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A Novel Industrially Promising Keratinase Isolated from *Aspergillus aureolatus* Changes its Substrate Specificity after pH-Mediated Autolysis

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Abstract: Microbial proteolytic enzymes are green catalysts with many potential applications. Keratinolytic enzymes (or keratinases) are crucial for efficient keratin degradation, making them essential in waste management, biomedicine, cosmetics, and various industrial applications. This study describes the production and characterization of a protease with keratinolytic activity from *Aspergillus aureolatus*. The protease was isolated from the culture fluid on the 5th day of cultivation with keratin hydrolysate through precipitation, dialysis, and isoelectric focusing. The enzyme exhibited limited self-cleavage at a moderately acidic pH range of 4.0-4.5. The molecular weight of the ancestral protease was identified as 27 kDa, while the self-cleaved form was 20 kDa. The self-cleaved protease showed increased specificity to keratin and maintained activity in acidic pH buffers, distinguishing it from its ancestral form. These properties suggest the enzyme's potential applications in biomedicine, biotechnology, and various industrial processes, where efficient keratin degradation is highly beneficial.

Keywords: Proteases, Fungal Proteases, Substrate Specificity, Keratinases, Keratinolytic Activity

Introduction

Fibrous Proteins, along with globular and membrane-bound, constitute one of the three main structural classes of proteins (Fibrous Proteins, 2006). Fibrous Proteins are characterized by their filamentous, robust, and often degradation-resistant structures and they serve crucial structural and protective functions in the organism, predominantly found in connective and epithelial tissues (Halper and Kjaer, 2014). The major and most abundant Fibrous Proteins include collagens, α-keratins, corneous β-proteins, elastin, fibrin, and fibroin.

Protein degradation is usually executed by proteolytic enzymes (proteases). Proteases targeting Fibrous Proteins are less common than those acting on globular proteins or those exhibiting broad substrate specificity. It is known that the activity towards Fibrous Proteins is controlled by several factors, such as the structure of the enzyme's active site, its size and structural organization, the overall charge of the molecule, and its domains (Hedstrom, 2002; Diamond, 2007). Despite potential homology, proteins belonging to the same family or clan can exhibit varying activity and specificity (Perona and Craik 1997; Luo and Hofmann, 2001).

Proteases with high activity towards Fibrous Proteins have various applications. In the industry, they are used to remove hair from animal hides and chemical tanning processes (Sundararajan *et al*., 2011; Abraham and Sánchez 2014). In medicine, they are used to create dressings that promote wound healing (Ye *et al*., 2020; Ye *et al*., 2022). In cosmetology, they are used as gentle skin peels (Gupta *et al*., 2013; Hassan *et al*., 2020). In molecular biology, they are used to purify nucleic acids from coprecipitated proteins (Su *et al*., 2020).

Among microbial producers of proteolytic enzymes, the genus *Aspergillus* is well-known for secreting active enzymes with a diverse range of activities, making it an intriguing and promising subject for research (Ward *et al*., 2005; Ntana *et al*., 2020). However, despite their recognized hydrolytic potential, significantly fewer proteases from fungi have been described than those from bacteria.

The lack of data on proteases from microfungi and their known biotechnological potential underscores the need to find and describe new proteases that are highly active and specific toward Fibrillar Proteins. This research is crucial for both fundamental understanding and practical applications.

Materials and Methods

Strain Management and Cultivation

Aspergillus aureolatus from the fungotheca of the Lomonosov Moscow State University was chosen as the study subject based on previous research (Shestakova *et al*., 2023). The strain was maintained on Malt Extract Agar (MEA) at 28°C in slant tubes and subcultured every two weeks. One-week-old slant cultures were used for inoculation.

For submerged cultivation of *A. aureolatus*, spores were washed into 100 mL of the inoculation medium (%: Malt extract-8.7, glucose-2.0, peptone-0.1) and the flask was placed in a shaking incubator (200 rpm, 28°C) for 48 h (Sharkova *et al*., 2015). After pre-cultivation, 3 mL of the biomass was aseptically transferred to 100 mL of the fermentation (biosynthetic) medium (%: Glycerol-7.0, glucose-3.0, keratin hydrolysate-0.5, KH_2PO_4-0.05, NaCl-0.05) and subsequently cultivated under the same conditions. Sterile samples were taken daily and the mycelial mass was paper-filtered from the culture fluid. The culture fluid was kept on ice and used for proteolytic profiling or protein precipitation.

Proteolytic Activity Assessment

The proteolytic activity of the strain or enzyme preparations was measured using different substrates prepared on 100 mM Tris-HCl buffer (pH 8.2) unless otherwise indicated (Timorshina *et al*., 2022). Each reaction was performed in triplicates. The reactions were carried out in 2 mL plastic tubes placed in rotating (600 rpm) incubators at 37°C. For daily sampling and constructing the proteolytic profiles of *A. aureolatus*, 0.2% solution of azocasein and 1.0% solution of keratin were used. For assessing substrate preferences, 0.2% suspension of azocoll, as well as fibrin blue and hemoglobin (0.2-1.0% in 100 mM acetate buffer, pH 4.7, respectively) were prepared. To initiate the reaction, 100 µL of the sample was mixed with 200 µL of substrate. After exactly 30 min, the reaction was stopped with 300 µL of 10% Trichloroacetic Acid (TCA). After centrifugation at 13400 rpm for 7 min, the optical density of the supernatants was measured at 280 nm (hemoglobin and keratin), 340 nm (azocasein), 520 nm (azocoll), 620 nm (fibrin blue). For native substrates (hemoglobin and keratin) one unit of activity was defined as the amount (µM) of tyrosine released in the reaction mixture per minute. For dyed substrates, an increase in absorbance by 0.01 was considered as an activity unit. Similar activities were measured for proteinase K (0.5 mg/mL), prepared in 100 mM Tris-HCl buffer (pH 8.2) and these were used for comparison with the results obtained in the study.

Protein Purification

After the proteolytic profiling, *A. aureolatus* was cultivated under the described conditions until peak activity was reached. To precipitate the proteins, the content of the flask was filtered to remove mycelia and supplemented with $(NH_4)_2SO_4$ to 80% saturation (608 g/L). After forming the precipitate at 4°C for 48 h, it was centrifuged, collected, transferred to a dialysis tube with a pore size of 3.5 kDa, and dialyzed against 1 mM Tris-HCl buffer, pH 8.2, for 16 h. The contents of the tube were then loaded onto a column for Isoelectric Focusing (IEF). IEF was performed using the method of Westerberg in sucrose (0-40%) and ampholine (pH 2-12) gradients (Allen *et al*., 1984). The column contents were then collected in fractions of 1.5 mL and for each fraction, pH and proteolytic activity were measured.

Gel Electrophoresis and Zymography

To study the homogeneity and potential molecular weight of the protease fractions, classical SDS-PAGE and casein zymography were used. The latter was performed according to Hu *et al*., with modifications: A 0.2% solution of Hammerstein casein was incorporated into the gel instead of fibrin and the digestion incubation time was reduced from 16 to 12 h (Hu *et al*., 2019). Casein is known as an easily digestible substrate, making it suitable for detecting even small amounts of proteolytic enzymes with different substrate specificities. For further research on hypothetical self-cleavage properties, the fraction containing a potential ancestral protease was incubated (1:1 v/v ratio) at 37°C for 1 h in 50 mM citrate buffer with pH 4 and 6, respectively and subsequently separated by SDS-PAGE.

Results and Discussion

Proteolytic Profiling of A. aureolatus

Aspergillus fungi are well-known for producing proteolytic enzymes with varying activities and specificities (Ntana *et al*., 2020; Ward *et al*., 2005). *A. aureolatus* was previously screened for proteolytic activity and preliminary tests (Shestakova *et al*., 2023) showed that it can cleave both fibrous and globular proteins. Since most proteases of fungi are known to be inducible, in this research *A. aureolatus* was cultivated in the liquid medium containing hydrolyzed keratin as a sole nitrogen source. For profiling, general proteolytic and keratinolytic activities were measured, with the results presented in Fig. (1).

As illustrated, peaks in general proteolytic and keratinolytic activity were observed on the 5^{th} day of cultivation. A visible time-dependant correlation between specific (keratinolytic) and non-specific proteolytic activities suggests that *A. aureolatus* produces proteases with specificities that include, but are not limited to, keratin. A significant decrease in proteolytic activity observed after the peak day is likely caused by aging and autolysis of the liquid culture. Interestingly, in the previous study *A. aureolatus* did not show any keratinolytic activity when cultivated in Petri dishes, in contrast to submerged fermentation conducted in this study (Shestakova *et al*., 2023). Thus, keratinase production by *A. aureolatus* is inducible and controlled by the environment of the fungal mycelia.

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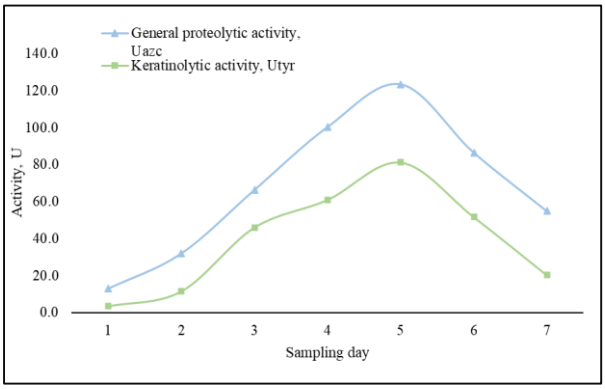

Fig. 1: Proteolytic profile of *A. aureolas* (general proteolytic and keratinolytic activity)

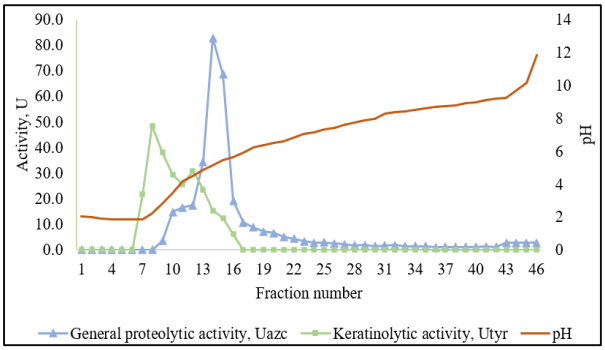

Fig. 2: IEF of *A. aureolas* extracellular protein preparation obtained after 5 days of cultivation with keratin hydrolysate

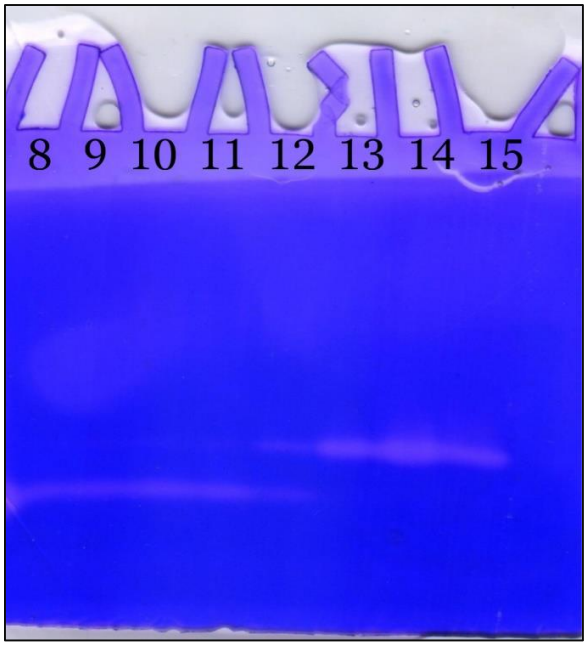

Fig. 3: Casein zymography of fractions with IEF-separated extracellular proteins of *A. aureolas*

Protease Purification and Visualization

For keratinase isolation, *A. aureolatus* was cultivated as previously described for 5 days and the culture fluid was used for extracellular protein precipitation. After collection and dialysis, proteins were separated by IEF. In the resulting fractions, pH and general proteolytic activity were measured in Fig. (2).

The highest proteolytic activity determined with azocasein was observed in fraction №14 while the highest keratinolysis values were found in fraction №8. Thus, we assumed that there are two enzymes with different substrate specificities with isoelectric points 4.9 and 2.9, respectively. To determine the true number of proteases, present on the active fractions, we performed casein zymography Fig. (3).

According to the zymogram, fractions 11-12 likely contain two proteases in different proportions and these proteases are predominant in other fractions with peak activities. Since some proteases have pH-dependent substrate specificity (Gillmor *et al*., 1997; Hedstrom, 2002) and others are known to undergo pH-mediated autolysis (Inomata *et al*., 1985; Lim *et al*., 2011; Salganik *et al*., 2012), we hypothesized that A. aureolatus produces one keratinase, which undergoes self-cleavage at lower pH. To test this hypothesis, we performed zymography on the extracellular protein concentrate (freeze-dried after dialysis) and additionally incubated fraction №14 (containing a potential ancestral protease) in buffers with pH 4 and 6 with subsequent SDS-PAGE analysis of the fragments. The results are shown in Fig. (4).

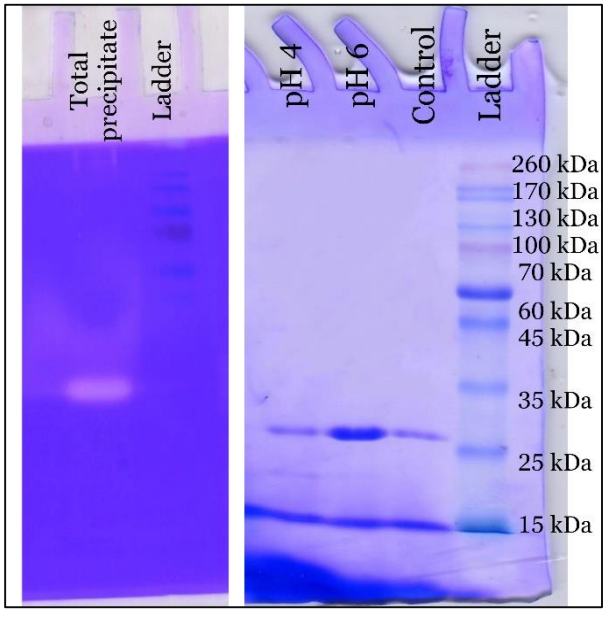

Fig. 4: Casein zymography of extracellular proteins precipitate (A) and SDS-PAGE of the fraction №12 incubated in buffers with different pH (B)

The total extracellular protein preparation of *A. aureolatus* obtained after submerged fermentation with keratin hydrolysate contains one protease (Fig. 4A) with a molecular weight of approximately 27 kDa. The culture fluid of fungi is usually alkaline due to deamination reactions (Cavello *et al*., 2013), supporting the hypothesis of pH-mediated autolysis, as different pH gradient zones are formed during IEF. It is also evident that at pH 4, a part of the protease's molecule is cleaved off (Fig. 4B). Since there is only one proteolytic enzyme in the fraction, we suggested, that self-cleavage occurred at low pH. The mass of the cleaved-off fragment was around 7 kDa and the self-cleaved protease has a molecular weight of around 20 kDa.

The enzyme's activity and substrate specificity depend on the active site structure, local and general charge, and conformation of the molecule, and these characteristics may have changed after pH-mediated autolysis. Therefore, we analyzed the proteolytic activity of both ancestral and self-cleaved proteases with diverse proteolytic substrates.

Substrate Specificity Assessment

Accurate substrate specificity assessment requires equal conditions for setting the reactions. This requires the protease to be purified and its total concentration to be known. However, both fractions containing the ancestral and self-cleaved protease have more than one protein. Thus, calculating relative (by protein content) or absolute activities would be incorrect. As protein purification is a separate and time-consuming task, we decided to calculate the ratio of specific activities determined with other proteinaceous substrates to general proteolytic activity. Additionally, we conducted the same measurements for a commercially available Proteinase K. The results are shown in the Table (1).

Table 1: Activities of *A. aureolas* protease (self-cleaved and ancestral forms) and Proteinase K

Substrate	Proteases		
	Ancestral protease of A. aureolas	*Self-cleaved protease of A. aureolas*	*Proteinase K*
1.0% keratin, pH 8.2	0.39	0.8	0.52
0.2% azocoll, pH 8.2	0	0	0.03
1.0% hemoglobin, pH 4.7	0	0.50	0.44
0.2% fibrin blue, pH 4.7	0	0.28	1.3

Overall, a higher value indicates a higher specificity and the table shows that the substrate specificity of the self-cleaved protease differs from that of the ancestral protease. Thus, pH-mediated autolysis alters the proteolytic activity of the enzyme. Interestingly, after self-processing, the protease of *A. aureolatus* showed activity at low pH, and globular substrates (hemoglobin) are hydrolyzed better than fibrous (fibrin blue). Additionally, its specificity for keratin (insoluble fibrous protein) also increased. However, none of the forms showed collagenolytic activity. Thus, pH-mediated limited proteolysis may have influenced the overall charge of the protease molecule and stabilized its binding to the substrate, thereby promoting better proteolysis. The keratinolysis rate of the self-processed protease of *A. aureolatus* was better than that of the commercially available keratinase Proteinase K, known as a reference keratinolytic enzyme (Ebeling *et al*., 1974; Li *et al*., 2013).

A proteolytic enzyme with the described features may have practical applications. For example, in wound therapy, such an enzyme would be useful for necrotic tissue removal, as it may be active against the major protein components of the wound (McCarty and Percival, 2013). In the leather industry, this enzyme may be applied for skin dehairing, preserving the skin from damage caused by chemical agents used in current protocols (Khambhaty, 2020). Additionally, since the dehairing process usually takes place at an alkaline pH that is subsequently decreased for tanning introducing a protease active at low pH is a promising and cost-effective alternative (Hasan *et al*., 2022). Other potential applications of the described enzyme include food and feed processing, keratin waste biodegradation, laundry detergent production, protein hydrolysate production, and others(dos Santos Aguilar and Sato, 2018; Razzaq *et al*., 2019; Nnolim *et al*., 2020; Li, 2021).

Conclusion

Thus, the production of protease with keratinolytic activity was demonstrated for *A. aureolatus* when submergedly fermented with keratin hydrolysate. Proteolytic profiling showed a peak of activity on the 5th day of cultivation. A novel protease with keratinolytic activity was isolated from the culture fluid of A. aureolatus using precipitation, dialysis, and IEF. It was shown that the enzyme undergoes limited self-cleaved at a moderately acidic pH (4.0-4.5). No limited self-cleaving keratinases and proteases, in general, were previously described for micromycetes, which highlights the importance and novelty of this study. The ancestral protease's molecular weight was 27 kDa, while that of a self-cleaved 20 kDa. It was demonstrated that, in contrast to the ancestral form, a self-cleaved form of the protease has a higher specificity to keratin and possesses activity in acidic buffers. The described features make the isolated enzyme a promising candidate for use in biomedicine,

biotechnology, and industry. However, more biochemical features (pH and temperature optima, stability, amino acid sequence, etc.) must be elucidated.

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Author's Contributions

Arina Mokhova: Made substantial contributions to the data acquisition, analysis, and visualization and prepared the manuscript.

Anna Shestakova: Contributed to conceptualizing the research project, proofread the article, and generally mentored the research.

Ethics

This article is original and contains unpublished material. The authors declare no conflict of interest.

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