



Purification and characterization of a keratinolytic metalloprotease from *Chryseobacterium* sp. kr6

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Abstract

The *Chryseobacterium* sp. kr6 strain has been described as a highly keratinolytic bacterium showing effective feather-degrading and de-hairing activities. A keratinase Q1 enzyme was purified from *Chryseobacterium* sp. kr6 culture by Phenyl Sepharose and Superose 12HR chromatography. This enzyme showed a specific activity of 967 U/mg for keratin azure. Electrophoresis under denaturing conditions showed a monomeric protein with approximately 64 kDa. The enzyme showed pH and temperature optima of 8.5 and 50 °C, respectively. The inhibitory effect of EDTA, EGTA and 1,10-phenanthroline characterized Q1 enzyme as a Zn-metalloprotease. Its activity was increased by three-fold in the presence of Ca²⁺. ESI-MS/MS analysis of peptides generated from a tryptic digestion revealed sequence homology which may characterize the Q1 keratinase as a member of the M14 metalloprotease family, with a consensus glycosylation region similar to proteins from *Chryseobacterium meningosepticum*. © 2006 Elsevier B.V. All rights reserved.

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1. Introduction

The breakdown of insoluble macromolecules, such as cellulose, lignin, chitin and keratin, depends on the

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microbial secretion of extracellular enzymes that have activities on the compacted surface of these molecules. Keratin, a structural protein found in feathers, wool and hair is resistant to degradation by common proteases such as trypsin, pepsin and papain (Papadopoulos et al., 1986; Shih, 1993). This property is conferred mainly by the composition and molecular conformation of the amino acids found in keratin, tightly packed in the α -helix or β -sheet into a supercoiled polypeptide chain due to its high degree of disulfide bridges, hydrogen bonds and hydrophobic interactions (Parry and North, 1998). Nevertheless, keratin does not accumulate in nature since it can be degraded by microorganisms (Onifade et al., 1998). To date, keratinolytic enzymes, so called keratinases, have been purified from different microorganisms with high proteolytic activity on insoluble substrates such as keratin (Lin et al., 1992; Böckle et al., 1995; Santos et al., 1996; Bressollier et al., 1999; Suh and Lee, 2001; Allpress et al., 2002; Nam et al., 2002; Farag and Hassan, 2004).

These enzymes may have relevant utilization in biotechnological processes, mainly for the poultry and leather industries, through the development of non-polluting processes (Shih, 1993; Onifade et al., 1998). In the poultry industry, feathers can be converted, after enzymatic hydrolysis, to feedstuffs, fertilizers, polymers or used in the production of rare amino acids such as serine, cysteine and proline (Papadopoulos et al., 1986; Yamauchi et al., 1996). As for the leather industry, the de-hairing chemical process, which produces a large amount of sulfide residues, has been slowly replaced by the use of keratinases (Thanikaivelan et al., 2004).

The properties of keratinases vary among the several microbial species. The genus *Chryseobacterium* has been associated with a strong proteolytic activity (Vandamme et al., 1994), however, little has been reported about the characterization of this proteases (Venter et al., 1999; Lijnen et al., 2000; Yamaguchi and Yokoe, 2000). Recently, *Chryseobacterium* sp. kr6 was isolated from an industrial poultry waste and characterized as a feather-degrading bacterium (Riffel and Brandelli, 2002; Riffel et al., 2003a) showing de-hairing activity in bovine pelts (Riffel et al., 2003b). Considering the potential of this enzyme to be used in biotechnological processes and the fact that keratin-degrading mechanism is still not understood, the current work presents the purification and

characterization of a keratinase obtained from *Chryseobacterium* sp. kr6.

2. Materials and methods

2.1. Microorganism, culture medium and growth conditions

The bacterial cultivation in the presence of whole poultry feathers was conducted according to Sangali and Brandelli (2000). Feathers were obtained from a Poultry Breeding Laboratory (USP/ESALQ, Piracicaba, Brazil) and they were, prior to use, washed in distilled water and completely air-dried. Briefly, as a starter culture, *Chryseobacterium* sp. kr6 (Genbank AY157745) was restored from frozen culture in 30% glycerol stock and, under aseptic conditions, pre-inoculated in a 250-mL Erlenmeyer containing 100 mL of autoclaved feather broth (FB) liquid medium (0.5 g/L NaCl, 0.3 g/L K_2HPO_4 , 0.4 g/L KH_2PO_4 , 10 g/L of whole chicken feathers, and pH adjusted to 7.5) followed by 18 h incubation at 30 °C and 150 rpm. Subsequently, 20 mL of the starter culture was transferred aseptically to 2 L of FB and the bacterial cells were grown for 18 h to reach the stationary phase at the same conditions for the production and further purification of keratinase.

2.2. Enzyme assays

The proteolytic activity was determined by using Hammerstein casein (Merck, Darmstadt, Germany) as substrate according to modification of the method of Kunitz (1947). Briefly, the reaction started by adding 150 μ L of the enzyme to 500 μ L of 0.5% (w/v) casein in 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.5, pre-heated to 50 °C. After 20 min at 50 °C, the reaction was stopped by adding 650 μ L of 10% (w/v) trichloroacetic acid (TCA), briefly homogenized and centrifuged at 13,000 $\times g$ for 15 min. The absorbance of the supernatant was read at 280 nm (Beckmann DU-640 spectrophotometer). The increase in absorbance at 280 nm above the blank was used as a measure of enzyme activity. TCA was added to the blank tubes just before the enzyme was added and then incubated for 20 min at 50 °C. A separate blank tube was prepared for each different enzyme fraction used. Assays

were conducted in triplicate. One unit of protease activity was defined as the amount of enzyme necessary to release 1 μmol of tyrosine/min under the conditions mentioned above. The molar extinction coefficient (ϵ) of tyrosine adopted was 1380 at 280 nm (Venter et al., 1999).

The keratinolytic activity was determined by using keratin azure (Sigma–Aldrich, St. Louis, USA) as the insoluble substrate (Bressollier et al., 1999). Aliquots of 500 μL of the enzyme samples were incubated in a solution of 5 mg of keratin azure in 500 μL of 100 mM Tris–HCl buffer, pH 8.5 for 1 h at 50 °C. Subsequently, the tubes were centrifuged at $13,000 \times g$ for 5 min and the absorbance of the supernatant was determined at 595 nm (Beckmann DU-640 spectrophotometer). Control samples were prepared in a similar manner except that the enzyme was replaced by the Tris–HCl buffer. The assays were conducted in triplicate. One enzymatic unit was defined as the amount of enzyme that resulted in an increase in absorbance at 595 nm (A_{595}) of 0.01 after reaction at 50 °C for 1 h with keratin azure.

2.3. Enzyme purification

Two liters of a 18 h culture were centrifuged at $12,000 \times g$ at 0 °C for 20 min and the supernatant was filtered through a 0.22- μm cellulose membrane (Millipore, Bedford, USA) for complete removal of cells and to be used as the primary source of crude enzyme. This filtrate was purified as follows. The filtrate was adjusted to 1 M of $(\text{NH}_4)_2\text{SO}_4$ pH 7.2 and applied to a Phenyl Sepharose CL4B (Amersham Pharmacia, Uppsala, Sweden) column (2.4 cm \times 8.0 cm) previously equilibrated with 50 mM phosphate buffer pH 7.2 containing 1 M $(\text{NH}_4)_2\text{SO}_4$. The column was eluted with the same buffer at a rate of 0.5 mL/min followed by 250 mL of a 1–0 M gradient of $(\text{NH}_4)_2\text{SO}_4$. Fractions of 5 mL were collected and the enzyme activity was monitored using casein as substrate as mentioned above. Fractions with confirmed enzyme activity were pooled, dialyzed overnight at 4 °C against 10 mM phosphate buffer pH 7.2, and lyophilized. The lyophilized fractions were resuspended in 10 mM phosphate buffer pH 7.2, applied in a Superose 12HR gel filtration column (Amersham Pharmacia, Uppsala, Sweden) using the HPLC-ÅKTA-Purifier system (Amersham Pharmacia, Uppsala, Sweden), and eluted at a rate 0.5 mL/min

using 50 mM phosphate buffer pH 7.2 containing 50 mM NaCl. Fractions of 500 μL were collected and the enzymatic activity was monitored. Fractions after gel filtration were used as pure enzyme for characterization. The keratinase activity was confirmed, from each step of the purification, by keratinolytic assay as described above.

2.4. Protein measurement

The concentration of soluble protein was determined by the Folin phenol reagent method (Lowry et al., 1951) with bovine serum albumin (BSA) as a standard protein. Simultaneously, the protein concentration was also determined by assuming that the absorbance of a 1% BSA solution at 280 nm is equal to 10.

2.5. Electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed with 12% polyacrylamide resolving gels and 4% stacking gels as described by Laemmli (1970). The relative molecular mass of the protease was determined by comparing with known standard molecular weights markers (10–170 kDa; Fermentas Inc., Canada). Samples were desalted by dialysis before electrophoresis whenever possible against distilled water or suitable buffer and concentrated, if necessary, by lyophilization. After electrophoresis, the protein was detected with silver staining (Switzer et al., 1979).

2.6. Zymography

The cell extract and the purified keratinase were submitted without previous heating to 7.5% SDS-PAGE in the cool room, as described by Laemmli (1970), with the following modifications. Reducing agent was absent and the resolving gel contained 1 mg/mL gelatin. After electrophoresis, the gel was washed twice in 50 mM Tris–HCl buffer, pH 8, supplemented with 2% Triton X-100 in cold distilled water. Each washing step was carried out for 30 min. The gel was covered with 50 mM Tris–HCl, 5 mM CaCl_2 , pH 8.5. After 60 min incubation at 50 °C, the gel was stained with Comassie blue R-250 and then destained. Protease bands appeared as clear zones on a blue background.

2.7. Determination of the optimal pH for activity

The optimal pH for keratinase was determined over a range of 6–10 with increments of 0.5 pH units. Phosphate buffer was used for pHs between 6.0 and 7.5; Tris–HCl for pHs between 8.0 and 9.0 and carbonate–bicarbonate buffer for pHs 9.5 and 10. To determine the optimal pH, 100 μL of 2.5% casein solution was added to 400 μL of each buffer. The different buffer–substrate solutions were incubated at 50 °C at which time the enzyme solution was added (150 μL) and the proteolytic activity measured in triplicate. The activity of the fraction at the pH with the highest activity was taken as 100%.

2.8. Determination of the optimal temperature for activity

The optimal temperature was determined at intervals of 10 °C over a range of 30–100 °C. The assay solutions were equilibrated at the required temperatures. Proteolytic activity was performed in triplicate with blanks at each temperature. The temperature with the highest activity was taken as 100%.

2.9. Thermal stability

The thermal stability was determined at 37, 50 and 80 °C for different time intervals (2, 10, 30, 60, 150 and 240 min). An aliquot of 5 mL of the enzyme solution was incubated in a water bath at the appropriate temperatures. Aliquots were withdrawn at the different time intervals and placed on ice. Assays were performed in triplicate with blanks of each aliquot. The activity of the aliquot that was not heat treated was taken to be at 100%. The half-life ($t_{1/2}$) of the enzyme was obtained from the equation:

$$t_{1/2} = \frac{\ln(2)}{k_d} \quad (1)$$

where the first-order rate constants for inactivation (k_d) of the enzyme at different temperatures were determined from the slopes of semi-logarithmic plots according to Eq. (2):

$$\ln A = -k_d t \quad (2)$$

2.10. Effect of chemicals on keratinase activity

An aliquot of 900 μL the purified keratinase was incubated for 1 h at 20 °C in the presence of the 100 μL of the following inhibitors at a final concentration in the reaction of: 100 μM iodoacetamide; 1 mM pepstatin; 10 mM and 50 mM EDTA; 10 mM and 50 mM EGTA; 1 mM phenylmethylsulfonyl fluoride (PMSF); 10 mM 1,10-phenanthroline. The effect of the compounds such as CaCl_2 , ZnCl_2 , MgCl_2 , AlCl_3 , CuCl_2 and CdCl_2 , at 2 mM, was also assayed using of the purified enzyme and incubated as mentioned above. Similar assay was conducted with 5% DMSO, 5% SDS, 5 mM dithiothreitol (DTT) and 5 mM 2-mercaptoethanol. The enzyme–chemical solution (150 μL) was added to the casein solution (500 μL) and assayed for proteolytic activity in triplicate. Appropriate blanks were prepared to each enzyme–chemical solution. Controls were prepared replacing the chemical solution with autoclave Milli-Q water or methanol. The rate of the inhibition or increase of the enzyme activity caused by each chemical was compared to that of control-enzyme as the relative proteolytic activity. The activity of control was taken as 100%. Since the inhibition of metalloprotease can be reversible, the same concentration of EDTA, EGTA, and 1,10-phenanthroline inhibitor was added to the casein solution.

2.11. Assay of protease activity with insoluble substrates

Proteolytic activities were also determined using the keratin azure (as described above) and washed native poultry feather as substrate. Purified protease was incubated with 0.5% (w/v) substrate in a 100 mM Tris–HCl buffer containing 2 mM CaCl_2 (pH 8.5). The addition of 1% of β -mercaptoethanol to the reaction was also tested. Assays were carried out at 50 °C with constant agitation for 24 h. The reactions were stopped by adding 10% TCA. After centrifugation at 10,000 $\times g$ the free amino groups were determined by the ninhydrin (Sigma–Aldrich) method at 570 nm (Moore, 1968).

2.12. Determination of metal ions

A volume of 100 μL of the purified protein was extensively dialyzed against fresh distilled water for 2

days at 4 °C. The presence of metal ions was determined by atomic absorption spectroscopy with the Varian SpectrAA-220 spectrometer with tungsten coil atomizer and hollow cathode lamps. The parameters used were based on the wavelength of maximal absorption at 324.8 and 213.9 nm for copper and zinc, respectively.

2.13. Identification of the protein

The keratinase protein band was excised from the SDS-PAGE gel and subjected to an *in-gel* trypsin digestion (Fontoulakis and Langen, 1997). The generated peptides were concentrated and subjected to mass fingerprinting using matrix-assisted laser desorption ionization-time of flight-MS (MALDI-TOF-MS) and sequences was obtained using liquid chromatography-quadrupole time of flight (Q-TOF)-tandem mass spectrometry by electrospray ionization (ESI-MS/MS).

2.14. Peptide Mass Fingerprint (PMF) by MALDI-TOF/MS

Analyses of all samples obtained by tryptic digestion were prepared using the dried droplet method. The sample was acidified by the addition of two sample volumes of 0.1% (v/v) trifluoroacetic acid (TFA) and was left at room temperature for a few minutes, to reduce the droplet volume through evaporation. The matrix [1% (w/v) α -cyano-4-hydroxycinnamic acid in 1:1 (v/v) H₂O/ACN solution containing 0.1% (v/v) TFA] was added and the sample was allowed to dry at room temperature. All measurements were performed on a MALDI-TOF-mass spectrometer (Waters-Micromass, UK) in a previously cleaned microplate. Analyses were performed exclusively for positively charged ions, in reflectron mode. For the subsequent data evaluation, including peptide identification, the software package Mascot Wizard (Matrix Science, London, UK) was used.

2.15. Peptide fingerprint by LC-MS/MS analysis

ESI-MS/MS analyses were performed in a Q-TOF (Micromass) coupled to a CapLC (Waters) chromatographic system. The tryptic peptides were on-line desalted using a Waters Opti-Pak C18 trap

column. The trapped peptides were eluted using a gradient of water/acetonitrile containing 0.1% formic acid and separated by a 75 μ m i.d. capillary column home-packed with C18 silica. Data were acquired in data-dependent mode (DDA), and multiply charged ions were subjected to MS/MS experiments. The MS/MS spectra were processed using MaxEnt3 and manually sequenced using the PepSeq software (Micromass). Typical conditions were: LC flow 200 nL/min, nanoflow capillary voltage at 3 kV, block temperature at 80 °C, cone voltage at 40 V.

3. Results

3.1. Keratinase purification

After bacterial growth in CP medium, the cell-free supernatant was submitted to hydrophobic interaction chromatography showing three peaks, HIC1, HIC2 and HIC3 that display proteolytic activity (Fig. 1A). These peaks were eluted through the ammonium sulfate gradient at approximately 0.5, 0.17 and 0M. Fractions from the first peak (HIC1) were pooled, freeze-dried and eluted through gel-filtration column. A single peak, which was denominated as Q1, showed the proteolytic and keratinolytic activity (Fig. 1B). Fractions of Q1 were used to characterize the proteolytic activity of keratinase. The overall purification factor was about 14-fold, and the final yield was 1.18%. The final product had a specific activity of about 2406 U/mg (Table 1). The specific activity on keratin azure as substrate was 967 U/mg protein.

3.2. Determination of the keratinase molecular mass

The relative molecular mass (M_r) of the Q1 protease was estimated based on its electrophoretic mobility and compared to standard molecular markers. The protease Q1 was subjected to SDS-PAGE and a unique band was observed, corresponding to a molecular weight of approximately 64 kDa (Fig. 2, lane B).

The production of extracellular proteases by strain kr6 was evaluated by zymogram analysis. Growth of *Chryseobacterium* sp. kr6 on feathers resulted in

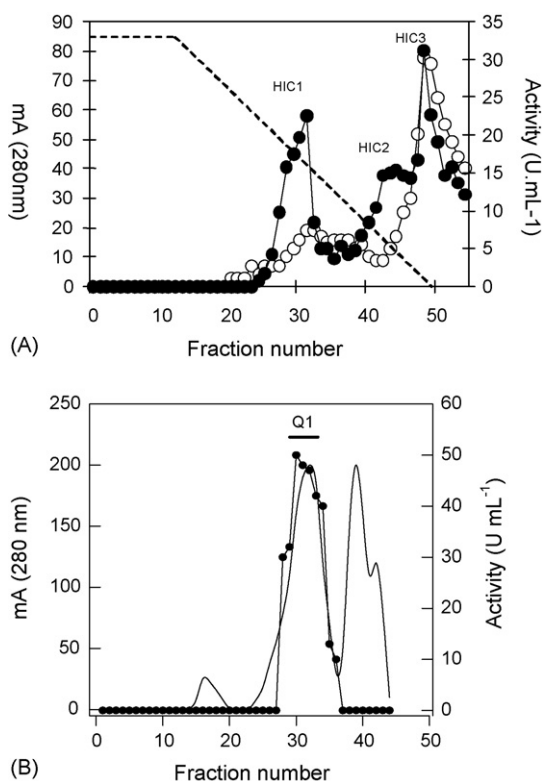


Fig. 1. Purification of keratinase produced by *Chryseobacterium* sp. kr6. (A) Crude culture filtrate was submitted to hydrophobic interaction chromatography on a Phenyl Sepharose column eluted with a gradient of 1–0 M $(\text{NH}_4)_2\text{SO}_4$ (dotted line) in 50 mM phosphate buffer pH 7.2. Proteolytic activity (●) was determined using casein as substrate; protein (○) was monitored by absorbance at 280 nm. (B) The peak HIC1 was submitted to gel filtration chromatography on a Superose 12HR column eluted with 50 mM phosphate buffer pH 7.2 containing 50 mM NaCl. Proteolytic activity (●) was determined using casein as substrate; protein (---) was monitored by absorbance at 280 nm.

the production of multiple proteases, as observed by a zymogram on gelatin (Fig. 2, lane C). At least four major bands were observed for fractions HIC1, HIC2 and HIC3 (Fig. 2, lanes D–F). A single band

was detected for the purified enzyme Q1 (Fig. 2, lane G).

3.3. Effects of temperature and pH on the activity and stability of Q1 protease

The keratinase was active in a range of 30–60 °C and pH between 7.5 and 9.5 with optimum activity at 50 °C and pH 8.5. The enzyme display stability at 37 °C, with more than 80% of its initial activity for up to 4 h. At 50 °C, the enzyme maintained 50% of its activity after 3 h, while at 80 °C, the keratinase was completely inactivated within the first 10 min of incubation (data not shown). The half-lives ($t_{1/2}$) of the enzyme at 37 and 50 °C were calculated as 976 and 347 min, respectively.

3.4. Effect of chemicals

The effect of various compounds on the proteolytic activity of the Q1 keratinase were examined. The results are summarized in Table 2. The serine (PMSF), cysteine (iodoacetamide) and aspartyl protease inhibitors had minor effects on keratinase activity. Chelating agents such as EDTA and EGTA had a different effect on the enzyme activity. EDTA at 10 and 50 mM inhibited 15 and 81%, respectively while EGTA at 10 or 50 mM inhibited 71 and 77%, respectively. Keratinase was completely inactivated by the presence of 1,10-phenanthroline. In the presence of 2 mM of ions Ca^{2+} , Cd^{2+} and Mg^{2+} the relative activity was 355, 123 and 114%, respectively. However, addition of 2 mM Al^{3+} , Zn^{2+} and Cu^{2+} ions resulted in relative activities of 72, 64 and 24%, respectively. The activity of the keratinase was unstable in the presence of reducing agents at 5 mM, i.e., no activity was observed in the presence of 2-mercaptoethanol, and only 23% of residual activity was detected with DTT. In the presence of DMSO, an organic solvent, there was a slight increase in the activity (Table 2).

Table 1
Purification of a keratinase produced by *Chryseobacterium* sp. kr6

Step	Total protein (mg)	Total activity (U) ^a	Specific activity (U/mg)	Purification fold	Yield (%)
Crude supernatant	2796	471,867	168.77	1	100
Phenyl Sepharose	11.15	6,162	552.45	3.27	1.31
Superose 12HR	2.3	5,545	2406.68	14.26	1.18

^a One unit of activity was defined as the amount of enzyme necessary to release 1 μmol of tyrosine/min, utilizing casein as substrate.

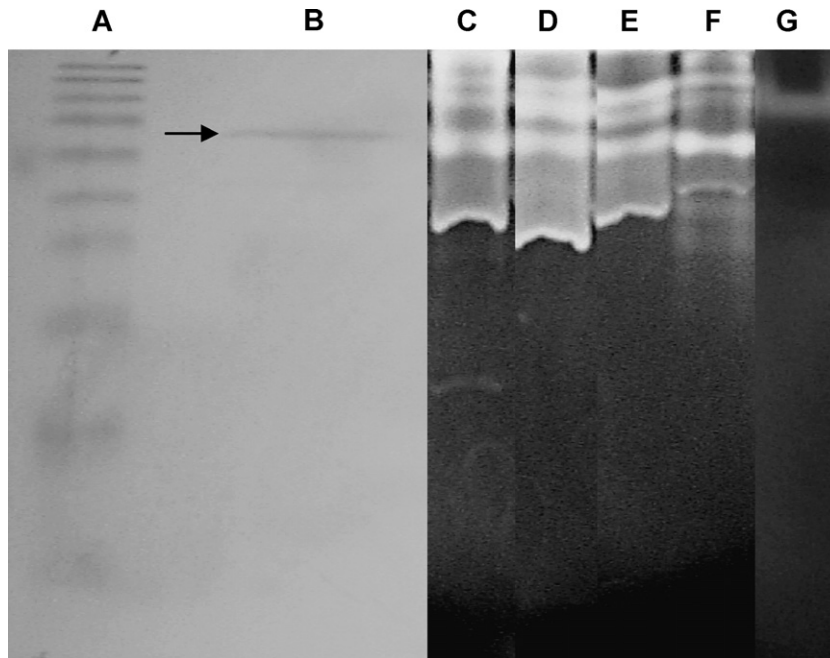


Fig. 2. Analysis of keratinase from *Chryseobacterium* sp. kr6 by SDS-PAGE and zymography. Samples of standard protein molecular markers (A; 170, 130, 100, 70, 55, 40, 35, 25, 15 and 10 kDa) and purified keratinase (B) were subjected to SDS-PAGE on 12% polyacrylamide gels and silver stained. Culture supernatant (C), HIC1 (D), HIC2 (E), HIC3 (F) and purified keratinase Q1 (G) were analyzed for proteolytic activity by gelatin zymography on 7.5% SDS-PAGE gels and stained with Coomassie blue R-250.

Table 2
Influence of inhibitors, ions and compounds on the proteolytic activity of a keratinase purified from *Chryseobacterium* sp. kr6

Chemical	Concentration	Residual activity (%)
None	–	100
EDTA	10 mM	85
	50 mM	19
EGTA	10 mM	29
	50 mM	23
1,10-Phenanthroline	10 mM	0
PMSF	1 mM	91
Iodoacetamide	0.1 mM	95
Ca ²⁺	2 mM	355
Mg ²⁺	2 mM	114
Al ³⁺	2 mM	72
Zn ²⁺	2 mM	64
Cu ²⁺	2 mM	24
Cd ²⁺	2 mM	123
2-Mercaptoethanol	5 mM	0
DTT	5 mM	33
DMSO	5% (v/v)	111
SDS	5% (v/v)	0

3.5. Hydrolysis of insoluble substrates

The Q1 keratinase was examined to determine its ability to solubilize native feathers. There was no feather keratin solubilization as determined by free amino acid detection. However, the purified keratinase could hydrolyze the substrate keratin azure.

3.6. Identification of keratinase protein

Attempts were made to identify the Q1 protein through mass spectrometry of the peptides generated by tryptic digestion of the keratinase. The fingerprinting analysis did not produce satisfactory data to identify the protein. Through the electrospray ionization, sequences of 23 peptides were obtained. These sequences did not show significant homology to any motifs of known proteins, based on searches of different protein data banks. On the other hand, the sequences of three internal peptides were homologous to the conserved sequence of the active residue and Zn binding site of the M14 metallopro-

					Family	Organism	
	↓ ↓	KHSFENFIEGK	--LVQLNRQ	--NTVSARIHR	--RRIDVEVEQGG		- <i>Chryseobacterium</i> sp. Kr6
145	↓ ↓	HSGEIDGKDA	--NLNLNRD	--PDLYIDIHV	--KGYDYRVEQSP	M14X	- <i>Shewanella frigidimarina</i>
111	↓ ↓	HPGEIPGKDA	--NLNLNRD	--PDLIVDTHD	--QGFETTLELSA	M14X	- <i>Gloeobacter violaceus</i>
186	↓ ↓	HSREWITHTAT	--GVDLNRN	--FKAMISIH	--IKYAFSFE [*] LRD	M14A	- <i>Mus musculus</i>

Fig. 3. Sequence of the tryptic peptides from *Chryseobacterium* sp. kr6 Q1 protease. The sequence has been aligned with those of other proteases belonging to the M14 metalloprotease family. Asterisk indicates the active site residue and the arrows (↓) the Zn binding site.

tease family (carboxypeptidase) and to a proposed consensus *O*-glycosylation site DS* in the peptide (KGSSADS*PNSEEK), which is generally found in *Chryseobacterium meningosepticum* proteins (Fig. 3).

4. Discussion

An extracellular keratinase isolated from *Chryseobacterium* sp. kr6 was purified and characterized. The detection of a unique band through the SDS-PAGE confirmed the purification of the Q1 keratinase. The proteolytic activity of the keratinase after the final purification (2406 U/mg) increased approximately 14-fold when compared to that of the crude extract (168 U/mg). The elution profile in the hydrophobic interaction chromatography and the gelatin zymography of the crude extract and HIC1, HIC2 and HIC3 peaks suggest that the strain kr6 secretes more than one protease. The presence of multiple keratinolytic proteases in bacterial crude enzyme has been reported for *Kytococcus sedentarius* (Longshaw et al., 2002) and *Streptomyces albidoflavus* (Bressollier et al., 1999).

When comparing the enzyme activity on different substrates, keratinase had high specific activity in the presence of casein (2406 U/mg) and keratin azure (967 U/mg) but was unable to solubilize native feather keratin. Disulfide bridges are an important structural feature of native keratin. Cleavage of these disulfide bonds seems to be indispensable to make the proteins available for the hydrolytic enzymes. A disulfide reductase was purified from the culture of a keratinolytic strain of *Stenotrophomonas* sp. (Yamamura et al., 2002) and increased thiol formation was detected during cultivation of *Chryseobacterium* sp. kr6 on feather keratin (Riffel et al., 2003a), suggesting that this enzyme is produced by the strain kr6 as well. This indicates that feather degradation could be a synergic mechanism

between the disulfide reductase and all secreted proteases by the *Chryseobacterium* sp. kr6, as described by some *Streptomyces* species (Böckle et al., 1995; Bressollier et al., 1999), *Trichophyton rubrum* (Monod et al., 2005) and *Microsporium gypseum* (Kunert, 1992).

The inhibitors of serine (PMSF) and cysteine (iodoacetamide) proteases did not inactivate the Q1 keratinase, which suggest that serine and cysteine residues are not involved in the catalytic mechanisms. Moreover, the chelating agents EDTA and EGTA inhibited the activity, thus suggesting that Q1 keratinase is a metalloprotease. The low inactivation rate caused by 10 mM EDTA (15%) might have been caused by a competition between the excess of metals present at non-active sites. As the concentration of EDTA increased to 50 mM, the inhibitory effect was similar to that found with EGTA, which has a lower stability constant value for other ions than EDTA (Auld, 1995). This taken together with the fact that Q1 keratinase was completely inactivated in the presence of 1,10-phenanthroline, suggested the enzyme was a Zn-metalloprotease. The presence of Zn was confirmed through atomic absorption spectroscopy. Most of the keratinases found to date have been reported to be serine proteases (Lin et al., 1992; Friedrich and Antranikian, 1995; Bressollier et al., 1999; Suh and Lee, 2001; Nam et al., 2002), and a few metalloproteases have shown keratinolytic activity (Brouta et al., 2001; Allpress et al., 2002; Farag and Hassan, 2004).

Keratinolytic metalloproteases may have great biotechnological promise, acting as secondary keratinases they may overcome the limited proteolysis on the surface of insoluble keratin particles because of restricted enzyme–substrate interaction (Allpress et al., 2002). In addition, the metalloenzyme nature presents a potential method of enzyme immobilization. Increased stability of immobilized keratinase has been reported because of a reduced autolysis (Wang et al., 2003),

which could be also achieved for keratinase Q1 by temporarily inactivation by chelating agents during storage.

Regarding the ions tested, the presence of Ca^{2+} in the reaction mixture caused a three-fold increase of the enzymatic activity similar to the *Flavobacterium psychrophilum* metalloprotease (Secades and Guijarro, 2001). Other metalloproteases have been reported with similar property including the ability of Ca^{2+} to restore the activity after inhibition by EDTA (Grimwood et al., 1994; Venter et al., 1999). This ion, as well as Mg^{2+} and Mn^{2+} , have been reported to be associated with metalloproteases and protect these enzymes against thermal denaturation (Kumar and Takagi, 1999). In addition, Ca^{2+} ions are known to be associated with the stabilization of the tertiary structure conformation and the protection of some metalloproteases against autolysis (Secades and Guijarro, 2001). Likewise, Cd^{2+} ions also stimulated the keratinase activity and this result could be attributed to its ionic radius (0.97 Å), which is closer to Ca^{2+} (0.99 Å) (Exterkate and Alting, 1999). On contrast, the presence of Zn^{2+} , Al^{3+} and Cu^{2+} caused an inhibitory effect on keratinase activity. Ions Ca^{2+} and Zn^{2+} have been reported as inducer and inhibitor, respectively, of keratinase enzymes isolated from *Vibrio* sp. kr2 (Sangali and Brandelli, 2000) and calpains (Sorimashi et al., 1997). Several zinc peptidases are known to be inhibited by excess zinc, e.g., carboxypeptidase A (Larsen and Auld, 1989), thermolysin (Holmquist and Vallee, 1974) and collagenase (Mallya and Van Wart, 1989). This effect has been attributed to the coordination of the hydroxide ion (ZnOH^+) to the catalytic zinc ions, particularly at alkaline pH (Larsen and Auld, 1989).

The reducing agents DTT and 2-mercaptoethanol caused important inhibition of keratinase Q1. This indicates that intact disulfide bonds might be important to maintain the molecular folding required for keratinase to be fully active. Moreover, DTT can chelate ions necessary to maintain the structure and activity of an enzyme (Cheng et al., 1999).

As for most of keratinases reported in the literature, the Q1 enzyme obtained in this study has also maximum activity in an alkaline milieu (Böckle et al., 1995; Santos et al., 1996; Bressollier et al., 1999; Nam et al., 2002). This mechanism is commonly found in proteases from microorganisms that use protein sources as nutrients. The protein degradation releases solu-

ble peptides and amino acids in the environment and the subsequent deamination liberates ammonium, thus increasing the local pH (Kunert, 1989). The stimulus of keratinase activity by alkaline pHs suggests a positive biotechnological potential. Alkaline proteases have been extensively used to formulate detergents, in leather industry and in bioremediation processes (Gupta et al., 2002).

The keratinase Q1 showed an optimum activity at 50 °C, which is similar to some keratinases isolated from the *Bacillus* genus (Lin et al., 1992; Kim et al., 2001), and remained 50% active after 3 h at this temperature. Its inactivation occurred at 80 °C, being less stable than keratinases from *Aspergillus oryzae* (Farag and Hassan, 2004), *Bacillus licheniformis* (Lin et al., 1992) and *Streptomyces* sp. KS₁₋₀₂ (Letourneau et al., 1998). The strain kr6 showed high keratin degradation at room temperature (Riffel et al., 2003a). The optimum activity at moderate temperature will be less energy-consuming than the currently used thermophilic bacteria and therefore meets the increasing consciousness for energy conservation. The molecular mass found for the Q1 keratinase (64 kDa) also corroborated with that reported for *A. oryzae* (Farag and Hassan, 2004), but it was slightly larger than the majority of keratinases, which vary from 20 to 50 kDa (Böckle et al., 1995; Cheng et al., 1999; Suh and Lee, 2001). Few reports on keratinases outside this range of molecular mass, include the keratinases produced by *Fervidobacterium pennavorans* of 130 kDa (Friedrich and Antranikian, 1995) and *Kocuria rosea* of 240 kDa (Bernal et al., 2006).

Analyses of the peptide fingerprinting (MALDI-TOF/MS) and the peptide sequences generated from the tryptic digestion (ESI-MS/MS) did not reveal homology or similarity to any known proteins from the database. However, among the 23 peptides sequenced, the sequence of the 3 internal peptides was homologous to the conserved sequence of the active residue and Zn-binding site of the metalloprotease M14 family (carboxypeptidase A). This family is characterized by the presence of a consensus sequence at the active site HXXE (where X is any amino acid), and with a histidine approximately 103–143 residues from the C-terminal of this motif (Barret et al., 2001). This family is widely distributed and is known in almost all types of organisms. Most of the peptidases in this family are carboxypeptidases, which hydrolyze single C-terminal

amino acids from polypeptide chains. They have a recognition site for the free C-terminal carboxyl group, which is a key determinant of specificity, but other kind of activity can be present into the family (Rawlings et al., 2006). To date, proteases of this family have not been associated with the *Chryseobacterium* genus or with keratinolytic activity. Moreover, other peptide sequences revealed the presence of a consensus site for O-glycosylation DS*, which has been found in proteins from the species *C. meningosepticum* (Plummer et al., 1995).

This study presents a putative new metallo-carboxypeptidase protein isolated from *Chryseobacterium* sp. kr6. This enzyme showed distinct properties and had no similarity to sequences of known keratinases or to other proteases, thus suggesting that this is a new protein. Further studies to investigate the molecular characterization of this protease should be conducted to validate this metalloprotease as a keratinolytic enzyme and to evaluate the biotechnological potential of this keratinase in processes involving keratin hydrolysis.

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