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ARTICLE

Purification and characterization of two thermostable protease fractions from *Bacillus megaterium*

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Abstract Protease enzyme from *Bacillus megaterium* was successively purified by ammonium sulfate precipitation, ion exchange chromatography on DEAE-cellulose and gel filtration chromatography on Sephadex G-200. The purification steps of protease resulted in the production of two protease fractions namely protease P1 and P2 with specific activities of 561.27 and 317.23 U mg^{-1} of protein, respectively. The molecular weights of *B. megaterium* P1 and P2 were 28 and 25 KDa, respectively. The purified fractions P1 and P2 were rich in aspartic acid and serine. Relatively higher amounts of alanine, leucine, glycine, valine, thereonine valine and glutamic acid were also present. The maximum protease activities for both enzyme fractions were attained at 50 °C, pH 7.5, 1% of gelatine concentration and 0.5 enzyme concentrations. P1 and P2 fractions were more stable over pH 7.0–8.5 and able to prolong their thermal stability up to 80 °C. The effect of different inhibitors on the protease activity of both enzyme fractions was also studied. The enzyme was found to be serine active as it had been affected by lower concentrations of phenylmethylsulfonyl fluoride (PMSF). Complete dehairing of the enzyme-treated skin was achieved in 12 h, at room temperature.
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1. Introduction

Proteases are one of the most important industrial enzymes produced by a wide range of microorganisms such as bacteria, yeasts, molds and are also found in plants and in various animal tissues [35]. Bacterial proteases are mostly extracellular, easily produced in larger amounts, thermostable, and active at a wider pH range. Protease from *Bacillus* has been purified and characterized, and significant activity, stability, broad substrate specificity, short period of fermentation, simple downstream purification and low cost have been demonstrated [15,24]. These properties make the bacterial proteases most suitable for a wider industrial application. Proteases represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes [3,12].

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Proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications [3,7]. *Bacillus* produces a wide variety of extra-cellular enzymes, including proteases. Several *Bacillus* species were involved in protease production i.e., *Bacillus cereus*, *Bacillus sterothermophilus*, *Bacillus mojavensis*, *Bacillus megaterium* and *Bacillus subtilis* [32,7,6,11]. The largest application of proteases is in laundry detergents, where they help in removing protein based stains from clothing [5,6]. For an enzyme to be used as a detergent additive it should be stable and active in the presence of typical detergent ingredients, such as surfactants, builders, bleaching agents, bleach activators, fillers, fabric softeners and other various formulation aids. In textile industry, proteases may also be used to remove the stiff and dull gum layer of sericine from the raw silk fiber to achieve improved luster and softness. Protease treatments can modify the surface of wool and silk fibers to provide new and unique finishes. Proteases have been used in the hide dehairing process, where dehairing is carried out at pH 8–10 [17]. Proteases are also useful and important components in biopharmaceutical products such as contact lens enzyme cleaners and enzymatic debriders [4]. The proteolytic enzymes also offer a gentle and selective debridement, supporting the natural healing process in the successful local management of skin ulcerations by the efficient removal of the necrotic material [31]. In this paper we aimed to purify and characterize neutral protease from local bacterial isolate and evaluate as dehairing enzyme.

2. Materials and methods

2.1. Isolation, screening and identification of protease-producing bacteria

Out of 30 bacteria isolated from the local soil, *B. megaterium* NRC had a higher enzyme production than the others used in this study. The potent protease producing bacterium has been identified based on morphological and physiological characteristics determined using Biolog GP2 MicroPlate™, as well as characteristic reaction pattern called a ‘‘metabolic fingerprint’’. The metabolic fingerprint patterns were compared and identified using the Micro Log™ database software [10]. The isolate was placed in the culture collection of the Microbial Biotechnology Department, National Research Center, Dokki, Cairo, Egypt. The isolates were cultivated on a gelatin liquid medium containing (g/L): gelatin, 10; yeast extract, 1.0; $KHPO_4$, 0.5; NH_4HPO_4, 0.5; $CaCl_2$, 0.1; $MgSO_4.5H_2O$, 0.05 at pH 7.0. A loopfull from each bacterial isolate was used to inoculate100-ml Erlenmeyer flasks each containing 20 ml liquid medium. Flasks were incubated at 50 °C for 72 h. The culture broth was centrifuged for 15 min at 5000 rpm and the supernatant was used to determine enzyme activity.

2.2. Protease enzyme production

One-hundred mL of gelatin liquid medium in a 1 L shake flask was inoculated with 1 mL of *B. megaterium* culture (24 h-old), and incubated at 50 °C with a shaking incubator (150 rpm) for 24 h. The cell-free enzyme supernatant was obtained by centrifugation at 5000 rpm for 30 min at 4 °C. The supernatant was used as the enzyme source.

2.3. Enzyme assay

Protease activity was measured by the method of Takami et al. [33] using gelatin as substrate. A 0.5 ml of enzyme was mixed with 0.5 ml of 1% gelatin solution in 0.2 M phosphate buffer pH 7.5, and the reaction mixture was incubated further for 60 min at 50 °C. The reaction was stopped by an addition of 0.5 ml of 10% TCA and the mixture was allowed to stand for 10 min at room temperature. Soluble peptides were separated by centrifugation for 10 min, using centrifuge (Sigma-Laborzentrifugen, 2K 215). The absorbance of the TCA-soluble peptides in the supernatant was measured at 280 nm. A control assay, without the enzyme in the reaction mixture, was done and used as the blank in all spectrophotometric measurements. One unit of enzyme activity was defined as the amount of enzyme, under given assay conditions that gave rise to an increase of 1 unit of absorbance at 280 per minute of digestion. The number of units of activity per mg protein was taken as the specific activity of the enzyme.

2.4. Protein determination

Protein content was determined by the dye binding assay according to Bradford [8] using bovine serum albumin as a standard protein. Absorbance at 280 nm was used for monitoring protein in column elutes [36].

2.5. Purification of protease enzyme

2.5.1. Step 1: precipitation by ammonium sulfate

Cell-free supernatant was collected by centrifugation. Ammonium sulfate was slowly added to the supernatant to 60% saturation and the mixture was incubated overnight at 4 °C. The precipitated protein was obtained by centrifugation for 20 min at 5000 rpm at 4 °C. The obtained pellet was re-suspended in a 10 mL of ice-cold 0.2 M phosphate buffer pH, 7.5 and then subjected to a process of dialysis against the same buffer to get rid of the excess of ammonium sulfate and then made up the final volume up to 30 mL. The protease activity and protein concentration were measured and the specific activity was calculated.

2.5.2. Step 2: chromatography on DEAE-cellulose

The dialyzed enzyme was applied to DEAE-cellulose column (50×2.0 cm, i.d.) previously equilibrated with phosphate buffer 0.2 M (pH 7.5). The adsorbed protein was eluted with a stepwise NaCl gradient ranging from 0.0 to 0.7 M prepared in 0.2 M phosphate buffer (pH 7.5) at a flow rate of 0.8 mL min^{-1}. Five ml fractions were collected wherein eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity. The peak with the highest protease activity was dialyzed against 0.2 M phosphate buffer (pH 7.5) and concentrated by dialysis against solid sucrose. The protease activity and protein concentration were measured and the specific activity was calculated.

2.5.3. Step 3: chromatography on Sephadex G-200

Active fractions, from DEAE-cellulose step, was desalted prior to loading into Sephadex G-200 column (90×2.4 cm, i.d.)

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