Biodegradation (2009) 20:727–736
DOI 10.1007/s10532-009-9260-4

ORIGINAL PAPER

Purification and characterization of an efficient poultry feather degrading-protease from *Myrothecium verrucaria*

Fabiana G. Moreira-Gasparin · Cristina G. Marques de Souza · Andréa M. Costa · Ana Maria Alexandrino · Cissa K. Bracht · Cinthia G. Boer · Rosane M. Peralta

Received: 20 October 2008 / Accepted: 31 March 2009 / Published online: 18 April 2009

Abstract The purpose of this work was to characterize an alkaline protease from the filamentous fungus *Myrothecium verrucaria* and to explore its capability to degrade native poultry feathers. The enzyme was purified to homogeneity using a single chromatographic step. Recovery was high, 62%, with a specific activity of 12,851.8 U/mg protein. The enzyme is a small monomeric protein with a molecular mass of 22 ± 1.5 kDa. It presented pH optimum of 8.3 and was stable over a broad pH range (5.0–12.0). The temperature optimum was 37°C, with thermal stability at temperatures up to 45°C. The enzyme presented an efficiency of 80.3% in the degradation of poultry feather meal, releasing amino acids and soluble peptides. It was able to hydrolyze β-keratin without necessity of chemical or enzymatic reduction of the disulphide bonds. Considering that, everyday, poultry-processing plants produce feathers as a waste products, this protease can be useful in biotechnological processes aiming to improve the transformation of poultry feathers through solubilization of β-keratin into usable peptides. Furthermore, it can also be useful in processes aiming to reduce the environmental pollution caused by the accumulation of feathers.

Keywords Biodegradation · Keratinase · Poultry feather · Protein purification · Submerged cultures

F. G. Moreira-Gasparin · C. G. M. de Souza · A. M. Costa · A. M. Alexandrino · C. K. Bracht · C. G. Boer · R. M. Peralta (✉)
Departamento de Bioquímica, Universidade Estadual de Maringá, Av. Colombo, 5790, Maringá, PR 87020-900, Brazil
e-mail: rmperalta@uem.br

Introduction

Worldwide, poultry-processing plants produce millions of tons of feathers annually as a waste product (Bertsch and Coello 2005). The large amount of feathers produced and their localized accumulation create a serious disposal problem leading to environmental pollution. The β-keratin content of the feathers is largely responsible for their high degree of recalcitrance to degradation: due to their high degree of cross-linking by disulphide bonds, hydrogen bonding, and hydrophobic interactions, keratins are resistant to degradation by common proteolytic enzymes such as trypsin, pepsin and papain (Onifade et al. 1998). However, in nature, keratins are hydrolyzed by some microorganisms that synthesize keratinases. These enzymes have the ability to degrade native keratin into smaller peptide entities that can be subsequently utilized by cells. Keratinolytic enzymes are widespread in nature and are produced by several microorganisms, most of them isolated from poultry wastes. They include bacteria, actinomycetes and fungi (Gupta and Ramnani 2006).

Studies on feather-degrading microorganisms focus either on animal diseases or on biotechnology for processing large amounts of waste by-products in poultry-processing plants. As keratinases from *Bacillus* spp. have greater potential for applications in commercial processes, biotechnological studies have focused on that genus (El-Refai et al. 2005; Grazziotin et al. 2006; Suntornsuk and Suntornsuk 2003). Among fungi, the keratinases from dermatophytic fungi have received more attention due to medical and veterinary applications, but enzymes of this kind have been identified also in non-dermatophytic fungi (Anbu et al. 2007; Cao et al. 2008; Gradisar et al. 2005; Marcondes et al. 2008; Santos et al. 1996). Potentially at least, fungi are as useful as bacteria in the conversion of poultry feathers into economically important products such as nitrogenous fertilizers, biodegradable films, glues, feed and foils (Bertsch and Coello 2005; Gupta and Ramnani 2006; Schrooyen et al. 2001).

Myrothecium verrucaria (Albertini and Schwein) Ditmar:Fr, is a filamentous fungus commonly found in soil and plant substrates worldwide. It has been proposed as a bioherbicide against a wide range of economically important weed species in agronomic, pasture and horticultural systems (Boyette et al. 2007). Recently, we described for the first time that *M. verrucaria* possesses the ability of completely disintegrate poultry feather when this material was offered as the only carbon and nitrogen sources to the fungus in submerged cultures (Moreira et al. 2007). The purpose of the present work was to obtain a homogeneous fraction of the *M. verrucaria* protease and to determine its effectiveness in degrading native feather keratin under various conditions. Most purified keratinases known to date do not degrade completely native keratin (Ghosh et al. 2008; Pillai and Archana 2008; Ramnani et al. 2005), but a highly efficient enzyme is very important if the purpose is to use it as a work tool for obtaining useful products from feather keratin.

Materials and methods

Microorganism

Myrothecium verrucaria (Albertini and Schwein) Ditmar:Fr CCT 1886 was obtained from the Collection Culture of the Fundação André Tosello, Campinas, SP, Brazil and was maintained on potato dextrose agar at 4°C. The conidial suspensions were prepared by adding 10 ml of sterilized water to slant cultures and the surface was gently rubbed with a sterilized wire loop.

Pretreatment of the feathers

White poultry feathers were supplied by a local industry. Freshly plucked wet feathers were washed extensively with water and detergent. Wet feathers were dried in a ventilated oven at 40°C for 72 h. The feathers were then milled in a ball mill and passed through a small-mesh grid to remove coarse particles.

Culture conditions

Myrothecium verrucaria spores (1×10^9) were transferred to 250 ml Erlenmeyer flasks containing 50 ml of mineral media and 1.0% (w/v) poultry feather powder as substrate. The mineral solution contained per liter: KH_2PO_4 5.0 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; $CaCl_2$ 1.0 g; yeast extract 1.0 g and 100 µl of microelements solution (Montenecourt and Eveleigh 1977). The medium was previously sterilized by autoclaving at 121°C for 15 min. After autoclaving, the pH of medium was found in the range of 5.8–6.0. The cultures were incubated at 28°C on a rotary shaker at 120 rpm. After 4 days of cultivation, the culture broth was filtered thorough Whatman no 1 filter paper to retain insoluble materials, and centrifuged (5,000*g* for 20 min at 4°C). The supernatants were used as sources of keratinases.

Determination of proteolytic activity

The protease activity in the culture filtrates was assayed as keratinase activity using poultry feather powder as substrate (Nickerson et al. 1963). The reaction mixture containing 10 mg of substrate, 1.0 ml of borate buffer (100 mM, pH 9.0), and 1.0 ml of a suitable diluted culture filtrate was incubated under agitation using a stirring bar for 1 h at 40°C. The reaction was stopped by boiling and the mixture was filtered through filter paper. The amount of tyrosine released was estimated by Lowry's method using a tyrosine standard curve. One unit of enzyme activity was defined as the

amount of enzyme producing 1 μg of tyrosine/min. Alternatively, the protease activity was determined as caseinolytic activity using 1.0% (w/v) casein in 100 mM borate buffer (pH 9.0) as substrate (Boer and Peralta 2000). One (1.0) ml casein was incubated at 40°C for 1 h with 1.0 ml of a suitable diluted culture filtrate. The reaction was stopped by the addition of 2.0 ml of 0.6 M trichloroacetic acid. After 15 min in an ice bath, the mixtures were mixed on a vortex mixer, and the tubes were centrifuged at 3,000 rpm for 10 min. The amount of tyrosine released was estimated by Lowry's method using a tyrosine standard curve. A unit of enzyme activity was defined as the amount of enzyme producing 1 μg of tyrosine/min.

Estimation of protein

Protein was estimated by the dye-binding method (Bradford 1976), using bovine serum albumin (BSA) as standard protein. In the column chromatography elution, the amount of protein was measured in terms of the absorbance at 280 nm. The specific activity was expressed as the enzymatic activity/mg of protein.

Enzyme purification

All purification steps were carried out below 15°C unless otherwise specified. The culture filtrate containing the crude protease was previously centrifuged at 15,000*g* for 30 min to remove particulate material. The supernatant was concentrated about tenfold by ultrafiltration (Amicon YM-10, 10 kDa cut-off). After removal of insoluble substances by centrifugation (5 min at 15,000*g*), the supernatant was dialyzed against water and concentrated by freeze-drying. The concentrate was re-suspended in 1.0 ml of 50 mM borate buffer, pH 9.0, and applied to a Sephadex G-100 gel filtration column (90 cm × 25 mm) equilibrated and eluted with the same buffer at a flow rate of 10 ml/h. The protease active fractions were pooled and concentrated by freeze-drying, and stored at −10°C. The purity of keratinase was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS–PAGE (Laemmli 1970). Protein bands were visualized by silver staining. The following standards (MW-70 kit-Sigma) were used to estimate the molecular weight of the enzyme: bovine serum albumin (66 kDa), ovalbumin (45 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa), and β-lactoglobulin (18.0 kDa). The protease MW was also estimated by gel filtration chromatography using Sephadex G-100 equilibrated with 50 mM borate buffer, pH 9.0 plus 100 mM Nacl. The column was calibrated with standard proteins (MWGF-70 kit-Sigma).

Effect of pH and temperature on the activity and stability of the enzyme

The effect of temperature and pH on the keratinase activity was carried out firstly using the traditional methodology which involves the variation of one parameter while keeping the other constant. The temperature was varied between 25 and 60°C at pH 8.0. The pH was varied using a series of McIlvaine's buffers (pH 5.0–8.3), 0.1 M tris–Hcl buffer (pH 8.0–9.5) and 0.1 M borate buffer (pH 8.5–12.0) at 40°C. Additionally, the effect of temperature and pH on the enzyme activity was studied using a chemometric approach (Response surface methodology, RSM) based on the use of a matrix of experiments by which the simultaneous variations of the factors can be studied (Box 1954; Myers and Montgomery 2002). A full 2^2 factorial design was used to study the effect of two factors on enzyme activity. This design was made up with its four points augmented with three replications of the centre points (all factors at level 0) and the four star points, that is, points having for one factor an axial distance to the center of $\pm\alpha$, whereas the other two factors are at level 0. The axial distance α was chosen to be 1.41 to make this design orthogonal. A set of 11 experiments was carried out. The central values (zero level) for the experimental design were pH 8.0 and temperature 40°C. For two factors the equation model is:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2 \quad (1)$$

Y is the response (keratinase activity, U/ml); x_1 is the pH and x_2 the temperature (°C). The b_i, b_{ii} and b_{ij} terms represent the parameters of the model. The results were analysed by the Experimental Design Module of the Statistica 7.0 software (Statsoft< Tulsa, OK, USA). The model allowed evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the chosen dependent variables. The quality of the fit of the polynomial

model equation was expressed by the coefficient of determination R^2 and its statistical significance was evaluated by an F-test.

For the measurement of pH stability, the enzyme was kept at 30°C for 6 h at different pH values (3–11) and the residual proteolytic activity was determined under standard assay conditions. The following buffer systems were used: citrate buffer, pH 5.0–6.0, phosphate buffer, pH 6.0–7.5; Tris–Hcl buffer, pH 8.0–8.5; borate buffer, pH 9.0–11.0.

Thermal inactivation was examined by incubating the purified enzyme at 30, 40, 50 and 60°C for 60 min. Aliquots were withdrawn at desired time intervals to test the remaining activity under standard conditions. The non-heated enzyme was considered the control.

Effect of enzyme inhibitors, solvents, detergents and metal ions

To study the influence of protease inhibitors, the purified enzyme (10 μg/ml) was pre-incubated with the following protease inhibitors: phenylmethanesulphonyl fluoride (PMSF, 1 mM), EDTA (5 mM), pepstatin A (0.7 μg/ml), iodoacetamide (0.05 mM), dithiothreitol (DTT, 1 mM), β-mercaptoethanol (βME, 1 mM); all of them in 100 mM borate buffer (pH 9.0) for 15 min at 30°C.

The effects of solvents and detergents on the keratinase activity were tested by incubating the purified enzyme (10 μg/ml) with isopropanol, methanol, ethanol, butanol, glycerol, acetonitrile, Triton X-100, SDS, Tween 80 and sodium azide previously mixed with 100 mM of borate buffer (pH 9.0). To determine the effect of cations on the keratinase activity, the purified enzyme was incubated in the presence of each cation (as chloride salt) at 5.0 mM.

Degradation of keratinous materials by the purified enzyme

Purified enzyme (final activity of 20 U/ml) was added to 100 mg of human hair, human nail, sheep wool and poultry feather powder in 9.0 ml of 100 mM borate buffer, pH 8.6 and the mixtures were maintained in a shaking 37°C-water bath at 130 rev/min for 3, 6, 12 and 24 h. Sodium azide (1 mM) was added to avoid contamination. Controls of keratinous materials were run in the same conditions, using purified enzyme solution previously boiled for 5 min. The dry weights of the remaining insoluble keratinous materials were determined after drying at 105°C for 12 h. The amount of degraded material was determined as the difference between the amount initially present and the amount still remaining after the incubation with the enzyme. The amino acids and soluble peptides resulting from the hydrolysis of the various keratins were quantified using the ninhydrin method with L-alanine as standard (Starcher 2001).

Statistical analysis

All analyses were performed at least in triplicate. The data were expressed as means ± standard deviations and the t test was carried out to assess significant differences between the means. The 5% level ($P < 0.05$) was adopted as a criterion of significance.

Results

Purification of *M. verrucaria* keratinase

The feather powder degradation was achieved during cultivation of *M. verrucaria*. Electrophoretic analysis of the culture filtrate concentrated by freeze-drying revealed that keratinase was the main and also smaller protein secreted by *M. verrucaria* under the conditions used in this work (data not shown). In order to purify the keratinase, the extracellular proteins were separated using a Sephadex G-100 column. Fractions with high keratinase activity were pooled, dialyzed and lyophilized without any apparent loss of activity. Table 1 presents a summary of a typical purification procedure. At the end of the process, keratinase was purified 6.1–fold with 68% recovery. Homogeneity of the purified enzyme was revealed by SDS–PAGE, showing a single protein band by silver staining (Fig. 1, lane 2). The molecular mass of the purified enzyme was 22 kDa as determined by SDS–PAGE and 23 kDa as determined by gel filtration chromatography using a column of Sephadex G-100 (data not shown).

Effect of pH and temperature on the activity and stability of the enzyme

Figure 2a and b shows the results obtained when the optima pH and temperature were determined by the

Table 1 Purification of *M. verrucaria* keratinase

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	26,000	18.8	1,383.0	1.00	1.00
After ultrafiltration concentration	25,100	12.6	1,992.1	1.44	96.5
After gel filtration in Sephadex G-100	17,680.4	1.43	12,363.6	8.94	68.0
Post gel filtration concentrate	17,350.0	1.35	12,851.8	9.29	66.7

Fig. 1 SDS–PAGE pattern of purified protease. *Lane 1* Standard proteins, (1) bovine serum albumin (66 kDa), (2) ovalbumin (45 kDa), (3) pepsin (34.7 kDa), (4) trypsinogen (24 kDa), (5) β-lactoglobulin (18.4 kDa); *Lane 2* purified *M. verrucaria* protease

conventional approach. Maximal keratinase activity was obtained at pH 8.0 and 40°C, respectively. The results of the central composite design experiments for studying the effects of pH and temperature on the keratinase activity are presented in Table 2.

Statistical analysis of the results revealed that, in the range studied, the two variables, as well as their interactions, have a significant effect on the keratinase activity. Analysis of variance (ANOVA) and Fisher's F-test resulted in the value of $F_{(5.5)} = 176.8$, which is 35 times higher than F tabulated $\mathrm{Ft}_{(5.5;\ 0.05)} = 5.05$, what demonstrates the significance of the regression model. The following regression equation was obtained to calculate the keratinolytic activity (K.A.):

$$\begin{aligned}\text{K. A. (U/ml)} = {} & 98.43 + 21.75(\text{pH}) - 44.47(\text{pH})^2 \\ & - 22.30(t) - 41.75(t)^2 \\ & - 16.50(\text{pH} \cdot t) \end{aligned} \quad (2)$$

The R^2 value was equal to 0.99805, indicating that only 0.195% of the total variation was not explained by the model. The contour graph of the keratinolytic activity observed as a response to the interaction of pH versus temperature is shown in Fig. 2c. The results indicate optimal pH and temperatures of 8.3 and 37°C, respectively, for the enzyme.

The enzyme was stable for 24 h at temperatures up to 37°C, while at 45°C, it was stable for 2 h (Fig. 3). Concerning pH, the enzyme was stable between pH 5.0–9.0 for at least 6 h (Fig 2a).

Effect of enzyme inhibitors, solvents, detergents and metal ions

Table 3 shows the results obtained when the *M. verrucaria* protease (as keratinase and caseinolytic activities) was incubated with different proteolytic inhibitors. The enzyme was strongly inhibited by PMSF and slightly inhibited by EDTA, but not by pepstatin A and iodoacetamide. DTT and β-mercaptoethanol improved the keratinolytic activity but they did not show any effect on the caseinolytic activity of the enzyme.

The effects of several compounds on the purified keratinase is shown in Table 4. The enzyme was stable in the presence of anionic (SDS) and non-ionic (Triton X-100) detergents even when they were present at high concentrations. Different organic solvents such as methanol, ethanol, isopropanol, butanol, glycerol, and acetonitrile at high concentrations had a relatively small influence on the enzyme activity. Most divalent cations tested in concentrations as high as 5 mM, had no influence on the enzyme. Ca^{2+} and Mg^{2+} caused a slight activation, whereas Ag^{+}, Cd^{+}, Pb^{+} and Hg^{+} had inhibitory effects. The enzyme was not affected by sodium azide.

Degradation of keratinous materials by the purified enzyme

The capabilities of the purified enzyme in hydrolyzing different keratins were evaluated under the

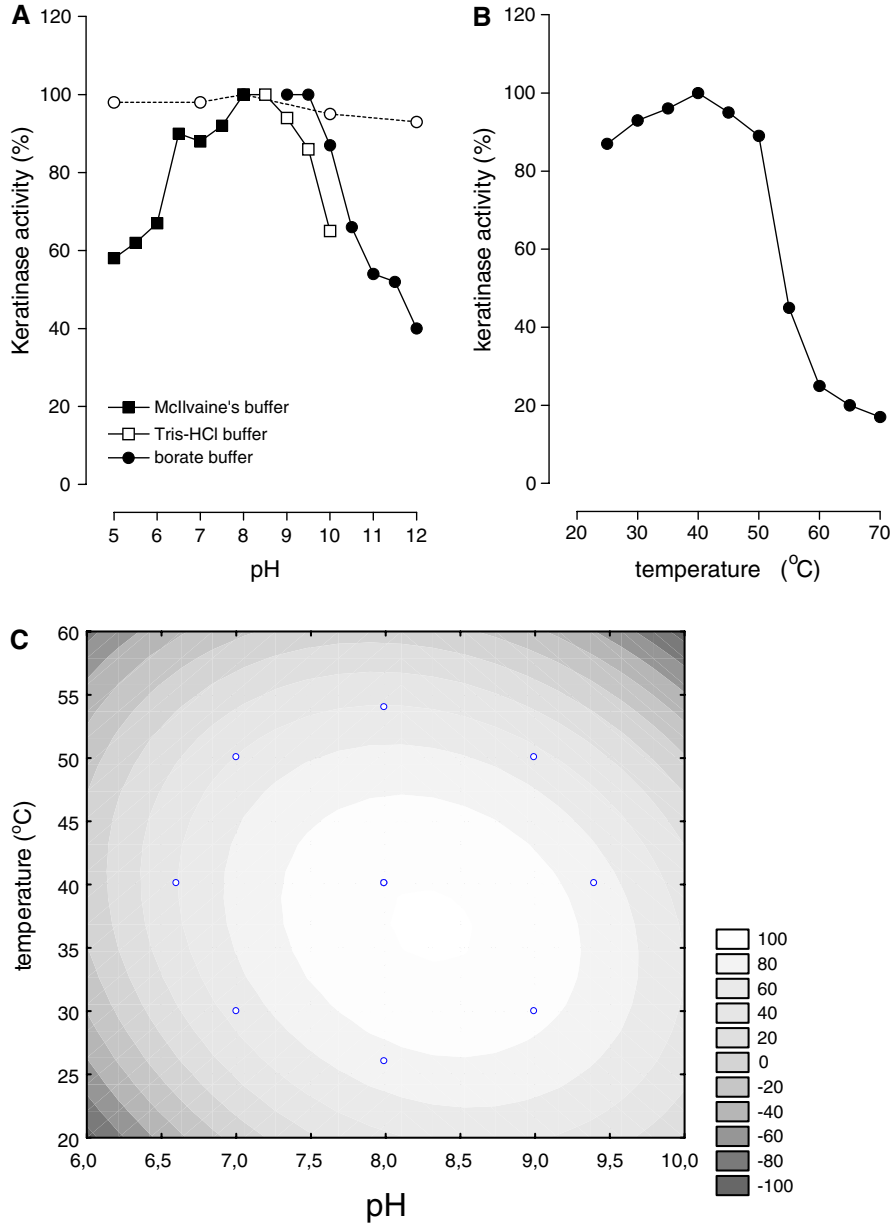

Fig. 2 Effect of pH and temperature on the keratinase activity. The optimum pH (**a**) and temperature (**b**) was determined using the conventional approach which involves the variation of one parameter and keeping the other constant. In **c** *Contour graph* of keratinolytic activity observed as a response to the interaction of pH versus temperature

optimal conditions of pH and temperature. Both the remaining insoluble material and the appearance of soluble amino acids and peptides were determined. At pH 8.3 and 37°C the purified *M. verrucaria* protease was able to hydrolyze efficiently keratin from poultry feather (83%). Keratin from sheep wool was partially hydrolyzed (37%), while human nail and hair keratins were poorly hydrolyzed (Fig. 4).

Discussion

In this paper we described the purification and partial biochemical characterization of a *M. verrucaria* keratinase produced in submerged cultures using poultry feather powder as the only substrate. The purification procedure was relatively simple; a single chromatographic step was enough to obtain a homogeneous fraction. Possibly the fungus secreted only a

Table 2 Experimental design and results of the 2^2 factorial design

Run	Coded values		Variables		Keratinase activity (%)	
	x_1	x_2	pH	t (°C)	Actual value	Predicted value
1	−1	−1	7.0	30	46.0	47.35
2	+1	−1	9.0	30	85.0	85.60
3	−1	+1	7.0	50	42.0	41.55
4	+1	+1	9.0	50	48.0	46.80
5	−1.41	0	6.6	40	39.5	38.89
6	+1.41	0	9.4	40	69.1	69.56
7	0	−1.41	8.0	26	74.0	72.65
8	0	+1.41	8.0	54	40.0	41.20
9	0	0	8.0	40	100	98.43
10	0	0	8.0	40	98.3	98.43
11	0	0	8.0	40	97.0	98.43

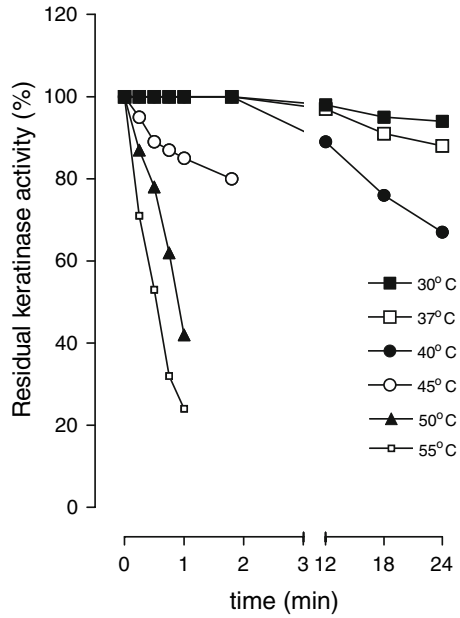

Fig. 3 Thermal stability of *M. verrucaria* keratinase

small amount of proteins in addition to the keratinase protein. It is equally possible that the exoprotease degraded at least in part the other extracellular proteins. If the phenomenon exists, however, it had a positive effect in the present work, because it facilitated the purification of the enzyme.

Considering the fact that the *M. verrucaria* keratinase was strongly inhibited by PMSF, it seems reasonable to classify the enzyme as a serine-protease.

Table 3 Effect of chemicals on the purified *M. verrucaria* protease

Chemical	Keratinolytic activity (%)	Caseinolytic activity (%)
None	100.0	100.0
1 mM PMSF	7.0 ± 2.0	11.8 ± 2.7
5.0 mM EDTA	38.6 ± 4.9	40.5 ± 2.1
0.7 μg/ml pepstatin A	98.7 ± 5.9	95.9 ± 6.9
0.05 mM iodoacetamide	98.4 ± 3.0	99.0 ± 4.9
1 mM DTT	134.9 ± 9.8	98.4 ± 3.7
1 mM β-mercaptoethanol	143.5 ± 12.0	97.0 ± 7.2

Table 4 Effect of several compounds on the purified *M. verrucaria* keratinase

Chemical	Concentration	Residual keratinase activity (%)
None	–	100
SDS	20 mM	96.4
Triton X-100	2.5%	86.9
Tween 80	2.5%	139.0
NaN_3	5 mM	98.9
Methanol	25%	90.0
Ethanol	25%	91.9
Isopropanol	25%	85.4
Butanol	25%	76.8
Glycerol	25%	81.0
Acetonitrile	25%	86.4
Ca^{2+}	5 mM	135.0
Mg^{2+}	5 mM	112.0
Cu^{2+}	5 mM	98.1
Zn^{2+}	5 mM	89.1
Co^{2+}	5 mM	89.3
Ba^{2+}	5 mM	87.6
Al^{3+}	5 mM	84.9
Mn^{2+}	5 mM	73.4
Pb^{2+}	5 mM	77.2
Hg^{2+}	5 mM	35.3
Ag^{2+}	5 mM	5.8
Cd^{2+}	5 mM	2.7
Fe^{3+}	5 mM	2.6

Actually, most keratinases described until now were classified into this category (Bressolier et al. 1999; Gupta and Ramnani 2006; Suh and Lee. 2001). It should equally be considered that partial inhibition of serine proteases by EDTA could also be caused by

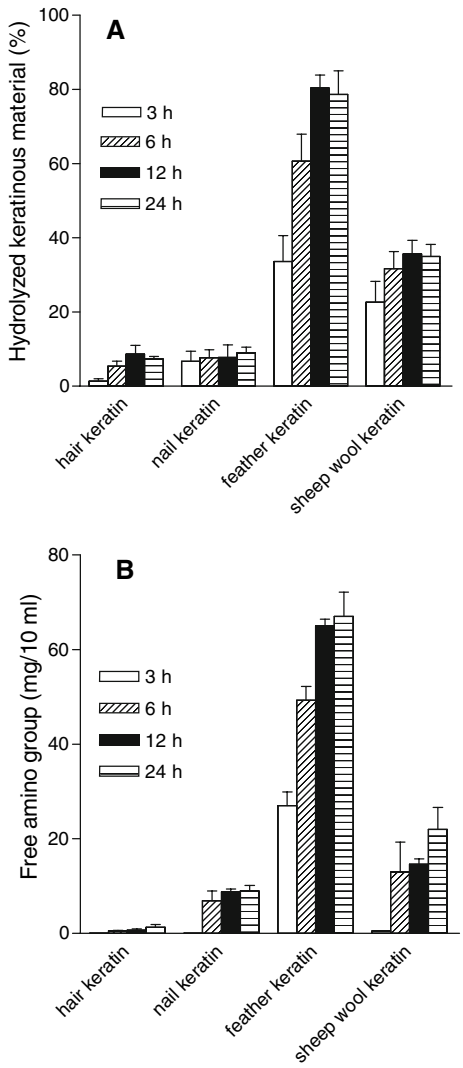

Fig. 4 Hydrolysis of several keratinous substrates by purified *M. verrucaria* keratinase. The purified enzyme was added to a final activity of 20 U/ml. In **a** the keratin degradation was estimated by determination of remaining insoluble material; In **b** the keratin degradation was estimated by determination of the appearance of soluble amino acids and peptides

chelation of cations, which are important as stabilizing agents (Gupta and Ramnani 2006). Corroborating this view, the activity of *M. verrucaria* keratinase was slightly improved by Ca^{2+} and Mg^{2+}.

It has been amply observed that reducing agents like DTT, βME, reduced glutathione, cysteine and sodium sulfite enhance the keratinolytic activity, indicating that these enzymes are thiol-activated (Gupta and Ramnani 2006). In the present work maximal improvement of the keratinolytic activity was found in the presence of the reducing agents DTT and βME. Since DTT and βME are known to cleave disulphide bonds, an influence either on the enzyme or on the substrate is possible. The reducing agents improved the enzyme activity when the substrate was keratin and not when the substrate was casein. Considering this, it is highly probable that the reducing agents act on the keratin and not on the enzyme.

RSM was used to study the effect of temperature and pH on the enzymatic activity. This methodology is a faster and less expensive method to find out the best conditions for a given enzyme. It was based on the RSM results that the efficiency of the enzyme in the degradation of keratinous materials was tested at pH 8.3 and 37°C, and not at pH 8.0 and 40°C, the optimal conditions determined by means of the conventional approach. In addition to this, the experiments to test the thermo-stability revealed that at 37°C the enzyme was stable for long periods (24 h), while at 40°C, the enzyme had already lost 30% of its initial activity after 24 h. It is worth to remember the sequence of decreasing hydrolyzing efficiency at pH 8.3 and 37°C: poultry feather keratin > sheep wool keratin > human nail keratin > human hair keratin. The keratin chain of hair (hard keratin) is similar to that of epidermis (soft keratin), since it is tightly packed in an α-helix, but differs from the latter in that it contains a several fold higher amount of cysteine. However, in feathers, the polypeptide assumes a β-conformation, which is more readily hydrolyzed compared with α-keratin (Ignatova et al. 1999).

A very expressive hydrolysis of feather meal was obtained by the action of keratinase (80.3%) after 12 h. Prolongation of treatment for up to 12 h, produced negligible improvements in the liberation of soluble peptides. Considering that the enzyme showed to be stable under the experimental conditions, it looks likely that it is probably not able to attack the portions of the keratin chain richer in disulphide bonds. The use of reducing agents to enhance keratin degradation by keratinases has been described (Bockle and Müller 1997; Riffel et al. 2003; Thys and Brandelli 2006), a strategy that can be tested in the future. This strategy can be useful also to improve the capability of the enzyme in degrading the other keratinous materials on which its action was inefficient.

Conclusion

In conclusion, the purified *M. verrucaria* keratinase presented a high efficiency in degrading poultry feather meal, liberating amino acids and soluble peptides without necessity of chemical or enzymatic reduction of disulphide bonds. Fungal keratinases, in the same way as bacteria, may play an important role in biotechnological processes aiming to improve the transformation of poultry feather through solubilization of β-keratin into usable peptides. Additionally, hydrolysis of poultry feathers with keratinase may contribute to reduce the environmental pollution caused by the accumulation of this potential pollutant. The enzyme can be easily purified to homogeneity using a single chromatographic procedure, a fact that facilitated considerably its characterization in the present work. However, for the future use of the enzyme in biotechnological processes, culture filtrates of the kind used in the present work seem to be much more adequate in economical terms.

Acknowledgments This work was supported by the Conselho Nacional de Pesquisa e Desenvolvimento-CNPq, Fundação Araucária and Fundo Paraná. R.M. Peralta is research fellow of CNPq. F.G. Moreira-Gasparin is a recipient of CAPES Fellowships. We thank A. Chaves and M.A.F. Costa for technical assistance.

References

Anbu P, Gobinath SCB, Hilda A, Lakshmipriya T, Annadurai G (2007) Optimization of extracellular keratinase by poultry farm isolate *Scopulariopis brevicaulis*. Bioresour Technol 98:1298–1303. doi:10.1016/j.biortech.2006.05.047

Bertsch A, Coello N (2005) A biotechnological process for treatment and recycling poultry feathers as a fed ingredient. Bioresour Technol 96:1703–1708. doi:10.1016/j.biortech.2004.12.026

Bockle B, Müller R (1997) Reduction of disulfide bonds by *Streptomyces pactum* during growth on chicken feathers. Appl Environ Microbiol 63:790–792

Boer CG, Peralta RM (2000) Production of extracellular protease by *Aspergillus tamari*. J Basic Microbiol 2:75–81. doi:10.1002/(SICI)1521-4028(200005)40:2<75::AID-JOBM75>3.0.CO;2-X

Box GEP (1954) The exploration and exploitation of response surfaces: some considerations and examples. Biometrics 10:16–60. doi:10.2307/3001663

Boyette CD, Hoagland RE, Abbas HK (2007) Evaluation of the bioherbicide *Myrothecium verrucaria* for weed control in tomato (*Lycopersicon esculentum*). Biocontrol Sci Technol 17:171–178. doi:10.1080/09583150600937451

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Bressolier P, Letourneau F, Urdaci M, Verneuil B (1999) Purification and characterization of a keratinolytic serine proteinase from *Streptomyces albidoflavus*. Appl Environ Microbiol 65:2570–2576

Cao L, Tan H, Liu Y, Xue X, Zhou S (2008) Characterization of a new keratinolytic *Trichoderma atroviride* strain F6 that completely degrades native chicken feather. Lett Appl Microbiol 46:389–394. doi:10.1111/j.1472-765X.2008.02327.x

El-Refai HA, AdelNaby MA, Gaballa A, El-Araby MH, Abdel Fattah AF (2005) Improvement of the newly isolated *Bacillus pumilus* FH9 keratinolytic activity. Process Biochem 40:2325–2332. doi:10.1016/j.procbio.2004.09.006

Ghosh A, Chakrabarti K, Chattopadhyay D (2008) Degradation of raw feather by a novel high molecular weight extracellular protease from newly isolated *Bacillus cereus* DCUW. J Ind Microbiol Biotechnol 35:825–834. doi:10.1007/s10295-008-0354-5

Gradisar H, Friedrich J, Krizaj I, Jerala R (2005) Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. Appl Environ Microbiol 71:3420–3426. doi:10.1128/AEM.71.7.3420-3426.2005

Grazziotin A, Pimentel FA, de Jong EV, Brandelli A (2006) Nutritional improvement of feather protein by treatment with microbial keratinase. Anim Feed Sci Technol 126:135–144. doi:10.1016/j.anifeedsci.2005.06.002

Gupta R, Ramnani P (2006) Microbial keratinases and their prospective applications: an overview. Appl Microbiol Biotechnol 70:21–33. doi:10.1007/s00253-005-0239-8

Ignatova Z, Gousterova A, Spassov G, Nedkov P (1999) Isolation and partial characterization of extracellular keratinase from a wool degrading thermophilic actinomycete strain *Thermoactinomyces candidus*. Can J Microbiol 45:217–222. doi:10.1139/cjm-45-3-217

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685. doi:10.1038/227680a0

Marcondes NR, Taira CL, Vandresen DC, Svidzinski TIE, Kadowaki MK, Peralta RM (2008) New feather-degrading filamentous fungi. Microb Ecol 56:13–17. doi:10.1007/s00248-007-9319-x

Montenecourt BS, Eveleigh DE (1977) Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. Appl Environ Microbiol 34:777–784

Moreira FG, Souza CGM, Costa MAF, Reis S, Peralta RM (2007) Degradation of keratinous materials by the plant pathogenic fungus *Myrothecium verrucaria*. Mycopathologia 163:153–160. doi:10.1007/s11046-007-0096-3

Myers RH, Montgomery DC (2002) Response surface methodology: process and product optimization using designed experiments. Wiley, New York

Nickerson WJ, Noval JJ, Robison RS (1963) Keratinase I. Properties of the enzyme conjugate elaborated by *Streptomyces fradiae*. Biochim Biophys Acta 77:73–86. doi:10.1016/0006-3002(63)90470-0

Onifade AA, Al-Sane NA, Al-Musallam AA, Al-Zarban S (1998) A Review: Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and others keratins as livestock feed resources. Bioresour Technol 66:1–11. doi:10.1016/S0960-8524(98)00033-9

Pillai P, Archana G (2008) Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel *Bacillus subtilis* isolate. Appl Microbiol Biotechnol 78:643–650. doi:10.1007/s00253-008-1355-z

Ramnani P, Singh R, Gupta R (2005) Keratinolytic potential of *Bacillus licheniformis* RG1: structural and biochemical mechanism of feather degradation. Can J Microbiol 51:191–196. doi:10.1139/w04-123

Riffel A, Lucas F, Heeb P, Brandelli A (2003) Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Arch Microbiol 179:258–265

Santos RMDB, Firmino ALP, de Sá CM, Felix CR (1996) Keratinolytic activity of *Aspergillus fumigatus* Fresenius. Curr Microbiol 33:364–370. doi:10.1007/s002849900129

Schrooyen PMM, Dykstra PJ, Oberthur RC, Bantjes A, Feijen J (2001) Partially carboxymethylated feather keratins 2. Thermal and mechanical properties of films. J Agric Food Chem 49:221–230. doi:10.1021/jf0004154

Starcher B (2001) A ninhydrin-based assay to quantify the total protein content of tissue samples. Anal Biochem 292:125–129. doi:10.1006/abio.2001.5050

Suh HJ, Lee HK (2001) Characterization of a keratinolytic serine protease from *Bacillus subtilis* KS-*1*. J Protein Chem 20:65–169. doi:10.1023/A:1011075707553

Suntornsuk W, Suntornsuk L (2003) Feather degradation by *Bacillus* sp FK46 in submerged cultivation. Bioresour Technol 86:239–243. doi:10.1016/S0960-8524(02)00177-3

Thys RCS, Brandelli A (2006) Purification and properties of a keratinolytic metalloprotease from *Microbacterium* sp. J Appl Microbiol 101:1259–1268. doi:10.1111/j.1365-2672.2006.03050.x