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# Partial characterization of protease from a thermophilic fungus, *Thermoascus aurantiacus*, and its hydrolytic activity on bovine casein

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**Abstract**

Proteases are one of the most important groups of industrial enzymes, with considerable application in the food industry. The aim of this work was to study a novel protease produced by the thermophilic fungus, *Thermoascus aurantiacus*, through solid-state fermentation (SSF). The enzyme acted optimally at pH 5.5 and 60 °C; it was stable up to 60 °C for 1 h and in the pH range 3.0–9.5. To elucidate the enzyme's proteolytic activity, its hydrolytic profile on bovine casein, an important protein in the food industry, was studied by enzymatic hydrolysis on skim milk, analyzed by gel electrophoresis (UREA-PAGE), which clearly showed that the protease does not have the same specificity as bovine chymosin.


*Keywords:* SSF; Protease; Wheat bran; Casein; Proteolytic activity; Characterization; Hydrolysis; Electrophoresis

## 1. Introduction

Proteases are a very important group of enzymes and represent nearly 60% of total enzyme sales, since they are used in the detergent, beer, meat, leather and dairy industries (Kumar, Sharma, Saharan, & Singh, 2005).

There has been many investigations aimed to obtain proteases from fungi, such as *Thermomyces lanuginosus* (Jensen, Nebelong, Olsen, & Reeslev, 2002; Li, Yang, & Shen, 1997), *Aspergillus* (Coral, Arikan, Ünaldi, & Güvenmez, 2003; Shata, 2005; Tunga, Shrivastava, & Banerjee, 2003), *Penicillium* (Germano, Pandey, Osaku, Rocha, & Soccol, 2003; Hashem, 2000), *Mucor* (Alves, Takaki, Porto, & Milanez, 2002; Maheshwari, Bharadwaj, & Bhat, 2000; Tubesha & Al-delaimy, 2003); *Rhizopus oryzae* (Kumar et al., 2005). However, their hydrolytic activity is not always clear.

Thermophilic fungi produce hydrolases with important characteristics, such as higher thermostability, optimum activity at higher temperatures and high rates of hydrolysis (Kalogeris et al., 2003). These thermostable enzymes play an important role as catalysts in technical processes that take place at high temperatures (Gey & Unger, 1995). The possibility of using these enzymes at elevated temperatures, especially in the food industry, implies increased reaction rates, improved solubility of reagents and a decrease of contamination by mesophilic microorganisms. Thermostable proteases, that act in the temperature range 65–85 °C for the bioconversions of proteins into aminoacids and peptides, have successful applications in baking, brewing, detergents and the leather industry (Haki & Rakshit, 2003).

This work presents, for the first time, studies on a protease produced from wheat bran by the thermophilic fungus, *Thermoascus aurantiacus*. The enzyme was partially characterized and its hydrolysis on bovine casein, an important protein in the food industry, was investigated to better understand its proteolytic activity.

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## 2. Materials and methods

### 2.1. Microorganism and inoculum

The fungus, *Thermoascus aurantiacus*, obtained from the Laboratory of Applied Biochemistry and Microbiology–IBILCE–UNESP, was inoculated in test tubes with Sabouraud dextrose agar medium (Oxoid) and incubated at 50 °C for 2 days for complete growth. Afterwards, it was kept at room temperature until further use.

The mycelium was suspended in 7 ml of sterilized nutrient solution, made up of 0.1% (w/v) $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$ and $NH_4NO_3$, and 1 ml of this mycelial suspension was used to inoculate the culture medium.

### 2.2. Fermentation medium and culture conditions

Media containing 10 g of wheat bran (WB) or 8 g of wheat bran and 2 g of casein (WBC), both hydrated with 15 ml of water, to approximately 60% moisture, were sterilized (120 °C/40 min) in 250 ml Erlenmeyer flasks.

The media were inoculated with mycelial suspension and cultivated at 45 °C for 4 days. The crude enzyme solution was obtained by adding 25 ml of water to the fermented material. Solids were removed by filtering through Whatman no. 1 filter paper and the clear solution was assayed.

### 2.3. Proteolytic activity

Proteolytic activity was determined according to Kembhavi, Kulkarni, and Panti (1993), with modification. The reaction mixture was made up of 0.4 ml of casein (Sigma), 0.5% (w/v) in distilled water and 0.4 ml 0.2 M acetate buffer, pH 5.5, to which 0.2 ml of the crude enzyme solution was added. The reaction was carried out at 60 °C and stopped after 30 min with 1 ml of 10% TCA (trichloroacetic acid). Test tubes were centrifuged at 5000 rpm/5 min and the absorbance of the supernatant was measured at 280 nm. An appropriate control was prepared, in which the TCA was added before the enzymatic solution. One unit of enzyme activity (U) was arbitrarily defined as the amount of enzyme required to cause an increase of 0.1 in absorbance at 280 nm, under the assay conditions. Enzymatic activity was calculated as follows: $U/ml = (\Delta Abs_{280\ nm} \times 10 \times \text{dilution factor})/0.2$. The specific activity was expressed as units of enzyme activity per mg of protein.

### 2.4. Protein content

Protein content was measured, following the method of Bradford (1976) and using bovine serum albumin (BSA) as standard.

### 2.5. Effects of pH and temperature on enzyme activity

To assay optimum pH, proteolytic activity was determined at 60 °C, at different pH values, using the following 0.2 M buffer solutions: acetate (pH 3.0–5.5), McIlvaine (pH 6.0–8.0) and glycine–NaOH (pH 8.5–10.0).

Optimum temperature was determined by incubating the reaction mixture at different temperatures ranging from 40 to 90 °C and assaying the activity at the pH determined as optimum.

### 2.6. Effect of pH and temperature on enzyme stability

For pH stability, the enzyme was dispersed (1:1) in the following 0.2 M buffer solutions: HCl–KCl (pH 1.0–2.0), acetate (3.0–5.5), McIlvaine (6.0–8.0) and glycine–NaOH (8.5–12.0), and maintained at 25 °C for 24 h. Afterwards, residual proteolytic activity was determined under optimum conditions of pH and temperature (5.5 and 60 °C, respectively).

Thermal stability was assayed by incubating the enzyme at different temperatures ranging from 30 to 75 °C for 1 h at pH 5.5. Afterwards, residual proteolytic activity was determined under optimum conditions of pH and temperature (5.5 and 60 °C, respectively).

### 2.7. Monitoring proteolytic activity during milk casein degradation: enzymatic hydrolysis of milk and electrophoresis

A 10% suspension of skim milk powder (Itambé) in 0.01 M $CaCl_2$ was subjected to hydrolysis at 45 °C. The hydrolysis was initiated by addition of 0.1 ml of enzyme solution, with specific activity of 39.8 U/mg proteins, to 0.9 ml of substrate solution. At selected times (5, 10, 20, 40, 60, 80, 100 and 120 min) the reaction was quenched by heating at boiling temperature for 6 min. After centrifugation (5000 rpm/5 min) the precipitate was used to monitor proteolysis, as described by Shalabi and Fox (1987). For this, 20 mg of the precipitate were incubated at 37 °C, in Eppendorf flasks, with 0.4 ml of 0.062 M Tris–HCl buffer, pH 6.7, containing 42% (w/v) urea for 1 hour. Afterwards, 10 μl of β-mercaptoethanol were added and the mixture again kept at 37 °C for 45 min. Finally, a drop of bromophenol blue was added. Electrophoresis was performed, according to Shalabi and Fox (1987), to these treated samples, using a Mini Protean 3 Cell vertical slab-gel unit (Bio Rad Laboratories). UREA-PAGE was performed at a constant voltage of 80V, using 0.046 M Tris–glycine, pH 6.7, as running buffer. Gels were stained overnight with Coomassie Brilliant Blue R-250 and destained with methanol/acetic acid/water 3:1:6 solution.

## 3. Results and discussion

### 3.1. Production and partial characterization

Fig. 1 shows the production profile for protease from *T. aurantiacus* in WB medium. It can be seen that maximum production occurred between the third and fourth

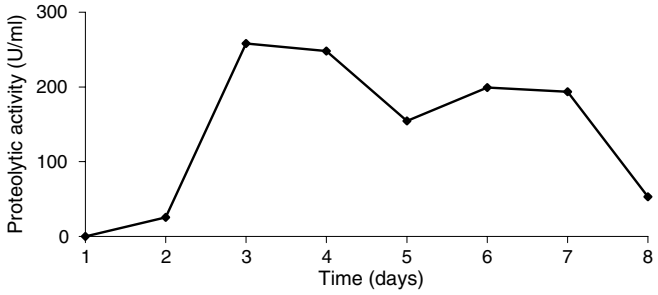


Fig. 1. Protease production by *T. aurantiacus* in WB medium

days (72 and 96 h, respectively), explaining why fermentation experiments were set on a 4-day period.

Investigations of protease production by fungal cultures have shown that there is a variation in the amount of enzyme produced according to the media used (Andrade et al., 2002), meaning that the microorganism will behave differently in different types of fermentation substrates. This was confirmed in this work, where WB and WBC presented 200 U/ml and 248 U/ml of proteolytic activity, respectively, on the fourth day of production. Better results for protease production by fungi, due to addition of casein to the fermentation medium, were also found by Silveira, Oliveira, Ribeiro, Monti, and Contiero (2005) and Tremacoldi and Carmona (2005).

Protease activity increased rapidly from 50 °C, reaching a maximum at 60 °C, with expressive loss at 75 °C (Fig. 2). Similar results, of optimum activity at elevated temperatures, were shown by proteases of the thermophilic fungi *Thermomyces lanuginosus* (70 °C) (Li et al., 1997) and *T. aurantiacus* var *levisporus* (55 °C) (Marcy, Engelhardt, & Upadhyay, 1984). Yet, proteases from the genera *Aspergillus* (Coral et al., 2003; Tunga et al., 2003) and from *Penicillium* sp. (Germano et al., 2003) showed optimum activities at lower temperatures, 40 °C and 45 °C respectively.

The enzymatic activity increased quickly from pH 3.5, reached maximal rate of reaction at pH 5.5, and started losing activity after pH 7.0 (Fig. 3). So, the enzyme appeared to be slightly acid, which is expected for proteases produced by fungi (Reed & Nagodawithana, 1993). Proteases from *Mucor*, described by Maheshwari et al. (2000), also exhibited low optimum pH. Calf rennet also shows a high rate of activity in the acidic range (Kumar et al., 2005).

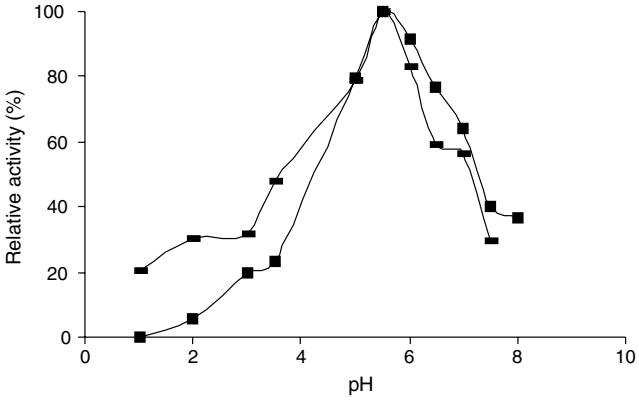


Fig. 3. Effect of pH on proteolytic activity. Wheat bran (WB ■), wheat bran with casein (WBC ▬).

The enzyme remained very stable up to 50 °C (Fig. 4). It showed a slightly lower thermal stability than did protease from *Penicillium*, which maintained 100% of its stability after a one hour treatment at 60 °C (Maheshwari et al., 2000). However, it was more thermostable than was *Penicillium* sp., which maintained only 20% of its activity after a one hour treatment at 50 °C (Germano et al., 2003) and *Aspergillus parasiticus*, which maintained 100% of activity at only 40 °C for 1 hour (Tunga et al., 2003).

The enzyme was stable over a wide range of pH, being highly active from 3.0 to 9.5. Below 3.0 and above 9.5 there was expressive loss of activity (Fig. 5). Proteases from *Aspergilus parasiticus* (Tunga et al., 2003) and from *Thermomyces lanuginosus* (Li et al., 1997) also exhibited a broad range of stability: from 5.0 to 10.0.

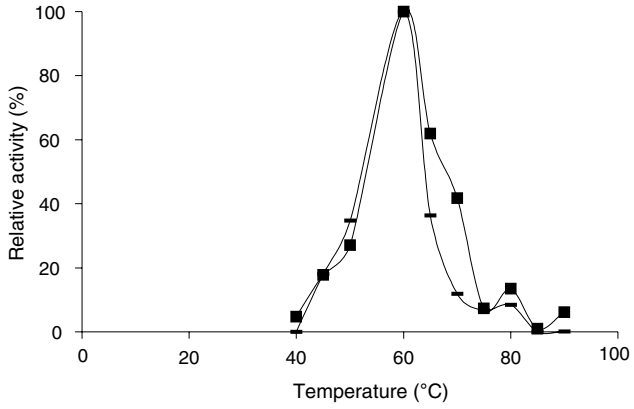


Fig. 2. Effect of temperature on proteolytic activity. Wheat bran (WB ■), wheat bran with casein (WBC ▬).

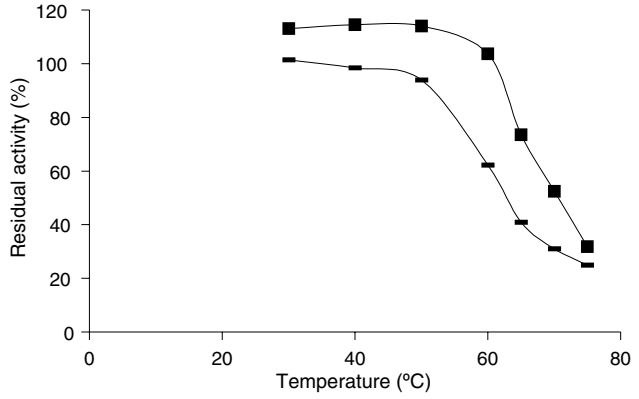


Fig. 4. Effect of temperature on protease stability. Wheat bran (WB ■), wheat bran with casein (WBC ▬).

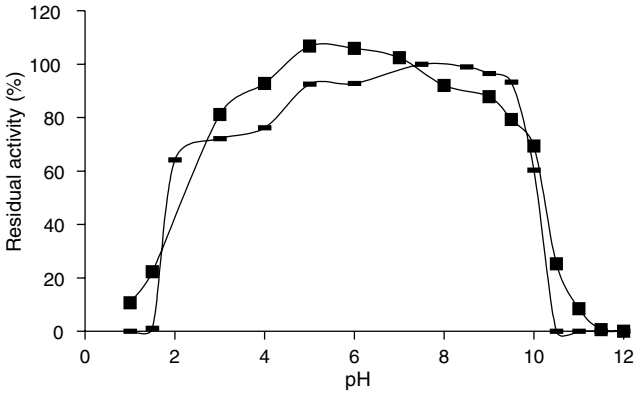


Fig. 5. Effect of pH on protease stability. Wheat bran (WB ■), wheat bran with casein (WBC ▬).

### 3.2. Enzymes hydrolytic behaviour

The hydrolytic behaviour of the protease in the enzyme solution from the fermentation medium WBC can be visualized by a frequently used method for monitoring proteolytic processes on caseins: urea–polyacrylamide gel electrophoresis. The hydrolysis for this experiment was performed on assay milk, to simulate real coagulation conditions. These results are shown in Fig. 6.

Two major groups of bands were identified in the UREA-PAGE: $\alpha_s$-casein, with higher mobility and β-casein, with lower mobility. The hydrolytic pattern is very clear and reveals that the protease is extremely proteolytic, breaking down all fractions of casein, especially the β fraction, after 10 min of incubation (lane 4, Fig. 6).

Some fungal acid proteinases have been studied for industrial application as calf rennet substitutes in cheese manufacture (Fernandez-Lahore et al., 1999). However, the enzyme studied in this work, produced by a thermophilic fungus, was more proteolytic than bovine chymosin, which implies lower yields in cheese production due to continued proteolysis following milk coagulation, leading to dissolution of the curd. Therefore this application would not be adequate for protease from *T. aurantiacus*.

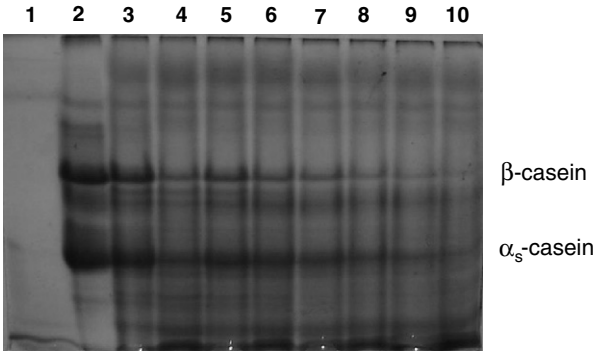


Fig. 6. UREA-PAGE of casein hydrolysis by protease. Lane 1: crude enzyme solution; line 2: assay milk; lanes 3–10: caseins after incubation for 5, 10, 20, 40, 60, 80, 100 and 120 min, respectively.

According to Rao, Tanksale, Ghatge, and Deshpande (1998), other possible uses of proteases, which require further studies, are as follows: in the leather industry, as an alternative to chemicals, for the hydrolysis of noncollagenous constitutents of the skin and for the removal of nonfibrillar proteins; in the food industry, in the baking segment to modify wheat gluten by limited proteolysis, in the manufacture of soy products, in debittering of protein hydrolysates by cleaving hydrophobic amino acids, and in the dairy industry for accelerating cheese maturation. Finally, they are used as ingredients for improving detergent action.

## 4. Conclusions

Partial characterization of a protease, produced by *Thermoascus aurantiacus* in an inexpensive medium, revealed that the enzyme had optimum pH at 5.5 and optimum temperature at 60 °C. It remained stable in a wide range of pH (from 3.0 to 9.5) and showed high stability up to 60 °C.

UREA-PAGE was an effective tool for showing that the enzyme caused excessive hydrolysis of milk caseins. Therefore, this low specificity, along with its high activity and stability at elevated temperatures, could make this protease useful for processes which require proteolytic action at high temperature.

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