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Calcium Chloride Can Influence the Stability Flexibility Complementarity and Consequently, Activity of Hydrolases: A Case Study on Porcine Alpha Amylase

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Aims: The specific aims of the research were i) to investigate the effects of extra "load" of calcium chloride at different temperatures and ethanol 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) 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.

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Methodology: Bernfeld method of enzyme assay was used. Controls were free from additives while the experimental test was either with calcium chloride or a mixture of alcohol and calcium chloride.
Results: The results presented graphically and in the Tables showed that the specific activity and kinetic parameters of the enzyme with Ca^{2+} - salt were higher than without Ca^{2+} - salt. The activation parameters, free energy ($\Delta G^{\#}$), enthalpy ($\Delta H^{\#}$), and entropy($\Delta S^{\#}$) for Ca^{2+} - salt treated enzyme were 77.36±0.11 kJ/mol, 54.95±0.96 kJ/mol, and – 68.39±3.03 J/mol.K respectively at 318.15 K. The enthalpy (ΔH) of enzyme substrate (ES) formation was – 41.10 for Ca^{2+} - salt treated enzyme. The *m* – values were positive in sign in the presence of Ca^{2+} - salt with all alcohols except local gin. All free energy values for folding – unfolding transition at infinite dilution of the Ca^{2+} - salt were negative for all alcohols except with local gin.
Conclusion: The presence of extra Ca^{2+} - salt can increase the specific activity, rate constant and catalytic efficiency of the enzyme. The ground state of Ca^{2+}-salt treated enzyme is less flexible 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.

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

1. INTRODUCTION

Porcine alpha amylases (α – 1, 4 – glucan – 4 – glucano – hydrolase E.C. 3.2.1.1) like any other homologue, catalyze the hydrolysis of the internal α - 1, 4 – glucosidic bond in starch and glycogen [1,2]. Glucano - hydrolases are very important because they are involved in biological reactions such as fermentation, germination or digestion and they are widely used in some industries for the production of glucose syrups, enhanced bread production by acting as anti-staling agents in baking or in detergents to remove starch based stains [3,4]. The human homologue of porcine alpha amylase (PPA), human salivary alpha amylase has diagnostic applications in the determination of the state of the nervous system, cognate mental state, and psychological state of individuals [5–7].

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

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

However, be it biotics, endogenous or exogenous enzyme supplements, the right thermal environment is essential for optimization of the very purpose of overall animal husbandry, poultry *etc* and ultimately human wellbeing without exception. This is against the backdrop of the fact that alpha amylase homologues belong to different classifications, psychrophiles, mesophiles, and thermophiles according to their the degree of dependence on the thermal environment which may be low, moderate and high as have been investigated in the past [13–16]. However**,** there is a school of taught that suggest that digestion of starch in the rumen is

essential to maximum utilization of feed grains by the ruminant [17]. This is contrary to the claim that in grain-fed animals, protozoa can exert an influence on ruminal starch hydrolysis rates in at least two respects namely ingestion of bacteria in numbers sufficient to decrease ruminal fermentation rates [18,19], and ingestion of starch granules and soluble sugars, with the result that the substrates needed for fermentation by the fast growing bacteria is diminished [20].

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

To this end therefore, and against the backdrop of the practice whereby hydrolases including alpha amylases are used for the pretreatment of starch of animal feeds to improve the digestibility of fibre and starch in particular [3], the objectives of this 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 folding – unfolding cooperativity (*m* – value) in the presence of a mixture of ethanol and calcium chloride.

2. MATERIALS AND METHODS

2.1 Materials

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

Equipment: *p*H meter (tester) was from Hanna Instruments, Italy; electronic weighing machine was from Wensar Weighing Scale Ltd, Chennai; Centrifuge, 800D model was from China; 721/722 visible spectrophotometer was from Spectrum Instruments Co Ltd, China.

2.2 Methods

A solution of enzyme was prepared by dissolving 0.01g in 20 mL tris – $HCl_{(aq)}$ buffer as stock at *p*H 7.4 to deliberately conform with biological human *p*H 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)/ε l, where ε ($181.1 M^{-1}cm^{-1}$), 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 3000 rpm (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 4 mM calcium chloride at absolute temperature ranging from 298.15 to 333.15 K 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 (Ea), enthalpy of activation ($\Delta H^{\#}$), entropy of activation ($\Delta S^{\#}$), and free energy of activation ($\Delta G^{\#}$) 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):

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

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

$$\Delta H^{\#} = Ea - RT \quad (2)$$

$$\Delta S^{\#} = \Delta G^{\#} - \Delta H^{\#} \quad (3)$$

$$\Delta G^{\#} = \ln (k_B T/hk_2) \quad (4)$$

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].

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

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

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

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

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

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

The determination of m – values (the slope of the protein folding stability with osmolyte concentration [27]) is determined by plotting free

energy of protein unfolding (ΔG^o) against co – solute concentration. Thus according to Rösgen et al. [27], *m* – value for the protecting osmolyte/additive (or a kosmotrope) is positive while the *m* – value for destabilizing osmolyte (or a chaotrope) is negative. The equation linking ΔG^o and *m* – values are presented in two ways. One approach is in terms of negative slope [28–30]:

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

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

$$\Delta G^o = \Delta G^o{}_{(N\rightarrow D)} + m\,[\text{co} - \text{solute}] \quad (8)$$

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

Meanwhile on the basis of two – state model,

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

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

2.3 Statistical Analysis

Values of parameters from duplicate assays are expressed as mean ± SD. Microsoft Excel was used to calculate standard deviation (SD).

3. RESULTS

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

Fig. 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℃ (318.15 K) and 50℃ (323.15 K) 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℃ (323.15 K) and 60℃ (333.15 K) 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.

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

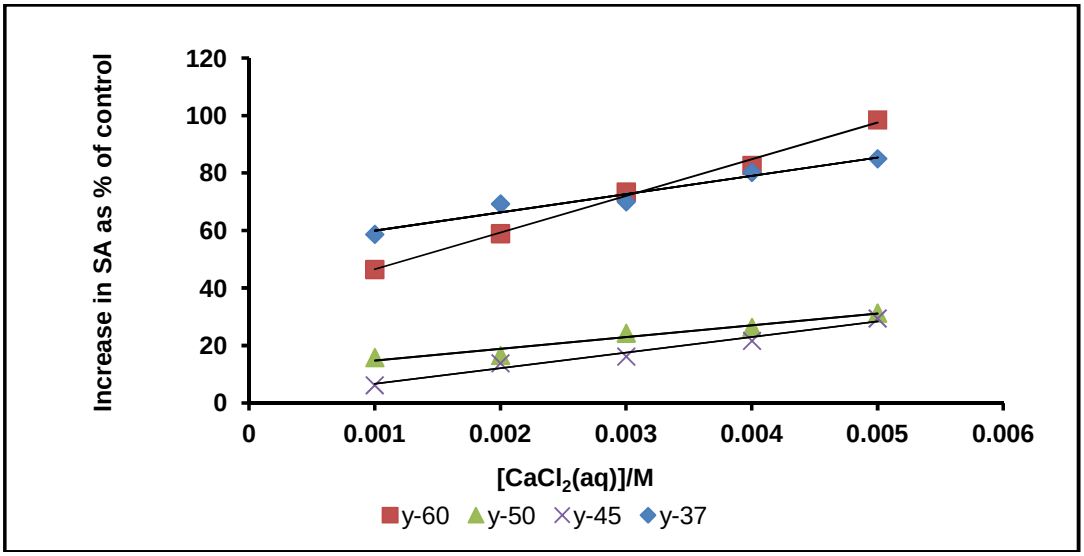

Fig. 1. Percentage increase in specific activity versus molar concentration of calcium chloride at different temperatures

The slopes which are 12768, 6351, 5413, and 4067 at 60, 37, 45, and 50℃ respectively may have some "physico-biochemical" implication. SA stands for specific activity of the enzyme. ■, ▲, x, and ◆ stand for assays at 60, 50, 45, and 37℃ respectively

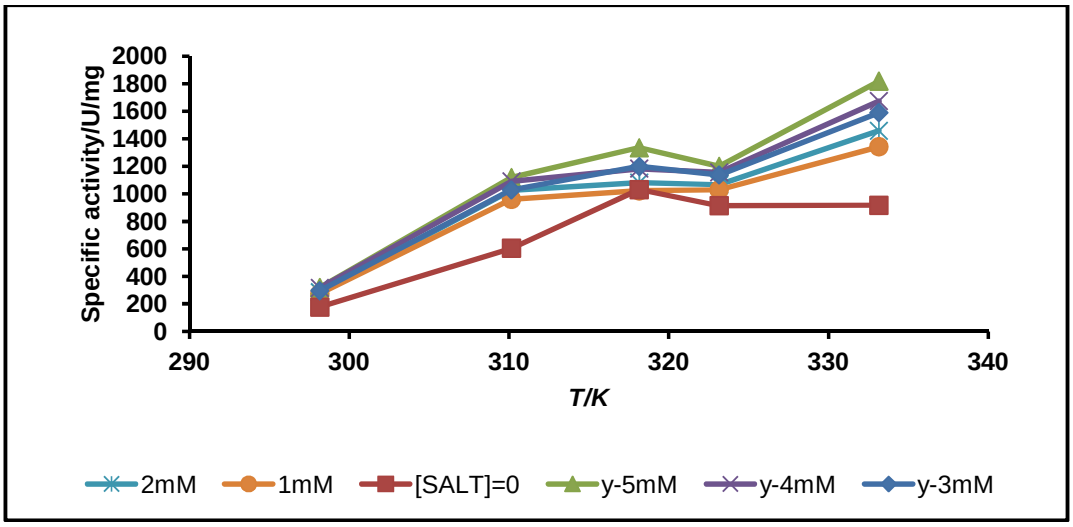

Fig. 2. Trend in specific activity of porcine alpha amylase with increasing temperature at different concentration of calcium chloride

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

As may be observed in Table 1, there was increasing trend in the values of K_m and k_2 with increasing temperature in the presence and absence of 4 mM Ca^{2+}-salt. The catalytic efficiency, k_2/K_m exhibited increasing trend on a regular basis in the absence of Ca^{2+}-salt unlike the irregular trend observed in the presence of Ca^{2+}-salt. The k_2 and k_2/K_m values for Ca^{2+}-salt treated enzyme, PAA, were higher than values for Ca^{2+}-salt free PPA. Except the value of K_m at 310.15K for Ca^{2+}-salt treated PPA, the values of K_m for Ca^{2+}-salt free PAA were lower than values for Ca^{2+}-salt treated enzyme.

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

T (K)	[$CaCl_2$(aq)] = zero			[$CaCl_2$(aq)] = 4 mM		
	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

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

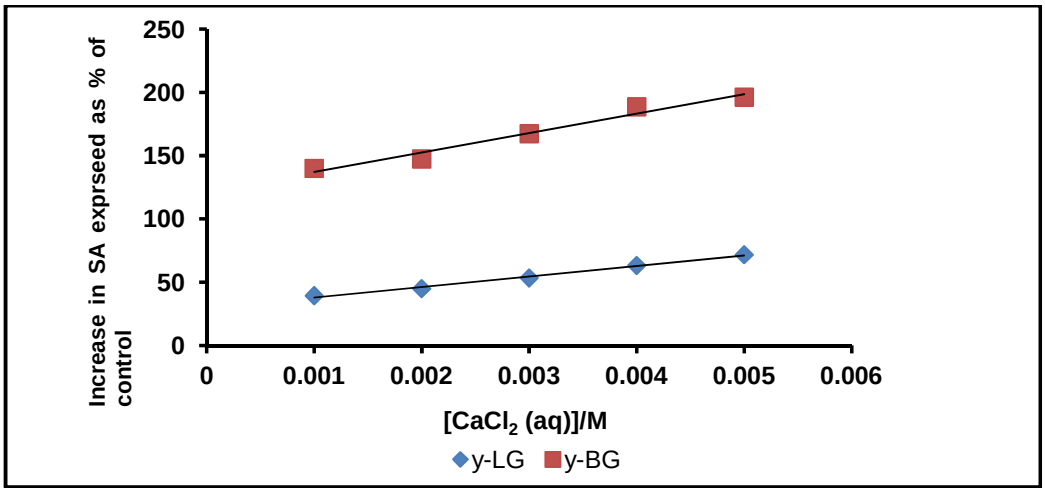

Fig. 3. Variation of increase in specific activity (SA) expressed as percentage of control without Ca^{2+}-salt versus molar concentration of Ca^{2+}-salt

The mean control velocity of hydrolysis (n = 2) for LG treated PPA without Ca^{2+}-salt is reported as mean±SD: (117.60±2.33 mU/mL); for BG treated PPA without Ca^{2+}-salt the value is: 132.50±23.4 mU/mL. The value for PPA free from any additive, i.e. as native enzyme, is 303.54±1.56 mU/mL. The control reaction mixture contains only alcohol. The slope in the presence of branded gin (Lord's gin) is 15372 with R^2 = 0.971 and in the presence of local gin, it is 8317 with R^2 = 0.992. *The symbols ◆ and ■ stand for assay in the presence of local gin (LG) and Branded gin (BG) – Lord's dry gin respectively*

In order to determine activation parameters for the hydrolysis of raw starch, in the presence of Ca^{2+}-salt, plots of Ink_2 versus 1/T (Arrhenius plot) (Fig. 4) were carried out for the determination of activation energy and enthalpy of activation for the hydrolysis of the starch. For the purpose of control, plots of Ink_2 versus 1/T (Fig. 4) were carried out for the determination of activation energy and enthalpy of activation for the hydrolysis of the starch for Ca^{2+}-salt free enzyme. The negative slope was in clear conformity with Arrhenius theory. The slope, $\partial \text{In}k_2/\partial 1/T$ for Ca^{2+}-salt treated enzyme is higher than for Ca^{2+}-salt free enzyme (Fig. 4). However, the coefficient of determination (R^2) for Ca^{2+}-salt free enzyme was higher than Ca^{2+}-salt treated enzyme.

Data obtained from plots of in (v/T) and In v versus 1/T were plotted versus molar concentration of Ca^{2+}-salt. The data from such plots, for $\Delta G^{\#}$, Ea, and $\Delta H^{\#}$ were plotted versus molar concentration of Ca^{2+}-salt (Fig. 5a). Also, data for $\Delta S^{\#}$ were plotted versus molar concentration of Ca^{2+}-salt (Fig. 5b). Finally the plot of In ($1/K_m$) versus 1/T (van't Hoff plot) is shown in Fig. 6. The activation energy for Ca^{2+}-salt treated PPA was higher than values for Ca^{2+}-salt free PAA as shown under Table 2. As shown in Table 2 the loss of entropy, negative $\Delta S^{\#}$, for Ca^{2+}-salt treated enzyme < values for Ca^{2+}-salt free enzyme. The same was applicable to free energy of activation in which values for Ca^{2+}-salt treated enzyme < values for Ca^{2+}-salt free PPA. However, values of enthalpy of activation, $\Delta H^{\#}$ for

Ca^{2+}-salt treated PPA was higher than values for Ca^{2+}-salt free PPA. Both Ca^{2+}-salt treated and Ca^{2+}-salt free PPA exhibited the same trend with increasing temperature in their values of activation parameters viz: decreasing trend in $\Delta H^{\#}$ (except departure at 318.15K) increasing trend in $\Delta S^{\#}$ and $\Delta G^{\#}$ (Table 2). Nonetheless, there was a departure from regular trend in the values of $\Delta S^{\#}$ and $\Delta G^{\#}$ for Ca^{2+}-salt treated PPA at 323.15K.

In order to have a broad coverage and insight, the effect of temperature at different molar concentration of Ca^{2+}-salt was investigated according to method adopted by EL – Hefnawy et al. [26] for the determination of *E*a, $\Delta S^{\#}$, $\Delta H^{\#}$, and $\Delta G^{\#}$. However, the values of the parameters were plotted versus molar concentration of Ca^{2+}-salt so as to establish the type of correlation between the activation parameters and Ca^{2+}-salt. With respect to $\Delta H^{\#}$ and *E*a (Fig. 5a), and $\Delta S^{\#}$ (Fig. 5b), the regression coefficients, the slopes, were positive pointing to the fact that there must have been a positive correlation between the activation parameters and Ca^{2+}-salt as evidenced with moderately high coefficient of determination R^2 which translated to correlation coefficient R gave respectively, 0.924, 0.922, and 0.945. On the other hand $\Delta G^{\#}$ showed negative slope (Fig. 5a) pointing to negative correlation with Ca^{2+}-salt given that R^2 translated to R gave high value equal to ~ 0.960. From the slope it can be seen that the magnitude of the entropic term $\partial\Delta S^{\#}/\partial[Ca^{2+}\text{-salt}]$ was very high (and much higher than other parameters if $\partial\Delta S^{\#}/\partial[Ca^{2+}\text{-salt}]$ is multiplied by *T*) (Fig. 5b) compared with $\partial\Delta H^{\#}/\partial[Ca^{2+}\text{-salt}]\times10^3$, $\partial\Delta G^{\#}/\partial[Ca^{2+}\text{-salt}]\times10^3$, and $\partial Ea/\partial[Ca^{2+}\text{-salt}]\times10^3$.

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

T (K)	$[CaCl_2(aq)]$ = zero			$[CaCl_2(aq)]$ = 4 mM		
	$\Delta S^{\#}$ ($Jmol^{-1}K^{-1}$)	$\Delta H^{\#}$ ($kJmol^{-1}$)	$\Delta G^{\#}$ ($kJmol^{-1}$)	$\Delta S^{\#}$ ($Jmol^{-1}K^{-1}$)	$\Delta H^{\#}$ ($kJmol^{-1}$)	$\Delta G^{\#}$ ($kJmol^{-1}$)
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

Activation energy (Ea): Ea values in the presence of 4 mM $CaCl_2$ (aq) and in the absence of $CaCl_2$(aq) were 57.59±0.96 kJ/mol and 45.05±1.25 kJ/mol; values were recorded as mean±SD where n = 2

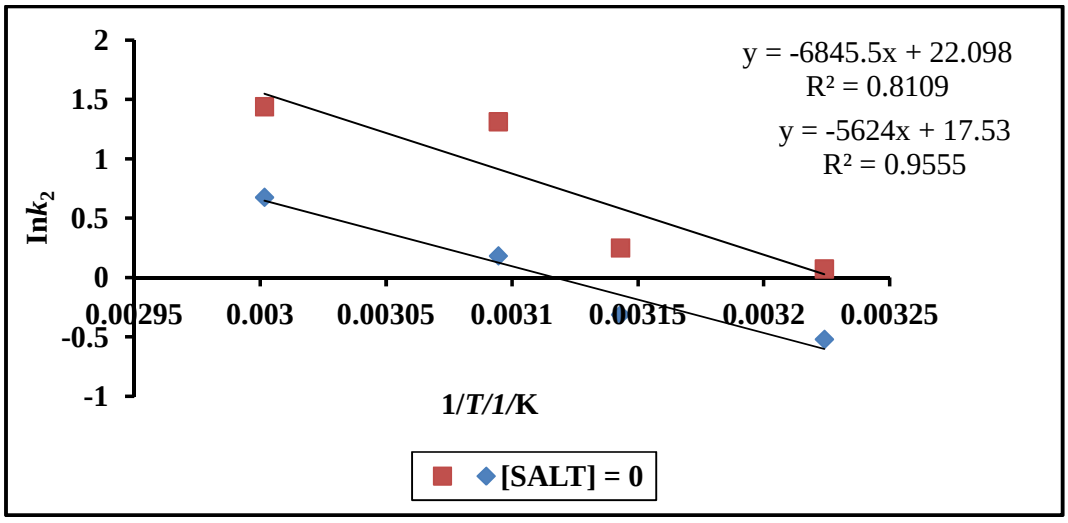

Fig. 4. Arrhenius plot for the determination of activation energy for the hydrolysis of starch in the absence and presence of calcium chloride

The symbols ◆ and ■ stand for assay in the absence and presence of calcium chloride respectively

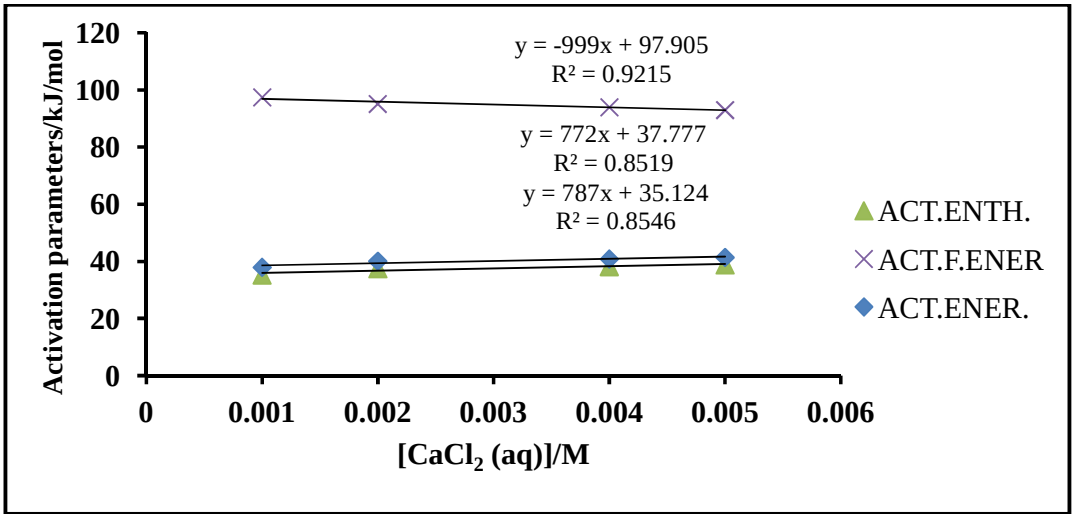

Fig. 5a. Variation of activation parameters – activation enthalpy, activation energy and activation free energy – with concentration of calcium chloride

(▲), (×), *and* (◆) *stands for activation enthalpy (ACT. ENTH: $\Delta H^{\#}$.), activation free energy (ACT.F.ENER: $\Delta G^{\#}$), and activation energy (ACT. ENER: Ea.)*

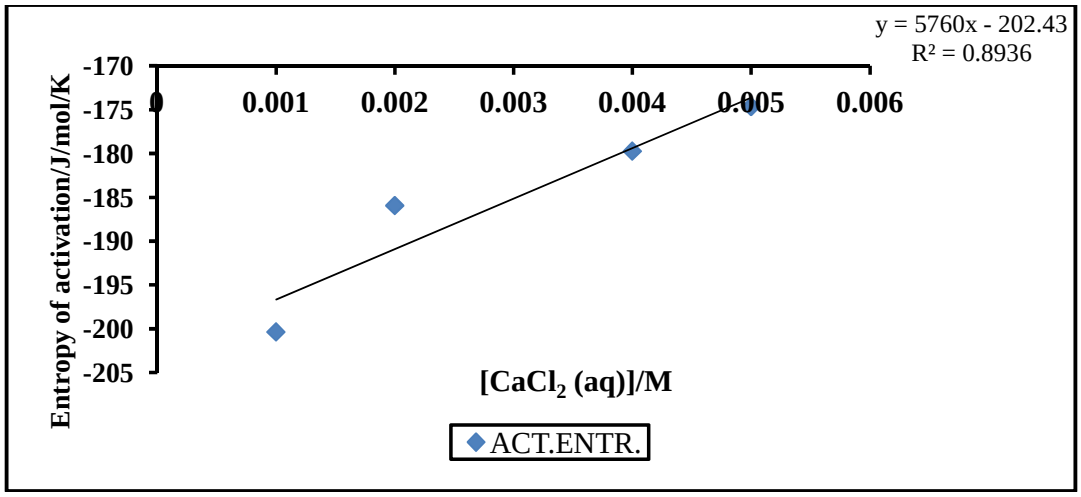

Fig. 5b. Variation of entropy of activation with calcium chloride concentration

The symbol ◆stands for activation entropy (ACT. ENTR: $\Delta S^{\#}$.)

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

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

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

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

The determination of *m* – values described as the slope of the protein folding stability with osmolyte concentration Rösgen 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.

4. DISCUSSION

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

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 ± SD where n = 2

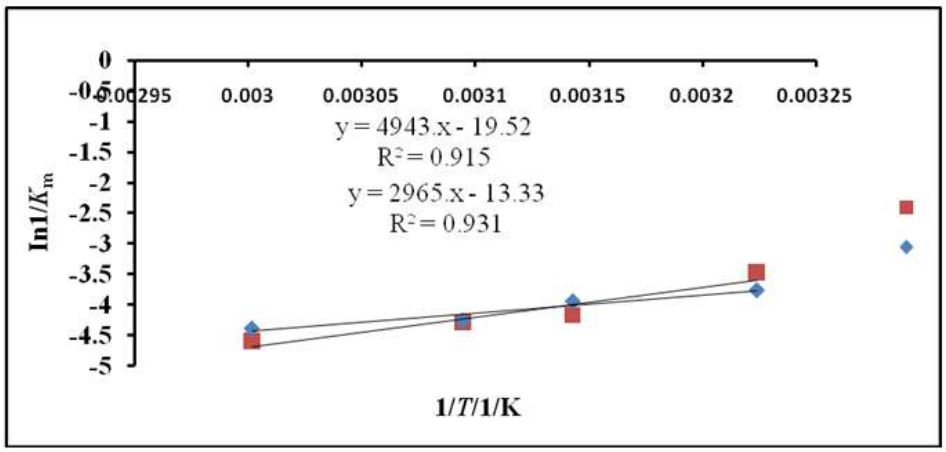

Fig. 6. Van't Hoff plot for the determination of enthalpy (Δ*H*) of hydrolysis of raw starch with porcine alpha amylase

The symbols ◆ and ■ stand for assay in the absence and presence of calcium chloride respectively

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℃ in the absence and presence of Ca^{2+}-salt seem to suggest that the enzyme has optimal temperature at *p*H 7.4 between 45 and 50℃. 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 perfectively evolved enzymes whose reaction rate is only diffusion controlled with the result that *E*a tends to zero as the exponential term *Ea*/*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 10ºC 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.

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

As shown in Table 1, there was increasing trend in the values of K_m with increasing temperature in the absence of Ca^{2+}-salt unlike previous report for HSαA, a very close homologue of PPA. Similar trend was observed in the presence of

Ca^{2+}-salt and except the value at 310.15 K for Ca^{2+} - salt treated PPA, other K_m values were higher than values for Ca^{2+} - salt free PPA unlike report for HSαA [23]. The implication is that PPA unlike HSαA, has lower affinity ($1/K_m$) for the soluble potato starch at higher temperature and in the presence of Ca^{2+}-salt. Often different substrate and homologous enzymes present different K_m values. Muscle and intestinal alpha amylase from pig parasite *Ascaris suum* have K_m values equal to 3.33 mg/L and 0.48 mg/L [45] whereas PPA has K_m values with native wheat, potato (not soluble potato), and waxy rice equal to 5.1, 5.8, and 6.8 g/L respectively [46]. The K_m values reported for PPA in this research are several folds higher than the reports in the past [46]. Expectedly, the rate constant k_2 and catalytic efficiency k_2/K_m exhibited increasing 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 184.09 L/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 HSαA [23], the increasing trend in k_2/K_m values for PPA in the presence of Ca^{2+}-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 10^7/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℃ 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 HSαA under well defined *p*H, 6.9 and temperature, 25℃ [50].

Fig. 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 Ca^{2+}-salt treated PPA and Ca^{2+}-salt free PPA. As shown under Table 2, the activation energy *E*a in the presence of Ca^{2+}-salt is higher than in the absence of the salt. This may imply that the presence of Ca^{2+}-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 HSαA[23] is less than values reported herein for PPA which in turn has value in the presence of Ca^{2+}-salt higher than 11.7 kcal/mol(~49.02 kJ/mol) [28] reported for itself. This is however, unlike in the absence of the salt. Also a value of 12 kcal/mol (~50.28 kJ/mol) had been reported for PPA while a value of 8.9kcal/mol (~37.29kJ/mol) was observed in respective of *Pseudoalteromonas haloplanktis* (AHA) alpha amylase, a cold adapted enzyme [49]. This simply suggests that AHA has greater conformational flexibility than PPA, a point that seems to be justified by the observed *E*a value of 17.4 kcal/mol (~72.91 kJ/mol) for *Bacillus amyloliquefacien* alpha amylase (BBA), a well known moderate thermophile) [28].

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

The higher negative value of entropy of activation ($\Delta S^{\#}$) for Ca^{2+}-salt free enzyme than for Ca^{2+}-salt treated enzyme seems to suggest that in the absence of the salt, the ground state of the enzyme had more flexibility such that transition to relative order in the activated complex may have resulted in greater loss of entropy than in the presence of the salt. This seems to be confirmed by the observation that the order of the strength and number of weak interactions among alpha

amylase homologues is: psychrophiles < mesophiles < thermophiles [28]. The $\Delta S^{\#}$ values for the hydrolysis of soluble starch with PPA in the presence of Ca^{2+}-salt is lower than in the absence of the salt unlike the report for HSαA [23]. While such report for HSαA is similar to the report for BLA in the presence and absence of SDS, it is not quite so for BAA in which treatment with SDS gave lower $\Delta S^{\#}$ than SDS free BAA at 25℃ [51] as was the case in this report for PPA in which treatment with Ca^{2+}-salt gave lower $\Delta S^{\#}$ than in the absence of Ca^{2+}-salt. However, D' Amico et al. [28] observed 1.8 kcal/mol (7.54 kJ/mol) a positive value for BAA unlike negative values reported for AHA – 5.5 kcal/mol (– 23.05 kJ/mol) and – 2.9 kcal/mol (–12.15 kJ/mol) reported for PPA at 10℃. This again is exemplified with the report which shows that $T\Delta S^{\#}$ for PAA and AHA are – 2.5 kcal/mol (~ –10.48 kJ/mol) and – 5.1 kcal/mol (~ – 21.37 kJ/mol) respectively [28]. The vital point to be made from these data is that the moderate and high temperature tolerant homologues undergo thermally enhanced transition from a less mobile ground state stabilized by multiple weak interactions to a more mobile or flexible activated complex. This may not imply absence of order. There may be relative order but the magnitude for thermophiles, mesophiles, and psychrophiles may be in the following order psychrophiles > mesophiles > thermophiles as the data portray.

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

In order to have an all-encompassing view of the effect of temperature in the presence of Ca^{2+}-salt, another approach for the determination of activation parameters referred elsewhere [26] as Eyring-Polanyi method entailed a plot of ln (v/T) versus $1/T$ and ln$v$ versus $1/T$ for the determination of Ea and, $\Delta H^{\#}$ and $\Delta S^{\#}$ 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 Ea and consequently $\Delta H^{\#}$. As Fig. 5a shows the positive regression coefficient or slope suggests that the Ea and $\Delta H^{\#}$ values were increasing with increasing concentration of Ca^{2+}-salt. This is therefore, a true reflection of the observed higher value of Ea and $\Delta H^{\#}$ for Ca^{2+}-salt treated PPA than for Ca^{2+}-salt free PPA, using usual Arrhenius method (lnk_2 versus $1/T$). Also the negative regression coefficient observed for the plot of $\Delta G^{\#}$ versus molar concentration of Ca^{2+}-salt (Fig. 5a) is another true reflection of the observed lower values $\Delta G^{\#}$ for Ca^{2+}-salt treated PPA than for Ca^{2+}-salt free PPA obtained using Eq. (4).

Like trend in Ea and $\Delta H^{\#}$ with increasing molar concentration of Ca^{2+}-salt, $\Delta S^{\#}$ 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 HSαA [23], it could be seen that PPA and the former showed similar trend with respect to the plot of Ea, $\Delta H^{\#}$, and $\Delta S^{\#}$ versus molar concentration of Ca^{2+}-salt. However, both enzymes differed in the trend of $\Delta G^{\#}$ versus molar concentration of Ca^{2+}-salt – increasing trend in the values of $\Delta G^{\#}$ with HSαA [23] unlike

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

Ultimately the moderately high thermal environment enabled the enzyme to thermally reduce the binding interactions including electrostatic interaction due to the presence of extra Ca^{2+}-salt. All these point to the fact that there must be some residual entropy of activation that must have a stimulating or activating effect on the enzyme and consequently a lower $\Delta G^{\#}$ value in the presence of Ca^{2+}-salt. Therefore, mesophiles and thermophiles under moderate temperature should be subject to thermal dissipative contribution of the unfavorable entropic term which provides the needed mobility of the active site in particular for function [37]. However as in the past [23] and report herein for PPA, the negative entropy of activation has been attributed to cooperative behavior of water molecules around the active site [51]. Besides it has been posited that the chloride component of Ca^{2+}-salt for chloride dependent enzyme, alpha amylase, for instance in the presence of a protonated state of Glu – 233 weakens the strength of calcium binding and ultimately the consequence of over rigidification [35]. This is contrary to the claim that chloride binding increases the affinity of the enzyme for calcium ion [53]. “Perhaps, this might be characterized by conditionality”.

It has been observed that the K_m values for Ca^{2+}-salt treated and Ca^{2+}-salt free PPA were increasing with increasing temperature. This simply implies that the affinity of the enzyme for the substrate gets weaker with increasing temperature. Except at 310.15 K, the K_m values for Ca^{2+}-salt treated PPA is higher than Ca^{2+}-salt free enzyme. These situations were unlike report for HSαA [23]. It appeared that at higher temperature the presence of the salt reduced the affinity of the enzyme, PPA for the substrate unlike the effect of SDS on the affinity of BAA and BLA for soluble amylose (DP = 18) [51].

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

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

Since the molar mass of the substrate is unknown the differences in free energy between Ca^{2+}-salt treated and Ca^{2+}-salt free PAA were calculated. But for the value at 310.15 K, the free energy for the hydrolysis of soluble potato starch with Ca^{2+}-salt treated 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 $\Delta G_{N\rightarrow U}$ 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 $\Delta G_{N\rightarrow U}$ 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 $\Delta G_{N\rightarrow U}$ 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 HSαA 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 Ca^{2+}-salt were higher in magnitude than the values for HSαA[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 HSαA [23]. It simply means that LG had greater effect on HSαA than it had for PPA.

5. CONCLUSION

Specific activities of the enzyme increased with increasing temperature and molar concentration of Ca^{2+}-salt. The rate constant (k_2) was higher for Ca^{2+}-salt treated than for Ca^{2+}-salt free enzyme and the values for both enzyme, treated and untreated, increased with increase in temperature. Catalytic efficiency for Ca^{2+}-salt treated enzyme is higher than for Ca^{2+}-salt free enzyme. The activation parameters *E*a and $\Delta H^{\#}$, for Ca^{2+}-salt treated enzyme, were higher than for Ca^{2+}-salt free enzyme while $\Delta G^{\#}$ for Ca^{2+}-salt treated enzyme was lower than for Ca^{2+}-salt free enzyme. The ground state of Ca^{2+}-salt treated enzyme is less flexible than Ca^{2+}-salt free enzyme (lower magnitude of negative $\Delta S^{\#}$ for Ca^{2+}-salt treated than Ca^{2+}-salt free enzyme). Positive *m* – values with ethanol from different sources showed that Ca^{2+}-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℃ (310.15 – 318.15K) should be made available to livestock.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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