

Extracellular acid protease from *Rhizopus oryzae*: purification and characterization

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Received 29 February 2004; accepted 15 June 2004

Abstract

Extracellular aspartate protease from *Rhizopus oryzae* was purified 91 times with 26% recovery using $(\text{NH}_4)_2\text{SO}_4$ fractionation, ion-exchange and size-exclusion chromatographic techniques. The enzyme was found to be monomeric in nature having a molecular mass of 34 kDa. The enzyme acts optimally at 60 °C with activation energy of 15.16 kcal/mol and was stable in the temperature range of 30–45 °C. The purified enzyme is an acid protease with optimum pH of 5.5 and retained 96% of residual activity between pH 5.5 to 7.5. Ca^{2+} activation (250 times) and varying substrate concentration gave an hyperbolic response. The Lineweaver–Burk plot showed K_m value of 5 mg/ml, when skim milk was used as substrate. The enzyme inhibition of 73 and 93% by pepstatin at 10 and 20 μM , respectively proved it to be an aspartate protease; however, the additional requirement of histidine residue for enzyme activity has been indicated by differential spectra of diethyl pyrocarbonate treated versus untreated enzyme.

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Keywords: Microbial enzyme; Aspartate protease; Milk clotting activity; Cheese; *Rhizopus oryzae*

1. Introduction

Enzymes have extensive applications in a range of industrial processes. Proteases account for approximately 60% of all enzyme sales because of their varied applications in food, pharmaceutical and number of other industries [1]. Rennet (EC 3.4.23.4), an aspartate protease is the chief enzyme employed in cheese production. Rennet not only clots the milk but also play an important role during cheese maturation, which is a vital and complex process for the balanced development of flavour and texture [2–4]. The scarcity and high price of traditional calf rennet has promoted research towards the alternate milk coagulants produced either by plant or by the native/genetically modified micro-organisms [5]. A suitable rennet substitute must have high specific caseinolytic activity (to promptly cleave the phe₁₀₅–met₁₀₆ bond of κ -casein) and small-generalized proteolytic activity [6–7]. After initial reports of isolation of milk clotting enzyme from fungi [8], analogous enzymes have been iso-

lated from number of micro-organisms. However, several of these could not produce good quality cheese with requisite flavour and taste and even some of them produce bitter peptides [9]. While screening a large number of bacteria and fungi for their capability to produce acid protease, a fungal strain was obtained which produced potential milk clotting protease (MCP), yielding better quality cheese when compared to commercialized enzymes. The fungal strain on identification from Institute of Microbial Technology, India was found to be *Rhizopus oryzae* Went and Prinsen-Geerlgs (MTCC 3690). Some of the kinetic characteristics of the purified enzyme preparation obtained from this fungus are reported.

2. Materials and methods

2.1. Materials

Column chromatography materials were purchased from Sigma Chemicals Co. St. Louis, MO, USA and molecular

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markers for electrophoresis were obtained from Genei, Bangalore, India. Other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Cultivation of fungal strain and enzyme production

R. oryzae isolated from the soil of an effluent treatment plant was maintained by weekly transfer on potato dextrose agar medium fortified with 10% skim milk at 30 °C. The extracellular acid protease was produced under solid-state fermentation conditions in wheat bran phosphate buffer medium (50 mM and pH 6.0) at 30 °C. The enzyme was harvested after 48 h of incubation with distilled water. The filtrate obtained was centrifuged at $10,000 \times g$ for 10 min at 4 °C. The supernatant so obtained was referred as crude extract. All steps of enzyme purification were carried out at 0–4 °C.

2.2.2. $(\text{NH}_4)_2\text{SO}_4$ fractionation

MCP in crude extract was precipitated between 20–60% saturation of $(\text{NH}_4)_2\text{SO}_4$. The precipitate obtained after centrifugation at $10,000 \times g$ for 30 min was suspended in 50 mM phosphate buffer, pH 6.0 and dialyzed overnight against repeated changes of the same buffer.

2.2.3. Ion-exchange chromatography

The enzyme preparation obtained from the above step was further purified by passing through a column (25 cm $\times$ 2.6 cm) of activated DEAE-cellulose previously equilibrated with 50 mM phosphate buffer, pH 6.0. The fractions of 3 ml each were eluted at the flow rate of 35 ml/h with linear gradient of 0–0.4 M KCl and analyzed for enzyme activity and protein content. The active fractions were pooled and concentrated by osmosis.

2.2.4. Size-exclusion chromatography

The concentrated enzyme was loaded on to Sephadex G-100 column (65 cm $\times$ 1.5 cm) pre-equilibrated with 50 mM phosphate buffer, pH 6.0. Enzyme fractions of 3 ml were eluted at 12 ml/h flow rate with the same buffer and were analyzed for enzyme activity and protein content. Active enzyme fractions were pooled and stored at 4 °C for further studies.

2.2.5. Milk clotting activity

The milk clotting activity of enzyme was determined as described elsewhere with modification [10]. Five milliliter of assay milk (10% skim milk and 0.01 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in distilled water) was taken in a test tube and the contents were brought to the temperature of 37 °C. 0.5 ml of enzyme extract was then added and the curd formation was observed while manually rotating the test tube from time to time. The end point was recorded when discrete particles were discernible. One milk clotting unit is defined as the amount of enzyme present in 1 ml of extract clotting 10 ml substrate in

40 min i.e.

$$\text{Milk clotting (U/ml)} = 2400/t \times \text{D.F.}$$

where t is clotting time and D.F. is dilution factor.

2.2.6. Caseinolytic activity

The proteolytic activity of the enzyme was assayed after Arima et al. [10]. To 2.5 ml of 1% (w/v) alkali soluble casein in 0.02 M potassium phosphate buffer (pH 6.5) 0.5 ml of enzyme extract was added. The reaction mixture was incubated at 37 °C in a water bath for 10 min and the reaction was terminated by adding 2.5 ml of 0.44 M trichloroacetic acid. The precipitates formed were removed by filtration through Whatman No. 1 filter paper. One milliliter of 1N Folin-Ciocalteu reagent and 2.5 ml of 0.55 M sodium carbonate solutions was added to 1 ml of the above clear filtrate. This was further incubated for 20 min at 37 °C for colour development. The optical density at 660 nm expresses activity in term of proteolytic units (PU).

2.2.7. Protein estimation

The protein content of individual fraction obtained after different steps of chromatography was monitored by measuring the extinction at 280 nm. Quantitative estimation of protein was done by the method of Lowry et al. [11].

2.2.8. Determination of purity and molecular mass

The purity of the enzyme preparation was judged by native PAGE and the molecular mass of the purified enzyme was estimated by gel filtration through a column of Sephadex G-100, which has previously been calibrated with the standard marker proteins viz. alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and Cyt. C (14.3 kDa). Subunit molecular mass was determined by SDS-PAGE according to the method of Laemmli [12]. The molecular weight markers used for electrophoresis were α -lactalbumin (14.3 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa) and phosphorylase b (97.4 kDa).

3. Results and discussion

3.1. Purification and molecular mass

The extent of purification and characteristic of an enzyme has a profound effect on product quality. The enzyme from *R. oryzae* was purified to electrophoretic homogeneity. The enzyme was fractionated between 20 and 60% $(\text{NH}_4)_2\text{SO}_4$ saturation; with 30-fold purification and 103% recovery. Higher enzyme recovery was probably due to removal of certain low molecular weight inhibitor. Passage from DEAE-cellulose column further purifies the enzyme to 79-fold with 32% recovery and finally on Sephadex G-100, the extent of purification was 91-fold with 26% recovery (specific activity 760 U/ml), having high specific

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