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Biochemical characterization of a novel fibrinolytic enzyme from *Cordyceps militaris*

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ABSTRACT

A fibrinolytic enzyme was produced by the medicinal mushroom, *Cordyceps militaris* using submerged fermentation. The enzyme was purified from culture supernatant by hydrophobic interaction, ion exchange and gel filtration chromatographies. It was purified by 36 fold, with a specific activity of 1,467.4 U/mg protein and the final yield was 5.8%. The molecular weight of the enzyme as determined by SDS-PAGE and gel filtration was 28 kDa and 24.5 kDa, respectively, and its isoelectric point (pI) was 9.0 ± 0.2. It was found to be a glycoprotein with carbohydrate content of 1.67% (w/v). The enzyme was optimally active at 37 °C and pH 7.2. The enzyme activity was strongly inhibited by soybean trypsin inhibitor (SBTI) and aprotinin which indicated it to be a serine protease, while other inhibitors like *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenyl methane sulfonyl fluoride (PMSF), pepstatin and metal chelator EDTA did not inhibit its activity. Amino acid sequences of the purified enzyme were determined partially by Q-TOF2 and they were IEDFPYQVDLR; ANCGGTVISEK; YVLTAGHCAEGYTGLNIR; TNYASVTPITADMICAGFPEGK; KDSCSGDSGGPLVTGGK; VVGIVSFGTGCAR; ANKPGVYSSVASAEIR. Sequences of the seven peptides completely matched with those of a trypsin-like serine protease from *Cordyceps militaris* CM01 (accession no. EGX95217.1). The purified enzyme degraded α chains of fibrinogen first and then β and γ chains and also activated plasminogen into plasmin. It can act as an anticoagulant and prevent clot formation by degrading fibrinogen. Based on these studies, the purified enzyme has great potential to be developed as a natural agent for prevention and treatment of thrombolytic diseases.

1. Introduction

Fibrin, the major insoluble protein component of thrombus is formed from soluble fibrinogen by the action of thrombin (EC 3.4.21.5). Fibrin monomers instantly polymerize and form fibrin clot. Fibrin not only gives the strength and structure to the clot, but also regulates fibrin clot formation and fibrinolysis [1]. Under normal healthy conditions, these processes are well balanced, however, when clots are not lysed due to some disorder, it results in thrombosis leading to myocardial infarction and other cardiovascular diseases [2]. Formation of thrombus is a very complicated physiological process which involves many factors. Fibrinolytic enzymes degrade fibrin clot into fibrin degradation products (FDP). According to their mode of action, fibrinolytic enzymes can be grouped into plasminogen activators and plasmin-like proteases. Plasminogen activators activate plasminogen into plasmin, whereas plasmin-like proteases (EC 3.4.21.7) can degrade fibrin clot directly [3–5].

Intravascular thrombosis is one of the major reasons of cardiovascular diseases (CVDs) including acute myocardial infarction, ischemic heart disease, and high blood pressure [2,3]. CVDs are one of the leading causes of death around the world. According to world health organization (WHO) report, in 2012, around 17.5 million people have died with CVDs, representing 31% of all global deaths. It is projected that CVDs remain the single leading cause of death and it is estimated that the death number will increase to reach 23.3 million by 2030 (http://www.who.int/mediacentre/factsheets/fs317/en/). Anticoagulants such as various inhibitors of coagulation factors and thrombolytic agents are used for the prevention and treatment of thrombosis [6].

Abbreviations: BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EDTA, ethylenediamine tetracetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; SBTI, soybean trypsin inhibitor; PMSF, phenylmethane sulfonyl fluoride.

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Thrombolytic agents such as tissue-type plasminogen activator (t-PA), a urokinase-type plasminogen activator (u-PA), and streptokinase (bacterial plasminogen activator) have been developed and widely used for treatment of CVDs [2,7,8]. Despite their widespread use, these agents have undesirable side effects such as excessive bleeding caused by depleting circulating fibrinogen and plasminogen, low specificity towards fibrin and are relatively expensive [4]. Therefore, researchers are actively searching for novel thrombolytic agents from different natural sources.

In recent years, researchers have reported several proteases with fibrinolytic activity from various microorganisms isolated from traditional fermented foods [6,9]. These enzymes extracted from food grade microorganisms have great potential to be developed as functional food additives and drugs in prevention and treatment of thrombotic diseases [3]. Mushrooms are reportedly used as food and also in traditional oriental medicine. However, in recent years, mushrooms have become an attractive source for various bioactive compounds. Fibrinolytic enzymes from non-toxic mushrooms received wide research attention for thrombolytic therapy. Several fibrinolytic enzymes have been reported from various mushrooms such as *Tricholoma saponaceum* [10], *Armillaria mellea* [11], *Fomitella fraxinea* [12], *Cordyceps sinensis* [13], *Flammulina velutipes* [8], *Schizophyllum commune* [5,14] and *Pleurotus ostreatus* [15].

Cordyceps militaris is an entomopathogenic fungus which invades insect larvae and pupae commonly known as caterpillar fungus. It belongs to families *Clavicipitaceae* and *Ascomycotina*, and has been used in oriental medicine for many years [16]. *Cordyceps militaris* received wide research attention as a nutraceutical and medicinal mushroom due to its various biological and pharmacological activities [16,17]. Various proteases play an important role during invasion of insects by the fungus. Some researchers have studied and reported fibrinolytic enzymes from fruiting bodies of *Codyceps militaris* [2,18]. However, little information is available on fibrinolytic enzymes from cultured supernatant of this mushroom [16]. Mushrooms are cultivated economically in a large scale however, artificial culturing of *Cordyceps* is time consuming and economically not feasible [19]. Submerged culturing has potential advantages and the productivity can be increased by optimizing culture conditions [14]. Macrofungal fibrinolytic enzymes produced by a submerged mycelia culture can be used for many medicinal applications [5,19,20]. In this study, submerged culturing method was used for the production of fibrinolytic enzyme. In our earlier study, our group reported a novel 32 kDa plasmin-like fibrinolytic enzyme from cultured supernatant of this mushroom [21]. In the present study, we describe production, purification and characterization of a novel dual functional fibrinolytic enzyme from the submerged culture supernatant of *Cordyceps militaris*.

2. Materials and methods

2.1. Materials and chemicals

Fibrinogen (bovine) and thrombin were bought from the Tianjin Blood Institute (Tianjin, China). Fibrinogen (human) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soybean trypsin inhibitor (SBTI), N-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenyl methane sulfonyl fluoride (PMSF), aprotinin, pepstatin and low molecular weight (LMW) protein standard kit for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Sangon (Shanghai, China). Chromatography matrices Sephadex G-25, Carboxy methyl (CM) Sepharose FF, Phenyl Sepharose HP, Superdex 75 16/60, and isoelectric focussing (IEF) calibration kit were procured from GE Life sciences (Pittsburgh, PA, USA).

2.2. Strain and culture conditions

In this study, the strain *Cordyceps militaris* was used for production of fibrinolytic enzyme. Stock cultures of the strain were maintained on potato dextrose agar slants and stored until use. The fermentation media is comprised of 2% sucrose and 5% soybean cake powder. Mycelia were inoculated into 50 mL of fermentation media in a 250 mL conical flask and incubated in a shaker for 5 days at 23 °C, 180 rpm. After fermentation, the broth was centrifuged at 9700 × *g* for 10 min, and the resultant supernatant was used as crude enzyme.

2.3. Fibrinolytic enzyme activity assay and protein estimation

Fibrinolytic enzyme activity was estimated according to the method of Astrup and Mullertz [22], with slight modifications. Fibrin plates were prepared by mixing 5 mL of fibrinogen (0.4%, (w/v) in 100 mM sodium barbital -hydrochloric acid buffer, pH 7.8,), 5 mL of agarose (0.5% (w/v)) and 1 mL of thrombin (200 U/mL). The mixture was poured into Petri dishes and kept at room temperature for 1 h for clot formation. 10 μL of enzyme sample was placed on the fibrin plate and incubated at 37 °C. After 6 h, the diameter of the lytic circle was calculated, which is directly proportional to enzyme activity. A calibrated standard curve was established using urokinase as standard. For standard curve calibration, urokinase (50000 U) was diluted with normal saline in the range 1–30 U/mL. (1, 5, 10, 15, 20, 25, 30). 10 μL of each concentration was analyzed using fibrin plate method.

Protein concentration of the samples was quantified by Lowry method [23], using bovine serum albumin as a standard. Purified protein was checked for glycoprotein analysis by Molish test, and carbohydrate content of the purified enzyme was estimated by anthrone colorimetric method using glucose as a standard [24].

2.4. Purification of fibrinolytic enzyme from culture supernatant

Crude enzyme was subjected to $(NH_4)_2SO_4$ precipitation (0–20%). After precipitation, the sample was centrifuged (9700 × *g*, 20 min, 4 °C) and the supernatant was taken for further purification. By using Sephadex G-25 (2.6 × 50 cm) column, the supernatant was buffer exchanged to 0.02 mol/L sodium phosphate buffer, (pH 7.4). Buffer exchanged sample was saturated with 30% $(NH_4)_2SO_4$ and loaded onto a Phenyl Sepharose HP column (2.6 × 30 cm) which was earlier equilibrated with the same buffer. Elution of bound proteins from the column was achieved by decreasing the concentration of $(NH_4)_2SO_4$ in linear fashion (30–0%). The fractions exhibiting enzyme activity were pooled, buffer exchanged using 0.02 mol/L sodium phosphate buffer (pH 6.5) and applied onto CM Sepharose FF (2.6 × 20 cm) column. Proteins were eluted with an increasing concentration of NaCl (0–0.8 M) in a linear fashion. Active fractions were pooled and further purified on a Superdex 75 column (1.6 × 60 cm) using 0.02 mol/L sodium phosphate buffer containing 0.3 mol/L NaCl, (pH 7.4). Active purified samples were lyophilized and used for further studies.

2.5. Determination of isoelectric point (pI) and molecular weight

Isoelectric point (pI) of the purified enzyme was determined by IEF using ampholytes (pH 3–9). In this study, a 7.5% polyacrylamide (3% cross linkage) gel was used for IEF. Pre-focusing of ampholytes was carried out at 60 V for 15 min. Purified enzyme (10 μL) was applied onto the pre-focussed gel. IEF of the sample was performed at 550 V, 8 mA and 10 °C. After electrophoresis, the protein bands were fixed using 15% TCA for 4 h. The protein bands were visualized by staining with Coomassie brilliant blue (CBB) R-250. The pI of the sample was calculated by comparing with the