PURIFICATION AND CHARACTERIZATION OF A NOVEL PROTEASE WITH FIBRINOLYTIC ACTIVITY FROM *Mucor subtilissimus* UCP 1262

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ABSTRACT - A novel protease with fibrinolytic activity was purified from *Mucorsubtilissimus*UCP 1262usinga two-step purification protocol.The enzyme was pre-purified using cetonic precipitation and adsorbed by ion exchange chromatography on DEAE-sephadex G50. System two-dimensional electrophoresis (2DE) coupled to SDS-PAGE showed a single protein band of15.3kDa approximatelyand isoeletric focusing point of 3.9, exhibiting a nature as an acidic enzyme.The activity was suppressed by Co^{2+} and $HgCl_2$, but slightly enhanced byCa^{2+}. Additionally, the activity was slightly inhibited by EDTA, but significantlyinhibited by PMSF. It exhibited fibrinolytic activity,which is weaker than that of plasmin, but also had a higher affinity for the N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (SAAPNA) and azocasein substrates, suggesting a chymotrypsin-like protease. These results demonstrated an economically viable protocol purification of a protease with fibrinolytic activity with high degree of purity that may represent a potential source of new therapeutic agents to treat thrombosis.

1. INTRODUCTION

Proteases constitute a large group of hydrolytic enzymes that cleave peptide bond of protein and degrade them into small peptides and amino acids. Among the different industrial enzymes, proteases make up 60% and they haveapplications mainly in food, detergents and leather processing industries (Anitha and Palanivelu, 2013).

The productionof microbial enzymes requires the investigation of the parameters that may affect enzyme yield, optimization of production and effective downstream processing techniques.Various natural-active enzymes purified from microbial sources have been extensively used in many fields. Among them, fibrinolytic enzymes have received attention fortheir potential medicinal use on thrombotic disease, which is becoming a leading cause of morbidity and mortality worldwide, yet most of current fibrinolytic agents available for clinic

are barely satisfactory (Bi*et al*, 2013).

The huge microbial diversity may potentially facilitate targeting novel fibrinolytic proteases with desired therapeutic attribute. In recent years, thrombolytic agents found in non-animal sources have received considerable attention because of their security(Kumar *et al*, 2013, Choi *et al*, 2013). Fibrin is formed from fibrinogen by the action of thrombin. Fibrin is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator. Hemostasis is a complex process that maintains the balance between fibrin formation and fibrinolysis. However, when fibrin hydrolysis is not complete because of a balance disorder, thrombosis, such as a myocardial infarction, can occur (Shirasaka *et al*., 2012).

The abnormal clot, called thrombus, within the vascular system obstructs the flow of blood and nutrient to vital tissues (Lu *et al*, 2010). Due to prevalence, these cardiovascular diseases are expected to impose an ever-increasing impact on our society emotionally, socially and financially (Kim *et al*., 2011).In this study, we therefore attempted to identify thrombolytic agent as a protease with fibrinolytic activity by fermentation from *Mucorsubtilissimus*UCP 1262. The purification and characterization of this protease are also described.

2. MATERIAL AND METHODS

2.1. Culture conditions for enzyme production

The culture conditions of *Mucorsubtilissimus*UCP 1262 for enzyme production were preliminarily optimized (30 °C, 120 rpm). A 6-day culture of *Mucorsubtilissimus*UCP 1262 in 7 mL Czapek Agar medium was used. The spores suspension at 10^4 of concentration was inoculated into 100 mL medium liquid described by (Porto*et al*, 1996)withminor modificationsso thatwheatwas addedas a nitrogen source.

The medium consisted of wheat bran filtrate (1%, w/v), K_2HPO_4 (0.435%, w/v), and mineral solution [$FeSO_4 \cdot 7H_2O$ (100 mg), $MnCl_2 \cdot 4H_2O$ (100 mg), and $ZnSO_4 \cdot H_2O$ (100 mg)] , NH_4Cl (0.1%, w/v), $MgSO_4 \cdot 7H_2O$ (0.06 %, w/v), and $CaCl_2$ (1% w/v) plus glucose (1%, w/v) dissolved in 100 mLof distilled water. The culture medium was added in a shaking flask (250 ml), which was incubated at 30°C on a shaker (120 rpm) for 4 days. The culture broth was obtained by filtration and used for enzyme purification.

2.2. Purification of protease with fibrinolytic activity

The liquid metabolic obtained by fermentation (300 mL) was submitted to precipitation using 70% acetone. The resulting precipitate was collected through centrifugation at 15,000xg,4°C, for 30 min. The pellet was dissolved in 100mM sodium acetate buffer, pH 7.5, and the solution was dialyzed against the same buffer overnight at 4°C. The dialysate was concentrated by lyophilization and subsequently loaded into a DEAE-Sephadex chromatography process, using a column (8.0 x 1.0 x 1.0 cm) equilibrated with Sodium acetate buffer (100 mM, pH 7.5). The adsorbent sample was then eluted with 0.5 M potassium

chloride in same thebuffer. The protein-containing fraction was pooled and the enzyme solution was concentrated for further analysis. All the process was monitored at 280 nm absorbance.

2.3. Determination of Total Protein

The protein content was determined according to the method of (Bradford, 1976) using bovine serum albumin to obtain a standard curve. Each experiment was performed in triplicate.

2.4. Determination of Fibrinolytic Activity

Fibrinolytic activity was measured using the fibrin degradation assay. Firstly, 0.4 ml of 0.72 % fibrinogen was placed in a test tube with 0.1 mL of 245 mM phosphate buffer (pH 7.0) and incubated at 37°C for 5 min. Then, 0.1 mL of a 20-U mL^{-1} thrombin was added. The solution was incubated at 37°C for 10 min, 0.1 mL of diluted enzyme solution was added, and incubation continued at 37°C. This preparation was mixed after 20 -40 min. At 60 min, 0.7 mL of 0.2 M trichloroacetic acid was added and mixed. The reaction mixture was centrifuged at 15,000 ×g for 10 min. After that, 1 mL of the supernatant was collected and the absorbance at 275 nm was measured. Each experiment was performed in triplicate and the average value was then calculated after correction of the corresponding blank. In this assay, 1 U (fibrin degradation unit) of enzyme activity was defined as a 0.01/min increase in absorbance at 275 nm of the reaction solution (Wang*et al*, 2011).

2.5. Proteolytic activity

Amidolytic activity was measured spectrophotometrically using the synthetic substrates: (S7388 Sigma) N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide –Chymotrypsin substrate, (G8148 Sigma) Gly-Arg-p-nitroanilide dihydrochloride - Urokinase and plasmin substrate. The mixture (0.8 mL) contained 30 µL of enzyme solution, 30 µL chromogenic substrate, and 140 µL of 20mM TRIS-HCl (pH 7.4). After incubation for 15 min at 37°C, the amount of liberated p-nitroaniline (pNA) was calculated by spectrophotometric absorption at 405 nm. One unit of amidolytic activity (AU) was expressed as micromoles of substrate hydrolyzed per minute and per milliliter by the enzyme. Each value is the mean of three determinations (Kim *et al*., 1996).

2.6. Determination of molecular mass and isoelectric point (IEF)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 12,5% polyacrylamide running gel according to (Laemmli, 1970). The molecular mass was approximately calculated using a molecular mass marker (Low-Range Rainbow Molecular Weight Markers - GE Healthcare) as a standard. Protein bands were detected by staining with Coomassie brilliant blue R-250.

The isoelectric focusing point (pI) of enzyme was determined by two-dimensional electrophoresis (2-DE) according to the manufacturer's procedure. Isoelectric 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 (GE Healthcare).

The purified enzyme was loaded by in-gel rehydration with a reselling 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 (GE Healthcare), 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.

2.7. Effect of inhibitors in protease activity

To evaluate the effect of inhibitors, the purified sample was exposed to the following inhibitors, the concentration of the solutions was standardized at 5 mM,: PMSF (fluoride methylphenylsulfonyl-$C_7H_7FO_2S$), mercuric chloride ($HgCl_2$), 2-mercaptoethanol (2-hydroxy-1-ethanethiol-C_2H_6SO) and EDTA (Ethylenediaminetetraacetic acid - acetic - $C_{10}H_{16}N_2O_8$) and incubated for 60 min at 37°C. The inhibitors were dissolved in 0,1 M Tris-HCl, pH 7.75, with 0.15M NaCl.

3. RESULTS AND DISCUSSION

3.1. Purification, determination of molecular mass and isoelectric point (IEF)

The fibrinolytic enzyme was purified using the steps described in table 1, including acetone precipitation and ion exchange chromatography with DEAE-Sephadex column. The specific activity of the enzyme after purification, in comparison with the crude extract, was increased 10-times, with a recovery of 1.71%. The yield of the purified enzyme after DEAE-Sephadex ionic exchange chromatography was about 3.63 % of the relative fibrinolytic activity of the culture supernatant and the enzyme had a specific activity of 47.28 U/mg of protein.

Many reports revealed the use of acetone at different volume concentrations: 5 volumes, 3 volumes, and 2.5 volumes, as a primary precipitation agent for the recovery of proteases. Precipitation was also reported by various workers with acetone at different concentrations: 80% (v/v), 66% (v/v); or 44, 66, and 83% (v/v), followed by centrifugation and/or drying. Precipitation of enzymes can also be achieved by the use of water-soluble, neutral polymers such as polyethylene glycol(Kumar and Takagi, 1999).

A single-step chromatographic procedure, anion-exchange chromatography, was performed for purification of the fibrinolytic enzyme. Due to the residues of the culture supernatant, it was directly subjected to the column after clarification pre-treatment. This was an advantage when the purification strategy used in this work was compared to the purification of proteolytic enzymes produced under solid state fermentation conditions, where a number of steps need to be applied to remove excess salt (Yegin*et al*, 2012). Anion-exchange chromatography was a particularly critical step to the success of fibrinolytic enzyme NJP purification (Wang *et al*, 2011) and fibrinolytic enzyme NJF purified from the second active fraction (Deng *et al*, 2010). The fibrinolytic enzyme purified from mycelium of *Perenniporiafraxinea* showed 14.2-fold with a yield of 0.8% (Kim *et al*, 2008).The purified

protease with fibrinolytic activity appeared as a single protein band on SDS-PAGE, and had a molecular weight of 15 kDa and isoeletric point of 3.9 (Figure 2). The purification of protease has been summarized in Table 1.

Table 1. Purification steps of protease with fibrinolytic activity from *Mucorsubtilissimus* UCP 1262

Steps	AF(U/mL	AF(U)	PT(mg/mL)	V(mL)	PT(mg)	SA(U/mg)	Fold	Yield (%)
Crude Extract	5.35	1605	0.006	300	1.8	891.66	1.00	100
Cetonic precipitate	5.41	162.51	0.0104	30	0.31	520.86	1.71	10.12
DEAE-Sephadex	1.96	5.90	0.0416	3	0.12	47.28	11.02	3.63

The fibrinolytic activity was eluted as two peaks as showed in Figure 1, the second peak with maximum fibrinolytic activity was collected, dialyzed and concentrated by lyophilization, and used as the purified enzyme preparation. The purified fibrinolytic protease was shown as a single band in SDS-PAGE showed in Figure 2A and one protein spot in 2-DE as showed in Figure 2C and the purity of the enzyme was thus confirmed.

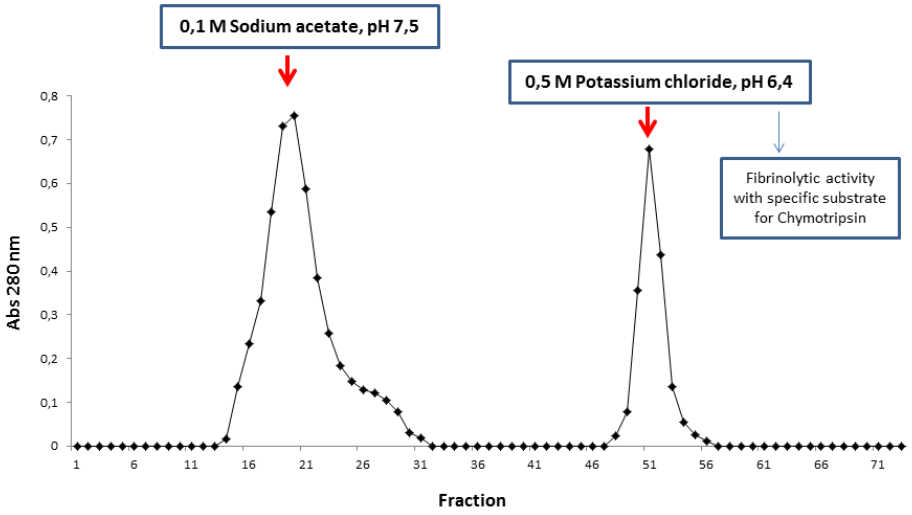

Figure 1 - Purification of fibrinolytic protease produced by *Mucor subtilissimus*UCP 1262. Anion-exchange chromatography on a DEAE-Sephadex column (8.0x1.0 cm) equilibrated with 0.1M Sodium acetate (pH 7.5), then eluted at a flow rate of 1.2 mL min $^{-1}$ with linear gradient of 0.5M Potassium chloride (pH 6.4).

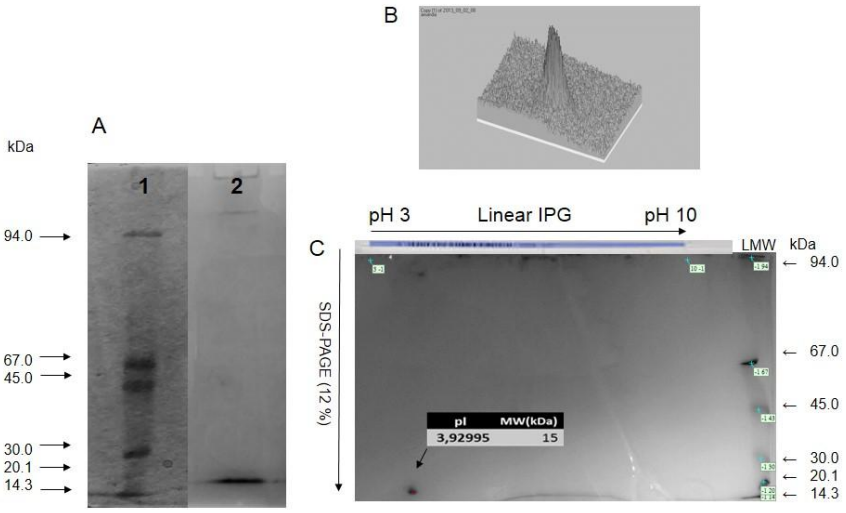

Figure 2 - SDS-PAGE (A), 3D-view of fibrinolytic protease obtained using Image Master software Platinum (GE Biosciences) (B) and 2-DE (C) of the purified fibrinolytic protease. (A) Lane 1, a standard LMW marker; Lane 2, purified fibrinolytic enzyme. (B) 3D-view of the purified fibrinolytic enzyme plot. (C) The first dimension was completed on immobilized pH 3–10 linear.

The purified fibrinolytic enzyme from *Mucorsubtilissimus* UCP 1262 with (15 kDa) is as small as the fibrinolytic enzyme purified from *Paecilomycestenuipes* (14 kDa). These enzymes have a molar mass lower than others fibrinolytic enzymes reported from fungi: *Paecilomyces tenuipes*(Kim *et al*, 2011), *Fusarium* sp. CPCC 480097(Wu*et al*, 2009), *Armillaria mellea*(Lee *et al*, 2005), *Aspergillus oryzae* KSK-3 (Shirasaka *et al*, 2012) and*Perenniporia fraxinea*(Kim *et al*, 2008).Comparative studies of thrombolytic enzymesfrom fungus with other emblematic thrombolytic agents revealed that these are low molar mass proteases, which may be an evidence for less immunogenicity character. Therefore, administration of these proteases in thrombolytic therapy, should not elicit the immune response against it. The therapeutic perspective of some of these agents is being evaluated in clinical trials (Arbind and Jagdeep, 2011).

The hydrolytic activity of the purified enzyme was measured using several chromogenic substrates. The highest level of fibrinolytic activity was observed with N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide suggesting that it is a chymotrypsin-like protease. This type of fibrinolytic enzymes has also been reported from *Armillariamellea*(Lee *et al*, 2005), *Perenniporiafraxineamycelia*(Kim *et al*, 2008) and*Fomitellafraxinea*(LEE*et al*, 2006).The purified sample with fibrinolytic activity was subjected to the protease inhibitors. The serinoprotease inhibitor, PMSF, showing a residual activity of 36.5%, significantly inhibited enzyme activity. It has also been inhibited by iodoacetic acid (54.5%) but was not inhibited in presence of pepstatin A, β- mercaptoethanol and EDTA with residual activity of 93.9, 98.6 and 132% respectively. These results allowed characterizing fibrinolytic protease as a serinoprotease. Similar results were obtained by (Shirasaka *et al*, 2012), when the enzyme activity was considerably inhibited by serine protease inhibitors PMSF, but not by chelator agent EDTA.

4. CONCLUSIONS

A chymotrypsin-like serineprotease with fibrinolyticactivity was purified from *Mucorsubtilissumus* UCP 1262. Its molecular weight was determined to be 15kDa which is one of the lowest among the so far reported from fungal enzymes. The purified fibrinolytic protease may represent a potential source of new therapeutic agents to treat thrombosis.

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