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STUDY OF THERMAL STABILITY OF BETA-GLUCOSIDASE FROM THE LAND CRAB DIGESTIVE JUICE (*CARDISOMA ARMATUM*): KINETIC AND THERMODYNAMIC ANALYSIS

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ABSTRACT

The thermal stability of beta-glucosidase from the land crab digestive juice (*Cardisoma armatum*) was investigated by studying the effect of heat treatment over a range of 60 to 75 °C. Thermal inactivation of this enzyme, evaluated by loss in activity, was apparently followed by first-order kinetics with k-values comprised between 0.0049 to 0.0391 min^{-1}. D and k-values decreased and increased, respectively, with increasing temperature, indicating faster inactivation of beta-glucosidase at higher temperatures. Ea and Z-values were estimated to 172.98 kJ mol^{-1} and 12.80 °C respectively. Thermodynamic parameters were also calculated. All the results suggest that enzyme was relatively resistant to long heat treatments up to 60 °C.

INTRODUCTION

Cellulases are an important class of enzymes which are used for the hydrolysis of cellulosic materials for the production of glucose, alcohol and cellulose acetate oligosaccharides. They have applications in various industries like fuel, textile, paper, feeds, fruits, vegetables (Zaldivar *et al*., 2001). These enzymes are also used for non-specific cleavage of chitosan to form low molar mass oligosaccharides (Xia *et al*., 2008). Now-a-days lignocellulosic materials are under intensive research due to depletion of fossil fuels and production of ecofriendly biofuels. Lignocellulosic materials mainly consist of cellulose which is available abundantly in nature. Hydrolysis of lignocellulosic wastes is of prime importance for its conversion into important industrial products. Beta-glucosidase (EC 3.2.1.21) is an important class of cellulolytic complex that completely breaks down various ligocellulosic wastes/materials by cleaving the beta-1, 4-glycosidic bond (Wallecha and Saroj, 2003). Despite beta-glucosidase are universally produced by prokaryotes and eukaryotes, including plants and animals, to date, the majority of beta-glucosidase used in industry are generally produced by bacteria and fungi (Saha *et al*., 2002; Saloheimo *et al*., 2002; Li *et al*., 2002; Wallecha and Saroj, 2003). Considering this importance of beta-glucosidase, many authors have attempted to understand the kinetic behaviour of these enzymes when exposed to high temperatures in some organisms such as *Aspergillus wentii* (Kvesitadze *et al*., 1990), *A. niger* (Rashid and Siddiqui, 1998), *Thermobifida fusca* (Spiridonov and Wilson, 2001) and *Cellulomonas biazotea* (Rajoka *et al*., 2004). In previous study, we purified to homogeneity one monomeric beta-glucosidase from the land crab digestive juice (*Cardisoma armatum*) (Ya *et al*., 2014), a West Africa land crab species which is living in a marshy and littoral middle (Zabi and Le Loeuf, 1992). This crab just as a wide variety of crab is herbivorous. It means that these animals consume plant material and are capable of assimilating significant cellulose and hemicellulose, which are there essential source of energy (Linton and Greenaway, 2004). This enzyme showed maximum activity at 60 °C and could possibly be used for industrial saccharification (Ya *et al*., 2014). In addition, this beta-glucosidase showed interesting properties to catalyse the transfer of glycosyl residues from *p*NP-beta-D-glucopyranoside onto maltose and cellobiose (Ya *et al*., 2014). This ability to synthesize higher oligosaccharides of up to six

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glucose residues from disaccharides could greatly extend the biotechnological applications of this enzyme. (Ya *et al*., 2014). Considering these important properties of beta-glucosidase, the present work aimed to investigate the effect of heat treatment over a range of temperatures from 60 to 75°C on this enzyme. So, determination and analysis of kinetic and thermodynamic parameters were undertaken.

MATERIALS AND METHODS

Enzyme

Beta-glucosidase used in this study was purified from the land crab digestive juice (*Cardisoma armatum*) (Ya *et al*., 2014). This enzyme was homogeneous on polyacrylamide-gel electrophoresis in absence of Sodium Dodecyl Sulphate (SDS).

Beta-glucosidase assay

Under standard test conditions, beta-glucosidase activity was measured at 37°C for 10 min in 100 mM acetate buffer (pH 5.6) containing 1.5 mM *para*-Nitrophenyl-beta-D-Glucopyranoside (*p*NPP). After pre-warming the mixture at 37°C for 5 min, the reaction was initiated by adding 50 µl (6 µg of protein) of enzyme solution. The final volume was 250 µl and the reaction was stopped by adding 2 ml of sodium carbonate (2 %, w/v). Absorbances were measured at 410 nm using a spectrophotometer (SHIMADZU) using *para*-Nitrophenol (*p*NP) as the standard. Under the above experimental conditions, one unit (U) of enzyme activity was defined as the amount of enzyme capable of releasing one µmol of *p*NP per minute. Specific activity was expressed as units per mg of protein (U/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 beta-glucosidase was investigated at various constant temperatures from 60 to 75°C after exposure to each temperature for a period of 5 to 60 min. The enzyme was incubated in 100 mM acetate buffer (pH 5.6). 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 beta-glucosidase can be described by a first-order kinetic model (Terebiznik*et al*., 1997; Guiavarc'h *et al*., 2002). The integral effect of 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 (A_0) 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 log (D) 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)

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

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

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

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

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

Where;

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

RESULTS AND DISCUSSION

Thermal inactivation kinetics of beta-glucosidase

The optimum temperature of beta-glucosidase purified from the land crab digestive juice (*Cardisoma armatum*) was 60 °C (Ya *et al*., 2014). In this study, the effect of heat treatment

over a range of temperatures from 60 to 75°C on beta-glucosidase was evaluated by determining the residual enzymatic activity. Thermal stability profile of this enzyme presented in the form of the residual percentage activity is shown in Table 1. The activity of beta-glucosidase was decreased with increasing heating time (5–60 min) and temperature (60–75°C). Indeed, at temperatures between 60-75°C, heat-denaturation of the enzyme occurred after 5 min of incubation (96.27 to 77.16 %). Although heating at 70°C for 25 min resulted in partial (50.03 %) inactivation, heating at 75°C for the same period strongly inactivated the enzyme (21.01 %). On one hand, beta-glucosidase from land crab digestive juice showed a typical temperature-dependent inactivation profile in the presence of the substrate used. At higher temperature, the enzyme most likely underwent denaturation and lost its activity. Stauffer (1989) states that denaturation is the heat induced spontaneous, irreversible breakdown of the secondary and tertiary structure of the enzyme protein such that the enzyme will no longer function and cannot re-activate. The results of the heat inactivation studies suggest that this enzyme belongs to the group of less thermo stable enzymes.

Table 1. Effect of treatment temperature and time on the inactivation of beta-glucosidase from land crab digestive juice (*Cardisoma armatum*)

Treatment time (min)	Relative activity (%) at each temperature (°C) of heat treatment			
	60	65	70	75
5	96.27	95.72	75.42	77.16
10	94.37	91.58	71.19	57.90
15	92.78	86.94	67.08	40.04
20	90.54	85.27	56.18	28.54
25	88.25	81.08	50.03	21.01
30	86.21	76.37	42.97	14.66
35	83.95	74.83	37.61	09.44
40	81.96	71.60	31.45	05.78
45	79.86	67.68	27.02	04.64
50	78.27	63.89	24.57	02.99
55	76.88	60.22	20.08	02.04
60	74.24	58.38	18.22	01.40

armatum) are reported in Table 2. According to the results presented in the Table 2, it was clear that the enzyme was less thermo stable at higher temperatures since a higher rate constant, k, means that the enzyme is less thermo stable (Marangoni, 2002). The half-life ($t_{1/2}$) is another parameter that plays an important role in the characterization of enzyme stability (Arogba *et al*., 1998). Based on the results shown in Table 2, the $t_{1/2}$ values in the temperatures ranging between 60°C and 75°C varied between 169.02 minutes and 4.31 minutes. The increasing temperature from 60°C to 75°C resulted in a decrease in $t_{1/2}$ values.

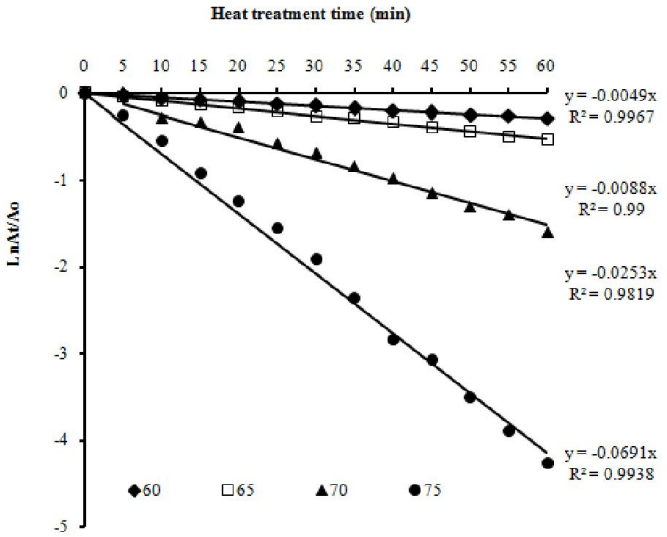

Figure 1. Thermal inactivation of beta glucosidase from the land crab digestive juice (*Cardisoma armatum*) in sodium acetate buffer pH 5.6 in the temperature ranged from 60 to 75 °C. A0 is the initial enzymatic activity and At the activity at each holding time

Inactivation rate constants were used to drawn the Arrhenius plot, from which slope activation energy was calculated and found to be 172.98 kJ/mol. This value of activation energy was much higher than that reported for an intracellular beta-glucosidase from a mutant-derivative of *C. biazotea* (57 kJ/mol (Rajoka *et al*., 2004) and a specific beta-glucosidase from the digestive fluid of larvae of the palm weevil (*Rhynchophorus palmarum*) (68.77 kJ/mol (Yapi *et al*., 2009), but lower than that for whole native or chemically modified beta-glucosidases (389 or 174 kJ/ mol) (Rashid and Siddiqui, 1998) and thermostable glucoamylase from a thermostabilized mutant derivative of *Aspergillus awamori* (Chen *et al*. 1994). Such a higher activation energy for *C. armatum* beta-glucosidase indicated that the conformation of the enzyme was still stable at the temperature on which the enzyme has been assayed (Rodrigo *et al*., 2007). In order to establish the link between treatment time and enzyme activity, the D-values

Table 2. k, D, Z and Ea-values for thermal inactivation of beta glucosidase from land crab digestive juice (*Cardisoma armatum*) at temperature range (60–75°C)

Temperature (°C)	Kinetic parameters				
	K (10^{-2}min^{-1})	$t_{1/2}$ (min)	D (min)	Z (°C)	Ea (kJ mol^{-1})
60	0.49	169.02	470		
65	0.88	54.57	261.70	12.80	172.98
70	2.53	10.19	91.03		
75	3.91	4.31	33.33		

The semi-log plots of residual activity versus treatment time (Figure 1) were linear at all temperatures, suggesting that the inactivation followed a simple first- order monophasic kinetic model (R^2 = 0.9819-0.9967). This result is in agreement with those obtained from *A. niger* (Rashid and Siddiqui, 1998) and *Fusarium solani* (Bhatti, 2013). From the slope of the lines in Figure 1, the inactivation rate constants (k) were calculated. The trend of changes in k values with increasing temperature indicates a faster inactivation of beta-glucosidase at higher temperatures. The rate constant values increased with temperature from 0.0049 to 0.0391 min^{-1} at 60-75°C, respectively. Estimated kinetic parameters for heat inactivation of beta-glucosidase from land crab digestive juice (*Cardisoma*

were calculated. The decimal reduction time (D value) was calculated according to equation 2. D value is the time, at a given temperature and pressure, needed for 90% reduction of the initial activity. As shown in table 2, D-values decreased by increased at temperature. D-values that obtained at 75 and 70°C were about 14.10 and 5.16 time lower than in comparison to D-value at 60 °C, respectively. This remarkable decrease at D-value between 70 and 75°C indicated a potential thermal denaturation of beta-glucosidase from *C. armatum*. The effect of temperature on D-values is shown in Figure 2, and from this representation, the Z-value was calculated and found to be 12.80 °C at 60–75°C (table 2). In general, high Z-values mean more sensitivity to the duration of heat treatment and lower Z-values mean more sensitivity to increase in temperature (Barrett *et al*., 1999). Therefore, the Z-value 12.80 °C for *C. armatum* beta-glucosidase indicated that this enzyme is more to sensitive to increase of temperature than to the extension of treatment time.

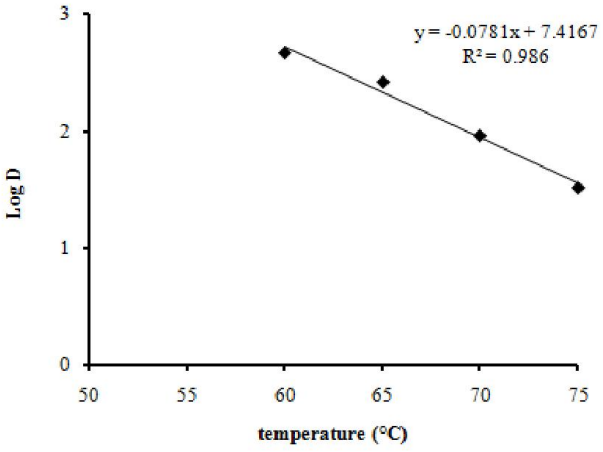

Figure 2. Effect of temperature on D-values for inactivation of beta glucosidase from the land crab digestive juice (*Cardisoma armatum*)

Thermodynamic studies of beta-glucosidase

Thermostability represents the capability of an enzyme molecule to resist against thermal unfolding in the absence of substrate, while thermophilicity is the ability of an enzyme to work at elevated temperatures in the presence of substrate (Georis *et al*., 2000). Irreversible thermal inactivation occurs in two steps as shown below:

N<—>U —>I

N is the native, U is the unfolded enzyme which could be reversibly refolded upon cooling and I is the inactivated enzyme formed after prolonged exposure to heat, and therefore, cannot be recovered on cooling. The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation (Daniel, 1996). The opening up of enzyme structure is accompanied by an increase in disorder, randomness or entropy of activation (Vieille and Zeikus, 1996). Concerning free energy ($\Delta G^{\#}$), which is a measure of spontaneity of inactivation processes. Beta-glucosidase from *C. armatum* presented a larger Gibbs free energy ($\Delta G^{\#}$) at all the temperatures. This value was positive at all temperatures for this enzyme (Table 3) and this indicates that inactivation processes was not spontaneous. However, when the temperature was increased from 60-75 °C there was reduction in $\Delta G^{\#}$ (Table 3), indicating destabilization of protein at this temperature (Rodrigo *et al*., 2007). At temperatures of 60–75°C, the average values of $\Delta H^{\#}$ and $\Delta S^{\#}$ were respectively 170.15 (kJ mol^{-1}) and 228.20 (J mol^{-1} K^{-1}). The $\Delta H^{\#}$ value of beta-glucosidase suggested that the numbers of non-covalent bonds broken in forming a transition state for enzyme inactivation were similar. The high values of change in enthalpy obtained for the different treatment temperatures indicate that enzyme undergoes a considerable change in conformation during denaturation. Positive values of $\Delta H^{\#}$ indicate the endothermic nature of the oxidation reaction (Anema and McKenna, 1996).

Table 3. Thermodynamics parameters of beta-glucosidase from the land crab digestive juice (*Cardisoma armatum*) under heat treatment between 60 to 75°C (assuming a 1st-order kinetic model)

Temperature(°C)	Thermodynamic parameters		
	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})
60	170.21	228.38	941.62
65	170.17	228.26	930.20
70	170.13	228.14	918.79
75	170.09	228.02	907.39
Mean	170.15	228.20	924.50

The $\Delta H^{\#}$ value was much higher than that reported for beta-Glucosidases from *F. solani* (50.43 kJ mol^{-1} (Bhatti *et al*., 2013) and *A. wentii* (65 kJ mol^{-1} (Kvesitadze *et al* 1990). The positive values found for $\Delta S^{\#}$ indicates that there are no significant processes of aggregation for beta-glucosidase, since, if this would happen, the entropy values 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 beta-glucosidase from *C. armatum* which is the main driving force of heat denaturation (D'Amico *et al*., 2003). Based on the results obtained for thermal inactivation, it is concluded that thermal inactivation of beta-glucosidase from *C. armatum* could be described by a first-order kinetic model. D, Z, k values and the high values obtained for activation energy, Ea and change in enthalpy indicated that a high amount of energy was needed to initiate denaturation of beta-glucosidase, most likely due to its stable molecular conformation.

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