

1 **Original Research Article**
 2 **“Calcium chloride can influence the**
 3 **stability-flexibility complementarity and**
 4 **consequently, activity of hydrolases: A case**
 5 **study on porcine alpha amylase”.**

6
 7 **ABSTRACT**

Aims: The specific aims of the research were i) to investigate the effects of extra “load” of calcium chloride at different temperatures on the specific activity of porcine alpha amylase (PPA), ii) characterize the effect of extra calcium chloride in terms of thermodynamic and activation parameters, and iii) determine the (un) folding cooperativity (m – value) in the presence of a mixture of ethanol and calcium chloride as well as free energy of folding – unfolding transition.

Study design: Experimental

.Place and Duration of Study: Research Division of Ude Concept International Limited (RC 862217), B. B. Agbor, Delta State, Nigeria and Ambrose Alli University Ekpoma, Nigeria. This study is part of a series of research that lasted for 7 months.

.Methodology: Bernfeld method of enzyme assay was used. Controls were free from additives while the experimental test reaction mixture contained either calcium chloride or a mixture of alcohol and calcium chloride.

Results: The results presented graphically, showed that specific activity enzyme (SA) of the enzyme increased with increasing temperature with and without Ca^{2+} - salt. The rate constant, k_2 and catalytic efficiency, k_2 / K_m were respectively, $79.24 \pm 3.16/\text{min}$ and

1.23±0.10L/g.min at 318.15K. The activation parameters, free energy ($\Delta G^\ddagger$), enthalpy ($\Delta H^\ddagger$), and entropy ($\Delta S^\ddagger$) for Ca^{2+} - salt treated enzyme were $77.36 \pm 0.11\text{kJ/mol}$, $54.95 \pm 0.96\text{kJ/mol}$, and $-68.39 \pm 3.03\text{J/mol.K}$ respectively at 318.15K. While for Ca^{2+} - salt free enzyme, the $\Delta G^\ddagger$, $\Delta H^\ddagger$, and $\Delta S^\ddagger$, were $78.93 \pm 0.01\text{kJ/mol}$, $43.27 \pm 1.25\text{kJ/mol}$, and $-112.09 \pm 3.94\text{J/mol.K}$ respectively. The enthalpy (ΔH) of enzyme substrate (ES) formation were -41.10 and -24.66 kJ/mol for Ca^{2+} - salt treated and Ca^{2+} - salt free enzyme. Except treatment with local gin, LG, the m – values were positive in sign in the presence of Ca^{2+} - salt with other sources of alcohol. In the same vein all free energy values for folding – unfolding transition at infinite dilution of the Ca^{2+} - salt were negative for all alcohol except with LG.

Conclusion: The presence of extra Ca^{2+} - salt can increase the specific activity, rate constant and catalytic efficiency of the enzyme. The presence of Ca^{2+} - salt rigidified the enzyme; lower $\Delta G^\ddagger$ for Ca^{2+} - salt treated enzyme presupposed that the chloride component might have promoted conformational flexibility of the active site of the enzyme. The ground state of Ca^{2+} -salt treated enzyme is less flexible than Ca^{2+} -salt free enzyme (lower magnitude of negative $\Delta S^\ddagger$ for Ca^{2+} -salt treated than Ca^{2+} -salt free enzyme). The hydrolysis of starch is enthalpically driven in the presence of Ca^{2+} -salt, while entropic factor is the case in the absence of the salt. The positive m – values implied that Ca^{2+} - salt can oppose the effect of a chaotrope like ethanol on the enzyme unlike in its absence as indicated by the negative sign of folding – unfolding free energy.

8

9 *Keywords: Porcine alpha amylase; calcium chloride; ethanol; temperature; kinetic*
10 *parameters; thermodynamic parameters; m-values.*

11 1. INTRODUCTION

12 Porcine alpha amylases (α – 1, 4 – glucan – 4 – glucano – hydrolase E.C. 3.2.1.1)
13 like any other homologue, catalyze the hydrolysis of the internal α - 1, 4 – glucosidic bond in

14 starch and glycogen [1, 2]. Glucano - hydrolases are very important because they are
 15 involved in biological reactions such as fermentation, germination or digestion and they are
 16 widely used in some industries for the production of glucose syrups, enhanced bread
 17 production by acting as anti-staling agents in baking or in detergents to remove starch based
 18 stains [3, 4]. The human homologue of porcine alpha amylase (PPA), human salivary alpha
 19 amylase has diagnostic applications in the determination of the state of the nervous system,
 20 cognate mental state, and psychological state of individuals [5 – 7].

21 Since native starch is water insoluble at room temperature (though the so-called
 22 soluble potato starch constitute a mere suspension in buffer and distilled water), many
 23 applications of amylases are carried out at high temperature and pressure where the starch
 24 is gelatinized. During gelatinization, the granular architecture and the molecular order
 25 (double helices) of the starch granule are disrupted [4] yielding different degree of
 26 gelatinization. This has important implication for the rate of enzymatic amylolysis of starch
 27 both for human and nonhuman animals. Most natural sources of starch are consumed in
 28 partially processed state, moderately gelatinized or outrightly in raw state. Boiled fresh maize
 29 cobs, cassava, yam, bread from wheat flour *etc* are examples.

30 While man needs food sources in processed form or much better in semi –
 31 processed form but this is not without cost in time, energy, and capital in general.
 32 Nonetheless, livestock is raised for human consumption, for growth, repair of body tissue,
 33 immune and hormone amplification and sustenance; livestock consume, when trained in
 34 particular, processed and natural sources of food, plants in particular, in totally raw state.
 35 Livestock feeds in processed state can raise the cost of animal production. Attempts have
 36 been made to improve animal productivity through various means ranging from welfare
 37 issues [8], application of exogenous enzymes, otherwise referred to as enzyme
 38 supplementation [9 – 11], and application of prebiotics, synbiotics, and probiotics in
 39 particular to the final formulation of animal feeds [12]. But keeping animal feeds at certain
 40 biologically tolerable temperature can enhance the digestibility of carbohydrate rich sources.

41 However, be it biotics, endogenous or exogenous enzyme supplements, the right
 42 thermal environment is essential for optimization of the very purpose of overall animal
 43 husbandry, poultry *etc* and ultimately human wellbeing without exception. This is against the
 44 backdrop of the fact that alpha amylase homologues belong to different classifications,
 45 psychrophiles, mesophiles, and thermophiles according to their the degree of dependence
 46 on the thermal environment which may be low, moderate and high as have been
 47 investigated in the past [13 – 16]. However, there is a school of taught that suggest that
 48 digestion of starch in the rumen is essential to maximum utilization of feed grains by the
 49 ruminant [17]. This contrary to the claim that in grain-fed animals, protozoa can exert an
 50 influence on ruminal starch hydrolysis rates in at least two respects namely ingestion of
 51 bacteria in numbers sufficient to decrease ruminal fermentation rates [18, 19], and ingestion
 52 of starch granules and soluble sugars, with the result that the substrates needed for
 53 fermentation by the fast growing bacteria is diminished [20].

54 Also research report has shown that protozoans reduce the rate of starch digestion
 55 and ruminal starch digestibility, shifting the site of starch digestion to the small intestine [21].
 56 All these observations point to the fact that digestion in the small intestine may preferably be
 57 more useful in the supply of fuel molecule than in the rumen. Incidentally pig may not be a
 58 ruminant but porcine alpha amylase has mesophilic attributes and it is one of such
 59 homologues that can fairly represent other homologues in mammal and birds. However,
 60 since ruminant mammals, raised for meat and poultry are indispensable sources of meat, the
 61 ruminants in particular need to be considered with respect to the ruminant state contribution
 62 to the nutritional status of the animals. Important as this contribution might be, volatile free
 63 fatty acids (VFAs) as products of rumen bacterial fermentation must have been preceded by
 64 the formation of alcohol of diverse chain length, aldehydes, and organic acids. These
 65 compounds, organic acid in particular can reduce the *pH* of the lumen to very high acidic
 66 levels (acidosis) with damaging consequences [22]. Therefore, it is imperative to ensure that
 67 starch in particular is exposed to the right condition in the small intestine of all live stock so

68 that much digestion could have taken place in that chamber before microbial activity
69 assumes prominence in the rumen.

70 To this end therefore, and against the backdrop of the practice whereby hydrolases
71 including alpha amylases are used for the pretreatment of starch of animal feeds to improve
72 the digestibility of fibre and starch in particular [3], the objectives of this research were i) to
73 investigate the effects of extra “load” of calcium chloride at different temperatures on the
74 specific activity of porcine alpha amylase (PPA), ii) characterize the effect of extra calcium
75 chloride in terms of thermodynamic and activation parameters, and iii) determine the folding
76 – unfolding cooperativity (m – value) in the presence of a mixture of ethanol and calcium
77 chloride.

78 **2. MATERIALS AND METHODS**

79 **2.1 Materials**

80 The chemicals: Porcine pancreatic alpha amylase (PPA) was purchased from Sigma, Aldrich,
81 USA; soluble potato starch was purchased from Sigma Chemicals Co, USA; dinitrosalicylic
82 acid (DSA) was purchased from Lab Tech Chemicals, India; 3, 5 – Sodium potassium tartrate
83 tetrahydrate was purchased from Kermel, China; Local gin (LG) was from local market;
84 branded gin (BG) (Lord’s dry gin) was from Nigerian Distilleries Ltd, Sango Otta, Ogun State,
85 Nigeria. Hydrochloric acid, ethanol, sodium hydroxide, and sodium chloride were purchased
86 from BDH Chemical Ltd, Poole England; Tris was from Kiran Light Laboratories, USA; calcium
87 chloride was from Lab Tech Chemicals, India; other chemicals were of analytical grade and
88 solutions were made in distilled water.

89 Equipment: pH meter (tester) was from Hanna Instruments, Mauritius; electronic weighing
90 machine was from Wensar Weighing Scale Ltd, Chennai; Centrifuge, 800D model was from
91 China; 721/722 visible spectrophotometer was from Spectrum Instruments Co Ltd, China.

92 **2.2. Methods**

93 A solution of enzyme was prepared by dissolving 0.01g in 20 mL tris – $\text{HCl}_{(\text{aq})}$ buffer
94 as stock at pH 7.4 to deliberately conform with biological human pH by choice as was the

case in previous research elsewhere [23]. The choice of concentration was at my discretion so as to enhance the detection of the occurrence of activity of the enzyme which has lower activity with raw starch [23]. The preparation of substrate, raw starch suspension, in buffer was as described in earlier research [23].

2.2.1 Assay of porcine pancreatic alpha amylase and determination of kinetic parameters

The control reaction mixtures were without co – solute. An *in vitro* assay of alpha-amylase was according to Bernfeld method [24]. The holoenzyme, porcine alpha amylase (PPA), was assayed for a short duration of 5 minutes and the final activity is obtained according to Beer – Lambert law as follows: corrected absorbance at wavelength, 540nm, $\times$ dilution factor (*i.e.* $3/\epsilon l$), where ϵ ($181.1\text{M}^{-1}\text{cm}^{-1}$), C , and l are molar absorption coefficient, molar concentration of product, and path length respectively. An assay of the enzyme was done with and without calcium chloride in a total reaction mixture of 3 millimetre (mL) composed of 1 mL of substrate (raw soluble potato starch), 1 mL of enzyme, 0.5 mL of calcium chloride and 0.5 mL of distilled water (or 1 mL of distilled water where calcium chloride is not included in the reaction mixture). Also, assay of the enzyme was carried out in a reaction mixture composed of 1 mL enzyme, 1 mL of substrate, 0.5 mL of ethanol, and 0.5 mL of calcium chloride. The duration of assay is 5minutes. Reaction mixture in the test tube was swirled to ensure mixing. Measurement of absorbance was taken after 5 minutes of centrifugation at 3000rpm (or at $1343 \times g$) using ordinary laboratory centrifuge (model 800D). Centrifugation was needed to prevent interference with spectrophotometric transmittance that could otherwise yield high absorbance that can best be described as an artifact. The increase or decrease in velocity of hydrolysis of raw starch due to the presence of additive of whatever kind, stabilizing or destabilizing as the case may be, is reported as percentage of control as follows: $100(v_c - v_{c=0})/v_{c=0}$, where, $v_{c=0}$ and v_c are the velocities of hydrolysis of raw starch in the absence of any additive tested and in the presence of additive tested respectively.

The determination of Michaelis – Menten constant (K_m) and maximum velocity (V_{max}) of hydrolysis of raw starch was according to Lineweaver – Burk method [25]. “The initial apparent unit of activity is M/mL.min (number moles of reducing sugar yielded per litre (L) of substrate per mL of enzyme per minute). Since 1 mL of substrate was hydrolyzed, the number of moles of reducing sugar yielded per minute in 1mL using 1 mL of enzyme and maltose as standard is xmmol/mL.min. Therefore, 1U = micromoles maltose released/mL enzyme in the reaction mixture/ 5 min” [23].

2.2.2. The determination of activation parameters

Two approaches were adopted for the determination of activation parameters as in past research [23]. One of the approaches entailed the use of rate constant when the enzyme is assayed in the absence and presence of 4mM calcium chloride at absolute temperature ranging from 298.15 to 333.15K and in order to give room for wider investigation within the limited time a method referred to as Eyring – Polanyi method was used to determine enthalpy of activation, entropy of activation, and activation energy as described elsewhere [26] and adopted in previous research [23]. The activation energy (E_a), enthalpy of activation ($\Delta H^\ddagger$), entropy of activation ($\Delta S^\ddagger$), and free energy of activation ($\Delta G^\ddagger$) were respectively calculated according to the following equations using rate constant k_2 ($k_2 = V_{max}/[E_T]$ where V_{max} and $[E_T]$ are maximum velocity of hydrolysis of raw starch and total concentration of the enzyme):

$$\ln k_2 = \ln A - E_a/RT. \quad (1)$$

where A , R , and T are pre – exponential factor, gas constant, and absolute temperature respectively.

$$\Delta H^\ddagger = E_a - RT \quad (2)$$

$$\Delta S^\ddagger = \Delta G^\ddagger - \Delta H^\ddagger \quad (3)$$

$$\Delta G^\ddagger = \ln (k_B T/hk_2) \quad (4)$$

where h and k_B are the Planck's constant and Boltzmann constant respectively.

The second approach is as described in the work of EL – Hefnawy *et al.* [26].

149 Since the molar mass of soluble starch is unknown, the difference between free energy in
150 the presence and in the absence of the salt was determined.

$$151 \quad \Delta(\Delta G) = -RT \ln (K_{m(c)}/K_{m(c=0)}) \quad (5)$$

152 where ΔG is the free energy for the hydrolysis of the soluble starch, subscripts (c) and (c = 0)
153 denote in the presence and absence of calcium chloride respectively. For the same reason,
154 the difference between the entropy in the presence and in the absence of the salt was
155 determined.

$$156 \quad \Delta(\Delta S) = -(\Delta(\Delta G) - \Delta(\Delta H))/T \quad (6)$$

157 where ΔS and ΔH are the entropy and enthalpy of hydrolysis of starch respectively.

158 **2.2.3 Determination of Gibbs free energy of unfolding in the absence of protecting** 159 **additive and m – values**

160 The determination of m – values (the slope of the protein folding stability with
161 osmolyte concentration [27]) is determined by plotting free energy of protein unfolding (ΔG^0)
162 against co – solute concentration. Thus according to Rösigen *et al.* [27], m – value for the
163 protecting osmolyte/additive (or a kosmotrope) is positive while the m – value for
164 destabilizing osmolyte (or a chaotrope) is negative. The equation linking ΔG^0 and m – values
165 are presented in two ways. One approach is in terms of negative slope [28 – 30]:

$$166 \quad \Delta G^0 = \Delta G^0_{(N \rightarrow D)} - m[\text{co – solute}] \quad (7)$$

167 The other approach is in terms of positive slope [31, 32]:

$$168 \quad \Delta G^0 = \Delta G^0_{(N \rightarrow D)} + m [\text{co – solute}] \quad (8)$$

169 where $\Delta G^0_{(N \rightarrow D)}$ is the Gibbs free energy of unfolding, native to denatured state transition
170 (N→D) in the absence of protecting co – solute/additive. Any of the equations chosen is
171 informed by reasons advanced elsewhere [23].

172 Meanwhile on the basis of two – state model,

$$173 \quad \Delta G^0 = -RT \ln U/1 - U \quad (9)$$

174 where U and $1 - U$ are the fractions of unfolded protein and fraction of native or folded
175 protein respectively and $U/(1-U)$ is the equilibrium constant (K_{eq}). The basis of Eq. (9) and
176 determination of U is as explained in literature [23].

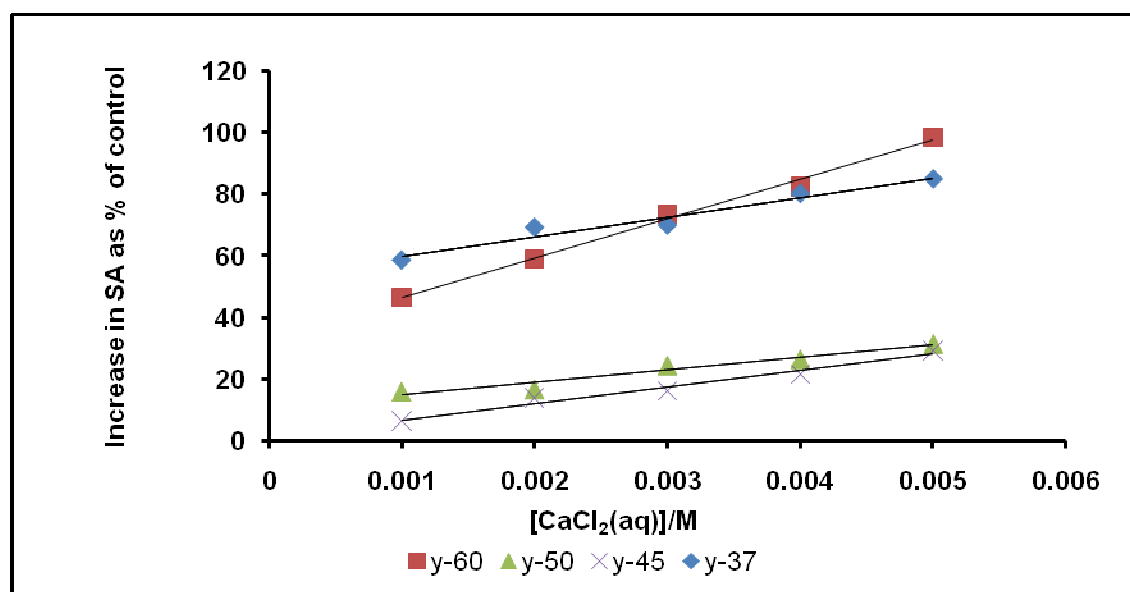
177 **2.3 Statistical analysis**

178 Values of parameters from duplicate assays are expressed as mean $\pm$ SD. Microsoft
179 Excel was used to calculate standard deviation (SD).

180 **3. RESULTS**

181 Several assays of the enzyme were carried out at different temperatures ranging
182 from 37 – 60°C in the absence and presence of calcium chloride in the reaction mixture. The
183 results of assays were presented graphically as shown in Fig. 1 in which the observed
184 increase in activities (expressed as a percentage of control) with increasing molar
185 concentration of calcium chloride at different temperatures were plotted versus molar
186 concentration of calcium chloride. Figure 1 clearly shows that the presence of calcium
187 chloride, hereinafter referred to as Ca^{2+} -salt at different temperature had variable influence
188 on the velocity of hydrolysis (activity) of soluble raw potato starch. In the first place, there
189 was a strong correlation between increasing trend in activity and increasing molar
190 concentration of Ca^{2+} -salt as can be attested to by high coefficient of determination R^2
191 ranging from 0.950 – 0.995 which if translated gives correlation coefficient (R) values
192 ranging from 0.975 – 0.997. The variation of the increase in activity with $[Ca^{2+}\text{-salt}]$,
193 $\partial v/\partial[Ca^{2+}\text{-salt}]$, the slope, shown under Fig. 1 at different temperatures is in the order:
194 60>37>45>50°C. Besides the value of slope at 60°C is ~ 2 times the value at 37°C. The
195 least value at 50°C has implication and demands interpretation. Nonetheless the point that
196 must be made is that the presence of Ca^{2+} -salt influenced the activity of the enzyme.

197



198

199 **Fig. 1: Percentage increase in specific activity versus molar concentration of calcium**
 200 **chloride at different temperatures.** *The slopes which are 12768, 6351, 5413, and 4067 at*
 201 *60, 37, 45, and 50°C respectively may have some “physico-biochemical” implication. SA*
 202 *stands for specific activity of the enzyme. ■, ▲, x, and ◆ stand for assays at 60, 50, 45, and*
 203 *37°C respectively.*

Figure 2 presents result in terms of increasing trend in specific activity (SA) as percentage of control versus temperature. The result in graphical format shows diverse features, rising increment in SA with increasing temperature but with sudden decrease between 45°C (318.15K) and 50°C (323.15K) in the presence and absence of calcium chloride. However, increment in activity expressed as percentage of control (100% activity without additive being tested) increased between 50°C (323.15K) and 60°C (333.15K) in the presence unlike in the absence of varying concentration of Ca^{2+} -salt. This observation once again, has “physico-biochemical” implication that deserves interpretation.

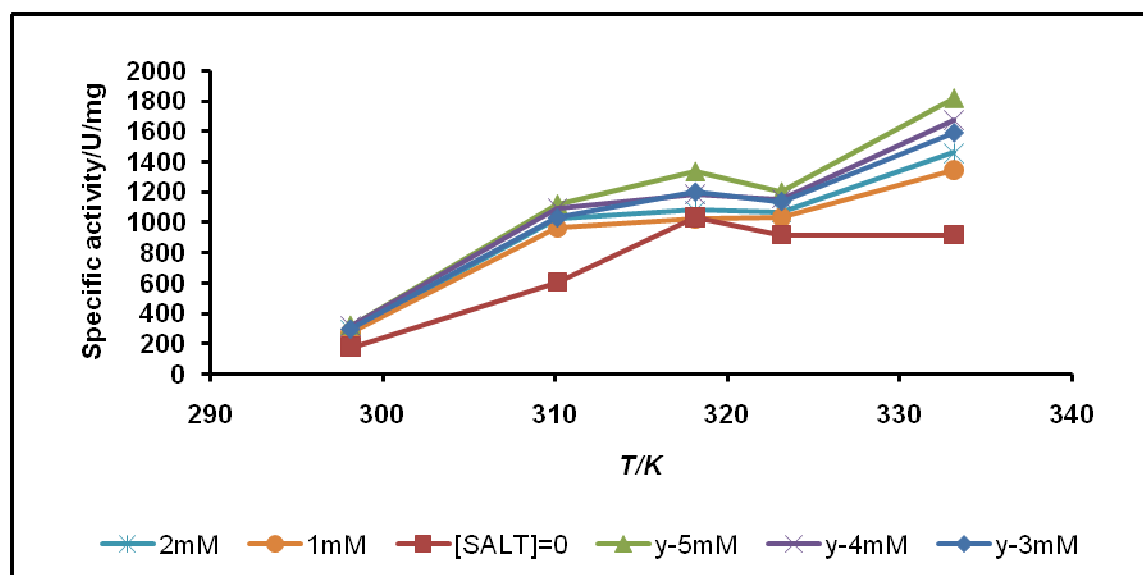
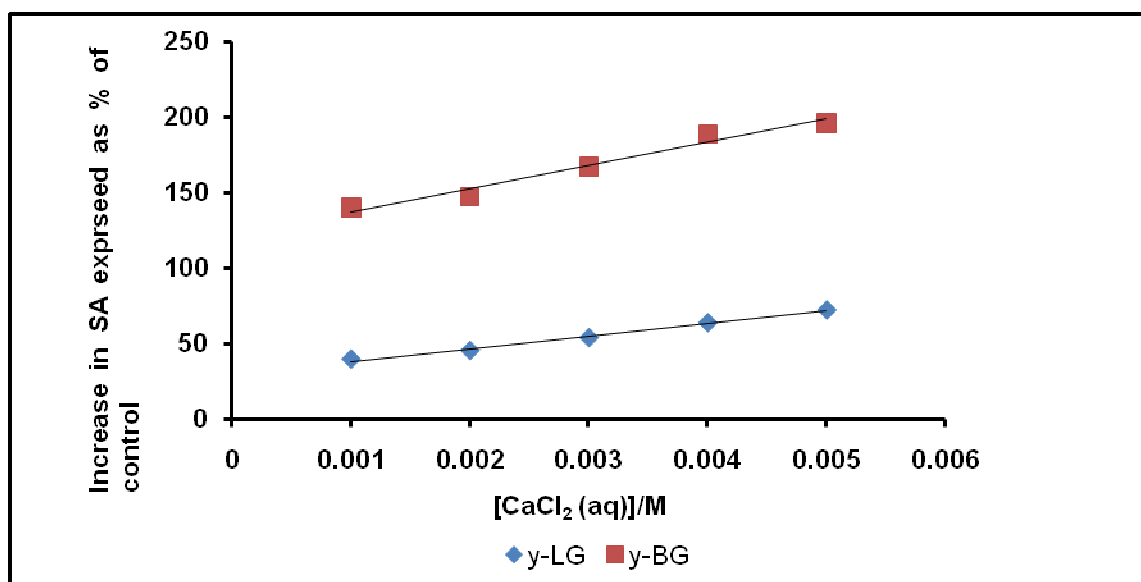


Fig. 2: Trend in specific activity of porcine alpha amylase with increasing temperature

at different concentration of calcium chloride. *, ●, ■, ▲, x, and ◆ stand for assays at different temperatures in the presence of 2mM, 1mM, zero concentration, 5mM, 4mM, and 3mM aqueous solution of calcium chloride respectively.

As shown in Fig. 3, the increase in activity as percentage of SA in control reaction mixture containing only alcohol without Ca^{2+} -salt, exhibited increasing trend with increasing molar concentration Ca^{2+} -salt. With branded gin (Lord's dry gin), the slope of such plot was 15372 ($R^2 = 0.971$) while with local gin, known as *kin kin* or *ogogoro*, the slope was 8317 (R^2

222 = 0.992). These values once again have important physico – biochemical implication that
 223 demands interpretation. The independent variable Ca^{2+} -salt clearly influenced the SA of
 224 alcohol treated enzyme. Also to be observed is the fact that the native PPA had velocity of
 225 hydrolysis which was higher than the value reported for LG and BG treated PPA as shown
 226 below Fig. 3. The velocity for BG treated PPA was higher than for LG treated PPA.



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228

229 **Fig. 3: Variation of increase in specific activity (SA) expressed as percentage of**
 230 **control without Ca^{2+} -salt versus molar concentration of Ca^{2+} -salt.** *The mean control*
 231 *velocity of hydrolysis ($n = 2$) for LG treated PPA without Ca^{2+} -salt is reported as mean $\pm$ SD:*
 232 *(117.60 $\pm$ 2.33mU/mL); for BG treated PPA without Ca^{2+} -salt the value is:*
 233 *132.50 $\pm$ 23.4mU/mL. The value for PPA free from any additive, i.e. as native enzyme, is*
 234 *303.54 $\pm$ 1.56mU/mL. The control reaction mixture contains only alcohol. The slope in the*
 235 *presence of branded gin (Lord's gin) is 15372 with $R^2 = 0.971$ and in the presence of local*
 236 *gin, it is 8317 with $R^2 = 0.992$. The symbols $\blacklozenge$ and $\blacksquare$ stand for assay in the presence of local*
 237 *gin (LG) and Branded gin (BG) – Lord's dry gin respectively.*

238 As may be observed in Table 1, there was increasing trend in the values of K_m and
 239 k_2 with increasing temperature in the presence and absence of 4mM Ca^{2+} -salt. The catalytic

240 efficiency, k_2/K_m exhibited increasing trend on a regular basis in the absence of Ca^{2+} -salt
 241 unlike the irregular trend observed in the presence of Ca^{2+} -salt. The k_2 and k_2/K_m values for
 242 Ca^{2+} -salt treated enzyme, PAA, were higher than values for Ca^{2+} -salt free PPA. Except the
 243 value of K_m at 310.15K for Ca^{2+} -salt treated PPA, the values of K_m for Ca^{2+} -salt free PAA
 244 were lower than values for Ca^{2+} -salt treated enzyme.

245 **Table 1. Kinetic parameters for the hydrolysis of raw starch with porcine pancreatic**
 246 **alpha amylase**

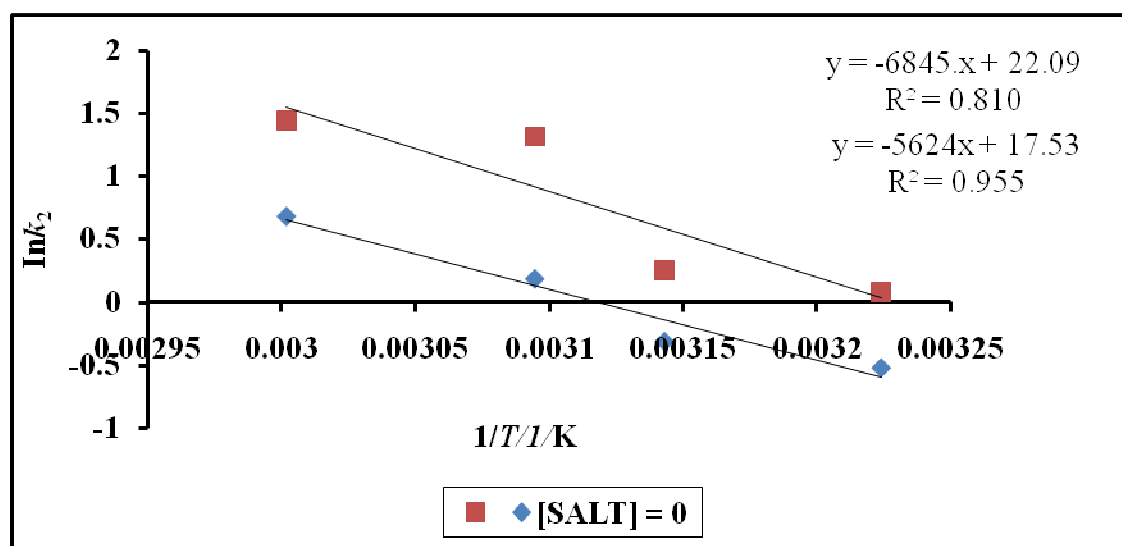
T (K)	[CaCl ₂ (aq)] = zero			[CaCl ₂ (aq)] = 4mM		
	K_M (g/l)	k_2 (min ⁻¹)	k_2/K_M (lg ⁻¹ min ⁻¹)	K_M (g/l)	k_2 (min ⁻¹)	k_2/K_M (lg ⁻¹ min ⁻¹)
310.15	42.34±1.01	36.18±5.26	0.85±0.03	31.76±0.00	64.58±0.00	2.03±0.00
318.15	48.54±3.59	43.85±0.28	0.90±0.10	64.18±1.27	79.24±3.16	1.23±0.10
323.15	69.15±2.89	74.32±3.25	1.07±0.10	72.69±0.00	222.88±0.00	3.07±0.00
333.15	80.52±0.68	113.51±3.13	1.39±0.04	98.95±0.07	259.75±9.31	2.63±0.09

247 *Values are recorded as Mean±SD where n = 2. The molar concentration of the enzyme PPA*
 248 *[E_T] = 9.26μM (mass conc. = 0.5g/L and molar mass = 54kg/mol).*

249 In order to determine activation parameters for the hydrolysis of raw starch, in the
 250 presence of Ca^{2+} -salt, plots of $\ln k_2$ versus $1/T$ (Arrhenius plot)(Fig. 4) were carried out
 251 respectively for the determination of activation energy and enthalpy of hydrolysis of the
 252 starch. For the purpose of control, plots of $\ln k_2$ versus $1/T$ (Fig. 4) were carried out
 253 respectively for the determination of activation energy and enthalpy of hydrolysis of the
 254 starch for Ca^{2+} -salt free enzyme. The negative slope was in clear conformity with Arrhenius
 255 theory. The slope, $\partial \ln k_2 / \partial 1/T$ for Ca^{2+} -salt treated enzyme is higher than for Ca^{2+} -salt free
 256 enzyme (Fig. 4). However, the coefficient of determination (R^2) for Ca^{2+} -salt free enzyme
 257 was higher than Ca^{2+} -salt treated enzyme.

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261 **Fig. 4: Arrhenius plot for the determination of activation energy for the**
 262 **hydrolysis of starch in the absence and presence of calcium chloride. The symbols ◆**
 263 **and ■ stand for assay in the absence and presence of calcium chloride respectively.**

264 Data obtained from plots of $\ln(v/T)$ and $\ln v$ versus $1/T$ were plotted versus molar
 265 concentration of Ca^{2+} -salt. The data from such plots, for $\Delta G^\ddagger$, E_a , and $\Delta H^\ddagger$ were plotted
 266 versus molar concentration of Ca^{2+} -salt (Fig. 5a). Also, data for $\Delta S^\ddagger$ were plotted versus
 267 molar concentration of Ca^{2+} -salt (Fig. 5b). Finally the plot of $\ln(1/K_m)$ versus $1/T$ (van't Hoff
 268 plot) is shown in Fig. 6. The activation energy for Ca^{2+} -salt treated PPA was higher than
 269 values for Ca^{2+} -salt free PAA as shown under Table 2. As shown in Table 2 the loss of
 270 entropy, negative $\Delta S^\ddagger$, for Ca^{2+} -salt treated enzyme < values for Ca^{2+} -salt free enzyme. The
 271 same was applicable to free energy of activation in which values for Ca^{2+} -salt treated
 272 enzyme < values for Ca^{2+} -salt free PPA. However, values of enthalpy of activation, $\Delta H^\ddagger$ for
 273 Ca^{2+} -salt treated PPA was higher than values for Ca^{2+} -salt free PPA. Both Ca^{2+} -salt treated
 274 and Ca^{2+} -salt free PPA exhibited the same trend with increasing temperature in their values
 275 of activation parameters viz: decreasing trend in $\Delta H^\ddagger$ (except departure at 318.15K)

276 increasing trend in $\Delta S^\ddagger$ and $\Delta G^\ddagger$ (Table 2). Nonetheless, there was a departure from regular
277 trend in the values of $\Delta S^\ddagger$ and $\Delta G^\ddagger$ for Ca^{2+} -salt treated PPA at 323.15K.

278 **Table 2. Thermodynamic activation parameters for the hydrolysis of raw starch with**
279 **porcine pancreatic alpha amylase.**

T (K)	[CaCl ₂ (aq)] = zero			[CaCl ₂ (aq)] = 4mM		
	$\Delta S^\ddagger$ (Jmol ⁻¹ K ⁻¹)	$\Delta H^\ddagger$ (kJmol ⁻¹)	$\Delta G^\ddagger$ (kJmol ⁻¹)	$\Delta S^\ddagger$ (Jmol ⁻¹ K ⁻¹)	$\Delta H^\ddagger$ (kJmol ⁻¹)	$\Delta G^\ddagger$ (kJmol ⁻¹)
310.15	-109.74±4.04	43.34±1.25	77.37±0.06	-67.24±3.13	55.02±0.97	75.87±0.00
318.15	-112.09±3.94	43.27±1.25	78.93±0.01	-68.39±3.03	54.95±0.96	77.36±0.11
323.15	-112.73±7.66	42.36±2.47	78.79±0.11	-64.59±7.25	54.91±0.97	75.84±0.00
333.15	-114.10±8.07	42.13±2.69	80.14±0.16	-68.52±2.91	54.83±0.97	77.84±0.10

280 *Activation energy (Ea): Ea values in the presence of 4mM CaCl₂ (aq) and in the absence of*
281 *CaCl₂(aq) were 57.59±0.96kJ/mol and 45.05±1.25kJ/mol; values were recorded as*
282 *mean±SD where n = 2*

283 In order to have a broad coverage and insight, the effect of temperature at different
284 molar concentration of Ca^{2+} -salt was investigated according to method adopted by EL –
285 Hefnawy *et al* [26] for the determination of E_a , $\Delta S^\ddagger$, $\Delta H^\ddagger$, and $\Delta G^\ddagger$. However, the values of
286 the parameters were plotted versus molar concentration of Ca^{2+} -salt so as to establish the
287 type of correlation between the activation parameters and Ca^{2+} -salt. With respect to $\Delta H^\ddagger$ and
288 E_a (Fig.5a), and $\Delta S^\ddagger$ (Fig. 5b), the regression coefficients, the slopes, were positive pointing
289 to the fact that there must have been a positive correlation between the activation
290 parameters and Ca^{2+} -salt as evidenced with moderately high coefficient of determination R^2
291 which translated to correlation coefficient R gave respectively, 0.924, 0.922, and 0.945. On
292 the other hand $\Delta G^\ddagger$ showed negative slope (Fig. 5a) pointing to negative correlation with
293 Ca^{2+} -salt given that R^2 translated to R gave high value equal to ~ 0.960. From the slope it
294 can be seen that the magnitude of the entropic term $\partial\Delta S^\ddagger/\partial[\text{Ca}^{2+}\text{-salt}]$ was very high (and

much higher than other parameters if $\partial \Delta S^\# / \partial [\text{Ca}^{2+}\text{-salt}]$ is multiplied by T) (Fig. 5b)
 compared with $\partial \Delta H^\# / \partial [\text{Ca}^{2+}\text{-salt}] \times 10^3$, $\partial \Delta G^\# / \partial [\text{Ca}^{2+}\text{-salt}] \times 10^3$, and $\partial E_a / \partial [\text{Ca}^{2+}\text{-salt}] \times 10^3$.

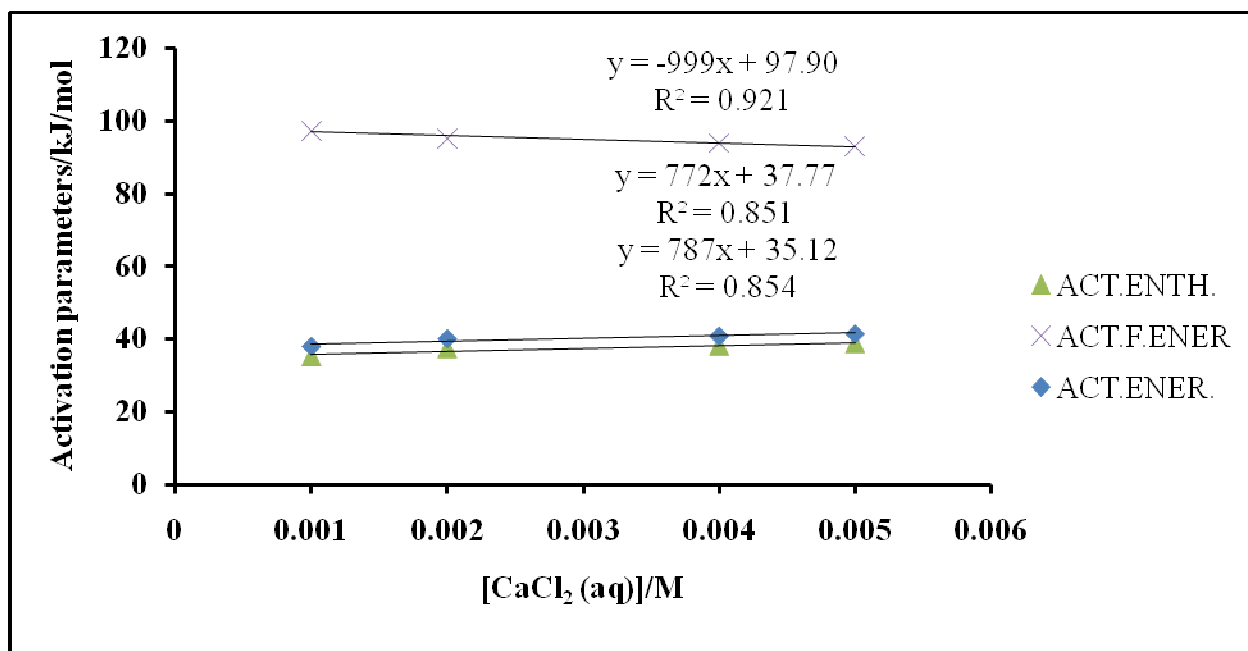
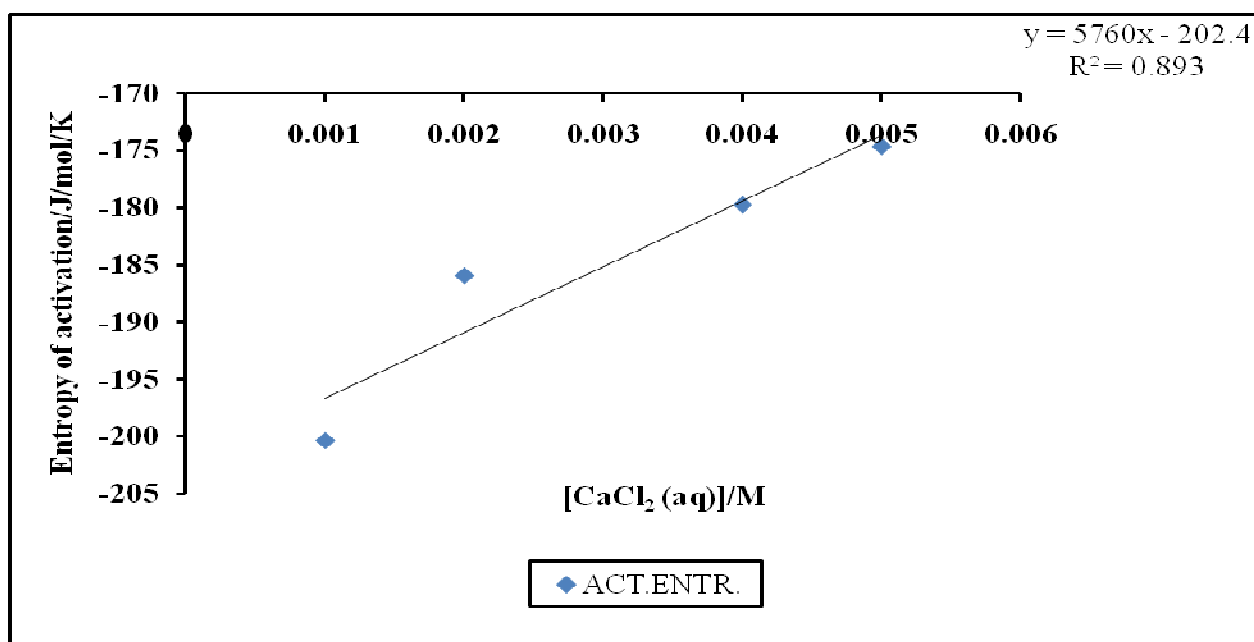


Fig. 5a: Variation of activation parameters – activation enthalpy, activation energy and activation free energy – with concentration of calcium chloride. (▲), (×), and (◆) stands for activation enthalpy (ACT. ENTH: $\Delta H^\#$), activation free energy (ACT.F.ENER: $\Delta G^\#$), and activation energy (ACT. ENER: E_a).



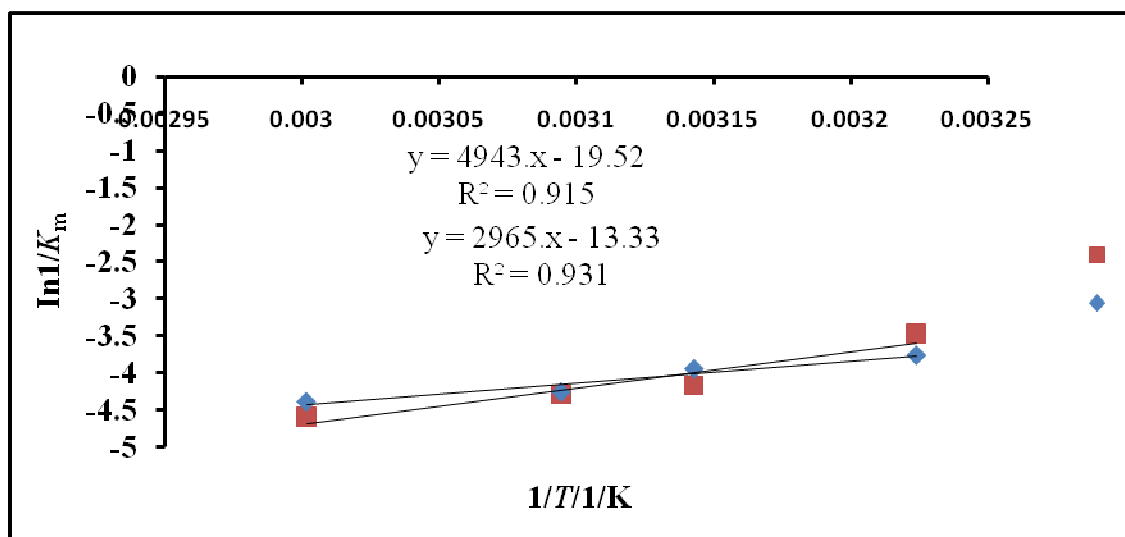
305

306

307 **Fig. 5b: Variation of entropy of activation with calcium chloride concentration.** *The*
 308 *symbol ♦ stands for activation entropy (ACT. ENTR: $\Delta S^\#$).*

309 In order to determine the temperature dependence of the velocity of raw starch, $\ln$
 310 $(1/K_m)$ was plotted versus $1/T$ (Fig.6). As a result of the use of so – called soluble potato
 311 starch as substrate whose molar mass is unknown and for the purpose of comparison,
 312 differences in thermodynamic parameters namely differences in free energy (ΔG) and
 313 entropy(ΔS) between Ca^{2+} -salt treated and Ca^{2+} -salt free PPA were determined. Thus as
 314 can be clarified in Table 3, and from the perspective of exothermic reaction, the thermal
 315 energy released in the presence of Ca^{2+} -salt is higher than the energy released in the
 316 absence of the salt. Given that the subscript c and c = 0 means Ca^{2+} -salt treated and Ca^{2+} -
 317 salt free PPA, then $-\Delta H_c > -\Delta H_{c=0}$. In the same vein, $-\Delta S_c > -\Delta S_{c=0}$ (loss of entropy for
 318 Ca^{2+} -salt treated PPA was $>$ loss for Ca^{2+} -salt free PPA). The magnitude of the loss
 319 decreased with increasing temperature. $\Delta G_c > \Delta G_{c=0}$ except at 310.5K (Table 3).

320



321

322 **Fig. 6: van't Hoff plot for the determination of enthalpy (ΔH) of hydrolysis of raw**
 323 **starch with porcine alpha amylase. The symbols $\blacklozenge$ and $\blacksquare$ stand for assay in the absence**
 324 **and presence of calcium chloride respectively**

325 **Table 3. Differences in thermodynamic parameters between calcium chloride free**
 326 **reaction mixtures and calcium chloride containing reaction mixtures.**

T (K)	310.15	318.15	323.15	333.15
$\Delta(\Delta S^0)$ (Jmol ⁻¹ K ⁻¹)	-53.04±0.06	-51.71±0.06	-50.91±0.08	-49.38±0.06
$\Delta(\Delta G^0)$ (kJmol ⁻¹)	-0.79±0.02	0.74±0.02	0.08±0.03*	0.57±0.02

327 *Enthalpy (ΔH^0) values in the absence and presence of calcium chloride are –*
 328 *24.66±0.42kJ/mol and – 41.10±0.20kJ/mol respectively; $\Delta(\Delta H^0) = -16.45±0.22$ kJ/mol.* is*
 329 *unusually different from general pattern for reason not quickly discernable. Values are*
 330 *recorded as mean±SD where n = 2.*

The determination of m – values described as the slope of the protein folding stability with osmolyte concentration Rösigen *et al* [27] and free energy of folding to unfolding transition, $\Delta G_{N \rightarrow U}$ gave values summarized in Table 4. There was increasing positive m – values with increasing concentration of ethanol pointing, perhaps, to the suggestion that Lord's dry gin, BG may fall between 50 – 90%(V/V) ethanol. Local gin, LG caused negative m – value corresponding to positive $\Delta G_{N \rightarrow U}$ unlike the effect of other concentrations of ethanol that showed or caused negative $\Delta G_{N \rightarrow U}$ in the absence of Ca^{2+} -salt.

Table. 4 The capacity of additive to force (un)folding of protein and the Gibbs free energy of folding – unfolding transition.

SOURCES OF ETHANOL	m – VALUES (kJmol ⁻¹ M ⁻¹)	$\Delta G_{N \rightarrow U}$ (kJmol ⁻¹)
BG	1,210.305±149.7	-7.20±0.21
LG	-625.07±25.53	3.50±0.01
18 (%(V/V))	510.84±18.05	-1.52±0.08
50 (%(V/V))	755.81±353.55	-6.45±0.53
90 (%(V/V))	1,690.58±66.74	-9.39±0.15

Values are recorded as mean $\pm$ SD where $n = 2$.

4. DISCUSSION

Unlike report in the past for human salivary alpha amylase (HS α A) [23], the SA of PAA increased with increasing concentration of Ca^{2+} -salt. PPA like its homologue HS α A is

calcium chloride dependent enzyme having both cation and anion binding sites [33]. However, the increasing trend in SA expressed as percentage of control without Ca^{2+} -salt plotted versus molar concentration of Ca^{2+} -salt (Fig. 1) shows that the enzyme is highly tolerant of extra load of calcium chloride. It appears therefore, that the proposition whereby binding of a 2nd calcium ion when in excess to the carboxyl group of Glu – 233 in a bidentate mode and of Asp – 197 in a univalent mode leading to inhibition [34] was not to be, due perhaps, to the fact that the protonated state of Glu – 233 in the presence of chloride ion weakened the strength of calcium ion binding and ultimately its inhibitory effect and consequently, conformational flexibility appeared to have overcome the forces of over rigidification of structure [35] unlike the case reported for HS α A[23].

The observed increase in specific activity (Fig. 2) with increasing concentration of Ca^{2+} -salt at every temperature under investigation is not unusual if cognizance is taken of the effect of Ca^{2+} -salt in the stability – conformational flexibility complementarity and temperature in the attainment of activated complex. As a well known mesophile with intermediate dependence on moderate thermal environment, suitable enthalpy of the environment is needed to reduce a large number of weak interactions being moderately high but fewer than those of thermophiles [36, 37]. However, a closer look at Fig. 2 reveal multiple features of rising, falling, and rising SA with increasing temperature in the presence of Ca^{2+} -salt. The decrease in SA at 50°C in the absence and presence of Ca^{2+} -salt seem to suggest that the enzyme has optimal temperature at pH 7.4 between 45 and 50°C. The failure to rise again in the absence of Ca^{2+} -salt, therefore, implies that the enzyme lost its stability at higher temperature if error in the concentration of the enzyme or substrate is precluded. However, the rise in specific activity at higher temperature in the presence of Ca^{2+} -salt suggests that the enzyme was stabilized by Ca^{2+} -salt thereby calling to question the doubt expressed.

Temperature above room temperature seemed to be suitable for the enzyme. This enables the enzyme attain suitable conformational flexibility needed for function. It is

therefore, not expected to achieve higher activity at low temperatures suitable to psychrophiles and not being what has been described as perfectly evolved enzymes whose reaction rate is only diffusion controlled with the result that E_a tends to zero as the exponential term E_a/RT tends to 1, leading to fast and essentially temperature – independent reactions [38], it is expediently expected therefore, to observe higher activity with increasing temperature, though not ad infinitum. However, it is not only diffusion controlled reaction that demands translational diffusion of interacting macromolecular reactants, starch and PPA for instant; otherwise this temperature dependent phenomenon cannot occur at all. “To bind at an enzyme’s active site, a ligand must diffuse or be transported to the enzyme’s surface, and, if the binding site is buried, the ligand must diffuse through the protein to reach it.”[39]. Also Berzzani *et al.* [40] posit that the enzymatic hydrolysis by alpha amylase is carried out by a side – by – side digestion mechanism after the enzyme diffuses and binds to the solid substrate. Lower activity at lower temperature is thus, in agreement with the observation that decrease in temperature will induce an exponential decrease in the reaction rate for most biological systems in which a decrease of 10°C may depress the velocity of amylolysis for instance by a factor ranging from 2 to 3, the exact value depending on the activation energy [38]. It is imperative therefore, to ensure that application of alpha amylase supplement and biotics [9 – 12] in animal feed preparation takes into account suitable thermal environment within and outside the animals’ digestive system. It should be pointed out that of special note is the use of probiotics *i.e.*, a live microbial food supplement (food supplements with brand name such as milk based hollandia yoghurts is an example) that beneficially affects the host animal by improving its intestinal microbial balance [12]. For human adult use without exemption health wise, this includes fermented milk products as well as over-the-counter preparations that contain lyophilized bacteria. Suitable ecological environment is vital for the realization of nutritional objectives in both human and nonhuman animals.

397 As shown below Fig. 3, the velocity of hydrolysis (SA) of raw soluble potato starch
 398 with native PPA is higher than LG and BG treated PPA. Similar report was made for HSαA
 399 [23] and it has also been shown that PPA and HSαA are similar in their hydrolytic action on
 400 raw starch and in their adsorption on starch granules [41]. This implied that there was
 401 inhibition of the activity of the enzyme due to factors such as lowered water activity, lowered
 402 solubility of both enzyme and substrate and unfolding of the enzyme [42] due to effect of
 403 ethanol. This view however, is parallel to the notion that a better solvent than water (the
 404 denaturant such as ethanol for instance) promotes substrate dissociation from the enzyme
 405 because the dissociated state provides greater surface exposure of both the protein fabric
 406 and substrate to favourable interaction with solvent [43]. Another reason that could be
 407 adduced as to the effect of ethanol from two sources is that ethanol as a chaotrope
 408 decreases the strength of hydrogen bond and other electrostatic interaction due to a change
 409 of the dielectric constant of the aqueous medium which may negatively affect the structure
 410 and function of the enzyme [44].

411 As shown in Table 1, there was increasing trend in the values of K_m with increasing
 412 temperature in the absence of Ca^{2+} -salt unlike previous report for HSαA, a very close
 413 homologue of PPA. Similar trend was observed in the presence of Ca^{2+} -salt and except the
 414 value at 310.15K for Ca^{2+} - salt treated PPA, other K_m values were higher than values for
 415 Ca^{2+} - salt free PPA unlike report for HSαA [23]. The implication is that PPA unlike HSαA,
 416 has lower affinity ($1/K_m$) for the soluble potato starch at higher temperature and in the
 417 presence of Ca^{2+} -salt. Often different substrate and homologous enzymes present different
 418 K_m values. Muscle and intestinal alpha amylase from pig parasite *Ascaris suum* have K_m
 419 values equal to 3.33mg/L and 0.48mg/L [45] whereas PPA has K_m values with native wheat,
 420 potato (not soluble potato), and waxy rice equal to 5.1, 5.8, and 6.8g/L respectively [46]. The
 421 K_m values reported for PPA in this research are several folds higher than the reports in the
 422 past [46]. Expectedly, the rate constant k_2 and catalytic efficiency k_2/K_m exhibited increasing
 423 trend with increasing temperature similar to the report for HSαA [23], though the k_2/K_m values

for the latter as well as values such as 704.41L/g/min for *Bacillus licheniformis* in which soluble potato starch was the substrate [47] and 184.09L/g.min for *B. licheniformis* EMS – 6 [2] were higher than values for PPA in this report. The kinetic parameters k_2 and k_2/K_m reported for PPA in literature [46] is difficult to interpret and cannot be compared with current result. Unlike the case with HS α A [23], the increasing trend in k_2/K_m values for PPA in the presence of Ca²⁺-salt was not regular, lowest value at 318.5 and highest value at 323.15 were observed. But the claim elsewhere [48] to the effect that “most enzymatic reactions have very large and remarkably similar apparent second-order rate constants, k_2/K_m , at mean values of about 10⁷/M/s with k_2 in the range of 10-1000/s” amount to over generalization because homogenous solution of a substrate such as solution of maltose, sucrose, and their corresponding enzymes cannot be anywhere comparable with the so-called soluble starch let alone insoluble starches and the corresponding enzymes in term of rate constant or turn over number or k_2/K_m . However, k_2 value reported for PPA at physiological temperature of 37°C is 518±22/s, [49] but it is not clear whether gelatinized soluble starch was the substrate. Also, k_2 value of 175/s was reported for HS α A under well defined pH, 6.9 and temperature, 25°C [50].

Figure 4 shows the Arrhenius plot for the determination of activation energy for the hydrolysis of soluble potato starch in native state and ultimately the enthalpy of activation. The plots were carried out following assay of Ca²⁺-salt treated PPA and Ca²⁺-salt free PPA. As shown under Table 2, the activation energy E_a in the presence of Ca²⁺-salt is higher than in the absence of the salt. This may imply that the presence of Ca²⁺-salt rigidified the enzyme resulting in higher activation energy so as to attain needed conformational flexibility for function [35]. Nonetheless, the E_a values reported for a HS α A[23] is less than values reported herein for PPA which in turn has value in the presence of Ca²⁺-salt higher than 11.7kcal/mol(~ 49.02kJ/mol)[28] reported for itself. This is however, unlike in the absence of the salt. Also a value of 12kcal/mol (~50.28kJ/mol) had been reported for PPA while a value of 8.9kcal/mol (~37.29kJ/mol) was observed in respective of *Pseudoalteromonas*

451 *haloplanktis* (AHA) alpha amylase, a cold adapted enzyme [49]. This simply suggests that
452 AHA has greater conformational flexibility than PPA, a point that seems to be justified by the
453 observed E_a value of 17.4kcal/mol (~72.91kJ/mol) for *Bacillus amyloliquefacien* alpha
454 amylase (BBA), a well known moderate thermophile) [28].

455 In Table 2, the enthalpy of activation ($\Delta H^\ddagger$) in the absence and presence of Ca^{2+} -salt
456 is recorded. As observed for E_a , $\Delta H^\ddagger$ for Ca^{2+} -salt treated PPA is higher than for Ca^{2+} -salt
457 free PPA. This could be attributed to stabilization of the ground state of the enzyme due to
458 the presence of extra Ca^{2+} -salt. Hence as the results depicts, $\Delta H^\ddagger$ which expresses
459 temperature dependence [28, 35], though moderate, is nevertheless related to the number of
460 enthalpically driven weak interactions that have to be broken to enable the enzyme reach the
461 activated complex for function[28]. This point of view is substantiated herein as in Table 2
462 and by reports that showed that $\Delta H^\ddagger$ for *Bacillus* sp (a thermophile) and *Alteromonas* sp (cold
463 adapted sp) are 99.6 and 73.6kJ/mol respectively. The observed values for PAA and AHA,
464 which were 11.5kcal/mol (~48.19kJ/mol) and 8.3kcal/mol (34.78kJ/mol) respectively [36] is
465 additional evidence.

466 The higher negative value of entropy of activation ($\Delta S^\ddagger$) for Ca^{2+} -salt free enzyme
467 than for Ca^{2+} -salt treated enzyme seems to suggest that in the absence of the salt, the
468 ground state of the enzyme had more flexibility such that transition to relative order in the
469 activated complex may have resulted in greater loss of entropy than in the presence of the
470 salt. This seems to be confirmed by the observation that the order of the strength and
471 number of weak interactions among alpha amylase homologues is: psychrophiles <
472 mesophiles < thermophiles [28]. The $\Delta S^\ddagger$ values for the hydrolysis of soluble starch with PPA
473 in the –presence of Ca^{2+} -salt is lower than in the absence of the salt unlike the report for
474 HS α A[23]. While such report for HS α A is similar to the report for BLA in the presence and
475 absence of SDS, it is not quite so for BAA in which treatment with SDS gave lower $\Delta S^\ddagger$ than
476 SDS free BAA at 25°C [51] as was the case in this report for PPA in which treatment with

477 Ca^{2+} -salt gave lower $\Delta S^\ddagger$ than in the absence of Ca^{2+} -salt. However, D' Amico *et al.* [28]
 478 observed 1.8kcal/mol(7.54kJ/mol) a positive value for BAA unlike negative values reported
 479 for AHA – 5.5kcal/mol (– 23.05kJ/mol) and – 2.9kcal/mol (–12.15kJ/mol) reported for PPA at
 480 10°C. This again is exemplified with the report which shows that $T\Delta S^\ddagger$ for PAA and AHA are
 481 – 2.5kcal/mol (~ –10.48kJ/mol) and – 5.1kcal/mol (~ – 21.37kJ/mol) respectively [28]. The
 482 vital point to be made from these data is that the moderate and high temperature tolerant
 483 homologues undergo thermally enhanced transition from a less mobile ground state
 484 stabilized by multiple weak interactions to a more mobile or flexible activated complex. This
 485 may not imply absence of order. There may be relative order but the magnitude for
 486 thermophiles, mesophiles, and psychrophiles may be in the following order psychrophiles >
 487 mesophiles > thermophiles as the data portray.

488 Unlike report for HSαA and report for *B. licheniformis* alpha amylase (BLA) in which
 489 sodium dodecyl sulphate (SDS) treated BLA alpha amylase had slightly higher $\Delta G^\ddagger$ than
 490 SDS free BLA., the free energy of activation $\Delta G^\ddagger$ for the hydrolysis of raw starch reported
 491 herein for Ca^{2+} -salt treated PPA is lower than for Ca^{2+} -salt free PAA. Like the report for SDS,
 492 it is most probable that Ca^{2+} -salt interacts not just with the enzyme but with the substrate
 493 also, leading to changes in the enzyme and substrate structure, the combined effect leading
 494 to a lowered energy barrier, that is, lower $\Delta G^\ddagger$. Transformation of a substrate to a product
 495 under influence of an enzyme proceed only after the formation of enzyme substrate complex
 496 which must be activated by the addition of Gibbs free energy of activation the magnitude of
 497 which constitute the energy barrier [52]. The role of the enzyme is to reduce the energy
 498 barrier a role that can be enhanced in the presence of stabilizers and activators.

499 In order to have an all-encompassing view of the effect of temperature in the
 500 presence of Ca^{2+} -salt, another approach for the determination of activation parameters
 501 referred elsewhere [26] as Eyring-Polanyi method entailed a plot of $\ln(v/T)$ versus $1/T$ and
 502 $\ln v$ versus $1/T$ for the determination of E_a and, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ respectively. These activation

parameters were plotted versus molar concentration of Ca^{2+} -salt in order to examine in its totality the influence of increasing Ca^{2+} -salt on the activation parameters. If the outcome follows from experimental experience then such outcome should be a reflection of the usual Arrhenius approach first for the determination of E_a and consequently $\Delta H^\ddagger$. As Fig. 5a shows the positive regression coefficient or slope suggests that the E_a and $\Delta H^\ddagger$ values were increasing with increasing concentration of Ca^{2+} -salt. This is therefore, a true reflection of the observed higher value of E_a and $\Delta H^\ddagger$ for Ca^{2+} -salt treated PPA than for Ca^{2+} -salt free PPA, using usual Arrhenius method ($\ln k_2$ versus $1/T$). Also the negative regression coefficient observed for the plot of $\Delta G^\ddagger$ versus molar concentration of Ca^{2+} -salt (Fig. 5a) is another true reflection of the observed lower values $\Delta G^\ddagger$ for Ca^{2+} -salt treated PPA than for Ca^{2+} -salt free PPA obtained using Eq. (4).

Like trend in E_a and $\Delta H^\ddagger$ with increasing molar concentration of Ca^{2+} -salt, $\Delta S^\ddagger$ showed similar trend (positive regression coefficient or slope) with increasing concentration of the salt (Fig.5b). "In line with mathematics, $0 > -1$ and $-1 > -2$ ". But negative values of entropy of activation simply mean diminution in entropy. Therefore, the positive correlation should be understood in terms of decreasing magnitude of the loss in entropy due to the increasing presence of the salt. There was increasing loss in entropy of activation with increasing temperature both in the presence and absence of Ca^{2+} -salt (Table 2); but the losses for Ca^{2+} -salt free PPA are higher than those reported for Ca^{2+} -salt treated PPA (Table 2) thereby justifying Fig. 5b. Therefore, the ground state entropy (the state of relative disorder) value for Ca^{2+} -salt treated PPA is less than the value for Ca^{2+} -salt free enzyme. This was exactly the case as reported in Table 2.

When compared with the result reported for HS α A [23], it could be seen that PPA and the former showed similar trend with respect to the plot of E_a , $\Delta H^\ddagger$, and $\Delta S^\ddagger$ versus molar concentration of Ca^{2+} -salt. However, both enzymes differed in the trend of $\Delta G^\ddagger$ versus molar concentration of Ca^{2+} -salt – increasing trend in the values of $\Delta G^\ddagger$ with HS α A [23]

529 unlike with PPA in this study. As stated earlier, as applicable to BAA, high temperature
 530 tolerant homologues undergo thermally enhanced transition from a less mobile ground state
 531 occasioned by multiple weak interactions to a relatively more mobile or flexible activated
 532 complex so that the difference in entropy between the ground state and the activated
 533 complex is less negative or assume small positive value. This is to say that the presence of
 534 the salt has impacted additional stabilizing force leading to extra rigidification totally
 535 unmatched by the opposing effect of the chloride components [23]. In support of this view is
 536 the proposition that a 2nd calcium ion when in excess bind to the carboxyl group of Glu-233
 537 leading to rigidification by electrostatic means [34].

538 Ultimately the moderately high thermal environment enabled the enzyme to
 539 thermally reduce the binding interactions including electrostatic interaction due to the
 540 presence of extra Ca²⁺-salt. All these point to the fact that there must be some residual
 541 entropy of activation that must have a stimulating or activating effect on the enzyme and
 542 consequently a lower $\Delta G^\#$ value in the presence of Ca²⁺-salt. Therefore, mesophiles and
 543 thermophiles under moderate temperature should be subject to thermal dissipative
 544 contribution of the unfavorable entropic term which provides the needed mobility of the
 545 active site in particular for function [37]. However as in the past [23] and report herein for
 546 PPA, the negative entropy of activation has been attributed to cooperative behavior of water
 547 molecules around the active site [51]. Besides it has been posited that the chloride
 548 component of Ca²⁺-salt for chloride dependent enzyme, alpha amylase, for instance in the
 549 presence of a protonated state of Glu – 233 weakens the strength of calcium binding and
 550 ultimately the consequence of over rigidification [35]. This is contrary to the claim that
 551 chloride binding increases the affinity of the enzyme for calcium ion [53]. “Perhaps, this
 552 might be characterized by conditionality”.

553 It has been observed that the K_m values for Ca²⁺-salt treated and Ca²⁺-salt free PPA
 554 were increasing with increasing temperature. This simply implies that the affinity of the
 555 enzyme for the substrate gets weaker with increasing temperature. Except at 310.15K, the

556 K_m values for Ca^{2+} -salt treated PPA is higher than Ca^{2+} -salt free enzyme. These situations
 557 were unlike report for HS α A [23]. It appeared that at higher temperature the presence of the
 558 salt reduced the affinity of the enzyme, PPA for the substrate unlike the effect of SDS on the
 559 affinity of BAA and BLA for soluble amylose (DP = 18) [51].

560 On grounds of thermodynamic stability of the outcome of a process, a negative
 561 enthalpy implies that the product may be stable compared to a product resulting from
 562 positive enthalpy change. The enthalpy (ΔH) for the formation of enzyme substrate complex,
 563 in this study showed that for the Ca^{2+} -salt treated and Ca^{2+} -salt free enzyme the ΔH values
 564 were negative that is they were exothermic. The magnitude of the exothermic heat change is
 565 higher for the Ca^{2+} -salt treated than for the Ca^{2+} -salt free PPA. This scenario is entirely
 566 different from the report for HS α A[23] and for BAA in the absence and presence of SDS [51]
 567 but similar to report for BLA, and unlike Ca^{2+} -salt treated PPA compared to Ca^{2+} -salt free
 568 PPA , the exothermic heat change for SDS treated BLA is less than SDS free enzyme [51].

569 If $-\Delta S_c - (-\Delta S_{c=0})$ (where subscript c and c=0 specify entropy change for Ca^{2+} -salt
 570 treated and Ca^{2+} -salt free PPA respectively.) is positive it means that $-\Delta S_{c=0}$ is higher than
 571 $-\Delta S_c$ in magnitude. This implies that the loss in entropy is higher for Ca^{2+} -salt free than Ca^{2+} -
 572 salt treated PPA. This might be as a result of looser internal packing of the 3-dimensional
 573 structure of $+\text{Ca}^{2+}$ -salt free PPA than for Ca^{2+} -salt treated PPA unlike earlier report for HS α A
 574 [23]. Unlike HS α A the reason that may be advanced for such observation with respect to
 575 PPA is that there may have been a decrease in the amount of dehydration required for ES
 576 formation [51] for Ca^{2+} -salt treated PPA. In other words the ES for the Ca^{2+} -salt free PPA
 577 was more hydrated than the Ca^{2+} -salt treated PAA.

578 Since the molar mass of the substrate is unknown the differences in free energy
 579 between Ca^{2+} -salt treated and Ca^{2+} -salt free PAA were calculated. But for the value at
 580 310.15K, the free energy for the hydrolysis of soluble potato starch with Ca^{2+} -salt treated
 581 PPA were found to be higher than with Ca^{2+} -salt free PPA. The difference is presented in

Table 3. This may not be unexpected because the presence of Ca^{2+} -salt has rigidified the enzyme resulting to less spontaneity in the formation of ES and transformation to product. This can also be adduced to what may be described as an increase in the degree of internal packing [51] occasioned by the effect of extra Ca^{2+} -salt, the binding of another Ca^{2+} to Glu – 233 and Asp – 197 partially opposed by the binding of Cl^{-1} [34, 35].

As Table 4 shows, the m – values of PPA in a reaction mixture containing any of the following, branded gin or Lord's dry gin (BG), 18% (V/V) ethanol, 50%(V/V) ethanol, and 90%(V/V) and Ca^{2+} -salt were positive. This implied that the presence of Ca^{2+} -salt impacted on the enzyme the capacity to resist folding – unfolding transition due to the effect of alcohol from different sources in line with the postulate that says that, the slope of the plot of free energy of folding stability versus osmolyte concentration is positive due to the presence of stabilizing osmolyte, (in) organic in nature [27]. The contrary was the case for the mixture of local gin (LG) otherwise known as *kin kin* or *ogogoro* and aqueous Ca^{2+} -salt in which the m – value was negative suggesting that the Ca^{2+} -salt was unable to offset the effect of LG. The free energy of folding – unfolding transition $\Delta G_{\text{N} \rightarrow \text{U}}$ values obtained from extrapolation to infinite dilution of Ca^{2+} -salt, were negative in the presence of BG, 18%(V/V), 50%(V/V), and 90%(V/V) ethanol. Thus the fact that $\Delta G_{\text{N} \rightarrow \text{U}}$ values were negative (which is indicative of spontaneity of unfolding process) in the absence of Ca^{2+} -salt or at its infinite dilution implied that the alcohol from the sources indicated was indeed destabilizing to the enzyme. However, the positive value of $\Delta G_{\text{N} \rightarrow \text{U}}$ in the presence of LG suggests the unusual compared to the effect of other alcohols. This lack of spontaneity may imply that LG had a combined effect with the chloride component of Ca^{2+} -salt to cause unfolding but cannot to do so in the absence of the Ca^{2+} -salt or at its infinite dilution with the result that total unfolding became less feasible or less spontaneous (Cl^{-1} is known to oppose the effect of Ca^{2+} [35]).

Looking back into literature one finds that the m – values reported for HSαA [23] were less than value reported herein for PPA in a reaction mixture containing BG, 18%

(V/V), and 90% (V/V); but the m – values for HS α A and PPA were positive in sign. Upon examination of $\Delta G_{N \rightarrow U}$ values, it could be observed that the negative values for PPA in the presence of BG, 18% (V/V), and 90% (V/V) (including 50 % (V/V), however) only or at infinite dilution of Ca²⁺-salt were higher in magnitude than the values for HS α A[23]. However, the presence of LG only showed different trend in that the $\Delta G_{N \rightarrow U}$ value for PPA was positive while it was negative in sign for HS α A [23]. It simply means that LG had greater effect on HS α A than it had for PPA.

4. CONCLUSION

Specific activities of the enzyme increased with increasing temperature and molar concentration of Ca²⁺-salt. The rate constant (k_2) was higher for Ca²⁺-salt treated than for Ca²⁺-salt free enzyme and the values for both enzyme, treated and untreated, increased with increase in temperature. Catalytic efficiency for Ca²⁺-salt treated enzyme is higher than for Ca²⁺-salt free enzyme. The activation parameters E_a and $\Delta H^\ddagger$, for Ca²⁺-salt treated enzyme, were higher than for Ca²⁺-salt free enzyme while $\Delta G^\ddagger$ for Ca²⁺-salt treated enzyme was lower than for Ca²⁺-salt free enzyme. The ground state of Ca²⁺-salt treated enzyme is less flexible than Ca²⁺-salt free enzyme (lower magnitude of negative $\Delta S^\ddagger$ for Ca²⁺-salt treated than Ca²⁺-salt free enzyme). Positive m – values with ethanol from different sources showed that Ca²⁺-salt can protect the enzyme against the effect of a chaotrope like ethanol. The negative values of $\Delta G_{N \rightarrow U}$ showed that unfolding in the presence of alcohol only is feasible. It is recommended that clean water at a temperature range of 37 – 45°C (310.15 – 318.15K) should be made available to livestock.

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