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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.