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A N I N T E R N A T I O N A L J O U R N A L

Kinetics and Thermodynamics of Thermal Inactivation of β-Galactosidase from *Aspergillus oryzae*

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ABSTRACT

For optimization of biochemical processes in food and pharmaceutical industries, the evaluation of enzyme inactivation kinetic models is necessary to allow their adequate use. Kinetic studies of thermal inactivation of β-galactosidase from Aspergillus oryzae were conducted in order to critically evaluate mathematical equations presented in the literature. Statistical analysis showed that Weibull model presented the best adequacy to residual enzymatic activity data through the processing time and its kinetic parameters as a function of the temperature, in the range of 58-66 ºC. The investigation suggests the existence of a non-sensitive heat fraction on the enzyme structure, which is relatively stable up to temperatures close to 59 ºC. Thermodynamic parameters were evaluated and showed that such β-galactosidase presents activation energy of 277 kJ mol^{-1} and that the enzyme inactivation is due to molecular structural changes. Results shown that the enzyme is quite stable for biotechnological applications.

Key words: β-Galactosidase, Enzyme Inactivation, Modelling, Protein Denaturation, Weibull.

INTRODUCTION

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β-Galactosidase (EC 3.2.1.23) is an important commercial enzyme used in the food and pharmaceuticals processes. It is mainly applied to produce low lactose dairy products for lactose intolerant people, and also to produce prebiotics like galactooligosaccharides (GOS), and a variety of additives for the pharmaceutical and cosmetic industries [1-4]. β-galactosidase from *Aspergillus oryzae* has been shown a particular importance in GOS production [5-7]. This enzyme can produce trisaccharides and higher saccharides, while β-galactosidases from *Kluyveromyces* sp. produces mainly trisaccharides [8].

GOS are formed by a glucose molecule attached to two to six galactose molecules through different glycosidic linkages, or only by galactose molecules linked by glycosidic bonds [9, 10], and its structure is primarily controlled by the identity of the enzyme used [10]. Beyond the enzyme source, the GOS synthesis can be influenced by the temperature, since at higher temperatures it is possible to achieve higher concentrations of lactose (600 g L^{-1}), increasing the GOS yield [10]. Moreover, the possibility of operating at higher temperatures offers the advantage of avoiding potential microbial contamination, which is greatly desirable in industrial scale. Then, in this case, thermal stability evaluations are essential in order to know the temperatures in which it is possible to work without losses in enzyme activity, enabling the development of the galactooligosaccharides synthesis reaction with higher efficiency.

The term "stability" refers to a protein's resistance to adverse influences such as heat or denaturants, that is, to the persistance of its molecular integrity or biological function in the face of high temperatures or other deleterious influences [11]. Inactivation of an enzyme can be of inter- or intramolecular nature. Intermolecular inactivation mechanisms may include autolysis and aggregation, whereas the intramolecular phenomena are due to the interaction of the protein with irreversible inhibitors, solvents, surfactants, salts, among others, or to extremes of pH and temperature. The mathematical simulation of the effect of these agents on the activity of the enzymes is an important approach to understand and to improve the stability of proteins as biocatalysts [4, 12].

A low number of studies on the inactivation of the *Aspergillus oryzae* β-galactosidase is available. Moreover, the kinetic modelling of thermal inactivation of this enzyme, and the determination of its thermodynamic parameters, that is of great interest for the GOS synthesis, is still lacking in the literature. Ladero and co-workers [4] studied the thermal and operational stability of a commercial β-galactosidase from *Kluyveromyces fragilis* (Lactozym) in several buffered solutions by testing different kinetic models for the thermal inactivation data of the enzyme. In the same way, Jurado and co-workers [13] have evaluated the influence that different experimental conditions (pH, temperature and ionic concentration) have on the activity of two commercial β-galactosidases (Lactozym - *Kluyveromyces fragilis* and Maxilact - *Kluyveromyces lactis*) by using two kinetic models. More recently, Guidini and co-workers [14] evaluated the thermal stability of the immobilized *Aspergillus oryzae* β-galactosidase during lactose hydrolysis; however, thermodynamics parameters were not determined. In this sense, the objective of the present work was to analyze the thermal stability of the *Aspergillus oryzae* β-galactosidase in different inactivating temperatures and to evaluate several proposed mathematical models to predict enzyme residual activity as a function of time. Moreover, temperature dependence and thermodynamic parameters for thermal inactivation were determined.

MATERIALS AND METHODS

Chemicals

β-Galactosidase from *Aspergillus oryzae*, which is encoded G5160 (CAS Number: 9031-11-2), and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were obtained from Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical grade.

Enzymatic Assay

β-Galactosidase activity was determined using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The reaction contained 0.45 mL of ONPG (15 mM, final concentration) dissolved in acetate buffer (0.1 M, pH 4.5) and 50 μl of conveniently diluted enzyme. After incubation (40 °C for 3 min), the reaction was stopped by adding 1.5 mL of 0.1 M sodium carbonate buffer (pH 10) and the absorbance was measured at 415 nm. One unit (U) of β-galactosidase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of ONPG to *o*-nitrophenol per minute at the defined assay conditions.

Thermal inactivation studies

Aliquots of buffered β-galactosidase solutions (0.5 mL; 0.015 mg mL^{-1} in 0.1 M acetate buffer, pH 4.5) were heated in sealed tubes with 1 mm of thickness, 9 mm of internal diameter and 4 cm of length in a thermostatically controlled water bath (Thermomix BM-S, B. Braun Biotech International, Melsungen, Germany) at temperatures ranging from 58 °C to 66 °C during up to 300 min. In order to avoid the effects of heating-up and cooling-down, the enzyme activity after 30 s of heating-up time (t = 0) was considered to be the initial activity, and after the exposure to heat, tubes were immediately immersed in an ice bath. Assays were done in duplicate and the average residual hydrolitic activities with respect to processing time at different temperatures were fitted to several kinetic models using non-linear regression by Statistica 7.0 (StatSoft Inc., Tulsa, OK).

Kinetic analysis

The mechanisms of the reactions involved in enzyme inactivation are complex, thus several inactivation equations have been proposed to mathematically express this kinetic behavior. In the equations, a represents residual β-galactosidase activity at time t (min) and a_0 is the initial enzyme activity.

First-order kinetics (eq. 1) suggests that the reaction happens at one inactivation rate (k-value) in a single step. It has been reported to model heat degradation of several enzymes, including β-galactosidase [15-17]

$$\frac{A}{Ao} = \exp(-kt) \quad (1)$$

Models that suggest the existence of more than one enzyme with similar activity but presenting different heat sensitivities (Eq. 2-6) can be described by the combination of exponential behaviors of the different fractions. The distinct isoenzymes model (Eq. 2) describes the sum of two exponential decays. A_L and A_R represent the residual activities for the labile and the resistant isoenzymes, respectively. k_L and k_R are the correspondent first-order reaction rate constants for each fraction, respectively [18].

$$\frac{A}{Ao} = A_L \exp(-k_L t) + A_R \exp(-k_R t) \quad (2)$$

The two-fraction model, represented by Equation 3, also describes the inactivation as a combination of two distinct groups of enzyme fractions, a stable and other sensitive to heat, where the coefficient *a* represents the active fraction of the heat labile group in relation to the total activity [19].

$$\frac{A}{Ao} = a \times \exp(-k_L t) + (1-a) \times \exp(-k_R t) \quad (3)$$

When there is an extremely thermal resistant fraction in the enzyme (A_r), fractional conversion model (Eq. 4) is employed to describe the residual activity as function of the processing time. It refers to a first-order degradation reaction and considers the non-zero enzyme activity upon prolonged heating [20].

$$\frac{A}{Ao} = A_r + (1-A_r) \times \exp(-kt) \quad (4)$$

Multi-component model (Eq. 5), equation proposed by Fujikawa and Itoh [21] uses the concept of cumulative of two distinct resistance fraction of the enzymes, where *r*-value is related to the combination of the initial activity of the resistant and labile form of the enzyme.

$$\frac{A}{Ao} = \frac{[\exp(-k_1 t) + r\exp(-k_2 t)]}{1+r} \quad (5)$$

The series-type model (Eq. 6) is based on a succession of first-order steps [22]. In the first step the protein unfolds (irreversibly or reversibly) from the native structure to yield an inactive or partially active intermediate, which is followed by an irreversible step that converts the intermediate in an inactive enzyme.

$$\frac{A}{Ao} = \alpha_2 + \left[1 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right] \exp(-k_1 t) - \left[\frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_1}{k_2 - k_1}\right] \exp(-k_2 t) \quad (6)$$

Applications of *n*th-order equation (Eq. 7) for the heat degradation of enzymes suggests that the activity decay through time is not necessarily exponential [23, 24].

$$\frac{A}{Ao} = \left\{A_o^{\,1-n} + (n-1) \times kt\right\}^{1/(1-n)} \quad (7)$$

Weibull distribution pattern (Eq. 8) [25] is based on the assumption that, under the conditions examined, the momentary rate of thermal sensitivity to heat is only a factor of the transient heating intensity and residual activity, but not of the rate at which the residual activity has been reached [26]. Weibull model is characterized by the values *n* and *b*-values; the former determines the shape of the distribution curve, whereas the later determines its scaling [26].

$$\frac{A}{Ao} = \exp(-bt^n) \quad (8)$$

Comparison of kinetic models

Residual activities with respect to heating time were fitted to the kinetic models, using the quasi-newton method for non-linear regression from statistica 7.0 (statsoft inc., tulsa, ok). For comparison of fits obtained, statistical and physical criteria were considered.

A physical criterion for rejection of a model is the estimation of negative kinetic parameters at a given temperature. The statistical criteria include coefficient of

determination (r^2), chi-square (λ^2) and Akaike's optimization criterion (AIC), like proposed by Sant'anna and co-workers [27].
Chi-square, used to compare the models, is mathematically given by equation 9:

$$\chi^2 = \frac{\sum\left(a_{mensured} - a_{predicted}\right)^2}{\left(n-p\right)} \quad (9)$$

When models with different numbers of parameters are compared, the residual sum of squares does not give enough information to discriminate between these models. The AIC produces ranking of parsimonious models when the number of experimental data is small, or when the number of fitted parameters is a moderate to large fraction of the number of data. This optimization criterion compares models by their sum of squares, corrected for the number of parameters and observations [28]. AIC is defined as

$$AIC = n\ln\left(\frac{\sum(a_{measured} - a_{predicted})^2}{n}\right) + \frac{n(n+p)}{n-p-2} \quad (10)$$

where n is the number of observations and p the number of parameters.
The model with the lowest λ^2, AIC, and higher r^2 for the adequacy of the experimental data to the kinectic equations was considered as the best choice from a statistical point of view.

Thermodynamics analysis
The Arrhenius equation is the most common mathematical expression to describe the temperature effect on the inactivation rate constants and the dependence is given by the activation energy (*Ea*):

$$\ln\left(k\right) = \ln(k_0) - \frac{E_a}{R.T} \quad (11)$$

where k_0 is the Arrhenius constant, E_a the activation energy, R (8.31 J mol^{-1} K^{-1}) the universal gas constant and T is the absolute temperature. The activation energy can be estimated by linear regression analysis of the natural logarithm of rate constant versus the reciprocal of the absolute temperature.
Activation enthalpy ($\Delta H^{\#}$), free energy of inactivation ($\Delta G^{\#}$) and activation entropy ($\Delta S^{\#}$) can be calculated according to the expressions:

$$\Delta H^{\#} = E_a - R.T \quad (12)$$

$$\Delta G^{\#} = -R.T.\ln\left(\frac{k.h}{K_B T}\right) \quad (13)$$

$$\Delta S^{\#} = \frac{\Delta H^{\#} - \Delta G^{\#}}{T} \quad (14)$$

were h (6.6262 x 10^{-34} J s) is the Planck´s constant and K_B (1.3806x10^{-23} J K^{-1}) is the Boltzmann´s constant.

Data analysis
Statistical analysis of the data was performed using the Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA) and plots were made using Microsoft Excel 2000 (MapInfo Corporation, Troy, NY, USA). Obtained *k*-values were compared using Tukey's approach, and a $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Kinetic analysis for β-galactosidase thermal inactivation

Residual hydrolytic activities in temperature range of 58-66 ºC are shown in Figure 1, where an exponential behavior can be observed. Eight inactivation kinetic models were tested to fit the experimental data for heat treatments of *Aspergillus oryzae* β-galactosidase. The statistical performance of these models is summarized in Table 1.

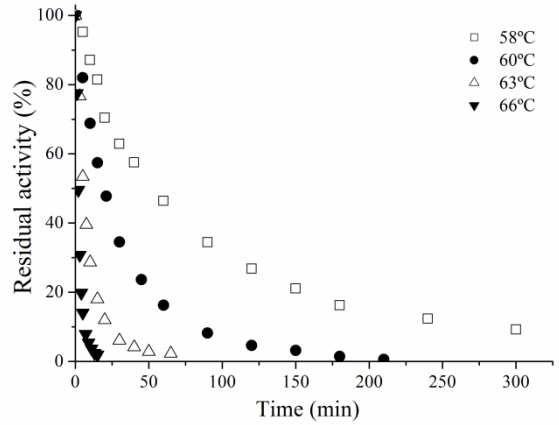

Figure 1. Thermal inactivation of beta-galactosidase at 58 (+), 60 (o), 63 (□), and 66 ºC (◊). Data presented are average values of two independent experiments and standard deviations were always lower than 5%.

Table 1. Statistical error analysis for fitting experimental data to different models.

Model (Equation)	r^2	λ^2	AIC
First-order (1)	[0.9784;0.9952]	[9.2E-4;1.0E-3]	[-80;-60]
Isoenzymes (2)	[0.9929;0.9998]	[5.9E-5;2.4E-3]	[-111;-46]
Two-fraction (3)	[0.9916;0.9998]	[2.2E-5;1.1E-3]	[-117;-52]
Fractional conversion (4)	[0.9912;0.9978]	[2.9E-4;1.2E-3]	[-82;-51]
Multi-component (5)	[0.9916;0.9998]	[2.2E-5;1.2E-3]	[-116;-55]
Series (6)	[0.6701;0.9912]	[3.2E-3;5.1E-1]	[-59;-23]
nth-Order (7)	[0.3541;0.9975]	[3.1E-04;1.7E-3]	[-90;2.9]
Weibull (8)	[0.9916;0.9992]	[1.0E-4;1.0E-3]	[-100;-57]

For multi-component, two-fractions and series models, negative parameter values were estimated, which is a physical criterion for rejection of the equations. For the distinct isoenzymes model, equal inactivation rate parameters, at 63 ºC and 66 ºC, were calculated, excluding this model for these temperatures. Fractional conversion, first order and Weibull distributions gave satisfactory description of the inactivation kinetics, with higher r^2-values and low values of λ^2 and AIC. From this, fractional conversion and Weibull equations presented the better adequacy for the experimental data, with the r^2-values higher than 0.99, and similar λ^2 and AIC values. The r^2 values for fractional conversion model ranged from 0.9912 and 0.9978, while the Weibull distribution's r^2 values ranged from 0.9916 and 0.9991. The AIC values for Weibull varied from -100 to -57 and from -82 and -51 for the fractional conversion model. The λ^2 ranged from 0.000299 and 0.001223 to fractional conversion and from 0.0001 to 0.001 for Weibull pattern, indicating a similar fit for both models. According to Schokker and co-workers [29], for predictive modeling, it is recommendable to choose the equation with fewer parameters to be estimate, because it is more stable, due to the parameters being less correlated, and easier to use the model. Then, it is suggested

that, in the temperatures range studied here, the Weibull model is the best model to explain the thermal inactivation for the β-galactosidase from *A. oryzae*.

Discussion and validation of the applicability and usefulness of Weibull model to explain the heat inactivation of enzymes like peroxidase and protease P7 [24, 30] have been recently published. The mathematical characteristics of enzyme populations during heat inactivation can be adequately described by continuous functions, and an alternative approach is to consider the survival curve as the cumulative form of a temporal distribution of lethal events. In heat processing, it is common to characterize Weibull reaction mechanisms in terms of the reliable life (t_R) (thermal death time concept) [31]. t_R (Eq. 15) is the necessary time to the enzyme activity decays 90% of its initial activity.

$$t_R = \left(\frac{2.303}{b}\right)^{1/n} \quad (15)$$

Table 2 shows the estimated values for *b*, *n*, t_R and *z* kinetic parameters for the β-galactosidase for heat treatments between 58 °C and 66 °C. The inactivation rate constants ranged from 0.3033 min^{-1} and 0.0272 min^{-1}, increasing with the higher processing temperatures, meanwhile t_R-values are between 103.15 min and 6.72 min at temperatures between 58 ºC and 66 °C, indicating faster inactivation at higher temperatures.

Table 2 Kinetic parameter values for thermal inactivation of β-galactosidase to Weibull model.

Temperature (ºC)	r^2	b (min^{-n})	n	t_R (min)	z' (º C)
58	0.9945	0.0272	0.8089	103.15	2.96
60	0.9992	0.0527	0.8666	49.65	
63	0.9916	0.1171	0.9959	19.73	
66	0.9945	0.3033	1.1726	6.72	

A Weibull distribution with *n* higher than 1 indicates that the semilogarithmic inactivation curve has a downward concavity, an upward concavity when *n* is lower than 1, and an exponential distribution when $n = 1$ [26, 32]. The *n*-values estimated for the inactivation of β-galactosidase ranged between 0.809 and 1.173. *n*-values lesser than 1 indicate the 'tailing' phenomena, which suggests that enzyme molecules showed different inactivation susceptibilities during heat treatment, corroborating to the idea of the isoenzyme and fractional conversion models, that also satisfactorily described the inactivation behavior (Table 1). Figure 2 shows that the increasing of the heating temperature implied in the linear enhance of the *n*-values. These results indicate that the shoulder behavior is attenuated with the increasing of the processing temperature, leading to an exponential behavior when the temperature process was 66 ºC. This is possibly because the temperature is high enough to the stable fraction of the enzyme to be degradated so faster as the labile fraction, leading to a single inactivation step.

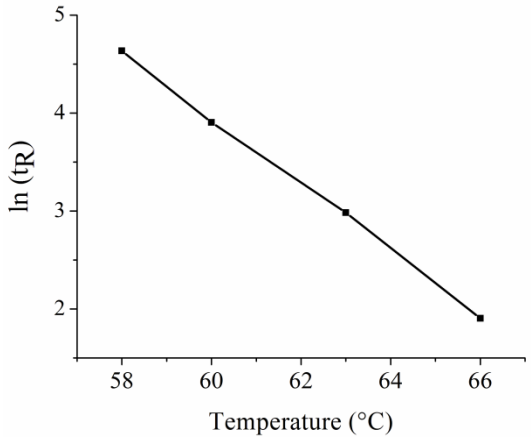

Figure 2. Temperature dependence of n-values in Weibull model. The regression equation was determined as n(T) = 0.047 T - 1.859 (r2 = 0.9823).

Dependence of enzyme activity with time and temperature

Time-temperature is an important binome in industrial applications. On this basis, the combination of the Weibull model, the Arrhenius equation and the linear behavior between the *n*-values and the processing temperature allow the relation among the enzymatic residual activity, time and temperature. Figure 3 shows the three-dimensional graphic of the enzymatic activity of the β-galactosidase as function of the two variables based on Equation 16.

$$\frac{A}{A_0} = \exp\left[-1.51410^{42}\exp\left(-\frac{277027}{8.314T}\right)t^{0.0457T-14.33}\right] \quad (16)$$

The 3D representation is an innovative approach in the enzyme thermal inactivation field, offering the possibility to evaluate the interaction of time and temperature on enzyme activity. It can be observed that this β-galactosidase is relatively stable at 332 K (59 ºC) for up to 50 min maintaining about of 50% of its initial activity. Also, Figure 3 shows that for temperatures higher than 334 K (62 ºC) the enzyme becomes very sensitive to heat leading to a reduction of the catalitic capacity in few minutes.

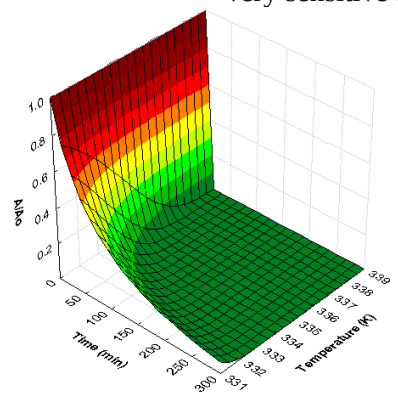

Figure. 3. Three-dimensional representation of the residual enzyme activity of β-galactosidase as a function of time and temperature, mathematically described by eq. 16.

Thermodynamic analysis for β-galactosidase thermal inactivation

Estimation of thermodynamic parameters is an important issue to determine biotechnological potential of enzymes and their structure-stability relationships. Activation energy (E_a), activation enthalpy ($\Delta H^{\#}$), activation entropy ($\Delta S^{\#}$) and free energy of inactivation ($\Delta G^{\#}$), calculated by the transition state theory, for the inactivation of β-galactosidase, are presented in Table 3. E_a can be defined as the

energy barrier that molecules need to cross in order to be able to react, and the proportion of molecules able to do that, usually increases with temperature, qualitatively explaining the effect of temperature on rates [33]. Therefore, the higher the E_a values, the higher the energy barrier to be transposed for enzyme inactivation, indicating an increased stability [15]. For the thermal inactivation of commercial β-galactosidase from *A. oryzae*, E_a was 277 kJ mol^{-1}, which is close to results observed by Ustok and co-workers [17]. These authors studied the inactivation of β-galactosidases from different strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, and their E_a values ranged from 200 to 215 kJ mol^{-1}.

Table 3. Thermodynamic parameter values of thermal inactivation of *β*-galactosidase activity.

Temperature (K)	E_a (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})
331	277.03	274.28	79.97	587.04
333		274.26	78.63	587.46
336		274.24	77.14	586.59
339		274.21	75.17	587.13

$\Delta H^{\#}$ and $\Delta S^{\#}$ are activation enthalpy and entropy, and are mainly related to the break of non-covalent bonds in enzymes, including hydrophobic interactions, and to the disorder change of molecules in the system, respectively [34]. Positive $\Delta H^{\#}$ values indicate that enzyme inactivation is an endothermic process [35] and, in turn, a positive $\Delta S^{\#}$ indicate that there is an increase in the molecule disorder during the exposure to high temperatures, and peptide chain unfolding might be the factor determining for the inactivation step. $\Delta G^{\#}$ represents the difference between the activated state and reactants [36], and the positive values mean that enzyme inactivation is not a spontaneous reaction. Then, the decrease observed in $\Delta G^{\#}$ values with increasing temperatures, indicate that the destabilization of the enzyme molecule is more spontaneous and faster [37].

Since $\Delta H^{\#}$ and $\Delta S^{\#}$ values are positive, there is an indication of the breakage of weak, non-covalent bonds and changes in the β-galactosidase structure (to a disordered polypeptide) at lower temperatures. In the temperature range of 58-66 ºC, the inactivation mechanism seems to be changed, since $\Delta H^{\#}$ and $\Delta S^{\#}$ did not present great variability (Table 3), although it happens faster, as the inactivation constants shows. The fully or partially unfolded enzyme might be non-active, since these intermediates can be non-correctly refolded upon cooling, producing molecules thermodynamically stable but inactives [15, 38, 39].

CONCLUSION

Commercial β-galactosidase from *A. oryzae* presented distinct active fractions with different heat sensibilities. The knowledge on the thermal stability is essential in evaluating the enzyme suitability for biotechnological applications. For *A. oryzae* β-galactosidase, this information is notably important, since this enzyme is recognised for their propensity to form GOS, which in turn, is favored at high temperatures. Weibull model showed to be the best equation to describe the changes on the residual activity through the incubation time and kinetic parameters as function of the temperature. Thermodynamic approach shows an enzyme relatively stable and suggests that inactivation mechanism is based on molecular structural changes.

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