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Production and biochemical characterization of an alkaline protease from *Aspergillus oryzae* CH93

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ABSTRACT

In this study, *Aspergillus oryzae* CH93 was isolated from soil sample and examined using molecular analysis. Following culture of *A. oryzae* CH93 under optimal enzyme production, a 47.5 kDa extracellular protease was purified using ammonium sulfate precipitation and Q-Sepharose chromatography. The optimal pH 8 and temperature of 50 °C obtained for the isolated protease. Sodium dodecyl sulfate (SDS), cetyltrimethyl ammonium bromide (CTAB), H_2O_2 decreased activity, while Triton X-100 and phenylmethanesulfonyl fluoride (PMSF) had no inhibitory effect on the enzyme activity; meanwhile, 2-mercaptoethanol and ethylenediaminetetraacetic acid (EDTA) declined the protease activity. Isoamyl alcohol and acetone (30%) enhanced activity whereas 2-propanol, isopropanol and dimethyl sulfoxide (DMSO) (30%) reduced protease activity. The enzyme exhibited a half-life of 100 min at its optimum temperature. Among five substrates of bovine serum albumin (BSA), *N*-acetyl-L-tyrosine ethyl ester monohydrate (ATEE), casein, azocasein and gelatin results showed that casein is the best substrate with V_{max} of 0.1411 ± 0.004 μg/min and K_m of 2.432 ± 0.266 μg/ml. In conclusion, the extracted protease from *A. oryzae* CH93 as a fungal source possessed biochemical features which could be useful in some application usages.

1. Introduction

Proteases are one of the essential enzymes in all forms of life, including prokaryotes, plants, fungi and animals. Nearly two third of commercial proteases are produced by yeasts, fungal and bacteria species [1]. Proteases have enormous applications in different industries such as laundry and detergents, food processing, leather, pharmaceuticals, textile, silk, brewing, photographic, bakery, dairy, bioremediation, biosynthesis, and biotransformation [2–4].

Aspergillus is a member of *Ascomycota* phylum and *Deuteromycetes* fungi. *Deuteromycetes* is a group without sexual generation; consequently, *Aspergillus* has asexual reproduction and germination. *Aspergillus* species are mainly aerobic and grow in all oxygen-rich environments, where they commonly live as molds on the surface of a substrate to easily access to high oxygen supply. Over 60 *Aspergillus* species are medically relevant pathogens [5] and some of them are useful in the production of citric acid and fermentation of soy sauce and chymosin [6–8]. *A. oryzae* genome was released by a consortium of Japanese biotechnology companies in late 2005. *Aspergillus oryzae* possesses eight chromosomes together comprise 37.1 million base pairs and 12.071 thousand predicted genes and its nucleotide sequences [9]. Moreover, *Aspergillus oryzae* is used to first break down starch into simpler sugars in rice, barley, sweet potatoes, and is also used for the production of rice vinegar [10]. In addition, many extracellular enzymes such as glycosidase, amylase and protease have been reported from *A. oryzae* by *Kurakake* et al. [11], *Zangirolamiand* et al. [12], and *Sivaramkrishnan* et al. [13], *Chancharoonpong* et al. [14,15]. *A. oryzae* is being utilized as an appropriate source for extraction of some nucleases such as S1 nuclease and ribonuclease in recombination technology [16]. Through alcoholic fermentation, *A. oryzae* can produce some metabolite such as lactic acid and acetic acid [10]. Furthermore a few sugars such trehalase and fructooligosaccharides have been extracted from conidia of *A. oryzae* [17,18].

In this study, a fungi strain was isolated from Charm-Shahr complex in Mashhad-Iran. After molecular analyses of the strain, an alkaline extracted protease was purified and biochemical features of the enzyme were studied.

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2. Materials and methods

Chemical reagents used in cultivation of microorganism were purchased from Titrachem (Tehran, Iran). Q-Sepharose was supplied by Pharmacia (GE Healthcare Life Sciences, Uppsala, Sweden). Bovine serum albumin (BSA) was purchased from sigma and casein from Merck. All other chemicals were of analytical grade.

2.1. Fungal isolation

Soil sample was taken from Charm-Shahr of Mashhad located in countryside of Mashhad-Iran. The sample was mixed with a small phosphate buffered saline (PBS) and microbial suspensions were inoculated onto a medium. The solid culture medium was composed of malt extract agar (3 g/dl), peptone (0.15 g/dl), casein (0.15 g/dl). The pH of the medium was adjusted to 6.0 [19].

After three-day incubation at 28 °C, fungal colonies with different shapes and colors appeared on the solid medium. Different colonies were isolated and grown up discretely on a freshly prepared solid medium and finally different fungal colonies transferred into a liquid medium and incubated in shaker incubator at 120 RPM and 28 °C. The composition of the culture medium is one of the most important factors influencing cell growth, physiology, and the formation of products [20]. The liquid medium was made up of malt extract (3 g/dl), peptone (1.0 g/dl), $MgSO_4$ (0.01 g/dl), $CaCl_2$ (5 mg/dl), KH_2PO_4 (0.28 g/dl), Na_2HPO_4 (0.07 g/dl) [19]. The growth of fungal strain and enzyme production was accomplished by this medium. To determine the concentration of fungus cells in this study, 0.5 McFarland standard was used corresponding $1–5 \times 10^6$ CFU/ml of fungal cells [21]. Strain possessing higher protease production was selected for subsequent studies.

2.2. Scanning electron microscope

To investigate the morphology of the strain, scanning electron microscopy was used. For this purpose, the strain was grown in liquid medium described in Section 2.1 under optimal conditions for 96 h. The cells were separated from medium by centrifugation and washed twice with phosphate buffered saline (PBS) and then freeze-dried by lyophilizator (ilShin TFD 5503, Seoul, South Korea). The cells were coated with thin layer of gold with a coater (E-1010, Hitachi). Scanning electronic microscopic of the strain were carried out by Nano laboratory (Tehran university, Iran) by using HITACHI S-4160 [14].

2.3. Identification of the strain

For PCR, universal primers called ITS1 and ITS4 were used as forward and reverse primer for 18 S rDNA gene amplification. The sequences of 5′-TCCGTAGGTGAACCTGCGG-3′ (T_m = 57 °C) and 5′-GCTGCGTTCTTCATCGATGC-3′ (T_m = 53 °C) were used as forward and reverse primer, respectively [22]. Amplification was carried out by Thermocycler (Bio-Rad, CA, USA) under following program: denaturation at 96 °C for 5 min followed by 35 cycles at 94 °C for 45s, 53 °C for 30s, and 72 °C for 90 s and 5 min-final extension at 72 °C. The PCR product was analyzed on 1% (w/v) agarose gel stained with 0.5 μg/ml ethidium bromide. The sequence of amplified gene was determined by Macogene Company (Seoul, South Korea).

2.4. Enzyme purification

In order to obtain enzyme for its characterization, the isolated fungal strain was grown in optimal conditions using liquid medium. Culture medium was separated from fungal cells by centrifuge at 12,500 g. By adding, 85% ammonium sulfate to the culture medium, protein content was precipitated at 4 °C for an overnight followed by dissolving in minimal volume of Tris-HCl 20 mM, pH 8. The resulting solution was dialyzed against the same buffer with several buffer changes. Subsequently, the sample was subjected to Q-Sepharose chromatography formerly equilibrated with Tris-HCl 20 mM, pH 8. By use of gradient salt of NaCl ranging from 0.0 to 1.0 M in the equilibrium buffer, the enzyme of interest was fractionized.

2.5. Effect some agents on enzyme production

The optimized production and various-affecting parameters on the enzymatic synthesis should always be investigated. In order to examine effect of pH on the growth, four different Erlenmeyer flasks containing liquid medium were adjusted at pH of 4, 5, 6 and 7. In each medium, amount of 0.5 McFarland of the strain was added and the amount of enzyme production through enzyme activity growth was measured at 120 RPM and 28 °C after 120 h incubation. To evaluate the influence of temperature, in three different liquid medium amount of 0.5 McFarland of the strain was added and the inoculated medium was incubated at 17 °C, 28 °C and 37 °C.

To determine effects of different carbon and nitrogen sources on protease production, in six different liquid mediums, amount 1% (w/v) of glucose, maltose, fructose, yeast extract, urea and ammonium sulfate was added [20]. Following addition 0.5 McFarland of the strain and incubation under optimal standard conditions, the enzyme activity was measured over five days.

2.6. Enzyme activity

The enzyme activity was conducted based on Moradian et al. with slight modification [23]. A volume of 100 μl enzyme along with 100 μl of 20 mM Tris-HCl buffer pH 8 as buffer assay was mixed with 400 μl of casein 0.6% dissolved in the same buffer. The mixture was incubated at 50 °C for 30 min. By adding 750 μl trichloroacetic acid (TCA) 10% (w/v) to the mixture, the protein content was precipitated and kept at 4 °C for 1 h. By use of centrifugation at 12,500*g* for 10 min, the precipitate was removed and the absorbance of the supernatant was measured at OD_{280} nm. One unit (U) of protease activity is amount of enzyme that hydrolyzes casein to release 1 μg tyrosine per 1 min under assay condition [14].

2.7. SDS-PAGE and zymography

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular weight and the purity of protease. This method was carried out using (5%, w/v) stacking gel and (12%, w/v) separating gel. Coomassie brilliant blue R-250 was prepared with water, methanol, acetic acid (5:4:1, v/v/v) to stain the gel. By comparing the mobility of standard proteins to the purified protease, the molecular weight of interest was estimated.

Native gel electrophoresis was used to show protease activity on the gel. Gelatin 0.1% (w/v) was copolymerized with separating gel for zymography analysis. Samples were mixed without beta-mercaptoethanol gel loading buffer and were loaded to the gel without heating. Electrophoresis was carried out by 120 V at 4 °C and the gel was soaked in renaturing buffer containing 2.5% (v/v) Triton X-100 for 60 min and then transferred to the developing buffer composed of 50 mM Tris-HCl, 0.2 M NaCl and 5 mM $CaCl_2$ at pH 8, and incubated for an overnight at 37 °C. Eventually, the gel was stained with 0.2% (w/v) Coomassie brilliant blue R-250 prepared in water, methanol, acetic acid (5:4:1, v/v/v) for 30 min and finally was decolorized with the same mixture but without Coomassie brilliant blue R-250. A clear band on the gel represents the presence of protease activity.