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Keratinolytic Activity of *Aspergillus fumigatus* Fresenius

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Abstract. *Aspergillus fumigatus* can utilize chicken feather keratin as its sole carbon and nitrogen source. Because enzymatic conversion of native keratin into readily usable products is of economic interest, this fungus was studied for its capacity to produce and secrete keratin-hydrolyzing proteinases. Substantial keratin-azure hydrolyzing activity was present in the culture fluid of keratin-containing media. Considerably lower activity was present in cultures containing glucose and nitrate as the carbon and nitrogen sources, or keratin plus glucose and nitrate. Secretion of keratin-hydrolyzing activity in *A. fumigatus* was induced by keratin but repressed by low-molecular-weight carbon and nitrogen sources. The amount of keratinolytic enzyme present in the culture fluid was dependent on the initial pH of the culture medium. The crude enzyme also hydrolyzed native keratin and casein in vitro. Hydrolysis was optimal at pH 9 and 45°C. The crude enzyme was remarkably thermostable. At 70°C, it retained about 90% of its original activity for 1.5 h. The obtained results indicated that the *A. fumigatus* keratinolytic enzyme may be suitable for enzymatic improvement of feather meal.

Feathers, which account for 5–7% of the total weight of mature chickens, are produced in huge quantities as a waste by-product at commercial poultry processing plants. Considering that millions of tons of feathers are produced annually worldwide and that they are made up primarily of keratin (90% or more), this by-product represents a potential alternative to more expensive dietary ingredients, as for example, for poultry production. However, it has long been recognized that feather protein is poorly digested by birds [21]. The tight packing of the protein chains in α-helix (α-keratin) or β-sheet (β-keratin) structures and their linkage by cystine bridges make the structural keratin highly stable and resistant to digestive enzymes such as trypsin and pepsin, or other proteolytic enzymes. Therefrom, feather waste is utilized on a limited basis as a dietary protein supplement for animal feedstuffs [25]. Nevertheless, feathers do not accumulate in nature, since structural keratin can be degraded by some species of saprophytic and parasitic fungi [2, 20, 22], a few actinomycetes [17, 18, 23], some *Bacillus* strains [25], the thermophylic *Fervidobacterium pennavorans* [8], and *Streptomyces pactum* DMS 40530 [3]. The keratinolytic enzymes characterized to date (see Ref. [3]) all act as proteinases and are active on keratin. The gene (*kerA*) encoding a keratinolytic protease of *Bacillus licheniformis* PWD-1 was sequenced and expressed in *Escherichia coli* cells [12]. It shares 97% sequence identity with the gene encoding subtilisin Carlsberg from *B. licheniformis* [12]. It has been suggested that keratinases may play an important role in biotechnological processes for the improvement of feather meal [3] through solubilization of the structural feather keratin into readly usable products or in the leather industry [6, 17, 19].

The fungus *Aspergillus fumigatus*, found ubiquitously in nature, is an opportunistic airborne pathogen affecting humans, birds, and other animals. It accounts for a variety of respiratory disorders and severe invasive infections. This fungus has been characterized as a producer of several proteolytic enzymes, such as the elastases [9, 14, 16], fibrinogenolytic serine proteases [11], and collagenases [15], which have been reported to be responsible for the key events involved in the physiopathology of *A. fumigatus* [10]. In our laboratory, this mold was found to utilize chicken feather keratin as its sole carbon and nitrogen sources. Considering that chicken feathers represent an alternative to more expensive

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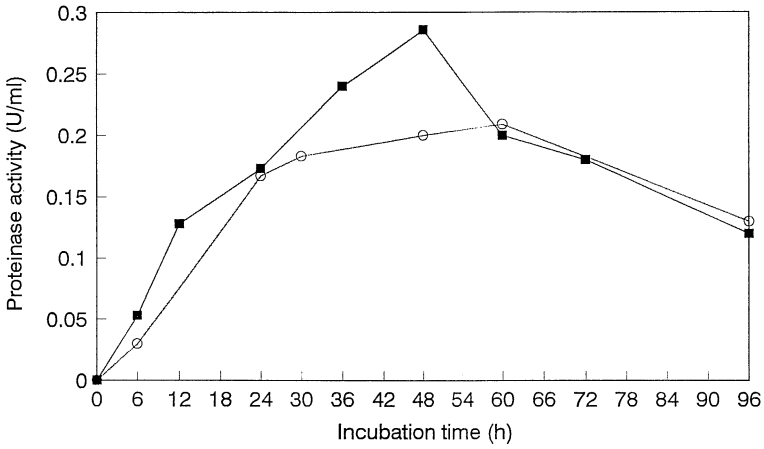

Fig. 1. Time course of keratinolytic enzyme production by *Aspergillus fumigatus* in a minimal medium containing 1% chicken feather flour. Cultures were inoculated with conidia (-o-) or with mycelial mass (-■-).

protein sources for animal feeding, we have started to examine the possibility of using this fungus to develop a rational biotechnological process to improve feather meal. It should be noted, however, that this fungus is a serious pathogen of fowl. Production of keratinases by genetically engineered heterologous cells may be the rational choice. Here, we report on the capacity of *A. fumigatus* to grow on chicken feather flour and on the production profile of its keratinolytic enzyme.

Materials and Methods

Organism and growth conditions. The fungus used in this study was *A. fumigatus*, isolated from decaying wood in a hot spring in the central east region (Caldas Novas) of Brazil. The fungus was maintained by serial passages in a potato broth–dextrose (10 g/L)–agar (18 g/L) medium.

Native chicken feather keratin preparation. Chicken feathers obtained from a commercial poultry processing plant were washed extensively with water and detergent, dried under sunlight, and then further dried for 72 h at 65°C. The feathers were then milled in a ball mill and passed through a small-mesh grid to remove coarse particles.

UV sterilization of chicken feather flour. The keratin used in the experiment of disintegration of native keratin was sterilized under ultraviolet light. Ten grams of chicken feather flour was exposed to UV irradiation at a distance of 10 cm from the light source (UV lamp, 220 V, 30 W) for 30 min. The flour was shaken by hand several times during irradiation. The irradiated flour was then immediately transferred to the experiment flasks containing the culture filtrate (see below).

Production and preparation of proteinase. For enzyme production, the cultures were inoculated with conidia or with mycelium. In the former case, samples of 10^9 conidia were transferred to Erlenmeyer flasks each containing 200 ml of autoclaved (121°C for 20 min) mineral liquid medium (in g/L: $MgSO_4 \cdot 7H_2O$, 0.52 g; KCl, 0.52 g; KH_2PO_4, 1.52 g, and traces of $FeSO_4 \cdot 7H_2O$ and $ZnSO_4$) supplemented with 1% native chicken feather keratin. In the latter case, the culture medium (see above) with or without 1% chicken feather flour was inoculated with mycelium produced in a liquid medium containing glucose (1%) and $NaNO_3$ (0.6%) for 24 h. It was then washed with sterile saline (0.15 M NaCl). For the experiment on utilization of native keratin, the chicken feather flour was sterilized under UV light for 30 min (see above) and then transferred to samples of mycelium-free culture filtrates obtained by filtration of 48-h-old cultures. The cultures were then incubated at 42°C for several time periods with constant shaking (120 rpm) and filtered through a paper filter. The culture filtrate was used immediately or kept at 4°C.

Determination of keratinolytic activity. The keratinolytic activity was determined with keratin-azure and native chicken feather keratin as substrates.

The activity against keratin-azure was monitored by measuring spectrophotometrically at 595 nm the liberation of the azo dye. The reaction medium contained 2 ml of crude enzyme and 4 mg of keratin-azure. The reaction was carried out at 42°C with constant agitation for several time periods and stopped by centrifugation of the reaction medium. As a control enzyme, samples boiled for 15 min were added to the reaction medium and were then incubated for the same time period as the nonboiled samples. The keratinolytic activities of the nonboiled samples were calculated by subtracting the absorbance recorded for the boiled samples. All assays were run in triplicate, and the data presented are mean values of the triplicate assays. The standard deviation values calculated for each set of three assays were always smaller than 16% of the mean values. One unit (U) of keratinolytic activity was defined as the amount of enzyme required to cause an increase of 1.0 A_{595nm} unit within 3 h.

The activity against native keratin was performed as for keratin-azure, except that the progress of the reaction was monitored by measuring spectrophotometrically at 280 nm the liberation of soluble peptides or amino acids from the chicken feather flour.

Protein and amino acid determinations. Protein in the culture supernatants was quantified by the Bradford method [4] with bovine serum albumin as standard. Amino acid was determined by the ninhydrin method [7]. The protein content of the chicken feather flour was determined by the Kjeldahl method [1].

Results and Discussion

Production of keratinase. The thermophilic fungus *A. fumigatus* grew strongly in the mineral medium containing either autoclaved or native chicken feather keratin as the sole carbon and nitrogen sources. Substantial keratin azure-hydrolyzing activity was present in the culture supernatant (Fig. 1). *Aspergillus fumigatus* is a well-

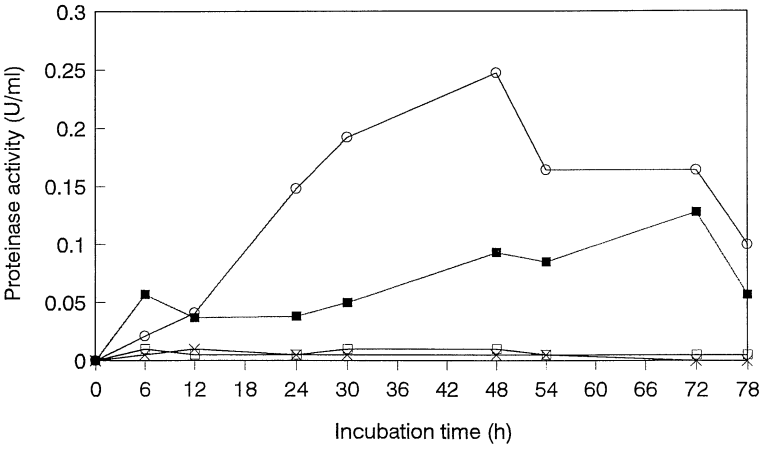

Fig. 2. Keratinolytic activity produced by *Aspergillus fumigatus* mycelium incubated in a minimal medium without carbon and nitrogen sources (-x-), or containing glucose and nitrate (-□-), keratin (-○-), and keratin plus glucose and nitrate (-■-).

known producer of several proteinases, such as elastase [9, 14, 16], fribrinogenolytic proteases [11], and collagenases [15], which have been reported to be virulence factors in invasive aspergillosis. However, none of them have been described as possessing keratinolytic activity, and the *A. fumigatus* culture filtrate also hydrolyzed native keratin (Fig. 5). It has been suggested that the ability of *A. fumigatus* to degrade elastin, which constitutes nearly 30% of the lung [24], may aid its invasion of and growth in lung tissue [14]. Since keratin constitutes or is present on the surface of many organisms, it might be that the keratinolytic activity, here first described for *A. fumigatus*, may represent a true keratinase which allows this ubiquitous fungus to survive in a noninvasive condition in nature.

Maximal enzyme production by *A. fumigatus* growing on keratin was achieved within 48 h (Fig. 1). In the cultures inoculated with conidia, complete dissolution of 2 g of chicken feather flour occurred within 72 h. However, in the cultures initiated with mycelium, the keratin was solubilized within 48 h. Complete dissolution of the keratin was even more evident in the cultures showing initial pH values of 6.5 or 7.5.

As expected, no proteinolytic activity was produced by the *A. fumigatus* mycelium in the absence of carbon and nitrogen sources. Glucose and nitrate supported mycelial growth but not keratinase production (Fig. 2). In the presence of keratin plus glucose and nitrate, the mycelial mass growth ratio was remarkable as assessed qualitatively, but the keratinolytic activity was considerably lower than that produced in the presence of keratin (Fig. 2). Therefore, unlike depression as for proteinases in *Aspergillus* species in the absence of carbon, nitrogen, and sulfur (5), induction seems to be the major mechanism controlling production of keratinase in *A. fumigatus.* Nevertheless, carbon and nitrogen repression is in command, and if induction of keratinase by keratin did occur in the presence of glucose and nitrate, it must be subordinate to catabolite repression. In contrast, supplementation of keratin-containing medium with glucose, ammonium, or sulfate did not repress keratinase production in *Chrysosporium queenslandicum* and *Scopulariopsis brevicaulis* [13].

Table 1. pH values of the culture filtrate of *Aspergillus fumigatus* grown for several time periods in a minimal medium containing 1% chicken feather flour

Incubation time (h)	pH values		
0	5.2	6.4	7.2
6	5.2	6.4	7.2
24	7.0	7.9	8.1
48	8.2	8.2	8.6
96	8.1	8.4	8.6
120	8.0	8.4	8.6

Aging of cultures was accompanied by an increase in the pH of the culture fluid. Independently of the initial value, at the time points of 48 h and 120 h the pH of all cultures was equivalent (Table 1). The cultures with an initial pH of 5.2 contained significantly higher amounts of enzyme after 96 h and 120 h of incubation (Fig. 3). As the pH values of all cultures were equivalent at these time points, the influence of pH on the stability of the enzyme was excluded. Acid conditions seem to sustain longer enzyme secretion, but neutral and alkaline conditions seem to favor hydrolysis of the keratin, i.e., the cultures showing higher initial pH values contained higher amounts of soluble proteins or peptides (Fig. 4), and amino acids (Fig. 5). In fact, as will be discussed below, the culture filtrate proteinase showed higher activity at an alkaline pH.

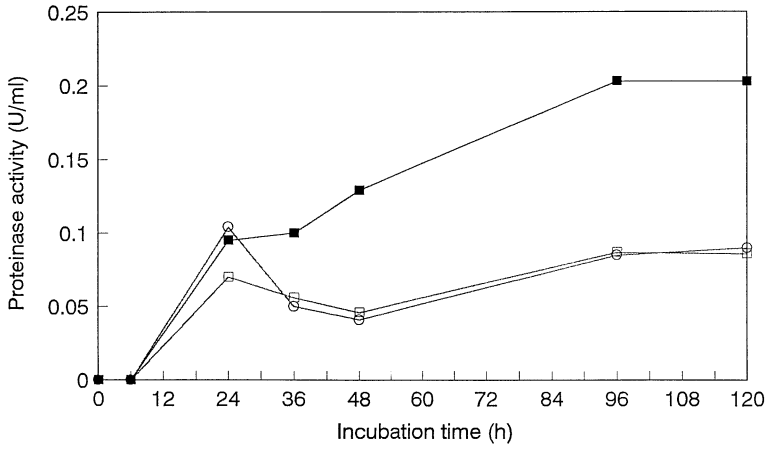

Fig. 3. Keratinolytic activity (U/ml) produced by *Aspergillus fumigatus* in liquid medium containing 1% chicken feather flour. Culture media with initial pH values of 5.2 (-■-), 6.4 (-○-), and 7.2 (-□-) were inoculated with conidia.

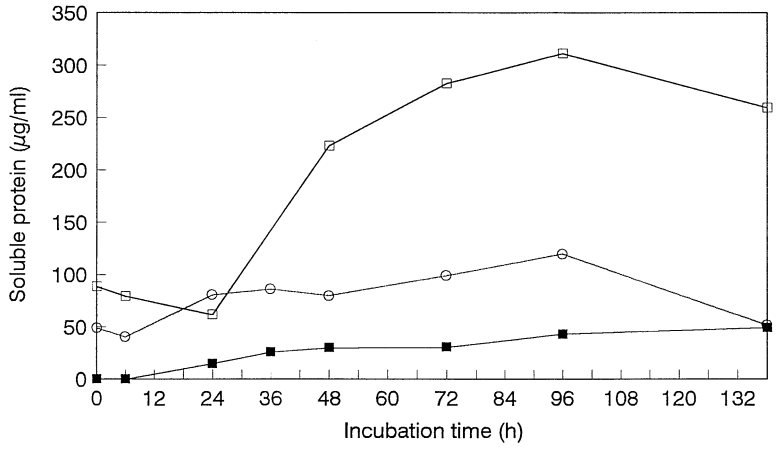

Fig. 4. Amounts of soluble protein or peptides in the culture fluid of *Aspergillus fumigatus* cultures containing 1% chicken feather flour with initial pH values of 5.2 (-■-), 6.4 (-○-), and 7.2 (-□-). (See Fig. 3).

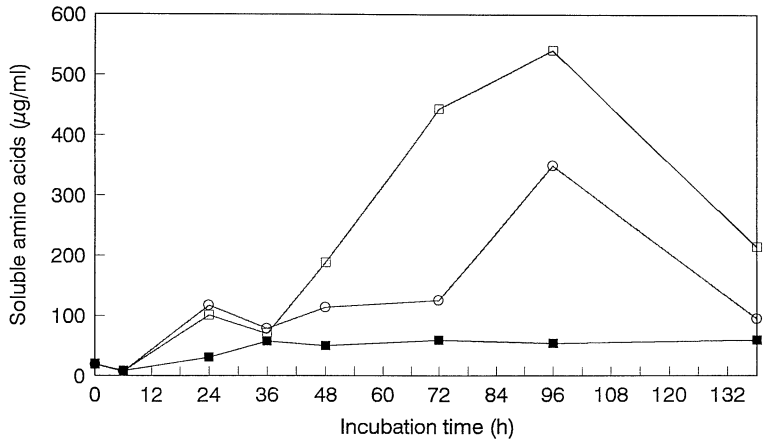

Fig. 5. Amounts of amino acids in the culture fluid of cultures of *Aspergillus fumigatus* containing 1% chicken feather flour, and with initial pH values of 5.2 (-■-), 6.4 (-○-), and 7.2 (-□-). See Fig. 3.

Keratinase properties. The *A. fumigatus* proteinase in vitro hydrolyzed autoclaved and native chicken feather keratin, the azo-substrate keratin-azure, and casein. The reaction followed Michaelis-Menten kinetics. Under our experimental conditions, the maximal hydrolysis rate of native keratin and keratin-azure was achieved within 2–10 h (Fig. 6). Casein was completely hydrolyzed within 20 min (not shown). At 42°C, the proteinase hydrolyzed keratin-azure in a broad pH range (Fig. 7). The highest activity was measured at pH 9, but substantial activities were recorded at the pH ranges of 6.5 to 8.5. At pH 8, maximal activity was recorded at 45°C (Fig. 8).

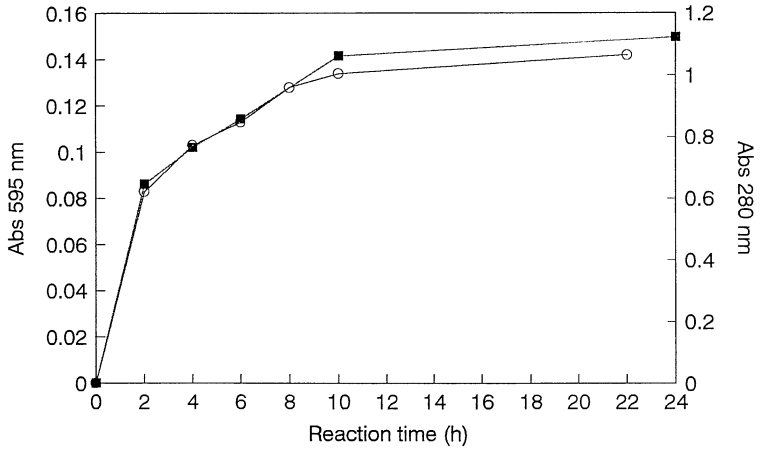

Fig. 6. In vitro hydrolysis of native chicken feather keratin (-■-) and keratin-azure (-○-) by the culture filtrate of *Aspergillus fumigatus* grown at 42°C, in a minimal medium containing 1% chicken feather flour for 48 h.

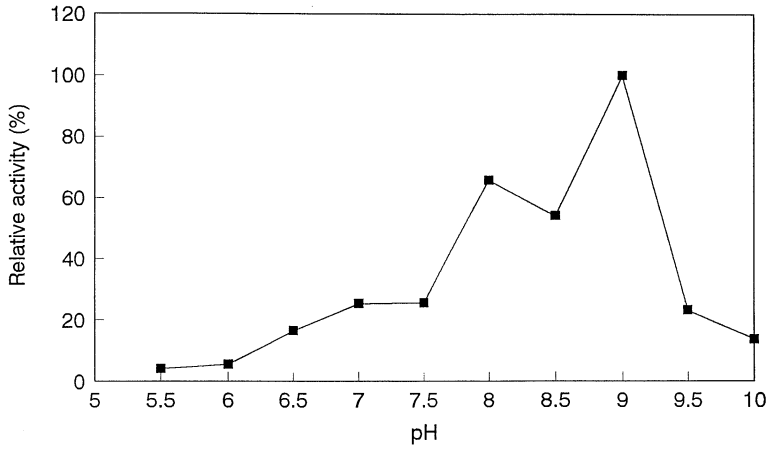

Fig. 7. Effect of the pH on hydrolysis of keratin-azure by the culture filtrate of *Aspergillus fumigatus* grown at 42°C in minimal medium containing chicken feather flour for 48 h.

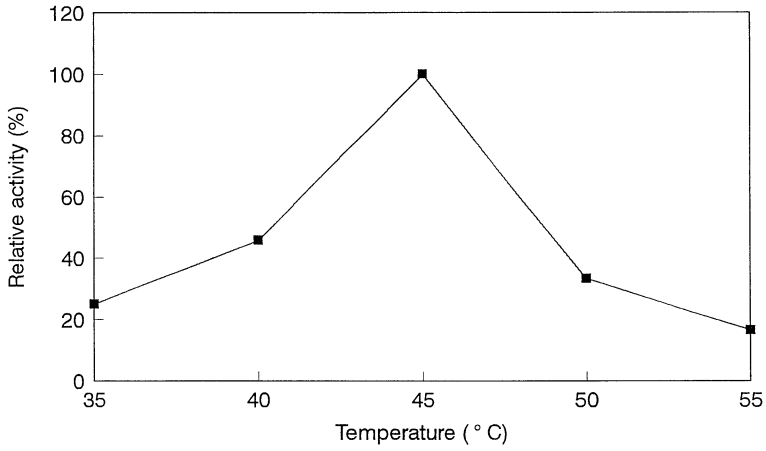

Fig. 8. Effect of temperature on the hydrolysis of keratin-azure by the culture filtrate of *Aspergillus fumigatus* grown at 42°C in a minimal medium containing chicken feather flour for 48 h.

Other proteinases produced by *A. fumigatus* showed maximal activities at temperatures of 45–55°C and at the pH range of 7–9 [9, 11, 14, 15].

The *A. fumigatus* proteinase secreted in the culture fluid was remarkably thermostable. At 70°C the culture filtrate retained about 90% of its original keratinolytic activity for 1.5 h. At 96°C it retained 20% of its activity for 20 min (Fig. 9). At room temperature the enzyme was fully active for several weeks. Such high thermostability is a common feature of the proteases produced by *A.*

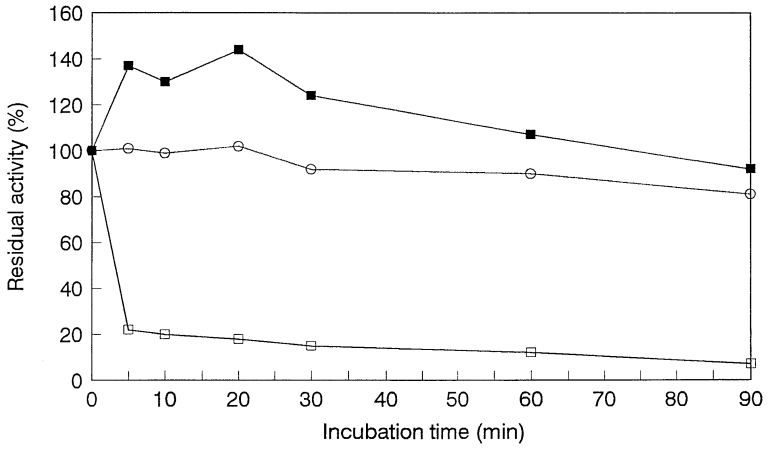

Fig. 9. Effect of the temperature on the stability of the proteinase produced by *Aspergillus fumigatus*. Enzyme samples were incubated at 50°C (-■-), 70°C (-○-), and 96°C (-□-) for different times and then tested for hydrolysis of keratin-azure as described in Materials and Methods. Residual activities were calculated with the activity of the nonincubated enzyme sample set as 100%.

fumigatus. Its purified elastase retained 50% of its original activity when held at 60°C for 1 h [14]. Moreover, incubation of the *A. fumigatus* culture filtrate at 50°C resulted in a slight increase in its keratinolytic activity (Fig. 9). This increase may be due to dissociation, at high temperature, of an enzyme inhibitor interacting with the proteinase, or to a transition of the active protein to a more appropriate conformation for forming the enzyme-substrate complex. Surprisingly, the original keratinolytic activity of the *A. fumigatus* culture filtrate, held at 96°C for 15 min, gradually recovered when the enzyme was reincubated at 4°C (not shown). This fact favors the hypothesis that a more appropriate conformation is achieved by the enzyme at 50°C.

The capacity to use keratin and to produce keratinases is not a feature restricted to *A. fumigatus* or to fungi in general. Several other microorganisms have been reported as true keratinolytics [2, 3, 8, 13, 17, 18, 20, 22, 23, 25]. Among them, *Streptomyces pactum* DSM 40530 was recently described as a keratin-degrading microorganism [3]. Its culture filtrate disintegrated whole chicken feathers at the temperature range of 40–70°C, but the purified enzyme, a serine protease, solubilized less than 10% of the native substrate. The capacity of the *A. fumigatus* culture filtrate to completely solubilize chicken feather flour and the remarkable thermostability of the involved proteinase encourage further investigation of the keratinolytic enzyme system of this fungus. Its keratinase may be helpful in the development of a rational process for the enzymatic improvement of feather meal. However, as *A. fumigatus* is a serious pathogen of fowl, this would probably negate its use for recycling keratin for feed. An enzyme purification protocol is now being carried out. The *A. fumigatus*-purified enzyme will then be studied for its biochemical properties and the keratinase-encoding gene used for genetic engineering of the transformation of heterologous cells.

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