Appl Biochem Biotechnol (2010) 162:548–560
DOI 10.1007/s12010-009-8835-1

Thermodynamics and Kinetics of Heat Inactivation of a Novel Keratinase from *Chryseobacterium* sp. Strain kr6

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Received: 20 June 2009 / Accepted: 25 October 2009 /
Published online: 21 November 2009

Abstract A novel keratinase from *Chryseobacterium* sp. strain kr6 was purified to homogeneity by $(NH_4)_2SO_4$ precipitation, gel permeation on Sephadex G-100, and Q-Sepharose Fast Flow anion-exchange chromatography. The molecular weight of the purified enzyme was around 20 kDa. Kinetic and thermodynamic parameters for thermal inactivation were determined. The influence of Ca^{2+} and Mg^{2+} ions and purification degree on the enzyme stability was evaluated in the range of 50 to 60 °C. The results showed that first-order kinetics explained well the thermal denaturation of the keratinase in this temperature interval. The presence of Ca^{2+} increases significantly the enzyme stability. Compared with the controls, the half-life of the purified enzyme after two purification steps in the presence of Ca^{2+} increased 7.3, 20.2, and 9.8 fold at 50, 55, and 60 °C, respectively. Thermodynamics parameters for thermal inactivation were also determined.

Keywords Biocatalysis · Enzyme · Kinetics · Microbial · Protease · Thermal inactivation

Nomenclature

C_0	Activity at time $t=0$
C_t	Activity at time t
k_d	First-order rate constant (min^{-1})
$E_{a,d}$	Activation energy for denaturation (kJ mol^{-1})
$\Delta G_d^{\#}$	Free energy (kJ mol^{-1})
$\Delta H_d^{\#}$	Activation enthalpy (kJ mol^{-1})
$\Delta S_d^{\#}$	Activation entropy (J mol^{-1} K^{-1})
H	Planck constant (J s)
K_B	Boltzmann constant (J K^{-1})
R	Universal gas constant (J mol^{-1} K^{-1})
r^2	Determination coefficient

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t Time (min)
$t_{1/2}$ Half-life time (min)
T Temperature (K)

Introduction

Keratinases (E.C. 3.4.99.11) are proteases able to degrade the scleroprotein keratin. Huge amounts of this protein are available as feathers, a waste by-product generated by the poultry production. Keratinases are very promising from a biotechnological point of view, since they could be applied to process keratin-containing wastes from leather and poultry industries and to improve the nutritional value of feather meal [1, 2].

Although keratinases from different microorganisms have been purified and characterized to date [3–6], thermodynamic data and kinetic studies about thermal inactivation of keratinases are not available. Nonetheless, data about the activity and stability of enzymes can be useful for enhancing biotechnological processes, providing information on the structure of this catalyst and help to optimize the economic feasibility of the industrial process.

Several techniques based on protein engineering [7], isolation of thermophilic organisms [8], chemically or enzymatically modified enzymes [9, 10], and use of additives [11] have been reported to successfully increase enzyme thermostability. It had been reported that the addition of Ca^{2+}, Mg^{2+}, or polyhydric alcohols caused an increase in thermal stability of some proteases [12, 13].

Recently, we isolated and characterized a new keratinolytic bacterium presenting a remarkable feather-degrading activity. This microorganism was identified as a *Chryseobacterium* sp. kr6 and its keratinase showed great potential for biotechnological applications [14]. A keratinolytic metalloprotease was recently purified from culture supernatants of strain kr6, being the first keratinase associated with the M14 family of peptidases [15]. In this paper, a novel keratinase from *Chryseobacterium* sp. strain kr6 was purified with only two chromatography steps. The aim of this work was to investigate the thermal stability of this keratinase. On this basis, an Arrhenius plot was derived and the thermodynamic parameters for thermal inactivation were investigated by kinetic method.

Materials and Methods

Microorganism, Culture Media, and Conditions

The keratinolytic *Chryseobacterium* sp. strain kr6 was isolated from feather waste produced by commercial poultry processing [14]. The culture was propagated and maintained on feather meal agar plates at 30 °C and subcultured at weekly intervals. The basal medium used for growth of the feather-degrading microorganism was feather meal broth: NaCl (0.5 g L^{-1}), KH_2PO_4 (0.4 g L^{-1}), $CaCl_2$ (0.015 g L^{-1}), and feather meal (10 gL^{-1}), pH was adjusted to 8.0. The medium was sterilized by autoclaving at 121 °C for 15 min. Cultures were incubated in an orbital shaker at 30 °C and 70 rpm, for 48 h. The culture was centrifuged at 4 °C and 10,000×g for 20 min to harvest the keratinase-containing supernatant.

Assay of Enzyme Activity

Proteolytic activity was assayed with azocasein as substrate by the following method [16]. The reaction mixture contained 120 μL of enzyme preparation and 480 μL of 10 g L^{-1}

azocasein (Sigma, St. Louis, MO, USA) in 50 mmol L^{-1} Tris-HCl buffer pH 8.0. The mixture was incubated for 40 min at 45 °C; the reaction was stopped by addition of 600 μL of 100 g L^{-1} trichloroacetic acid. After separation of the un-reacted azocasein by centrifugation, 800 μL of clear supernatant was mixed with 200 μL of 1.8 mol L^{-1} NaOH, resulting in a yellow-colored complex that was measured spectrophotometrically at 420 nm. One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01, under these conditions. Keratinolytic activity was also determined. The reaction mixture, containing 100 μL of enzyme sample and 500 μL of 20 g L^{-1} azokeratin suspension in 50 mmol L^{-1} Tris-HCl buffer pH 8.0, was incubated for 30 min, at 45 °C. The reaction was terminated by addition of 600 μL of 100 g L^{-1} trichloroacetic acid, centrifuged, and the supernatant measured spectrophotometrically at 450 nm. One unit of keratinolytic activity was defined as an increase in the A_{450} of 0.01, under the specified conditions [17].

Enzyme Purification

The supernatant containing the enzyme was collected after centrifugation at 10,000×*g* for 20 min. The enzyme was precipitated from the supernatant by gradual addition of solid ammonium sulphate, with gentle stirring, to 50% saturation, allowed to stand for 3 h and centrifuged at 10,000×*g* for 20 min. The pellet was dissolved in 50 mmol L^{-1} Tris-HCl buffer pH 8.0. The concentrated sample was applied to a Sephadex G-100 gel filtration column (Pharmacia, Uppsala, Sweden), equilibrated and eluted with 50 mmol L^{-1} Tris-HCl buffer pH 8.0, at a linear rate of 1 cm min^{-1} (flow rate 0.5 cm^3 min^{-1}). The fractions were monitored by measuring the absorbance at 280 nm and enzyme activity. The fractions with high enzyme activity from gel permeation were pooled and dialyzed overnight against 20 mmol L^{-1} Tris-HCl buffer pH 8.5. The dialyzed enzyme solution was applied to Q-Sepharose Fast Flow column (3.5×15 cm) equilibrated with Tris-HCl 20 mmol L^{-1} buffer pH 8.5. The unbound proteins were washed with 20 mmol L^{-1} Tris-HCl buffer pH 8.5. The bound proteins were eluted at a linear rate of 0.074 cm min^{-1} (flow rate 0.712 cm^3 min^{-1}), with a linear gradient of NaCl (0–1 mol L^{-1}) in 20 mmol L^{-1} Tris-HCl buffer pH 7.0. The fractions were monitored by measuring the absorbance at 280 nm and enzyme activity. The concentration of soluble protein was determined by the Folin phenol reagent method [18], using bovine serum albumin as standard.

Electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed with 12% polyacrylamide gels essentially as described elsewhere [19]. After electrophoresis the protein was detected with silver staining [20].

Thermal Inactivation

Kinetics of thermal inactivation of *Chryseobacterium* kr6 keratinase was determined by incubating the enzyme with different purifications grades, in the absence (control) or presence of Ca^{2+} or Mg^{2+} ions (5 mmol L^{-1} final concentration) at 50, 55 or 60 °C. Enzyme solutions (1.0 mL) were heated in sealed tubes, which were incubated in a thermostatically controlled thermal block (Labnet International, Woodbrige, NJ, USA). Tubes were withdrawn at each time intervals and the residual activity was determined as described earlier, with azocasein as substrate. The activity after 1 min of heating-up time (t=0) was

considered to be the initial activity, thereby eliminating the effects of heating-up. Assays were done in triplicate.

Kinetic Model and Statistical Analysis

The first-order rate constants for denaturation (k_d) of the enzyme at different temperatures were determined from the slopes of semi-logarithmic plots according to Eq. 1.

$$1n\ (C_t/C_0) = -k_d t \tag{1}$$

where C_t is the enzyme activity at the time t, C_0 the initial enzyme activity, k_d is the first-order rate constant for denaturation, and t is the time.

The activation energy for denaturation ($E_{a,d}$) was obtained from the slope ($-E_{a,d}/R$) of Arrhenius plot of ln k_d verus *1/T*. Free energy ($\Delta G_d^{\#}$), enthalpy ($\Delta H_d^{\#}$) and entropy ($\Delta S_d^{\#}$) of activation for denaturation of the enzyme were calculated from Eq. 2–4, respectively.

$$\Delta G^{\#}_{d} = -\mathrm{RTln}\frac{k_d h}{K_B T} \tag{2}$$

where h (Planck constant)=6.626×10^{-34} J s, and K_B (Boltzman constant)=1.381×10^{-23} J K^{-1}.

$$\Delta H^{\#}_{d} = E_{a,d} - RT \tag{3}$$

where R (gas constant)=8.314 J mol^{-1} K^{-1}

$$\Delta S^{\#}_{d} = \frac{\Delta H^{\#}_{d} - \Delta G^{\#}_{d}}{T} \tag{4}$$

The half-life ($t_{½}$) of the enzyme was obtained from Eq. 5.

$$t_{(1/2)} = 1n(2)/k_d \tag{5}$$

The values of k_d and $E_{a,d}$ were estimated by regression analysis using the statistical package of Microsoft Excel® (Microsoft, Seattle, WA). Data were compared by the Tukey's test using the Statistica 5.0 software (Statsoft, Tulsa, OK) and values were considered different each other when $p<0.05$.

Results and Discussion

Keratinase Purification

The culture supernatant was submitted to precipitation with ammonium sulphate, gel filtration, and ion exchange chromatography. Purification with gel filtration chromatography separated two peaks, where only one presented proteolytic activity against azocasein. While in the Q-Sepharose anionic exchange chromatography it was observed two peaks with enzymatic activity. These peaks were eluted through the NaCl gradient at approximately 0.27 and 0.61 mol L^{-1} (Fig. 1). Fractions of the first peak, showing prominent activity, were pooled and used to thermal inactivation studies. This fraction was subjected to SDS-PAGE and a unique band was observed, corresponding to a molecular mass of approximately 20 kDa (Fig. 1, inset). The overall purification factor was about 40.2

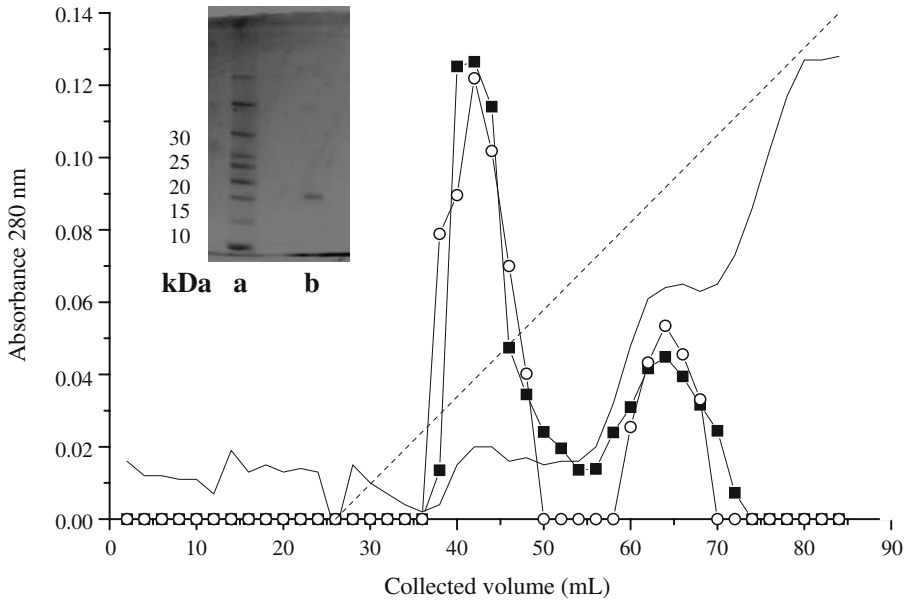

Fig. 1 Elution profile of keratinase from *Chryseobacterium* sp. kr6 on Q-Sepharose Fast Flow equilibrated with 20 mmol L^{-1} Tris-HCl buffer pH 8.5 and eluted with an NaCl gradient (0–1 mol L^{-1}). Proteolytic activity (*filled square*), keratinolytic activity (*empty circle*), absorbance at 280 nm (*solid line*), and NaCl concentration (*broken line*). *Inset* silver-stained gel showing **a** molecular mass standards and **b** purified enzyme

fold, and the final yield was 7.1%. The final product had a specific activity of about 21 466 U mg^{-1} (Table 1).

These results indicate that a novel keratinolytic enzyme was purified, since the earlier purified keratinase Q1 from *Chryseobacterium* sp. kr6 showed a molecular mass of 64 kDa [15]. The molecular mass of this novel enzyme is in agreement with most keratinases of mesophilic microorganisms, which range from 20 to 50 kDa [1, 4].

Kinetics of Thermal Inactivation

The extent of inactivation after heating the enzyme solution for 10 min at various temperatures was measured. Initially, residual activity was compared after 10 min of thermal treatment in the range from 50 to 80 °C, using crude and purified enzyme samples. The enzyme retained about 40% its initial activity after 10 min at 50 °C, but it

Table 1 Purification of the keratinase from *Chryseobacterium* sp. strain kr6.

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Crude enzyme	89,995	168.3	534	1	100
$(NH_4)_2SO_4$ precipitation	68,501	7.9	8,671	16.2	76.1
Sephadex G-100	18,280	1.3	14,061	26.3	20.3
Q-Sepharose Fast Flow	6,440	0.3	21,466	40.2	7.1

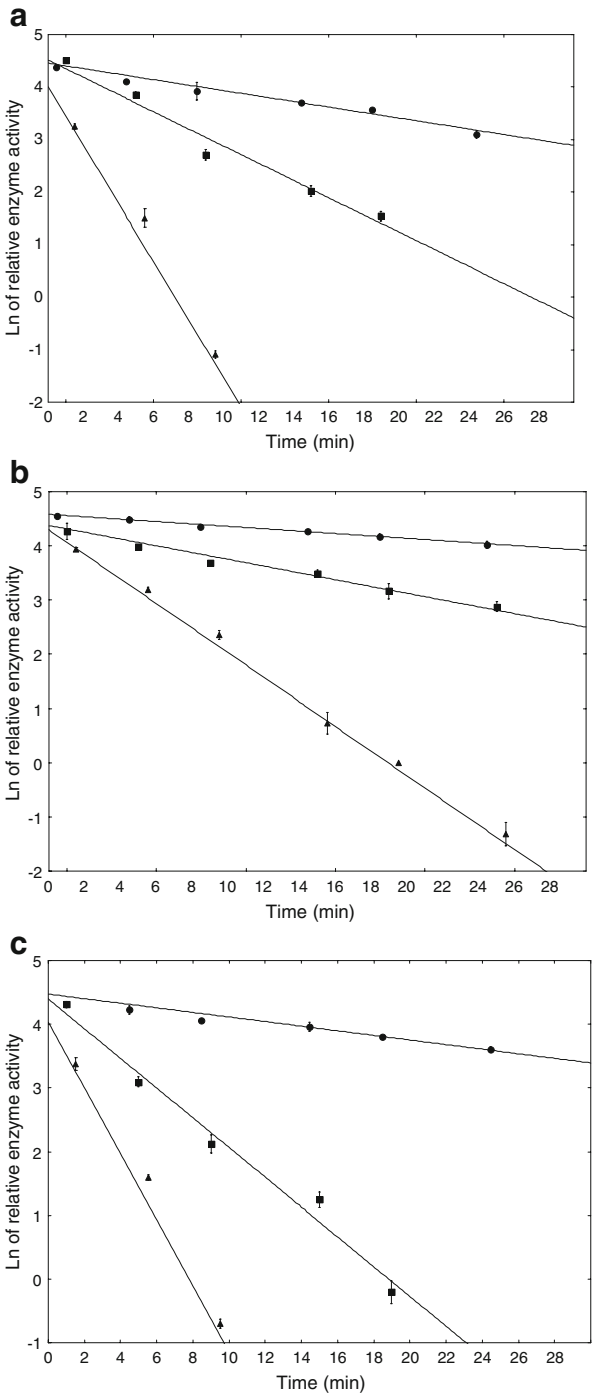

Fig. 2 First-order plots for thermal inactivation of crude enzyme. **a** Control; **b** enzyme with Ca^{2+}; and **c** enzyme with Mg^{2+}. The r^2 values for 50 °C (*filled circle*), 55 °C (*filled square*), and 60 °C (*filled triangle*) were respectively: **a** 0.95, 0.97, and 0.99; **b** 0.99, 0.97, and 0.99; and **c** 0.95, 0.98, and 0.99

was completely inactivated at 65 °C or more (results not shown). Residual activity of the different enzyme preparations was then compared over the temperature range from 50 to 60 °C. Kinetic studies about thermal inactivation of proteases are often more complex due to the autolysis effect [21]. However, the first-order kinetic model has been used, mainly at low enzyme concentration, to describe the thermal inactivation of proteases [21, 22].

The influence of temperature on inactivation of the crude enzyme is shown in the Fig. 2 (a, b, c), being the straight lines in these figures the linear fit for each condition, which were well fixed by the Eq. 1. The slopes in these figures are the first-order rate constants for enzyme denaturation (k_d), estimated by linear regression of the experimental data. The k_d values and the half life ($t_{½}$) are given in Table 2. Comparing the rate constants (k_d) for enzyme inactivation, smaller values were observed with addition of Ca^{2+} at the different temperatures tested. In addition, excepting for the enzyme with Ca^{2+}, the rate constants increased considerably with the increase of temperature. Compared with control, the $t_{1/2}$ was increased around 2.5 fold at different temperatures in the enzyme samples with calcium.

After 25 min at 50 °C the crude enzyme maintained about 22.1% its initial activity, in the same conditions in the presence of calcium the residual activity was 55.8%. When magnesium was added, 36.5% residual activity was observed.

The effect of temperature on inactivation of the partially purified enzyme (after one chromatography step) is shown in the Fig. 3 (a, b, c), the calculated k_d values and the half life are listed in Table 3. Control enzyme maintained 10.9% its initial activity after 25 min, while in the presence of calcium and magnesium it retained 66.5% and 60.8% initial activity, respectively. In the samples of this purification grade, it has been observed that calcium ion has a remarkable effect on the enzyme stability, and the increase on $t_{1/2}$ ratio was 5.6, 7.6 and 8.4 fold for 50, 55, and 60 °C, respectively.

Compared with control, the $t_{1/2}$ of the purified enzyme (after two chromatography steps) in the presence of Ca^{2+} was increased by 7.3, 20.2, and 9.8 fold at 50, 55, and 60 °C, respectively (Table 4). The Fig. 4 (a, b) illustrates the denaturation kinetics of the purified enzyme. After the second chromatographic step, the control enzyme maintained 52% its initial activity, while in the presence of calcium the sample maintained 90.3% residual

Table 2 Kinetic and thermodynamic parameters for thermal denaturation of crude enzyme.

T (K)	k_d (min^{-1})	$t_{1/2}$ (min)	ΔG_d (kJ mol^{-1})	ΔH_d (kJ mol^{-1})	ΔS_d (J mol^{-1} K^{-1})
Control ($E_{a,d}$=211.6±3.5 kJ mol^{-1})					
323 (50 °C)	0.053±1.41E^{-3}	13.0	87.2	208.9	376.7
328 (55 °C)	0.164±7.07E^{-4}	4.2	85.5	208.8	376.0
333 (60 °C)	0.56±7.07E^{-3}	1.2	83.4	208.8	376.5
Ca^{2+} ($E_{a,d}$=208.7±15.3 kJ mol^{-1})					
323 (50 °C)	0.022±3.54E^{-3}	31.2	89.5	206.0	360.6
328 (55 °C)	0.063±2.12E^{-3}	10.9	88.1	205.9	359.3
333 (60 °C)	0.231±2.83E^{-3}	3.0	85.9	205.9	360.3
Mg^{2+} ($E_{a,d}$=239.5±3.7 kJ mol^{-1})					
323 (50 °C)	0.036±7.07E^{-4}	19.0	88.2	236.8	460.0
328 (55 °C)	0.235±7.78E^{-3}	2.9	84.5	236.7	464.0
333 (60 °C)	0.528±1.20E^{-2}	1.3	83.6	236.7	459.7

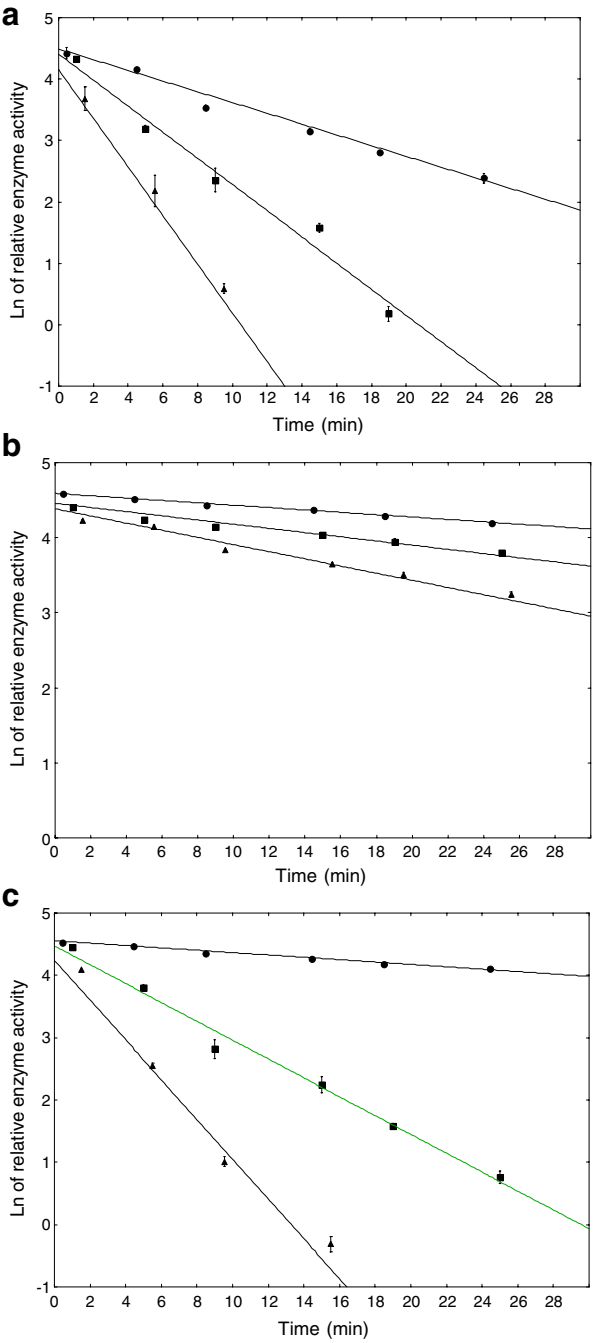

Fig. 3 First-order plots for thermal inactivation of partially purified enzyme. **a** Control; **b** enzyme with Ca^{2+}; and **c** enzyme with Mg^{2+}. The r^2 values for 50 °C (*filled circle*), 55 °C (*filled square*), and 60 °C (*filled triangle*) were respectively: **a** 0.99, 0.98, and 0.98; **b** 0.99, 0.97, and 0.99; and **c** 0.98, 0.99, and 0.98

Table 3 Kinetic and thermodynamic parameters for thermal denaturation of partially purified enzyme.

T (K)	k_d (min^{-1})	$t_{1/2}$ (min)	ΔG_d (kJ mol^{-1})	ΔH_d (kJ mol^{-1})	ΔS_d (J mol^{-1} K^{-1})
Control ($E_{a,d}$=135.4±1.5 kJ mol^{-1})					
323 (50 °C)	0.089±7.07E^{-4}	7.7	85.8	131.9	142.6
328 (55 °C)	0.214±4.95E^{-3}	3.2	84.8	131.8	143.4
333 (60 °C)	0.402±3.54E^{-3}	1.7	84.4	131.8	142.3
Ca^{2+} ($E_{a,d}$=99.1±3.8 kJ mol^{-1})					
323 (50 °C)	0.016±7.07E^{-4}	43.3	90.4	96.4	18.7
328 (55 °C)	0.028±7.07E^{-4}	24.3	90.3	96.4	18.6
333 (60 °C)	0.048±2.12E^{-3}	14.3	90.2	96.4	18.4
Mg^{2+} ($E_{a,d}$=255.0±5.7 kJ mol^{-1})					
323 (50 °C)	0.019±7.07E^{-4}	35.5	89.9	252.3	502.9
328 (55 °C)	0.155±2.83E^{-3}	4.5	85.7	252.3	508.0
333 (60 °C)	0.335±9.19E^{-3}	2.0	84.9	252.2	502.6

activity for a duration of 25 min at 50 °C. At 55 °C for 25 min, only 1.4% residual activity is observed, while 80.5% is maintained in the presence of calcium. The control enzyme was totally inactivated at 60 °C for 10 min, but a residual activity of 39% was observed after 25 min in the presence of calcium. The protective effect caused by the presence of calcium became more pronounced as the purification degree increased.

The presence of Ca^{2+} and Mg^{2+} ions have been associated with an increase thermal stability of some bacterial proteases. The extracellular protease of *Pseudomonas fluorescens* T20 contained Mg^{2+}, which plays an important role on enzyme stability [23]. The presence of calcium on thermolysin, a typical bacterial metalloprotease, contributes to heat stability, prevention of autolysis, and maintenance of structural integrity [24]. In this work, only Ca^{2+} appeared to increase the thermal stability of keratinase.

It has been reported that the addition of Ca^{2+} caused an increase in the thermal stability of alkaline proteases. For example, the half-life was increased 2.5 fold at 50 °C, and 10-fold at 60 °C by adding Ca^{2+} to the alkaline proteases from *Bacillus* sp. GX6638 and *B. sphaericus*, respectively [25, 26]. The addition of $CaCl_2$ increases the half-life of an alkaline protease of *Bacillus mojavensis* from 15 min to 57 min, showing the importance of this ion to increase the thermo resistance [11]. In agreement, the half-life at 50 °C of an alkaline protease from *Conidiobolus coronatus* increases from 17 to 47 min by adding Ca^{2+}

Table 4 Kinetic and thermodynamic parameters for thermal denaturation of purified enzyme.

T (K)	k_d (min^{-1})	$t_{1/2}$ (min)	ΔG_d (kJ mol^{-1})	ΔH_d (kJ mol^{-1})	ΔS_d (J mol^{-1} K^{-1})
Control ($E_{a,d}$=239.0±3.8 kJ mol^{-1})					
323 (50 °C)	0.025±1.41E^{-3}	27.7	89.2	236.3	455.4
328 (55 °C)	0.174±2.83E^{-3}	4.0	85.3	236.3	460.1
333 (60 °C)	0.360±3.61E^{-2}	1.9	84.7	236.2	455.1
Ca^{2+} ($E_{a,d}$=202.7±8.5 kJ mol^{-1})					
323 (50 °C)	0.003±7.07E^{-4}	202.1	94.5	200.0	326.5
328 (55 °C)	0.008±7.07E^{-4}	81.8	93.6	199.9	324.3
333 (60 °C)	0.037±1.41E^{-3}	18.7	91.0	199.9	327.1

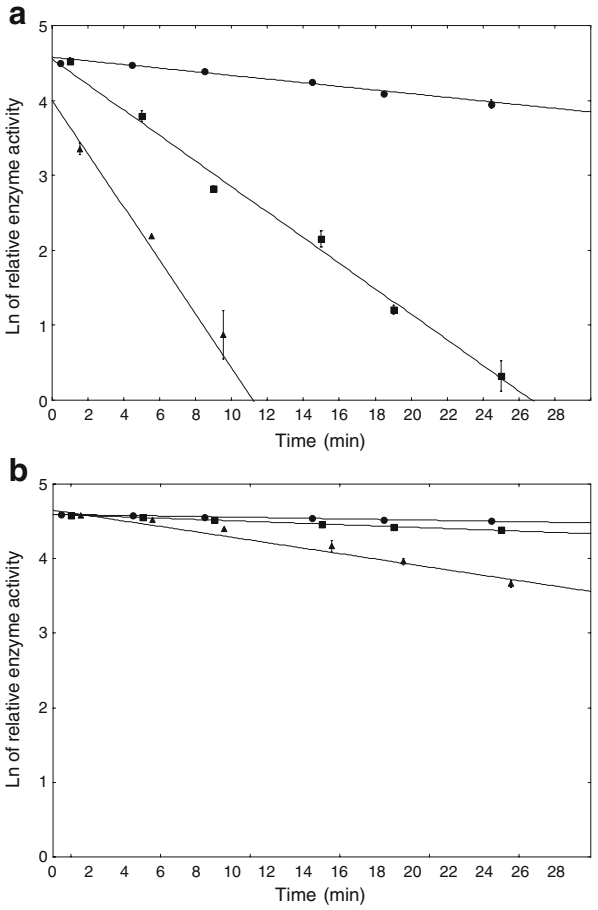

Fig. 4 First-order plots for thermal inactivation of purified enzyme. **a** Control; and **b** enzyme with Ca^{2+}. The r^2 values for 50 °C (*filled circle*), 55 °C (*filled square*), and 60 °C (*filled triangle*) were respectively: **a** 0.98, 0.99, and 0.96; and **b** 0.98, 0.99, and 0.96

[12]. The effect of calcium on the improvement of thermal stability against heat inactivation may be explained by the strengthening of interactions inside the protease molecules and by the binding of Ca^{2+} to the autolysis site [12, 13].

Thermodynamic Parameters

From the values obtained for the denaturation rate constants, it was possible to establish a relationship between the value of k_d and temperature by fitting the data to the Arrhenius equation, which relates rate constants to temperature.

The activation energy ($E_{a,d}$), free energy ($\Delta G_d^{\#}$), enthalpy ($\Delta H_d^{\#}$) and entropy ($\Delta S_d^{\#}$) for enzyme inactivation at different temperatures are listed in Tables 2, 3, and 4. The thermal denaturation of enzyme is accompanied by weakening or disruption of non-covalent linkages with simultaneous increase in the enthalpy of activation, and opening of the enzyme structure is accompanied by an increase in the disorder or entropy [27, 28].

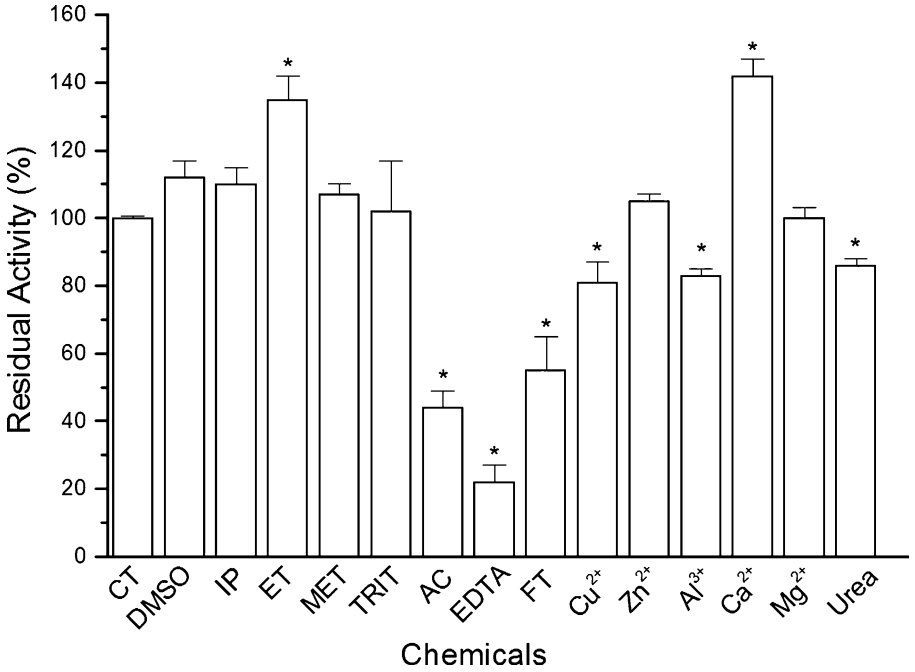

Fig. 5 Effects of various chemicals on enzyme activity. The enzyme was first incubated for 30 min at room temperature. Residual activity was measured as described elsewhere, with azocasein as substrate. CT (control), DMSO, IP (1% *v*/*v* isopropyl alcohol), ET (1% *v*/*v* ethanol), MET (1% *v*/*v* methanol), TRIT (1% *v*/*v* Triton X-100), AC (1% *v*/*v* acetonitrile), EDTA (5 mmol L^{-1}), FT (5 mmol L^{-1} 1,10 phenanthroline), Cu^{2+}, Zn^{2+}, Al^{3+}, Ca^{2+}, Mg^{2+}, urea: 5 mmol L^{-1}. *significantly different from control at $p<0.05$

For crude enzyme, the variation of the $\Delta H_d^{\#}$ was observed to be insignificant for the different conditions evaluated (Table 2). However, the presence of calcium reduced the $\Delta H_d^{\#}$ values from 131 kJ mol^{-1} to 96.4 kJ mol^{-1} for partially purified enzyme (Table 3) and from 236 kJ mol^{-1} to 200 kJ mol^{-1} for purified keratinase (Table 4).

The smaller activation energy for denaturation of the enzyme was obtained with the partially purified enzyme with Ca^{2+}, and this result differs significantly of the other conditions ($p<0.05$).

The values of entropy ($\Delta S_d^{\#}$) of activation for denaturation of the purified enzyme with Ca^{2+} were remarkably reduced, around 7-fold, changing from 142.6 J mol^{-1} K^{-1} to 18.7 J mol^{-1} K^{-1}. Viewed from thermodynamics, decrease in entropy means decrease in disorder degree of molecules [28]. The ΔG value is directly related with the protein stability: the higher ΔG is higher will be the enzyme stability [29]. Results for $\Delta H_d^{\#}$ show that the enthalpy is practically independent of temperature, thus, there is no change in enzyme heat capacity [30].

The purity of the enzyme is reported to have a strong effect on the inactivation of pseudomonal proteases [31]. The addition of sodium caseinate to an extracellular proteinase from *P. fluorescens* caused an increase in inactivation rate, probably caused by aggregation of the enzyme molecules with caseinate [22]. Though, when the authors compared the kinetic parameters of purified proteinase with the unpurified sample they did not detect statistically significant difference.

Some proteases are protected against thermal inactivation by companion proteins [28]. Possibly, the thermostability of purified enzymes may be reduced due to partial

denaturation during the purification process, and the effect of autoproteolysis has been also associated with inactivation at moderate temperatures [22].

There is no specific information available on kinetics and thermodynamic parameters for keratinase heat-inactivation. The thermodynamic parameters estimated here were similar to those reported by Vicente et al. [32] for thermal inactivation of an extracellular aspartic protease from *Phycomyces blakesleeanus*. The fact that no specific studies were found to report kinetics and thermodynamic parameters for heat-inactivation of keratinase gives the present investigation an innovatory character in the analysis of these important proteolytic enzymes.

Effects of Various Chemicals

To further characterize the *Chryseobacterium* sp. kr6 keratinase, we examined the effects of some chemicals on the enzyme activity. Protease activity was investigated after preincubation of the enzyme with several chemicals for 30 min at room temperature. The effects of various inhibitors, metal ions, and solvents on the enzyme activity are summarized in Fig. 5. The protease activity of the sample without any reagent (control) was taken as 100%. Tukey's test was carried out to verify if the difference was significantly at 95% of confidence level.

Among the solvents, ethanol was the only one that caused a significant increase on the enzyme activity, while acetonitrile inhibited the proteolytic activity.

The enzyme was significantly inactivated by the presence of 1,10-phenanthroline and EDTA. The inhibitory effect of metal chelator EDTA and Zn-specific chelator 1,10-phenanthroline characterized the enzyme as a metalloprotease. In the presence of divalent ions Zn^{2+} and Mg^{2+} significant effects on the enzyme activity were not detected. However, Ca^{2+} promoted an increase on proteolytic activity while Cu^{2+} provide a reduction, when compared with the control. Finally, the urea also did not exert significantly inhibition, at the same significance interval.

Conclusions

We reported here for the first time the thermodynamic and kinetics data for thermal denaturation of a keratinase from *Chryseobacterium* sp. strain kr6. An increase in the enzyme stability with the purification degree and an increase in stability in the presence of calcium were observed. Calcium seems to play an important role in the maintenance of thermal stability of this keratinase.

Acknowledgements This work was supported by CAPES and CNPq (Brazil).

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