Kinetic and Thermodynamic Investigation on Ascorbate Oxidase Activity and Stability of a *Cucurbita maxima* Extract

Tatiana S. Porto,*,† Camila S. Porto,‡ Maria T. H. Cavalcanti,‡ José L. Lima Filho,‡ Patrizia Perego,§ Ana L. F. Porto,|| Attilio Converti,§ and Adalberto Pessoa Jr.†

Department of Biochemical and Pharmaceutical Technology, University of São Paulo, Av. Prof. Lineu Prestes, 580, B. 16., 05508-000 São Paulo-SP, Brazil, Laboratory of Immunopathology Keizo Asami (LIKA), 50670-901 Recife-PE, Brazil, Department of Chemical and Process Engineering, Genoa University, I-16145 Genoa, Italy, and Department of Morphology and Animal Physiology, Federal Rural University of Pernambuco, 52171-900 Recife-PE, Brazil

The kinetic and thermodynamic properties of ascorbate oxidase (AO) activity and stability of a *Cucurbita maxima* extract were investigated. Activity tests performed at 25 °C using initial ascorbic acid concentration in the range 50−750 μM allowed estimating the Michaelis constant for this substrate (K_m = 126 μM) and the maximum initial rate of ascorbic acid oxidation ($A_{0,max}$ = 1.57 mM min^{-1}). The main thermodynamic parameters of the enzyme reaction (ΔH^* = 10.3 kJ mol^{-1}; ΔG^* = 87.2 kJ mol^{-1}; ΔS^* = −258 J mol^{-1} K^{-1}) were estimated through activity tests performed at 25−48 °C. Within such a temperature range, no decrease in the initial reaction rate was detected. The long-term thermostability of the raw extract was then investigated by means of residual activity tests carried out at 10−70 °C, which allowed estimating the thermodynamic parameters of the irreversible enzyme inactivation as well (ΔH^*_D = 51.7 kJ mol^{-1}; ΔG^*_D = 103 kJ mol^{-1}; ΔS^*_D = −160 J mol^{-1} K^{-1}). Taking into account the specific rate of AO inactivation determined at different temperatures, we also estimated the enzyme half-life (1047 min at 10 °C and 21.2 min at 70 °C) and predicted the integral activity of a continuous system using this enzyme preparation. This work should be considered as a preliminary attempt to characterize the AO activity of a *C. maxima* extract before its concentration by liquid−liquid extraction techniques.

1. Introduction

Ascorbate oxidase (AO) (EC 1.10.3.3) is a multicopper oxidase that catalyzes one-electron oxidation of L-ascorbic acid with concomitant four-electron reduction of biatomic oxygen to two water molecules. AO is a glycoprotein widely distributed in higher plants and microorganisms, the main sources being members of the *Cucurbitaceae* (cucumber, zucchini, pumpkin, squash, melon, etc.) (*1*).

Recently, this enzyme has been used for biosensors as well as for clinical and food analyses of ascorbic acid (*2*). Plant AOs are homodimers with each subunit containing one Type (T)1 copper and one T2/T3 trinuclear cluster. T1 copper is responsible for the intense blue color of AO, while T2 copper is responsible for electron transfer to O_2 (*3*). Two T3 copper ions are linked through a OH bridge (*3*) and are believed to act as electron acceptors (*4*).

The biological function of AO is not entirely known. AO activity shows a significant increase during a specific period of fruit development, which suggests that it might play an important role during its ripening. AO participation in cell expansion has recently been demonstrated, but the mechanism by which it affects cell elongation is still unclear (*5*). It is believed that the enzyme protects the fruit against oxidative damage resulting from any injury.

AO activity from *Cucurbita maxima* was shown to follow the Michaelis−Menten law with an independent binding of ascorbic acid and oxygen and was completely lost after 1 min at 100 °C (*6*). AO from *C. maxima* is considered to be relatively heat-resistant: its activity remained almost unaltered when it was incubated at 0−40°C for 30 min (*6*), and the dimer from cucumbers was shown to be the form most resistant to heat inactivation (*7*). However, no systematic thermodynamic study has been performed up to now on this enzymatic system, to the best of our knowledge. The aim of this work was to perform a kinetic−thermodynamic characterization of AO activity and thermostability of a raw extract of *C. maxima*. These results could be of crucial importance either to establish possible effects of impurities on kinetic and thermodynamic properties or to concentrate and partially purify *C. maxima* AO by extraction with an aqueous two-phase system. This technology has in fact great potential mainly for industrial separation of enzymes, because it is a cheap and efficient downstream method and offers many advantages, among which are low process time, low energy consumption, and mild conditions (*8*). The final part of this study was devoted to the prediction of the integral activity of a continuous system simulating the functioning of a biosensor useful for a variety of applications of concern for the food industry (*2*).

* To whom correspondence should be addressed. Tel: +55-11-30217642. Fax: +55-11-38156386. E-mail: portots@usp.br.
† University of São Paulo.
‡ LIKA.
§ Genoa University.
|| Federal Rural University of Pernambuco.

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2. Materials and Methods

2.1. Extract Preparation. The fresh fruits of the Brazilian variety *Cucurbita maxima* var. jerimum caboclo were obtained from a local food supplier. In order to minimize any variation in the AO content of the raw extract, all experiments were made using always the same batch of fruits harvested just before their ripening. The fruits were knife-peeled to a depth of about 3 mm. The tissue was blended in 0.1 M citrate/phosphate buffer, pH 6.0, for 5 min at 4 °C, keeping the ratio of 1 g tissue per 1.5 mL buffer. After homogenate centrifugation at 13,000*g* for 10 min at 0−4 °C, the precipitate was discarded, and the supernatant, denominated as raw extract, was stored at −10 °C.

2.2. AO Activity Determination. The AO activity of the extract (*A*) was measured at 268 nm (*5*, *6*) through the 3-min oxidation of ascorbic acid (AA) at 25 °C in a UV−vis spectrophotometer, model Ultrospec 2000 (Amersham Pharmacia Biotech, Piscataway, NJ); a Peltier device was utilized for temperature control (*6*). One AO unit was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of AA per minute under the assay conditions.

For tests, aliquots (25 μL) of the extract previously stored at −10 °C were thawed and immediately used. Tests for determination of kinetic parameters of AO activity were performed at 25 °C and using different initial AA concentrations ($50 \leq S_0 \leq 750$ μM), whereas those for estimation of thermodynamic parameters of enzyme reaction were carried out at $S_0 = 50$ μM and different temperatures ($25 \leq T \leq 48$ °C).

The residual activity was determined by diluting known volumes of defrosted extracts in the above citrate-phosphate buffer contained in 10-mL glass tubes and exposing them at the selected temperature range (10−70 °C) for variable time. Aliquots (25 μL) were withdrawn at different times (1, 10, 30, and 60 min), rapidly cooled at 25 °C, to ensure efficient refolding of the enzyme molecules eventually reversibly inactivated, and then assayed for AO activity as described above.

All tests were performed in triplicate, and the results are expressed as means values. Statistical analysis was done using standard deviations of the experimental data from the means values.

3. Theory

3.1. Enzyme Activity. ***3.1.1. Kinetics of Enzyme Activity.*** The kinetics of AO action obeys the Michaelis−Menten law with an independent binding of substrates (*6*, *9*). Under conditions where AA was the limiting substrate, a Michaelis−Menten-type equation has been used to fit the experimental data of the initial rate of AA oxidation, A_0 (mM min^{-1}).

A nonlinear least-squares method was used in a common program for error minimization (Table Curve Jandel for Windows) to estimate with good accuracy the values of the maximum initial rate, $A_{0,max}$, and the Michaelis constant, K_m (μM), from the experimental data of A_0 and starting AA concentration, S_0 (μM).

3.1.2. Thermodynamics of the Enzyme Reaction. The thermodynamics of the enzyme reaction was investigated in the temperature range 25−48 °C under conditions where AA was the limiting substrate. The specific reaction rate, k_v (min^{-1}), was defined as to be proportional to the rate of optical density decrease under the conditions selected for the initial activity determination. This rate increased with temperature along the whole temperature range under investigation, according to the equation of Eyring (*10*):

$$\ln\frac{k_v h}{k_B T} = -\frac{\Delta H^*}{RT} + \frac{\Delta S^*}{R} \quad (1)$$

where k_B is the Boltzmann constant, h the Planck constant, R the ideal gas constant, ΔH^* the activation enthalpy, and ΔS^* the activation entropy.

Semilog plots of $\ln(k_v h/k_B T)$ versus $1/T$ allowed for estimation of ΔH^* from the slope of the resulting straight line and ΔS^* from the intercept on the ordinate axis, respectively. Finally, the activation free energy (ΔG^*) was calculated at a reference temperature of 25 °C according to the equation

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (2)$$

3.2. Enzyme Thermal Inactivation. Enzyme thermal inactivation is generally described by a reversible unfolding of its native form to give an intermediate less stable unfolded form, which is subject to irreversible denaturation to an inactivated form (*11*−*15*).

As far as plant AOs are concerned, these enzymes are stable homodimers (E2A) (*2*), whereas their monomeric form (E1I) is inactive (*16*); therefore, a first-order reaction mechanism can be proposed:

$$\text{E2A} \xrightarrow{k_d} 2\ \text{E1I} \quad (3)$$

where k_d (min^{-1}) is the specific rate of AO irreversible inactivation.

Therefore, the rate of E2A inactivation can be described by the equation

$$\ln \psi = -k_d t \quad (4)$$

where

$$\psi = \frac{C_{E2A}}{C_{E2A_0}} \quad (5)$$

is the so-called residual activity coefficient (dimensionless) and C_{E2A} and C_{E2A_0} are the actual and the starting concentrations of E2A.

According to this model, already proposed for other enzymatic systems (*13*, *15*, *17*), the values of k_d were estimated at different temperatures by semilog plots of ln ψ versus time. Like the thermodynamic parameters of the enzyme reaction, those of the enzyme inactivation were estimated by Eyring semilog plots of ln k_d versus $1/T$.

3.3. Enzyme Half-Life. The enzyme half-life ($t_{1/2}$) was defined as the time after which the enzyme activity was reduced to one-half of the initial activity and then calculated, according to Rashid and Siddiqui (*13*), from the k_d values as

$$t_{1/2} = \ln 2/k_d \quad (6)$$

3.4. Enzyme Integral activity. Because of the enzyme denaturation, the enzyme activity becomes a function of the operating time; therefore, the integral activity, P (mM), can be predicted for a continuous process by integrating the product of A_0 to the activity coefficient (*18*, *19*):

$$P(t) = \int A_0 \psi\, dt = \int A_0 \exp(-k_d t)\, dt = \frac{A_0}{k_d}[1 - \exp(-k_d t)] \quad (7)$$

According to the above model, this parameter achieves, up to the enzyme half-life ($t_{1/2}$), the value

$$P_{1/2} = \frac{A_0}{2k_d} \tag{8}$$

and twice this value up to infinity of time (P_∞).

4. Results and Discussion

4.1. Kinetic Characterization of AO Activity. Figure 1 shows the Lineweaver−Burk plot of the initial rate of AA oxidation by *C. maxima* extract versus the initial substrate concentration at 25 °C. However, for more accuracy, the values of the biochemical kinetic parameters ($A_{0,max} = 1.57 \pm 0.14$ mM min^{-1}; $K_m = 126 \pm 11$ μM) were estimated, with satisfactory correlation (average standard deviation of 0.0301) using a nonlinear least-squares method.

The estimated value of $A_{0,max}$ is much higher than those reported for AO from extracts of *Cucumis sativus* (0.15 mM min^{-1}) (*20*), *Cucurbita pepo* var. *condensa* (0.010 mM min^{-1}) (*21*), and *Cucumis anguria* (0.012 mM min^{-1}) (*22*). Besides, the value of K_m is lower than those already reported for either raw or concentrated extracts of *C. maxima* ($K_m = 200$ μM) (*6*), *Cucumis sativus* (157 μM) (*20*), and *Acremonium* sp. (290 μM) (*23*). These results on the whole demonstrate the interesting activity of the raw extract of the *C. maxima* variety under consideration, in spite of the simultaneous presence of many other proteins.

4.2. Thermodynamics of the Enzyme Reaction. Figure 2 shows the semilog Eyring plot of the specific reaction rate versus the reciprocal absolute temperature, which allowed estimating with good correlation ($r^2 = 0.993$) the activation enthalpy (ΔH^*) and entropy (ΔS^*) of the reaction of AA oxidation by the *C. maxima* raw extract. The temperature range investigated (25−48 °C) was selected on the basis of previous literature reports as well on the optimum temperature of AO activity (30−35 °C) (*20*, *24*−*26*). As is well known, such a graphical estimation of entropies is rough, a rigorous attempt requiring quantum mechanical approaches, which would be almost impossible to apply to biological systems and out of the purposes of this study. The estimated values of these parameters are listed in Table 1 together with those of ΔG^* calculated through eq 2.

The specific reaction rate increased with temperature according to eq 1 within the whole temperature range selected for this study. Contrary to what has been observed for similar enzyme preparations (*15*, *17*, *18*), no decrease in the initial activity versus temperature was detected, which confirms the interesting thermal pattern of the raw extract of *C. maxima* for possible industrial application. This result suggests an optimum activity temperature higher than those for purified AOs from *C. pepo medullosa* (35 °C) (*25*), *Acremonium* sp. HI-25 (45 °C), and cucumber (30 °C) (*24*).

It is likely that the natural environment of the extract might have provided AO with a sort of protection against thermal inactivation, which suggested us to check the long-term enzyme stability by residual activity tests (see the next section).

The activation enthalpy of the enzyme reaction ($\Delta H^* = 10.3$ kJ mol^{-1}) is very low and in agreement with the high efficiency of AA oxidation by AO. Besides, it is close to the values estimated for the activity (10 kJ mol^{-1}) (*25*) and internal electron transfer from T1 to T3 Cu(II) of purified AO from *C. pepo medullosa* ($\Delta H^* = 6.8$−9.1 kJ mol^{-1}) (*9*). This result should not be surprising taking into account that such an electron transfer could have become, under the selected conditions, the rate-determining step of the overall AO activity. The above ΔH^* value does not differ much from those estimated for the AO activity of the soluble fraction of cabbage leaves (*Brassica oleracea*) (18.4 kJ mol^{-1}) and of vegetable marrow (*Cucurbita pepo*) (14.6−16.7 kJ mol^{-1}), whereas those from their respective cell walls ($\Delta H^* = 50.2$ and 28 kJ mol^{-1}, respectively) (*28*) and from 30-fold purified *C. maxima* extract ($\Delta H^* = 36.8$ kJ mol^{-1}) (*6*) were significantly higher. Relatively low values of the activation enthalpy are typical of most oxidoreductases implying internal electron transfer. For instance, Farver et al. (*26*) reported an activation enthalpy of 22.7 ± 3.4 kJ mol^{-1} for nitrite reductase.

The ΔH^* value estimated in this work is about 8 times lower than the energy of activation required for the reaction catalyzed by $CuSO_4$ ($\Delta H^* = 92.1$ kJ mol^{-1}) (*6*), thus confirming the interesting AO activity of the raw extract of *C. maxima* var. jerimum caboclo.

The negative and large value of ΔS^* (−258 J mol^{-1} K^{-1}) is consistent with the formation of a transition state with more rigid structure with respect to the separate reactants (AO and AA) and agrees with those reported for most enzymatic systems (*18*, *29*, *30*), in particular those estimated for purified AO from *C. pepo medullosa* (from −215 to −170 J mol^{-1} K^{-1}) (*9*, *26*). Because the entropy of activation includes an electronic factor

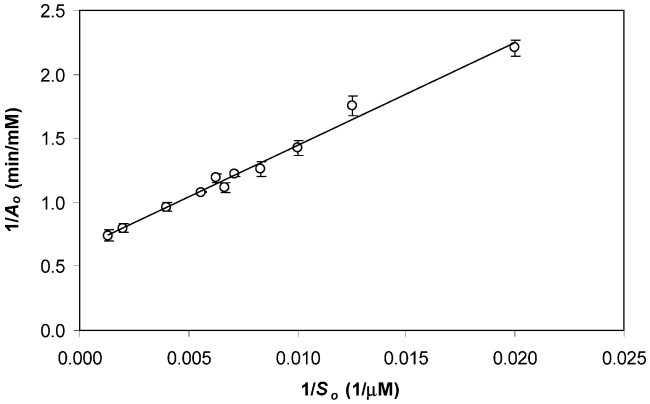

Figure 1. Lineweaver−Burk plot of the initial rate of AA oxidation by *C. maxima* extract versus the initial substrate concentration.

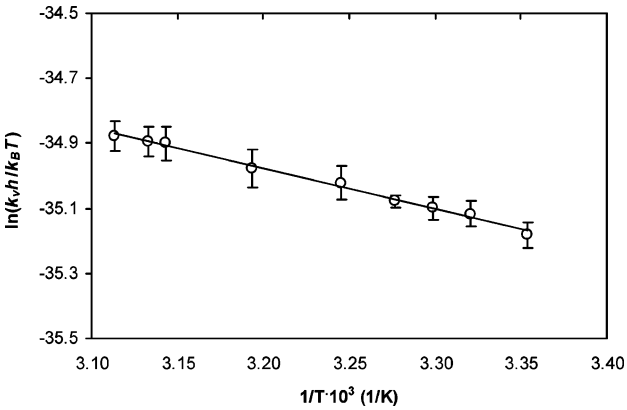

Figure 2. Semilog Eyring plot for the determination of the thermodynamic parameters of the enzyme reaction.

Table 1. Thermodynamic Parameters of the Enzyme Reaction and Thermal Inactivation of the Raw Extract of *C. maxima*

	ΔG^* (kJ mol^{-1})	ΔH^* (kJ mol^{-1})	ΔS^* (J mol^{-1} K^{-1})	r^2
AO activity[a]	87.2	10.3	−258	0.993
AO Inactivation[b]	103	51.7	−160	0.967

[a] Reference temperature: 25 °C. [b] Reference temperature: 50 °C.

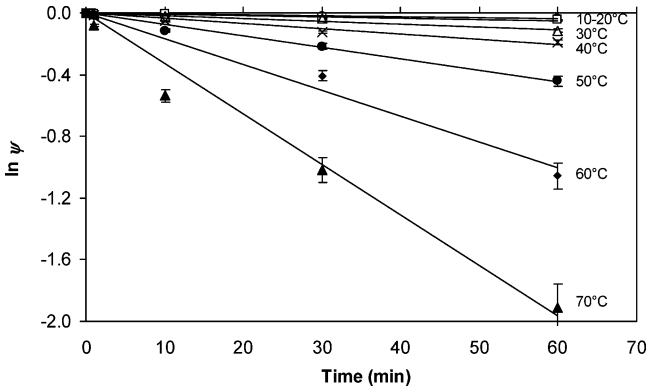

Figure 3. Semilog Eyring plot of the residual AO activity coefficient (ψ) of *C. maxima* raw extract.

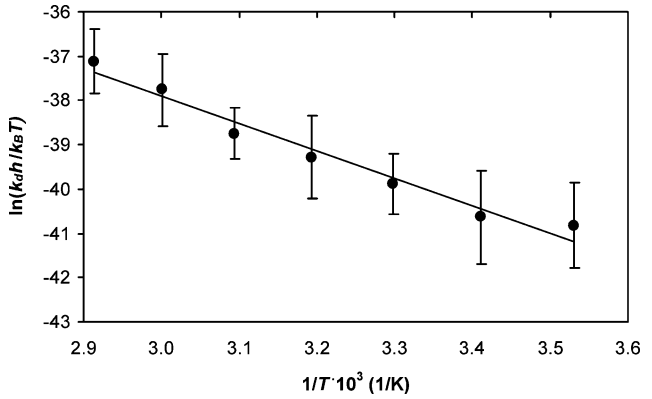

Figure 4. Semilog Eyring plot for the estimation of the thermodynamic parameters of AO irreversible thermal inactivation.

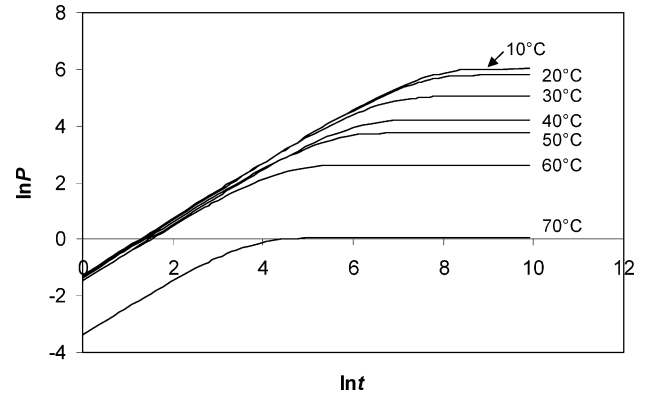

Figure 5. Integral AO activity (P, mM) versus time (t, min) of the raw extract of *C. maxima* at different temperatures.

(i.e., the transmission coefficient), an additional cause of the above low values of the kinetic constants might be a combination of the low driving force and the changes taking place in the structures of the respective electron-accepting sites. In other words, the disadvantageous entropic contribution could have almost completely counterbalanced the favorable enthalpic situation. As a result, the system exhibited a relatively high and positive value of the activation free energy ($\Delta G^* = 87.2$ kJ mol^{-1}). This value is higher than those that can be estimated from literature data for purified AO ($\Delta G^* = 60-70$ kJ mol^{-1}).

Comparing the thermodynamic parameters estimated in this work with those of pure AO, it is noteworthy that the presence of AO in the natural extract of *C. maxima* made the enzyme peculiarly resistant to initial heat stress, while obviously decreasing its activity. However, it is not clear if the impurity content of the extract can exert any influence on the thermodynamic parameters of the enzyme reaction, thus requiring further investigation using a partially purified extract or even pure AO from this variety.

Table 2. Results of the Specific Rate of AO Denaturation (k_d), Half-Lives ($t_{1/2}$), and Integral Activities up to $t_{1/2}$ ($P_{1/2}$) and Infinity of Time (P_∞) Estimated for the Raw Extract of *C. maxima* at Different Temperatures

	T (°C)						
	10	20	30	40	50	60	70
$k_d \times 10^3$ (min^{-1})	0.662	0.822	1.80	3.40	6.00	16.7	32.7
$t_{1/2}$ (min)	1047	844	385	204	115	41.5	21.2
$P_{1/2}$ (mM)	205	166	78.6	34.2	21.3	6.89	0.535
P_∞ (mM)	410	332	157	68.3	42.6	13.8	1.07

4.3. Thermodynamics of AO Thermal Inactivation. This part of this study was addressed to the effect of temperature on the long-term thermostability of AO, which was suggested by the above relative insensitivity to temperature of the initial AO activity of the raw extract at 25−48 °C. For this purpose, residual activity tests were carried out within a larger temperature range (10−70 °C), whose results in terms of the residual AO activity coefficient (ψ) are illustrated in Figure 3. The lowest temperature (10 °C) was selected as a reference temperature at which the enzyme thermal inactivation could be considered almost negligible. Similar irreversible inactivation by heat in a linear fashion has been observed for purified AO from *C. pepo medullosa* (*25*).

Within the tested temperature range, the AO activity followed the typical decay of the classic first-order denaturation pattern, like that observed by Moreno et al. (*31*) for both native and immobilized purified lipases.

The kinetic results of these tests summarized in Table 2 show that the specific rate of AO denaturation (k_d) progressively increased with temperature, which means that the inactivation became progressively more significant, likely due to breaking of strong electrostatic bonds among intersubunits of E2A and formation of totally inactive monomers (E1I).

It should be noticed that the values of k_d were particularly low (from 6.62×10^{-4} min^{-1} at 10 °C to 0.0327 min^{-1} at 70 °C) and those of $t_{1/2}$ particularly high (1047 min at 10 °C and 21.2 min at 70 °C) if compared with those of pure AO from *C. pepo medullosa* thermally inactivated ($k_d = 0.032$ min^{-1} and $t_{1/2} = 21.5$ min at 55 °C; complete activity loss at $T > 65$ °C) (*25*). These results would be consistent either with good long-term thermostability of the raw enzyme preparation or with slower degradation of AO at lower temperature. However, this last effect has been excluded by nearly negligible protease activity under all the conditions tested in this work (results not shown), with unappreciable dependence of this activity on temperature.

On the other hand, at $T \geq 40$ °C, the half-life values were much lower than at low temperature, i.e., the enzyme was denaturated quickly. As a consequence of these effects, the overall time-dependent AO activity was affected by a temperature increase more than the initial activity was favored. Even better thermostability parameters ($t_{1/2}$ ca. 128 min at 60 °C) can be calculated from the data reported for thermostable monomeric AO from *Acremonium* sp. HI-25 cloned into *Aspergillus nidulans* (*32*).

Figure 4 shows the semilog plot of k_d versus the reciprocal absolute temperature, which allowed estimating the thermodynamic parameters of AO irreversible inactivation (Table 1).

The enzyme inactivation was characterized by an activation enthalpy of 51.7 kJ mol^{-1} and a negative and relatively large activation entropy ($\Delta S^*_D = -160$ J mol^{-1} K^{-1}), resulting in particularly high activation free energy ($\Delta G^*_D = 103$ kJ mol^{-1}),

which is consistent with the high thermostability of the raw extract. The estimated value of ΔH^*_D is comparable with those reported in the literature for several biological systems. Surprisingly, it lies in the range of values reported for thermal reversible unfolding (56−150 kJ mol^{-1}) (*15*, *18*) and is much lower than those reported for the irreversible denaturation of different enzymes (220−235 kJ mol^{-1}) (*19*, *33*).

Nevertheless, some interesting studies on AO inactivation with different methods highlighted that this phenomenon takes place irreversibly (*34*). The negative value estimated for ΔS^*_D provides some information on possible time-dependent inactivation mechanism. At first, heat absorption by the extract could have irreversibly inactivated the stable dimer to give inactive unfolded monomers; in a subsequent step, two molecules of this could have aggregated giving a transition state with more rigid structure than the separate molecules.

The one-step fashion observed in this work for AO thermoinactivation is qualitatively different from the multistep ones described in the literature for well known thermostable enzymes, such as psychrotroph lipases (*35*) and glucose-6-phosphate dehydrogenase (*17*).

4.4. Integral Activity. As is well known, an increase in thermostability usually will be accompanied by a decrease in activity and vice versa. An optimum compromise between these opposite tendencies should then be searched to select the best conditions to perform an industrial process. For this purpose, the integral activity up to the half-lives ($P_{1/2}$) has been calculated by eq 8 (Table 2).

In view of possible industrial exploitation of the extract AO activity, we investigated the time behavior of the integral activity, $P(t)$, defined in eq 7, which represents the amount of AA oxidized by 1 g of biocatalyst under continuous feed conditions (*36*). This system can simulate an oxidase column functioning as a biosensor useful for a variety of applications of concern for the food industry (*37*). The possibility of using with success raw extracts for biosensor preparation has been in fact demonstrated (*38*).

The pattern described by eq 7 is illustrated in Figure 5, where the integral activity estimated in the temperature range 10−70 °C is plotted versus time in a bi-log plot. All curves grew linearly with nearly the same starting rate, as a consequence of the fact that the enzyme denaturation became significant only after a relatively long time. Besides, this time was shorter at higher temperature because of higher k_d values; therefore, the activity achieved progressively lower P_∞ values.

The only exception in this trend is given by the run performed at 70 °C, which exhibited remarkably less initial activity than the others. This result, which is consistent with the observations of Maccarrone et al. (*25*), suggests the occurrence of an initial activity decay at this temperature, not yet evidenced in the above investigated temperature range (25−48 °C). Finally, the long duration of this linear increase in all cases (≥50 min) is in accordance with the very low k_d values, i.e., with the satisfactory thermostability of the extract.

Conclusions

The kinetic and thermodynamic parameters of AO activity and stability of a *Cucurbita maxima* extract were estimated in this work. The Michaelis constant for the ascorbic acid and the maximum activity were 126 μM and 1.57 mM min^{-1}, respectively. Initial activity tests performed at 25−48 °C allowed estimating the following thermodynamic parameters of the enzyme reaction: $\Delta G^* = 87.2$ kJ mol^{-1}; $\Delta H^* = 10.3$ kJ mol^{-1}; $\Delta S^* = -258$ J mol^{-1} K^{-1}. Residual activity tests carried out at 10−70 °C allowed estimating very long enzyme half-lives ($t_{1/2} \geq 204$ min at $T \leq 40$ °C) as well as the thermodynamic parameters of the irreversible AO inactivation ($\Delta G^*_D = 103$ kJ mol^{-1}; $\Delta H^*_D = 51.7$ kJ mol^{-1}; $\Delta S^*_D = -160$ J mol^{-1} K^{-1}). The estimated values of the enzyme half-life revealed the interesting thermostability of this enzyme preparation for future applications. To this purpose, the integral activity of a continuous system containing such an extract has also been predicted.

The next effort will deal with liquid−liquid extraction with an aqueous two-phase system to remarkably increase the AO activity without affecting its thermostability, as well as the identification of possible interference of impurities contained in the raw extract on the AO thermodynamic properties.

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Notation

A	AO activity, mM min^{-1}
A_0	initial AO activity, mM min^{-1}
$A_{0,max}$	maximum initial AO activity, mM min^{-1}
C_{E2A}	concentration of the active AO dimer, μM
C_{E2A_0}	starting concentration of the active AO dimer, μM
h	Planck constant, J min
k_B	Boltzmann constant, J K^{-1}
K_m	Michaelis constant, μM
k_v	specific reaction rate, min^{-1}
k_d	specific rate of AO irreversible denaturation, min^{-1}
P	integral AO activity, mM
$P_{1/2}$	integral AO activity up to $t_{1/2}$, mM
P_∞	integral AO activity up to infinity of time, mM
R	molar ideal gas constant, J mol^{-1} K^{-1}
r^2	determination coefficient, dimensionless
S_0	initial AA concentration, μM
t	reaction time, min
T	temperature, °C or K
$t_{1/2}$	AO half-life, min

Abbreviations

AA	ascorbic acid
AO	ascorbate oxidase
E2A	active AO dimer
E1I	inactive AO monomer

Greeks

ΔG^*	activation free energy of the enzyme reaction, kJ mol^{-1}
ΔG^*_D	activation free energy of AO inactivation, kJ mol^{-1}
ΔH^*	activation enthalpy of the enzyme reaction, kJ mol^{-1}
ΔH^*_D	activation enthalpy of AO inactivation, kJ mol^{-1}
ΔS^*	activation entropy of the enzyme reaction, J mol^{-1} K^{-1}
ΔS^*_D	activation entropy of AO inactivation, J mol^{-1} K^{-1}
ψ	residual activity coefficient, dimensionless

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