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Isolation and Characterization of a Novel Feather-Degrading Bacterial Strain

Sidnei Sangali and Adriano Brandelli*

Departamento de Ciência de Alimentos, ICTA, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, Brazil, E-mail: abrand@vortex.ufrgs.br

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Abstract

Feather waste, generated in large quantities as a byproduct of commercial poultry processing, is almost pure keratin, which is not easily degradable by common proteolytic enzymes. Feather-degrading bacteria were isolated from a Brazilian poultry industrial waste. Among these isolates, a strain identified as kr2 was the best feather-degrading organism when grown on basal medium containing 10 g/L of native feather as a source of energy, carbon, and nitrogen. The isolate was characterized according to morphological characteristics and biochemical tests belonging to the *Vibrionaceae* family. Keratinolytic activity of this isolate was monitored throughout the cultivation of the bacterium on raw feather at different temperatures. The optimum temperature for growth was about 30°C, at which maximum enzyme and soluble protein production were achieved. The enzyme had a pH and temperature optima of 8.0 and 55°C, respectively.

Index Entries: *Vibrioneceae*; feather digestion; keratinase; poultry waste; protease.

Introduction

Animal wastes have been used as a nutrient source in crop production. During the last three decades, research has been conducted to improve agronomic utilization of animal waste, including poultry waste. The large increase in size of individual production facilities and the total poultry industry has resulted in enormous increases in waste, particularly litter, to be managed. Feather waste, generated in large quantities as a byproduct of industrial poultry processing, is nearly pure keratin protein *(1)*. Keratin in its native state is not degradable by common proteolytic enzymes such

*Author to whom all correspondence and reprint requests should be addressed.

as trypsin, pepsin, and papain. However, this protein does not accumulate in nature, and keratinolytic activity has been reported for species of *Streptomyces (1,2)*, *Bacillus (3,4)*, and some other microorganisms *(5,6)*.

Currently, feather waste is utilized on a limited basis as a dietary protein supplement for animal feedstuffs (feather meal). Prior to use, the feather waste is steam pressure cooked or chemically treated to make it more digestible. These treatment processes require significant energy and destroy certain amino acids *(7)*. Enzymatic hydrolysis by keratinolytic microorganisms represents an alternative method to improve the nutritional value of feather waste. It has already been demonstrated that feather-lysate obtained by *Bacillus lichineformis* PWD-1 has similar nutritional features to soybean protein for feed use *(8)*. Indeed, the development of bioprocesses using keratinolytic microorganisms addresses three important needs: ecological, because the degradation of feathers in the environment leads to production of sulfur compounds; nutritional, by generating a product with better digestibility and nutritional value; and economic, because the current processes are expensive.

In this article, we describe the isolation and characterization of a novel keratinolytic bacterial strain able to completely disintegrate chicken feathers.

Materials and Methods

Media and Culture Conditions

The basal medium used for isolation, maintenance, growth, and fermentation analysis of the feather-degrading microorganism contained 0.5 g/L of NaCl; 0.3 g/L of Na_2HPO_4; 0.4 g/L of NaH_2PO_4, supplemented with 10 g/L of whole feather or 10 g/L of feather meal; and 15 g/L of agar for plates. Feathers were washed in distilled water prior to being sterilized by autoclaving (120°C at 101 kPa for 15 min). For growth determination, the microorganisms were cultured in 500-mL Erlenmeyer flasks containing 100 mL of whole-feather medium. Growth was followed at 25, 30, 37, and 42°C with aeration by the rotation of the flasks at 3.6*g*. Aliquots of 0.5 mL were removed at different times, and cell counts were determined by the plate count on nutrient agar (Difco BRL, São Paulo, Brazil), following standard procedures *(9)*. Samples were prepared for plating as described previously *(10)*.

Isolation of Microorganisms

Feather were collected from several sites at a local poultry company (Avipal, Porto Alegre, Brazil). Feathers were flooded in phosphate-buffered saline, and the suspension was used to streak the feather-meal agar plates, which were allowed to grow at 37°C for 1–5 d. Single colonies were screened for their ability to hydrolyze keratin in feather-meal agar plates. Colonies producing clearing zones in this medium were selected for further work. The purity of isolates was checked by repeated streaking.

Identification of Feather-Degrading Isolate

A feather-degrading isolate was identified according to morphological examinations and several biochemical tests *(11)*.

Enzyme Preparation

The isolate was cultivated for 80 h in whole-feather medium, from a 10^6 CFU/mL culture. The culture medium was centrifuged at 10,000*g* for 10 min, and the supernatant was used as enzyme preparation. The protein concentration of this enzyme preparation was 2.2 mg/mL.

Keratinolytic Activity

Keratinolytic activity was determined as described previously *(12)*, using azokeratin as a substrate. The reaction was initiated by pipeting 150 µL of enzyme into 250 µL of azokeratin (2% in 50 m*M* Tris, pH 8.0). The reaction mixture was incubated for 30 min at 50°C, and then 1.2 mL of 10% trichloroacetic acid was added. The contents were mixed and allowed to stand for 15 min to ensure complete protein precipitation. Samples were centrifuged at 10,000*g* for 5 min, 1.2 mL of the supernatant was transferred to a clean tube, and absorbance at 440 nm was determined. One unit of enzyme is the amount of enzyme that causes a change of absorbance of 0.01 at 440 nm/30 min at 50°C. To study the effect of different chemicals on enzyme activity, substances were added to the buffer before incubation. Azokeratin was synthesized as described previously *(13)*.

Analysis of Soluble Protein

Cell-free supernatant of each feather culture was used for the determination of soluble protein by the Folin phenol reagent method *(14)*.

Comparison with Commercial Proteases

The activity of kr2 keratinase on azokeratin and azocasein (Sigma, St. Louis, MO) was compared with commercially available proteases such as pronase (Sigma), trypsin (Sigma), papain (Merck, Darmstadt, Germany), alcalase (Novo Nordisk, Bagsvaerd, Denmark), and flavorzyme (Novo Nordisk). Enzymes were dissolved at 1 mg/mL in 50 m*M* Tris, pH 8.0, and then assayed for azoproteins as described in Keratinolytic Activity subsection.

Results and Discussion

Isolation and Adaptation of Feather-Degrading Microorganisms

After an initial enrichment on mineral-feather medium, feather-degrading cultures were screened on feather-meal agar. At least five distinct colony morphologies were observed when streaked onto feather-meal agar plates. Three produced clearing zones in feather-meal agar, and one pale-pink, rod-shaped bacterium demonstrated the most pronounced clear-

ing zones and the best feather-degrading activity. The isolate was selected for the purpose of identification and to characterize further its feather-degrading properties. Cells of the isolate were grown on whole feathers and transferred at frequent intervals to the basal medium, containing whole feathers. Eventually, after repeated transfer on steam-treated feathers, the isolate was able to degrade non-steam-treated feathers.

Characterization of Feather-Degrading Isolate

The identification of the feather-degrading isolate was based on cell morphology, colony morphology, growth characteristics, and several biochemical tests. Microscopic observation of the isolate showed a straight rod (2 to 3 μm by 0.5 μm) without endospores. The bacterium grew aerobically, was Gram-negative, and strongly sensitive to Vibrio static agent O/129. Collectively, these characteristics indicated that the isolate was of the family *Vibrionaceae*. The strain grew very slowly on sheep-blood agar or human-blood agar, and did not cause β-hemolysis. We propose kr2 as the strain designation for this keratinolytic strain.

Degradation of Feathers by Isolate

It was observed that aerobic growth by the isolate on whole feathers, with the feathers as its primary source of carbon, energy, nitrogen, and sulfur, resulted in complete degradation of the keratin protein after 5 d of incubation at 30°C (Fig. 1). Note that all feather barbule and almost all feather rachis were degraded after 2 d.

Figure 2A shows the growing curves for the bacterial isolate at several temperatures. The microorganism grew well at both 25 and 30°C, but did not at 37 and 42°C. Generally, the keratinolytic microorganisms described have higher optimum temperatures of growth and feather degradation *(4,6,10)*.

The production of soluble protein at several temperatures was monitored (Fig. 2B). Higher amounts of soluble protein were produced at 30°C. The maximum protein concentration was 2.5 mg/mL.

The production of keratinase by the organism at several temperatures was determined during feather biodegradation, based on the hydrolysis of azokeratin. Generally, keratinolytic activity increased with cultivation time (Fig. 2C), with the maximum activity coinciding with the start of the stationary phase. This was similar to results observed for *Streptomyces fradiae* growing on wool *(15)*.

Feather-waste biodegradation using microbial sources represents an alternative way to improve the nutritional value of feather, because feather waste is currently utilized on a limited basis as a dietary protein supplement for animal feedstuffs. The amino acid composition of keratin is characterized by a low content of lysine, which is the main limiting essential amino acid in food. Protein concentrates from keratin sources show a deficit of lysine, which can be enhanced by the addition of lysine-rich protein or by plastein reaction *(16)*. Indeed, a feather-lysate produced

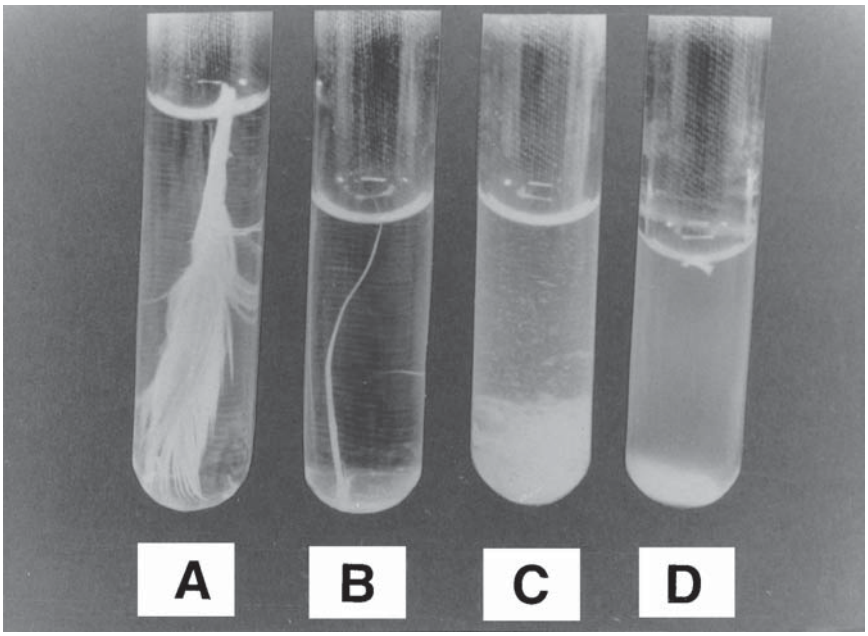

Fig. 1. Feather degradation by kr2 microorganism after (A) 1, (B) 48, (C) 72, and (D) 96 h.

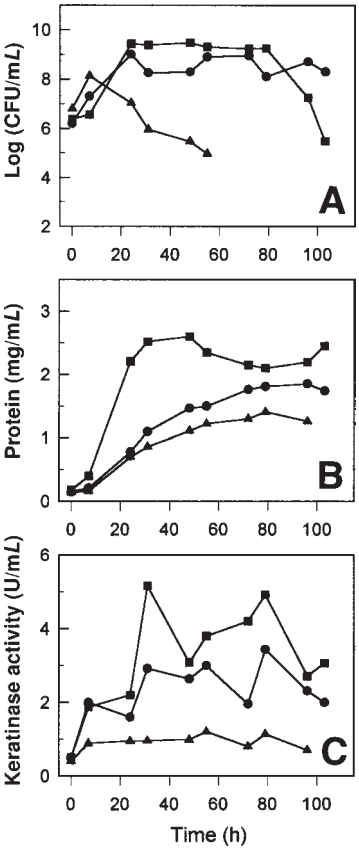

Fig. 2. Effect of temperature on **(A)** microorganism growth, **(B)** keratinase production, and **(C)** protein production. (●) 25°C; (■) 30°C; (▲) 37°C.

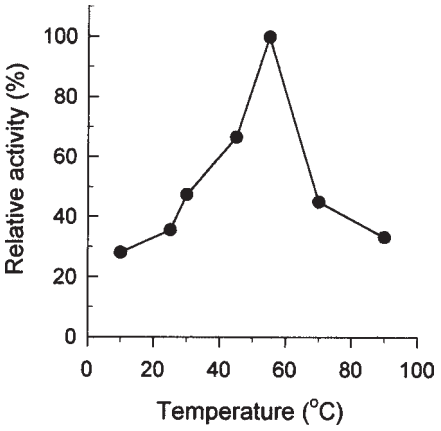

Fig. 3. Effect of temperature on activity of kr2 keratinase. Activity was measured at different temperatures against azokeratin in 50 m*M* Tris, pH 8.0.

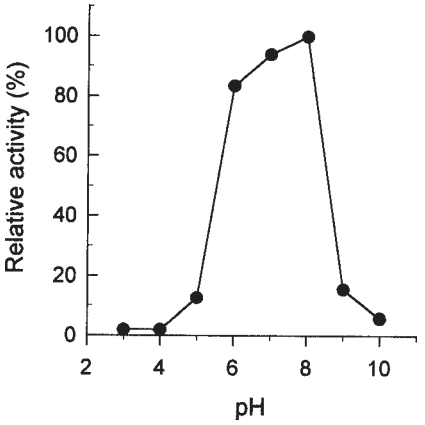

Fig. 4. Effect of pH on activity of kr2 keratinase. Activity was measured at 50°C against azokeratin in 50 m*M* citrate-phosphate buffer (pH 3.0–7.0) or 50 m*M* Tris (pH 7.0–10.0).

by *B. lichineformis* PWD-1 was demonstrated to be digestible and nutritionally equivalent to soybean protein for feed use *(8)*.

Keratinase Properties

The effect of pH and temperature on keratinolytic activity was investigated. Maximum activity was observed between pH 6.0 and 8.0, with an optimum pH at 8.0 (Fig. 3). The enzyme mixture was active between 30 and 90°C, with an optimum pH at 55°C (Fig. 4). This optimum pH for activity was in good agreement with other known keratinolytic enzymes from *S. fradiae (17)*, and *Bacillus* spp. *(18)*.

Keratinase produced by kr2 was partially inhibited by EDTA and β-mercaptoethanol, and completely inhibited by phenylmethysulfonyl

Table 1
Effect of Chemicals on kr2 Keratinase

Compound	Relative activity (%)
None	100
PMSF (2 m*M*)	0
EDTA (10 m*M*)	89
β-Mercaptoethanol (10 m*M*)	87
$CaCl_2$ (10 m*M*)	130
$MgCl_2$ (10 m*M*)	151
$ZnCl_2$ (10 m*M*)	22
$HgCl_2$ (10 m*M*)	0

Table 2
Hydrolysis of Azoproteins with kr2 Keratinase and Commercial Proteases

Enzyme	Activity on azocasein (U/mg protein)[a]	Activity on azokeratin (U/mg protein)[a]	Keratin:casein ratio
Keratinase kr2	37.0	14.4	0.39
Trypsin	25.1	5.6	0.22
Papain	21.9	2.5	0.11
Pronase	26.5	6.7	0.25
Alcalase	36.9	11.4	0.30
Flavorzyme	23.6	0	0

[a]Results are the mean of triplicate assays.

fluoride (PMSF) (Table 1), suggesting that the supernatant fluid could contain several enzymes affecting keratinolysis. Similar results were observed for species of *Streptomyces (19,20)*. Among the metal ions tested, Hg^{2+} and Zn^{2+} strongly inhibited the enzyme, whereas Ca^{2+} and Mg^{2+} increased proteolytic activity. This last observation resembles the characteristics of calpains, which are involved in the aging of meat *(21)*. The effect of Hg^{2+} inhibiting the enzyme suggests that a free cysteine is present at or near the active site. The enzyme preparation was also active on benzoyl arginine-*p*-nitroanilide (BAPNA), indicating that a serine-protease activity is present, as in keratinases produced by *Streptomyces pactum (2)* and *B. lichineformis (3)*. These results suggest that a serine-protease and a cysteine-protease may be involved in keratin degradation by kr2.

The hydrolysis of azokeratin and azocasein by kr2 protease was compared with commercial enzymes (Table 2). Data were expressed as a ratio of hydrolysis rates of insoluble vs soluble protein realized at 50°C and pH 8.0. Under these conditions, this enzyme preparation was superior in hydrolysis of keratin substrates compared with other commercially available proteases such as pronase, derived from *Streptomyces griseus*, and alcalase, from *B. lichineformis*.

Acknowledgments

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