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Research paper

# Starase: A bi-functional fibrinolytic protease from hepatic caeca of *Asterina pectinifera* displays antithrombotic potential

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ABSTRACT

A bi-functional fibrinolytic serine protease, Starase exhibiting thrombolytic potency was purified from hepatic caeca of *Asterina pectinifera*. Starase showed a single band of approximately 48 kDa by SDS-PAGE and fibrin zymography. The N-terminal sequence of Starase was AIPTEFDARTKKHNN, which does not match with any known fibrinolytic enzyme. Starase had optimum amidolytic activity at 50 °C and pH 8.0 and the activity was inhibited by PMSF and APMSF. Starase showed the highest specificity toward the substrate H-D-Val-Leu-Lys-pNA for plasmin followed by pyroGlu-Gly-Arg-pNA for urokinase. The apparent $K_m$ and $V_{max}$ values of Starase toward a chromogenic substrate for plasmin H-D-Val-Leu-Lys-pNA were determined as 1.37 mM and 6.8 mM/min/mg respectively. The fibrinolytic activity of Starase by fibrin plate assay displayed that it could not only directly degrade fibrin clot but also activate plasminogen. Starase showed a strong fibrinogenolytic activity, cleaving all three major chains of fibrinogen rapidly. In addition, Starase with more than 1 μg could cleave extracellular matrix component type VII collagen, and plasma proteins such as bovine albumin and bovine gamma globulin. It could also inhibit factor Xa and thrombin activity. Starase at a dose of 0.8 mg/kg was devoid of hemorrhagic activity and it demonstrated antithrombotic effect in three animal models; $FeCl_2$-induced carotid arterial thrombus model, carrageenan-induced tail thrombosis model and collagen and epinephrine induced pulmonary thromboembolism mice model. These results suggest that Starase has the potential to be a potent thrombolytic agent due to its bi-functional properties (containing both direct-acting and plasminogen-activating activities) and lack of hemorrhagic activity. Although Starase might interfere with the normal composition of the plasma proteins, it may be used not only for the treatment and prevention of thrombosis, but also in a number of biomedical applications.



## 1. Introduction

Cardiovascular diseases (CVD) are the leading causes of morbidity and death in modern societies worldwide. The underlying pathophysiological process associated with circulatory disorders such as myocardial infarction, stroke, deep-vein thrombosis, and pulmonary embolism is the formation of thrombus (fibrin clot) inside the blood vessels. The formation of thrombus is a highly dynamic process and is dependent on the activation of the coagulation cascade [1]. The coagulation cascade is the sequential process of interaction and activation of coagulation factors, resulting in generation of fibrin, the major protein component of thrombus [2]. Fibrin clots are dissolved by plasmin, a serine protease, which is generated from its zymogen plasminogen by the action of plasminogen activators (PAs) such as tissue plasminogen activator (t-PA) and urokinase (u-PA), which has the unique ability to dissolve the blood clots via the activation of intrinsic factors of the coagulation cascade [3]. Although PAs are effective in thrombolytic therapy, the risk of bleeding complications, as well as low fibrin specificity, short-half-lives and resistance to repercussion limit their use [4–6]. Therefore, the search for safe and effective thrombolytic agent from various sources continues.

Serine proteases, which are found in diverse organisms, are known to have a wide range of physiological functions and affect

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several processes such as the hemostasis, fibrinolysis and immune response [7,8]. Over the last decade, several proteases which can interfere with blood homeostasis have been purified and characterized from variety of animals, such as earthworms [9,10], snake venoms [11], insects [12], leeches [13], shark [14] and lampreys [15]. Serine proteases from animal sources are multifunctional proteases and have been extensively investigated over the last decade for potential therapeutic use in thrombolytic therapy. However, their undesirable side effects, such as the risk for internal hemorrhagic, have prompted researchers to look for alternative sources of anticoagulant and antithrombotic agent. *A. pectinifera* is known to have invaded aquaculture farms [16]. This species causes serious damage to commercial shellfish mariculture operations and fisheries in Korea [17]. The Korean government spent about 3.73 million US$ from 2006 to 2011 to remove the seastar from coastal area [18]. Hence, it is important to find a way to utilize this unused marine resource. Previously, cortical granule serine protease, anti-inflammatory protein and anticoagulant collagen protein have been reported from echinoderms [19–21]. The bioactive substances derived from *Asterina pectinifera* have recently attracted much attention of the scientific community. Studies have shown that extracts obtained from *A. pectinifera* had antimicrobial, anticancer, antimelanogenic, anti-inflammatory activities [22–26]. Recently Mita et al. [27] has been reported the relaxin-like peptide from radial nerves of *A. pectinifera*. Proteolytic enzymes, such as cathepsins, can degrade extracellular matrix proteins and can activate the precursor form of urokinase plasminogen activator [28]. Cathepsins have been proven to play an important role in cardiovascular diseases [29]. There are several reports on cathepsins from different body parts of echinoderms including *A. pectinifera* [16,30,31]. Previously, proteolytic enzymes were reported from the pyloric caeca of echinoderm [32] and cathepsin, a proteolytic enzyme, was found to be highly expressed from the liver cells of *A. pectinifera* [16]. However there is no report on antithrombotic protease from *A. pectinifera.* Based on these studies, we speculate that hepatic caeca of this echinoderm might contain such type of protease. Therefore, in this study, we attempted to isolate a fibrinolytic protease from hepatic caeca of *A. pectinifera* and analyze its biochemical properties and tested its antithrombotic ability on human blood and in rodent models.

## 2. Materials and methods

### 2.1. Animals

The seastar, *A. pectinifera*, was collected from the coastal area of Wando, Republic of Korea and was identified using taxonomic keys [33]. A voucher specimen was deposited at Chosun University. The collected samples were washed with distilled water and the hepatic caeca were manually isolated by forceps, then quickly frozen on dry ice and kept at a −70 °C before use.

Male ICR (Imprinting Control Region), the outbred strain of mice (20–30 g), approximately 4–6 week old and male Sprange-Dawley (SD) rats (220–300 g) were used in the experiments. The mice were housed four per cage and rats were housed two per cage. All animals were maintained under controlled environmental conditions (22 ± 2 °C, 12 h light/dark cycle). Food (Certified Rodent Diet 5002, Orient Bio, Seongnam, Korea) and tap water were available *ad libitum*. All efforts were made to minimize animal suffering and to reduce the number of animals used. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23 revised 1996) and the Guiding Principles for the Care and Use of Animals approved by the internal Ethics Committee of Chosun University.

### 2.2. Blood

In the present experiment, the blood samples were collected from healthy human volunteers, in accordance with the approval by the Chosun University's internal ethics committee. The informed consent from all volunteers was obtained in written and the consent procedure was also approved by the committee.

### 2.3. Reagents

Human fibrinogen, thrombin, plasmin, plasminogen, κ-carrageenan, urokinase, acrylamide, trizma base, trizma HCl, phenylmethylsulphonyl fluoride (PMSF), tosyllysine chloromethyl ketone (TLCK), aprotinin, tosylphenylalnine chloromethyl keptone (TPCK), ethyleneglycolbis-(2-aminoethyl)-*N*,*N*,*N*′,*N*′ tetraacetic acid (EGTA), ethylenediaminetetra acetic acid (EDTA), diethylaminoethyl (DEAE) sepharose CL6B, and Phenyl sepharose CL4B were purchased from Sigma–Aldrich (St. Louis, MO, USA). Mono Q HR 5/5 was obtained from GE-Healthcare Life Sciences (Uppsala, Sweden). Para-nitroanilide (pNA) chromogenic substrates were obtained from Chromogenix (Milano, Italy). PageRuler™ Plus Prestained protein ladder was purchased from Thermo Scientific (Rockford, IL, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), penicillin and streptomycin were obtained from Gibco BRL (Gaithersburg MD, USA). All other reagents were of analytical grade and procured commercially.

### 2.4. Preparation of hepatic caecum extract

The frozen caeca were ground in liquid nitrogen by using a mortar and pestle. Subsequently, a double volume of distilled water was added, and the ground tissue was homogenized on ice by using a motorized homogenizer and stirred overnight at 4 °C. The homogenate was then centrifuged at 14 000 *g* for 40 min at 4 °C. The Supernatant was filtered using filter paper (110 mm, Toyo Roshi Kaisha Ltd., Japan), and an equal volume of −70 °C pre-chilled ethanol was added, drop wise, with constant stirring on ice. After stirring for 1 h, precipitated protein was removed by centrifugation at 10 000 *g* for 30 min at 4 °C. The ethanol-soluble fraction was added with increased concentration (75%) of −70 °C pre-chilled ethanol drop wise by constant stirring. Stirring continued for 1 h after which the precipitated protein was recovered by centrifugation at 10 000 *g* for 30 min. The precipitated protein was used as a crude extract.

### 2.5. Enzyme purification

All purification steps were carried out at 4 °C unless otherwise stated. The crude extract was dissolved in 20 mM Tris–HCl (pH 7.4), and loaded on DEAE-sepharose CL6B column (2.5 × 10 cm) equilibrated with same buffer. The elution was performed with a linear gradient of KCl from 0 to 0.1 M at a flow rate of 0.1 ml/min. The absorbance of each fraction was measured at 280 nm and screened for protease activity using a chromogenic substrate S-2251 (H-D-Val-Leu-Lys-pNA) and by fibrin plate method [34] as described below. Fractions having high protease activity were pooled and concentrated using a Vivaspin 20 (Sartorius Stedim Biotech, Gottingen, Germany). The concentrated sample was applied to a Sepharose CL4B column (1.5 cm × 75 cm) equilibrated with 20 mM Tris–HCl (pH 7.4) at a flow rate of 1.0 ml/min. The elution was measured at 280 nm and assayed for protease activity. Fractions with high protease activity were pooled, concentrated and further purified by fast protein liquid chromatography (FPLC) using a Mono Q HR 5/5 column equilibrated with the same buffer. Elution was performed with a linear gradient of KCl (0–0.4 M) at a flow rate of



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