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Catalytic properties and thermal stability of a crude protease from the keratinolytic *Bacillus* sp. CL33A

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ABSTRACT

Protease production by a feather-degrading mesophilic *Bacillus* sp. CL33A was performed through submerged cultivations using feather meal as low-cost organic substrate. Soluble protein concentration increased from 1.15 mg/mL at the beginning of cultivations (0 h) to 4.39 mg/mL after 72 h, indicating effective substrate solubilization. Crude protease, recovered as culture supernatants after 72 h of cultivation, was characterized. Optimal temperature and pH conditions for proteolytic activity were 48–62 °C and pH 7.2–9.2. Presence of $MgCl_2$ (1–5 mM), $MnCl_2$ (1 mM), Tween-20 and dimethyl sulfoxide (0.5–1.0%, v/v) increased protease activity at optimal conditions (55 °C; pH 8.0). Contrarily, $CoCl_2$, $CuSO_4$, $FeSO_4$, $ZnCl_2$, $MnCl_2$ (5 mM), sodium dodecyl sulfate (0.05–1.00%, w/v), and β-mercaptoethanol (0.1–1.0%, v/v) decreased enzyme activity. Inhibition by phenylmethylsulfonyl-fluoride (25%), ethylenediaminotetraacetic acid (35%) and 1,10-phenanthroline (31%) indicates that crude protease contains serine and/or metalloproteases. Thermal inactivation of the crude protease at 50–60 °C followed apparent first-order kinetics. Inactivation rates (k) increased at higher temperatures, resulting in half-lives of 38.6 and 2.7 min, and D-values of 128.4 and 8.9 min, at 50 and 60 °C, respectively. Activity decay was modeled as a function of time and temperature by combining first-order and Arrhenius equations. Thermal inactivation was an endothermic process, resulting from conformational destabilization due to a highly positive apparent entropic contribution. Activity at mild conditions and low thermal stability might be beneficial for biocatalytic processes. Crude protease specificity might be explored for the hydrolysis and modification of food proteins, particularly casein and soy protein isolate.

1. Introduction

Proteases are hydrolases that cleave peptide bonds in proteins and peptides. Extracellular proteases produced by microorganisms play essential roles on microbial nutrition, since protein hydrolysis results in amino acids and peptides that could be absorbed by cells. From a higher level of complexity, proteolysis performed by microorganisms and their enzymes participate on important environmental processes such as nitrogen cycling, acting as a rate-limiting step of nitrogen mineralization, particularly in soils (Vranova et al., 2013). Beyond ecological significance, microbial proteases constitute a major group of industrial enzymes, accounting for two-thirds of the global enzyme sales. These enzymes are employed by the food, feed, leather, detergent, among other industries, and might also find applicability in green synthesis, in a market that is continuously expanding (Kasana et al., 2011; Lateef et al., 2015a; Adelere and Lateef, 2016).

Diverse microorganisms are investigated as sources of proteases for commercial and industrial applications (Bach et al., 2011; Gupta et al., 2015; Rathod and Pathak, 2016). Since ecological conditions act by selecting microbial functionalities, environmental prospection represents one of the most important approaches to obtain microorganisms and microbial enzymes for biotechnological purposes (Lateef et al., 2010). Bacteria from the genus *Bacillus* receive special interest, owing to their physiological versatility and the ability to secrete large amounts of extracellular enzymes (Schallmey et al., 2004; Badoei-Dalfard and Karami, 2013; Lateef et al., 2015b; Sathishkumar et al., 2015).

Industrial production of microbial enzymes requires suitable conditions, such as adequate substrates to sustain microbial growth and elicit the secretion of desired enzyme(s). Considering that the composition of culture media represents approximately 40% of the enzyme production costs, the utilization of agro-industrial wastes and by-products could be beneficial from both financial and environmental aspects (Chaturvedi et al., 2014; Sathishkumar et al., 2015; Adelere and Lateef, 2016; Bhargavi and Prakasham, 2017). For instance, feather meal is a protein- and nitrogen-rich product obtained from hydrothermal treatment of waste feathers generated by poultry processing. Although representing

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a major strategy presently utilized for feathers management, feather meal has limited utilization as an ingredient of animal feed due to poor protein quality and digestibility. However, feather meal could also be regarded as a widely available low-cost organic substrate for microbial growth aiming to obtain useful and added-value products in a cost-effective manner, such as proteases and protein hydrolysates (Daroit and Brandelli, 2014).

A feather-degrading mesophilic bacterium isolated from a site containing waste feathers in Southern Brazil, identified as *Bacillus* sp. CL33A and related to *Bacillus aerius*, was previously demonstrated to produce extracellular proteases during submerged cultivations using feather meal as growth substrate (Oliveira et al., 2016). Considering the wide range of protease applications, characterization of enzyme activity and stability are of major relevance from industrial and commercial standpoints (Kasana et al., 2011). In this context, this study aimed to characterize the activity and thermal stability of the crude protease produced by *Bacillus* sp. CL33A during submerged cultivations performed using feather meal as the only organic substrate for microbial growth.

2. Material and methods

2.1. Microorganism and protease production

Bacillus sp. CL33A, previously isolated from a site where organic residues (including feathers) were discarded, was employed in this study. For protease production, the bacterium was inoculated into Erlenmeyer flasks (250 mL) containing 50 mL of feather meal (FM) broth, composed by 0.5g/L NaCl, 0.3g/L K_2HPO_4, 0.4g/L KH_2PO_4, 10g/L feather meal, initial pH 7.0. Cultivations were performed at 30 °C in an orbital shaker (125 rpm) for 72 h (Oliveira et al., 2016). After this period, cultures were centrifuged (10,000*g* for 5 min) and supernatants were employed as crude extracellular protease.

Additionally, the soluble protein (mg/mL) released during cultivations performed for up to five days on feather meal medium was determined on culture supernatants through the Folin-phenol method, using bovine serum albumin as standard.

2.2. Assay of proteolytic activity

Proteolytic activity assays were performed using azocasein as substrate. Reaction systems were composed by 100 μL of convenient buffer (100 mM; see Section 2.3), 300 μL of azocasein solution (10*g*/L) prepared in the same buffer, and 100 μL of appropriately diluted crude protease. After incubation (10 min) at the specified temperature, reaction was terminated by adding 600 μL of trichloroacetic acid solution (TCA; 10%, w/v). Following centrifugation (10,000*g* for 5 min), 800 μL of the supernatant were mixed with 200 μL of NaOH solution (1.8 M), and the absorbance of this mixture was evaluated at 420 nm. One unit (U) of proteolytic activity was arbitrarily defined as the amount of enzyme required to increase 0.1 absorbance unit at the assay conditions.

2.3. Determination of crude protease catalytic properties

Optimal conditions of temperature and pH for azocasein hydrolysis by the crude protease were evaluated using a 2^2 rotatable central composite design (CCDR). In this experiment, five levels of temperature and pH were employed (Table 1). To attain the respective pH values, the following buffers (100 mM) were utilized: sodium phosphate buffer (pH 6.0 and 6.6), and Tris-HCl buffer (pH 8.0, 9.4, and 10.0). Protease activity was taken as dependent variable, and the results were analyzed using the Experimental Design module of the Statistica 5.0 software (Statsoft, USA). Coefficients were obtained through regression analysis, and model fit was assessed through the coefficient of determination (R^2) and analysis of variance (ANOVA).

Crude protease activity was evaluated at optimal conditions of pH and temperature in the presence of different azocasein concentrations, from 0.06 to 6.00 mg/mL, with suitable blanks and controls. Obtained results were fitted, using nonlinear regression, to the Michaelis-Menten equation aiming to obtain K_m and V_{max} values.

The effect of salts ($CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $MnCl_2$, $MgCl_2$, $ZnCl_2$), Triton X-100, Tween 20, sodium dodecyl sulfate (SDS), β-mercaptoethanol, ethanol, and dimethyl sulfoxide (DMSO) on crude protease activity was evaluated at optimal conditions of temperature and pH. Phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) and 1–10 phenanthroline were evaluated as protease inhibitors. Crude protease was initially incubated with the respective inhibitor for 20 min at 25 °C. After this period, the azocaseinolytic assay was carried out at optimal conditions of temperature and pH.

2.4. Thermal inactivation of the crude protease

Inactivation of the crude protease as a function of temperature was investigated by pre-incubating the crude protease in Tris-HCl buffer (100 mM, pH 8.0), without substrate, at distinct temperatures (50–60 °C) for different periods (0–20 min). Following pre-incubation, reaction was terminated by immediately placing test tubes on an ice bath. Then, azocaseinolytic assay was performed at optimal conditions. Obtained data were fitted, through nonlinear regression, to the first-order model:

$$\frac{A}{A_0} = \exp(-kt)$$

where A/A_0 was the residual enzyme activity, t is time(min), and k (min^{-1}) is the inactivation rate constant at a given temperature. A is activity measured after pre-incubation at a given temperature (°C) and t (min), and A_0 is the activity at the same temperature at $t=0$. From the data, the kinetic parameters D (time needed for a tenfold reduction of initial activity), z (temperature required to decrease D value by one log unit) and $t_{1/2}$ (enzyme half-life) values were calculated. Thermodynamic parameters were also obtained. Association between temperatures and the respective inactivation rate constants (k) might be expressed by the Arrhenius' equation:

$$k = k_0 \exp\left(\frac{-E_a}{RT}\right)$$

where k_0 is the Arrhenius constant, E_a the activation energy for the inactivation process, T is the absolute temperature (K), and R the universal gas constant (8.314 J/mol·K). The E_a can be estimated by the slope of linear regression analysis of the natural logarithm of rate constant versus the reciprocal of the absolute temperature. A good fit to the Arrhenius equation enables calculation of activation enthalpy for inactivation ($\Delta H^{\#}$), free energy of inactivation ($\Delta G^{\#}$), and activation entropy of inactivation ($\Delta S^{\#}$) for each temperature (Daroit et al., 2011).

2.5. Hydrolysis of protein substrates

The capability of the crude protease to hydrolyze chicken feathers, human hair, feather meal, bovine serum albumin, casein and soy protein isolate was also evaluated. Crude protease (0.5 mL) was added to 25 mL of substrate suspensions (10g/L) prepared in Tris-HCl buffer (100 mM; pH 8.0). Controls were performed by adding buffer instead of crude protease. Reaction systems were incubated at 50 °C for up to 4 h. At specified intervals, 0.5 mL samples were collected and hydrolysis reactions were terminated through the addition of 0.5 mL TCA (10%, w/v). After centrifugation at 10,000*g* for 5 min, absorbance at 280 nm was measured (Giongo et al., 2007).