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Kinetic and Thermodynamic Characterization of two Polygalacturonases Isolated from the Digestive Juice of the Snail *Limicolaria flammea*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Polygalacturonases are extensively used in food industries for pectic substances degradation. In this paper, we investigate on thermal stability parameters of two Polygalacturonases previously isolated from digestive juice of the snail *Limicolaria flammea* for several industrial applications such as fruit juice clarification. Thermal inactivation was carried out in the temperature range of 55°C to 80°C from 15 to 120 min. All results were statistically analysed. The results shown that thermal inactivation of studied acid phosphatases follows first order kinetics. At their optimum temperatures, these enzymes showed high half-lives ranging from 462.06 to 630.10 min and D values from 1535.00 to 2093.64 min suggesting that these two enzymes had a large thermal stability. The high values of $\Delta G^{\#}$ (93.96 to 94.97 kJ/mol) reveal a better resistance to denaturation. The relatively high activation energies (from 120.35 to 129.13 kJ/mol) and average enthalpy values (from 117.67 to 126.44 kJ.mol−1) could corroborate the good stability of these biocatalyst. All these results suggest that Polygalacturonases from digestive juice of the snail *Limicolaria flammea* may be profitably exploited in future food industrial applications.

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1. INTRODUCTION

Pectinases are a versatile group of enzymes that degrade or modify pectin substances, which are complex polysaccharides present as the major components in the middle lamella and primary cell walls in plants [1,2]. Known pectinases are classified into three groups, pectinesterases, depolymerizing pectinases, and protopectinases [3], which represent around 25% of the commercial sales of food enzymes. These enzymes (pectinases) are widespread in nature and are produced by bacteria, filamentous fungi, plants, and animals [4,5]. Pectinases have many applications in the industry, especially in the extraction of fruit juices. These enzymes reduce viscosity by degrading pectin in the middle layer, which facilitates subsequent processes like juice filtration [1]. The incorporation of pectinolytic enzymes also preserves the nutritional value, original color, and flavor [6].

Among these pectinases, polygalacturonases have more important industrial applications due to their stability to various physicochemical and thermodynamic parameters [7]. Polygalacturonase (E.C. 3.2.1.15) is a hydrolytic pectinase with affinity for galacturonic acid glycosidic linkages [8]. Numerous studies have already been carried out on polygalacturonase production and its applications in the industrial and other bioprocess fields [9-13]. So, it is well known that temperature is a key variable in bioprocesses using enzymes [14]. According to Silva et al. [15] thermodynamic studies determine the thermal stability and economic viability of enzymes in food and non-food systems. Indeed, the enzyme that remains active in wide temperature ranges for longer time are usually considered as practicable and economical from industrial point of view [16]. Thus, high stability is generally considered an economic advantage because it reduces the enzyme loss and consequently the costs of the process [17]. Following Souza et al. [17], thermodynamic and kinetic studies can provide valuable information about the thermostability of enzymes at a given operating temperature. Mozhaev [18] and Illanes [19] argue that enzyme stability represents a critical point in most bioprocesses.

Most of the polygalacturonases used in bioprocesses are of microbial origin [4,12]. To our knowledge, very few studies have been carried out to polygalacturonases animal sources. As regards polygalacturonases PG1 and PG2 previously isolated from the digestive juice of the snail *Limicolaria flammea* by Kanga et al. [20], no study has been devoted to their thermal stability parameters. Therefore, objective of the current study was to determined thermal stability parameters (Speed consistency, activation energy, half-life time, decimal reduction time, temperature of destruction, temperature coefficient, enthalpy, entropy et Gibbs free energy) of the two polygalacturonases purified from digestive juice of the snail *Limicolaria flammea* [20] for several industrial applications such as fruit juice clarification.

2. MATERIALS AND METHODS

2.1 Enzymatic Source and Crude Extract Preparation

Snail *Limicolaria flammea* were collected locally in Côte d'Ivoire at the National Floristic Center of the University Felix Houphouet-Boigny (5°21′ N and 3°54′ W). After isolation of the digestive tract, the digestive fluid was collected in a jar placed in ice, filtered through cotton wool, and then centrifuged at 10,000 × g for 30 min at 4°C. The supernatant constituted the crude extract.

2.2 Enzyme Activity Assay

The polygalacturonase activity was determining by using the 3.5-dinitrosalicylic acid (DNS) method [21] with D-(+)-galacturonic acid as the standards. All reactions contained 200 µL of 0.5% of polygalacturonic acid (PGA), 200 µL of 100 mM sodium acetate buffer (pH 5.6) and 50 µL of enzyme solution. The reaction mixture was incubated at 37 °C for 30 min. After incubation, 300 µL DNS was added to terminate the reaction, followed by 5-min incubation in boiling water bath. The absorption at 540 nm was measured at room temperature. All experiments were performed in triplicate. One unit (U) of polygalacturonase was defined as the amount of enzyme that released reducing sugars equivalent to 1 µmol of D-(+)-galacturonic acid per min under the standard conditions. The specific activity was expressed as unit of activity per mg of protein.

2.3 Proteins Estimation

Protein concentrations and elution profiles from chromatographic columns were determined by

the Folin method [22]. Bovine serum albumin (BSA) was used as the standard protein.

2.4 Thermal Inactivation

The thermal inactivation of each purified polygalacturonases was determined at various constant temperatures from 50 to 70 °C. Enzymes in sodium acetate buffer (pH 5.6) were exposed to each temperature for a period of 15 to 120 minutes. Then, aliquots were withdrawn at intervals (15 minutes) and immediately cooled at 4ºC for 20 minutes. Residual activities, determined at 37°C under the standard enzyme assay conditions, were expressed as percentage of activity of zero-time control of untreated enzymes.

2.5 Residual Activity

$$Residual\ activity\ (\%) = (At / A0) \times 100 \quad (1)$$

where *At* and *A*0 are the activities of the treated and untreated enzyme solution, respectively.

2.6 Kinetic Data Analysis

2.6.1 Denaturation constants of the enzyme fraction k

Thermal inactivation of each phosphatase can be described by a first-order kinetic model [23]. The integral effect of an inactivation process at constant temperature, where the inactivation rate constant is independent of time, is given in Equation /(1) as follows:

$$ln\ (At/A0) = -kt\ (first\text{-}order\ kinetics) \quad (2)$$

where *At* is the residual enzyme activity at time *t* (minutes), *A*0 is the initial enzyme activity, *k* (minute−1) is the inactivation rate constant at a given condition. *k* values were obtained from the regression line of ln *(At /Ao)* versus time as slope.

2.6.2 Determination of half-life of the enzyme solution t1/2

The *t*1/2 was obtained from the following equation:

$$t1/2 = ln2/k \quad (3)$$

2.6.3 Calculation of activation Energy Ea

The *Ea* was obtained from the Arrhenius equation:

$$k = A.e^{-(EaR/T)} \quad (4)$$

where *k* is the first-order rate constant of thermal inactivation of the enzyme activity, obtained through the slopes of the plots described above, R = 8.314 J K-1 mol-1 is the universal gas constant and T represents the absolute temperature (K).

2.6.4 Calculation of temperature coefficient (Q10)

The temperature coefficient (Q10) is the rate of change of the enzymatic catalytic reactions for each 10°C rise in temperature. It is the ratio of the percentage of activity X10 of the reaction at temperature T +10 °C to the percentage of activity X at temperature T °C using the following formula:

$$Q10 = X10 / X \quad (5)$$

With:

X10 = % activity at T + 10 °C
X = % activity at T °C

2.6.5 Calculation of D values of the enzyme fractions

D-value is defined as the time needed, at a constant temperature, to reduce the initial enzyme activity (*A*0) by 90 % (or decimal reduction time or time required to preincubate the enzyme at a given temperature to maintain 10% residual activity). For first-order reactions, the *D*-value is directly related to the rate constant *k* (Eq. 2) [24,25]:

$$D = ln\ (10)/k = 2.303/k \quad (6)$$

2.6.6 Calculation of z values of the enzyme fractions

The *z* value (°C) is the temperature increase needed to induce a 10-fold reduction in *D* value [25]. This *z* value follows the Eq. (7):

$$log\ (D1/D2) = (T2 - T1)/z \quad (7)$$

where *T*1 and *T*2 are the lower and higher temperatures in °C or K. Then, *D*1 and *D*2 are *D* values at the lower and higher temperatures in minute, respectively.

The *z* values were determined from the linear regression of log (*D*) vs temperature (T).

2.6.7 Thermodynamic parameters

The enthalpy of reaction (Δ*H#*), which is the amount of energy required to bring the enzyme to the activated state for the subsequent denaturation at a given temperature, Gibbs free energy (Δ*G#*) and the entropy (Δ*S#*) were calculated through the following equations [26,27].

$$\Delta H\# = Ea - RT \quad (8)$$

$$\Delta G\# = -RT \times \ln (k \times h / Kb \times T) \quad (9)$$

where

Δ*H#* = Change in enthalpy;
Ea = activation energy of inactivation;
R = universal gas constant;
T = absolute temperature(K).

$$\Delta S\# = (\Delta H\# - \Delta G\#) / T \quad (10)$$

where

Δ*G#* = free energy change of inactivation;
h = Plank's constant (6,62607 x 10-34 m2.kg/s);
Kb = Bolztman constant (1,3807 x 10-23 J/K).
Δ*S#* = Change in entropy.

2.7 Statistical Analysis

All tests for the different analyses were performed in triplicate and the data obtained were expressed as the arithmetic mean with the corresponding statistical standard deviation. The one factor analysis of variance (ANOVA) was performed on all the results obtained in order to determine the existence of statistically significant differences between the values of the calculated means. Statistically significant differences were highlighted by Duncan's test at 95% confidence level. This statistical analysis was performed using Statistica 7.1 software.

3. RESULTS AND DISCUSSION

In this study, the effect of heat treatment over a range of temperature from 50 to 70°C on both polygalacturonases PG1 and PG2 was evaluated by determining the residual percentage activity (Table 1).

The activity of both polygalacturonases was decreases with increasing heating time (5-120min) and temperature (50-70°C). Indeed, it is observed that between 50 and 70°C thermal denaturation of PG1 and PG2 occurred after 15 min incubation (97.63% to 73.20% and 98.12% to 81.55% respectively). Heat treatment at 50°C for 120 min resulted in partial inactivation of polygalacturonases PG1 and PG2 (activity of 82.53% and 87.37% respectively). Partial inactivation for PG1 (activity: 53.85%) and PG2 (activity: 49.18%) was also observed at 60°C. However, a strong inactivation of the activity of both enzymes was observed at 70°C during the 120 min incubation time (activity of 7.64% for PG1 and 20.60% for PG2).

The graphical representations of lnAt/Ao as a function of the pre-incubation time of polygalacturonases at temperatures between 50 and 70 °C gave affine lines translating a first order thermal inactivation kinetics (Fig. 1).

The inactivation rate constant (*k*) value and half-life (t1/2) of polygalacturonases PG1 and PG2 from digestive juice snail *Limicolaria flammea* are presented in Table 2.

The rate constant values increased with temperature from (0.0015 to 0.0207 min^{-1} for PG1 et 0.0011 to 0.0146 min^{-1} for PG2) at 50-70°C, respectively. While the half-life (t1/2) decreases with increasing temperature and presents values between 462.00 and 33.48 min for PG1 and between 630.00 and 47.47 min for PG2. The K values obtained in this study are lower than those reported in the studies by Adedeji et Ezekiel [28] with the purified polygalacturonases PUo- PG, PCPOF- PG, PUp-PG and PCPPF- PG and those of Faiza et al. [29] with the free exo-polygalacturonase. Polygalacturonases PG1 and PG2 are thus more thermostable than these reported biocatalysts. According to Marangoni [30], a lower rate constant means that the enzyme is more thermostable.

Table 1. Effect of the treatment temperature and time on the inactivation of PG1 and PG2 polygalacturonases of digestive juice snail *Limicolaria flammea*

Time (min)	Residual activity (%) at each temperature (°C) of heat treatment									
	PG1					PG2				
	50	55	60	65	70	50	55	60	65	70
0	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}	100±0^{a}
15	97.63±0.58^{b}	94.65±0.01^{b}	91.48±0.01^{b}	81.79±0.04^{b}	73.20±0.02^{b}	98.12±0.01^{b}	97.04±0.02^{b}	91.30±0.01^{b}	84.24±0.03^{b}	81.55±0^{b}
30	95.89±0.17^{c}	90.03±0.03^{c}	84.96±0.01^{c}	70.33±0.03^{c}	54.06±0.01^{c}	96,95±0.01^{c}	95.31±0.02^{c}	84.20±0.02^{c}	70.87±0^{c}	65.91±0.02^{c}
45	93.43±0.03^{d}	85.13±0.02^{d}	78.58±0.02^{d}	57.64±0.05^{d}	41.90±0.01^{d}	95.60±0.03^{d}	92.97±0.04^{d}	76.26±0.02^{d}	60.62±0^{d}	54.55±0.01^{d}
60	91.48±0.08^{e}	81.38±0.01^{e}	72.40±0.03^{e}	48.43±0.02^{e}	29.43±0.02^{e}	94.08±0.01^{e}	90.66±0.02^{e}	71.32±0.01^{e}	54.01±0.02^{e}	44.89±0.01^{e}
75	89.40±0.03^{f}	77.58±0.03^{f}	66.17±0.02^{f}	40.90±0.01^{f}	21.85±0.02^{f}	92.13±0.02^{f}	87.89±0.04^{f}	64.47±0.01^{f}	45.16±0.01^{f}	36.79±0^{f}
90	87.02±0.03^{g}	73.48±0.06^{g}	62.65±0.01^{g}	33.49±0.04^{g}	16.12±0^{g}	90,99±0.02^{g}	86.61±0.01^{g}	59.35±0.04^{g}	39.53±0.04^{g}	30.36±0.02^{g}
105	85.07±0.01^{h}	69.05±0.02^{h}	58.04±0.01^{h}	27.84±0.02^{h}	11.28±0.02^{h}	89,40±0,05^{h}	83.44±0.01^{h}	55.72±0.05^{h}	33.12±0.03^{h}	24.71±0.04^{h}
120	82.53±0.01^{i}	66.69±0.01^{i}	53.85±0.07^{i}	21.98±0.01^{i}	7.64±0^{i}	87,37±0,02^{i}	82.28±0^{i}	49.18±0.01^{i}	27.78±0.01^{i}	20.60±0.01^{i}

The averages assigned to the same lowercase letters in a column are not statistically different with a threshold α = 0.05

The half-life values (t1/2) obtained in our study are lower than those reported by Faiza et al. [29] with exo-polygalacturonase immobilized by covalent binding on a sodium alginate matrix using glutaraldehyde as cross-linking agent, and exo-polygalacturonase immobilized by adsorption on a sodium alginate matrix but higher than those of Rapidase C80, Pectinase CCM, Pectinex 3XL and Grindamyl 3PAa [31].

The values of the temperature coefficients (Q10) increase with increasing temperature for PG1, while they decrease with increasing temperature for PG2. Thus between 50-60°C and 60-70°C, the values of Q10 are respectively 3.4667 and 3.9808 for PG1 and 5.2727 and 2.5172. These Q10 values are higher than Q10 values in general during enzymatic reactions (1-2). This implies that the hydrolysis reaction is controlled by factors other than temperature [32].

To establish the link between treatment time and enzyme activity, the D-values were calculated. The decimal reduction time (D value) was calculated according to equation 5. As shown in table IV, D-values decreased by increased at temperature, indicating a faster inactivation of polygalacturonases at higher temperatures. These values vary respectively from 1535 to 111 min and from 2093.64 to 157.74 min for PG1 and PG2.

The effect of temperature on D-values is shown in Fig. 2, and from this representation, the Z-value was calculated and found to be 17.60°C et 16.45 for PG1 and PG2 respectly at 50–70°C (Table 3). These relatively high values imply a lower heat sensitivity of the polygalacturonases PG1 and PG2 and could therefore be used in long term industrial applications (60-120 min) subjected to heat treatments at temperatures between 50 and 70 °C. According to Barrett et al [33], when the Z value is very low, the enzyme is very sensitive to heat.

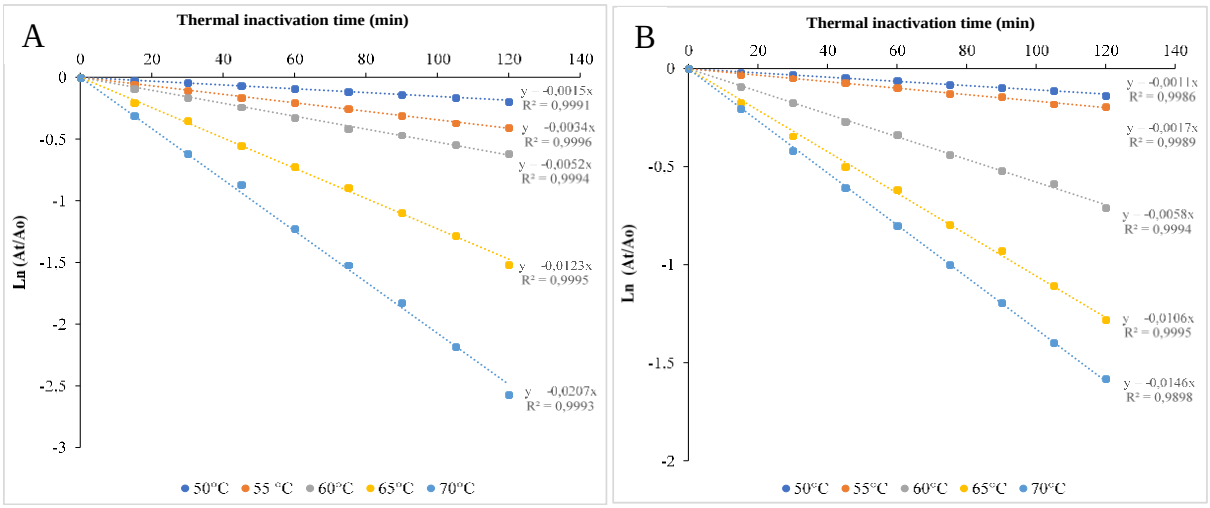

Fig. 1. Thermal inactivation curves of PG1 and PG2 polygalacturonases of digestive juice snail *Limicolaria flammea* at temperature range 50-70°C

A: PG1. B: PG2

A0 : the initial enzymatic activity, At : the activity at each holding time

Table 2. K-values and $t_{1/2}$ for thermal inactivation of PG1 and PG2 polygalacturonases of digestive juice snail ***Limicolaria flammea* at temperature range (50-70°C)**

Temperatures (°C)	PG1		PG2	
	K-Values (min^{-1})	$t_{1/2}$ (min)	K-Values (min^{-1})	$t_{1/2}$ (min)
50	0.0015±0.00^{a}	462.00±0.20^{e}	0.0011±0.00^{a}	630.00±0.20^{e}
55	0.0034±0.00^{ab}	203.82±0.02^{d}	0.0017±0.00^{a}	407.65±0.01^{d}
60	0.0052±0.00^{b}	133.27±0.20^{c}	0.0058±0.00^{b}	119.48±0.01^{c}
65	0.0123±0.00^{c}	56.34±0.02^{b}	0.0106±0.00^{c}	65.38±0.02^{b}
70	0.0207±0.00^{d}	33.48±0.01^{a}	0.0146±0.001^{d}	47.47±0.00^{a}

The averages assigned to the same lowercase letters in a column are not statistically different with a threshold α = 0.05; t1/2 is the half-life constant of polygalacturonases

In order to determine the thermodynamic parameters for thermal stability, the energy of activation (Ea) for thermal denaturation was determined by applying the Arrhenius plot. Graphical representations of lnk as a function of the inverse of temperature in degrees Kelvin of polygalacturonases PG1 and PG2 also yielded affine lines with negative slopes (Fig. 3). The slopes of these plots (slope = -Ea/R) allowed us to determine the activation energies during thermal inactivation of our polygalacturonases. The values of activation energy (Ea) recorded in Table 4 are 120.35 and 129.13 KJ.mol-1 for PG1 and PG2 respectively. These high activation energy values indicate that polygalacturonases PG1 and PG2 are highly resistant to high temperatures [34,35]. These activation energy values are higher than those reported by Rodrigo et al. [36] with the free and immobilized pectinase extracted from Aspergillus aculeatus (85.1 and 101.6 kJ.mol-1), also higher than that of the polygalacturonase peh 28 [37].

Table 3. Values of the temperature coefficients (Q10) during the thermal inactivation (50-70 °C) of PG1 and PG2 polygalacturonases of digestive juice snail *Limicolaria flammea*

	Temperature coefficients (Q10)	
Temperature interval (°C)	**PG1**	**PG2**
50 – 60	3.4667±0.02^{a}	5.2727±0.01^{b}
60 – 70	3.9808±0.01^{b}	2.5172±0.01^{a}

The averages assigned to the same lowercase letters in a column are not statistically different with a threshold α = 0.05

Table 4. D-, Z-, and Ea-values for thermal inactivation of PG1 and PG2 polygalacturonases of digestive juice snail *Limicolaria flammea* at temperature range (50-70°C)

Kinetic parameters	PG1	PG2
D-Values (min)		
D_{50}	1535±2.64^{e}	2093.64±0.86^{j}
D_{55}	677±1.00^{d}	1354.71±4.97^{i}
D_{60}	443±0.17^{c}	397.07±0.02^{h}
D_{65}	187±0.46^{b}	217.26±0.23^{g}
D_{70}	111±0.86^{a}	157.74±0.65^{f}
Z-Values (°C)	17.60±0.02	16.45±0.02
Ea (KJ.mol^{-1})	120.35±0.03	129.13±0.04

The averaged affected of the same tiny letters in a column are not statistically different with a threshold α = 0.05. D-, Z- and Ea were respectively the decimal reduction time, the constant thermal resistance, and the activation energy of polygalacturonases

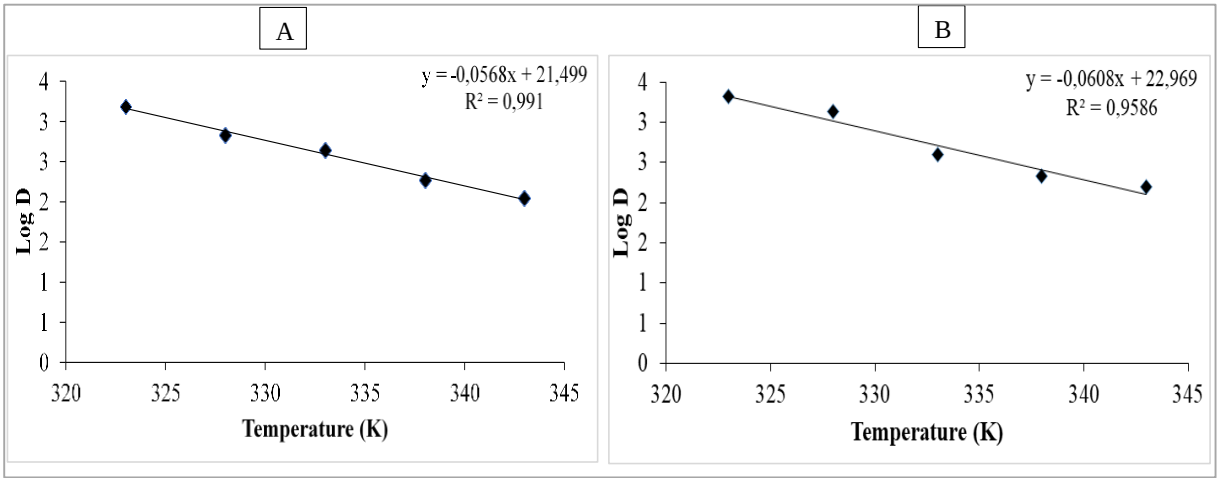

Fig. 2. Effect temperature on D-values for inactivation of PG1 and PG2 polygalacturonases of digestive juice snail *Limicolaria flammea*

A: PG1; B: PG2

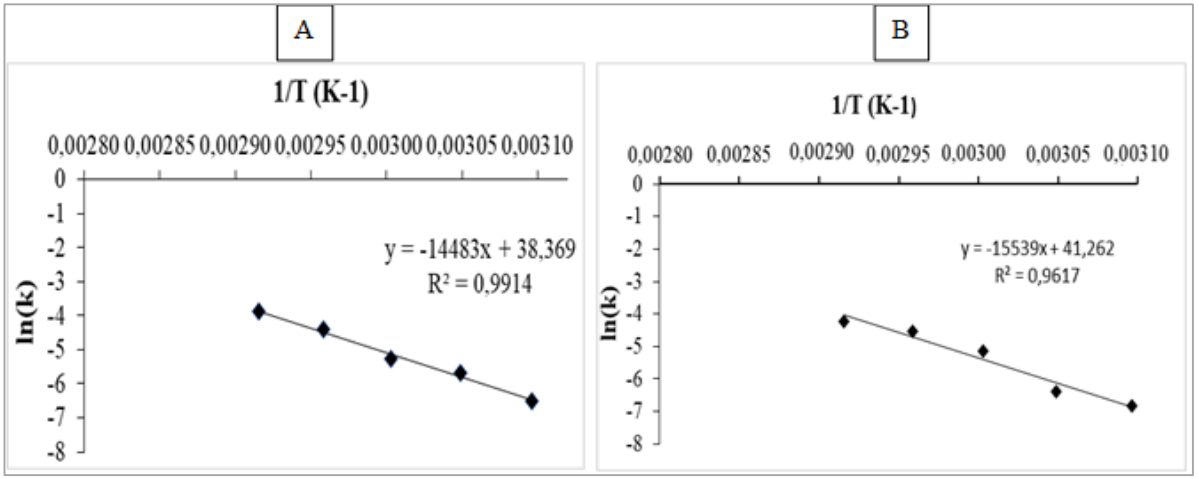

Fig. 3. Effect of the temperature on the constant of the speed (K) during the inactivation of PG1 and PG2 polygalacturonases of digestive juice snail *Limicolaria flammea*

A: PG1; B: PG2

Table 5. Thermodynamic parameters of PG1 and PG2 polygalacturonases of digestive juice snail *Limicolaria flammea* under heat treatment between 50 at 70°C

Temperature (°C)	Thermodynamic parameters					
	$\Delta H^{\#}$(kJ/mol)		$\Delta S^{\#}$ (J $mol^{-1}K^{-1}$)		$\Delta G^{\#}$ (kJ/mol)	
	PG1	PG2	PG1	PG2	PG1	PG2
50	117.67±0.010^{e}	126.44±0^{e}	73.40±0.043^{e}	97.44±0^{e}	93.96±0.016^{e}	94.97±0^{e}
55	117.63±0.017^{d}	126.40±0.020^{d}	73.27±0.010^{d}	97.31±0.017^{d}	93.59±0.017^{d}	94.48±0.023^{d}
60	117.59±0^{c}	126.36±0.010^{c}	73.14±0.010^{c}	97.18±0.010^{c}	93.23±0.003^{c}	93.99±0.007^{c}
65	117.54±0.010^{b}	126.32±0.010^{b}	73.02±0.020^{b}	97.06±0.036^{b}	92.86±0.015^{b}	93.51±0.008^{b}
70	117.50±0.026^{a}	126.28±0.020^{a}	72.89±0.026^{a}	96.94±0.010^{a}	92.49±0.038^{a}	93.03±0.023^{a}
Mean	117.59±0.003^{c}	126.36±0.006^{c}	73.14±0.017^{c}	97.19±0.002^{c}	93.23±0.008^{c}	93.99±0.006^{c}

The averaged affected of the same tiny letters in a column are not statistically different with a threshold α = 0.05

ΔH#, ΔS# and ΔG# were respectively variations in enthalpy, entropy and Gibbs free energy

The thermodynamic values of the variation of enthalpy (ΔH#), entropy (ΔS#) and Gibbs' free energy (ΔG#) of the polygalacturonases PG1 and PG2 of the digestive juice of the snail Limicolaria flammea calculated at different temperatures are shown in Table 5. The values of enthalpy (ΔH#), entropy (ΔS#) and Gibbs free energy (ΔG#) during the inactivation of our enzymes are positive. At temperatures between 50 and 70°C, the mean values of ΔH#, ΔS# and ΔG# were 117.59 kJ.mol-1, 73.14 J mol-1K-1 and 93.23 kJ.mol-1 for PG1 and 126.36 kJ.mol-1, 97.19 J mol-1K-1 and 93.99 kJ.mol-1 for PG2, respectively.

The high enthalpy values of PG1 and PG2 suggest that a large amount of energy is required for the denaturation of our biocatalysts because the breakdown of covalent bonds including interactions is associated with increased enthalpy. This positive value from ΔH# states that the catalytic reaction is endothermic [38]. The positive values found for ΔS# indicate that there is no significant aggregation process for PG1 and PG2, the reaction proceeds with less speed and is characterized by poor regularity [39]**.** In addition, these high entropy values probably reflect an increased disorder of the active site or structure of our biocatalysts [40]. The variation of ΔS with increasing temperature could be due to a change in the conformation of the enzyme structure [41]. The values of ΔG# were positive, indicating that the processes were endergonic and not spontaneous. The high values of ΔG* reveal a better resistance to denaturation. However, when the temperature increased from 50 to 70°C; there was a reduction in ΔG#, indicating a destabilization of the protein at this temperature [36].

4. CONCLUSION

The study of thermodynamic parameters for the inactivation of polygalacturonases PG1 and PG2 in the temperature range of 50°C to 70°C has shown that our biocatalysts are stable at high temperatures. The D-, Z-, k values of half life, Q_{10} indicate that polygalacturonases PG1 and PG2 are heat stable. The free energy (Ea) and enthalpy (ΔH#) values indicate that a large amount of energy is required to initiate denaturation of these biocatalysts. These interesting results suggest that polygalacturonases PG1 and PG2 can be used in the industrial field for the hydrolysis of polygalacturonic acid, especially for juice clarification.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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