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Characterization of a keratinase produced by *Bacillus* sp. P7 isolated from an Amazonian environment

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ABSTRACT

The Amazonian bacterium *Bacillus* sp. P7 efficiently degraded feather keratin during submerged cultivations, producing extracellular keratinolytic enzymes. Keratinase produced during growth on feather meal broth was partially purified by ammonium sulphate precipitation, gel filtration, and ion-exchange chromatography, resulting in a purification factor of 29.8-fold and a yield of 27%. Zymography revealed two proteolytic bands, mainly inhibited by phenylmethylsulfonyl fluoride (PMSF). Partially purified keratinase had optimal activity at 55 °C and pH 9.0, was stimulated by Ca^{2+} and Mg^{2+}, and was inhibited by Hg^{2+}, Cu^{2+} and Zn^{2+}. Organic solvents 2-mercaptoethanol and Triton X-100 slightly affected the enzyme activity, whereas SDS stimulated it. PMSF and ethylenediaminetetraacetic acid (EDTA) inhibited proteolytic activity, which suggests its serine-protease feature, with the requirement of metal ions for maximum activity and/or stability. Alkaline keratinase might be employed in detergent formulations, in leather processing, and in other processes involving protein hydrolysis. The maintenance of enzyme activity in the presence of reducing agent (2-mercaptoethanol) makes this partially purified keratinase interesting for application in the breakdown of recalcitrant keratin wastes.

1. Introduction

Keratins are insoluble fibrous proteins highly cross-linked with disulfide bridges, hydrogen bonds, and hydrophobic interactions. The tightly packed supercoiled polypeptide chains result in high mechanical stability and resistance to proteolysis by common proteases such as trypsin, pepsin, and papain (Onifade et al., 1998). Keratin is the major component of feathers, which represent up to 10% of the total weight of mature chickens. Since feathers are generated as a waste product in commercial poultry-processing plants, reaching millions of tons per year worldwide (Brandelli, 2008), keratinous wastes are increasingly accumulating and there is a demand for developing alternatives for their recycling (Gupta and Ramnani, 2006). Waste feathers are currently converted to feather meal by a hydrothermal process; however, this process destroys certain amino acids, yielding a product with poor digestibility and variable nutrient quality (Wang and Parsons, 1997).

Despite the recalcitrant character of keratins, diverse bacteria, actinomycetes, and fungi have been identified and reported to degrade and utilize keratin because they produce keratinolytic enzymes (Gupta and Ramnani, 2006; Brandelli, 2008). Thus, keratinolytic microorganisms may have important biotechnological applications in the environment-friendly processing of keratinous wastes from the poultry industry (Shih, 1993; Onifade et al., 1998). Particularly, since poultry feathers and also feather meal are inexpensive, abundant, and readily available, the utilization of such byproducts as substrates by keratinolytic microorganisms represents a feasible microbial technology for obtaining proteolytic enzymes (Bernal et al., 2003; Thys et al., 2006), microbial biomass, and protein hydrolysates (Daroit et al., 2009) with lower production costs.

Alkaline proteases are among the most important industrial enzymes; they are primarily employed as detergent additives, accounting for 40–60% of the global enzyme market. The genus *Bacillus* provides most of the alkaline proteases with commercial value, offering the advantages of being easy to culture and maintain (Rao et al., 1998; Kumar and Takagi, 1999; Gupta et al., 2002). Keratinolytic microorganisms and microbial keratinases have a wide spectrum of industrial applications, as they are potentially valuable in the bioconversion of keratinous wastes; in the detergent, fertilizer, biopolymer, pharmaceutical, and animal feed industries; and in leather processing and hydrolysis of prion proteins as well (Gupta and Ramnani, 2006; Brandelli, 2008). Therefore, such microorganisms and enzymes have been the focus

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in several studies (Bressollier et al., 1999; Macedo et al., 2005; Thys and Brandelli, 2006; Anbu et al., 2007; Riffel et al., 2007; Kumar et al., 2008) and *Bacillus* sp. are reported to be among the most efficient keratin degraders (Kumar et al., 2008; Son et al., 2008; Daroit et al., 2009).

Microbial biodiversity is a major resource for novel biotechnological products and processes (Gupta et al., 2002). The Amazonian environment represents a vast biodiversity that might lead to the isolation of strains producing diverse metabolites with potential commercial applications and, in this context, a keratinolytic *Bacillus* sp. P7 was isolated from the intestine of the Amazon basin fish, *Piaractus mesopotamicus* (Giongo et al., 2007). The aims of this work were to investigate the production of extracellular alkaline keratinases by *Bacillus* sp. P7 in submerged cultivations with keratinous substrates and to determine the properties of the keratinase.

2. Materials and methods

2.1. Bacterial strain and culture media

Bacillus sp. P7 was isolated from the intestines of the teleost fish Jaraqui (*P. mesopotamicus*), which was kindly provided by the Universidade Federal do Amazonas, Brazil. This strain was characterized based on 16S rDNA sequencing, clustering with *Bacillus velesensis*, *Bacillus subtilis*, and *Bacillus amyloliquefaciens* (Giongo et al., 2007).

The media utilized in this study were feather meal broth (FM), whole feather broth (WF), and human hair broth (HH), which contained, per liter: 0.5 g of NaCl, 0.3 g of K_2HPO_4, 0.4 g of KH_2PO_4, and 10 g of either feather meal (for FM), whole feathers (for WF), or human hair (for HH). The pH was adjusted to 8.0 before autoclaving. The strain was inoculated, and incubation was performed at 30 °C for up to 96 h with shaking at 120 rpm. The substrate in which the higher keratinolytic activity was achieved was selected for enzyme production.

2.2. Keratinolytic potential

Keratin degradation by *Bacillus* sp. P7 was evaluated during growth on FM, FB, and HH by the dry weight of the keratinous substrates remaining during cultivation (Daroit et al., 2009), and by the soluble protein in culture supernatants, which was determined by the Folin phenol reagent method (Lowry et al., 1951), using bovine serum albumin as standard.

2.3. Enzyme activity

Keratinolytic and proteolytic activities were determined using azokeratin or azocasein (Sigma Co., USA), respectively, as substrates. Azokeratin was synthesized as described elsewhere (Riffel et al., 2003). The reaction mixture contained 100 μl of enzyme preparation and 100 μl of 10 mg ml^{-1} azokeratin (or azocasein) in 20 mM Tris–HCl buffer, pH 8.0. The mixture was incubated for 30 min at 37 °C; the reaction was stopped by adding 500 μl of 10% (w/v) trichloroacetic acid. After centrifugation (10,000 × *g* for 5 min) of the reaction mixture, 800 μl of the supernatant were mixed with 200 μl of 1.8 M NaOH, and the absorbance at 420 nm was measured. One unit of enzyme activity was considered as the amount of enzyme that caused a change in absorbance of 0.01 units at the above assay conditions.

2.4. Purification of extracellular keratinase

After 48 h of incubation on FM, the culture supernatant was obtained by centrifugation (10,000 × *g* for 15 min at 4 °C) and used as an enzyme source for the purification protocol (Table 1). The enzyme was precipitated from the culture supernatant by the gradual addition of solid ammonium sulphate to 60% saturation in an ice bath with gentle stirring. This mixture was allowed to stand for 1 h, it was centrifuged (10,000 × *g* for 15 min at 4 °C), and the resulting pellet was dissolved in 20 mM Tris–HCl buffer pH 8.0.

Table 1
Summary of keratinase purification protocol.

Purification step	Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Purification factor
Crude enzyme	1446	3.33	434.2	1.0
$(NH_4)_2SO_4$ precipitation	2192	3.60	608.8	1.4
Sephadex G-200	1109	0.70	1584.3	3.6
SP-Sepharose	840	0.35	2400.0	5.5
DEAE-Sepharose	389	0.03	12,966.6	29.8

The enzyme sample from ammonium sulphate precipitation was applied to a gel filtration Sephadex G-200 (Pharmacia Biotech, Sweden) column (25 × 0.5 cm) previously equilibrated with Tris–HCl buffer (20 mM, pH 8.0). The flow rate was maintained at 0.33 ml min^{-1}, and 30 fractions of 1 ml were collected. The absorbance at 280 nm was recorded and enzyme activity was determined as mentioned in the previous section.

The active fractions were pooled and applied to an ion-exchange SP-Sepharose (Pharmacia Biotech, Sweden) column (10.5 × 0.65 cm) equilibrated with Tris–HCl buffer (20 mM, pH 8.0). Elution was performed with the same buffer with a linear gradient of NaCl (0–2 M) at a flow rate of 0.6 ml min^{-1}, and fractions of 2 ml were collected. In each fraction, the absorbance at 280 nm and the azocaseinolytic activity were measured. Active fractions were pooled and loaded to a DEAE-Sepharose FF (GE Healthcare, Sweden) column (15 × 1 cm) equilibrated with Tris–HCl buffer (20 mM, pH 8.0). Elution was carried out with the same buffer containing a linear gradient of NaCl (0–2 M), at a flow rate of 0.6 ml min^{-1}, and fractions of 2 ml were collected. In each fraction, the measurement of absorbance at 280 and the enzyme assay were performed. Fractions with proteolytic activity were pooled and submitted to enzyme assay and protein determination. This resulting pool was utilized for enzyme characterization.

The soluble protein content in each stage of purification was determined by the Folin phenol reagent method (Lowry et al., 1951), using bovine serum albumin as standard.

2.5. Zymography

Zymogram analysis was based on a previously described procedure (Giongo et al., 2007), with modifications. The partially purified keratinase was electrophoresed at room temperature on 16% polyacrylamide gels containing gelatin (1 mg ml^{-1}). After electrophoresis, the gels were washed twice with Tris–HCl buffer (20 mM, pH 8.0) containing 2.5% (v/v) Triton X-100 for 30 min, and then with the same buffer without detergent for 60 min. After 12 h of incubation at 30 °C in the last buffer, the gels were stained with Coomassie Brilliant Blue R-250 and then destained. Protease bands appeared as clear zones on a blue background due to gelatin hydrolysis. Alternatively, gels were incubated in the presence of either 10 mM EDTA or 10 mM PMSF.

2.6. Effects of pH and temperature on enzyme activity, and thermostability

For pH optimum determination, azocaseinolytic activity was assayed at 37 °C in a pH range from 5 to 12 using the following buffers (20 mM): phosphate (pH 5.0–6.5), Tris–HCl (pH 7.0–9.0),