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Biochemical and enzymatic properties of a fibrinolytic enzyme from *Pleurotus eryngii* cultivated under solid-state conditions using corn cob

Wol-Suk Cha [a,1], Sang-Shin Park [b,1], Sung-Jin Kim [a], DuBok Choi [c,*]

[a] Department of Biochemical and chemical Engineering, Chosun University, Gwangju 501-759, Republic of Korea
[b] Department of Biotechnology, Dongguk University, Gyeongju 780-714, Republic of Korea
[c] Department of Environmental Health, Chodang University, Chonnam 534-701, Republic of Korea

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ABSTRACT

Biochemical and enzymatic properties of a fibrinolytic enzyme purified from *Pleurotus eryngii* cultivated under solid-state conditions using corn cob as energy source were investigated. The molecular mass of the enzyme was estimated to be 14 kDa by SDS–PAGE. The enzyme exhibited the highest activity (28.96 mol/min/mg) for the substrate tosyl-Gly-Pro-Lys-*p*-nitroanilide. K_m and V_{max} values were 0.18 mM and 53.5 U/ml, respectively. The enzyme was completely inhibited by 1.0 mM phenylmethylsulfonyl fluoride (PMSF). The N-terminal sequence was A-M-D-S-Q-T-D-A-S-Y-G-LA-N-D. This sequence exhibited a high degree of similarity to the N-terminal sequences of the subtilisin-like serine proteases. The enzyme was very stable at pH 4.0–6.0 with an optimum pH 5.0 at 40 °C. The enzyme rapidly hydrolyzed the A α-chain of fibrinogen within 5 min of incubation, followed by the B β-chain after 10 min. The fibrinolytic enzyme from *P. eryngii* cultivated under solid-state conditions using corn cob could be potentially exploited in thrombolytic therapy.

1. Introduction

Mushroom extracts are widely used as nutritional supplements and medicines, with claimed human health benefits (Borchers et al., 1999). *Pleurotus eryngii* is a saprophytic mushroom that is taxologically related to *Basidomycotina*, *Agricales*, *Pleurotaceae*, and *Pleeurotus*. While most agaric fungal fruiting bodies naturally adhere to the stump or trunk of decayed trees as part of their decomposition activity, *P. eryngii* can grow gregariously as a mycelium or bundle in grassland oil of subtropical regions (Zadrazil, 1974). *P. eryngii* is known as the king oyster mushroom in Europe, where it is commonly cultivated, and development of an artificial cultivation technique has allowed the establishment of mushroom cultivating farmhouses equipped with automatic facilities in Korea (Kim et al., 1997). *P. eryngii* has been reported to exhibit anti-aging and anti-tumor (Guillen et al., 2000), and blood glucose-lowering properties (Kang et al., 2001). It also inhibits angiogenesis-related enzymes (Kang et al., 2003) and the proliferation of human colon cancer cells (Hawang et al., 2003), has antioxidant and free radical scavenging activities (Hui et al., 2002), and activates immune cells (Kang et al., 2004). In Korea, *P. eryngii* fruiting body is being produced on an industrial scale by sawdust obtained from various wood sources. However, alternative processes have been extensively explored because of the high production cost and environmental impact associated with the excessive utilization of natural wood sources. Of these alternatives, mushroom production from agricultural wastes such as cheese whey for *Ganoderma lucidum*, ginseng by-product for *Phellinus linteus* and *Lentinus edodes*, citrus juice processing waste for *Corious versicolor*, and green tea processing waste for *Fomitopsis pinicola* cultivation is attractive (Lee et al., 2003; Park et al., 2005; Jang et al., 2005). Among the various agricultural waste material, corn cob is a promising agricultural resource for mushroom cultivation due to the extensive cultivation of corn and because corn cobs are rich in hemicelluloses.

In this study, the biochemical and enzymatic properties of a fibrinolytic enzyme purified from fruiting bodies of *P. eryngii* grown on corn cob for effective fibrinolytic enzyme production were investigated.

2. Methods

2.1. Strain and cultivation

P. eryngii was originally provided by Hampyoung Mushroom Co., Korea and was cultivated on agar disks placed on potato dextrose agar medium at 15 °C in a controlled humidity (80%) atmosphere. After 10 d, disks were individually placed in the center of

* Corresponding author. Tel./fax: +81 61 450 1979.
E-mail address: choidubok@yahoo.co.kr (D. Choi).
[1] These authors contributed equally to this work.

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a heat-resistant polypropylene bottle filled with corn cob waste and rice bran as energy sources. Cultivation was continued for 30 d.

2.2. Chemicals

Fibrinogen, human fibrinogen, thrombin, plasmin, bovine serum albumin (BSA), azocasein, trichloroacetic acid (TCA), azocasein, phenylmethylsulfonyl fluoride (PMSF), N-α-tosyl-L-lysine chloromethyl ketone (TLCK), N-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), ethylenediaminetetra acetic acid (EDTA), diisoproopylfuorophosphate (DFP), *Streptomyces* subtilisin inhibitor (SSI), pepstatin A, phenantrolin, and low molecular weight makers for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were purchased from Sigma–Aldrich (St. Louis, MO). Molecular weight markers were SeeBlue® Plus2 Pre-Stained. Standard molecular weight electrophoresis gel markers were purchased from Invitrogen (Carlsbad, CA). Ammonium sulfate was purchased from Duksan Pure Chemical (Duksan, Korea). Bio-Lyte 3/10 Ampholyte 40% and Bio-Lyte 4/6 Ampholyte 40% were purchased from Bio-Rad Laboratories (Hercules, CA). DEAE-Sepharose Fast Flow and Sephacryl S-300 HR columns were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were the highest grade commercially available.

2.3. Preparation of crude extract

P. eryngii was cleaned, cut into 1.0 cm pieces, and dried at 40 °C in a hot-air drier. The dried pieces were ground to a powder <0.1 mm in size and stored in a sealed 0.2 mm polyethylene film bag at −20 °C. For extraction, 20 mg of the material was retrieved from the freezer and added to 100 ml of sodium phosphate buffer (50 mM, pH 7.0). The suspension was microwaved and centrifuged (7000*g*, 10 min, and 4 °C). The supernatant containing the crude extract of the fibrinolytic enzyme was recovered.

2.4. Enzyme purification

Solid ammonium sulfate was slowly added to the supernatant to 20% saturation.

The mixture was stored at 4 °C overnight, centrifuged (7000*g*, 10 min, 4 °C) to remove particulates and the supernatant was adjusted to 80% ammonium sulfate by addition of solid ammonium sulfate and stored overnight at 4 °C. The precipitate was collected by centrifugation (7000*g*, 10 min, and 4 °C), resuspended in 50 mM sodium phosphate buffer (pH 7.4) and dialyzed against the same buffer for 24 h before ultrafiltration using an Amicon YM 10 membrane (Millipore, Billerica, MA). After removal of insoluble materials by centrifugation at 10,000*g* for 10 min, the concentrated suspension was applied to a 2.5 × 30 cm DEAE-Sepharose Fast Flow ion-exchange column (Amersham Bioscience) equilibrated with 50 mM sodium acetate (pH 7.0). The bound proteins were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 5 ml/min. Fractions exhibiting fibrinolytic activity were pooled, concentrated by ultrafiltration, and desalted with a PD-10 column (Amersham Bioscience). The desalted active fraction was applied to a 16 × 60 cm Sephacryl S-300 HR column (Amersham Bioscience) that had been pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.4). Protein was eluted with the same buffer at a flow rate of 1.0 ml/min by fast protein liquid chromatography (FPLC; Amersham Bioscience). The active fractions were pooled and concentrated by ultrafiltration. The fraction was further purified by FPLC using a 16 × 60 cm Sephacryl S-300 HR column (Amersham Bioscience) equilibrated with same buffer at a flow rate of 0.5 ml/min by FPLC. The fractions exhibiting fibrinolytic activity were pooled, concentrated, and used as the purified enzyme preparation.

2.5. Enzyme assay

Fibrinolytic activity was determined using a slight modification of the fibrin plate assay (Astrup and Mullertz, 1952). Briefly, 5 ml of fibrinogen solution (0.4%) in 100 mM barbital sodium chlorhydric acid buffer (pH 7.8) was mixed in a Petri dish with 5 ml of a 0.5% agarose solution along with 1 ml of a thrombin solution (200 U/ml). The solution was left for 1 h at room temperature to allow formation of a fibrin clot layer. Then, 10 μl of the sample solution was recovered and added to a fibrin plate. The plate was incubated at 37 °C for 6 h, and the fibrinogenolytic activity was quantified by a modified fibrinogenolytic assay by measuring the area of lysis on the plate. The fibrinogen solution (200 μl of human fibrinogen in 0.05 mol/l Tris–HCl buffer, pH 7.6) was mixed with the purified enzyme solution (60 μl of 0.2 mol/l) and incubated at 37 °C. After designated time intervals, aliquots were transferred to ice and separated by 12% SDS–PAGE to examine the cleavage pattern of the fibrinogen chains. Amidolytic activity was measured using an S-3100 UV–vis spectrophotometer (Scinco, Seoul, Korea) using synthetic substrates. The substrates used were succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, benzoyl-Phe-Val-Arg-*p*-nitroanilide, tosyl-Gly-Pro-Lys-*p*-nitroanilide, benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, benzoyl-Val-Gly-Arg-*p*-nitroanilide, tosyl-Gly-Pro-Arg-*p*-nitroanilide, succinyl-Ala-Ala-Ala-*p*-nitroanilide, and benzoyl-Pro-Phe-Arg-*p*-nitroanilide. All assays were done in 20 mM sodium phosphate (pH 7.0) using 600 μl of each substrate (3.0 mM), and purified enzyme (1 μg/200 μl). The mixture was incubated at 37 °C for 5 min and the reaction was stopped by adding 0.1 ml of 50% acetic acid. Formation of *p*-nitroanilide was measured at 405 nm. One unit of amidolytic activity was defined as nmol substrate hydrolyzed per min per ml of enzyme. Protease activity was determined by measuring the release of acid-soluble material from azocasein using the aforementioned UV–vis spectrophotometer. Purified enzyme (50 μl) was mixed with 200 μl of 50 mM Tris–HCl buffer (pH 7.0) containing 0.1% azocasein. After incubation at 37 °C for 60 min, 400 μl of ice-cold 10% trichloroacetic acid (TCA) was added to the mixture and immediately vortexed. The mixture was placed on ice for 10 min and centrifuged (10,000*g*, 10 min, 4 °C). The absorbance of the supernatant was measured at 366 nm. One unit (U) of protease activity was defined as the amount of the enzyme causing an increase in absorbance of 0.001 min^{-1}.

2.6. Determination of molecular weight

SDS-PAGE was performed at room temperature by the method of Laemmli (Laemmli 1970), using 12% (w/v) polyacrylamide stacking and separating gels. SeeBlue® Plus2 Pre-Stained Standard molecular weight markers were included in each gel.

2.7. Analysis of N-terminal amino acid sequence

N-terminal amino acid sequence of the purified fibrinolytic enzyme was determined by the automated Edman degradation method. SDS–PAGE purified enzyme was transferred to a polyvinylidine difluoride (PVDF) membrane using a Minitransblot electroblotting system (Bio-Rad) and stained with Coomassie Blue. The stained band was excised and used directly for N-terminal sequencing by a Precise 491 amino acid sequencer (Applied Biosystems, Foster City, CA).

2.8. Effects of pH on fibrinolytic activity

The influence of pH on fibrinolytic activity of the enzyme was assayed from pH 2.0–10.0. The buffers used and their pH ranges were 50 mM glycine–HCl buffer (pH 2.0–3.0), 50 mM sodium ace-