

Purification and characterization of a novel thermoacid-stable fibrinolytic enzyme from *Staphylococcus* sp. strain AJ isolated from Korean salt-fermented Anchovy-*jeot*

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Abstract A novel fibrinolytic enzyme (AJ) was purified from *Staphylococcus* sp. strain AJ screened from Korean salt-fermented Anchovy-*jeot*. Relative molecular weight of AJ was determined as 26 kDa by using SDS-PAGE and fibrin zymography. Based on a 2D gel, AJ was found to consist of three active isoforms (pI 5.5–6.0) with the same N-terminal amino acid sequence. AJ exhibited optimum pH and temperature at 2.5–3.0 and 85°C, respectively. AJ kept 85% of the initial activity after heating at 100°C for 20 min on the zymogram gel. The Michaelis constant (K_m) and K_{cat} values of AJ towards α -casein were 0.38 mM and 19.73 s⁻¹, respectively. AJ cleaved the A α -chain of fibrinogen but did not affect the B β - and γ -chains, indicating that it is an α -fibrinogenase. The fibrinolytic activity was inhibited by diisopropyl fluorophosphate, indicating AJ is a serine protease. Interestingly, AJ was very stable at acidic condition, SDS, and heat (100°C), whereas it was easily

degraded at neutral and alkaline conditions. In particular, AJ formed an active homo-dimer in the pH range from 7.0 to 8.0. To our knowledge, a similar combination of acid and heat stability has not yet been reported for other fibrinolytic enzymes.

Keywords Fibrinolytic enzyme · *Staphylococcus* sp. strain AJ · Anchovy-*jeot* · Thermoacid-stable · Fibrin zymography

Introduction

Fibrin is the primary protein component of a blood clot, which is formed from fibrinogen by the activated thrombin (EC 3.4.21.5). It is dissolved by fibrinolytic enzyme, plasmin (EC 3.4.21.7), which maintains blood flow at vascular injury sites and is an important component of the normal hemostatic response. When clots are not lysed, they accumulate in blood vessels, and cause thrombosis leading to myocardial infarction and other cardiovascular diseases [1, 2]. The major thrombolytic agents are classified into two types. The plasminogen activators, such as urokinase, tPA (tissue type plasminogen activator), and streptokinase, which activate plasminogen to plasmin, and the plasmin-like proteins, such as nattokinase [3] and lumbrokinase [4], which can directly degrade the fibrin.

Many bacterial fibrinolytic enzymes were discovered from fermented foods, such as *natto* [3, 5–7], *shiokara* [8] in Japanese food, *Chungkook-Jang* [1], *Doen-Jang* [2], and *Jeot-Gal* [9] in Korean food, *douchi* [10] in Chinese food, and *Tempeh* [11] in Indonesian food. Of them *Shiokara* (Japan) and *Jeot-Gal* (Korea) are fish-fermented foods, which are used as important additive for improving the taste of other foods as well as being foods themselves [12].

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In addition, some fibrinolytic enzymes were discovered from root tissue of *Stemona japonica* (Blume) Miq, a Chinese traditional medicine [13], and marine alga (e.g., *Codium latum*, *C. divaricatum*, and *C. intricatum* [14–16]. In particular, regarding nattokinase produced by *Bacillus natto* screened from *natto* it was reported that the enzyme not only hydrolyzed thrombi in vivo, but also converted plasminogen to plasmin [6]. Oral administration of the enzyme showed that it could enhance fibrinolytic activity in plasma and the production of tPA, and its fibrinolytic activity was retained in the blood for more than 3 h.

Anchovy-*jeot* is a typical and popular fish-fermented food and is considered safe for long-term consumption. Recently, we isolated a halotolerant *Staphylococcus* strain, which produce an extracellular fibrinolytic enzyme, and designated as *Staphylococcus* sp. strain AJ. Here we report the purification, some characteristics and primary structure of the fibrinolytic enzyme (AJ) produced by *Staphylococcus* sp. strain AJ.

Materials and methods

Materials

Human fibrinogen, thrombin, plasmin, and diisopropyl fluorophosphates were purchased from Sigma (St Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane and electrophoretic reagents were purchased from Bio-Rad (Hercules, CA, USA). Other chemicals were of analytical grade.

Bacterial strain and cultural conditions

The bacterium isolated from Anchovy-*jeot*, which was confirmed by the Korean Collection for Type Cultures (KCTC) as a strain of *Staphylococcus* sp. strain AJ was grown in tryptic soy broth (TSB, Difco, Sparks, MD, USA) at 37°C for 2 days.

Purification of the fibrinolytic enzyme

The fibrinolytic enzyme (AJ) was purified from the culture supernatants of *Staphylococcus* sp. strain AJ. All purification procedures were performed at 4°C and with 20 mM sodium phosphate buffer, pH 6.4 (buffer A). Two liters of the crude enzyme was concentrated by ultrafiltration with the PM-10 membrane (Amicon Inc., USA). The concentrated solution was dialyzed against 20 volumes of buffer A for 1 day with three buffer changes. The dialyzed solution was loaded onto the DEAE-cellulose column (2.0 × 10 cm) (Pharmacia Biotech, Sweden), and the proteins were fractionated with 300 ml linear gradient from 0 to 1 M NaCl.

Fractions showing the fibrinolytic activity were pooled and dialyzed against buffer A, and then were concentrated by lyophilization. The fibrinolytic enzyme was further purified by the TSK gel filtration (Toyopearl HW-55F, TOSOH, Japan) using the buffer A containing 0.1 M NaCl.

Fibrin/casein plates

Fibrinolytic activity was determined by using fibrin plate method [17]. Fibrinogen (5 ml of 0.6% [w/v]) solution in a 50 mM sodium phosphate buffer (pH 7.4) was mixed with the same volume of 2% (w/v) agarose solution and 0.1 ml of thrombin (10 NIH U/ml) in a Petri dish. The solution was left for 1 h at room temperature to form a fibrin clot layer. Caseinolytic activity was assayed by using the casein plate. Casein (5 ml of 0.6% [w/v]) solution in a 50-mM sodium phosphate buffer (pH 7.4) was mixed with the same volume of 2% (w/v) agarose solution in a Petri dish. Twenty microliters (3 µg) of the sample solution was then dropped into holes that had been made in the fibrin plate by a capillary glass tube (5-mm diameter), and the plate was incubated at 37°C for 12 h. An equal volume of plasmin solution (1 NIH U/ml) also was incubated as a control. Enzyme activity was estimated by measuring the width of the clear zone. Each value represents the mean of four determinations.

Enzyme activity

Enzyme activity was assayed by the following procedure. A mixture (1.0 ml) containing 0.7 ml of 50 mM glycine–NaOH buffer (pH 10.0), 0.1 ml of 2% α -casein (w/v), and 0.1 ml of enzyme solution was incubated at 37°C for 10 min, mixed with 0.1 ml of 1.5 M trichloroacetic acid, allowed to stand at 37°C for 30 min, and then centrifuged at room temperature. The absorbance at 275 nm for the supernatant was measured and converted to the amount of tyrosine equivalent. The kinetic constants (K_m) for the hydrolysis by the AJ were determined by Lineweaver–Burk plot using the various concentrations (1–10 mg/ml) of α -casein. One unit of caseinolytic activity was defined as the amount of enzyme releasing 1 µmol of tyrosine equivalent per minute.

Effect of pH on activity

The pH effect on the enzyme was assayed from pH 2.0 to 12.0. The enzyme in 20 mM sodium phosphate buffer (pH 6.4) was mixed with different buffers. The buffers used (and their pH ranges) were 0.1 M citrate–phosphate buffer (pH 2.0–5.0), sodium phosphate buffer (pH 6.0–7.0), Tris–HCl buffer (pH 8.0–9.0), and glycine–NaOH buffer (pH 10.0–12.0). The reaction mixtures were incubated at 37°C for

1 h, and the enzyme activities were measured using α -casein. Maximum activity was expressed as 100%, and the others were compared to the maximum activity. Each value represents the mean of four determinations.

Effect of temperature on activity

The effect of temperature on activity was assayed at various temperatures at pH 3.0. The enzyme in 20 mM citrate–phosphate buffer (pH 3.0) was incubated at different temperatures varying from 25 to 100°C for 20 min. The enzyme activities were assayed using α -casein, and the relative activities were calculated as percentage of the maximum activity.

Effects of protease inhibitors and metal ions

Each inhibitor and various divalent ions were mixed with purified enzyme (in 20 mM citrate–phosphate buffer, pH 3.0) at 37°C for 10 min. The enzyme activities were assayed using α -casein, and the relative activities were calculated on the basis of the activity of the pure enzyme without any inhibitor or metal ions under the same experimental conditions.

Zymography

Fibrin zymogram gel electrophoresis was carried out as described previously [18]. The separating gel solution (12%, w/v) was prepared in the presence of fibrinogen (0.12%, w/v) and 100 μ l of thrombin (10 NIH U/ml). After electrophoresis in a cold room (at 10 mA constantly), the gel was incubated for 30 min in 50 mM Tris (pH 7.4), which contained 2.5% Triton \times -100. The gel was washed with distilled water for 30 min to remove Triton \times -100, and then incubated in zymogram reaction buffer (30 mM Tris, pH 7.4, and 0.02% [w/v] NaN_3) at 37°C for 12 h. The gel was stained with Coomassie blue for 1 h and then destained. The bands indicating areas where fibrin was digested were visualized as the non-stained regions of the zymogram gel.

Two-dimensional gel electrophoresis

Isoelectric focusing (IEF) was carried out using 7-cm linear immobilized IPG gels with pH range of 3–10 (Bio-Rad, Hercules, CA, USA). Purified protein was loaded by in-gel rehydration with a reswelling solution containing 8 M urea, 0.3% DTT (w/v), and 0.2% (v/v) pH 3–10 IPG buffer. IEF was carried out for 4,000 Vh at 20°C in a IPGphor IEF system (Bio-Rad), wherein the voltage was linearly increased from 250 to 4,000 V over the first 3 h and then was maintained at 4,000 V for the final 10 h. After the IEF, the strips

were equilibrated with the SDS equilibration buffer (62.5 mM Tris–HCl, pH 6.8, 2.3% [w/v] SDS, 30% [v/v] glycerol, and 0.05% [w/v] bromophenol blue) for 15 min. The equilibrated strips were loaded into the stacking gel of the SDS gel and fibrin gel, and the 2D gel electrophoresis was performed.

Fibrinogenolytic activity

Fibrinogenolytic activity [19] was assayed by incubating 0.1 ml of a 0.2% human fibrinogen solution (w/v) with 0.05 ml of enzyme solution (100 ng) in 50 mM Tris–HCl buffer (pH 7.4) at 37°C. At different times (0–5 min), 0.15 ml of a denaturing SDS sample buffer (0.125 M Tris–HCl, pH 6.8, 0.1% [w/v] SDS, and 1% [w/v] β -mercaptoethanol) was added and the mixture heated at 95°C for 4 min. Twenty-five microliters (about 15 μ g of fibrinogen) of each sample was subsequently analyzed by SDS-PAGE.

Determination of N-terminal amino acid sequence of AJ

After SDS-PAGE was done, purified protein on the gel was transferred to a PVDF membrane by electroblotting [20] and stained with Coomassie blue. The stained portion was excised and used for N-terminal sequencing directly by the automated Edman degradation method using a gas-phase protein sequencer (model Procise 491, ABI, USA).

PCR cloning of AJ gene

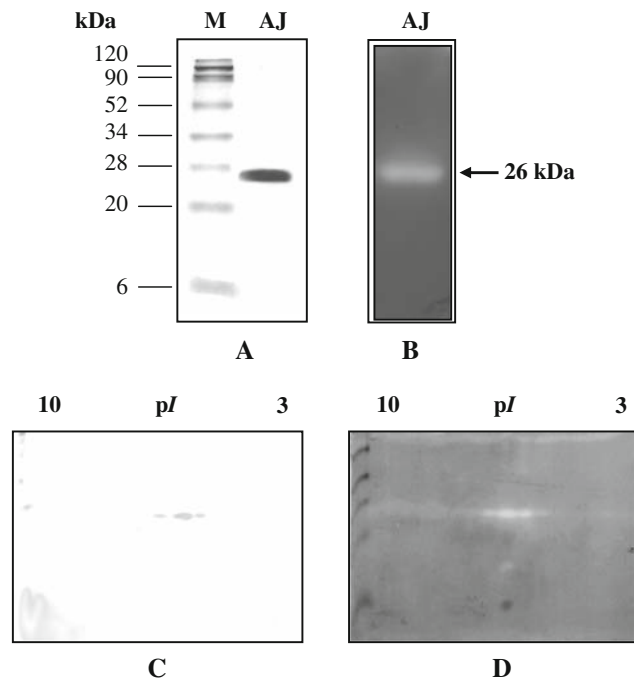
Using the amino acid sequence of AJ, we searched the nucleotide sequence database of National Center for Biotechnology Information for a AJ homologue and found the glutamyl endopeptidase clone (Accession No.: *AB096695.1*). Chromosomal DNA from *Staphylococcus* sp. strain AJ was prepared by the method of Rochelle [21], and used as the template for PCR. AJ gene was amplified by PCR using *Nde* I-linked sense primer (5'-GGAATTCATATGTAATATTACCTAATAATAATAGAC-3') and *Xho* I-linked antisense primer (5'-CCGCTCGAGTTACTGAA TATTTATATCAGGTATA-3'). PCR amplification was performed under the following conditions: 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min. The PCR-amplified 651 bp DNA fragment was extracted from agarose gel and then ligated into pGEM-T Easy vector (Promega) to generate pT-AJ plasmid.

Thrombolytic activity of the purified enzyme

For the thrombolytic test by purified enzyme, the actual fibrin clots (thrombi) of whole blood isolated from a rat's vena cava were used [22]. Thrombi were prepared by

Table 1 Purification steps of AJ from *Staphylococcus* sp. strain AJ

Step	Total Protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Culture broth	168.0	2,500	14.9	1	100
Ultrafiltration (YM10)	52.4	2,317	44.2	3.0	92.7
DEAE-cellulose	35.5	2,143	60.4	4.1	85.7
Toyopearl HW-55F	27.8	2,012	72.4	4.9	80.5

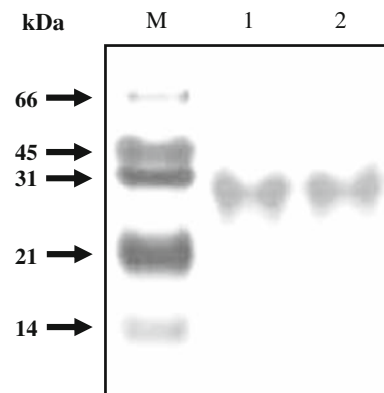
**Fig. 1** 1D and 2D SDS-PAGE (a, c) and fibrin zymography (b, d) of purified fibrinolytic enzyme (AJ) by gel filtration on Toyopearl HW-55F. AJ was subjected to electrophoresis on 12% gels

adding of thrombin (10 NIH U/ml) to rat whole blood and then washing three times with 0.1 M Tris–HCl (pH 7.4) containing 0.9% (w/v) NaCl.

Results

Purification of the AJ from *Staphylococcus* sp. strain AJ

A bacterial strain producing extracellular fibrinolytic enzyme was isolated from Anchovy-jeot, a popular fish-fermented food in Korea. AJ was purified from the culture supernatants of *Staphylococcus* sp. strain AJ. Its purification procedure is summarized in Table 1. The final specific activity of the purified enzyme increased by more than 4.9-fold. AJ migrated as a single band, and its apparent molecular mass was estimated to be approximately 26 kDa by SDS and zymogram gel (Fig. 1a, b). Using a 2D gel, AJ was found to consist of three active isoforms (pI 5.5–6.0) with the same N-terminal amino acid sequence (Fig. 1c, d). Plus,

**Fig. 2** Analysis of AJ protein on a native gel (6%). After incubation of AJ at pH 3.0 or 7.0 for 2 h, the samples were diluted with five times sample buffer (without SDS and reducing agent). (312.5 mM Tris–HCl, pH 6.8, 50% glycerol, and 0.05% bromophenol blue). 1, 2 mean AJ at pH 3 and 7, respectively. M markers from the Sigma nondenatured protein molecular weight marker

AJ migrated as a single band on a native gel, which indicates they are homogenous protein (Fig. 2).

Enzymatic characterization of AJ

The effect of pH on the activity of purified enzyme was examined. The enzyme was active in the broad pH range (Fig. 3a), and the optimum pH and temperature for the fibrinolytic activity were at 2.5–3.0 and 85°C, respectively (Fig. 3a, b). The thermal stability of the enzyme was examined by assaying the residual enzyme activity after the incubation of the enzyme at the various temperatures in 0.1 M citrate–phosphate buffer (pH 3.0). After incubation for 20 h at 25°C, the enzyme remained relatively stable (Fig. 3c), and also after incubation for 60 min at 70–80°C, the enzyme was fairly stable (Fig. 3d). Finally, the effect of protease inhibitors was studied at pH 3.0. The activity of the enzyme was inhibited by 1 mM DFP, but not affected by PMSF, EDTA, EGTA, E-64, or leupeptin (Table 2). In view of the effects of pH, temperature, and inhibitors, the enzyme can apparently be classified as a thermoacid-stable serine protease. Enzyme activity was also assayed in the presence of different metal ions at a concentration of 5 mM. Most of the metal ions studied had no significant effect on the enzyme, with the exception of Fe²⁺ with which the activity was about twofold lower (Table 2).

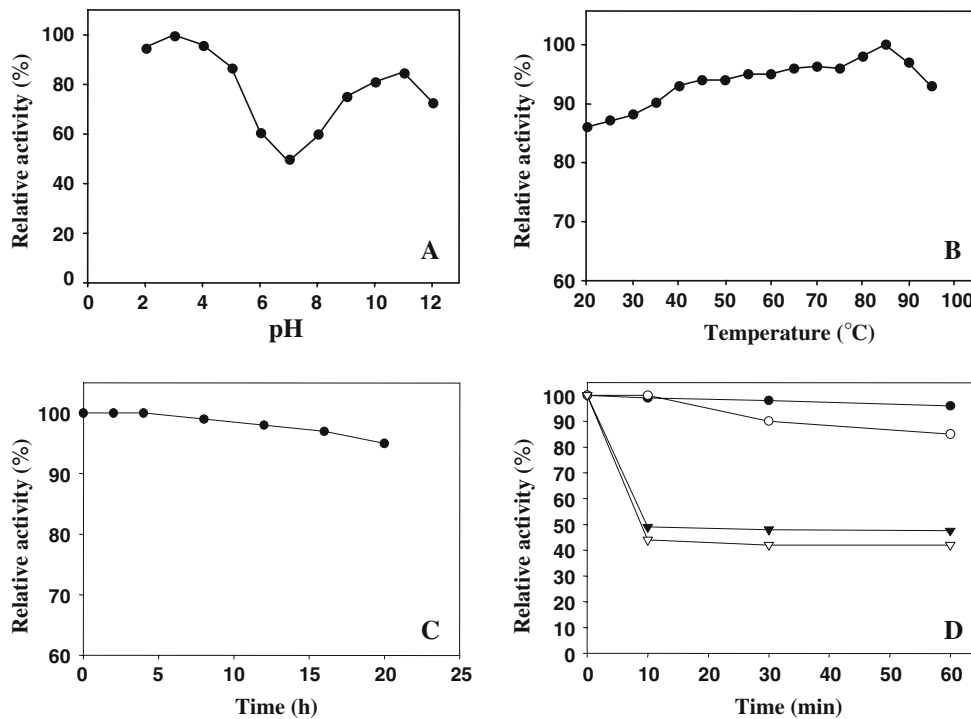


Fig. 3 Effects of pH (a) and temperature (b) on enzyme activity of AJ from *Staphylococcus* sp. strain AJ. Effect of temperature on the activity and stability of AJ at pH 3.0. After incubation for 20-h at 25°C, the residual activity was measured (c). To establish the thermostability of

AJ (d), the residual enzyme activity was measured after incubation for 60 min. Filled circle 70, open circle 80, filled inverted triangle 90, open inverted triangle 100°C

Table 2 Effects of cation ions and inhibitors on activity of AJ

Matal ions (5 mM)/inhibitors (1 mM)	Activity (%)
None	100
CdCl ₂	91.8
CoCl ₂	92.1
CuSO ₄	94.0
FeCl ₂	42.3
MgCl ₂	97.8
MnCl ₂	85.5
NiCl ₂	94.5
ZnCl ₂	96.4
PMSF	94.8
EDTA	99.2
EGTA	97.5
E-64	102.4
Leupeptin	98.5
Diisopropyl fluorophosphate	38.4

Stability of AJ on the zymogram and SDS gels

To study the primary structure of AJ under various pH conditions, SDS gel and fibrin zymography were performed. AJ kept 85% of the initial activity after heating at 100°C for 20 min on the zymogram gel (Fig. 4a). In particular, AJ

was very stable at acidic condition, whereas it was easily degraded at neutral and alkaline conditions (Fig. 4b). Interestingly, in the pH range from 7.0 to 8.0, AJ formed a dimer, showing an activity on SDS gel and fibrin zymogram gel (Fig. 4b, c). However, when AJ was incubated with SDS solution (1–5%) or at 100°C for 20 min at pH 7.0, it was not degraded and showed the same pattern with acid condition (Figs. 5, 6).

Comparison of AJ with other proteases for fibrinolytic activity

The fibrinolytic activity of AJ was compared with those of other proteases. AJ was 1.2- and 1.6-fold less efficient than those of subtilisin Carlsberg and subtilisin BPN', respectively (Table 3). On the other hand, the specific activity (F/C, the ratio of fibrinolytic to caseinolytic activity) was 2.8 and 1.6 times higher than those of subtilisin Carlsberg and subtilisin BPN', respectively (Table 3). The K_m and K_{cat} values of AJ were 0.38 mM and 19.73 s⁻¹, respectively (Table 4).

Fibrinogenolytic activity of AJ

AJ cleaved the A α -chain of fibrinogen, but did not affect the B β - and γ -chains, indicating that AJ is a α -fibrinogenase

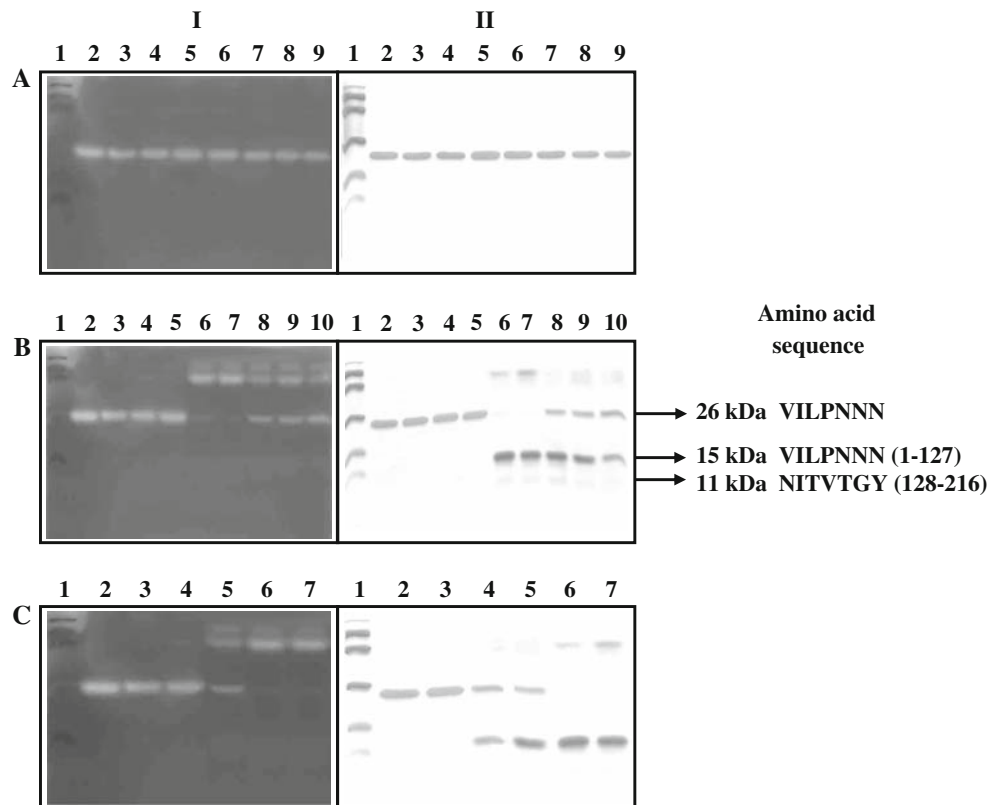


Fig. 4 Stability of AJ on the pH and temperature by using fibrin zymography (I) and SDS-PAGE (II). **a** After incubation of AJ at the indicated temperature for 20 min, the samples were applied on the 12% gels. I Molecular mass marker; 2–9 30, 40, 50, 60, 70, 80, 90, 100°C. **b** After incubation of AJ at the various pH for 1 h. I Molecular mass

marker, 2–10 pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0. **c** Stability of AJ at pH 7.0. I Molecular mass marker; 2–7 after incubation of AJ at pH 7.0 for 0, 10, 20, 30, 40, 60 min. The positions of amino acid sequence were determined based on the AJ protein (deposited in GenBank under accession number AY829230)

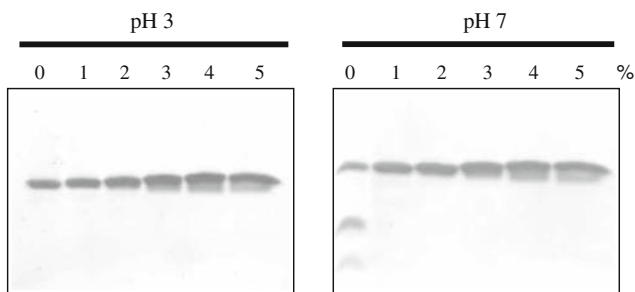


Fig. 5 Effect of SDS on the stability of AJ at pH 3.0 or 7.0. After incubation of AJ in the indicated concentration of SDS for 20 min, the mixtures were subjected to electrophoresis on 12% gel

(Fig. 7a). Even after a prolonged incubation of 10 min, no fibrinogen-clotting activity was observed (Fig. 7b).

N-terminal amino acid sequence of the purified enzyme

The N-terminal amino acid sequence of the purified enzyme up to residue 17 was Val-Ile-Leu-Pro-Asn-Asn-X-Arg-His-Gln-Ile-Phe-X-Thr-Thr-Gln-Gly-, and shared a high degree of similarity with the N-terminal sequences of a glutamyl endopeptidase (also known as V8 serine prote-

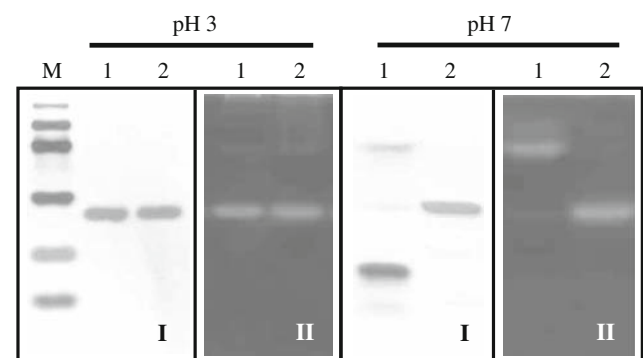


Fig. 6 Effect of heat treatment on AJ at pH 3.0 or 7.0. After boiling (100°C) of AJ for 20 min, AJ was incubated at pH 3.0 or 7.0 for 2 h. The activity and stability of AJ were assayed by SDS-PAGE (I) and fibrin zymography (II). I Without boiling and 2 boiling of AJ

ase) from *S. epidermidis* ATCC 12228 [23], *S. aureus* ATCC 12600 [24], and *S. warneri* M [25].

Cloning of AJ

AJ gene of *Staphylococcus* sp. strain AJ was cloned and its nucleotide sequence was determined (deposited in GenBank

Table 3 Comparison of AJ with other proteases for enzyme activity

Protease sources	Fibrinolytic activity (U)	Casinolytic activity (U)	F/C
AJ from <i>Staphylococcus</i> strain AJ	59.92	57.70	1.03
Subtilisin BPN' from <i>B. amyloliquefaciens</i>	53.36	102.62	0.52
Subtilisin Carlsberg from <i>B. licheniformis</i>	38.12	131.56	0.27

Mean values of three measurements are presented

Table 4 Kinetic constants of the AJ from *Staphylococcus* sp. strain AJ and NK from *Bacillus natto*

AJ from <i>Staphylococcus</i> sp. strain AJ			NK from <i>B. natto</i>		
K_{cat} (s ⁻¹)	K_m (mM)	K_{cat}/K_m (s ⁻¹ M ⁻¹)	K_{cat} (s ⁻¹)	K_m (mM)	K_{cat}/K_m (s ⁻¹ M ⁻¹)
19.73	0.38	5.2×10^4	17.85	0.52	3.4×10^4

under accession number AY829230). The nucleotide sequence revealed only one large open reading frame. The amino acid sequence of AJ was compared with the published sequences of the other proteins, and AJ was found to be identical to V8 protease from *S. epidermidis* ATCC 12228 [23], except for one amino acid substitution Ser44Asn (Fig. 8, arrow marked).

Thrombolytic activity of the purified enzyme

For the thrombolytic test by purified enzyme, the actual fibrin clots (thrombi) of whole blood isolated from a rat's vena cava were used. As shown in Fig. 9b, the purified

enzyme dissolved actual fibrin clots, indicating that AJ is a potent thrombolytic agent.

Discussion

This article describes the purification, some characteristics and primary structure of AJ produced from *Staphylococcus* sp. strain AJ for assessment of its application as a thrombosis agent. On the basis of this report, bacterial strain producing fibrinolytic enzyme was isolated from Anchovy-joet, a popular fish-fermented food in Korea, and was identified as *Staphylococcus* sp. strain AJ. AJ was purified from supernatant of *Staphylococcus* sp. strain AJ culture broth and it showed thermoacid-stable and strong fibrinolytic activity. But, no effect on plasminogen activator activity was observed.

In the pH range from 7.0 to 8.0, AJ formed an active homo-dimer on SDS gel and fibrin zymogram gel (Fig. 4b, c). We found that AJ consists of three isoforms (pI 5.5–6.0) with the same molecular mass and N-terminal amino acid sequence (Fig. 1c, d). Therefore, it is supposed that these three isoforms interact and form a dimer of AJ protein. Also, AJ was easily degraded as two fragments, 15 and 11 kDa, at neutral and alkaline conditions. However, no activity was found in both fragments. The N-terminal amino acid sequences of two fragments were determined (GenBank under accession number AY829230).

AJ showed unusual thermostable at acid condition, as shown in Figs. 3d and 4a, whereas it was unstable at neutral and alkaline conditions (Fig. 4b, c). We also examined the stability of AJ in SDS (1–5%) or incubation at 100°C for 20 min. Interestingly, AJ was not degraded and showed the same pattern with acid condition (Figs. 5, 6). This means

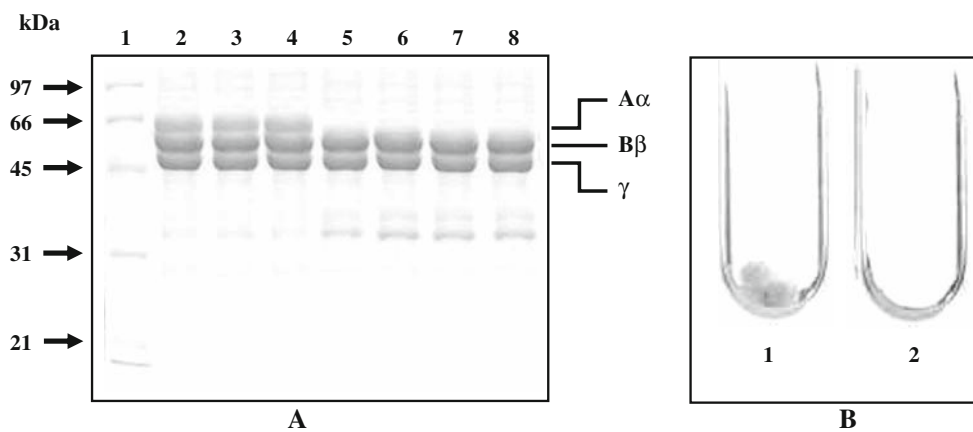


Fig. 7 SDS-polyacrylamide gel electrophoresis of the digested human fibrinogen by AJ under reducing conditions. **a** Fibrinogen consisted of three-polypeptide chains: A α (66,000), B β (54,000), and γ (48,000). Fifteen microliters of fibrinogen incubated with enzymes were subjected to electrophoresis on 10% gel: 1 molecular mass marker; 2

fibrinogen incubated without enzyme for 5 min; 3–8 fibrinogen samples after incubation with enzymes for 0, 1, 2, 3, 4, 5 min. **b** After incubation of AJ with fibrinogen for 10 min, 100 μ l of thrombin (1.0 NIH U/ml) was added. 1 Without AJ and 2 AJ (1.0 μ g)

Fig. 8 Comparison of the amino acid sequence of AJ with those of other proteases. AJ, *Staphylococcus* sp. strain AJ (this study); V8, from *S. epidermidis* ATCC 12228 [23]; glutamic, glutamic acid-specific endoprotease from *S. aureus* ATCC 12600 [24]; glutamyl, glutamyl endopeptidase from *S. warneri* M [25]. Underline indicates the N-terminal amino acid position of 11 kDa fragment of AJ

		***** **	* ** **	*		*		*	****	** ↓	****
AJ		YILPNNNRHQ	IFNTTQGHYD	AVSFIYIPID	GGYMSGSGVY	VGSEILTNK					
V8		YILPNNNRHQ	IFNTTQGHYD	AVSFIYIPID	GGYMSGSGVY	VGSEILTNK					
glutamyl		YILPNNDRHQ	INDTTLGHYA	PVTFYQVQSN	EGTFIASGVY	VGKDKLLTNK					
glutamic		YILPNNDRHQ	ITDTTNGHYA	PVTYIQVEAP	TGTFIASGVY	VGKDTLLTNK					
		*** **	*** *	**** *	***** *	***** *					
AJ		HVVNGAKGNP	RNISVHPSAK	NENDYPNGKF	VGQEIPYPG	NSDLAILRVS					
V8		HVVNGAKGNP	RNISVHPSAK	NENDYPNGKF	VGQEIPYPG	NSDLAILRVS					
glutamyl		HVVDATEGNP	RALKAFPSAV	NQNNYPNGGF	TGEQITKYPG	NADLAIVKFS					
glutamic		HVVDATEGDP	HALKAFPSAI	NQDNYPNGGF	TAEQITKYSG	EGDLAIVKFS					
		** * **	** ** *	* * **	*****	***** **					
AJ		PNEHNQHI GQ	VYKPATISSN	TDTRINENIT	VTGYPGDKPL	ATMWESYGKY					
V8		PNEHNQHI GQ	VYKPATISSN	TDTRINENIT	VTGYPGDKPL	ATMWESYGKY					
glutamyl		PNNQNQNI GE	VYTPATLSDN	ADTSQNQPIT	VTGYPGDKPL	ATMWESRGI					
glutamic		PNEQNKHI GE	VYKPATMSNN	AETQVNQNI	VTGYPGDKPV	ATMWESKGI					
		** * *	**** *****	***** * *	***** ** *	* * **					
AJ		YYIGGEELRY	DLSTVGGNSG	SPVFNGKNQV	IGIHYGGVDN	KYNSSVYIND					
V8		YYIGGEELRY	DLSTVGGNSG	SPVFNGKNQV	IGIHYGGVDN	KYNSSVYIND					
glutamyl		TQIQGEDMHY	DLSTTGGNSG	SPVFNSRNEV	IGIHWGGAAN	SYNGAVFINN					
glutamic		TYLKGEAMQY	DLSTTGGNSG	SPVFNEKNEV	IGIHWGGVYPN	QFNGAVFINE					
		* ** *	**								
AJ		FVQQFLRNNI	PDINI Q....					
V8		FVQQFLRNNI	PDINI Q....					
glutamyl		NVQNFLKQNI	EDIHFSNSDN	NDD.....	..NDNNN..					
glutamic		NVRNFLKQNI	EDINFANDDH	PNNPDNPDP	NNDNNPNDP	NPNNPDNPDP					
AJ						
V8						
glutamyl		.GTDDNNNN.NDDDD	NNYDNPDA A					
glutamic		PNNPDNPNNP	DNPNNPDQPN	NPNNPDNGDN	NNSDNPDA A					

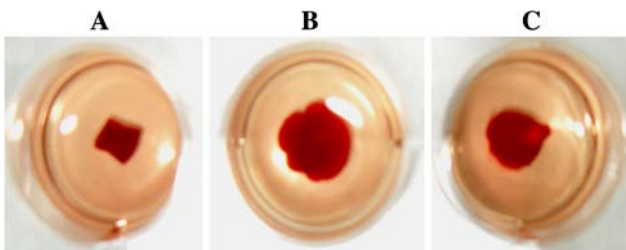


Fig. 9 Thrombolytic activity of the AJ for thrombi in a rat's vena cava. Freshly drawn thrombi was placed with 10 μ g of AJ (b) and human plasmin (c) in saline, and was incubated at 37°C for 24 h. a Saline without of the enzyme was used as a control

that SDS and heating may act as a stabilizing factor. To our knowledge, a similar combination of acid and heat stability has not yet been reported for other fibrinolytic enzymes.

As mentioned above, AJ cleaved the α -chain of fibrinogen but did not affect the β - and γ -chains (Fig. 7). In general, fibrin (ogen)olytic enzymes belong to as two classes, the $\alpha(\beta)$ -fibrinogenases (known as zinc-metalloproteinases) and the β -fibrinogenases (known as thermostable serine proteinases) [26, 27]. On the basis of these results, AJ is a new type of serine α -fibrinogenase.

In general, *S. aureus* secretes four major extracellular proteases: serine protease (V8 protease; SspA), cysteine protease (SspB), metalloprotease (aureolysin; Aur), and second cysteine protease (Scp). All four proteases are produced as proenzymes, which are proteolytically cleaved to form the active mature enzymes [28]. V8 serine protease of them was one of the first isolated enzymes of *S. aureus* to be purified and characterized in detail [29]. Its molecular mass was estimated to be 11.4 kDa by SDS gel, and it exhibited maximum proteolytic activity at pH 4.0 and 7.8. In recent, the glutamyl endopeptidase (designated GluSE), which showed 78.9% (15 residues) homology to that of mature GluV8, was purified from *S. epidermidis* ATCC 14990 [30]. GluSE was a 27 kDa and the optimum pH for activity was 8.0, and was low when under pH 6.0 or over pH 9.0, showing quite different enzymatic properties with AJ.

AJ gene of *Staphylococcus* sp. strain AJ was cloned and its nucleotide sequence was determined. The nucleotide sequence revealed only one large open reading frame. The deduced amino acid sequence of AJ was 99.5 similar to that of V8 protease from *S. epidermidis* ATCC 12228 (Fig. 8) [23]. But, two proteins showed different optimum pH

condition, thermostability and substrate specificity. Clearly, change in amino acid at position 44 (Ser44Asn) may account for these differences. It is reported that the amino acid substitutions of Asp44Glu, Asn71Ser, and Lys147Arg of V8 protease increased the half-life of the enzyme stability against 5 M urea [31, 32]. Thus, site-directed mutagenesis and functional analysis of AJ will be continued to identify the amino acid critical for the thermoacid stability and substrate specificity.

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