The Journal of Microbiology (2011) Vol. 49, No. 6, pp. 1018-1021
DOI 10.1007/s12275-011-1376-7

NOTE

Detection of a Unique Fibrinolytic Enzyme in *Aeromonas* sp. JH1[§]

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(Received August 1, 2011 / Accepted November 2, 2011)

A fibrinolytic enzyme was found in a Gram-negative bacterium, *Aeromonas* sp. JH1. SDS-PAGE and fibrin-zymography showed that it was a 36 kDa, monomeric protein. Of note, the enzyme was highly specific for fibrinogen molecules and the hydrolysis rate of fibrinogen subunits was highest for α, β, and γ chains in that order. The first 15 amino acids of N-terminal sequence were X-D-A-T-G-P-G-G-N-V-X-T-G-K-Y, which was distinguishable from other fibrinolytic enzymes. The optimum pH and temperature of the enzyme were approximately 8.0 and 40°C, respectively. Therefore, these results provide a fibrinolytic enzyme with potent thrombolytic activity from the *Aeromonas* genus.

***Keywords*:** Amidolytic activity, *Aeromonas* sp. JH1, fibrinogen subunits, fibrinolytic enzyme, N-terminal amino acid sequence

The main protein component of the blood clot, fibrin, is normally formed from fibrinogen by the action of thrombin (EC 3.4.21.5) (Blann *et al*., 2002). Fibrinogen is an acute phase protein and elevated levels of fibrinogen are involved in inflammation, trauma, and malignancy. The accumulation of fibrin in the blood vessels usually increases thrombosis, leading to myocardial infarction and other cardiovascular diseases. Insoluble fibrin fiber is normally hydrolyzed by plasmin generated from plasminogen activator (Bode *et al*., 1996).

The typical fibrinolytic enzymes for clinical use are mostly plasminogen activators, such as tissue-type plasminogen activators (t-PA), a urokinase-type plasminogen activator, and the bacterial plasminogen activator streptokinase (Blann *et al*., 2002). These agents show undesired side effects, exhibit low specificity for fibrin, and are also relatively expensive. Therefore, potent fibrinolytic enzymes have been identified from a wide variety of sources, such as earthworms (Mihara *et al*., 1991), snake venoms (Jia *et al*., 2003), insects (Ahn *et al*., 2003), food-grade microorganisms (Kim *et al*., 1997; Jeong *et al*., 2001), and fermented food products such as pickled anchovies (Jeong *et al*., 2004b). A variety of fibrinolytic enzymes isolated from bacteria originated from Gram-positive strains. For instance, streptokinase (Medved *et al*., 1966), staphylokinase (Arai *et al*., 1995), and a variety of proteases, including BPN´ (Smith *et al*., 1968), Carlsberg (Smith *et al*., 1968), Nattokinase (Nakamura *et al*., 1992), Sub E (Yang *et al*., 2000), CK (Kim *et al*., 1996), and BK (Jeong *et al*., 2004a) have been isolated from Gram-positive bacterial strains. However, only a few studies have reported on fibrinolytic enzymes from Gram-negative bacteria (Jeffries and Buckley, 1980). In this paper, we describe the detection of a unique fibrinolytic enzyme in a newly isolated Gram-negative bacterium, *Aeromonas* sp. JH1, which is associated with human diseases such as gastroenteritis and bacteremia (Altwegg and Geiss, 1989).

Earthworms living in the sediments of Korean swine farms in Yangsan, Korea were isolated, and the gut was extracted and homogenized in a saline solution. The gut homogenates were suspended in the solution at a dilution rate of 1:1. One hundred microliter of diluted sample was spread on a Luria-Bertani (LB) medium consisting of 1% Bacto-tryptone (Sigma, USA), 1% sodium chloride, and 0.5% Bacto-yeast extract (Sigma). The LB plates were aerobically incubated at 37°C overnight. Six microbial strains with fibrinolytic activity were isolated from the earthworm gut. Among them, a strain possessing substantial fibrinolytic activity and protein-degrading capability on the LB plate supplemented with 1% skim milk was selected for identification and physical characterization. Cell morphology was determined by scanning electron microscopy and motility was assessed by direct microscopic observation during growth (Supplementary data Fig. 1). Gram staining was conducted using a bioMériux Gram stain kit (Medical Chemical Corp., USA) according to the manufacturer's instructions. The isolated strain was a Gram-negative, motile, and facultative anaerobic bacterium. The strain was

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§ Supplemental material for this article may be found at http://www.springer.com/content/120956

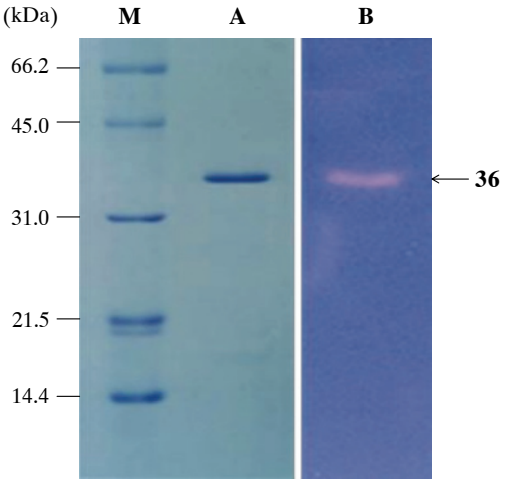

Fig. 1. SDS-PAGE and fibrin-zymography of the fibrinolytic enzyme (AK) purified from *Aeromonas* sp. JH1. Lane M, protein molecular mass markers; lane A, AK protein on SDS-PAGE; lane B, AK protein on fibrin-zymography. The AK protein showed only a single polypeptide on SDS-PAGE and fibrin-zymography, which was in the same position. The arrow indicates the fibrinolytic enzyme purified from *Aeromonas* sp. JH1.

carefully identified using the Easy 24E Plus kit (Komed, Korea), which is generally used to identify Gram-negative bacteria including *Enterobacteriaceae* and *Vibrio* strains. The bacterial strain showing strong fibrinolytic activity was designated as *Aeromonas* sp. JH1 and used as the source of a unique fibrinolytic enzyme in this study. Physiological and biochemical properties of the microbial strain are listed in Supplementary data Table 1.

Cultured cells were removed by centrifuging at 12,000 rpm for 15 min and ammonium sulfate was slowly added to the supernatant to 80% saturation. The mixture of supernatant and ammonium sulfate was allowed to stand at 4°C for 12 h to harvest the extracellular proteins. The precipitate was obtained by centrifuging at 12,000 rpm for 15 min and dialyzed using 5 L of 20 mM Tris-HCl (pH 7.5) three times. All purification steps were performed at 4°C. Protein concentration was measured according to the method of Bradford (1976). The enzyme solution was fractionated by DEAE-Sephadex A-50 column chromatography using a linear gradient from 0.1 to 0.5 M sodium chloride, Sephadex G-75 gel filtration and Fast Performance Liquid Chromatography (FPLC) using Superdex 75 HR 10/30 gel filtration (GE-Healthcare, USA) and was then lyophilized. To determine the molecular mass of the protein purified by FPLC, SDS-PAGE of the lyophilized protein was performed according to the method of Laemmli (1970) by using a 10-15% gradient polyacrylamide gel and 4% stacking gel at 4°C. The fibrin-zymography analysis of the protein was conducted according to the method described by Markus *et al*. (1984), except a 0.12% fibrinogen solution instead of distilled water was used in the preparation of the separating gels. The activity band was recognizable as a colorless area in a fibrin-depletion gel against a blue background.

Fibrinolytic activity was determined by both a plasminogen-free fibrin plate method and a plasminogen-rich fibrin plate method (Astrup and Müllertz, 1952). The activity of the fibrinolytic enzyme was determined by measuring the dimension of the clear zone on the fibrin plate and plotting to the standard curve obtained by varying the quantity of plasmin, which was used as a standard substrate. In addition, fibrinogenolytic activity was measured as follows: 80 µl of 1% human fibrinogen dissolved in 20 mM Tris-HCl (pH 7.5) was mixed with 10 µg of a purified enzyme and incubated at 37°C for 60 min. At various intervals, a portion of the reaction solution was withdrawn and analyzed by SDS-PAGE. The fibrinolytic protein purified on an SDS-PAGE gel was electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad, USA) and stained with Coomassie Brilliant Blue R-250 (Sigma). The stained protein band was excised and the amino acids of the N-terminal sequence were determined with the automated Edman method using a gas-phase protein sequencer (Applied Biosystems, USA). The analysis of the N-terminal amino acid sequence was performed by the BLAST program of NCBI (http://www.ncbi.nlm.nih.org).

The optimum temperature for the enzyme activity was determined by conducting the assay at various temperatures (20, 30, 40, 50, 60, 70, and 80°C) in 10 mM phosphate buffer (pH 7.4) for 30 min. The thermostability of the enzyme was measured after preincubating the enzyme in the same buffer but at various temperatures for different incubation periods. Relative fibrinolytic activity was measured under the same conditions. The optimal pH for the fibrinolytic activity of the enzyme was determined within a pH range of 4.0-10.0, using the following buffer systems: 0.05 M citrate buffer (pH 4.0), 0.05 M sodium phosphate (pH 5.0-7.0), 0.05 M Tris-HCl (pH 8.0-9.0) and 0.05 M glycine-NaOH (pH 10.0), respectively. Fibrin plates of different pH values were prepared and the pH of the enzyme was adjusted to the same value as that of the respective fibrin plate. The relative activities were expressed as a percentage of maximum enzyme activity. All experiments were carried out in duplicate three times.

The fibrinolytic enzyme was purified to electrophoretic homogeneity by the steps listed in Supplementary data Table 2. Compared to the culture supernatant, the specific activity of

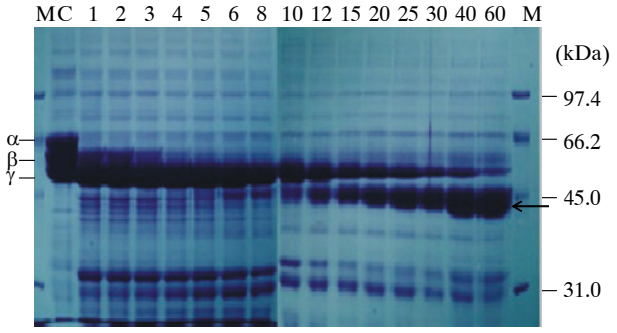

Fig. 2. Fibrinogenolysis of AK on the fibrinogen plate (B). Lane M, protein molecular mass markers, Lane C, human fibrinogen molecules consisting of α, β, and γ subunits. The numbers on lanes indicate incubation periods (min) of the AK protein mixed with the fibrinogen molecules. The arrow represents γ subunits liberated from fibrinogen molecules.

Protease	1				5					10				15
AK	X	D	A	T	G	P	G	G	N	V	X	T	G	K
BPN′	A	G	S	V	P	Y	G	V	S	G	I	V	A	P
Carlsberg	A	Q	T	V	P	Y	G	I	P	L	I	V	A	A
CK	A	Q	T	V	P	Y	G	I	P	L	I	K	A	D
SubE	A	Q	S	V	P	Y	G	I	S	L	I	K	A	P

Fig. 3. Comparison of the fibrinolytic enzyme (AK) purified from *Aeromonas* sp. JH1 with other known proteases from Gram-positive bacteria with regard to the N-terminal amino acid sequence. AK, 36 kDa fibrinolytic enzyme from *Aeromonas* sp. JH1; BPN´, subtilisin BPN´ from *B. amyloliquefaciens*; Carlsberg, subtilisin Carlsberg from *B. licheniformis*; CK, extracellular protease from *Bacillus* sp. strain CK 11-4, and Sub E, subtilisin E from *B. subtilis*. The consensus sequences of BPN´, Carlsberg, CK, and SubE are indicated by the box.

the enzyme purified by FPLC was increased approximately 1986-fold, with a recovery of 14%. As shown in Fig. 1, FPLC gel filtration yielded only a single polypeptide showing high fibrinolytic activity on an SDS-PAGE gel as well as by fibrinzymography. The apparent molecular mass of the purified fibrinolytic enzyme, designated as Aerokinase (AK) was estimated to be approximately 36 kDa on the gels. The proteolytic activity of the enzyme was observed in a fibrin plate assay for 4 h. As shown in Supplementary data Fig. 2, fibrinolytic activity of the purified enzyme, AK was observed in both plasminogen-rich and plasminogen-free fibrin plates to a similar degree and its relative activity was approximately 1.4 U/ml (Supplementary data Fig. 2C). These data may suggest that the AK protein exerts a dual function in the fibrinolytic pattern in that the protein not only degrades fibrin by forming plasmin from plasminogen (*plasminogen activator type*) but also digests fibrin in a direct manner

In addition, the fibrinogenolytic activity of the fibrinolytic enzyme was investigated. Human fibrinogen is a 340 kDa plasma protein composed of two identical molecular halves, each consisting of three non-identical subunit polypeptides designated as α, β, and γ-chains held together by multiple disulfide bonds (Dobrovolsky and Titaeva, 2002; Mosesson, 2005). As seen in Fig. 2, the α and β subunits of the fibrinogen molecule were completely digested in 1 and 4 min of incubation, respectively. However, γ subunits of fibrinogen tended to be degraded in a time-dependent manner throughout the 60 min. These data suggest that the AK protein was highly specific for fibrinogen molecules and the hydrolysis rate of fibrinogen subunits was highest for the α, β, and γ chains in that order. Of note, in the incubation range between 10 and 60 min, the amount of γ subunits liberated from the fibrinogen molecule tended to be augmented in a time-dependent fashion. Thus, these findings may support that when fibrinogen is activated by the fibrinolytic enzyme, α and β subunits tend to be degraded first and the remaining γ units mainly polymerize into fibrin strands, forming the basic structure of a blood clot (Mosesson *et al*., 2001; Matsuda and Sugo, 2002).

The first 15 amino acid residues of the AK N-terminal sequence were X-D-A-T-G-P-G-G-N-V-X-T-G-K-Y (Fig. 3). No significant homology in the N-terminal amino acid sequence was found between the AK protein and other known fibrinolytic enzymes. Of note, BlastP analysis shows that the N-terminal sequence of AK is highly homologous to elastase of *A. caviae*, *A. hydrophila*, and *A. salmonicida*, which digests elastic fibers, where elastin determines the mechanical properties of connective tissue. Elastase also plays an important immunological role by digesting *Shigella* virulence factors through the cleavage of peptide bonds in the target proteins (Weinrauch *et al*., 2002). In addition, the N-terminal sequence of AK is significantly homologous to protease sequences of *A. punctata*, *A. veronii*, and *A. sobria* (Kawakami *et al.*, 2000; Khan *et al*., 2007; Li *et al*., 2011), as shown in Supplementary data Fig. 3. These results may suggest that the proteolytic enzymes AK, elastase, and proteases of the *Aeromonas* genus play important roles in digesting proteins such as fibrin and fiber.

The temperature activity profile showed that the optimum temperature for the AK protein was 40°C (Supplementary data Fig. 4A). The protein maintained nearly 98% of enzyme activity at a temperature range between 30 and 50°C. The pH activity profile of fibrinolytic enzyme, AK showed that it was significantly active at pH values ranging from 7.0 to 9.0 (Supplementary data Fig. 4B), but significantly decreased at pH values below 5.0 and at pH 10.0. These results suggest that the AK protein is relatively stable in neutral and mild alkaline pH regions.

In conclusion, the 36 kDa fibrinolytic enzyme from *Aeromonas* sp. JH1 detected in earthworm gut was purified and analyzed for its biochemical characteristics. The enzyme was highly specific for fibrinogen molecules and the hydrolysis rate of fibrinogen subunits was highest for the α, β, and γ chains in that order. Of note, the AK protein was distinguished from other known fibrinolytic enzymes since it was purified from a Gram-negative bacterium and had a unique N-terminal sequence. The purified fibrinolytic enzyme, AK was not only a plasminogen activator type, degrading fibrin clots by forming plasmin activated from plasminogen but was also capable of degrading fibrin in a direct manner. Therefore, these findings demonstrate a unique fibrinolytic enzyme from *Aeromonas* sp. JH1, which may contribute to the development of a potent thrombolytic agent.

This research was supported by the Dong-A University Research Fund, Korea.

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