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Purification and characterization of a novel extracellular alkaline protease from *Cellulomonas bogoriensis*

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ABSTRACT

An extracellular alkaline protease produced by the alkali-tolerant *Cellulomonas bogoriensis* was purified by a combination of ammonium sulfate precipitation and cation exchange chromatography. The purity of the protease was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and its molecular weight was confirmed to be 18.3 kDa. The enzyme showed optimum activity at 60 °C and pH 11. The stability of the protease was maintained at a wide temperature range of 4–60 °C and pH range of 3–12. Irreversible inhibition of the enzyme activity by phenylmethylsulfonyl fluoride and tosyl-L-phenylalanine chloromethyl ketone demonstrated that the purified enzyme is a chymotrypsin of the serine protease family. The K_m and V_{max} of the protease activity on casein were 19.2 mg/mL and 25000 μg/min/mg, respectively. The broad substrate specificity and remarkable stability in the presence of organic solvents, salt, and commercial detergents, as well as its excellent stain removal and dehairing capability, make the purified alkaline protease a promising candidate for industrial applications.

1. Introduction

Proteases dominate the worldwide industrial enzyme market and account for nearly 60% of the total worldwide enzyme sales. Among them, alkaline proteases occupy an important position because of their broad applications in detergents, food, photography, leather, cosmetics, pharmaceuticals, and waste treatment [1–4].

Microorganisms produce a large number of extracellular proteases and are considered the main source of commercial alkaline proteases. Several studies have recently characterized alkaline proteases from different microorganisms, including bacteria, actinomycetes, and fungi. Bacteria are the most dominant group of alkaline protease producers. *Bacillus* sp., *Pseudoalteromonas* sp., *Stenotrophomonas* sp. and *Caldicoprobacter* sp. have been exploited for alkaline protease production [5–8]. *Streptomyces* species are also predominant producers; some novel alkaline proteases from *Streptomyces* were recently reported [9,10]. Certain fungi exhibit excellent alkaline protease activity, including *Aspergillus* sp. and *Alternaria* sp., although most of the fungus-derived enzymes are acidic [11,12].

Despite the long list of protease-producing microorganisms, only a few of them are considered feasible for wide-scale commercial applications because of their weak stability at wide temperature and pH ranges or their poor compatibility with surfactants, salts, and organic solutions, among others. Therefore, alkaline proteases with superior characteristics are still being sought [13].

In this study, we reported the purification and characterization of an alkaline protease produced by the alkali-tolerant *Cellulomonas bogoriensis*. This work provides information on the potential use of the purified enzyme in further research and its possible industrial applications.

2. Materials and methods

2.1. Chemicals

Phenylmethylsulfonyl fluoride (PMSF), 4-bromophenacyl bromide (4-BPB), tosyl-L-lysine chloromethyl ketone (TLCK), tosyl-L-phenylalanine chloromethyl ketone (TPCK), casein, gelatin, hemoglobin, collagen, and bovine serum albumin (BSA) were purchased from the Sigma–Aldrich Chemical Company. Keratin was purchased from the Tokyo Chemical Industry. Unless otherwise stated, all chemicals used were of analytical grade.

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2.2. Microorganism and medium

The *C. bogoriensis* used in this study was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ). The strain was isolated from the sediment of the littoral zone of Lake Bogoria, Kenya. The production medium contained 1.5% sucrose, 0.1% KH_2PO_4, 2.67% NaCl, 0.75% beef extract, and 0.75% yeast extract; the pH of the medium was adjusted to 10.5.

2.3. Purification of protease

A 1.6 L culture of *C. bogoriensis* was fermented at 30 °C for 5 d, and the supernatant was collected by centrifugation at 10,000 rpm for 20 min. Fractional ammonium sulfate saturation at 30% and 80% was performed to remove some unwanted proteins. The precipitate at 80% saturation was dissolved in a Na_2HPO_4–NaH_2PO_4 buffer (pH 7.9) and dialyzed over night against the same buffer. The sample was further applied to a CM Sepharose Fast Flow (FF) column (1 cm × 10 cm). The column was eluted with the series of salt concentrations from 0.02 M to 0.5 M NaCl at a flow rate of 1 mL/min. All active fractions were collected and kept at −80 °C.

2.4. Enzymatic assay

The reaction mixture with a total volume of 2 mL was composed of 1 mL enzyme solution and 1 mL of 2% casein dissolved in 20 mM potassium phosphate buffer (pH 8.0), except otherwise indicated. The mixtures were incubated at 40 °C for 10 min before 2 mL of trichloroacetic acid was added to terminate the reaction. The reaction mixtures were allowed to stand for 20 min at 40 °C and then centrifuged at 10,000*g* for 15 min to remove the precipitate. The absorbance of the supernatant was measured at 280 nm. A unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μg of tyrosine residue per minute [14].

2.5. Protein measurement

The purity of the fractions was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each gel was stained with Coomassie brilliant blue R-250 to visualize the protein bands. The molecular weight of the purified enzyme was determined by comparison with a standard low-molecular-weight marker in 12% SDS-PAGE gels. The protein content was quantified by Protein Assay Kits (KeyGEN, China) with BSA as the standard.

2.6. Effects of pH and temperature on the protease activity of the purified enzyme

The activity of the purified protease was assayed in 0.05 M buffers of various pH levels (phosphate buffer, pH 6–8; Tris–HCl buffer, pH 8–9; Gly-NaOH buffer, pH 9–13) to determine the optimum pH. The optimum temperature for enzyme activity was determined by performing the enzyme reaction at temperatures from 10 °C to 80 °C. To evaluate the pH stability and thermostability of the enzyme, the purified protease was kept either at a pH range from 3 to 13 at 4 °C for 24 h or at temperatures from 4 °C to 80 °C for 2 h, then residual activity was assayed under standard conditions as previously described.

2.7. Effects of inhibitors, metal ions, chemicals, and organic solvents on the protease activity of the purified enzyme

The inhibitors PMSF, 4-BPB, TLCK, TPCK, and ethylenediaminetetraacetic acid (EDTA) were tested to determine the type of protease. Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Na^{+}, Fe^{3+}, Mg^{2+}, Zn^{2+}, K^{+}, Fe^{2+}, Cu^{2+}, and Ca^{2+} were added to the reaction to evaluate their effects on the enzyme activity. Each inhibitor or metal ion was added to a final concentration of 1 and 10 mM. To determine the effect of chemicals on the activity of the purified protease, surfactants (Triton X-100, Tween-80 and SDS) and urea were added to the enzyme reaction with concentrations of 1% (v/v) and 10% (v/v). The organic solvents methanol, ethanol, acetonitrile, isopropanol, glycerin, and acetone were also assayed with concentrations of 1% (v/v) and 10% (v/v) to determine their effects. The enzyme activity of the controls ample without any inhibitor, metal ion, chemical, or organic solvent was taken as 100%.

2.8. Effect of salt on the activity of the purified enzyme

To detect the halostability, the purified enzyme was incubated in a final concentration of 1 M–5 M NaCl for 1 d–15 d at 4 °C. The relative activity of the residue was tested under the optimal reaction conditions in 24 h intervals. The enzyme solution without any added salt was used as the control with 100% activity.

2.9. Activity of the purified enzyme on various substrates

The substrate specificity of the purified protease was assayed by different substrates, namely, casein, gelatin, hemoglobin, collagen, BSA, and keratin dissolved in 50 mM glycine–NaOH buffer (pH 10.0) to a concentration of 20 mg/mL.

2.10. Determination of kinetic constants

The kinetic assays were performed under the optimal temperature and pH conditions with casein (0.5–20 mg/mL) as the substrate. K_m and V_{max} were calculated according to the Lineweaver–Burk equation.

2.11. Detergent compatibility assay

The compatibility of protease with commercial detergents in the local market, such as DIAO® and Supra® (Nice Group Co., Ltd., China), WhiteCat® (Shanghai Hutchison WhiteCat Co., Ltd., China), Liby® (Guangzhou Liby Enterprise Group Co., Ltd., China), and Luowa® (Beijing Luowa Chemical Co., Ltd., China), was tested. The detergent solutions with a 7 mg/mL concentration were pre-heated at 100 °C for 1 h to destroy the endogenous protease activity. Enzyme activity in the presence of the detergent was assayed at

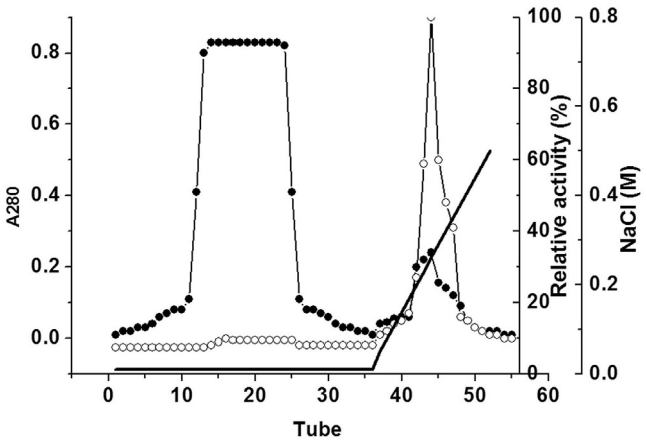

Fig. 1. Elution profile of alkaline protease by CM Sepharose FF chromatography. ● A280, ○ relative activity of the enzyme, black line is the concentration of the NaCl.