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Effect of pH and temperature on stability and kinetics of novel extracellular serine alkaline protease (70 kDa)

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ABSTRACT

A novel extracellular serine protease (70 kDa by SDS-PAGE) was purified and characterized. This enzyme retained more than 93% of its initial activity after preincubation for 30 min at 37 °C in the presence of 25% (v/v) tested organic solvents and showed feather degradation activity. The purified enzyme was deactivated at various combinations of pH and temperature to examine the interactive effect of them on enzyme activity. The deactivation process was modeled as first-order kinetics and the deactivation rate constant (k_d) was found to be minimum at pH 9 and 37 °C. The kinetic analysis of enzyme over a range of pH values indicated two p*K* values at 6.21 and at 10.92. The lower p*K* value was likely due to the catalytic histidine in the free enzyme and higher p*K* value likely reflected deprotonation of the proline moiety of the substrate but ionization of the active site serine is another possibility. Inhibition kinetic showed that enzyme is serine protease because enzyme was competitively inhibited by antipain and aprotinin as these compounds are known to be competitive inhibitors of serine protease. The organic solvent, thermal and pH tolerances of enzyme suggested that it may have potential for use as a biocatalyst in industry.

1. Introduction

Enzymes play an important role in modern biotechnology because of their specificity, selectivity, efficiency and sustainability. One of the most frequently industrially used groups of biocatalysts is protease. Proteases are exploited in various processes, such as leather, food, pharmaceutical, textile, organic chemical synthesis, waste water treatment and other industries [1]. The synthesis of specific products by enzymes is a fundamental aspect of modern biotechnology. This biocatalytic approach has several advantages over traditional chemical engineering, such as higher product purity, fewer waste products, lower energy consumption and more selective reactions due to the high regio- and stereoselectivity of enzymes [2].

The most important features of an enzyme are stability and better catalytic performance [3]. One of the major drawbacks of the proteases is their instability in alkaline pH and higher temperature [4]. It is desirable to overcome this problem by searching for new proteases with novel properties from different sources. The stability of an enzyme is judged by the residual activity while catalytic performance is defined as the ability of an enzyme to catalyze a process. Stability and activity are affected by certain parameters, which may be either physical (temperature and pH) or chemical (the presence of inhibitors or activators etc.) [3,5,6]. For effective use in industries, proteases needs to be stable and active at high temperature and pH [7]. These conditions are detrimental to most enzymes and therefore there is a growing demand for enzymes with an improved stability [3].

Enzyme deactivation plays a significant role in biotechnological processes. Enzymes may be deactivated in many ways and it represents a major constraint in many biotechnological processes [8,9]. Deactivation studies help to characterize enzyme stability and control over the deactivation process. Deactivation studies help investigate the relationship between the structure and function of a particular enzyme which will provide a valuable physical insight into the structure and function of enzymes [6].

As per our knowledge, the deactivation kinetics of protease has been poorly studied. Therefore, in the present paper, the influence of different parameters on protease stability and activity is investigated. The initial part of this communication deals with purification and characterization of extracellular protease. The influence of different parameters on protease stability and activity is studied in the second part. The results consequently provide a better understanding on stabilization and catalytic properties of protease.

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2. Materials and methods

2.1. Chemicals

Bradford Reagent (Sigma, USA), bovine serum albumin (BSA) (Himedia, India), dialysis sacks (Sigma, USA), trichloroacetic acid (Merck, India) and casein for protease assay (Himedia, India) was used in this study. All other chemicals used were of analytical grade and commercially available in India.

2.2. Microorganism and seed culture

The protease producing *Bacillus licheniformis* NCIM-2042 was procured from NCL, Pune, India. The microorganism was grown on nutrient agar slants at 37 °C at pH 7.4. It was maintained by subculturing on nutrient agar slants kept at pH 7.4. For production experiments, the culture was revived by adding a loop full of pure culture into 50 ml of sterile nutrient broth (pH 7.4).

2.3. Protease production

A 2% fresh culture ($OD_{550} \approx 0.2$) was inoculated in 50 ml complex media of 250 ml Erlenmeyer flask, containing optimized media (g/l); starch, 30.8; soybean meal, 81.6; K_2HPO_4, 3; KH_2PO_4, 1; $MgSO_4$, 0.5 and NaCl, 5.3. The culture was centrifuged at 10,000 × *g* for 10 min at 4 °C. The cell pellet was discarded and the supernatant was used for assay of protease activity.

2.4. Enzyme assay and determination of protein concentration

Protease activity was determined by a modified method of Folin and Ciocalteu [10]. Protein concentration was determined by the method of Bradford using bovine serum albumin (BSA) as the standard [11]. All experiments were done in triplicate.

2.5. Purification and characterization of purified enzyme

The source of enzyme in the present investigation is crude alkaline protease from *Bacillus* sp. Purification of enzyme sample was done in the laboratory following the protocol describe below. The fermented broth was centrifuged at 10,000 rpm for 30 min at 4 °C. Solid ammonium sulphate was added to the supernatant for 30% saturation and centrifuged at 4 °C for 30 min. Again solid ammonium sulphate was added to the supernatant for 60% saturation and centrifuged at 4 °C for 30 min. The precipitated sample was desalted by dialysis through semi permeable membrane (molecular weight cutoff 12 kDa, Sigma) against 50 mM phosphate buffer (pH-9) for overnight.

After dialysis with Phosphate buffer (pH 9), the dialysate was applied to Sephadex G-100 column (3 cm × 100 cm) and equilibrated with 25 mM Tris–HCl pH 8.0 containing 0.05% (v/v) Triton X-100. Enzyme fractions of 5 ml were collected at a flow rate of 25 ml/h with the same buffer. Protein content and protease activity were measured. Fractions showing protease activities were pooled.

The active fractions were applied to a CM-Sepharose column (3 cm × 30 cm) equilibrated with 25 mM Tris–HCl buffer pH 8.0. After being washed with the same buffer, bound proteins were eluted with a linear gradient of sodium chloride in the range of 0–0.5 M in the equilibrating buffer. Fractions (5 ml each) were collected at a flow rate of 120 ml/h. The fractions with high protease activity were pooled and then concentrated by lyophilization and purity was checked by SDS-PAGE and HPLC analysis. All the purification steps were conducted at temperatures not exceeding 4 °C.

2.5.1. Molecular weight determination by SDS-PAGE

SDS-PAGE was performed to estimate the molecular weight of the purified enzyme according to method of Laemmli [12]. Molecular weight was estimated by comparing the relative mobility of proteins of different molecular size using a standard molecular weight marker (Fermentas, USA).

2.5.2. HPLC analysis of purified enzyme

The purified sample was qualitatively analyzed using HPLC. For the analysis of purity of sample, HPLC system (Waters™ 600) equipped with UV/visible detector was used. Chromatographic separation of protease was performed on a C_{18} hypersil column (4.6 mm × 250 mm; 5 μm particle size; Waters, USA). Mobile phase used was acetonitrile–water (70:30 v/v), at a flow rate of 1 ml/min. Temperature of the column oven was maintained at 30 °C. The sample (20 μl) was injected and analyzed at 280 nm using UV–visible detector.

2.5.3. Effect of protease inhibitor on protease activity

Preincubation of enzyme solutions with PMSF (2.5 and 5 mM), EDTA (2.5 and 5 mM) and E-64 (0.1 and 0.2%, w/v) were carried out at room temperature for 30 min. The remaining activity was measured at optimum assay condition. Activity of the enzyme solution without inhibitor was considered as control (100%).

2.5.4. Effect of metal ions on protease activity

The effect of metal ions (2.0, 5.0 and 10 mM) on protease activity were investigated using Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}. The enzyme solutions with metal ions were incubated for 30 min at room temperature. The remaining activity was measured at optimum assay condition. Activity of the enzyme solution without metal ion was considered as control (100%).

2.5.5. Organic solvent stability of purified protease

Stability of the enzyme in organic solvents was studied by incubating the enzyme solution (3 ml) with various organic solvents (1 ml) namely DMSO, methanol, ethanol, ACN, 2-propanol, benzene, toluene and hexane at 37 °C with shaking at 150 rpm for 30 min. The remaining activity was measured. The activity of the enzyme solution without organic solvent was considered as control (100%).

2.5.6. Degradation of chicken feathers by purified proteases

Chicken feathers were collected from a local slaughter-house, rinsed thoroughly with tap water to remove excess blood and autoclaved. Disintegration of whole chicken feathers was assessed by incubation with purified enzyme (6000 U casein activities) at 37 °C for 24 h.

2.5.7. Effect of temperature and pH on enzyme stability

Experiments were conducted to study the thermal stability of purified protease enzyme. The purified enzyme was incubated at different combinations of pH and temperature. The pHs selected to study the deactivation of protease were 6, 7, 8, 9, 10, 11 and 12 of Britton–Robinson buffer and at each pH the deactivation was carried out at temperature of 37, 40, 50, 60, 70, 75 and 80 °C. The enzymes were deactivated at various combinations of pH and temperature as discussed above and aliquots of samples were taken at different intervals of time. Residual enzyme activity of samples after deactivation was measured in standard assay conditions.

The deactivation of protease enzymes is assumed to follow first-order kinetics. This is called single step two-stage theory [5,9]. The two-state mechanism is as follows:

$$E_A \rightarrow E_D \quad (1)$$