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# Study of thermal stability of α-amylases sourced from digestive tract of the tropical house cricket Gryllodes sigillatus (orthoptera: gryllidae): kinetic and thermodynamic analysis

**Eugène Jean Parfait Kouadio*, Hubert Kouassi Konan, Soumaïla Dabonné, Edmomd Ahipo Dué and Lucien Patrice Kouamé**

Laboratoire de Biocatalyse et des Bioprocédés, Unité de Formation et de recherche des Sciences et Technologie des Aliments, Université Nangui Abrogoua (Ex Université d'Abobo-Adjamé), Abidjan, Côte d'Ivoire

**Corresponding author Email :** nkouadiop@yahoo.fr

**ABSTRACT:** The thermal stability of α-amylases Amy A1 and Amy A2 from digestive tract of tropical house cricket *G. sigillatus* was investigated by studying the effect of heat treatment over a range of 55 to 70 °C. Thermal inactivation of each enzyme, evaluated by loss in activity, was apparently followed by first-order kinetics with k-values comprised between 0.014–0.833 and 0.030–0.219 $min^{-1}$ for Amy A1 and Amy A2, respectively. D and k-values decreased and increased, respectively, with increasing temperature, indicating faster inactivation of Amy A1 and A2 at higher temperatures. Ea and Z-values were estimated to 245.89 kJ/mol and 8.77 °C for Amy A1, 182.92 kJ/mol and 11.63 °C for Amy A2. Thermodynamic parameters were also calculated. All the results suggest that both α-amylases are relatively resistant to long heat treatments up to 60 °C.

***Keywords*:** α-amylases; Gryllodes sigillatus; heat treatment; kinetic and thermodynamic parameters; thermal inactivation.

## INTRODUCTION

α-Amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyse starch by cleaving the internal α-1,4-glucosidic bonds. Theses enzymes are important, particularly in the food, paper, textile and detergent industries (Norman, 1982; Guzman-Maldonado *et al.*, 1995, Ramachandran *et al.*, 2004; Behal *et al.*, 2006; Thippeswamy *et al.*, 2006). α-Amylases are well-known to play an important role in starch degradation and represent about 25 to 33% of the enzyme world market, in second place after proteases (Ben Abdelmalek-Khedher *et al.*, 2008; Rasooli *et al.*, 2008). Despite α-Amylases are universally produced by prokaryotes and eukaryotes, including plants and animals, to date, the majority of α-amylases used in industry are generally produced by bacteria and fungi (Hoque *et al.*, 2006; Sivaramakrishnan *et al.,* 2006).

Considering this importance of α-Amylases, many authors have attempted to understand the kinetic behaviour of these enzyme when exposed to high temperatures in bacteria such as *Bacillus cereus* (Sugumaran *et al.*, 2012), *Bacillus licheniformi*s (Violet and Meunier, 1989; Declerck *et al.*, 1997), *Bacillus amyloliquefaciens* (Tanaka and Hoshino, 2002), *Bacillus subtilis*, *Aspergillus oryzae* (Duy and Fitter, 2005) and even in plants (Kumari *et al.*, 2011). Moreover, generally speaking, comparative studies of thermophilic and mesophilic enzymes have demonstrated that weak interactions such as hydrogen bonds (Macedo-Ribeiro *et al*., 1996)' disulfide bonds (Hopfner *et al.*, 1999), ion pairs (Vetriani *et al*., 1998), salt bridges Criswell et al., 2003), hydrophobic interactions (Elcock,1998)

and compactness (Russell *et al*., 1997) are of importance for stability. In addition, it is a known fact that a thermostability of an enzyme depends on the flexibility of each molecule that comprises it (Vihinen *et al*., 1990) and on improved conformational rigidity, which is indispensable for enhancing the stability on an enzyme against heat denaturation **(**Zavodsky *et al*., 1998).

Enzyme thermal inactivation occurs in two steps according to the general model proposed by Lumry and Eyring (1954):

$$N \rightleftarrows U \rightarrow I$$

where N is the native catalytically active enzyme, U is the reversibly unfolded catalytically inactive enzyme and I is the irreversibly inactivated enzyme. In the first step there is a partial loss of activity due to the disruption of the non-covalent interactions maintaining the native conformation. This process is reversible, because the enzymatic activity is completely recovered when the enzyme is cooled down (Tandford, 1968; Violet and Meunier, 1989). Then, upon prolonged heating, another process occurs, leading to an irreversibly inactivated enzyme (Ghosh and Nanda, 1993; Gnangui *et al*., 2009).

In previous study, we purified to homogeneity two dimeric α-amylases from the digestive tract of the tropical house cricket *Gryllodes sigillatus*, an insect belonging to the family of Gryllidae (Kouadio *et al*., 2010). This insect is often found in large numbers in and around human habitation in tropical areas (Smith and Thomas,1988). Both amylases termed Amy A1 and Amy A2 showed maximum activity at 55 °C and could be useful for starch saccharification in the production of syrup of oligosaccharides mixture and baking (Kouadio *et al*., 2010, 2012). In addition, these α-amylases showed interesting properties to catalyse the transfer of glycosyl residues from starch onto phenol (Kouadio *et al*., 2012). This could be an alternative to carry out the glycosylation of phenolic compounds. Indeed, Glycosylation of the phenolic compounds constitute a valuable approach to improve their characteristics for enhancing their usefulness as food and cosmetic ingredients (Sugimoto *et al*., 2007; Kouadio *et al*., 2012).

Considering these important properties of Amy A1 and Amy A2, the present work aimed to investigate the effect of heat treatment over a range of temperatures from 55 to 70°C, on these α-amylases. So, determination and analysis of kinetic and thermodynamic parameters were undertaken. This will better apprehend their use in the processes listed above.

## MATERIALS AND METHODS

### *Enzyme*

α-amylases used in this study were purified from the digestive tract of the tropical house cricket *G. sigillatus* (Kouadio *et al*., 2010). Theses enzymes were homogeneous in on polyacrylamide-gel electrophoresis in the absence of Sodium dodecyl sulphate (SDS).

### *α-amylase assay*

The two enzymes assays were performed as described below (Bernfeld, 1955). Briefly, each purified α-amylase extract (50 µl) was incubated with 0.5 % solution starch (80 µl) on 100 mM phosphate buffer pH 6.6 (170 µl) at 37°C for 30 min. The amount of reducing sugars produced was determined by dinitrosalicylic acid (DNS) method with maltose as the standard. One unit of the enzyme activity (UI) is defined as the amount that liberated 1 µmol of maltose equivalent per min under the above conditions. Specific activity was expressed as units per mg of protein.

### *Protein determination*

Protein was determined according to Lowry method (Lowry *et al*., 1951) using bovine serum albumin as standard.

### *Thermal inactivation*

The thermal inactivation of each α-amylase was investigated at various constant temperatures from 55 to 70°C after exposure to each temperature for a period of 5 to 60 min. Each enzyme was incubated in 100 mM phosphate buffer (pH 6.6). Aliquots were drawn at intervals and immediately cooled in ice-cold water. Experiments were performed in triplicate. The residual enzymatic activity, determined in both cases at 37°C under the standard test conditions, was expressed as percentage activity of zero-time control of the untreated enzyme.

***Kinetic data analysis***

Thermal inactivation of each α-amylase can be described by a first-order kinetic model (Terebiznik *et al*., 1997; Guiavarc'h *et al*., 2002). The integral effect of an inactivation process at constant temperature, where the inactivation rate constant is independent of time, is given in Eq. 1:

$$\ln (A_t/A_0) = -kt \quad (1)$$

where;

$A_t$ is the residual enzyme activity at time t, Ao is the initial enzyme activity; k is the reaction rate constant ($min^{-1}$) at a given condition. k values were obtained from the regression line of ln ($A_t$ /$A_0$] versus time as -slope.

The D-value is defined as a time required, at a constant temperature, to reduce the initial enzyme activity (Ao) by 90 %. For first-order reactions, the D-value is directly related to the rate constant k (Eq. 2) (Stumbo, 1973):

$$D = 2.303/ k \quad (2)$$

Z (°C) is the temperature increase necessary to induce a 10-fold reduction in D-value and follows the Eq 3:

$$\log (D1/D2] = (T2-T1)/Z \quad (3)$$

where;

T1 and T2 are the lower and higher temperatures in °C or °K; D1 and D2 are D-values at the lower and higher temperatures in min.

The Z-values were determined from the linear regression of logD and temperature (T).

The temperature of treatment and the rate constant in a denaturation process was related according to the Arrhenius equation (Eq 4 or 5)

$$k = Ae^{(-Ea/RT)} \quad (4)$$

$$\text{or } \ln k = \ln A – Ea/R \times T \quad (5)$$

where;

k is the reaction rate constant value, A is the Arrhenius constant, Ea is the activation energy (energy required for the inactivation to occur), R is the gas constant (8.31 $Jmol^{-1}K^{-1}$), T is the absolute temperature in °K.

When lnk is plotted versus the reciprocal of the absolute temperature, a linear relationship should be observed in the temperature range studied. The slope of the line obtained permitted to calculate the activation energy and the ordinate intercept corresponds to ln A (Dogan *et al*., 2002).

The values of the activation energy (Ea) and Arrhenius constant (A) allowed the determination of different thermodynamic parameters[38] such as variations in enthalpy, entropy and Gibbs free energy, $\Delta H^{\#}$, $\Delta S^{\#}$ and $\Delta G^{\#}$, respectively, according to the following equations ((Eq. 6; 7; 8) [39]

$$\Delta H^{\#} = Ea - RT \quad (6)$$

$$\Delta S^{\#} = R (\ln A - \ln K_B/h_P - \ln T) \quad (7)$$

$$\Delta G^{\#} = \Delta H^{\#} - T \Delta S^{\#} \quad (8)$$

Where;

$K_B$ is the Boltzmann constant (1.38 x 10-23 J/K), $h_P$ is the Planck constant (6.626 x $10^{-34}$ J.s) and T is the absolute temperature.

## RESULTS AND DISCUSSION

The optimum temperature of the α-amylases purified from the digestive tract of cricket *G. sigillatus* was 55 °C (Kouadio *et al*., 2010). Thus, theses amylases are mesophilic enzymes. In this study, the effect of heat treatment over a range of temperatures from 55 to 70°C on both α-amylases was evaluated by determining the residual enzymatic activity. The thermal stability profiles of α-amylases Amy A1 and Amy A2 presented in the form of the residual percentage activity are shown in Table 1.

Table 1. Effect of treatment temperature and time on the inactivation of α-amylases Amy A1 and Amy A2 [Values represent mean ± SD triplicate measurements]

| Treatment time (min) | Residual activity (%) at each temperature (°C) of heat treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 55 | | 60 | | 65 | | 70 | |
| | Amy A1 | Amy A2 | Amy A1 | Amy A2 | Amy A1 | Amy A2 | Amy A1 | Amy A2 |
| 5 | 92.6 ± 2.4 | 83.3 ± 2.5 | 83.9 ± 5.0 | 74.3 ± 7.1 | 68.0 ± 2.8 | 33.3 ± 7.5 | 1.5 ± 0.4 | 0 |
| 10 | 86.7 ± 2.8 | 75.0 ± 6.6 | 73.4 ± 5.2 | 52.6 ± 1.2 | 42.4 ± 3.4 | 11.1 ± 6.7 | 0 | 0 |
| 15 | 80.3 ± 2.3 | 64.3 ± 8.1 | 62.8 ± 4.0 | 37.0 ± 2.4 | 17.3 ± 1.6 | 0 | 0 | 0 |
| 20 | 76.0 ± 2.5 | 54.9 ± 6.0 | 53.5 ± 5.2 | 0 | 6.3 ±1 1.1 | 0 | 0 | 0 |
| 25 | 71.8 ± 4.2 | 47.4 ± 9.2 | 46.0 ± 3.2 | 0 | 0 | 0 | 0 | 0 |
| 30 | 66.1 ± 3.9 | 39.3 ± 9.4 | 41.1 ± 0.5 | 0 | 0 | 0 | 0 | 0 |
| 35 | 62.9 ± 3.7 | 34.4 ± 1.4 | 0 | 0 | 0 | 0 | 0 | 0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 40 | 57.2 ± 2.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45 | 51.7 ± 1.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 50 | 48.5 ± 1.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 55 | 44.8 ± 2.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 60 | 41.9 ± 3.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

The heat treatment during 25 min at 55 °C caused the partial inactivation (47.47±9.25%) of Amy A2 whereas for Amy A1, the partial inactivation (46.06±3.29%) during the same period occurred at 60 °C. There was complete loss of the two *α*-amylases activities after 5 min of heat treatment at 70 °C. On one hand, the decrease of percentage residual activity at temperatures higher than 55 °C could be explained by the unfolding of tertiary structure of these enzymes to form a secondary structure and on other hand, it could be explained by the chemical modification (Tanaka and Hoshino, 2002).

Considering the logarithmic linear relationship between amylases Amy A1 and Amy A2 activities and heat treatment time for the temperatures ranged from 55 to 70 °C (Fig. 1), it can be concluded that the thermal inactivation of each amylase can be adequately described by a first order reaction. Theses results were consistent with those reported for amylases from *Bacillus* licheniformis (Declerck *et al*., 1997), *Bacillus amyloliquefaciens* (Tanaka and Hoshino, 2002) and *Aspergillus oryzae* (Samborska *et al*., 2007).

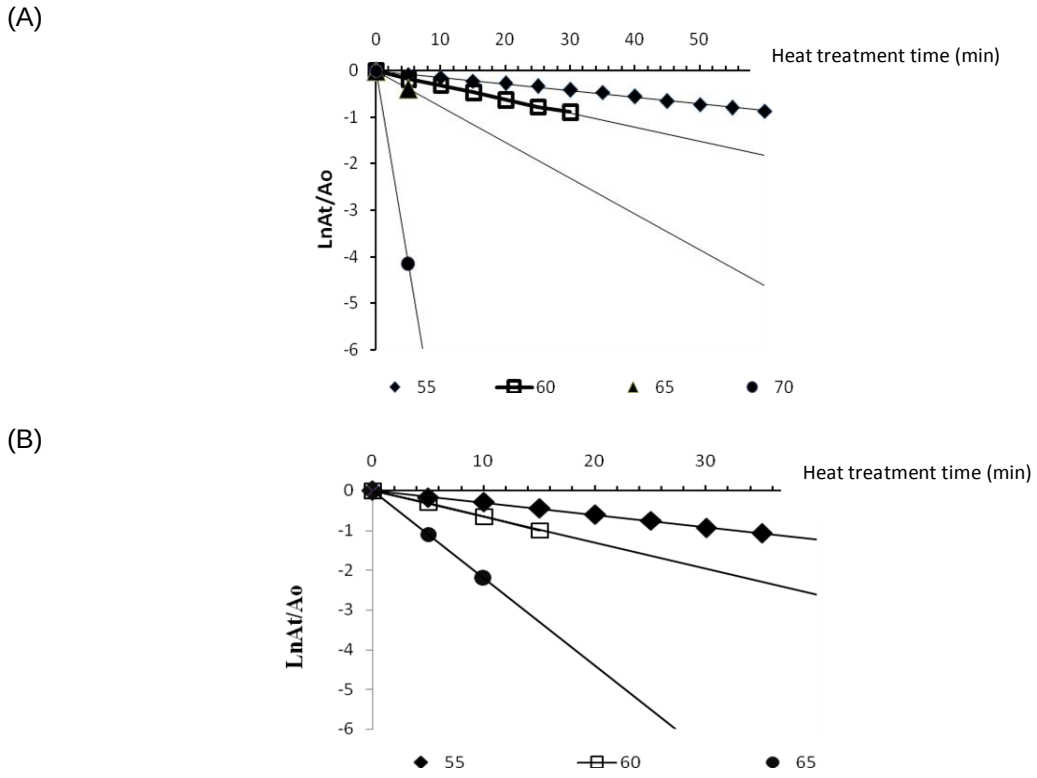



Figure 1. Thermal inactivation of α-amylases Amy A1 and Amy A2 in sodium phosphate buffer pH 6.6 in the temperature ranged from 55 to 70°C. $A_0$ is the initial enzymatic activity and $A_t$ the activity at each holding time. (A): α-amylase Amy A1; (B): α-amylase Amy A2

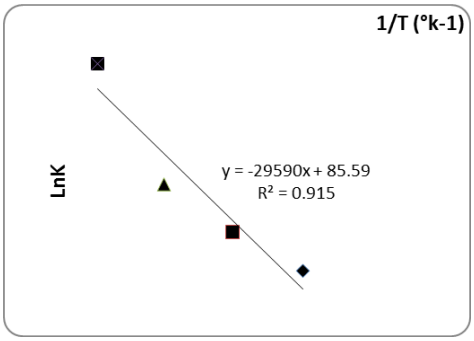
Rate constants of each amylase thermal inactivation increased with the temperature (Table 2). This result showed clearly that Amy A1 and Amy A2 were completely unstable at high temperatures (65-70 °C) since a higher rate constant means that the enzyme was less thermostable (Marangoni, 2002).

Table 2. k-values for thermal inactivation of α-amylases AmyA1 and Amy A2 at temperature range (55–70°C) [Values represent mean ± SD three independent determinations]

| Temperature (°C) | Amy A1 | | Amy A2 | |
|---|---|---|---|---|
| | k values | $R^2$ | k values | $R^2$ |
| 55 | 0.014 ± 0,001 | 0.996 | 0.030 ± 0.006 | 0.997 |
| 60 | 0.030 ± 0,002 | 0.997 | 0.065 ± 0.031 | 0.998 |
| 65 | 0.077 ± 0,005 | 1 | 0.219 ± 0.043 | 1 |
| 70 | 0.833 ± 0,077 | 1 | - | - |

Rate of Amy A1 and Amy A2 inactivation, after logarithmic transformation, decreased linearly with the inverse of temperature (Fig. 2).

(A)




(B)

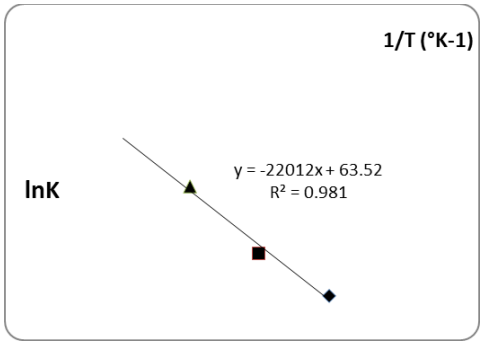



Figure 2. Temperature dependence of inactivation rate constant for thermal inactivation of α-amylases Amy A1 and Amy A2. 1/T represents the reciprocal of the absolute temperature. (A): α-amylase Amy A1; (B): α-amylase Amy A2

This relationship is described by the equation:
ln k = - 29590 (1/T) + 85.59 ($R^2$ = 0.91) and ln k = - 22012 (1/T) + 63.52 ($R^2$ = 0.98) for Amy A1 and Amy A2, respectively, where T represents absolute temperature (°K). From 55 to 70°C, the activation energy (Ea) value for thermal inactivation of the amylases Amy A1 and Amy A2 was calculated to be 245.89 ± 3.74 and 182.92 ± 4.23 kJ/mol respectively (Table 3).

Table 3. D, Z and Ea-values for thermal inactivation of α-amylases AmyA1 and Amy A2 at temperature range (55–70°C). [Values represent mean ± SD three independent determinations]

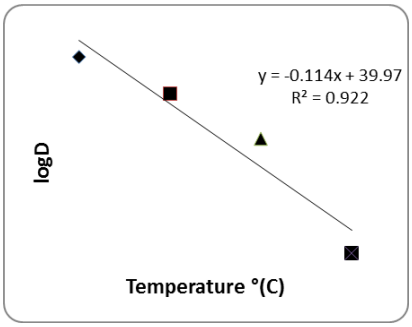
| | D values | |
|---|---|---|
| | Amy A1 | Amy A2 |
| $D_{55}$ | 164.50 ± 6.33 | 76.76 ± 41.78 |
| $D_{60}$ | 76.77 ± 6.81 | 35.43 ± 14.76 |
| $D_{65}$ | 29.91 ± 10.31 | 10.51 ± 2.94 |
| $D_{70}$ | 2.76 ± 0.22 | - |
| Z value (°C) | 8.77 ± 0.17 | 11.63 ± 2.29 |
| Ea (kJ/mol) | 245.89 ± 3.74 | 182.92 ± 4.23 |

These values of activation energy were much higher than that reported for *Bacillus licheniformis* α-amylase (53.1 kJ/mol)[11], but lower than that for whole α-amylases from *Bacillus subtilis* (262.0 ± 15.5 kJ/mol), *Aspergillus oryzae* (317.9 ± 11.6 kJ/mol), *Bacillus amyloliquefaciens* (255.4 ± 13.9 kJ/mol) (Duy and Fitter, 2005). These relative high values of activation energy found for the two amylases mean that an important amount of energy is needed to initiate denaturation of each enzyme. This also indicates the relative stability of these proteins (Timasheff, 1993). This is probably due to the presence of $Ca^{2+}$ in molecule of both amylases Amy A1 and Amy A2 (Kouadio *et al*., 2010). Indeed, the role of $Ca^{2+}$ in maintaining stability of structure of α-amylases has been

demonstrated (Kim *et al*., 1995; Mishra *et al*., 1996; Hagihara *et al*. 2001; Murakami *et al*., 2007). Furthermore, a large number of reports have showed the effect of $Ca^{2+}$ in the enhancement of thermostability of amylases (Violet and Meunier, 1989; Khajeh et *al.*, 2001; Tanaka and Hoshino, 2002; Saboury, 2002; Nielsen *et al*., 2003).

D-values for thermal inactivation of amylases Amy A1 and Amy A2 were calculated using equation (2) and are presented in Table 3. As expected the decimal reduction time decreases with temperature increase for both amylases (Fig. 3).

**(A)**




**(B)**

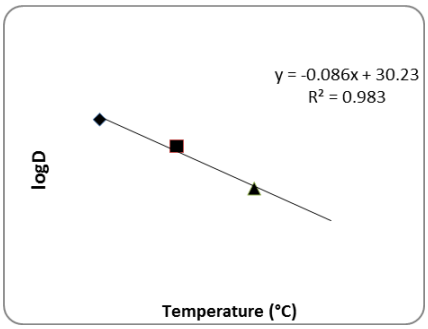



Figure 3. Effect of temperature on D-values for inactivation of α-amylases Amy A1 and Amy A2 Activities. (A): α-amylase Amy A1; α-amylase Amy A2

At 65°C the D-values are 30 and 11 min for Amy A1 Amy A2, respectively. Thus, Amy A1 is more heat-resistant than Amy A2. Moreover, these D-values of amylases from *G. sigillatus* at 65 °C are relatively higher compared to D-value of 2.4±0.2 min at 68 °C for amylase from *Aspergillus oryzae* (Samborska, 2007).

The calculated Z-values for Amylases Amy A1 and Amy A2 were 8.77 ± 0.17°C and 11.63 ± 2.29°C at 55-70 °C, respectively. These Z-values of Amy A1 and AmyA2 inactivation were in agreement with the Z-values of other proteins reported in the literature which range from 8.5 to 10.1°C (Vamos-Vigyazo, 1981; Râpeanu and Bulancea, 2005). According to Barrett *et al*. (1999), low Z-values are thought to indicate greater sensitivity to heat.

The thermodynamic parameters for thermal inactivation of amylases Amy A1 and Amy A2 from digestive tract of tropical house cricket *G. sigillatus* are shown in Table 4.

Table 4. Thermodynamic parameters of α-amylases Amy A1 and Amy A2 under heat treatment between 55 to 70°C (assuming a 1st-order kinetic model) [Values represent mean ± SD three independent determinations]

| Temperatures (°C) | Thermodynamic parameters | | | | | |
|---|---|---|---|---|---|---|
| | $\Delta H^{\#}$ ( kJ/mol) | | $\Delta S^{\#}$ (J $mol^{-1}$ $K^{-1}$ ) | | $\Delta G^{\#}$ ( kJ/mol) | |
| | Amy A1 | Amy A2 | Amy A1 | Amy A2 | Amy A1 | Amy A2 |
| 55 | 243.16±3.74 | 180.19±4.23 | 465.67±11.80 | 282.27±32.40 | 90.42±0.14 | 87.61±0.81 |
| 60 | 243.12±3.74 | 180.15±4.23 | 465.54±11.80 | 282.14±32.40 | 88.09±0.20 | 86.19±0.15 |
| 65 | 243.08±3.74 | 180.11±4.23 | 465.42±11.80 | 282.02±32.40 | 85.77±0.25 | 84.78±0.52 |
| 70 | 243.04±3.74 | 180.06±4.23 | 465.30±11.80 | 281.89±32.40 | 83.44±0.31 | 83.37±1.17 |
| Mean | 243.10±3.74 | 180.13±4.23 | 465.48±11.80 | 282.08±32.40 | 86.93±0.23 | 85.49±1.19 |

At temperatures of 55–70°C, the average values of $\Delta H^{\#}$, $\Delta S^{\#}$ and $\Delta G^{\#}$ were respectively 243.10 ± 3.74 kJ/mol, 465.48 ± 11.80 J.mol/K and 86.93 ± 0.23 kJ/mol for Amy A1 and 180.13 ± 4.23 kJ/mol, 282.08 ± 32.40 J.mol/K, 85.49 ± 1.19 kJ/mol for Amy A2. The high values of change in enthalpy obtained for the two amylases at the different treatment temperatures indicate that these enzymes undergo a considerable change in conformation during denaturation. Moreover, positive values of ΔH indicate that denaturation of each amylase Amy A1 and Amy A2 is an endothermic reaction. Positive values of ΔH have also reported for many amylases (Declerck et *al*., 1997; Nielsen *et al*., 2003; D'Amico *et al*., 2003). The positive values found for ΔS indicate that there are no significant processes of aggregation for both amylases, since, if this would happen, the values of entropy would be negative (Anema and McKenna, 1996). Furthermore, these high values of entropy variation probably reflect an increased disorder of the active site or of the structure of each amylase which is the main driving force of heat denaturation (D'Amico et al., 2003). Concerning the free energy (ΔG), which is a measure of the spontaneity of the inactivation processes, the value for each amylase decreased slightly with increasing temperature between 55 and 70 °C. These values are positive at all temperatures for both amylases and this indicates that the inactivation processes are not spontaneous.

Based on above results, it is concluded that thermal inactivation of each α-amylase Amy A1 and Amy A2 of cricket *G. sigillatus* digestive tract could be described by a firs-order kinetic model. It was found that the heat treatment at 70 °C during 5 min caused the complete inactivation of both amylases. However, the high values obtained for activation energy and change in enthalpy indicate that a high amount of energy is needed to initiate denaturation of these enzymes, most likely due to the presence of $Ca^{2+}$ in their structure. In general, both α-amylases are relatively resistant to long treatments up to 60 °C. This fact should be taken into account in the application of α-amylases Amy A1 and Amy A2 for the saccharification of starch in the production of syrup of oligosaccharides mixture and when used for glycosylation of phenolic compounds.

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