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Kinetic and thermodynamic studies of a novel acid protease from *Aspergillus foetidus*

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ABSTRACT

The kinetics of a thermostable extracellular acid protease produced by an *Aspergillus foetidus* strain was investigated at different pH, temperatures and substrate concentrations. The enzyme exhibited maximal activity at pH 5.0 and 55 °C, and its irreversible deactivation was well described by first-order kinetics. When temperature was raised from 55 to 70 °C, the deactivation rate constant increased from 0.018 to 5.06 h^{-1}, while the half-life decreased from 37.6 to 0.13 h. The results of activity collected at different temperatures were then used to estimate, the activation energy of the hydrolysis reaction (E^* = 19.03 kJ/mol) and the standard enthalpy variation of reversible enzyme unfolding (ΔH°_U = 19.03 kJ/mol). The results of residual activity tests carried out in the temperature range 55–70 °C allowed estimating the activation energy (E^*_d = 314.12 kJ/mol), enthalpy ($311.27 \leq (\Delta H^\circ_d \leq 311.39$ kJ/mol), entropy ($599.59 \leq \Delta S^*_d \leq 610.49$ kJ/mol K) and Gibbs free energy ($103.18 \leq \Delta G^*_d \leq 113.87$ kJ/mol) of the enzyme irreversible denaturation. These thermodynamic parameters suggest that this new protease is highly thermostable and could be important for industrial applications. To the best of our knowledge, this is the first report on thermodynamic parameters of an acid protease produced by *A. foetidus*.

1. Introduction

Microbial proteases (EC 3.4) are among the most important hydrolytic enzymes, representing one of the largest groups of industrial enzymes and accounting for approximately 60% of the total enzyme sales in the world [1]. The wide specificity of the hydrolytic action of proteases finds extensive applications in different industries such as the food, laundry detergent, leather, pharmaceutical and silk ones, for recovery of silver from exhausted X-ray films and for waste management, as well as in the structural elucidation of proteins, whereas their synthetic action is exploited in the synthesis of proteins [2–5].

Fungi are commonly used in the industrial practice because of their ability to release extracellular enzymes at high concentration; moreover, they offer the additional advantage over bacteria of making the downstream processing easier [6]. Filamentous fungi of the *Aspergillus* genus are able to produce a variety of extracellular proteases, some of which are used as models in thermostability studies [7].

Thermodynamic and kinetic studies can provide valuable information about the thermostability of enzymes at the operating temperature. The enzyme is supposed to undergo a first-order deactivation reaction, which is responsible for its irreversible denaturation, whose kinetics is usually expressed in terms of enzyme half-life ($t_{1/2}$) [8]. On the other hand, the activation energy and the changes in Gibbs free energy, enthalpy and entropy between the folded and unfolded states of the enzyme are the parameters most often reported in the literature to describe denaturation thermodynamics [9].

The activity and thermostability of enzymes are important issues that must be taken into account to assess the economic

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feasibility of enzyme-based industrial processes. High stability is generally considered an economic advantage because of reduced enzyme consumption; therefore, before proceeding to develop enzyme formulations, accumulating information on enzyme stability under different conditions is needed [10,11]. A number of reports on heat-stable fungal proteases are found in the literature; however, thermodynamic properties of the protease are poorly described, especially in the case of acid proteases.

On the basis of this background, the aim of the present work is the determination of kinetic and thermodynamic parameters of the activity and thermostability of an extracellular acid protease produced by a new *Aspergillus foetidus* strain isolated from the Brazilian Savannah (*Cerrado*).

2. Materials and methods

2.1. Microorganism and culture conditions

The strain of *Aspergillus foetidus* used in this work was isolated from the soil of Brazilian Savannah (*Cerrado*) and kindly provided by the Culture bank of the Enzymology Laboratory of Cell Biology Department, University of Brasilia (Brasilia, DF, Brazil). It was maintained at 4 °C and cultured in Petri dishes containing 4% (w/v) potato dextrose agar (PDA) for 5–7 days at 28 °C.

As suggested in a previous optimization study (results not published), submerged cultures were carried out for 7 days at 28 °C on a rotary shaker (150 rpm), using peptone 2% (w/v) as a substrate. To this purpose, mycelia plugs (4 mm) from a 7 days-old PDA culture were used as inoculum. Culture medium and mycelia were vacuum filtered through Whatman No. 1 filter paper on Büchner funnel, and the resulting supernatant was used as crude enzyme preparation.

2.2. Protease activity assay

Protease activity was determined by the method proposed by Charney and Tomarelli [12]. Briefly, after digestion of azocasein obtained from a casein chromophore containing a dinitrogenated arylamine, protease activity was quantified based on released peptides by absorbance at 430 nm. The crude enzyme extract (0.05 mL) was mixed with 0.05 mL of 0.5% (w/v) azocasein dissolved in 0.2 M sodium acetate buffer (pH 5.0) and incubated at 37 °C for 40 min. The reaction was then stopped by adding 0.05 mL of cold 10% (w/v) trichloroacetic acid (TCA), and non-hydrolyzed proteins were removed by centrifugation at 4000 *g* for 10 min. To intensify the azo-associated color, 1.0 mL of the supernatant was mixed with 1.0 mL of 0.5 M KOH. A blank was prepared in a similar way, but TCA was added before the enzyme. One unit (U) of enzyme activity was assumed to be the amount of enzyme needed to raise the absorbance at 430 nm by 0.01 unit per minute under the standard assay conditions.

To investigate the effect of pH (5.0–10.0) on protease activity, the 0.5% (w/v) azocasein solution was dissolved, according to the selected pH, in 0.2 M sodium acetate (pH 5.0), 0.2 M sodium phosphate (pH 6.0–8.0), or 0.2 M sodium carbonate (pH 9.0–10.0). All the experiments were carried in triplicate and the results expressed as mean values ± SD (standard deviation).

2.3. Determination of kinetic parameters

The Michaelis constant (K_m) and the maximum rate (v_{max}) of the enzyme-catalyzed reaction were determined through a double reciprocal (Lineweaver–Burk) plot of protease activity versus azocasein concentrations (1.25–15.0 g/L) at pH 5.0.

2.4. Thermodynamic study

As described by Converti, Del Borghi, Gandolfi, Lodi, Molinari and Palazzi [13], the overall phenomenon of enzyme thermal inactivation can be described by an equilibrium of enzyme unfolding (equilibrium constant K_U) followed by an irreversible step leading to its denaturation (first-order rate constant k_d). At temperature (T) lower than the optimum (T_{opt}), the above equilibrium is shifted toward the left side, the enzyme inactivation is negligible, and the initial specific rate of protein hydrolysis (k_0) can be described by the Arrhenius equation. On the other hand, at $T > T_{opt}$, it is shifted toward the right side [14]; thus, substituting the K_U definition in the enzyme material balance and applying the Gibbs and Michaelis–Menten equations, we obtain:

$$k_0 = \frac{A \exp\left(-E^*/RT\right)}{1 + B \exp\left(-\Delta H_u^\circ/RT\right)} \quad (1)$$

where E^* is the activation energy of protease-catalyzed reaction, R the ideal gas constant, A the Arrhenius pre-exponential factor, ΔH°_U the standard enthalpy variation of the inactivation equilibrium and B an additional pre-exponential factor.

Under these conditions, the contribution of the unfolded form of the enzyme becomes predominant owing to K_U increase with temperature, therefore Eq. (1) simplifies to:

$$k_0 = \frac{A}{B} \exp\left(\frac{\Delta H_U^\circ - E*}{RT}\right) \quad (2)$$

Despite of the impossibility to get, for a new enzyme system like that investigated in this study, the actual vales of k_0, we exploited the proportionality existing between such a parameter and the initial enzyme activity (v_0). Therefore, E^* for the protease-catalyzed reaction and ΔH°_U for its reversible unfolding were estimated from the slopes of the straight lines describing, according to the Arrhenius equation and Eq. (2), respectively, the dependence of $\ln v_0$ on $1/T$.

On the other hand, the irreversible thermoinactivation process (denaturation) can kinetically be described by first-order kinetics:

$$v_d = k_d E \quad (3)$$

where v_d is the rate of enzyme inactivation and E the concentration of its active form.

Defining the activity coefficient as the ratio of E to the enzyme concentration at the beginning of thermal treatment ($\psi = E/E_o$), k_d was estimated at different temperatures from the slopes of the straight lines obtained plotting the experimental data of $\ln\psi$ vs time. Finally, plotting according to Arrhenius $\ln k_d$ vs $1/T$, we estimated from the slope of the resulting straight line the activation energy of the irreversible enzyme inactivation (denaturation) (E^*_d). The remaining thermodynamic parameters of protease denaturation were estimated, as described by Melikoglu, Lin and Webb [15] by the equations:

$$\Delta H_d^* = E_d^* - RT \quad (4)$$

$$\Delta G_d^* = -RT \ln\left[\frac{k_d h}{k_B T}\right] \quad (5)$$

$$\Delta S_d^* = \frac{\Delta H_d^* - \Delta G_d^*}{T} \quad (6)$$

being ΔH^*_d, ΔG^*_d and ΔS^*_d the changes of enthalpy, Gibbs free energy and entropy between the denatured and active forms of the enzyme, respectively, while k_B and h are the Boltzmann and Planck constants, respectively.

The enzyme half-life ($t_{1/2}$) was defined as the time after which E was reduced to one half the initial value and then calculated as $t_{1/2} = \ln 2/k_d$ [15].

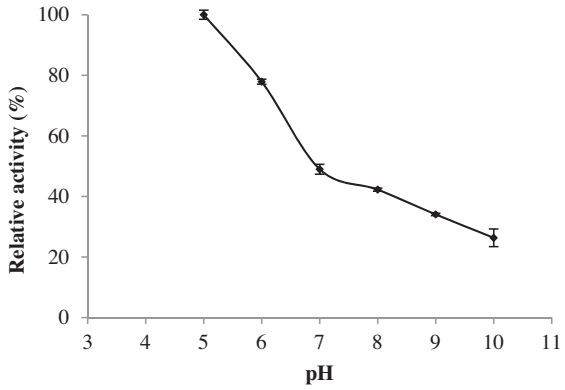

Fig. 1. Effect of pH on the activity of the extracellular acid protease produced by *A. foetidus*. The error bars represent 95% confidence limits for the measurements.

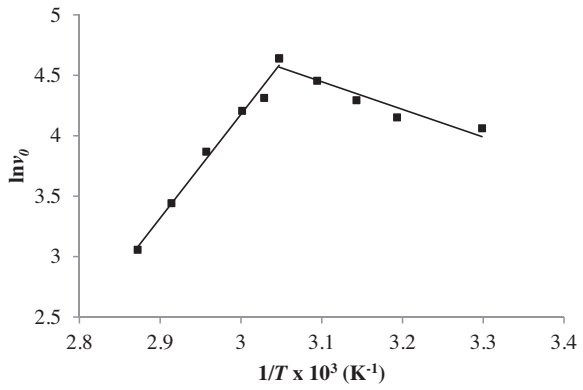

Fig. 3. Arrhenius-type plot of initial activity of *A. foetidus* acid protease using azocasein as a substrate.

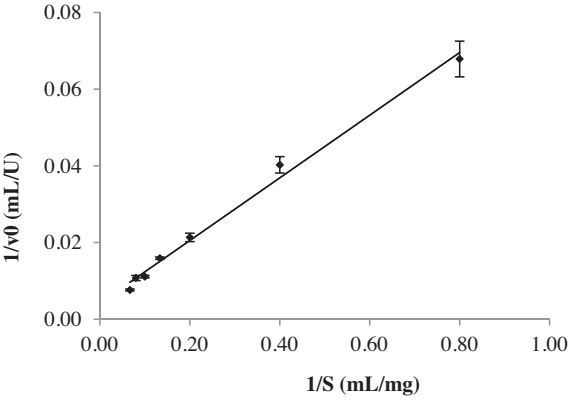

Fig. 2. Double reciprocal (Lineweaver-Burk) plot of the initial rate of azocasein hydrolysis by *A. foetidus* acid protease versus azocasein concentration. The error bars represent 95% confidence limits for the measurements.

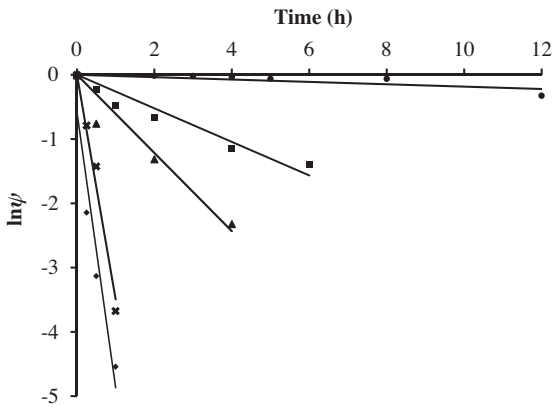

Fig. 4. Semi-log plots of irreversible denaturation of acid protease from *A. foetidus*. *T* (°C): 55 (●); 60 (■); 65 (▲); 67 (×); 70 (♦). pH_0 5.0; starting protease activity = 50 U/mL; starting substrate concentration = 1 mM azocasein.

3. Results and discussion

To select the optimal pH for the activity of *Aspergillus foetidus* protease, activity tests were performed at a temperature (*T*) of 37 °C in the initial pH (pH_0) range of 5.0-10.0, using an initial azocasein concentration (S_0) of 5 g/L (Fig. 1). Protease showed satisfactory activity from pH 5.0 to 7.0, with the highest value at pH 5.0 (55.65 U/mL). Different pH_0 values were reported for optimal activity of proteases from *Aspergillus tamari* (pH 6.0 to 9.5) [16] and *Aspergillus awamori* (pH 4.0 and 5.0) [17]. Based on these results, the next set of experiments was performed at pH_0 = 5.0.

To estimate the kinetic parameters K_m and v_{max}, additional tests were carried out at 37 °C and pH_0 5.0 varying S_0 from 1.25 to 15 g/L. Plotting the results according to Lineweaver-Burk (Fig. 2), the enzyme was shown to follow, with good correlation (R^2 = 0.994), Michaelis-Menten-type kinetics with K_m = 1.92 mg/mL (0.8 mM) and v_{max} = 357.14 U/mL. It is difficult to compare these results with those reported in the literature, because the activity of any enzyme is well known to depend on the type of substrate used. Nevertheless, although the above K_m value is higher than those reported for serine protease from *A. niger* (0.44 mg/mL) [18] and for acid protease from *A. awamori* (1.08 mg/mL) [17] using azocasein as substrates, the one of v_{max} is two orders of magnitude higher (126.5 U/mL for the former and 8.8 U/mL for the latter), thus indicating better kinetics.

The optimum temperature of protease activity was identified by carrying out activity tests at pH_0 5.0, S_0 = 100 mg/L and varying *T* from 30 to 75 °C. To minimize the influence of activity loss due to irreversible denaturation of protease, only the initial values of enzyme activity (v_0) were taken into consideration. The protease proved to be active over a relatively wide *T* range (30–65 °C) with an optimal value at 55 °C. The semi-log plot of $\ln v_0$ vs $1/T$ showed a linear increase below 55 °C, whereas an opposite trend was observed over this threshold value (Fig. 3). Such an optimum temperature is consistent with that reported by other researchers for extracellular proteases of other aspergilli, namely *A. fumigatus* [7,15].

From the slopes of the straight lines of Fig. 3, an activation energy of the hydrolysis reaction (E^*) of 19.03 kJ/mol and a standard enthalpy variation of enzyme unfolding (ΔH°_U) of 90.35 kJ/mol were estimated with satisfactory correlation (R^2 = 0.908 and 0.988, respectively). The activation energy is lower than those of similar enzymes in water; for instance, E^* values of 62, 36.8 and 31.97 kJ/mol were reported for a neutral serine protease from *A. fumigatus* [7,15] and an alkaline protease from *Nacordiopsis alba* [11], respectively. The very low E^* value estimated for the protease under investigation indicates that a few energy is required to form the activated complex of azocasein hydrolysis, thus highlighting an especially-effective hydrolytic capacity. Finally, the above comparison as a whole suggests that also its acidic nature may have played a leading role from both viewpoints.

As far as protease thermostability is concerned, long-term residual activity tests were carried out in the temperature range 55–70 °C using higher E_0 (50 U/mL) to speed up the reaction, whose results in terms of the residual activity coefficient (ψ) are illustrated in Fig. 4. Protease activity followed the classical decay of the first-order denaturation pattern already observed for other fungi belonging to the *Aspergillus* genus [7,15,19].

Table 1
Thermodynamic and kinetic parameters of the irreversible thermal deactivation (denaturation) of the acid protease from *A. foetidus*.

T (°C)	k_d (h^{-1})	$t_{1/2}$ (h)	ΔH^*_d (kJ/mol)	ΔG^*_d (kJ/mol)	ΔS^*_d (J/mol K)
55	0.018	37.6	311.39	113.87	601.92
60	0.261	2.65	311.35	107.96	610.49
65	0.608	1.14	311.31	107.59	602.45
67	0.836	0.82	311.29	107.34	599.59
70	5.06	0.13	311.27	103.18	606.41

As is well known, the half-life ($t_{1/2}$), which is the time required for the enzyme activity to drop down to 50% of the starting value at a given temperature, is an important economic parameter in many industrial applications, because the higher its value, the higher the enzyme thermostability. The kinetic results of these tests summarized in Table 1 show that $t_{1/2}$ progressively decreased and the specific rate of first-order protease thermoinactivation (k_d) progressively increased with temperature, which means that its irreversible denaturation became more and more significant. The value of k_d obtained at 55 °C (0.018 h^{-1}) was significantly lower, and that of $t_{1/2}$ (37.64 h) higher, than those reported for proteases from *A. awamori* (k_d = 0.04 h^{-1}; $t_{1/2}$ = 17.1 h; T = 55 °C) [15] and *A. fumigatus* (k_d = 0.026 h^{-1}; $t_{1/2}$ = 1.08 h; T = 50 °C) [7]. Thus, the acid protease produced in the current study appears to be more thermostable than at least some of similar enzymes reported in the literature.

Semi-log plot of lnk_d vs 1/T allowed estimating an activation energy of protease denaturation (E^*_d) of 314.12 kJ/mol (R^2 = 0.946). This value is one order of magnitude higher than those reported for alkaline proteases from *A. fumigatus* (69 kJ/mol) [7], *Nocardiopsis alba* (74.8 kJ/mol) [11] and *Bacillus licheniformis* (32.8–48.5 kJ/mol depending on pH) [20], providing a further proof of the excellent thermostability of *A. foetidus* protease, likely associated to its acidic nature. One can also realize from this comparison that there is a very large variability of E^*_d data in the literature, likely due to large differences in the source and purity of the enzymes as well as the substrates used.

E^*_d is directly related, through Eq. (4), to the activation enthalpy of denaturation (ΔH^*_d), which is another important thermodynamic parameter expressing the total amount of energy required to denature the enzyme; therefore, large and positive values of E^*_d and ΔH^*_d should be associated with high enzyme thermostability [8,9]. The acid protease investigated here has a ΔH^*_d value at 55 °C (311.39 kJ/mol) (Table 1) much higher than those of the above-mentioned alkaline or neutral proteases from *A. awamori* [15], *A. fumigatus* [7] and *N. alba* [11], confirming the earlier conclusions.

The extent of thermal enzyme denaturation also depends on the activation entropy (ΔS^*_d) of this event, which expresses the amount of energy per degree involved in the transition from a native to a denatured state [8]. As shown in Table 1, consistently with the increase in the disorder or randomness degree consequent to such a transition, the ΔS^*_d values were positive (599.59–610.49 J/mol K) at all temperatures at which *A. foetidus* protease was tested. In addition, they were higher than those reported for the above proteases from *A. awamori* ($-93.5 \leq \Delta S^*_d \leq 126.5$ J/mol K) [15], *N. alba* ($\Delta S^*_d = -69.99$ J/mol K) [11] and *A. fumigatus* ($-69.5 \leq \Delta S^*_d \leq -68.2$ J/mol K) [7].

Another important thermodynamic parameter, the Gibbs free energy of denaturation (ΔG^*_d), includes both enthalpic and entropic contributions; therefore, it is a more accurate and reliable predicting tool to evaluate enzyme stability. A smaller or negative value of such a parameter is associated with a more spontaneous process, that is to say, the enzyme becomes less stable and more easily undergoes denaturation [8]. On the contrary, an increase in ΔG^*_d reveals an increase in resistance to denaturation, i.e. increased thermostability [11]. Consistently with the above-discussed thermostability, ΔG^*_d for *A. foetidus* acid protease was quite high, reaching 113.8, 107.9, 107.5, 107.3 and 103.1 kJ/mol at 55, 60, 65, 67 and 70 °C, respectively (Table 1). Comparable ΔG^*_d values were found in previous reports for proteases from *A. awamori* (111.7 kJ/mol) [15] and *A. fumigatus* (103. 07 kJ/mol) [7].

4. Conclusions

This work is aimed at the determination of the kinetic and thermodynamic properties of extracellular acid protease produced by *A. foetidus*, which, to the best of our knowledge, has not been done to date. Maximal activity was observed at pH 5.0 and 55 °C. Residual activity tests carried out at 10–70 °C allowed estimating very long enzyme half-lives ($t_{1/2}$ = 37.74 h at 55 °C) as well as the thermodynamic parameters of the irreversible denaturation of the enzyme. Such event was characterized by an activation energy, Gibbs free energy and enthalpy as high as 314.12, 111.7 and 311 kJ/mol and an activation entropy of 601.9 J/mol K. These values as a whole highlight an excellent thermostability of the enzyme that could be profitably exploited for future industrial applications, especially in the cosmetic and pharmaceutical sectors.

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