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Catalytic and Thermodynamic Properties of *Digitaria exilis* β -amylase

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Abstract: β -Amylase (E.C. 3.2.1.2) from Fonio millet Acha *Digitaria exilis* was purified by acid treatment, anion exchange chromatography on DEAE cellulose and gel filtration on Sephadex G-100. The pH and temperature optima were 6.0 and 50°C, respectively using soluble starch as substrate. The enzyme was active within the pH range of 4.0 -10.0 and was most stable between 30 and 50°C. The Km value for soluble starch was 5.892 mg mL⁻¹. The activation energy for catalysis by *Digitaria exilis* β -amylase was 10.49 kJ mol⁻¹. The free energy change (ΔG°), enthalpy change (ΔH°) and entropy change (ΔS°) for inactivation were 53.18, 23.7 and -101.02 kJ mol⁻¹, respectively. The enzyme was activated and stabilized by Na⁺ and Ca²⁺ and inhibited by Hg²⁺ and EDTA. pH dependence of the temperature stability of the enzyme was studied in the temperature range of 40 - 70°C at pH 5.0, 6.0, 7.0 and 8.0. The pH dependent thermal activation was investigated between 25-50°C at 5.0-8.0 pH. At pH 6.0 the activation energy (Ea) for *Digitaria exilis* β -amylase was 10.49 kJ mol⁻¹ while the activation enthalpies (ΔH^\ddagger) and activation entropies (ΔS^\ddagger) were 8.01 and -96.12 J mol⁻¹K⁻¹ at 25°C, respectively. The result showed that thermal stability and thermal activation were pH dependent. *Digitaria exilis* seed β -Amylase was very stable at alkaline pH and neutral pH at higher temperatures of 60 and 70°C but less stable at acidic pH of 5 and 6. Thermodynamic activation parameters derived from thermal activation data suggested the reaction was faster at pH 6. The thermodynamic data also suggest that hydrogen bond might play a role in stabilization of the enzyme at higher temperature in alkaline medium. The enzyme recovered 68% of its original activity following renaturation brought about by the removal of the 4 M guanidine hydrochloride, the denaturing agent.

Key words: *Digitaria exilis*, β -amylase, fonio millet, acha seed, thermal activation, inactivation, thermostability

Introduction

β -Amylase (E.C. 3.2.1.2 α -1,4,-D-glucan maltohydrolase) plays an important role in the biogeochemical cycle of carbon and it also has a wider application in the biotechnological-based pharmaceutical, food and beverage industries in the conversion of starch into maltose solution (Mohapatra *et al.*, 1998). β -Amylase hydrolyses α -1,4-glucosidic linkages of the starch-type substrate in an exo-fashion from the non-reducing end to β -maltose (Visko-Neilsen *et al.*, 1997). Substrates for the enzyme include α - (1,4)-glucans e.g., amylose, amylopectin and limit dextrans with a minimal chain length of four glucose units (Lizotte *et al.*, 1990). Commercial β -amylase may be obtained from plants or microbial sources. Most of the microbial enzymes are not thermostable enough to replace the plant β -amylase (Hyun and Zeikus, 1985).

In plants, β -amylase occurs in free and combined forms (Evans *et al.*, 1997). It has been isolated and studied extensively in different plant species. β -Amylase has previously been purified and characterized from sweet potatoes, soybean, barley, rye and pea (Brena *et al.*, 1996). This

enzyme together with other hydrolytic enzymes are said to be involved in the breakdown of starch during germination of seeds (Guglieminetti *et al.*, 1995) and in the development of embryo in the seed (Silva *et al.*, 1997).

Fonio millet Acha *Digitaria exilis* also known as hungry rice is an annual grass which produces numerous small brownish yellow seeds (Nzelibe and Agbojo, 2005). It is grown as cereal throughout the savannah zone of West Africa. It is grown as complimentary cereal where the annual rainfall exceeds 400 mm. It has an attractive flavour when used as porridge (Hulse *et al.*, 1980). It is an underutilized and understudied cereal (NAQAS, 2001). Fonio millet is used for brewing (Novelle, 1977). Recently, Nzelibe *et al.* (2000) and Nzelibe and Nwasike (1995) studied the brewing potentials and malting characteristics of Fonio millet and reported β -amylase as the major degrading enzyme. It is therefore the objective of this study to isolate, purify and determine some of the catalytic and thermodynamic properties of this enzyme with a view to determining its possible industrial relevance.

Materials and Methods

Materials

Potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, acetic acid, acetone, 3, 5-dinitrosalicylic acid were products of BDH Chemical Limited, Poole, England. Hydrochloric acid, ammonia, soluble starch, potassium sodium tartarate (Rochelle salt), sodium hydroxide, sodium carbonate, copper sulphate, maltose, glycine and TRIS were products of Eagle Scientific, Beeston, Nottingham, England. Ethylene Diamine Tetraacetic Acid (EDTA) and DEAE cellulose were products of Sigma Chemical Company, St. Louis Mo. USA. Other chemicals and reagents used were of analytical grade.

Acha seeds (*Digitaria exilis*) used were obtained from a local market in Jos, Nigeria. The research was carried out in our laboratory at the Federal University of Technology, Akure, Nigeria.

Methods

Enzyme Extraction

Ground seed (70 g) was mixed and stirred in cold 210 mL 0.016 M acetate buffer pH 4.2 for 5 h. The homogenate was filtered using cheesecloth. The filtrate was centrifuged at 4000 g for 10 min with Automatic super speed centrifuge at 4°C. The supernatant was filtered through a loose plug of glass wool to remove the floating lipids. The filtrate was the crude enzyme extract.

Acidification

The pH of the crude extract (150 mL) was slowly adjusted to 3.6 with ice-cold 1 N HCl. The acidified enzyme solution was allowed to stand for 10 min so as to selectively denature the α -amylase present in the extract. The pH was adjusted to 8.0 with 3% ammonium hydroxide solution (Matsui *et al.*, 1977). The resulting solution was called the acid-treated sample and was kept at 4°C for subsequent use.

Purification

Crude extract (acid treated) (25.0 mL) was applied to a column packed with DEAE cellulose (2.5×27 cm) that had previously been equilibrated with 0.016 M Tris-HCl buffer pH 8.0. The unadsorbed proteins were eluted with the same buffer. Fractions of 5.0 mL at a flow rate of 20 mL h⁻¹ were collected. The bound proteins were eluted with a linear gradient of 0-1.0 M NaCl in the same eluting buffer.

The pooled bound β -amylase was dialyzed for 24 h with four changes of the buffer (0.016 M acetate buffer pH 4.2, a total volume of 4 L). This was further purified on a column packed with

Sephadex G-100 (2.5×68 cm) that had previously been equilibrated with 0.016 M acetate buffer pH 4.2. Fractions of 5.0 mL were collected at a flow rate of 18 mL h⁻¹. The fractions containing β -amylase activity were pooled and stored at -5°C. Unless otherwise stated all purifications were carried out at 4°C in the cold laboratory.

Enzyme Assay

β -Amylase activity was measured as described by Oboh and Ajele (1997) using 3,5-dinitrosalicylic acid (DNS) colour reagent. A unit of β -amylase activity was defined as that amount of enzyme, which released one micromole of maltose from starch in a minute at 25°C and pH 6.0. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Determination of Kinetic Parameters

The apparent kinetic parameters: Michaelis-Menten constant, K_m and the maximum velocity, V_{max} and the catalytic constant K_{cat} were determined for the purified β -amylase by determining initial velocity of the reaction at varying concentrations of soluble starch (0-20 mg mL⁻¹). The assay was carried out according to the standard assay procedure. The values of K_m and V_{max} were estimated from the Eadie-Hofstee plot. Activation and inactivation energies and thermodynamic parameters of activation and inactivation were evaluated from the Arrhenius' plot and Eyring's absolute equation derived from transition state theory.

Effect of pH on Enzyme Activity and Stability

Buffers of different pH ranging from 4-10 at a concentration of 0.01 M were prepared using different buffer systems: acetate buffer pH 4 and 5; phosphate buffer, pH 6 and 7; Tris-HCl buffer pH 8-10. Each buffer solution was used to prepare the 1% soluble starch solution used as substrate in assaying the enzyme. The assay was carried out according to the standard assay procedure. The effect of pH on the stability of the enzyme was determined by incubating the enzyme at room temperature for 4 h in the buffer solution of the desired pH. Aliquot (0.5 mL) was withdrawn and assayed for β -amylase residual activity at one-hour intervals.

Effect of Temperature on Enzyme Activity and Stability

Enzyme activity was determined according to the standard assay procedure at different temperatures ranging from 20-80°C. The effect of temperature on the stability of the enzyme was determined by incubating the enzyme solution at various temperatures (20-80°C) without substrate for 1 h. At interval of 10 min, aliquot (0.5 mL) was withdrawn and assayed for β -amylase residual activity.

Effect of Some Salts on the Enzyme Activity

Effect of some salts on the activity of the enzyme was determined at a final concentration of 100 mM in the assay mixture as described by Oboh and Ajele (1997).

Effect of Metallic Chloride on the Half-Life of the β -amylase.

The enzyme was incubated in 0.1 M phosphate buffer pH 6.0 (optimum pH) in the presence of 0.1M of the salt of interest in the assay mixture at 50°C (optimum temperature). Residual activity was measured at 50°C at 10 min interval. The blank contained the buffer solution with the metal ion under consideration. The control consisted of the enzyme and the substrate without any chloride.

The half-life was determined from the relationship:

$$t_{1/2} = \frac{\ln 2}{k_d}$$

where k_d is the deactivation rate constant that can be derived from:

$$v = v_0 e^{-k_d t}$$

where v_0 is the initial enzyme activity while V is the enzyme activity at time t of incubation; and k_d can be obtained from the plot of:

$$\ln v = \ln v_0 - k_d t$$

pH Dependent Temperature Stability of β -amylase

The dependence of the temperature stability on pH was investigated at 40, 50, 60 and 70 °C. The enzyme was diluted 3-fold with the appropriate buffer. An aliquot of the diluted enzyme solution (3.0 mL; 0.96 mg protein) was incubated at the desired temperature for 1 h. Aliquot (0.5 mL) was withdrawn at 15 min interval and assayed for enzyme activity according to the standard assay procedure.

pH Dependent Thermal Activation

β -amylase was assayed as described earlier at various temperatures ranging from 25 to 50 °C at pH 4-8. Values of $\ln V_{max}$ estimated from the Eadie-Hofstee plots, were plotted against the Arrhenius temperatures. Activation energies of catalysis of *Digitaria exilis* β -amylase was calculated from the slope of the above plot at different pH values and thermodynamic data (enthalpy change of activation, entropy change of activation and Gibbs' free energy change of activation) were calculated by rearranging Eyring's absolute equations derived from the transition state theory as described earlier (Eyring and Stearn, 1939)

Denaturation and Renaturation Studies

A denaturation and renaturation experiment was performed according to the methods of Ajele and Afolayan (1992) using 4 M guanidine hydrochloride to denature the enzyme. Renaturation of the enzyme was effected by diluting the unfolded enzyme 25 fold with the renaturation buffer.

Results

Kinetic Parameters

The β -amylase from Fonio millet seed (*Digitaria exilis*) showed typical Michaelis-Menten kinetics for soluble starch. The Michaelis-Menten constant, K_m , estimated from the Eadie-Hofstee plot (Fig. 1) was 5.892 mg mL⁻¹ at 25 °C and pH 6.0. The maximum velocity, V_{max} , also estimated from the Eadie-Hofstee plot was 1.479 μ mol min⁻¹ mL⁻¹.

Thermodynamic Parameters of Heat Activation and Inactivation

From the $V_{max,app}$ data at pH 6.0 as a function of temperature, the Arrhenius energy of activation (ΔE_{ac}) between 20-50 °C and Arrhenius energy of inactivation between 50-80 °C from the slope of $\log V_{max,app}$ against $1/T$ was evaluated as shown in Fig. 2. The thermodynamic parameters for activation and

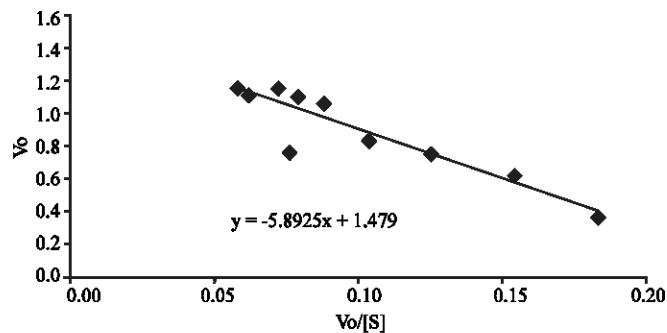


Fig. 1: Eadie-Hofstee plots for the determination of the kinetic parameters (K_m and V_{max})

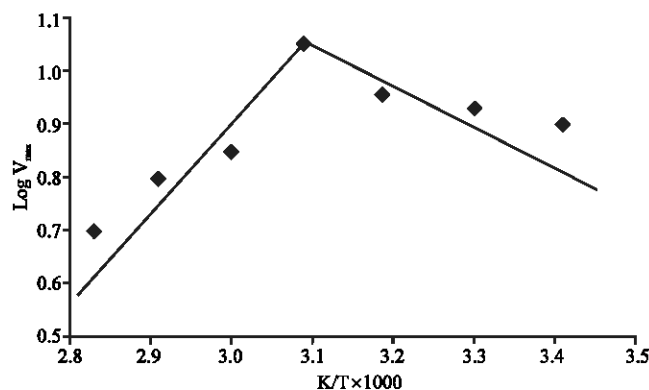


Fig. 2: Arrhenius plot for the determination of activation energy and inactivation energy of catalysis of *Digitaria exilis* β -amylase. The inflexion point corresponds to change in conformation of *Digitaria exilis* β -amylase

inactivation for the catalysis according to Eyring absolute reaction rate theory were determined. The activation energy of catalysis was $10.49 \text{ kJ mol}^{-1}$. The free energy change (ΔG°), enthalpy change (ΔH°) and entropy change (ΔS°) were $36.66 \text{ kJ mol}^{-1}$, $8.013 \text{ kJ mol}^{-1}$ and $-96.12 \text{ J mol}^{-1}\text{K}$, respectively. The inactivation energy of catalysis was $26.17 \text{ kJ mol}^{-1}$. The free energy change (ΔG°), enthalpy change (ΔH°) and entropy change (ΔS°) for inactivation were 53.18 , 23.7 kJ mol^{-1} and $101.02 \text{ J mol}^{-1} \text{ K}$. The kinetics of thermal inactivation was found to be first order within the thermo inactivation temperature range of between $50\text{-}80^\circ\text{C}$.

Effect of pH on the Activity of β -amylase

The effect of pH on the activity of β -amylase is illustrated in Fig. 3a. The optimum activity was obtained at pH 6. The enzyme was active in the pH range of 4-10. An increase in activity was observed as the pH increased to the optimum pH 6. There was a gradual decline in the activity thereafter. The effect of pH on the stability of the enzyme is illustrated in Fig. 3b. The enzyme was stable for 4 h. at the weakly acidic pH 5 and 6 at 25°C . The enzyme was unstable at pH 4 and pH 8 at 25°C .

Effect of Temperature on the Enzyme Activity

The influence of temperature on the enzyme activity is illustrated in Fig. 4a. The enzyme had optimum activity at 50°C . A gradual increase in activity was noticed between $20\text{-}50^\circ\text{C}$, while there

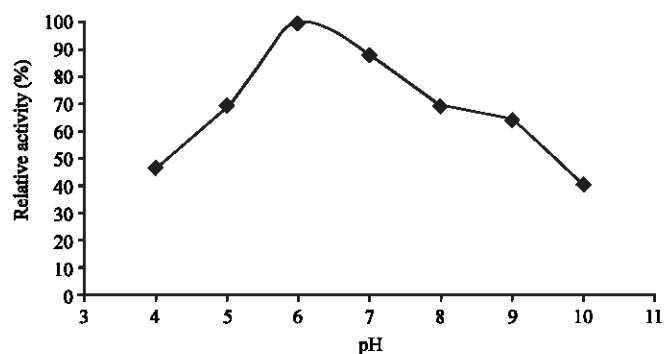


Fig. 3a: Effect of pH on activity of purified *Digitaria exilis* β -amylase

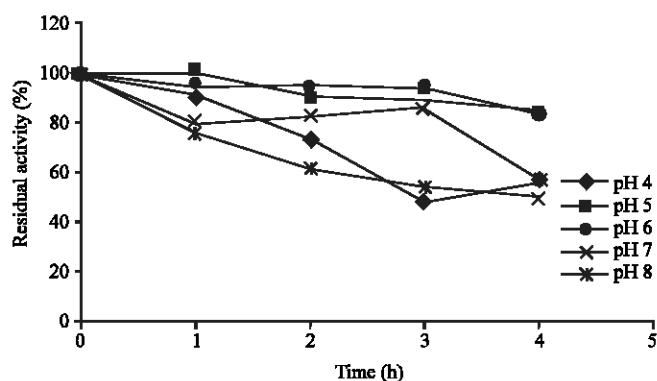


Fig. 3b: Effect of pH on the stability of *Digitaria exilis* β -amylase

was a decline in the activity from above 50°C. The effect of temperature on the stability of β -amylase at pH 6.0 is illustrated in Fig. 4b. The enzyme was very stable for 1 h at 30 and 40°C and fairly stable at 50°C for 1 h. The stability was drastically reduced at 60 and 70°C.

Effect of Salts on the Activity and Half Life of β -amylase

The effect of some salts on the activity of *Digitaria exilis* β -amylase is shown in Table 1. Only the salts of sodium, calcium and potassium had a slight activating effect on the enzyme. Copper sulphate, magnesium sulphate and Zinc sulphate (hydrated) marginally inhibited the enzyme. EDTA also marginally inhibited the enzyme. However, aluminum chloride (hydrated) and mercury chloride strongly inhibited the enzyme. The half-life of the enzyme was slightly increased in the presence of chlorides of sodium, calcium and potassium and decreased by aluminum chloride all at a concentration of 100 mM (Table 2).

pH Dependent Temperature Stability of β -amylase and pH Dependent Thermal Activation

The plot of the percentage residual activity of the enzyme at pH 5-8, against time of incubation at the different temperatures (40, 50, 60 and 70°C) are illustrated in Fig. 5(a-d), respectively. At all the pH examined, the enzyme was very stable at 40°C; however, the percentage residual activity of the enzyme dropped with time at higher temperatures of 50, 60 and 70°C. At pH 5 and pH 6, the drop in the residual relative activity was noticeable at 60 and 70°C compared to less noticeable change at higher pH of 7.0 and 8.0. There was a slight thermal activation of the enzyme in the first 15 min of incubation at pH 5.0 at 50°C and thereafter the relative activity fell below 100%. The enzyme retained

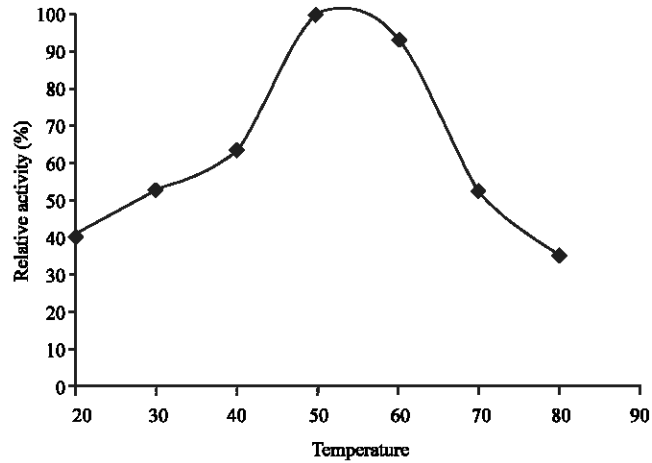


Fig. 4a: Effect of temperature on activity of purified *Digitaria exilis* β-amylase

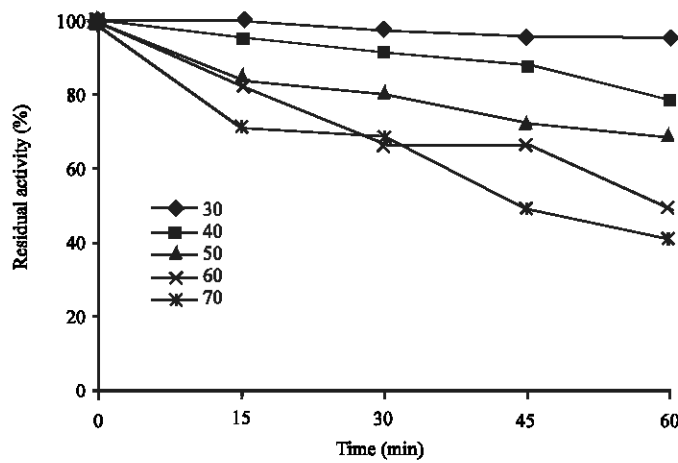


Fig. 4b: Effect of temperature on the stability of *Digitaria exilis* β-amylase at 30, 40, 50, 60 and 70°C

Table 1: Effects of metal ions and some inhibitors on partially purified *Digitaria exilis* β-amylase

Metals/Inhibitors	Conc. (mM)	Relative activity
None	0	100
CuSO ₄	100	68
MgSO ₄	100	82
Na ₂ SO ₄	100	113
ZnSO ₄ ·7H ₂ O	100	73
NaCl	100	125
CaCl ₂	100	121
KCl	100	108
NH ₄ Cl	100	85
AlCl ₃ ·3H ₂ O	100	27
HgCl ₂	100	7
EDTA	100	86
CaCO ₃	100	100
NaNO ₃	100	108

more than half (50%) of its original activity at 60°C after incubation for 1 h at pH 5.0. Less than 50% of the activity was recovered at 70°C after incubation for 45 min. The stability of the enzyme was enhanced in neutral medium (pH 7.0) and alkaline medium (pH 8.0). The enzyme was however less stable in acidic medium (pH 5.0 and 6.0) at higher temperature of 60 and 70°C.

The results of the pH dependent thermal activation thermodynamic parameters are presented in Table 3. At pH 6.0 and 25°C, the activation energy (Ea) for *Digitaria exilis* β-amylase reaction was 10.49 kJ mol⁻¹ while the activation enthalpies (ΔH[#]) and activation entropies (ΔS[#]) were 8.01 kJmol⁻¹ and -96.12 J mol⁻¹K⁻¹, respectively. Activation energy (Ea), enthalpies (ΔH) and entropies (ΔS[#]) were higher at pH 8. Generally, there were less energy requirements for catalysis to optimize product yield at acidic pH of 5 and 6 compared to alkaline pH of 8.0 and neutral pH (7.0). *Digitaria exilis* β-amylase reaction has positive ΔH[#] and ΔG[#] but negative ΔS[#] of activation.

Denaturation and Renaturation Kinetics

The β-amylase completely lost its activity in the presence of 4M-guanidine hydrochloride. The renaturation curve of the denatured enzyme at 15°C and pH 6.0 is presented in Fig. 6. The relative activity obtained at each data point therefore is the activity of the renaturing enzyme expressed as a percentage of the control. At 15°C the recovered enzyme activity as a function of time was hyperbolic. Sixty eight percent of the enzyme activity was recovered after 120 min at 15°C.

Table 2: Effects of Metallic chlorides on half life of *Digitaria exilis* β-amylase

Metallic chloride	Half-life (min)
Control	26.50
Na ⁺	28.44
Ca ²⁺	28.50
NH ₄ ⁺	26.42
Al ³⁺	19.58
K ⁺	27.10

Table 3: Thermodynamics data for thermo-inactivation of *digitaria exilis* β-amylase at various pH

pH	Ea kJ mol ⁻¹	ΔH kJ mol ⁻¹	ΔS J mol ⁻¹ K ⁻¹	ΔG kJ mol ⁻¹
5.0	12.38	9.90	-98.73	35.93
6.0	10.49	8.01	-96.12	36.66
7.0	13.26	10.78	-101.42	35.04
8.0	16.08	13.60	-120.16	32.12

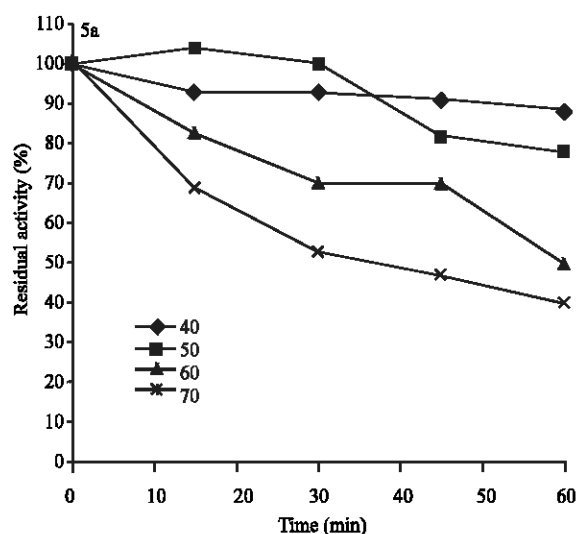


Fig. 5: Continue

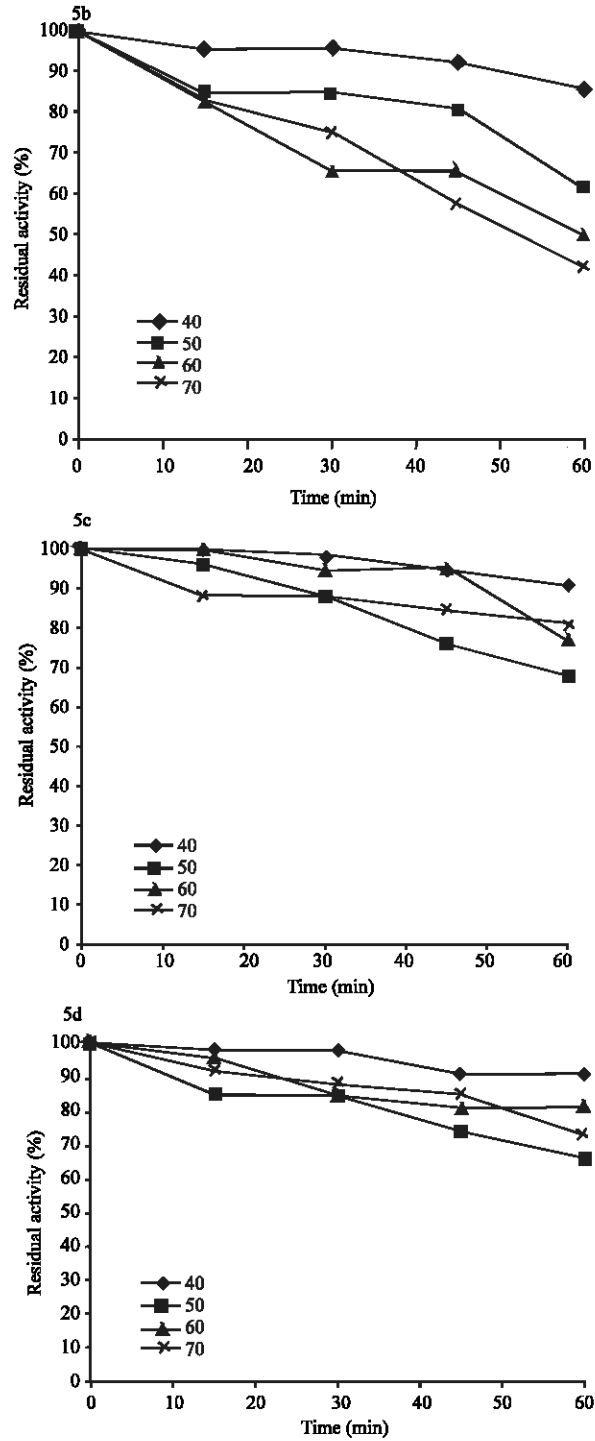


Fig. 5: pH dependence of the temperature stability of *Digitaria exilis* β -amylase. pH dependent study was investigated at pH 5 (a), pH 6 (b), pH 7 (c) and pH 8 (d). At each pH the influence of temperature on the enzyme stability was investigated at 40, 50, 60 and 70°C.

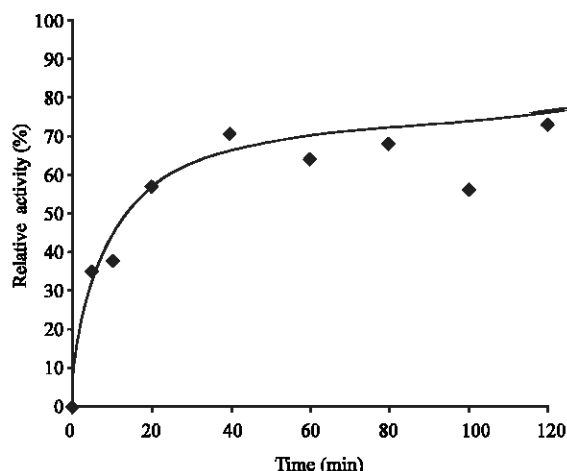


Fig. 6: Renaturation curve of *Digitaria exilis* β -amylase

Discussion

The Michaelis-Menten constant, K_m , value of 5.893 mg mL^{-1} obtained in this report was higher than the value of 3 mg mL^{-1} for the β -amylase from rice reported by Matsui *et al.* (1977) and 1.0 mg mL^{-1} for *Clostridium thermosulfurogenes* (Hyun and Zeikus, 1985). This K_m value reported for Fonio millet β -amylase is distinct and therefore suggests that the enzyme has a lower affinity for the soluble starch substrate and that soluble starch is a poor substrate for the enzyme. This might suggest that the β -amylase from ungerminated Fonio millet is not involved in starch degradation during germination but its physiological role may merely be to improve the seed nutritional quality. It may be a storage protein and may be insignificant for germination (Yamasaki, 2003)

Most commonly described β -amylase lack significant activity at the extreme acidic pH (Eke and Oguntimehin, 1992). The fact that the β -amylase from *Digitaria exilis* was active over a wide pH range may imply that the enzyme will be useful in processes that are subjected to wide pH change from the acidic to alkaline. The optimum pH 6.0 obtained for this enzyme agreed with the general view that most plant β -amylases have their pH optima in the acidic region while microbial β -amylases have theirs in the alkaline region. Some workers have reported different pH optima for β -amylases from various sources. Ajele (1997) reported pH 4.5 for soybean β -amylase, Babu *et al.* (1996) reported pH 4.6-6.0 for sweet potato β -amylase while Matsui *et al.* (1977) obtained an optimum pH of 5.5-6.5 for rice β -amylase while the optimum pH of 6-7 was reported by Okolo *et al.* (2000) for *Aspergillus carbonarium* β -amylase. The stability of the enzyme particularly at the weakly acidic pH had also been demonstrated in other enzymes (Martinez *et al.*, 2002). β -Amylase from *Clostridium thermosulfurogenes* was very stable at the acidic region after 1 h of incubation (Hyun and Zeikus, 1985).

The optimal activity of the enzyme at 50°C is in agreement with what was reported for β -amylase from other sources. The optimal temperature for β -amylase activity from potato leaf was 40°C . This value of 40°C was also reported by Okolo *et al.* (2000) for *Aspergillus carbonarium* β -amylase. Microbial β -amylases such as *Bacillus polymyxa* 26-1 β -amylase had an optimum temperature of 45°C , *Clostridium thermocellum* SS8 β -amylase was optimally active at 60°C (Swamy *et al.*, 1994). *Bacillus circulans* β -amylase was also reported by Ray *et al.* (1996) to have an optimum temperature of 60°C . The values of thermodynamic parameters of thermoinactivation between 50 and 80°C suggest that *Digitaria exilis* β -amylase was substantially stable. The large

negative value of entropy change (ΔS^\ddagger) of activation of catalysis ($-96.12 \text{ J mol}^{-1} \text{ K}^{-1}$) which is almost the same with entropy change (ΔS^\ddagger) of inactivation of catalysis ($-101.02 \text{ J mol}^{-1} \text{ K}^{-1}$) might reflect the high ordering of the enzyme itself during catalysis. This also suggests that potato starch might be a poor substrate for the enzyme and this was reflected in a fairly high K_m value.

The slight effect of sodium and calcium salts on the activity of β -amylase from *Digitaria exilis* is similar to that observed by Matsui *et al.* (1977) for the β -amylase from rice. The fact that EDTA could only slightly inhibit the enzyme is further evidence that the enzyme does not have an absolute requirement for any of the metallic ions for activity. It can be reasoned that the chloride ion might not play any major role in the stabilization because if it did, the degree of stabilization will be expected to be the same for all the salts. An increased half-life in the presence of both Ca^{2+} and Na^+ ions is an indication of enzyme stabilization. This is in agreement with the observation of Pfuellar and Elliott (1969) who reported that Ca^{2+} and Na^+ showed a protective action against thermal inactivation of β -amylase of *Bacillus stearothermophilus* Ca^{2+} being more effective. Some metals not only activate but also stabilize β -amylase. Inhibitory effect of mercury on *Digitaria exilis* β -amylase is in agreement with the report of Okolo *et al.* (2000) that mercury inhibited the activity of β -amylase.

The enhancement of the stability in neutral medium (pH 7.0) and alkaline medium (pH 8.0) as opposed to the observed reduced stability in acidic medium (pH 5.0 and 6.0) at higher temperature of 60 and 70 °C had earlier been observed for the β -amylase from soybeans (Ajele and Sanni, 1998). The index of enzyme stability in alkaline environment at high temperature is the resistance of conformation to exchange of the disulphide bonds and the destruction of cystine residues by β -elimination (Ahern and Manning, 1992). One can therefore suggest that disulphide bonds might play a role in the stabilization of *Digitaria exilis* β -amylase in alkaline medium at high temperatures. Observed activation at pH 5.0 is in consonance with the reported view that most plant β -amylases show enhanced higher activity in acidic medium while the microbial β -amylases have higher activity in the alkaline region (Muraio *et al.*, 1979).

Good thermal stability is necessary to maintain high specific activity of β -amylase due to multistage and long time process involved in the saccharification of starch at temperature at or below 60 °C (Saha and Zeikus, 1989). Thus the high stability of *Digitaria exilis* β -amylase at alkaline pH at high temperatures could imply high industrial utility potential for the production of various high conversion maltose syrups from raw and liquefied starch where high value is placed on the thermostability and thermoactivity of the enzyme because of high temperature operating conditions (Swamy *et al.*, 1994).

The effect of pH on the activation of *Digitaria exilis* β -amylase can be expressed by usual thermodynamic values and may provide clues as to the mechanism involved in the reaction, the maximum possible extent of the reaction and the energy requirements. Thermal activation is pH dependent. Enzymes catalyze their reactions by entropic effect which is given by the negative activation entropies, ΔS^\ddagger (Page and Williams, 1997). The negative entropy effect of catalysis is associated with the restriction of the motion of the reacting fragments or associated with solvation entropies. Reactions involving the creation of charge give rise to negative entropies of activation because of electrostriction of solvent molecules (Page and Williams, 1997). This indicates that large negative entropies of activation of *Digitaria exilis* β -amylase might be due at least in part to the solvation of enzyme and substrate with water molecules and creation of charge is very specific at acidic pH and might not require hydroxyl group. The lowered entropy of activation at pH 6.0 indicated that the reactant (the potato soluble starch substrate) required less ordering in the opening of the enzyme structure at transition state than they would at pH 5-8.

If one accepts Ragone estimates of hydrogen-bond energies (Ragone, 2001), that is, approximately 7.5 kJ mol^{-1} at 298 K, the enthalpy required to disrupt 1 mole of hydrogen bonds, it would permit at least 1 mole of hydrogen bond to be broken at the transition state at pH 5, pH 6 and pH 7 but two at pH 8 during the catalysis by *Digitaria exilis* β -amylase. Hydrogen bond must have been created at alkaline pH that might confer the stability at higher temperature.

The free energy of activation, ΔG^\ddagger , is directly related to the reaction rate and is the sum of those energetic factors that influence the reaction rate (Piszkewicz, 1977). The better the stabilization of the transition state in the enzyme, the smaller is the activation free energy change (ΔG^\ddagger) and consequently a faster reaction rate. The ΔG^\ddagger of *Digitaria exilis* β -amylase catalysis of soluble starch at various pH indicated that the reaction was faster at pH 6.0 compared to pH 8.0 implying that catalysis rate is faster in acidic than in alkaline medium. The low value of ΔG^\ddagger at pH 6.0 is as a result of the sum of the low value of activation entropy (ΔS^\ddagger) and low value of enthalpy of activation (ΔH^\ddagger). The most important part of catalytic effect results from the reduction of ΔG^\ddagger by transition state stabilization.

As proposed by Tombs (1985), an index of enzyme stability apart from maintenance of conformation is the inherent ability of the enzyme to refold after denaturation. The pH value is a crucial factor in oxidative folding; at pH 6 denatured *Digitaria exilis* β -amylase refolded to retain about 70% of the activity of the native enzyme. The yield at this higher pH might be due to faster disulphide-bond formation and/or shuffling. The refolding temperature is an important factor as aggregation, proteolytic degradation and chemical modification are enhanced at higher temperatures (Tombs, 1985). The folding at 15°C was chosen where neither proteolytic degradation nor modification could be detected.

In sum, the kinetic and thermodynamic data generated in this report showed that *Digitaria exilis* β -amylase is less efficient in bioconversion of starch to maltose but still has some industrial application. The thermodynamic activation parameters have given a possible reason for the high stability of *Digitaria exilis* seed β -amylase at high temperature at alkaline pH condition.

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