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Cloning and characterization of a novel intracellular serine protease (IspK) from *Bacillus megaterium* with a potential additive for detergents

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ABSTRACT

A new intracellular serine protease gene of *Bacillus megaterium*, *isp*K, encoding a protein composed of 332 amino acid residues with a predicted pI of 4.7 was cloned into *Escherichia coli*. The deduced amino acid sequence of IspK showed 49–56% similarity with the other microbial intracellular serine proteases described in the literature. The enzyme was effectively purified by one-step chromatography after heat-treatment, and showed a homogeneous band corresponding to 35 kDa by SDS-PAGE analysis. Amino acid analysis showed that 16 amino acids of the N-terminus of IspK were removed by post-translational protease activity. The optimum pH and temperature of IspK were 6.0–7.0 and 50 °C, respectively. In the presence of 2 mM of Ca^{2+} ion, the optimum temperature was increased to 65 °C and thermostability ($t_{1/2}$) increased 32.9-fold from 3.3 min to 108.5 min at 60 °C. The enzyme was activated by Ca^{2+} and Mg^{2+}, almost completely inhibited by phenylmethanesulfonyl fluoride (PMSF) and EDTA, but tolerant to nonionic surfactants, such as, Triton X-100 or Tween 80. IspK efficiently hydrolyzed natural proteins, such as, casein and hemoglobin, and improved blood stain removal. These results suggest IspK can be used as a useful additive for detergent formulations and for deproteinizations.

1. Introduction

Bacterial proteases play important roles in cellular and metabolic processes, and are used for leather processing, brewing, and in foods, cosmetic, and pharmaceuticals [1], and in particular, alkaline proteases are used as detergent additives [2]. Recently, the diagnostic, therapeutic, and antimicrobial properties have attracted the attention of researchers [3,4].

Bacillus species have been extensively used to produce various industrial enzymes, including proteases [5]. *Bacillus subtilis* is the most widely used, and an eight protease-deficient mutant WB800 strain has been developed [6], and used to produce several heterologous proteins [7–10]. *Bacillus megaterium* has attracted research attention for more than 50 years because it produces useful enzymes, has high secretion capacities for recombinant proteins due to the lack of extracellular alkaline proteases unlike *B. subtilis*, and high plasmid stability [11–13].

Intracellular proteases of *Bacillus* species are relatively poorly characterized as compared with its extracellular proteases. *B. megaterium* produces two types of extracellular proteases [14], and a major protease was cloned and characterized from ATCC 14581 and DSM319 strains [15,16]. In fact, at least three types of intracellular proteases have been identified in *B. megaterium* [17]. The role played by a major intracellular protease in growth and sporulation, and its activation and regulation have been explored [18–20]. However, no gene of the three types of enzymes identified in *B. megaterium* has yet been cloned or characterized.

Relatively few intracellular protease genes of *Bacillus* sp. have been cloned and analyzed. Those that have been investigated are; *B. subtilis* [21], *B. polymyxa* [22], *Bacillus* sp. NKS-21 [23], and *Bacillus* sp. WRD-2 [24], and of these, three enzymes from *B. subtilis*, *Bacillus* sp. NKS-21, and *Bacillus* sp. WRD-2 have been characterized [21,23,24].

Here, we report the cloning of a new intracellular serine protease (*isp*) gene, *isp*K, from *B. megaterium*, the production of protease IspK using an *E. coli* transformant, the purification of the protease using simple and efficient methods, and unique properties of ispK.

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2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

Bacillus megaterium ATCC 14945 (KCTC 3071) was used as protease gene source and *Escherichia coli* DH5α as a cloning host, and these two strains were grown aerobically in Luria-Bertani (LB) broth media at 37 °C, respectively. If necessary, ampicillin (50 μg/ml) was added to medium. Plasmid pUC19 was used as a cloning vector.

2.2. Cloning of the ispK gene from B. megaterium

B. megaterium chromosomal DNA was prepared as described previously [25]. Fragments of 3 ~ 5 kb in length from chromosomal DNA digests obtained using *Eco*RI were eluted from 0.7% (w/v) agarose gel, ligated to *Eco*RI-digested pUC19, and transformed into *E. coli* DH5 (Yeastern Biotech. Co., Taipei, Taiwan). *E. coli* transformants were primarily grown on LBS agar plates (LB medium containing 1.0% skim milk) supplemented with ampicillin for about two days, screened by tooth-picking for all ~1200 transformants, and grown for about two days. A transformant with a clear zone around its margin was selected for further study.

2.3. DNA sequencing and analysis

The DNA sequence of the inserted fragment was determined at Solgent (Daejeon, Korea) and analyzed using DNAMAN software (Lynnon Biosoft, version 4.11, Quebec, Canada). Homologies of amino acid sequences were searched using BLAST at NCBI (http://www.ncbi.nlm. nih.gov), and multiple sequence alignments were conducted using COBALT at NCBI and DNAMAN. The signal peptide was predicted using SignalP 4.1 in CBS (http://www.cbs.dtu.dk/services/SignalP/) [26]. The molecular mass and pI of the encoded protein were determined at the ExPASy site (http://www.expasy.ch/tools/protparam.html).

2.4. Determination of protease activity

The protease activity was measured using a modification of a previously described method [27]. Briefly, a reaction mixture (0.2 ml) containing 1.25% azocasein (Sigma Chemical Co.) and 2 mM $CaCl_2$ in 50 mM Tris-HCl (pH 7.5) was incubated at 60 °C for 30 min, and this reaction was used as standard condition in this study. The reaction was then stopped by adding 0.6 ml of 10% trichloroacetic acid and undigested azocasein was pelleted by centrifugation for 10 min at 15,000×*g*. NaOH (0.7 ml, 1.0 N) was then added to 0.7 ml of the supernatant and absorbance was measured at 440 nm. One unit of the enzyme was defined as the amount that produced an absorbance change of 1.0 in 30 min at 60 °C.

2.5. Production of protease in the transformant using different carbon and nitrogen sources

The positive transformant, *E. coli* (pHK65), was grown on LB medium supplemented with 2% of different carbon sources: D-mannitol, D-xylose, D-sorbitol, D-fructose, D-glucose, sucrose, maltose, lactose, or soluble starch. Separately, the nitrogen source (tryptone) in LB medium was replace with different nitrogen sources; peptone, casein, yeast extract, malt extract, beef extract, casitone, tryptose, soy bean, $NaNO_3$, $(NH_4)_2SO_4$, or NH_4Cl. Cells were harvested after 21 h of culture at 37 °C with shaking at 220 rpm, disrupted twice with a sonicator (Model VCX600, Sonics & Materials, USA) for 1 min in an ice bath, and crude extracts were then obtained by centrifuging at 15,000×*g* for 15 min.

2.6. Purification of cloned IspK

The transformant was aerobically cultured in 200 ml of LB broth in 1-l Erlenmeyer flask containing ampicillin at 37 °C for 21 h and harvested at the stationary phase by centrifugation for 10 min at 6000×*g*. Cells were then washed with 50 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 2 mM $CaCl_2$, resuspended in 6 ml of the same buffer, and disrupted as described above. The crude supernatant was heat treated at 60 °C for 45 min and heat-labile proteins were removed by centrifugation for 15 min at 15,000 *g*. The heat-treated sample was applied to a High-Q cartridge (5 ml bed volume, Bio-Rad, USA). Proteins were eluted using a linear gradient of high salt buffer (20 mM Tris-HCl, pH 8.0, and 1 M NaCl) at a flow rate of 1 ml/min. Collected fractions (1.0 ml each) were checked for activity, active fractions were pooled, dialyzed against 50 mM Tris-HCl buffer (pH 7.5), and stored at −20 °C in aliquots until required. Protein concentrations were determined using the Bradford method [28], and molecular mass of IspK was determined by SDS-PAGE using 11.5% gel [29].

2.7. Effects of pH and temperature on the enzyme activity

The optimum pH of IspK was determined using 50 mM sodium acetate buffer (pH 3.5–5.0), sodium phosphate buffer (pH 5.0–7.0) and Tris-HCl buffer (pH 7.0–9.0). pH stability was investigated by preincubating the enzyme at each pH for 30 min at 40 °C, and then measuring the residual activities. Optimum temperature was analyzed over the 30–80 °C range and thermostability was investigated by preincubating the enzyme in the absence of substrate for indicated times at 50 and 60 °C, and then measuring the residual activities. To investigate the effects of Ca^{2+} on optimum temperature and thermostability, 2 mM of Ca^{2+} ion was added to the enzyme solution which was extensively dialyzed against Ca^{2+}-free 50 mM Tris-HCl buffer.

2.8. Effects of metal ions, surfactants, and other chemicals

The influences of various cations and other chemicals on the enzyme activity of IspK were analyzed at a concentration of 5.0 mM for Na^+ (as well as 1.0 and 3.0 M), K^+, Mg^{2+}, Mn^{2+}, Ca^{2+} (as well as 1.0 and 2.0 mM), Cu^{2+}, Co^{2+}, Ba^{2+}, Fe^{2+}, Zn^{2+}, phenylmethanesulfonyl fluoride (PMSF) (Sigma Chemical Co.), and ethylenediaminetetraacetic acid (EDTA), and at concentrations of 0.5, 1.0, and 2.0% for SDS, Triton X-100, and Tween 80. To investigate tolerances, the enzyme was preincubated with NaCl (1.0 M and 3.0 M), Triton X-100, and Tween 80 (0.5, 1.0, and 2.0%) for 24 h at 4 °C, residual activities was measured under the standard condition.

2.9. Substrate specificity of IspK

Protease activities for natural proteins, such as, casein, hemoglobin, bovine serum albumin (BSA), gelatin, and collagen, were assayed as described by Anson [30] with slight modification using Folin-Ciocalteau reagent [31]. The reaction mixture, which contained 2% of substrate in Tris-HCl buffer (pH 7.5) and 0.1 ml of enzyme, was incubated at 60 °C for 30 min. The reaction was terminated by adding an equal volume of 10% (w/v) of trichloroacetic acid and centrifuged at 15,000×*g* for 5 min. The supernatant (1 ml) was added to 2.5 ml of 0.5 M Na_2CO_3 solution, and 0.5 ml of Folin-Ciocalteau reagent was then added and mixed thoroughly. Color development after 30 min of incubation at room temperature was measured at 660 nm.

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