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Purification and Characterization of a Novel Fibrinolytic Protease from *Schizophyllum commune*

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ABSTRACT

Schizophyllum commune, a widely distributed medicinal mushroom, was found with fibrinolytic activity from its basidiomycetes. The fibrinolytic activity of *S. commune* was to be beneficial for antithrombotic therapy. In this study, *S. commune* was cultured with fermentation technology, and protease purification was carried out by cross-flow filtration and fast performance liquid chromatography (FPLC) system. The specific activity of *S. commune* fibrinolytic protease increased 9.29-fold over the culture broth after purification. The fibrinolytic protease shows superior fibrinolytic activity than human plasmin and is inhibited by EDTA. Characterizations of this protease showed 21.32 kDa in molecular mass and monomeric form in protein structure. The optimal protease activity reveals at pH 5.0 and 45°C, and is enhanced by magnesium.

Key words: *Schizophyllum commune*, mushroom, cross-flow filtration, fibrinolytic protease, antithrombotic

INTRODUCTION

Blood clot is composed of fibrin, which is triggered from a precursor fibrinogen via the action of thrombin. The abnormal clot, called thrombus, within the vascular system obstructs the flow of blood and nutrient to vital tissues. It causes a variety of diseases such as acute myocardial, valvular heart disease and stroke, and is the leading cause of death in developed countries[(1)]. The life-threatening thrombus has to be eliminated rapidly by fibrinolysis for homeostasis[(2)]. In clinical therapy, thrombolytic agents are plasminogen activator, which stimulates fibrinolysis depended on the plasmin activation[(3)]. But limited efficacy, expensive costs and potentially life-threatening side effects are defects of current thrombolytic agents[(4)]. Thus, investigators are trying to overcome these problems by looking for superior thrombolytic agents with high thrombolytic activity and thrombus specificity.

Thrombolytic agents are classified according to fibrinolysis mechanisms. One is plasminogen activator which activate the zymogen plasminogen to generate plasmin for fibrinolysis system[(5)]. Another is plasmin-like protein, which perform fibrinolytic actions directly. Plasmin-like proteins were discovered from snake venom[(6,7)], earthworm[(8)], microorganisms[(9)] and fermented foods[(10)]. They were proven with fibrinolytic, anti-thrombotic activities and attracted much attention in investigators. Recently, production of plasmin-like proteins by fermentation technology and genetic engineering were investigated extensively[(9)]. These production processes have given rise to economical manipulation of thrombolytic agents.

Mushroom-derived proteins with bioactive functions have been tested for their pharmacological effect and were believed of great benefits for human health[(11,12)]. In the past, a fermented cheese-like food produced by *Schizophyllum commune* was found with anti-thrombosis activity[(13)]. *Schizophyllum commune* is one of medicinal mushrooms with high fungi protein content and fibrinolytic activity. However, the fibrinolytic mechanism is still unclear. In this study, we described the purification and characterization of fibrinolytic protease from *S. commune* culture broth. In addition, the prospective uses of the medical mushrooms protease were predicated.

MATERIALS AND METHODS

I. *Culture of Schizophyllum commune*

Schizophyllum commune was obtained from

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Bioresource Collection and Research Center (BCRC, Taiwan, R.O.C.). Culture of *S. commune* was carried out on Difco YM agar (comprising peptone, malt extract, dextrose, yeast extract and 0.2% agar) in Petri dish at 25°C. Two square centimeters plate agar covered with grown mycelia was transferred into 50 mL of YM broth as seed culture and incubated at 25°C for 5 days. Twenty milliliters of seed culture broth was transferred into 2 L of YM broth for mass fermentation culture. Fermentation culture was processed with stirring and capped with silicosen for air exchange at 28°C for 10 days.

II. *Cross-Flow Filtration of Fermentation Broth*

Mycelia in broth were removed by centrifugation, 8,200 ×g at 4°C for 30 min. Supernatant was filtered with 0.45 μm membranes and separated by cross-flow filtration system using a 100 kDa ceramic column (Advanced Biotechnology Laboratories, Taiwan, R.O.C.). The small molecules solution (< 100 kDa) was then concentrated by flow through 3 kDa ceramic column and for precipitation of proteins.

III. *Azocasein Assay for Protease Activity*

Protease activity to azocasein is an index for fibrinolytic activity in advance. It was determined by measuring the release of acid-soluble material after protease digest azocasein[14]. One hundred microliters of protein fraction was mixed with the same volume of 0.5% azocasein solution, which was prepared in 0.1 M sodium phosphate buffer (pH 8.0) and incubated at 37°C for 20 min. In order to stop reaction, 350 μL of ice-cold 10% (w/v) trichloroacetic acid was added and the mixture stood for 3 min. The mixture was then centrifuged for 15 min, 10,000 ×g at 4°C. Supernatant was withdrawn and mixed with an equal volume of 0.5 N NaOH and measured absorbance by OD_{440nm}. The sample in which the protease activity was inactivated by boiling and had followed the same procedure was used as a blank. One unit of the protease activity was defined as an absorbance difference of 0.001 at OD_{440nm} per minute to blank. Protease specific activity was calculated as the protease activity (U) per mg of protein.

IV. *Purification of Fibrinolytic Enzyme*

Fast performance liquid chromatography purification process was performed and protein concentration of each eluted fraction was detected with A_{280} in real time by ÄKTA purifier 10 (GE Healthcare). Proteins smaller than 100 kDa in concentrated solution were precipitated by adding ammonium sulfate to 80% of saturation with stirring at 4°C for 8 hours. After centrifuged at 4°C (10,000 ×g, 30 min), pellet of precipitated proteins was dissolved in a minimum volume of ddH_2O. The proteins solution was then dialyzed against ddH_2O at 4°C for an additional 24 hours as 1st dialysis substrate and for protein concentration determination. This 1st dialysis substrate was then equilibrated with column buffer, 50 mM sodium phosphate buffer (pH 8.0) containing 1 M $(NH_4)_2SO_4$ and for next step of chromatography.

Phenyl Sepharose™ High Performance beaded packing column was preequilibrated with column buffer. Equilibrated 1st dialysis substrate was loaded on column with column buffer at flow rate of 1 mL/min. Elution processes was started with column buffer without $(NH_4)_2SO_4$ in a stepwise manner of 60 min interval at flow rate of 1 mL/min. Eluted fractions were analyzed for protease activity to azocasein. The active fractions were pooled; protein precipitated and dialyzed against 20 mM Tris (pH 8.0) as 2nd dialysis substrate for protein concentration determination and next step of chromatography.

Mono Q™ 5/50 GL column was preequilibrated 20 mM Tris buffer (pH 8.0). The 2nd dialysis substrate was loaded on Mono Q™ 5/50 GL column at flow rate of 1 mL/min. Elution was carried out with the same buffer but containing 1 M NaCl, by linear gradient of 20-fold column volume to 40% 1 M NaCl. After examination of protease activity to azocasein, the active fractions were pooled; protein precipitated and dialyzed against 50 mM sodium phosphate buffer (pH 8.0) as 3rd dialysis substrate for protein concentration determination and next step of chromatography.

Superdex 75 10/300 GL column was preequilibrated with 50 mM sodium phosphate buffer. The 3rd dialysis substrate was loaded with same buffer at flow rate of 1.2 mL/min. Eluted fractions were analyzed for protease activity to azocasein, protein concentration determination and fibrin plate assay.

V. *Protein Concentration Determination*

Protein concentration was estimated by Bio-Rad Protein Assay kit according to Bradford method[15]. An acidic dye was added to protein solution following 5 min reaction and absorbance was measured by OD_{595nm}. Protein concentration was determinate based on a standard curve of bovine serum albumin that provides a relative measurement.

VI. *Fibrin Plate Assay for Fibrinolytic Activity*

Fibrinolytic activity to fibrin was determined by artificial fibrin plate assay, which modified from the method described by Astrup and Mullertz[16]. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fibrin clot was made in Petri dish at room temperature by 1.5% agarose, 0.2% human fibrinogen and 10 U of human thrombin. Twenty microliters of eluted fractions from Superdex 75 10/300 GL column was loaded in aperture on fibrin plate at 20°C for 24 hours. Plasmin from human plasma (≧ 3 units/mg protein) was used as a positive control.

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VII. *SDS-PAGE and Gel Staining*

Dialysis substrate (1st, 2nd and 3rd) and fractions eluted from Superdex 75 10/300 GL column were mixed with 4X SDS-PAGE sample buffer, followed by boiling denaturation and performed on polyacrylamide (10%) gel. After electrophoresis, polyacrylamide gel was stained with coomassie brilliant blue R-250.

VIII. *Molecular Weight Determination*

To estimate the native molecular weight, the protease was loaded to Superdex 75 10/300 GL column at flow rate of 1.2 mL/min with 50 mM phosphate buffer. Ribonuclease A (13.5 kDa), carbonic anhydrase (29 kDa), apoferritin (44.3 kDa) and bovine serum albumin (67 kDa) were used as standard markers with flow through retention volume.

IX. *Glycoprotein Staining*

After SDS-PAGE electrophoresis, polyacrylamide gel was stained with GelCode Glycoprotein Staining Kit (Pierce Biotechnology, USA). Horseradish peroxidase was used as positive control and soybean trypsin inhibitor was used as negative control.

X. *Effect of pH on Protease Activity of Fibrinolytic Enzyme*

The optimum pH for protease activity of fibrinolytic enzyme was determined in the pH range of 2.0-10.0, using various buffers. Three replicates of fibrinolytic enzyme was prepared in 50 mM glycine-HCl (pH 2.0, 3.0), acetate (pH 4.0, 5.0), Tris-HCl (pH 6.0), and glycine-NaOH (pH 7.0, 8.0, 9.0, 10.0) buffer. After incubation at 37°C for 2 hours, the protease activity was measured by azocasein assay.

XI. *Effect of Temperature on Protease Activity of Fibrinolytic Enzyme*

Three replicates of fibrinolytic enzyme solution were incubated at various temperatures (37 to 98°C) for 2 hours, and then protease activity was examined by azocasein assay.

XII. *Effect of Divalent Cations and Protease Inhibitors on Protease Activity of Fibrinolytic Enzyme*

Three replicates of fibrinolytic enzyme solution were mixed with $HgCl_2$, $C_4H_6O_4Zn{\cdot}2H_2O$, $CuSO_4$, $MgCl_2$, $CoCl_2$, $CaCl_2$, $Pb(NO_3)_2$, ddH_2O (control) and protease inhibitors of phenylmethylsulfonyl fluoride (PMSF), ethylenedinitrilotetraacetic acid (EDTA), benzamidine hydrochloride hydrate, pepstatin A, aprotinin and phosphoramidon respectively. The protease activity of mixed solution was examined by azocasein assay. Relative protease activity was expressed as index to the control (100%).

XIII. *N-terminal Sequence*

After SDS-PAGE electrophoresis, fibrinolytic enzyme was transferred to polyvinylidine difluoride (PVDF) membrane by electro blotting. The N-terminal sequence of the bound-purified protein was identified with Edman degradation method by Perkin-Elmer/Applied Biosystems Procise 494 microsequencer (Überlingen, Germany) in National Taiwan University.

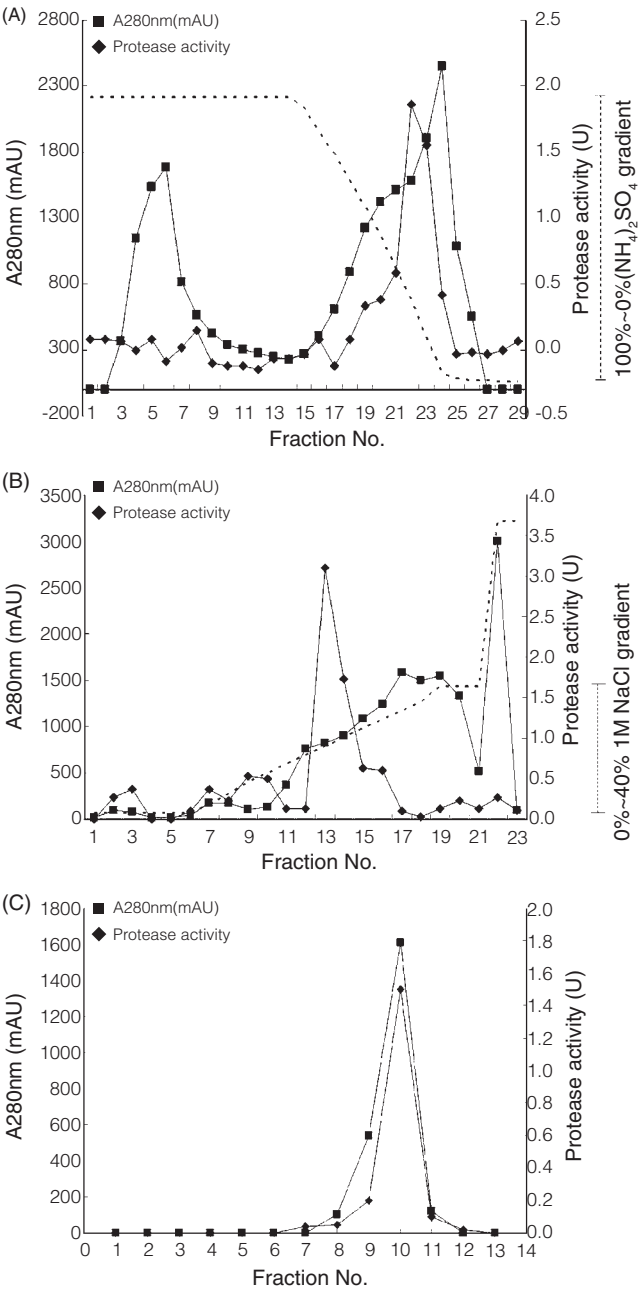

Figure 1. Chromatography and protease activity examination in steps of purification. (A) Eluted fractions of Phenyl Sepharose™ High Performance column; (B) Eluted fractions of Mono Q column; (C) Eluted fractions of Superdex 75 10/300 GL column.

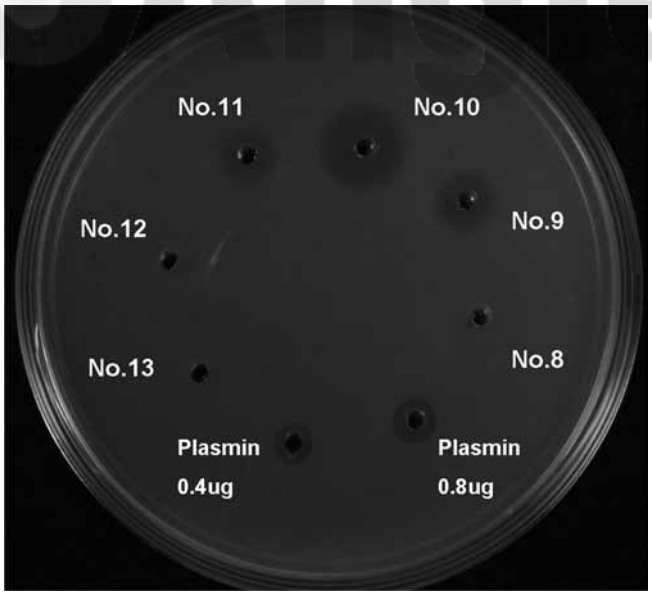

Figure 2. Fibrin plate assay of chromatography eluted fractions. No. 8-12: Fractions No. 8-12 eluted from Superdex 75 10/300 GL column chromatography. Positive control: 0.4 μg and 0.8 ug of human plasmin.

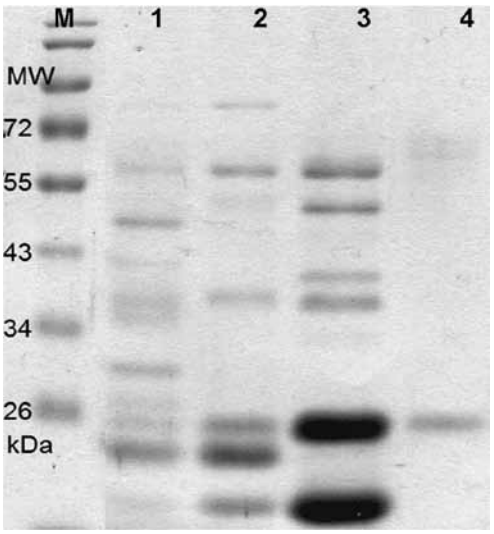

Figure 3. SDS-PAGE of each purification chromatography steps; M: protein markers; Lane 1: 1st dialysis substrate; Lane 2: 2nd dialysis substrate; Lane 3: 3rd dialysis substrate; Lane 4: Protein with highest protease activity eluted from Superdex 75 10/300 GL column.

RESULTS AND DISSCUSSION

I. *Purification of Fibrinolytic Protease from S. commune*

Separation of protease from *S. commune* culture broth was interfered with schizophyllan, which was secreted within homopolysaccharide structure and huge molecular mass[17]. In this study, cross-flow filtration system was used to eliminate schizophyllan in broth at first step, following by FPLC to separate and purify extracellular proteins. As the results, significant protease activities were observed in fractions No. 21-24 separated by Phenyl Sepharose™ High Performance column (Figure 1A), in fractions No.13-14 separated by Mono Q column (Figure 1B) and in fractions No. 9-11 from Superdex 75 10/300 GL column (Figure 1C). In fibrin plate assay, digestive zones were visible in fraction No. 9 to 11 of Superdex 75 10/300 GL column. The fraction No. 10 showed the highest fibrinolytic activity. After protein concentration estimated, fraction No. 9 showed higher fibrinolytic activity than human plasmin even in lower protein dosage (Figure 2). A purified protein in fraction No. 10 was identified by SDS-PAGE electrophoresis and showed single domain (Figure 3).

Purification and recovery rate of fibrinolytic protease from *S. commune* in this study is summarized in Table 1. Though the acquisition rate of fibrinolytic protease was low (0.07%), but specific protease activity of the fibrinolytic protease increased 9.29-fold from 15.7 U/mg to 145.8 U/mg.

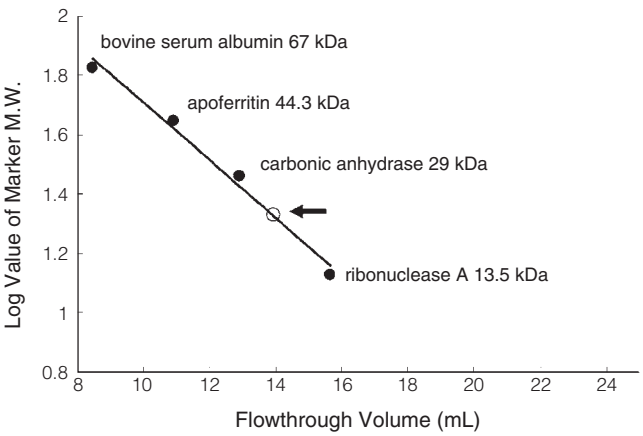

Figure 4. Semi-logarithmic plot of the protein marker flow through on Superdex 75 10/300 GL column. Arrowhead shows the molecular mass of the fibrinolytic protease from *S. commune*.

III. *Characterization of Fibrinolytic Protease from S. commune*

The non-reducing molecular weight of fibrinolytic protease from *S. commune* was 21.32 kDa (Figure 4) and shows similarity to the fibrinolytic metalloprotease from *Aremillariella mella* fruit-body[18]. After glycoprotein staining test, the fibrinolytic enzyme from *S. commune* showed a negative response. It indicated that no carbohydrate contains in the protein structure (Figure 5).

Viscoelastic and fibrinolytic properties of clot are affected by the arising structure, biological, physical and chemical factors in blood[19]. Therefore, mushroom derived fibrinolytic proteases are believed to be able to apply in thrombolytic therapy [20-23]. Among them,

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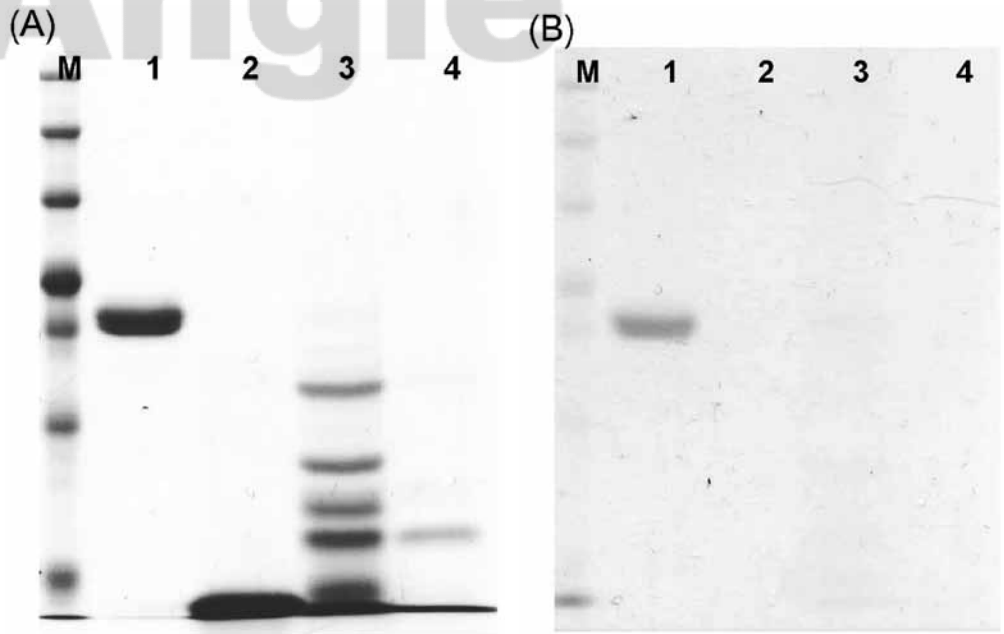

Figure 5. Glycoprotein staining of SDS-PAGE. (A) SDS-PAGE stained with CBR-250; (B) SDS-PAGE stained with GelCode Glycoprotein Staining. M: protein markers; Lane 1: positive control, horseradish peroxidase; Lane 2: negative control, soybean trypsin inhibitor; Lane 3: crude proteins of the 2nd dialysis substrate; Lane 4: Protein with highest protease activity eluted from Superdex 75 10/300 GL column.

Table 1. Purification and recovery ratio of fibrinoytic protease from *S. commune*

Purification steps	Protein (mg) (± SD)	Protease activity (U)	Specific protease activity (U/mg)[1]	Purification folds[2]	Acquisition rate (%)[3]
Culture broth	173.4 (± 2.39)	2730.0 (± 256.32)	15.7	1	100.00
1st dialysis substrate	135.7 (± 12.18)	6392.0 (± 1427.41)	46.9	2.98	78.28
2nd dialysis substrate	15.9 (± 0.78)	1244.0 (± 384.97)	79.1	5.04	9.14
3rd dialysis substrate	0.5 (± 0.03)	56.6 (± 14.81)	116.0	7.39	0.28
Fibrinolytic enzyme from Superdex 75	0.1 (± 0.02)	18.5 (± 3.77)	145.8	9.29	0.07

1. Specific protease activity was calculated with protease activity over protein content.
2. Specific protease activity of each steps compare to culture broth.
3. Protein content in each steps compare to culture broth.

proteases purified from mycelia or fruiting bodies have the optimal protease activity in the range of pH 6.0 to 8.0[24,25]. Two extracellular proteases isolated from *Flammulina velutipes* and *Cordyceps sinensis* have the optimal activity at pH 6.0 and 7.0[22,23]. The fibrinolytic protease from *S. commune* showed the highest protease activity at pH 5.0, and activity decreased from pH 5.0 to 10.0 (Figure 6A). We suggested a better acid-resisting activity of the protease from *S. commune*, as the potential to be developed in food additives and drugs[26]. The suitable temperature of fibrinolytic protease from *S. commune* is around 45°C and the activity disappeared after 2 hours incubated at 85°C and 98°C (Figure 6B). In previous resports, fermentation culture technology is a convenient and efficient method to obtain large quantities of proteins from mushrooms[10]. The

Table 2. N-terminal amino acid sequence of fibrinoytic protease from *S. commune* compared to metalloendopeptidases

Enzyme[1]	N-terminal amino acid sequence									Fibrinolytic activity
GFMEP		T	Y	N	G	C	S	S	S	Non-identify
POMEP	A	T	F	V	G	C	S	A		Non-identify
AMMEP	X[2]	X	Y	N	G	X	T	X		+
TSMEP	A	L	Y	V	G	X	S	P		+
Fibrinolytic enzyme from *S. commune*	A	S	Y	N	G	X	S	S		+

1. GFMEP, POMEP, AMMEP and TSMEP are metalloendopeptidases from *Grifola frondosa, Pleurotus ostreatu, Armillariella mellea* and *Tricholoma saponaceum*.
2. "X" means amino acid undetermined.

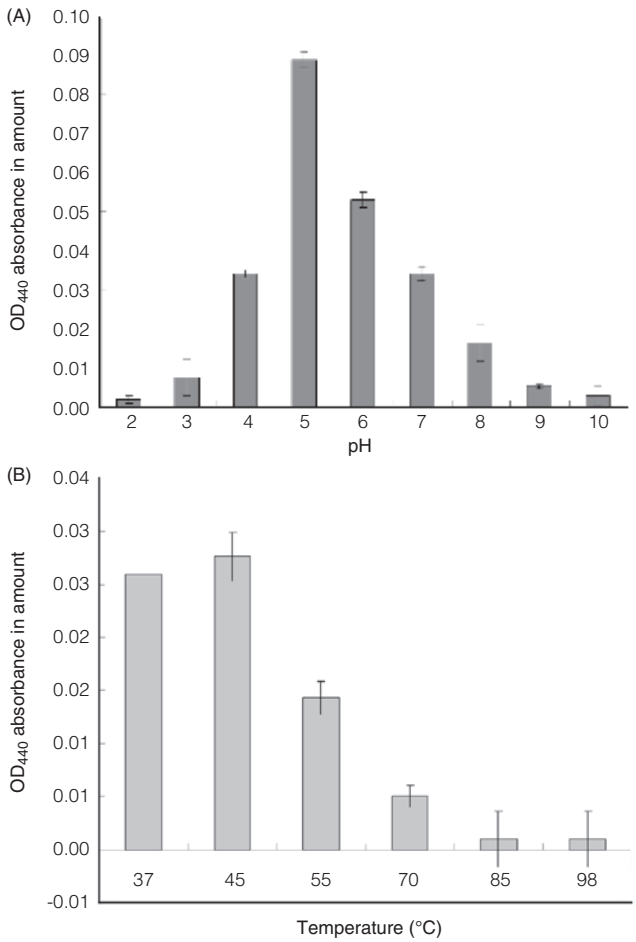

Figure 6. The effects of (A) pH and (B) temperature on protease activity of fibrinolytic protease from *S. commune*.

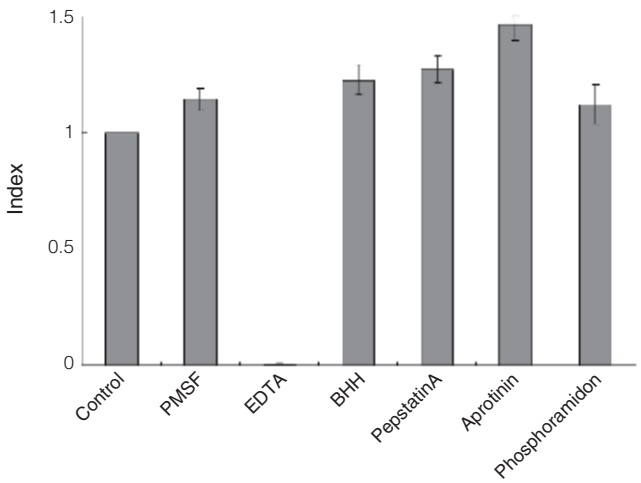

Figure 7. The effects of protease inhibitors on protease activity of fibrinolytic protease from *S. commune*.

temperature-resistance of the protease from *S. commune* was found to prevent losing activity in production and purification processing. Furthermore, thrombolysis consists of pharmacological dissolution of blood clot by administration of thrombolytic agents that promote fibrinolysis[3]. Once the protease enters circulating blood in pH between 7.35-7.45 and at physiological temperature, the protease will maintain its activity unless neutralizing antibodies are elicited. Further efforts are still required to study any potential side effects of fibrinolytic protease.

II. *Comparison of Fungi Fibrinolytic Proteases*

Two protease from *Armillariella mellea* (AMMEP) and *Tricholoma saponaceum* (TSMEP) have been proven with fibrinolytic activity[27,28]. Both of them belong to the metalloendopeptidase (MEPs) family, which have the identical features, such as sensitivity to EDTA, specificity to cleave peptide bonds, zinc-binding site structure and similarity in N-terminal sequence[29]. In this study, the fibrinolytic activity and the EDTA inhibition effect of the protease from *S. commune* are identical to MEPs. The results of N-terminal sequence were summarized in Table 2, it demonstrated the distinctive feature of the protease from *S. commune* to MEPs[27-29]. Moreover, in protease inhibitors examination, phosphoramidon

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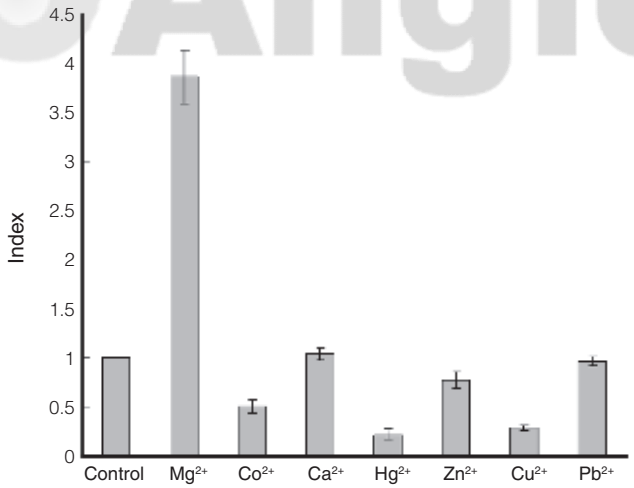

Figure 8. The effects of divalent cations on protease activity of fibrinolytic protease from *S. commune.*

(endopeptidase inhibitor) showed no inhibition to the fibrinolytic protease from *S. commune* (Figure 7). As the results, we predicate that the fibrinolytic protease from *S. commune* does not belong to MEPs family.

Activity of the fibrinolytic protease from *S. commune* was enhanced nearly four folds in the presence of Mg^{2+} ($MgCl_2$) as shown in Figure 8. Magnesium is an known essential factor for platelet activation and exposed vessel components at sites of vessel injury[30]. In model experiments, infusion of $MgSO_4$ significantly reduced stent thrombosis occurrence in dogs and swine[31,32]. Although the magnesium therapy is still controversial, the beneficial effects of magnesium have been documented in clinical trials to patients who suffered from thrombus[33-35]. The activity of fibrinolytic protease from *S. commune* was significantly induced by magnesium supplementation in this study. Therefore, the potential use in administration of the protease with magnesium for thrombolytic therapy may be considered in the future research.

CONCLUSIONS

A rare fibrinolytic metalloprotease was purified from *S. commune* culture broth by cross-flow filtration and FPLC system. The optimal protease activity displayed at the condition of pH 5.0 and 45°C. The activity was reinforced by magnesium and inhibited by EDTA. Although the production ratio and acquisition rate was still low, the fibrinolytic protease revealed 9.29-fold in specific protease activity after purification, and showed superior activity than human plasmin. For mass production of fibrinolytic protease from *S. commune,* a better manipulative fermentation culture processes will be required in the future.

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