytic activity was assayed using the fibrin plate method. Both the plasminogen-rich and plasminogen-free fibrin plates were used to investigate whether NJP was able to convert plasminogen to plasmin. Similar-sized clear lysis zone were observed on both fibrin plates (Fig. 5B). The fibrinolytic activity measured from the two plates showed a difference of less than 2%. The results indicated that NJP was not a plasminogen activator, since it could directly degrade fibrin. So, the secondary effects such as platelet activation related plasmin formation caused by plasminogen activators such as UK, streptokinase (SK) and t-PA, could be avoided (Wu et al., 2009). This is a particular advantage of NJP over the clinically used plasminogen activators.

NJP had stronger fibrinolytic activity compared with UK, the fibrinolytic activity of NJP was about 12,000 U (urokinase units)/mg protein (Fig. 5C). The units for the activity of fibrinolytic enzymes have been defined differently in many studies, making it difficult to compare absolute values. The measured activity of NJP was lower than that of NJF (30,000 U/mg protein) (Wang et al., 2011). However, we noticed that the activity of NJP were much higher than that of recombinant earthworm fibrinolytic enzyme-3 (EFE-3, 2600 U/mg protein) (Dong et al., 2004) and the seven EFE (a–g) (250 to 644 U/mg protein) (Wang et al., 2003), which were assayed by the same method. NJP has relatively high substrate specificity for fibrin. The strong activities of NJP on fibrin and fibrinogen indicate that it might be a potential fibrinolytic agent.

3.5. Effects of temperature and pH on enzyme activity

Azocasein was used as the substrate in the assay of the effects of temperature and pH on the protease activity in the purified enzyme. The effect of temperature on the proteolytic activity revealed that the enzyme was active between 20 °C and 70 °C (Fig. 6A). The optimum temperature was at 40 °C. It is noteworthy that NJP retained around 17% of its optimal activity even at a lower temperature as 10 °C, while retained more than 40% to 74% of that from 20 °C to 30 °C. On the other hand, the activities decreased markedly when temperature was above 60 °C, mainly due to the thermal denaturation (Klomklao et al., 2007). This optimum temperature was similar to those of CPM-2 (40 °C) (Ahn et al., 2005), and was lower than that reported for NJF (60 °C) (Deng et al., 2010), *Ef*P-III-1 and *Ef*P-II (45–50 °C) (Zhao et al., 2007), but higher than that of MEF (30 °C) (Hahn et al., 1999). The enzyme activity was stable between 30 °C and 60 °C.

The enzyme activity was measured at various pH values in buffers with the same ionic concentrations. It was found that NJP have azocaseinolytic activity within a broad range of pH values, from acidic to alkaline. The optimum pH was found to be 9.0 (Fig. 6B). The total proteolytic activity of NJP for azocasein hydrolysis increased when pH

Table 2
Effects of protease inhibitors on enzyme activity of NJP.

Inhibitors	Concentration	Residual activity (%)
None		100 ± 3.7
PMSF	1 mM	0
EDTA	5 mM	97 ± 1.7
EGTA	5 mM	87 ± 0.8
Iodoacetate	0.05 mM	100 ± 4.8
Aprotinin	1 μg/mL	91 ± 5.1
Pepstatin A	1 μg/mL	93 ± 2.4
SBTI	1 μg/mL	89 ± 3.0
Benzamidine	15 mM	92 ± 0.8
β-Mercaptoethanol	10 mM	85 ± 3.5

Activities were assayed after NJP incubated with different protease inhibitors at 37 °C, pH 9.0 for 1 h, and azocasein was used as the substrate. Data were expressed as mean ± SD (n = 3).
SBTI: soybean trypsin inhibitor.

Table 3
Amidolytic activity of NJP on two chromogenic substrates.

Chromogenic substrates	Amino acid sequence	Characteristics	mmol/min/mg
S-2238	H-D-Phe-Pip-Arg-pNA	For thrombin	10.11 ± 2.7
S-2251	H-D-Val-Leu-Lys-pNA	For plasmin and streptokinase-activated plasminogen	1.89 ± 0.8

Activities were assayed at pH 7.4, 37 °C for continuous measurement of 5 min when S-2238 or S-2251 was used as the substrate. Data were expressed as mean ± SD (n = 3).

was increased from 5.0 to 11.0. Two major groups of activity were observed in this pH range, with a low activity at pH 5.0 to 7.0, and a high activity at pH 7.0 to 11.0, respectively. The relatively low activity at acidic pH was probably because the charge distribution and conformation were changed and enzymes could not bind to substrates properly (Simpson and Haard, 1984). A wide pH range of proteolytic activity against proteinaceous substrates has also been reported for some enzymes from earthworm species, such as *E. fetida* (Zhou et al., 1988) and *L. rubellus* (Nakajima et al., 1993). This optimum pH of NJP was almost the same as NJF (9.0) (Deng et al., 2010), but was lower than that of CPM-2 (9.8) (Ahn et al., 2005) and lumbrokinase (F-I-0, F-I-1, F-I-2, F-II, F-III-1, and F-III-2) (9.0–11.0) (Nakajima et al., 1993). NJP showed at least 80% of the maximum activity over a pH range from pH 7.0 to 11.0, but below 7.0, enzyme activity reduced abruptly. Therefore, NJP can be classified as an alkaline protease.

Optimal temperature and pH of serine proteases may vary within different species from which they were extracted. Cho et al. (2004) purified six fibrinolytic serine proteases from earthworm *L. rubellus* lysates, which had an optimal temperature at 50 °C and wide optimal pH values ranging from pH 4.0 to 12.0. Another serine protease with strong antiviral activities was purified from the coelomic fluid of the earthworm *E. fetida* (Ueda et al., 2008), which showed maximal activity at pH 9.5 and 40 °C to 50 °C.

3.6. Effects of protease inhibitors on enzyme activity

The effects of various protease inhibitors on the fibrinolytic activity of NJP were summarized in Table 2. The protease activity of NJP was completely inhibited by the typical serine protease inhibitor PMSF, while another serine protease inhibitor SBTI, did not exhibit the effective inhibition on NJP. Additionally, EDTA and EGTA (metallo-proteinase inhibitor), iodoacetate and β-mercaptoethanol (cysteine protease inhibitors), aprotinin (inhibitor of trypsin-like proteases), pepstatin A (aspartic protease inhibitor) and benzamidine (competitive inhibitor of trypsins), were not effective or had a weak inhibitory effect on NJP. These results suggest that NJP is a unique serine protease. Most known fibrinolytic enzymes from animals and microbes belong to serine proteases, such as earthworm fibrinolytic enzymes (EFEa–g) (Wang et al., 2003), nattokinase (Fujita et al., 1993), and *Bacillus lichenifomis* KJ-31 (Hwang et al., 2007).

3.7. Amidolytic activity on chromogenic substrates

The amidolytic activity of NJP was assessed using two chromogenic substrates. As shown in Table 3, the enzyme showed a higher degree of specificity for the substrate S-2238 for thrombin. The substrate specificity of NJP clearly differed it from other serine proteases from earthworm *E. fetida*, including the antiviral serine protease (Ueda et al., 2008) and the earthworm fibrinolytic enzyme (EFE-d, EFE-e, and EFE-f) (Wang et al., 2003), which represent as chymotrypsin-like enzymes. The earthworm protease has different activities for the different substrates. Nakajima et al. (1993) reported that F-III-2 and F-III-1 were almost reactive for several substrates and showed highest amidolytic activity to S-2238, while F-I-1 and F-I-2 were most reactive to H-D-Ile-Pro-Arg-pNA (S-2288, for t-PA). Mihara et al. (1991) considered F-I as a chymotrypsin-like enzyme, F-II as a strong thrombin-like, moderate elastase-like, and weak chymotrypsin-like serine protease, and F-III as a trypsin-like enzyme. From these results including the substrates and inhibitor specificities, the purified NJP was identified to be a thrombin-like serine protease.

4. Conclusions

In conclusion, we have purified and characterized a novel protease (NJP) from *N. japonica* (Izuka) which was clearly different from the previous isolated NJF and other fibrinolytic enzymes. NJP is an alkaline thrombin-like serine protease with a much higher fibrinolytic activity than the UK and earthworm fibrinolytic enzymes. It can directly degrade fibrin distinct from the plasminogen activators. Taking account of these results, NJP might be a potential candidate for the thrombosis prevention and thrombolytic therapy. Further studies of NJP on the physiological function and the effectiveness of *in vivo* thrombolysis are required.

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References

Ahn, M.Y., Hahn, B.S., Ryu, K.S., Hwang, J.S., Kim, Y.S., 2005. Purification and characterization of a serine protease (CPM-2) with fibrinolytic activity from the dung beetles. Arch. Pharm. Res. 28, 816–822.
Astrup, T., Mullertz, S., 1952. The fibrin plate method for estimating fibrinolytic activity. Arch. Biochem. Biophys. 40, 346–351.
Beynon, R.J., Kay, J., 1978. The inactivation of native enzymes by a neutral proteinase from rat intestinal muscle. Biochem. J. 173, 291–298.
Bortoleto, R.K., Murakami, M.T., Watanabe, L., Soares, A.M., Arni, R.K., 2002. Purification, characterization and crystallization of Jararacussin-I, a fibrinogen-clotting enzyme isolated from the venom of *Bothrops jararacussu*. Toxicon 40, 1307–1312.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Cho, I.H., Choi, E.S., Lim, H.G., Lee, H.H., 2004. Purification and characterization of six fibrinolytic serine-proteases from earthworm *Lumbricus rubellus*. J. Biochem. Mol. Biol. 37, 199–205.
De-Simone, S.G., Correa-Netto, C., Antunes, O.A., De-Alencastro, R.B., Silva Jr., F.P., 2005. Biochemical and molecular modeling analysis of the ability of two *p*-aminobenzamidine-based sorbents to selectively purify serine proteases (fibrinogenases) from snake venoms. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 822, 1–9.
Deepak, V., Pandian, S.R., Kalishwaralal, K., Gurunathan, S., 2009. Purification, immobilization, and characterization of nattokinase on PHB nanoparticles. Bioresour. Technol. 100, 6644–6646.
Deng, Z., Wang, S., Li, Q., Ji, X., Zhang, L., Hong, M., 2010. Purification and characterization of a novel fibrinolytic enzyme from the polychaete, *Neanthes japonica* (Iznka). Bioresour. Technol. 101, 1954–1960.
Dong, G.Q., Yuan, X.L., Shan, Y.J., Zhao, Z.H., Chen, J.P., Cong, Y.W., 2004. Molecular cloning and characterization of cDNA encoding fibrinolytic enzyme-3 from earthworm *Eisenia foetida*. Acta Biochim. Biophys. Sin. (Shanghai) 36, 303–308.
Fujita, M., Nomura, K., Hong, K., Ito, Y., Asada, A., Nishimuro, S., 1993. Purification and characterization of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan. Appl. Microbiol. Biotechnol. 197, 1340–1347.
Ge, T., Fu, S.H., Xu, L.H., Tang, Q., Wang, H.Y., Guan, K.P., Liang, G.D., 2007. High density fermentation and activity of a recombinant lumbrokinase (PI239) from *Pichia pastoris*. Protein Expr. Purif. 52, 1–7.
Hahn, B.S., Cho, S.Y., Ahn, M.Y., Kim, Y.S., 2001. Purification and characterization of a plasmin-like protease from *Tenodera sinensis* (Chinese mantis). Insect Biochem. Mol. Biol. 31, 573–581.
Hahn, B.S., Cho, S.Y., Wu, S.J., Chang, I.M., Baek, K., Kim, Y.C., Kim, Y.S., 1999. Purification and characterization of a serine protease with fibrinolytic activity from *Tenodera sinensis* (praying mantis). Biochim. Biophys. Acta 1430, 376–386.
Howard, S.C., Algra, A., Rothwell, P.M., 2008. Effect of age and glycaemic control on the association between fibrinogen and risk of acute coronary events after transient ischaemic attack or stroke. Cerebrovasc. Dis. 25, 136–143.
Hu, R., Zhang, S., Liang, H., Li, N., Tu, C., 2004. Codon optimization, expression, and characterization of recombinant lumbrokinase in goat milk. Protein Expr. Purif. 37, 83–88.

Hwang, K.J., Choi, K.H., Kim, M.J., Park, C.S., Cha, J., 2007. Purification and characterization of a new fibrinolytic enzyme of *Bacillus licheniformis* KJ-31, isolated from Korean traditional *Jeot-gal*. J. Microbiol. Biotechnol. 17, 1469–1476.

Kim, S.H., Choi, N.S., 2000. Purification and characterization of subtilisin DJ-4 secreted by *Bacillus* sp. strain DJ-4 screened from *Doen-Jang*. Biosci. Biotechnol. Biochem. 64, 1722–1725.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B.K., 2007. Trypsin from the pyloric caeca of bluefish (*Pomatomus saltatrix*). Comp. Biochem. Physiol. B 148, 382–389.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lee, S.Y., Kim, J.S., Kim, J.E., Sapkota, K., Shen, M.H., Kim, S., Chun, H.S., Yoo, J.C., Choi, H.S., Kim, M.K., Kim, S.J., 2005. Purification and characterization of fibrinolytic enzyme from cultured mycelia of *Armillaria mellea*. Protein Expr. Purif. 43, 10–17.

Leonardi, A., Gubensek, F., Krizaj, I., 2002. Purification and characterisation of two hemorrhagic metalloproteinases from the venom of the long-nosed viper, *Vipera ammodytes ammodytes*. Toxicon 40, 55–62.

Li, R.G., Qian, D.M., Guo, D.S., Du, G.C., Yan, Z.Y., Wang, B., 2006. Isolation of a cDNA encoding a protease from *Perinereis aibuhitensis* Grube. Acta Biochim. Biophys. Sin. (Shanghai) 38, 543–548.

Li, S., Guo, D., Zhao, B., Ye, J., Tian, J., Ren, W., Ju, Y., Cui, P., Li, R., 2010. Cloning and expression of cDNA encoding a cysteine protease inhibitor from clamworm and its possible use in managing *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae). J. Microbiol. Biotechnol. 20, 1243–1250.

Mihara, H., Sumi, H., Yoneta, T., Mizumoto, H., Ikeda, R., Seiki, M., Maruyama, M., 1991. A novel fibrinolytic enzyme extracted from the earthworm, *Lumbricus rubellus*. Jpn J. Physiol. 41, 461–472.

Mine, Y., Wong, A.H.K., Jiang, B., 2005. Fibrinolytic enzymes in Asian traditional fermented foods. Food Res. Int. 38, 243–250.

Murray, V., Norrving, B., Sandercock, P.A., Terent, A., Wardlaw, J.M., Wester, P., 2010. The molecular basis of thrombolysis and its clinical application in stroke. J. Intern. Med. 267, 191–208.

Nakajima, N., Mihara, H., Sumi, H., 1993. Characterization of potent fibrinolytic enzymes in earthworm, *Lumbricus rubellus*. Biosci. Biotechnol. Biochem. 57, 1726–1730.

Pinto, A.F., Dobrovolski, R., Veiga, A.B., Guimaraes, J.A., 2004. Lonofibrase, a novel alpha-fibrinogenase from *Lonomia obliqua* caterpillars. Thromb. Res. 113, 147–154.

Simpson, B.K., Haard, N.F., 1984. Trypsin from Greenland cod, Gadus ogac. Isolation and comparative properties. Comp. Biochem. Physiol. B 79, 613–622.

Ueda, M., Noda, K., Nakazawa, M., Miyatake, K., Ohki, S., Sakaguchi, M., Inouye, K., 2008. A novel anti-plant viral protein from coelomic fluid of the earthworm *Eisenia foetida*: purification, characterization and its identification as a serine protease. Comp. Biochem. Physiol. B 151, 381–385.

Wang, C.T., Ji, B.P., Li, B., Nout, R., Li, P.L., Ji, H., Chen, L.F., 2006. Purification and characterization of a fibrinolytic enzyme of *Bacillus subtilis* DC33, isolated from Chinese traditional *Douchi*. J. Ind. Microbiol. Biotechnol. 33, 750–758.

Wang, F., Wang, C., Li, M., Gui, L., Zhang, J., Chang, W., 2003. Purification, characterization and crystallization of a group of earthworm fibrinolytic enzymes from *Eisenia fetida*. Biotechnol. Lett. 25, 1105–1109.

Wang, S.H., Li, Q., Deng, Z.H., Ji, X., Jiang, X., Ge, X., Bo, Q.Q., Cui, J.Y., Zhang, L.Z., Liu, J.K., Hong, M., 2011. *Neanthes japonica* (Iznka) fibrinolytic enzyme reduced cerebral infarction, cerebral edema and increased antioxidation in rat models of focal cerebral ischemia. Neurosci. Lett. 489, 16–19.

Wu, B., Wu, L., Chen, D., Yang, Z., Luo, M., 2009. Purification and characterization of a novel fibrinolytic protease from *Fusarium* sp. CPCC 480097. J. Ind. Microbiol. Biotechnol. 36, 451–459.

Wu, J.X., Zhao, X.Y., Pan, R., He, R.Q., 2007. Glycosylated trypsin-like proteases from earthworm *Eisenia fetida*. Int. J. Biol. Macromol. 40, 399–406.

Xu, Z.R., Yang, Y.M., Gui, Q.F., Zhang, L.N., Hu, L., 2010. Expression, purification, and characterization of recombinant lumbrokinase PI239 in *Escherichia coli*. Protein Expr. Purif. 69, 198–203.

You, W.K., Sohn, Y.D., Kim, K.Y., Park, D.H., Jang, Y., Chung, K.H., 2004. Purification and molecular cloning of a novel serine protease from the centipede, *Scolopendra subspinipes mutilans*. Insect Biochem. Mol. Biol. 34, 239–250.

Zhao, J., Xiao, R., He, J., Pan, R., Fan, R., Wu, C., Liu, X., Liu, Y., He, R.Q., 2007. In situ localization and substrate specificity of earthworm protease-II and protease-III-1 from *Eisenia fetida*. Int. J. Biol. Macromol. 40, 67–75.

Zhou, Q., Li, M., Xi, T., 2009. Cloning and expression of a clamworm antimicrobial peptide perinerin in *Pichia pastoris*. Curr. Microbiol. 58, 384–388.

Zhou, Y.C., Zhu, H., Chen, Y.C., 1988. The isolation and purification of earthworm fibrinolytic protease from *Eisenia fetida*. Acta Biochim. Biophys. Sin. (Shanghai) 20, 35–42.