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Thermostability and thermodynamic characterization of sprouted pearl millet α-amylases for its biotechnological applications

K. U. Agbo*, P. C. Okwuenu, A. L. Ezugwu, S. O. O. Eze and F. C. Chilaka

Department of Biochemistry, University of Nigeria, Nsukka, Nigeria

Abstract

There is an increasing demand for amylolytic products such as glucose syrup in biotechnological industries. Starch liquification by α-amylase must take place at temperatures higher than the gelatinization temperatures and the use for thermostable α-amylase is therefore required. Two (or) isotypes namely amy-1 and amy-2 of α-amylase from pearl millet have been investigated for their thermostability and thermodynamic characterization.Thermal inactivation of α-amylase follows first order kinetics which varies with time and product during inactivation process. The half-life of α-1 was 198 mins at 50ºC. The *Ea (inact)* was calculated using Arrhenius plot gave 22.00 KCalmol^{-1}K^{-1} for α-1 and 19.44 KCalmol^{-1}K^{-1} for α-2. The Z-values were 22.52ºC and 25.38ºC for α-1 and α-2, respectively. The thermodynamic parameters: the enthalpy change of inactivation ΔH (inact) had 87.815KJmol^{-1}K^{-1} at 50ºC for α-1 and 78.56KJmol^{-1}K^{-1} at 50ºC for α-2. The change in free energy of inactivation ΔG (inact) of α-1 and α-2 were 105.5 KJMol^{-1}K^{-1} and 104.33KJMol^{-1}K^{-1} at 50ºC, respectively. The entropy of inactivation ΔS (inact) values for α-1 and α-2 are calculated as -54.80JMol^{-1}K^{-1} and -79JMol^{-1}K^{-1} at 50ºC. This suggest that the α-1 and α-2 are thermostable.

Keywords : Thermostability; Biocatalysts; Thermodynamic; Millet; Inactivation

Introduction

Catalyst speeds up the turning of reactants to products but doesn't undergo any permanent change. Catalyst acts by reducing the energy barrier of chemical reactions, by producing a dramatic increase in reaction rates from 10^6-10^{24}. Catalysts found in a living system are called biocatalysts. Amylases are enzymes that catalyze the conversion of starch and other related polysaccharides possessing α-1,4 glycosidic bonds into simpler derivatives such as oligosaccharides or monosaccharides. The enzyme has been purified from few plants such as barley, sorghum, soybeans, millet, wheat (Thoma *et al.,* 1971), wheat germ (Sharma *et al.,* 2000), maize (Warner and Knutson, 1991). Carbohydrate based agricultural products such as starch occur abundantly in some developing countries of the tropics (Okolo *et al.,* 1995). Pearl millet *(Pennisetum glaucum)* belongs to the family of *Poaceae.* It is grown under conditions of high temperature (thermophile) and low rainfall (Ambasht and Kharkrang, 2012). Cereal α-amylases have gained importance in processes due to their suitability for biotechnological application in industries like food, brewing, detergents, paper and textile. The enzyme is important for diagnosis of pancreatitis (Ambasht and Kharkrang, 2012). Conversion of starch to various sweeteners is achieved through a chemical (acid) or enzymatic process. However,

the use of enzyme is preferred to acid, because it produces high yields of desirable products and less formation of undesirable products such as toxic compounds (Sanjust *et al.,* 2004; Chaplin, 2004). Temperature and chemicals promote enzyme inactivation. Temperature variation may opposed effects on enzyme activity and stability and it is therefore a key variable in any biocatalytic process (Wasserman, 1984). Biocatalyst stability i.e the capacity to retain activity over time is undoubtedly the limiting factor in most bioprocesses, biocatalyst stabilization being a central issue of biotechnology (Illanes, 1999). Biocatalyst thermostability allows a higher operation temperature, which is clearly advantageous because of higher reactivity, higher process yield, lower viscosity and fewer contamination problem (Mozhaev, 1993). Enzyme thermal inactivation is the consequence of weakening the intermolecular forces responsible for the preservation of its 3D-structure, leading to a reduction in its catalytic capacity (Misset, 1993). Inactivation may involve covalent or non-covalent bond disruption with subsequent molecular aggregation or improper folding (Bommarius and Broering, 2005). Knowledge of enzyme inactivation kinetics under process condition is an absolute requirement to properly evaluate enzyme performance (Illanes *et al.,* 2008).Various thermostability parameters, such as half life of the enzyme preparation $(T_{1/2})$, the activation energy of thermal

denaturation *(Ea$_D$),* enthalpy of activation of the thermal denaturation *(ΔH$_D$),* entropy of activation of thermal denaturation *(ΔS$_D$)* and the Gibbs free energy of activation of thermal denaturation *(ΔG$_D$)* were used to evaluate the thermostability of α-amylase extracted from germinating pearl millet. The search for new or improved heat stable α-amylase has been the subject for research and discussion. Though thermophilic microorganisms seems to be promising in the production of thermostable enzymes, the technological use of these organisms is faced with challenges since knowledge on physiology and genetics of such organism is poor, they are fastidious, grow slowly and not recognized as safe (Illanes, 1999). Hence, an alternative source of thermally stable ∝ - amylase suitable for biotechnological industries is required for production processes.

Materials and methods

The ∝ - amylase used in this study was donated by the enzymology research unit, Department of Biochemistry, University of Nigeria, Nsukka as requirement for the work. Unless indicated otherwise, all chemicals used in the present investigation were of high quality analytical grade.

Processing of cassava starch

Cassava starch was processed using the method described by Corbishley and Miller (1984).

Protein content determination

Protein content of the enzyme solution was determined by the method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard.

Determination of enzyme activity

Amylase activity was determined using dinitrosalicyclic acid (DNSA) as described by Bernfeld (1951). One unit of amylase activity was expressed as the amount of enzyme which released one micromole (μmole) of the reducing equivalent (maltose) per minute under assay conditions. Amylase activity was estimated by the amount of reducing equivalent (maltose) released during the hydrolysis of the starch.

Thermal inactivation studies

The activity of each enzyme fraction was assayed at 50ºC without preheating. This was carried out by using 0.5 ml of each of the enzyme fractions, 1%(w/v) starch solutions, and 1ml of the sodium acetate buffers of pHs 4.5 and 5.5 for the α-1 and α-2 respectively for 30mins at optimum temperature (50ºC) to obtain the A$_0$ and these were called heat untreated

samples. While the test mixtures were incubated for 2hours at varying temperatures, 40º, 50º, 60º and 70ºC and at every 30 minute interval, 0.5 ml was taken from each of the fractions, cooled at 4ºC for 20minutes so that the enzyme's secondary and tertiary structures became stable after the heat treatment any reversible unfolding reverted back to the native forms (Griffin *et al.,* 1984) then, were assayed at optimum conditions (pH of 4.5 and 5.5 for α-1 and α-2 and then 50ºC for both amylases) as was done for heat untreated samples. The activity obtained from the samples is called the *A$_t$.*

Determination of percentage residual activity

The % residual activity of the enzyme fraction was calculated using the equation 1 below:

At/Ao x 100% ---------------- Equation 1

Where At = the enzyme activity at time, *t*

   Ao = the initial enzyme activity at time, 0

Denaturation constants of the enzyme fraction k$_d$

The k_d of the α-amylase preparations was estimated as follows. The calculated values of the % residual activity were tabulated and k_d was determined from the slope of plots of *In* of %residual activity of various temperatures against time *(t)* from the equations 2 below:

At/Ao = exp$^{(-kdt)}$ -------------- Equation 2

$In(\frac{At}{Ao})$ = *-kdt* first order kinetics

Where *kd* = denaturation constant of the enzyme

   t = time

Half-life of the enzyme solution t$_{1/2}$

The $t_{1/2}$ was obtained from the following equation:

$t_{1/2}$ = *In2/k$_d$* ----------------- Equation 3

Activation energy of inactivation E$_{(inact)}$

The Ea$_{(inact)}$ was obtained from the Arrhenius equation:

Ink = Ae$^{-EaR/T}$ ----------------Equation 4

where *kd* is the first order rate constant of thermal inactivation of the enzyme activity, obtained through the slopes of the plots described above.

Calculation of D-values of the enzyme fractions

The *D*-values (decimal reduction time or time required to

preincubate the enzyme at a given temperature to maintain 10% residual activity) of the enzyme fractions were calculated from the formula below:

$$D = \frac{In\ 10}{kd}$$ --------------------Equation 5

Calculation of Z-Values of the enzyme fractions

The *Z*-value (temperature rise necessary to reduce the *D* value by one logarithmic cycle (Pal *et al.,* 2011) was calculated from the slope of the graph between logD vs temperature (°C) using the equation and the results were presented in the table below:

Slope = (- $\frac{1}{z}$)-----------------equation 6

Thermodynamics parameters

The enthalpy of activation of thermal denaturation *ΔH$_{(inact)}$,* 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 of activation of the thermal denaturation *(ΔG$_{(inact)}$)* and the entropy of activation of the denaturation *(ΔS$_{(inact)}$)* were calculated through the following equations (Siddiqui *et al.,* 1997; Riaz *et al.,* 2007).

ΔH$_{(inact)}$=Ea$_{(inact)}$ - RT ----------------- Eqaution 7

Where *ΔH$_{(inact)}$* = change in enthalpy of inactivation

   Ea$_{(inact)}$ = activation energy of inactiavation

   R= general gas constant

   T= temperature

ΔG$_{(inact)}$= -RT x *In* ($\frac{Kd\ x\ h}{Kb\ x\ T}$)---------------- Equation 8

Where *ΔG$_{(inact)}$* = free energy change of inactivation

h= Plank's constant

Kb= Bolztman constant

$$ΔS_{(inact)} = \frac{ΔH_{(inact)} - ΔG_{(inact)}}{T}$$ ------------------- Equation 9

ΔS= change in entropy of inactivation

Results and discussion

From the gel filtration profile of the purified enzyme, it was observed that the enzyme has three different isotypes, two of them (amy1 and amy2) were used for further studies.

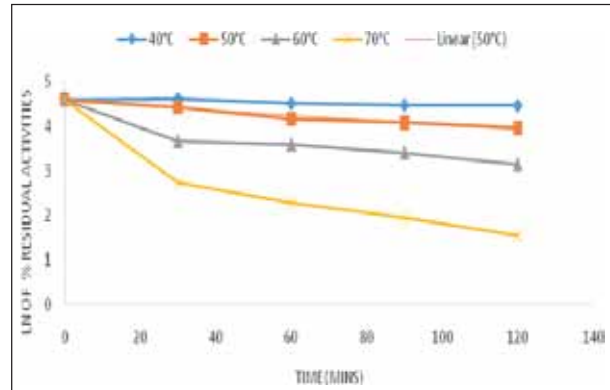*Determination of denaturation constant (Kd)*

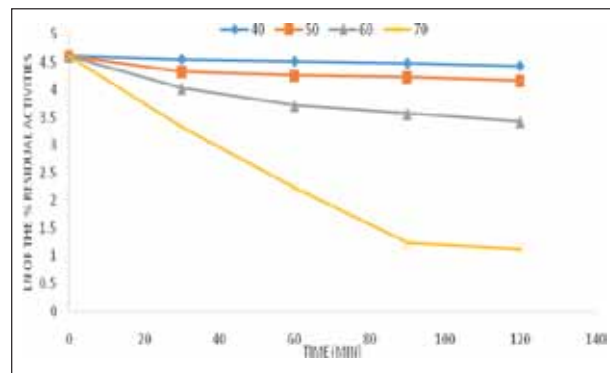**Fig. 1. Percentage residual activity against time for fraction one (α-1)**

Fig. 2. Percentage residual activities against time for fraction two (α-2)

Determination of Z-values of the two isotypes

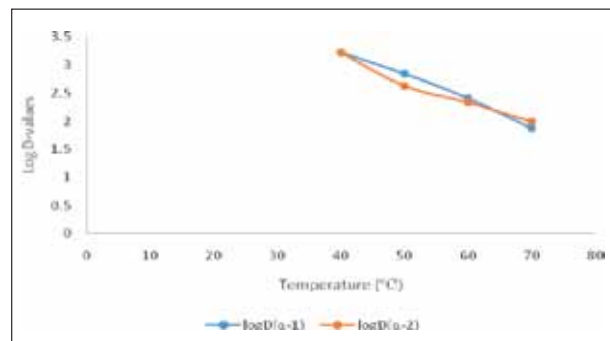**Fig. 3. Calculation of Z-value for the two isotypes (α-1 and α-2)**

Determination of activation energy of inactivation of the two enzyme isotypes
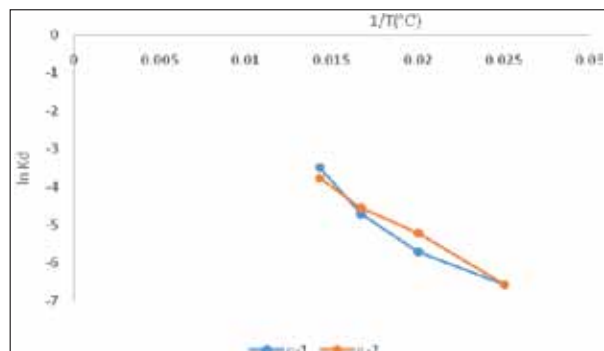

Fig. 4. Arrhenius plot for determination of activation energy of inactivation for α-1 and α-2

Various data were obtained from the plots above and summarized in the tables below:

Enzymes in general are protein molecules with precise primary, secondary and tertiary structures based on their amino acid sequences. Heating can denature the enzymes, i.e., the enzymes lose their secondary, tertiary and quaternary (if present) structures which are essential for their activities (Petsko and Ringe, 2004). Thermostability of an enzyme refers to its resistance to unfolding upon heating. Thermal denaturation occurs in two steps.

$$N \longleftrightarrow U \longleftrightarrow D$$

N is the native, U is the unfolded enzyme which could be reversibly refolded upon cooling and I (D) is the inactivated enzyme formed after prolonged exposure to heat and therefore cannot be recovered by cooling. Various kinetics and thermodynamics parameters have been effectively used

Table I. Summary of the thermoinactivation and thermodynamics of the enzyme denaturation for fraction one (α-1)

Temperature(°C)	$T_{1/2}(min)$	$K_d^{(min-1)}$	D-values (mins)	$\Delta H°_{(D)}$ (kJ/mol/K)	$\Delta G°_{(D)}$ (kJ/mol/K)	$\Delta S°_{(D)}$ (J/mol/K)
40	495	0.0014	1645	87.898	104.54	-0.0532
50	198	0.0035	658	87.815	105.50	-0.0548
60	72.94	0.009	242.42	87.731	106.09	-0.0551
70	22.90	0.032	76.26	87.648	106.05	-0.0536
Ea	22.00 KCalmol^{-1} K^{-1} = 92.4 KJMol^{-1}K^{-1}					
Z-value	22.52°C					

Table II. Summary of the thermoinactivation and thermodynamics of the enzyme denaturation for fraction two (α-2)

Temperature(°C)	$T_{1/2}(min)$	$K_d^{(min-1)}$	D-values (mins)	$\Delta H°_{(D)}$ (kJ/mol/K)	$\Delta G°_{(D)}$ (kJ/mol/K)	$\Delta S°_{(D)}$ (J/mol/K)
40	495	0.0014	1645	78.65	104.54	-0.083
50	128.33	0.0054	426.48	78.56	104.33	-0.079
60	65.38	0.0106	217.26	78.47	105.79	-0.082
70	30.13	0.023	100.13	78.39	106.85	-0.083
Ea	19.44KCalmol^{-1} K^{-1} = 81.243KJMol^{-1}K^{-1}					
Z-value	25.38°C					

in assessing enzyme thermostability and such parameters are: half-life ($t_{1/2}$), activation energy of denaturation ($Ea_{(D)}$), the enthalpy of denaturation ($\Delta H_{(D)}$), the change in entropy associated with thermal denaturation ($\Delta S_{(D)}$) and the change in Gibbs free energy during the thermal denaturation ($\Delta G_{(D)}$) (Maragoni, 2003).The plots of the residual activities vs incubation time (Figs. 1 and 2) for the enzymes are linear, showing that the inactivation could be expressed as first order kinetics in the temperature range of 40ºC-70ºC.

The half-life ($t_{1/2}$) determinations was accurate and reliable especially when computing the stability properties of an enzyme at different temperatures. On the half-life each of the two peaks, a longer half-life was observed at lower temperatures, indicating that the enzyme is more thermally stable at lower temperature. Comparing the two enzymes at their optimum temperature, (50ºC), α-1 has half-life of 210 minutes while α-2 has 128.33 minutes (Tables I and II). This is an indication that α-1 is more thermally stable than α-2. The half-life of 23.10 minutes was observed in α-amylase sourced from digestive tract of tropical house cricket at 60ºC (Kouadio, 2013) while the half-life of 8.5minutes was observed in the α-amylase from sorghum bicolar at 60ºC (Kumar, 2008). Comparing the data above with the present study, 72.94 minutes and 65.38 minutes for α-1 and α-2 at 60ºC (Tables I and II), suggesting that the enzyme under study maybe more thermally stable than the cited ones.

Also, the time required to reduce the enzyme activity by 90% i.e., the D-value is another reliable parameter in assessing enzyme thermostability. Just like the $t_{1/2}$, the D-values decreased with increase in temperature, showing a decrease in denaturation time as the temperature increases. This implies that the enzyme is thermally unstable at higher temperatures. α-1 has D-value of 658 minutes (Table I) while α-2 has the D-value of 462.48 minutes (Table II) at their optimal temperature. This indicates that α-1 is more stable than α-2 at their optimum temperature. The D-values of α-1 and α-2 from digestive tract of tropical house cricket at 60ºC were obtained 76.77 minutes and 35.43 minutes while in the present study, 242.42 minutes and 217.26 minutes for α-1 and α-2, respectively at the same temperature (Tables I and II). This could be an indication of high thermostability of this enzyme. Thermal denaturation of enzymes requires an input of a minimum amount of energy known as activation energy of denaturation ($Ea_{(D)}$) which must be supplied to the native enzyme in order to initiate the denaturation process. The denaturation process proceeds through an unstable intermediate transition (or activated) state, U (Siddiqui *et al.,* 1997). As long as the initial input of energy is less than the

$Ea_{(D)}$ the unstable intermediate (U) can fold back into the native state (N) upon cooling (Saqib *et al.,* 2010). This activation barrier is crucial to the stability and survival of the biomolecules in nature. Once the $Ea_{(D)}$ barrier has been overcome, the enzyme is denatured (I) and cannot refold to the native form. This is known as the irreversible thermal denaturation of the enzyme (Siddiqui *et al.,* 1997; Javed *et al.,* 2009). The $Ea_{(D)}$ was calculated from the lines of best fit from the Arrhenius plots of the rate of thermal denaturation (K_d) versus temperature (Fig. 4) which were 22.00kcalmol^{-1}k^{-1} (92.4KJmol^{-1}K^{-1})and 19.44Kcalmol^{-1}k^{-1} (81.243KJmol^{-1}k^{-1}) (Tables I and II) for α-1 and α-2 respectively. $Ea_{(D)}$ of 2510KJmol^{-1}k^{-1} was obtained from peroxidase from white yam (Eze *et al.,* 2010). $Ea_{(D)}$ of 245.89KJmol^{-1}K^{-1} and 182.92KJmol^{-1}K^{-1} were obtained from the digestive tract of tropical house cricket α-amylase (Kouadio *et al.,* 2013). Duy and Fitter, 2005 reported that the $Ea_{(D)}$ of α-amylase from *Bacillus licheniformis* to be 53.1KJmol^{-1}K^{-1}. The $Ea_{(D)}$ is directly related to another important thermodynamic parameter, the enthalpy of the activation of the denaturation, $\Delta H_{(D)}$. It is the total amount of energy required to bring the enzyme from the ground state to the activated intermediate. In the present study, $\Delta H_{(D)}$ values were found to be decreasing as the temperature increased (Tables I and II). The high values of the change in enthalpy of denaturation obtained for thermoinactivation of the α-amylases from pearl millet indicated that the enzyme would undergo a considerable change in conformation during denaturation (Marin *et al.,* 2003). The fact that ΔH values decreased with increase in temperature reveals that less energy is required to denature the enzyme at high temperature (Bhatti *et al.,* 2006). A similar trend in the values of ΔH was observed in peroxidase from white yam (Eze *et al.,* 2010) though with different magnitude, α-amylase from digestive tract of tropical house cricket (Kouadio *et al,* 2013), α-amylase from sorghum bicolar (Kumar, 2008). As a rule of thumb, the higher the $Ea_{(D)}$, the higher the $\Delta H_{(D)}$. Comparing the $\Delta H_{(D)}$ and $Ea_{(D)}$ of α-1 and α-2 obtained in this work, (Tables I and II), the $Ea_{(D)}$ of α-1 was higher than the $Ea_{(D)}$ of α-2 likewise their mean $\Delta H_{(D)}$ values. This is an indication that α-1 will not easily undergo conformational changes like the α-2.

Although a high enthalpy ($\Delta H_{(D)}$) and activation energy of denaturation ($Ea_{(D)}$) would imply that the enzyme would be thermostable, it is important to look at the other parameters, $\Delta G_{(D)}$ and $\Delta S_{(D)}$ as well. The Gibbs free energy of activation of denaturation $\Delta G_{(D)}$ is in fact a more reliable indicator of stability (Maragoni, 2003). A smaller or negative value of change in Gibbs free energy indicates spontaneity of a reaction, in this case, enzyme thermal denaturation hence

lower stability (Maragoni, 2003; Damodaran, 2006). In the present study, there was increase in $\Delta G_{(D)}$ of both enzyme isotypes (Tables I and II) which is an indication that the enzyme is thermally stable at high temperature. An opposite trend was observed in α-amylase from the digestive tract of tropical house cricket (Kuoadio, 2013). A similar trend was also observed in α-amylase from sorghum bicolar (Kumar, 2008). Comparing α-1 and α-2 in the present study, both isotypes gave equal $\Delta G_{(D)}$ at 40ºC but experienced a change as the temperature increased (Tables I and II). At their optimum temperature (50ºC), their $\Delta G_{(D)}$ was calculated to be 105.50 KJmol^{-1}K^{-1} and 104.33 KJmol^{-1}K^{-1} respectively (tables I and II).   This suggests that α-1 could be more thermally stable than α-2. It is also noteworthy that $\Delta G_{(D)}$ value, which is a measure of the spontaneity of the inactivation process is higher than the $\Delta H_{(D)}$ values in both α-1 and α-2. This is due to the negative entropic contribution during the process (Tanaka and Hoshino, 2002; Pal and Khanum, 2011). The values obtained here are close to the ones obtained from crude endoglucanase from *Aspergillus fumigatus* grown under submerged fermentation (Saqib *et al.,* 2010). This is an indication that the two enzymes may have similar amino acid sequence. However, Eze *et al.* (2010) obtained a negative $\Delta G_{(D)}$ values from peroxidase of white yam showing that the enzyme is less thermostable when compared to the one studied herein. Also the difference could be attributed to the fact that two enzymes might have different primary structure. The Gibbs free energy of a substance results from the stabilizing forces present in a protein structure.

Thermal denaturation of enzymes also results in an increase in entropy. This results in positive entropies of activation of denaturation, $\Delta S_{(D)}$, because the randomness or disorder is increased in the transition from ground state to the unstable intermediate state (U), but α-amylases from pearl millet had negative entropy which revealed that the enzyme was in more ordered state (Tables I and II). The negative values for change in entropy could have some implications which include; compactation of the protein, ordering of water molecules in the vicinity of the hydrophobic residues and possible aggregation during denaturation (Gummadi, 2003; Marin *et al.,* 2003). A similar trend was observed in peroxidase, lipoxygenase and polyphenol oxidase from white yam (Eze *et al.,* 2010) and xylanase from *Aspergillus niger* (Pal and Khanum, 2011) but the magnitude was higher in enzymes from white yam, showing that they undergo more aggregation and compactation during inactivation. The differences observed here could be as a result of change in the conformation of the enzymes studied (Gouda *et al.,* 2003).

In conclusion, it is obvious from the ongoing that the pearl millet ∝ - amylase is thermally stable and also suitable for its application for biotechnological processes but needs to be improved for the maximization of this attribute.

Acknowledgement

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