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A novel serine protease with human fibrino(geno)lytic activities from *Artocarpus heterophyllus* latex

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ABSTRACT

A protease was isolated and purified from *Artocarpus heterophyllus* (jackfruit) latex and designated as a 48-kDa antimicrobial protease (AMP48) in a previous publication. In this work, the enzyme was characterized for more biochemical and medicinal properties. Enzyme activity of AMP48 was strongly inhibited by phenylmethanesulfonyl fluoride and soybean trypsin inhibitor, indicating that the enzyme was a plant serine protease. The N-terminal amino acid sequences (A-Q-E-G-G-K-D-D-D-G-G) of AMP48 had no sequence similarity matches with any sequence databases of BLAST search and other plant serine protease. The secondary structure of this enzyme was composed of high α-helix (51%) and low β-sheet (9%). AMP48 had fibrinogenolytic activity with maximal activity between 55 and 60 °C at pH 8. The enzyme efficiently hydrolyzed α followed by partially hydrolyzed β and γ subunits of human fibrinogen. In addition, the fibrinolytic activity was observed through the degradation products by SDS-PAGE and emphasized its activity by monitoring the alteration of secondary structure of fibrin clot after enzyme digestion using ATR-FTIR spectroscopy. This study presented the potential role to use AMP48 as antithrombotic for treatment thromboembolic disorders such as strokes, pulmonary emboli and deep vein thrombosis.

1. Introduction

Proteases (proteolytic enzymes) are one of the important enzymes which play a significant role in various biochemical mechanisms to maintain metabolic processes of all organisms. Proteases are widely used in various industries, e.g., food, textile, leather industry, detergents and pharmaceutical. In pharmaceutical, proteases can be used as therapeutic enzymes, for instance, L-asparaginase (L-ASP) for cancer treatments [1], nattokinase for fibrinolysis [2,3] and papain for burns and wound healing [4]. Plant, proteases are implicated in many processes, for instance, seed germination, defense mechanisms, senescence and proenzymes activations [5]. Latex is a source of proteases derived from laticifer cells of several plants such as families: Apocynaceae, Caricaceae, Euphorbiaceae and Moraceae [6,7]. Tropical areas including Thailand have several rubber-producing plants. The present work focuses on characterization of the biochemical properties of a proteolytic enzyme from *Artocarpus heterophyllus* (jackfruit) latex. *A. heterophyllus* is a rubber-producing plant that belongs to the family Moraceae widely distributed in Thailand [8]. A 79.5-kDa serine protease (artocarpin) has been isolated from jackfruit latex [9]. Its proteolytic activity could be enhanced by thiol reducing agent, and inhibited by phenylmethylsulphonylfluoride (PMSF) [9]. However, the medicinal properties of this protein have not been investigated. In our previous publication, a 48-kDa protease (AMP48) was isolated from jackfruit latex and characterized as an antibacterial and antifungal agent [10]. In this study, the AMP48 protein was further characterized for its biochemical and medicinal properties.

2. Materials and methods

2.1. Materials

Q Sepharose Fast Flow (1.5 cm × 3 cm) column, SP Sepharose™ Fast Flow (1.5 cm × 3 cm) column, molecular weight standard marker for SDS-PAGE, 7 cm immobilized pH gradient strip pH 3–10, Coomassie brilliant blue R-250, polyvinylidene fluoride (PVDF) membrane and acrylamide were purchased from GE Healthcare (Sweden). Bicinchoninic acid (BCA) assay kit was purchased from Pierce (USA). Endoglycosidase H (Endo H) and N-glycosidase F (PNGase F) were purchased from New England Biolabs (USA). Human fibrinogen, human plasmin, human thrombin, phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), iodoacetic acid (IAA) and soybean trypsin inhibitor (SBTI) were purchased from Sigma (USA). Other analytical grade chemicals were purchased from Acros Organics (Belgium). The latex used for

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purification and characterization in this work was collected from a jackfruit tree located in Tambon Suranaree, Muang district, Nakhon Ratchasrima province, Thailand.

2.2. Purification of AMP48 protein

The AMP48 protein was isolated and purified from the crude latex of jackfruit following the method as described in our previous report [10].

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

One dimensional SDS-PAGE (1D SDS-PAGE) was performed following the method of Laemmli [11]. The polyacrylamide gel contained 0.1% (w/v) sodium dodecyl sulfate (SDS) using a Tris–glycine buffer, pH 8.8. Protein bands were observed by staining with Coomassie brilliant blue R-250.

Two dimensional SDS-PAGE (2D SDS-PAGE) was performed as described in the previous report [10]. Protein was examined by staining with Coomassie brilliant blue R-250. For N-terminal sequencing, the protein was transferred into a PVDF membrane and the membrane was stained with Coomassie brilliant blue R-250. The protein spots of AMP48 were cut from stained membrane and subjected to N-terminal protein sequencing.

2.4. Protein concentration determination

Protein concentrations were analyzed using the BCA assay kit following the manufacture's instruction. Protein (25 μl) was added to microwell plate and mixed with BCA reagent (200 μl). The plate was incubated at 37 °C for 30 min and then measured at 540 nm with a microwell plate reader (Labsystems iEMS, Labsystems Ltd. Oy, Finland) using Ascent software™ version 2.4 (Labsystems Ltd. Oy, Finland). Various concentrations of bovine serum albumin (BSA) (0.025–2.0 mg/ml) were used as a standard calibration curve.

2.5. N-terminal protein sequencing

The protein spots of AMP48 from 2D-SDS as described above were cut and analyzed for N-terminal sequence by the automated Edman degradation method. N-terminal sequencing was carried out by the Proteomics International Pty Ltd., Australia. N-terminal sequence homology was matched against BLAST database search [12].

2.6. Mass spectrometry

The intact molecular mass of AMP48 was detected by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonics MALDI-TOF/TOF Mass Spectrometer Autoflex II, Bruker Daltonics®, Germany) and using sinapinic acid (SA) as MALDI matrix. Mass spectrometric analyses were performed in the linear, positive mode at 20 kV with a pulsed nitrogen laser, emitting at 337 nm.

2.7. Secondary structure determination

The secondary structure of AMP48 was performed using circular dichroism (CD) spectroscopy with a Jasco J-715 spectropolarimeter (Japan) calibrated with CSA (nonhygroscopic ammonium (+)-10-camphorsulfonate). The CD spectrum of AMP48 (1 mg/ml in 25 mM Tris–HCl, pH 8.8) was recorded at room temperature with a scan speed of 20 nm/min, 2 nm bandwidth, 100 mdeg sensitivity, an average response time of 2 s and an optical path length of 0.2 mm. The CD spectrum of the protein was subtracted with a baseline buffer (25 mM Tris–HCl, pH 8.8) and analyzed using the K2D program.

2.8. Human fibrinogenolytic activity

Human fibrinogenolytic activity was assayed by the method of Satake et al. [13] with some modification. The reaction mixture contained 40 μl of human fibrinogen (10 mg/ml human fibrinogen in 25 mM Tris–HCl buffer, pH 8.8) and 4 μg of AMP48 (in a total volume of 80 μl). Then the mixture was incubated at 37 °C for 2 h. The reaction was stopped by adding 40 μl of 10% (w/v) trichloroacetic acid (TCA) and left to stand for 10 min. Then the reaction mixture was centrifuged at 10,000×g for 20 min. The supernatant containing digested peptides was transferred to a new tube. The supernatant (25 μl) was subjected to determine the peptide concentration using the BCA assay kit (as described in the method 2.4). Blank (control assay) was done using the reaction without adding AMP48. One unit of enzyme activity was defined as the amount of AMP48 required to give an increase in absorbance of 0.01 at 540 nm/min (at 37 °C). The specific activity was expressed as units of activity per milligram of protein (units/min/mg of protein).

2.9. Effects of various inhibitors and metal ions on fibrinogenolytic activity

Various inhibitors that are specific to serine proteases (PMSF and SBTI), cysteine protease (IAA and $HgCl_2$), and metalloproteases (EDTA and *O*-phenanthroline) [7] (10 mM each except 1 mM for SBTI) were used to identify the specific class of AMP48 protease. Some metal cations (10 mM of each metal ion: Ca^{2+}, Mg^{2+}, Fe^{3+} and Zn^{2+}) were used to identify their effects on AMP48 activity. The AMP48 was incubated with an inhibitor or a metal ion (total reaction volume of 40 μl) for 30 min at room temperature. Then the fibrinogenolytic activity was determined using human fibrinogen as substrate following the method (Section 2.8) described above. A control assay was done in the reaction without inhibitors, and the activity was taken as 100%. All reactions were done in triplicate.

2.10. Effects of pH and temperature on fibrinogenolytic activity

The optimal pH was examined by varying the pH of reaction mixture between 3 and 12. The buffers used were 25 mM sodium acetate (pH 3–5), 25 mM sodium phosphate (pH 6–7), 25 mM Tris–HCl (pH 8–9), and 25 mM glycine–NaOH (pH 10–12). Human fibrinogen substrate was dissolved with the respective pH buffers. Then the optimal pH of AMP48 on fibrinogenolytic activity was assayed following the method (Section 2.8) described above. The optimum temperature was determined in the temperature range of 10 to 95 °C and the enzyme activity of AMP48 was assayed following the method in Section 2.8.

The pH stability was determined by incubating AMP48 (4 μg) with respective pH buffers (range of 3 to 12) for 24 h. After incubation, the activity of the treated enzyme was determined by the standard method (Section 2.8). The temperature stability was determined by incubated enzyme (4 μg) at each temperature (range of 10 to 95 °C) for 4 h. Then the activity of treated enzyme was assayed by the standard method (Section 2.8).

All determinations described above were done in triplicate. The residual activity of the pretreated sample was done using the standard condition (Section 2.8) and taken as 100%.

2.11. Subunit specific of fibrinogenolytic activity

Specific subunit of human fibrinogen digested by AMP48 was analyzed by the method of Rajesh et al. [14] with slight modification. Human fibrinogen (50 μg in 25 mM Tris–HCl buffer pH 8.8) was mixed with different amounts of AMP48 (1–32 μg) in total reaction volume of 40 μl and incubated at 37 °C for 2 h. The reaction was stopped by adding 20 μl of denaturing buffer (1 M urea, 4% SDS and 4% β-mercaptoethanol). The digested products were analyzed by

SDS/10% PAGE and the protein bands were stained with Coomassie brilliant blue R 250. In addition, the fibrinogenolytic activity of AMP48 (2 and 4 μg) was compared with human plasmin (1 and 2 μg). The time-dependent digestion assay was determined following the method described above by incubating AMP48 (4 μg) with human fibrinogen at37 °C for various incubation time (5 min to 4 h).

2.12. Fibrinolytic activity

2.12.1. Detection of Fibrinolytic activity using SDS PAGE

The fibrin clot was prepared by the method of Rajesh et al. [14] with slight modification. One hundred fifty microliters of human fibrinogen (1 mg/ml) was mixed with 5 μl of 0.5 M $CaCl_2$ and 10 μl of thrombin (0.25 NIH Units). The reaction was incubated at 37 °C for 20 min. Then the fibrin clot was transferred to the new tube and incubated with different amounts of AMP48 in 40 μl of 25 mM Tris–HCl, pH 8.8 at 37 °C for 2 h. The reaction was stopped by adding 20 μl of buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol. The degradation patterns were detected using SDS/7.5% PAGE and the protein bands were stained with Coomassie brilliant blue R 250. In addition, the fibrinogenolytic activity of AMP48 (4 μg) was compared with human plasmin (2 μg).

2.12.2. Detection of fibrinolytic activity using Fourier transform infrared (FT-IR) spectroscopy

Fibrinolytic activity of AMP48 was determined by the time-dependent digestion assay using FTIR spectroscopy. The infrared spectra were collected using the attenuated total reflectant (ATR)-FTIR spectroscopy with a single reflection ATR sampling module coupled with an MCT detector cooled with liquid nitrogen over the measurement range from 4000 to 600 cm^{-1}. The fibrin clot was prepared on a standard sample cell (a Pike Miracle single-bounce attenuated total reflectance (ATR) cell equipped with a ZnSe single crystal, Bruker, Germany). The fibrin clot was prepared by a slightly modified method described above (Section 2.12.1). The samples were composed of 150 μl of human fibrinogen (20 mg/ml) mixed with 5 μl of 0.5 M $CaCl_2$ 10 μl of thrombin (0.25 NIH Units) and then left to clot at room temperature for 20 min. Then the measurements were performed with a spectral resolution of 4 cm^{-1} with 64 scans co-added. The enzyme reaction was performed by adding 40 μl (20 μg) of AMP48 into the fibrin clot and spectra were collected at room temperature for 2 h. IR different spectra were analyzed using all other sets of spectra corrected within the experiment (the fibrin clot with enzyme) minus their individual spectra with the first spectra of scans corrected as the internal reference (the fibrin clot without enzyme).

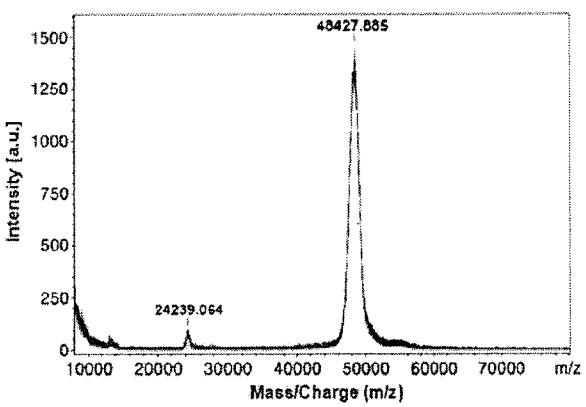

Fig. 1. Mass spectrometry of AMP48. The intact molecular mass of AMP48 was analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and using sinapinic acid (SA) as MALDI matrix.

Table 1
Comparison of the N-terminal amino acid sequences of AMP48 with various plant serine proteases.

Serine protease	Sequence of 10–11 amino acids at N-terminus	Similarity (%)	Reference
AMP48	AQEGGKDDDGG		
Lilly LIM9	TTHTPDYLGIQ	-	[15]
Cucumisin melo L.	TTRSWDFLGFP	-	[16]
Tomato P69B	TTRSPTFLGLE	-	[17]
White gourd	TTRSWDFLNFP	-	[18]
Arabidopsis	TTRTPLFLGL	-	[19]
Bamboo protease	TTRTPSFLRLS	-	[20]
Euphorbia supine protease B	TTRTPNFLGL	-	[21]
SCSI (soy bean) (D)	TTRSWDFLKS	-	[22]
Port CI (soy bean)	TTRSWDFIGH	-	[22]
Cucumis trigonus Roxburghi	TTRSWDFLSG	-	[23]

(–) No significant similarity found.

Reactions were conducted in buffers containing 25 mM Tris–HCl, pH 8.8. The fibrinolytic activity was performed by determining time dependence of the alteration of the secondary structure of a human fibrin clot. The fibrinolytic activity of human plasmin (20 μg) was used as positive control. All spectra were recorded and analyzed using the OPUS 6.5 software (Bruker, Germany).

3. Results and discussions

3.1. Protein purification, molecular mass determination, amino acid sequencing and homology search

A 48 kDa protein purified from jackfruit latex by acid precipitation and ion exchange chromatography was previously reported as a protease using gelatin- and casein-zymography [10]. The protein had antimicrobial activity, especially antifungal and antibacterial activities. Therefore, the protein was signatured as a 48-kDa antimicrobial protease or AMP48 [10]. The intact molecular mass of AMP48 was analyzed by MALDI-TOF mass spectrometry, and its molecular mass was 48427.885 Da (Fig. 1). The first 11 amino acid residues of its N-terminal analyzed by Edman degradation method were A-Q-E-G-G-K-D-D-D-G-G. The N-terminal amino acid sequences of AMP48 had no sequence similarity matches with any sequence databases of BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) and other plant serine proteases (Table 1). Based on the results of proteomic analysis and N-terminal sequencing, AMP48 was identified as a novel protease.

3.2. Secondary structure study

CD spectroscopy was used for estimation of secondary structure of AMP48. The spectrum is shown in Fig. 2. Far UV spectrum was analyzed using K2D program (available at URL: http://www.embl.de/~andrade/k2d.html). The secondary structure of AMP48 was composed of high α-helix (51%) and low β-sheet (9%). These results were different from other proteases purified from plant latex that showed high content of the β-sheet (between 34 and 48%) and low content of the α-helix (between 7 and 13%) [14,24]. However, CD spectra of these enzymes were measured at different pH conditions (estimated at about pH 7–7.4) of AMP48 (at pH 8.8).

3.3. Effects of inhibitors and metal ions on fibrinogenolytic activity

The specific class of AMP48 and effects of some metal cations on fibrinogenolytic activity were analyzed (Table 2). The activity of enzyme was significantly decreased by the serine protease inhibitors (PMSF and SBTI). The activity of the enzyme remained at only 1% in

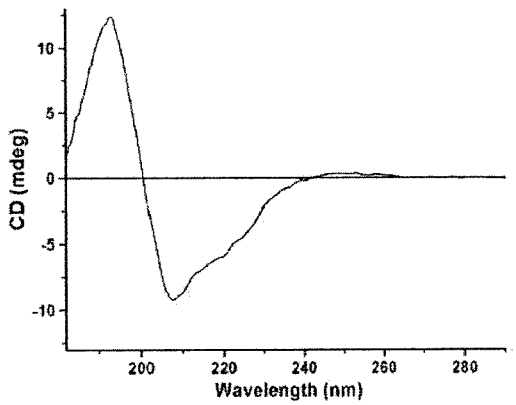

Fig. 2. Circular dichroism (CD) spectrum of AMP48. Far-UV CD spectrum of AMP48 (1 mg/ml in 25 mM Tris-HCl, pH 8.8) was recorded at room temperature with an optical path length of 0.2 mm.

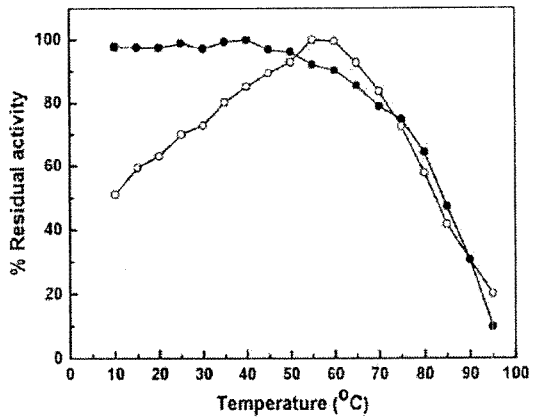

Fig. 3. Effects of temperature on the activity (○) and stability (●) of AMP48. The optimum temperature on fibrinogenolytic activity of AMP48 was determined by digesting human fibrinogen with the protein (40 μg) at different temperatures (10–95 °C). The temperature stability was determined by incubating the protein at different temperatures (10–95 °C) for 4 h and measuring its activity with the standard condition (Section 2.8).

the condition containing 10 mM PMSF. In contrast, AMP48 could not be inhibited by the cysteine protease inhibitor (IAA) except for $HgCl_2$ that almost absolutely blocked the enzyme activity (remaining activity 7%) at 10 mM $HgCl_2$. The enzyme is partially blocked by metalloprotease inhibitors (EDTA and *O*-phenanthroline) and metal cations (Mg^{2+}, Fe^{3+} and Zn^{2+}) except Ca^{2+}. Since AMP48 could be significantly inhibited by serine protease inhibitors, it was classified in the family of serine protease [8,25,26]. In addition the effect of serine protease inhibitors and metal ions on AMP48 was different from other plant latex serine proteases in the family Moraceae, for instance, benghalensin isolated from *Ficus benghalensis* [27]. This protease could not inhibit by SBTI and $HgCl_2$ [27]. Although AMP48 could be inhibited by $HgCl_2$ which was a cysteine protease inhibitor and partially inhibited by some metalloprotease inhibitors, the enzyme was still classified as a serine protease. Several plant serine proteases which were similarly blocked by inhibitors and metal ions as AMP48, have been reported, such as a heat stable glycoprotein from *Synadenium grantii* latex [14] and a serine protease with fibrinolytic activity from *Euphorbia hirta* latex [24]. Combining the results of N-terminal sequencing and inhibitor assay emphasized that AMP48 was a novel serine protease with fibrinogenolytic activity.

3.4. Effects of pH and temperature on fibrinogenolytic activity

The optimal temperature of AMP48 on fibrinogenolytic activity was between 55 and 60 °C and the activity was almost lost at 95 °C (Fig. 3). Thermostability analysis showed that AMP48 could maintain its activity up to 50 °C (Fig. 3).

The optimum pH for AMP48 was 8 (Fig. 4). The activity remained at least at 80% when incubated at pH 7 and 10 for 24 h (Fig. 4).

Table 2
Effects of inhibitors and metal ions on the fibrinogenolytic activity of AMP48.

Type of inhibitor and metal ion	Inhibitor and metal ion	Concentration (mM)	Relative activity (%)
None			100
Serine protease	PMSF	10	1±2
	SBTI	1	16±3
Cysteine protease	Iodoacetic acid	10	109±4
	$HgCl_2$	10	7±3
Metalloprotease	EDTA	10	80±3
	O-phenanthroline	10	50±2
Metal ion	Ca^{2+}	10	105±2
	Mg^{2+}	10	80±3
	Fe^{3+}	10	13±3
	Zn^{2+}	10	34±4

Relative activity represents the mean ± SD of three individual experiments.

Effects of pH and temperature on enzymatic activity of AMP48 were similar with some plant latex serine proteases, for instance, a glycosylated protease from *Euphorbia milii* [28] and a highly stable protease from *F. benghalensis* [27]. A serine protease from *E. milii* latex had optimum temperature and thermostability at 60 and 65 °C, respectively. Moreover, it had optimum pH at 8 and stability at pH 5–12 [28]. A stable protease from *F. benghalensis* can hydrolyze casein substrate with optimum pH and temperature at 8 and 55 °C respectively [27].

3.5. Specific fibrinogenolytic activity

Human fibrinogen generally composes of two sets of three polypeptide chains (or subunits) termed α, β and γ which are joined by disulfide bonds within the N-terminal of E domain [29]. The estimated molecular weights of the three subunits (α, β and γ) are 63, 56 and 47 kDa respectively [30]. The human fibrinogen could be hydrolyzed by AMP48 predominantly at the α subunit (Fig. 5) and partially hydrolyzed at β and γ subunits. However, the enzyme could hydrolyze the β subunit better than the γ subunit. The major hydrolytic products were polypeptide chains with molecular weights of about 35, 33 and 28-kDa, respectively (Fig. 5A). AMP48 completely hydrolyzed

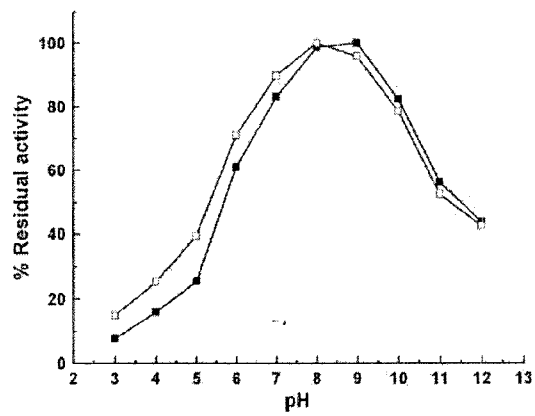

Fig. 4. Effects of pH on the activity (□) and stability (■) of AMP48. The optimum temperature on fibrinogenolytic activity of AMP48 was determined by digesting human fibrinogen with the protein (40 μg) at various pH between 3 and 12. The pH stability was determined by incubating the protein at different pH (3–12) for 24 h and measuring its activity with the standard condition (Section 2.8).

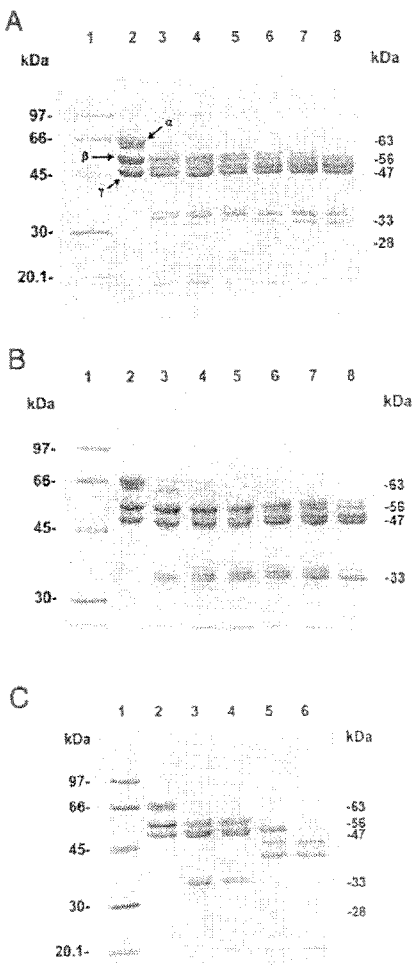

Fig. 5. Fibrinogenolytic activity of AMP48 analyzed by dose-dependent (A), time-dependent (B) and comparison with activity of human plasmin (C). All hydrolyzing patterns of human fibrinogen are shown on SDS/10% PAGE. Lane 1 of all experiments contains protein molecular weight markers. A. Human fibrinogen (50 μg) was incubated with AMP48 at different amounts: 0 μg (lane 2), 1 μg (lane 3), 2 μg (lane 4), 4 μg (lane 5), 8 μg (lane 6), 16 μg (lane 7) and 32 μg (lane 8) for 2 h at 37 °C. B. Human fibrinogen (50 μg) was incubated with AMP48 (4 μg) for different times: 0 min (lane 2), 5 min (lane 3), 15 min (lane 4), 30 min (lane 5), 60 min (lane 6), 120 min (lane 7) and 240 min (lane 8) at 37 °C. C. Human fibrinogen (50 μg) was incubated with AMP48 at amounts: 2 and 4 μg (lanes 3 and 4, respectively) and separately with human plasmin at amounts: 1 and 2 μg (lanes 5 and 6) for 2 h at 37 °C. Lane 2 is the reaction without any enzyme.

α subunit of human fibrinogen after incubation for 30 min (Fig. 5B). The β and γ subunits had begun to be hydrolyzed at 60 min after incubation (Fig. 5B). Human plasmin is a plasma serine protease which plays an important role in the dissolution fibrin blood clots. Plasmin (2 μg) could almost completely hydrolyze all subunits of fibrinogen (Fig. 5C). The activities of AMP48 and plasmin were compared as shown by the pattern of hydrolytic products on SDS-PAGE (Fig. 5C). The major products from plasmin digestion had higher molecular weights and more digested polypeptide chains than those from AMP48 digestion. The products from plasmin digestion were between 45–42 kDa and 22–28 kDa.

The fibrinogenolytic activity of AMP48 was similar to the other potential therapeutic enzymes isolated from snake venoms, for instance, a metalloprotease from Colombian *Bothrops atrox* [31], a fibrinogenase from Taiwan habu [32] and a serine protease (α-mucrofibrase from

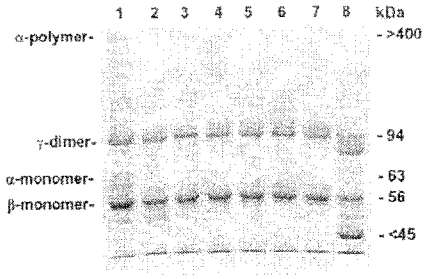

Fig. 6. Fibrinolytic activity of AMP48. The partial human fibrin clot was prepared by thrombin inducing clot and incubated with AMP48 at different amounts: 0 μg (lane 1), 1 μg (lane 2), 2 μg (lane 3), 4 μg (lane 4), 8 μg (lane 5), 16 μg (lane 6), 32 μg (lane 7) and with 2 μg of human plasmin (lane 8) at 37 °C for 2 h.

Chinese habu) [33]. Moreover, its activity was similar to the other proteases isolated from plant latex, for example, a serine protease from the medicinal herb *E. hirta* [24]. These enzymes efficiently hydrolyzed α followed by partially hydrolyzed β and γ subunits.

3.6. Fibrinolytic activity

A partially cross-linked human fibrin clot prepared from human thrombin was incubated with different concentrations of AMP48 to determine the effect of the enzyme on a fibrin clot. The fibrin degradation products were examined on SDS/7.5% PAGE in the reducing condition as shown in Fig. 6. AMP48 could completely hydrolyze the α-chain and α-polymer (molecular weight more than 400 kDa [30]), and partially hydrolyze the β-chain and the γ-dimer. The pattern of hydrolytic products from AMP was different from human plasmin (Fig. 6). Human plasmin could completely hydrolyze all α-chains and its polymers but partially hydrolyzed the β-chain and γ-dimer showing digested products with the major chain at molecular weight under 45 kDa (Fig. 6). Since the degradation products of human fibrinogen digested with AMP48 were smaller than 40 kDa (Fig. 5), the digested products could be visualized by the resolution of SDS/7.5% PAGE analysis (Fig. 6).

The efficiency of AMP48 on fibrin clot degradation was emphasized using attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy. The fibrin clot digested by AMP48 was monitored for its secondary structure alteration within 2 h, at room temperature. The alterations of the β-sheet and the α-helix of the

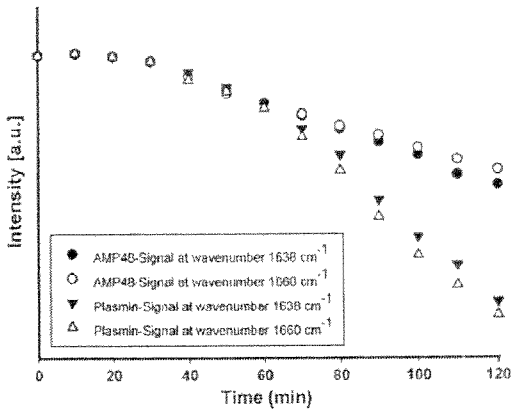

Fig. 7. The time dependences of secondary structural changes of human fibrin clot digested by AMP48 and human plasmin. The hydrolyzing reaction of AMP48 and human plasmin were followed by using ATR-FTIR spectroscopy to monitor the decrease in absorbance of the β-sheet (centered at 1638 cm^{-1}) and the α-helix (centered at 1638 cm^{-1}). The experiments were performed in buffers containing 25 mM Tris-HCl, pH 8.8 at room temperature.

fibrin clot were recorded at wavenumbers 1638 and 1660 cm^{-1}, respectively [34]. Fig. 7 showed that AMP48 could digest fibrin by decreasing its secondary structure for both β-sheet and α-helix when the incubation times increased. The feature of the fibrin clot was also changed from solid to partial liquid state. In addition, the IR spectra of fibrin digested by plasmin showed a reaction similar to that of AMP48 by decreasing its secondary structures after plasmin cleavage (Fig. 7).

Summarizing all data from N-terminal sequencing, fibrinogenolytic and fibrinolytic activities including ATR-FTIR spectroscopy, the results suggested that AMP48 was a novel plant serine protease which had fibrino(geno)lytic activities. Based on AMP48 activities, it might be used as medicinal plant protease like other natural sources, for instance, venom protease from snake in the genus *Bothrops* for thromboembolic disorder treatment (e.g. deep vein thrombosis, strokes and heart attacks) [35].

4. Conclusions

In our previous report, a protease was isolated from jackfruit latex and characterized as an antimicrobial agent. The protein was designated as AMP48. In this study, more biochemical and biological properties of the protein were characterized. AMP48 was classified as a serine protease and intact molecular mass was 48427.885 Da. The enzyme had fibrinogenolytic and fibrinolytic activity as shown by decreasing of all fibrin clot's secondary structures after enzyme digestion detected by ATR-FTIR spectroscopy. Based on N-terminal sequencing, AMP48 was a novel serine protease of jackfruit* latex which has fibrino(geno)lytic activity. In addition, this work also presented a potential to use AMP48 for treatment of thromboembolic disorders. Consequently, the properties of AMP48, which involve human blood coagulation factors and blood coagulation time, will be further investigated.

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