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Original Research Articles

The effect of additive (s) and temperature on the velocity of hydrolysis of raw starch with human salivary α - amylase.

ABSTRACT

Aim: The research was carried out to determine the effect of increasing concentration of calcium chloride at different temperatures and a mixture of aqueous ethanol and calcium chloride at 37°C on the activity of human salivary α – amylase (HSαA) and to characterize the effects of the additives (co – solutes) separately, and as a mixture, in terms of activation and thermodynamic parameters.

Study Design: Experimental

Place and Duration: Research Division of Ude Concept International Limited B. B. Agbor and Owa Sec Sch, Owa Oyibu, Delta, Nigeria. The research spanned a period of six months.

Methodology: Bernfeld method of enzyme assay was used. Controls were free from additive(s) while the experimental tests reaction mixture contained either a single additive or a mixture of additives. The additives are different sources of ethanol and calcium chloride.

Results: The values of Gibbs free energy of activation ($\Delta G^{\#}$), are 75.90 ± 0.14 kJ/mol, and 77.28 ± 0.15kJ/mol, in the absence and presence of the salt respectively. The enthalpies of hydrolysis of the starch are 20.18 ± 0.27 kJ/mol and 9.58 ± 0.35 kJ/mol in the presence and absence of the salt respectively. The positive *m* – value ($(113 \pm 3)\times10^4$ J/mol.M) being lower in the presence of local gin (LG) in the reaction mixture than *m* – value ($(121.5 \pm 0.05)\times10^4$ J/mol.M) in the presence of Lord's dry gin (BG), implies that LG is a stronger alcoholic beverage than BG.

Conclusion: The increase in activation parameter resulted in a decrease in activity due to extra stabilizing effect of excess calcium ion. The hydrolysis of starch is enthalpically driven in the absence of the salt, while entropic factor is the case in the presence of the salt. The presence of calcium chloride opposed the inhibiting effect of lower concentration (« 90% (V/V)) of ethanol.

Keywords: Activation parameters; calcium chloride; ethanol; Human salivary alpha amylase; thermodynamic parameters.

1. INTRODUCTION

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Alpha amylase (E. C. 3, 2.1.1 1, 4 - α - D – glucanohydrolase) is known for its capacity to hydrolyze starch by cleaving the internal α - 1, 4 glucosidic bonds [1]. The hydrolysis occurs in a random fashion yielding soluble maltose dextrin, maltose, and glucose [2]. The enzyme such as plasma, pancreatic and salivary alpha amylase has medical application. Thus the enzyme is used for diagnostic purpose [3]. Although the digestive role of mammalian alpha amylase is well known, the thermal environment of the enzyme, apart from well known *p*H and human body temperature, has not been seen as a non-trial issue during dinner. There seems to be little concern for the digestive significance of this enzyme in the light of the fact that on most domestic dining table in affluent homes and hospitality establishment a good number of persons may like to eat but not without pure cold water. It has been observed that direct entry of starch into the small intestine, by passing partial digestion in the oral cavity results in substantially less starch digestion in the small intestine and glucose absorption [4, 5]. However, temperature adaptation of enzymes has been of interest to most researchers [6 – 9]. Nonetheless, it has been shown that while some enzymes are stable and very active at high temperatures, as expected of thermophiles, some of the enzymes are much more stable and active at low temperatures and, other enzymes are stable and very active at moderate thermal environment [9]. Also, the effect of stabilizers such as calcium ion in particular, have been investigated in the light of effect of moderate and extreme thermal environment and the presence of chemical denaturant such as dodecyl trimethylammonium bromide (DTAB) on the catalytic function of alpha amylases [10]. However, one of the most studied natural occurring destabilizing osmolyte urea has its effect on rabbit muscle lactate dehydrogenase opposed by a stabilizer, trimethlamine – N – Oxide (TMAO) [11]. Also polar destabilizers such as ethanol have been investigated for their effect on enzymes. *Bacillus licheniformis* alpha amylase had its activity reduced when exposed to increasing concentration of ethanol [12].

The initial speculation is that calcium ions may oppose the effect of ethanol on alpha amylase. Any delay in the digestion of food in gastrointestinal tract for whatever reason amount to temporary indigestion. Therefore, the duration of assay in this research was limited to five (5) minutes since long – term effect of high temperature, ethanol of whatever concentration, and calcium chloride was not intended so as to conform, without exception, to the usual transient duration of most dinner and most importantly, to the fact that digestion of starchy food in particular begins in the mouth. Therefore, the objectives of research were (1) to determine the apparent effect of increasing concentration of calcium

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chloride on the activity of human salivary α – amylase (HSαA) at different temperatures (2) to determine the apparent effect of a mixture of aqueous ethanol and calcium chloride on the activity of the enzyme, (3) to characterize the effects of the co – solutes separately, and as a mixture, in terms of thermodynamic and activation parameters as the case may be.

2. Materials and Methods

2.1. Materials

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

Equipment used were: *p*H meter (tester) from Hanna Instruments, Mauritius; electronic weighing machine from Wensar Weighing Scale Ltd, Chennai; Centrifuge, 300D model from China; 721/722 visible spectrophotometer from Spectrum Instruments Co Ltd, China.

2.2. Methods

A solution of enzyme was prepared by dissolving 8.5mg in 50cm^3 tris – $HCl_{(aq)}$ buffer as stock. Appropriate dilution was carried out to give ~ 0.223g/dm^3 while soluble starch solution (herein referred to as solution for simplicity sake otherwise it was a mere suspension) was prepared by dissolving 1g in a mixture of tris – $HCl_{(aq)}$ buffer (90cm^3) whose *p*H was 7.4, 5 cm^3 of 6% (W/V) $NaCl_{(aq)}$, and 5cm^3 of distilled water to give 1g% as stock solution. Approximate dilutions of the stock solution of the substrate carried out to give substrate concentrations ranging from 0.5 to 1g% were for the determination of K_m and V_{max} at 25 °C, 37°C, and higher temperatures up to 60 °C in the presence and absence of 4mM calcium chloride.

2.2.1. Assay of 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 [13]. The holoenzyme, human salivary alpha amylase (HSαA), was assayed for a short duration of 5 minutes and the final activity is obtained according to Beer –

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Lambert law as follows: corrected absorbance at wave length, 540nm, × 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. The duration of *in vitro* assay is intended deliberately so as to be similar to the duration of mastication and ingestion of at least a typical fast food few hours before lunch. Most unfolding assay in the presence of denaturant is held for a long time, usually for a period ranging from 1/2 to 24hours. An assay of the enzyme was done with and without calcium chloride in a total reaction mixture of 3 cm^3 composed of 1 cm^3 of substrate (raw soluble potato starch), 1 cm^3 of enzyme, 0.5 cm^3 of calcium chloride and 0.5 cm^3 of distilled (or 1 cm^3 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 cm^3 enzyme, 1 cm^3 of substrate, 0.5cm^3 of ethanol, and 0.5 cm^3 of calcium chloride. Reaction mixture in the test tube was swirled to ensure mixing.

Measurement of absorbance was taken after 3minutes of centrifugation at 3000rpm so as to ensure that no suspension interferes with transmittance. The *p*H was 7.4. N.B.: The initial apparent unit of activity is M/mL.min (mol of reducing sugar yielded per litre (L) of substrate). Since 1mL 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/5min.

The percentage decrease in activity in reaction mixture containing calcium chloride only was calculated as follows: velocity of hydrolysis in the absence of calcium chloride minus velocity of hydrolysis of raw starch in the presence of calcium chloride divide by velocity in calcium chloride – free reaction mixture. The percentage increase in velocity of hydrolysis of raw starch in a reaction mixture containing a mixture of ethanol and calcium chloride was calculated as follows: rate of hydrolysis of raw starch in a reaction mixture containing ethanol and calcium chloride minus velocity of hydrolysis in a reaction mixture containing only ethanol as the only co – solute divide by velocity of hydrolysis in the presence of ethanol only. The percentage decrease and increase in rate of hydrolysis were plotted versus molar concentration of calcium chloride. Determination Michaelis – Menten constant (K_m) and maximum rate of enzymatic hydrolysis (V_{max}) was according to Lineweaver – Burk plot [14]. Since consistency in methodology is vital to reproducibility of any research outcome, explanation and modification of models used in literature had to be made in the proceeding paragraphs.

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2.2.2. The determination of activation parameters.

The determination of activation parameters were carried in two ways. One of the ways involve 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 [15] was used to determine enthalpy of activation and entropy of activation as reported in highly respected journal[15] while activation energy was determined as described in the same paper [15].

The need to determine rate constant and catalytic efficiency for product formation, the maximum velocity of hydrolysis of starch had to be determined and consequently, all cognate activation parameters for the hydrolysis of native soluble starch are calculated using the following equations:

$$\Delta H^{\#} = Ea - RT \quad (1)$$

where R and T are gas constant and absolute temperature respectively and the activation energy (Ea) is given as:

$$\ln k_{cat} = \ln A - Ea/RT. \quad (2)$$

where $k_{cat} = V_{max}/[E_T]$ and k_{cat} is the rate constant, V_{max} is the maximum velocity of enzyme catalyzed hydrolysis, and $[E_T]$ is the total concentration of the enzyme. The entropy of activation ($\Delta S^{\#}$) for the hydrolysis of starch is calculated using the following equation:

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

where $\Delta G^{\#}$ is Gibbs free energy of activation given as:

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

The second approach is as described in the work of EL – Hefnawy *et al* [15].

Since the molar mass of soluble starch is unknown, the difference between the change in 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) denotes in the presence and absence of calcium chloride respectively. For the same reason, the difference between the change in entropy in the presence and in the absence of the salt was determined.

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

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

2.2.3 Determination Gibbs free energy of unfolding and m – values.

The determination of *m* – values described as the slope of the protein folding stability with osmolyte concentration [16] is determined by plotting free energy of protein unfolding (ΔG^o) against co – solute concentration. But a slope is either positive (positive correlation) or negative (negative correlation). Thus according to Rösgen *et al* [16], *m* – value for the protecting osmolyte (or a kosmotrope) is positive while the *m* – value for destabilizing osmolyte (or a chaotrope) is negative. Yet the equation linking ΔG^o and *m* – values is, as often cited in literature in terms of the presence of minus sign [9, 17, 18]:

$$\Delta G^o = \Delta G^o_{(W)} - m[\text{co} - \text{solute}] \quad (7)$$

Other scholars[19, 20] use the equation in which the plus sign is the case:

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

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 co – solute. However, results obtained from reaction mixture containing ethanol and calcium chloride, plotted versus molar concentration of calcium chloride, leads to $\Delta G^o_{[Ethanol(aq)]}$ by extrapolation to infinite dilution of calcium chloride. The subscript [Ethanol (aq)] implies, in line with proposition by Baskakov and Bolen [21], that, the aqueous solution of ethanol serves two purposes: one, as co – solute whose effect on enzyme is presumably opposed by calcium chloride and two, as a co – solvent. “Scholars at both ends, Eq(7) and Eq (8) need not hotly disagree on what may be considered as an appropriate equation, but if on the contrary, the grass, preferably *Oryza sativa*, burns off”.

Meanwhile on the basis of two – state model,

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

where *U* and *N* are the fractions of unfolded protein and fraction of native or folded protein respectively and *U*/*N* is the equilibrium constant (K_{eq}). However, it has been shown that fluorescence and specific activity parameters used to monitor stability of proteins yields similar results, ΔG^o and *m* –

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values. This is possible because, it has been assumed that specific activity, SA, of the native enzyme represents the amount of folded protein, SA_{min} is the minimum specific activity in the absence of stabilizer, and SA_{max} is the maximum specific activity represented by the total amount of folded protein in the limit of high concentration of stabilizer [21].

$$U = (SA_{max} - SA)/(SA_{max} - SA_{min}) \quad (10)$$

But it is not unlikely that the observed activity in a mixture of stabilizer and denaturant, though higher than minimum activity is nevertheless lower than the activity of the native enzyme. Hence,

$$U = (SA - SA_{obs})/(SA - SA_{min}) \quad (11)$$

Equation (11) follows original Pace's equation [22] that depends on fluorescence data. The equation is:

$$U = (A_N - A_{obs})/(A_N - A_D) \quad (12)$$

were A_{obs} is the observed absorbance used to follow unfolding in the transition region, and A_N and A_D are the values of absorbance of the native and denatured conformation of the protein, respectively. This therefore, justifies the assertion of Baskakov and Bolen [21]. Thus according to Pace [22],

$$K_{eq} = U/(1-U) \quad (13)$$

Substituting Eq (13) into Eq (9) gives,

$$\Delta G^o = -RT \ln U/(1-U) \quad (14)$$

It should be noted that K_{eq} can be determined directly according to the following equation [21].

$$K_{eq} = (SA_{max} - SA)/(SA - SA_{min}) \quad (15)$$

However, Eq (15) depends on the relationship: $SA_{max} > SA > SA_{min}$.

2.3 Statistical analysis

All values are expressed as mean ± SD. The sample size, *n*, is equal to 2 (assay was in duplicate). Microsoft Excel was used to calculate standard deviation (SD).

3. RESULTS AND DISCUSSION

Assays of the enzyme human salivary alpha amylase (HSαA) were conducted. The effect of increasing absolute temperature in the absence and presence of aqueous solution of calcium chloride

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was investigated. Effect of ethanol alone from different sources and the effect of a mixture of it and aqueous calcium chloride were also investigated. The results obtained are either presented as in Tables 1a and 1b or graphically presented. In most cases the results, as expected, are compared with results in literature. As Fig 1 shows, the percentage decrease in activity of the enzyme measured at all temperatures except 310.15K (37°C) varies directly with the concentration of calcium chloride which for the sake of brevity is subsequently referred to as salt. This means that at all temperatures (except 310.15K) there was increasing trend in percentage drop in activity with increasing concentration of salt compared with control without the salt. In other words the activity of the enzyme decreased with increase in concentration of the salt. At 310.15K the percentage decrease in activity varied inversely with increasing salt concentration. This implied that the activity showed increasing trend with increasing concentration of the salt, similar to the report that HSαA had, to a small extent, enhanced activity by calcium ion at 37°C [23].

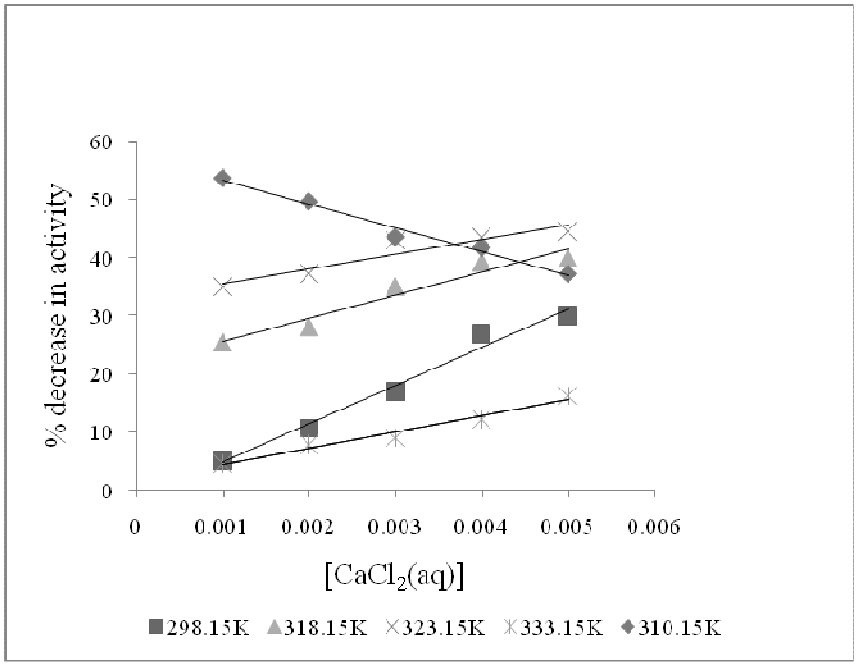

Fig. 1. Variation of percentage (%) decrease of the activity of human salivary alpha amylase (HSαA) with different concentrations of calcium chloride at different temperatures. The concentration of calcium chloride ranges from 1–5mM.

Earlier research [24] has shown that nonmetallic chloride (NH_4Cl (aq)) has inhibitory effect on pig pancreatic alpha amylase activity. The fact is that the salt ($CaCl_2$ (aq)) has dual effect on the

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enzyme. The chloride component described as a physiological effector ligand causes a strong activation (or stimulation) of the α - amylolytic activity of pancreatic amylase and HSαA [25]. On the other hand calcium ion stabilizes the enzyme against environmental stress by subjecting the enzyme to less conformational flexibility [17]. On the contrary, ammonium ion is not known as stabilizer, but, this notwithstanding, this research has shown that, unlike ammonium ion [24] the salt ($CaCl_2$) reduced the activity of the enzyme, due perhaps, to a greater loss of conformational flexibility occasioned by the presence of calcium ion, despite the presence of the chloride component at absolute temperature greater than and less than 310.15K. It makes sense, therefore, to suggest that increasing activity at 310.15K implied in Fig. 1 and as observed elsewhere [23] may be as a result of increasing conformational flexibility with increasing molar concentration of the salt (the chloride component in particular). The observed chloride free HSαA exhibition of 8% of original activity [26] with soluble starch as substrate points to the fact that the increasing percentage loss in activity in the presence of increasing concentration of calcium chloride may have been due to increasing rigidification of the enzyme unmatched by conformational flexibility that should be made possible by the anions. This supports the view that the chloride ion protects *Alteromonas haloplanctis* α-amylase from calcium ion inhibition [27]. The reason advanced for calcium ion inhibition is that the 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 unidentate mode in the enzyme leads to inhibition [28]. On the other hand a protonated state of Glu – 233 in the presence of chloride ion weakens the strength of calcium ion binding and concomitantly its inhibitory effect also [27]. This is despite the claim to the contrary that chloride ion binding increases the affinity of amylase for calcium ion [25]. It seems, in line with the views of Feller *et al* [27] that, there is a context between forces of rigidification of structure and conformational flexibility in which the outcome is net rigidification leading to activities that were less than controls (Fig 1). Also, as shown in Table 1, the maximum velocity of hydrolysis of native (raw) starch in the presence of the salt is lower than in the absence of the salt. Yet it has been reported that calcium ion was stimulatory at 1 and 5mM to another homologue – acidophilic alpha amylase from *Bacillus cereus* MTCC1025 [29]. However, in line with structure – function complementarity, the term stimulatory, a property of chloride ion, for the chloride dependent enzyme, is similar to the term activating which results from conformational flexibility needed for function as against rigidification of 3 – dimensional structure of

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the enzyme for the purpose of stability against adversities of the environment, made possible by the binding of calcium ion.

As shown under Fig. 2, the percentage decrease in velocity of hydrolysis of starch or activity of the enzyme in 18% and 90% (volume / volume (V/V)) absolute ethanol free from calcium chloride are 12.97±1.78% and 53.07±1.53% respectively. Also percentage decrease (or inhibition) in activity of the enzyme in the presence of LG known in various names such as *ogogoro*, *kinkin etc* and BG free from calcium chloride is 16.38±2.38 and 10.16±2.25% respectively. Therefore, LG may contain higher concentration of ethanol than BG.

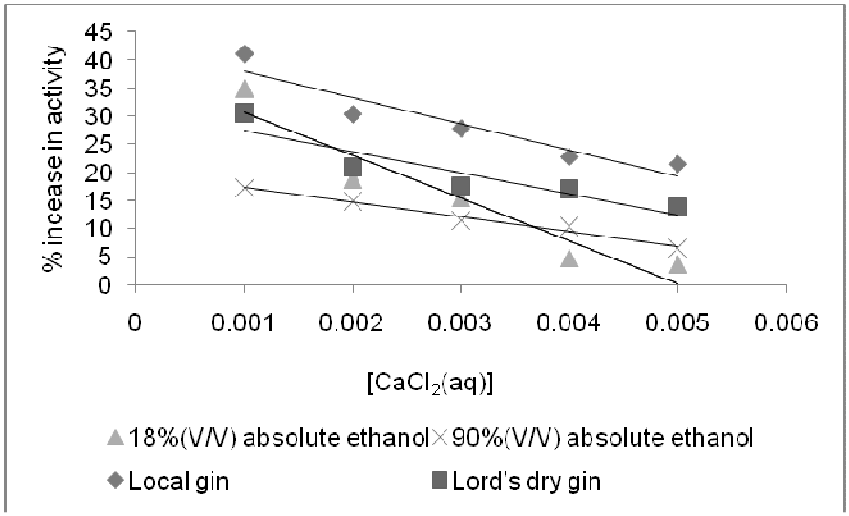

Fig. 2. Variation of % increase in the activity of HSαA in aqueous 18% and 90% absolute ethanol (95% (*V*/*V*)), local gin (LG) and Lord's dry gin (BG) mixed with different concentration of calcium chloride at 37°C. % decrease in activity of the enzyme is reported as mean±SD from duplicate assays (*n* = 2): The values in the presence of 18% and 90% absolute ethanol free from calcium chloride are 12.97±1.78% and 53.07±1.53% respectively. Also % decrease (or inhibition) in activity of the enzyme in LG and BG free from calcium chloride is 16.38±2.38 and 10.16±2.25% respectively.

Effect of ethanol ought not to be in doubt because past research has shown that, the enzyme, *Bacillus licheniformis* retained full activity measured reductometrically in (3:7, V/V) ethanol aqueous buffer mixtures, more than 50% of its activity in (1:1, (V/V)) mixtures and 18% of its activity in (7:3, V/V) mixtures [12]. The enzyme had no activity in ethanol-aqueous buffer (4:1, V/V) [12]. In a mixture

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of ethanol-aqueous buffer (1:1, V/V) the enzyme retains half the activity shown in water alone while in ethanol – aqueous buffer (7:3, V/V) about 20% (perhaps to one significant figure) of the activity is retained [12]. In the presence of varying concentration of calcium chloride, in a mixture of ethanol and the salt, there were rather increases in activities above the activities in the presence of ethanol only. As Fig. 2 shows, the percentage increases were however, decreasing with increasing concentration of the salt. It has also be shown that the presence of calcium chloride dihydrate lead to 115% relative activity compared with the control (100%) [30]. Despite these observations, Fig. 2, and percentage decrease shown below the figure and report in the past, there was a report to the effect that alpha amylase in the saliva and plasma of habitual alcohol drinkers is significantly higher than in non – drinking controls [3]. It is probable that ethanol stimulates the tissues and glands via the nervous system to synthesize and secrete the enzyme. Otherwise it is probable the assay of the enzyme from saliva and plasma may have been assayed *in vitro* by the researcher without ethanol the outcomes of which are 185IU/L and 144 IU/L for habitual alcohol drinkers and non – drinkers respectively [3]. Unfortunately, the authors did not state the kind of substrate used and the number of times the samples were diluted.

The decrease in activity of HSαA in the presence of ethanol only and in the presence of ethanol-calcium chloride mixture, if the activity in the mixture is compared to activity of the enzyme without any co – solute, has different reasons as may be found in literature. The decrease is attributable to the low water activity, lowered solubility of both enzyme and substrate, and denaturation of the enzyme by organic solvent [12] This is slightly different from what has been described as a better solvent than water – the denaturant – such as urea and by extension ethanol, which promotes substrate dissociation from the enzyme because the dissociated state provides greater surface exposure of both the protein fabric and substrate to favourable interactions with the solvent [21]**.** Beyond the consequence of lowered water activity [12, 31] is the suggestion that chaotropic compound like ethanol, decreases the strength of hydrogen bond and other electrostatic interaction due to a change of the dielectric constant of the aqueous medium which, may compromise the structure and function of the enzyme [29, 32]

As may be observed in Table 1a, there was decreasing trend in the values of Michaelis – Menten (K_m) in the presence and absence of calcium chloride (4mM) with increasing temperature. However, the values of the K_m in the absence of the salt were higher than values in the presence of

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the salt. It seems therefore, that the native soluble potato starch has higher affinity for the enzyme in the presence of the salt and at higher temperatures.

Table 1a. Kinetic parameters for human salivary alpha amylase hydrolyzing native soluble potato starch at pH, 7.4.

T/(K)	**298.15**	**310.15**	**318.15**	**323.1**	**333.15**
$V_{max(c=0)}$/mU/mL	103±18	222±16	228±16	260±5	336±11
$K_{m(c=0)}$/g/L	27±0	24.9±0.3	23.2±1.4	19.6±0.4	18.7±0.3
$k_{2(c=0)}$/min	29.61±5.04	63.84±4.67	65.4±4.7	74.74±1.58	96.54±3.11
$k_2/K_{m(c=0)}$/L/min.g	1.08±0.18	2.56±0.19	2.8±0.23	3.81±0.08	5.16±0.17
$V_{max(c)}$ /mU/mL	87±5	130±7	135±16	163±11	298±12
$K_{m(c)}$ /g/L	20.4±2.0	20.3±0.7	18.1±1.5	14.59±0.52	8.2±0.1
$k_{2(c)}$ /min	24.89±1.75	37.37±2.1	38.93±4.62	46.73±3.15	85.64±3.57
$k_2/K_{m(c)}$ /L/min.g	1.22±0.09	1.8±0.1	2.15±0.26	3.29±0.22	10.44±0.44

$V_{max(c=0)}$, $K_{m(c=0)}$, $k_{2(c=0)}$, and $k_2/K_{m(c=0)}$ are maximum velocity of hydrolysis of starch, Michaelis – Menten constant, rate constant for the formation of product, and specificity constant respectively in the absence of calcium chloride; $V_{max(c)}$, $K_{m(c)}$, $k_{2(c)}$, and $k_2/K_{m(c)}$ are maximum velocity of hydrolysis of starch, Michaelis – Menten constant, rate constant for the formation of product, and "specificity constant" respectively in the presence of calcium chloride. Therefore, 1U = micromoles maltose released/mL enzyme in reaction mixture×5min. Using example at 310.15K: 222U ≡ 2.22×10^{-4} M/mL.min (2.22×10^{-4} mmol /mL.min). This is intended to avoid confusion.

With blue starch, as substrate, the K_m values for HSαA A and B are 19.3g/L and 16.6g/L respectively while the K_m value for pancreatic amylase is 12.5g/L [33]. All these values with corresponding V_{max} values such as 421, 433, and 228I.U/mg respectively, are said to be similar but there is a clear substantial difference between value for pancreatic amylase and HSαA [33]. It is not certain what the nature of blue starch is in terms of solubility; texture *etc* and whether it was used in raw state or in gelatinized state. Nevertheless, these values of K_m reported by Takeuchi [33] fall within the range of values shown in Table 1a, in the presence of calcium chloride in particular. As indicated in Table 1a, the K_m at physiological temperature, 37°C and *p*H 7.4, in the presence and absence of the salt are 20.31 and 24.85g/L with corresponding V_{max} values of 130 and 222 mU/mL respectively which, for the purpose of clarity, are equivalent to 0.13 and 0.222(mol/L)/mL.min which are the

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velocities of hydrolysis of 1mL (millilitre) of starch with 1mL of enzyme. Report for B. licheniformis shows that with soluble starch the K_m and V_{max} are 3.4g/L and 19.5U respectively [30]. All these values show that homologous enzymes from different sources show different affinity for the same substrate if such substrate is of the same kind. Also, report in 2015, shows that, the K_m and V_{max} of post carbohydrate meal immobilized HSαA are 1.6mg/mL and 16.4µmol /min (16.4U) respectively [34]. The V_{max} values (Table 1a) obtained were not expected to be high, though the magnitude depends on the concentration of the enzyme, when other factors are held constant, because raw or granular starch and gelatinized starch in aqueous medium may not be digested at the same rate. Evidence to this issue can be found in a research which shows that despite only 1.6U of alpha amylase in dissolved – starch digests and 12U in starch granule digests, the action of 1.6U yielded twice the percentage hydrolysis of starch granules at 35°C in 30 minutes [35]. Also, soluble potato called lintner potato was subjected to 8.0% and 10.10% hydrolysis in 0.5hr and 2hr respectively [35].

The rate constant (k_2) (Table 1a) showed increasing trend with increasing temperature in the presence and absence of the salt. In the presence of the salt k_2 values ranged from 24.89 ± 1.75 – 85.64 ± 3.57/min. In the absence of the salt k_2 values ranged from 29.61 ± 5.04 – 96.54 ± 3.11/min. It is obvious that the k_2 values in the absence of the salt were higher than in its absence. The values were however, much less than 2395/min reported for alkaline *B. licheniformis* (RT7PE1) [30] Alpha amylase from *B. licheniformis* EMS – 6 has k_2 value equal to 152.8/min [2]. The catalytic efficiency otherwise called specificity constant (k_2/K_m) (2.56 ± 0.19 – 3.81 ± 0.081L/g.min) at different temperatures ranging from 310.15 to 323.15K in the absence of calcium chloride is higher than the values (1.84 ± 0.10 – 3.20 ± 0.22L/g.min) in the absence of the salt (Table 1a). At 288.15K, k_2/K_m values in the presence of the salt are slightly higher than without the salt while at 333.15K the k_2/K_m value (10.44 ± 0.44L/g.min) in the presence of the salt is higher than 5.16 ± 0.17 L/g.min in the absence of the salt. These values seem to be much lower than 2395L/3.4g.min; instead of 704.41L/g.min, 7043/0.34% (W/V).min or 7043/% (W/V).min was shown in the Table 3 of that report for *B. licheniformis* in which soluble potato starch was the substrate [30]. According to Ikrah – Ul – Haq, *et al* [2] the catalytic efficiency of alpha amylase from *B. licheniformis* EMS – 6 is 184.09L/g.min.

Although research has shown that enzyme unfolds irreversibly when depleted of its calcium ion at low temperatures and when exposed to excess calcium ion at high temperatures [36], not all unfolding is functionally undesirable. Thus at moderately high temperatures, in the presence and

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absence of calcium chloride, the mesophiles and thermophiles should be subject to the thermal dissipative contribution of the unfavourable entropic term which provides the required mobility for enzyme function [37] made possible primarily by the moderate or high temperature environment as the case may be. Thus activation parameters and apparent enthalpy of hydrolysis of starch were primarily determined according to well known methods. When the enzyme is at the optimal conformational flexibility the enzyme – substrate complex ([ES]) is activated by the addition of Gibbs free energy activation ($\Delta G^{\#}$) whose magnitude constitutes the "energy barrier" to the reaction [6]. The effectiveness of the enzyme in its catalytic function depends on its capacity to reduce $\Delta G^{\#}$. As Table (1b) shows the $\Delta G^{\#}$ values ranges from 74.77±0.13 – 80.58±0.10 kJ/mol in the absence of the salt. In the presence of the salt the range is 75.2±0.19 – 80.92±0.05kJ/mol. Therefore, the capacity of the enzyme to reduce the "energy barrier" diminished in the presence of the salt.

Table 1b. Activation parameters for the ES complex formation and the hydrolysis of native soluble potato starch with human salivary alpha amylase.

T/(K)	**298.15**	**310.15**	**318.15**	**323.15**	**333.15**
$\Delta G_{(c=0)}^{\#}$/ kJ/mol	74.8±0.1	75.9±0.1	77.9±0.3	78.8±0.2	80.6±0.1
$\Delta H_{(c=0)}^{\#}$/ kJ/mol	23.7±3.8	23.6±3.5	23.5±3.5	23.5±3.8	23.4±3.3
$\Delta S_{(c=0)}^{\#}$/ J/mol.K	–171.3±12.6	–168.6±11.4	–170.8±11.1	–171±11	–171.6±10.6
$\Delta G_{(c)}^{\#}$ /kJ/mol	75.2±0.2	77.3±0.2	79.2±0.1	80.0±0.1	80.9±0.1
$\Delta H_{(c)}^{\#}$/ kJ/mol	24±2	24±1	23.9±2.4	23.9±1.1	23.8±1.1
$\Delta S_{(c)}^{\#}$/J/mol.K	–171.5±3.6	–171.9±3.5	–173.9±3.3	–173.8±3.2	–171.5±3.2
$\Delta(\Delta G)$/J/mol	–719.8±1.5	–525.4±0.1	–656.6±0.3	–793.1±0.1	–2283.5±0.4
$\Delta(\Delta S)$/J/mol.K	37.97±0.45	35.9±1.2	35.4±0.6	35.3±0.4	38.7±1.3
Ethanol	**BG**	**LG**	**18%(V/V)**		**90%(V/V)**
$\Delta G^{o}_{N\rightarrow D}$ /kJ/mol	–2.64±0.22	– 2.04±0.93	– 2.23±0.53		– 3.58±0.12
m – values (10^4J/mol.M)	108±33	113±3	58±8		66.0±0.3

$\Delta G_{(c=0)}^{\#}$, $\Delta H_{(c=0)}^{\#}$, and $\Delta S_{(c=0)}^{\#}$ are the Gibbs free energy, enthalpy, and entropy of activation respectively in the absence of calcium chloride; $\Delta G_{(c)}^{\#}$, $\Delta H_{(c)}^{\#}$, and $\Delta S_{(c)}^{\#}$ are the Gibbs free energy,

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enthalpy, and entropy of activation respectively in the presence of calcium chloride. BG and LG are branded gin (Lord's dry gin) and local gin popularly called *ogogoro* respectively. *E*a (activation energy) values in the presence and absence of calcium chloride are: 26.56±1.07 and 26.18±3.52kJ/mol respectively.

The Arrhenius plot for the determination of activation energy and consequently enthalpy of activation is shown in Fig. 3 and, in Table 1b, the increasing trend in $\Delta G^{\#}$ parallels the trend in the values of enthalpy of activation.

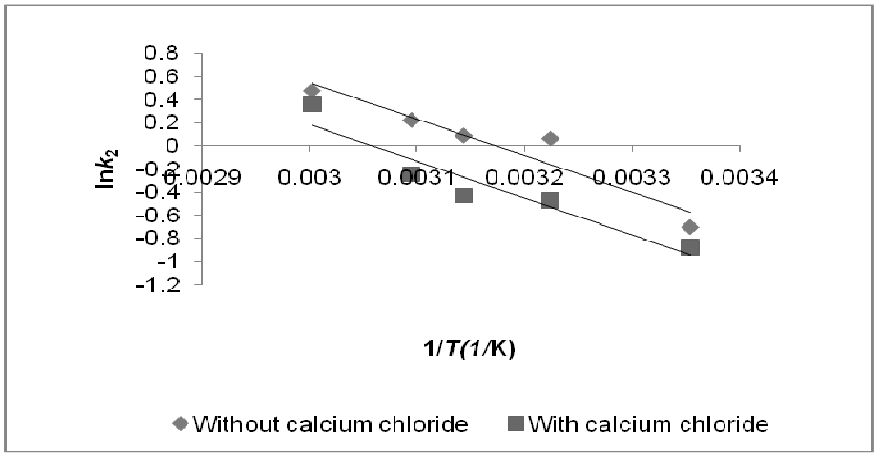

Fig. 3. Arrhenius plot: determination of activation energy for the enzymatic hydrolysis of raw soluble potato starch. Activation energy, *E*a, is presented as mean±SD from duplicate assay (n = 2). *E*a in the absence of $CaCl_2$ (aq) = 26.18±3.52kJ/mol and *E*a in the presence of $CaCl_2$(aq) = 26.56±1.07kJ/mol.

The trend in $\Delta S^{\#}$ is irregular in the absence and presence of the salt. The values of $\Delta G^{\#}$, $\Delta H^{\#}$, and $\Delta S^{\#}$ at 37°C (310.15) are 75.90±0.14kJ/mol, 23.6 ± 3.52kJ/mol, and – 168.68 ± 0.36 J /mol.K respectively and values at 45°C (318.15K) are 77.86 ± 0.26 kJ/mol, 23.53 ± 3.50 kJ/mol, –170.77 ± 11.07 J/mol.K respectively in the absence of the salt (Table 1b). In the presence of the salt the values of$\Delta G^{\#}$, $\Delta H^{\#}$ and $\Delta S^{\#}$ at 37°C are 77.28 ± 0.15kJ/mol, 23.98 ± 1.10 kJ/mol, and –171.85 ± 3.50 J/mol.K respectively, and at 45°C the values are 79.24 ± 0.05 kJ/mol, 23.91 ± 2.43 kJ/mol, and –173.91 ± 3.50 J/mol.K respectively. These temperatures and the corresponding thermodynamic activation parameters are of interest because they represent normal body temperature and moderate

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temperatures within the limit of tolerance of human body – oral cavity and intestinal lumen. The activation energy (*E*a) values in this research are 26.18 ± 3.52 kJ/mol and 26.56 ± 1.07kJ/mol in the absence and presence of the salt respectively. However, research in the past has shown that the *E*a value for the hydrolysis of starch (whose nature is unknown) by immobilized HSαA is 31.kJ/mol. Study by Ikram – Ul – Haq *et al* [2] shows that the *E*a for the hydrolysis of starch by *B. licheniformis* EMS – 6 is 25.14kJ/mol. The *E*a values reported for porcine pancreatic alpha amylase (PPA) and *B. amyloliquefaciens* (BAA) are 11.7 and 17.4 kcal/mol (*ie* 49.02 and 72.91kJ/mol) respectively [9]. The authors reported $\Delta G^{\#}$, $\Delta H^{\#}$ and $\Delta S^{\#}$ at a temperature below the lower limit of the temperature range of the present study. The order of magnitude is however, AHA (*Pseudoalteromonas haloplanktis*) < PPA < BAA at 10°C. PPA is a very close homologue to HSαA and human pancreatic alpha amylase. The values of $\Delta G^{\#}$, $\Delta H^{\#}$, and $\Delta S^{\#}$ reported for BAA with amylose (degree of polymerization = 17) as substrate, are 52.5 ± 0.2 kJ/mol, 26.6 ± 1.6 kJ/mol and – 87.0 ± 0.2 J/mol.K in the absence of SDS at 25°C [38]. The values in the presence of SDS are not inconsequential. The values of $\Delta G^{\#}$, $\Delta H^{\#}$, and $\Delta S^{\#}$, in the absence of the salt as in Table 1b, reported in this study, are 74.77 ± 0.13 kJ/mol, 23.70 ± 3.78 kJ/mol, and –171.29 ± 5.04 J/mol.K respectively. The issue of interest is the magnitude in particular and signs of the parameter(s) where applicable. The free energy stated by [2] was not clearly specified as activation parameter so it cannot be quoted or referred to but the enthalpy and entropy of activation reported for *B. licheniformis* EMS – 6, alpha amylase, are 22.53 kJ/mol and –110.95 J/mol.K respectively.

The results from this current research and in the past should not be a surprise. This is so because, psychrophilic, mesophilic, and thermophilic alpha amylases function catalytically at low, moderate and high thermal environment respectively [9]. Hence as the results show, $\Delta H^{\#}$ which depicts temperature dependence [39] is moderate for mesophilic alpha amylase (HSαA). $\Delta H^{\#}$ values are also related to a number of enthalpically driven weak interactions that have to be broken to enable the enzyme reach the activated complex for function [40] The number of weak interactions that has to be broken are in the following order of magnitude, thermophiles > mesophiles > psychrophiles.

The entropy of activation for the hydrolysis of soluble starch with BAA is positive while it is negative for AHA and PPA similar to the negative values reported in this research and the report for *B. licheniformis* EMS – 6 by Ikrah-Ul-Haq, [2]. The simple reason is that the magnitude of the ground state entropy of the ES (enzyme – substrate complex) is in the order, psychrophiles > mesophiles >

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thermophiles. The activated complex is far more ordered than the ground state ES for all homologues but the magnitude of order is in the following order psychrophiles > mesophiles > thermophiles. This is to say that the magnitude of transition of structural motions from a flexible and mobile active site in the ground state, to a more ordered active site in the activated complex is in the order psychrophiles > mesophiles > thermophiles [9, 41]. This implies that the 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 so that the difference in entropy between activated complex and ground state may be positive as was the case for BAA at low temperature [9]. But this was unlike PAA and AHA as well as HSαA in this research, whose ground state has fewer weak interactions and does not need as much thermal energy to achieve activated complex like thermophiles. However, at higher temperature, 25°C and above all homologues exhibit relatively large negative entropy of activation for the hydrolysis of starch because the ground state has become more mobile but achieved higher relative order in the activated complex. This scenario seems to be prompted due to cooperative behavior of water molecules around the active site [38].

Additional supportive evidence for the effect of extra calcium ions in a reaction mixture on HSαA is exemplified in Figs 4 and 5. The Figures clearly show that the activity of the enzyme was increased with increase in temperature. But the activation parameters exhibited increasing trend with increasing concentration of the salt. This presupposes that the energy barrier has increased resulting in lower activity in the presence of the salt as illustrated in Fig. 1 which shows that the percentage decrease in activity increased with increase in the concentration of the salt at all temperatures except at 310.15K where, the percentage decrease decreased with increasing concentration of the salt.

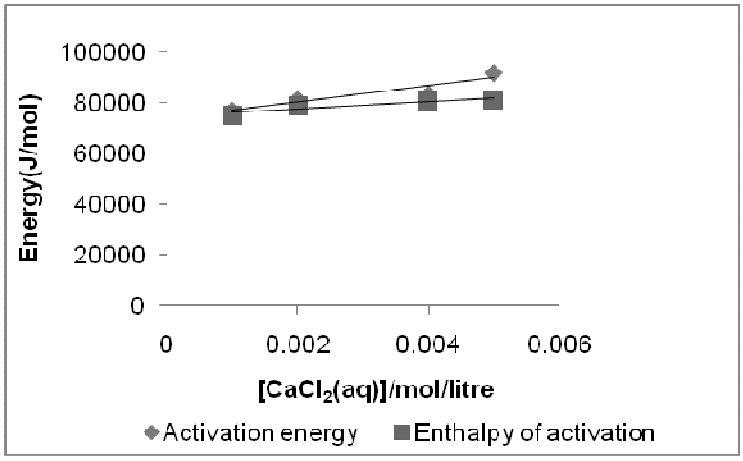

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Fig.4. Variation of enthalpy of activation ($\Delta H^{\#}$) and activation energy (Ea) obtained from the plot of lnv/T and ln$v$ respectively versus 1/T with molar concentration of calcium chloride.

Thus as Fig.4 shows the activation energy and consequently enthalpy of activation increased with increase in the concentration of the salt and, as such, the number of weak interaction that should be broken to reach the transition state has increased [40] due perhaps to additional electrostatic interaction within the 3 – dimensional structure of the enzyme as a whole and active site in particular. The larger coefficient of determination (Fig.5) for the plot of $T\Delta S^{\#}$ versus the concentration of the salt than the value for the plot of $\Delta G^{\#}$ versus the concentration of the salt, suggests that the salt indeed promoted large decease in ground state entropy of the ES following transition to activated ES [9, 41] in concentration dependent manner. Although there was increase in $\Delta G^{\#}$ due to the presence of the salt, it was not strongly concentration dependent manner (R^2 = 0.541) 'probably due to large entropy change largely matched by the enthalpy of activation'.

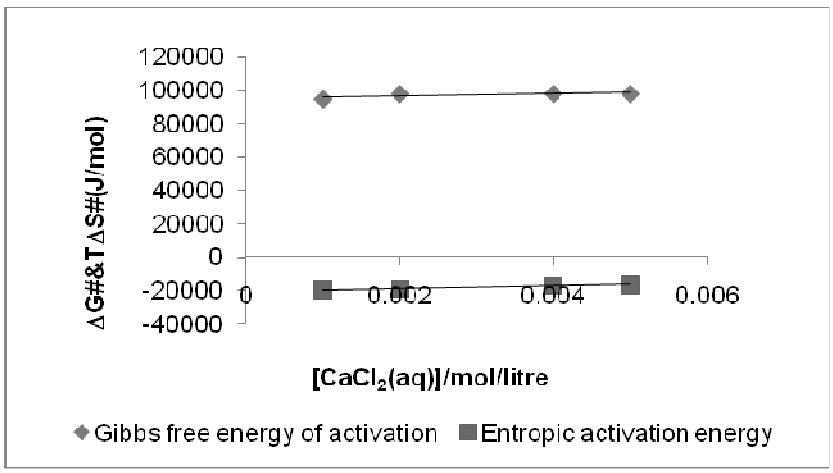

Fig.5. Variation of Gibbs free energy of activation ($\Delta G^{\#}$) and entropic energy ($T\Delta S^{\#}$) of activation with molar concentration of calcium chloride.

The van't Hoff plot for the determination of enthalpy of hydrolysis is shown in Fig. 6. From the plot it can be seen that the enthalpy of hydrolysis in the presence of the salt is higher than in the absence of the salt. However, both enthalpies of hydrolysis of starch with HsαA were endothermic.

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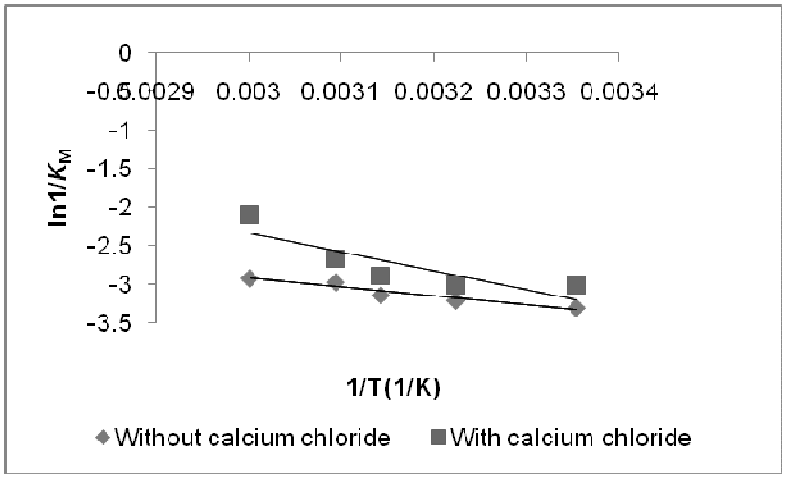

Fig. 6. van't **Hoff plot for the determination of Enthalpy (ΔH^o) of hydrolysis of raw soluble potato starch with human salivary alpha amylase.** ΔH^o values obtained without and with $CaCl_2$ (aq) in the reaction mixture are, 9.58±0.35kJ/mol and 20.18±0.27kJ/mol respectively.

Since the molar mass of soluble potato starch is not known the difference in free energy ($\Delta(\Delta G)$) of enzymatic hydrolysis of starch between calcium chloride containing system and calcium chloride free system was determined. As shown in Table 1b**,** the magnitude of $\Delta(\Delta G)$ values, – 2283.48J/mol which was the highest at 333.15K and – 625.42J/mol which was the least at 310.15K implies that the Gibbs free energy is lower in calcium chloride containing reaction mixture than in calcium chloride free reaction mixture. This is as may be expected because K_m values in presence of the salt were lower than in the absence of the salt thereby suggesting that the enzyme has higher affinity for the substrate in the presence of the salt. Similar to observation in the presence of SDS [38] the presence of calcium chloride led to larger entropy than in its absence because $-\Delta S_{(c)}$ for calcium chloride containing system minus ($-\Delta S_{(c = 0)}$) for calcium chloride free system has negative values. In order words, $\Delta S_{(c)}$ may be larger than $\Delta S_{(c = 0)}$ ($\Delta S_{(c)} > \Delta S_{(c = 0)}$). This difference $\Delta(\Delta S)$ (Table 1b), may have been occasioned by the increase in the amount of dehydration required for ES formation thereby implicating the entropic factor to have made greater contribution to the formation of ES complex [38].

The *m* – values (Table 1b) which express the capacity of a co – solute or co – solvent otherwise generally called osmolyte, to force (un)folding, showed that a mixture of BG and calcium chloride and a mixture of LG and calcium chloride presented a case in which a destabilizer like ethanol is unable to force unfolding due to the opposing presence of a stabilizer like calcium ion. Meanwhile it is not out of place to wonder why less attention is given to *m* – values of ethanol, calcium

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chloride, and sucrose with respect to mammalian (human in particular) enzymes. Thus the positive *m* – value ((113 ± 3)×10^4 J/mol.M) being lower in the presence of LG in the reaction mixture than *m* – value ((121.5 ± 0.05)×10^4 J/mol.M) in the presence of BG, implies that LG is a stronger alcoholic beverage than BG. This is clearly evidenced (so long as Baskakov and Bolen postulation [21] remains reasonable) in the values of $\Delta G^{o}_{([Ethanol(aq)])}$, which in this case represents free energy of unfolding in the absence of calcium chloride that leaves ethanol alone as the only co – solvent; these are – 2.04 ± 0.09J/mol.M and – 2.64 ± 0.22J/mol.M in the presence of LG and BG respectively. The positive sign of *m* – value confirms the postulation that the *m* – value of protecting organic osmolyte such as sucrose, sorbital *etc* is positive as exemplified by *m* – values of organic protecting osmolyte such as $m_{Sucrose}$ = 1.62(±0.1) kcal/mol.M, $m_{Sorbitol}$ = 1.38(±0.18) kcal/mol.M *etc* in respect of organic osmolyte – induced folding of RCAM-T1 (reduced and carboxyamidated RNase T1) [19]. In the presence of 18% (V/V) and 90 % (V/V) absolute ethanol, the *m* – values are (58 ± 8)×10^4J/mol.M and (66 ± 3) ×10^4J/mol.M respectively. The corresponding $\Delta G^{o}_{([Ethanol(aq)])}$(or $\Delta G^{o}_{N\rightarrow D}$) values are –2.23 ± 0.53 kJ/mol and –3.58 ± 0.12 kJ/mol in the presence of 18% (V/V) and 90 % (V/V) absolute ethanol respectively. It is obvious that in the presence of very high concentration of absolute ethanol (90 % (V/V) and calcium chloride, the calcium ion component was unable to totally oppose unfolding or inhibiting effect of ethanol. Hence the negative free energy simply suggests that unfolding was highly favoured or feasible. It ought not to be an overemphasis to state that the positive *m* – value due to 18% (V/V) – calcium ion mixture is another evidence of the capacity of the cation to oppose unfolding or inhibition arising from adverse chemical factor. Also, it is clear that in the absence of the salt (or cation), ethanol at concentrations of 18% (V/V) and 90% (V/V) may inhibit the activity of the enzyme because the $\Delta G^{o}_{N\rightarrow D}$ values being negative, is an expression of the spontaneity of native to denatured state transition. In being negative, the *m* – value in this report, is another confirmation of the claim that regardless of the parameter used in monitoring the stability of the protein (fluorescence or specific activity), the sign and magnitude of the ΔG^{0} values shows RCAM-T1is thermodynamically unstable in buffer solution (ΔG^{0} = – 2.23 kcal/mol (*m* = –1.77) from fluorescence data; ΔG^{0} = – 2.48 kcal/mol (*m* = –1.70) from specific activity measurement) [21].

The significance of this research outcome is not farfetched. People whose work involves long hours of sedentary life – style may not consume heavy carbohydrate meal, but, whatever is consumed can serve the best nutritional interest of such individuals if such meal is taken while very warm, and,

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perhaps with warm none alcoholic beverage followed by warm water for quick digestion and assimilation. This issue is even very important for the athlete, in which a lot of calories need to be consumed. Therefore, a well programmed nutritional regime need to be advocated and adopted such that diet consumed at the right time is such that must be digested under the right thermal internal environment so that source of energy like glycogen in particular can be sufficiently stored before major event. This can enhance the eradication of doping as a means of boosting energy need of the athletes.

4. CONCLUSION

The salt partially decreased the activity of human salivary α – amylase (HSαA) at different temperatures compared to control activity. The presence of calcium chloride increased the activity of the enzyme in the presence of lower concentration of ethanol (absolute ethanol ((18%V/V), LG, and BG) to values of activity higher than control values in the absence of ethanol and the salt. The salt may not totally oppose the effect of much higher concentration of absolute ethanol (90%V/V). The affinity (in terms of $1/K_m$) of the enzyme for the substrate in the presence of the salt is higher than in the absence of the salt. The rate constant, k_2, or maximum velocity of hydrolysis, in the presence of the salt were lower than values in the absence of the salt. The catalytic efficiency otherwise called specificity constant (k_2/K_m) was higher at higher temperature (60°C) and at 25 ^{o}C in the presence of the salt. The energy barrier in the presence of the salt was higher than in the absence of the salt. The formation of ES complex and hydrolysis of starch was entropically driven in the presence of the salt, while in the absence of the salt enthalpic factor was the main driving force. The positive *m* – value (positive slope) showed that at much lower and moderately low concentration of ethanol, calcium chloride can effectively oppose or reverse the effect of ethanol. The free energy of unfolding in the absence of the salt being negative, implied that low, moderate, and high concentration ([Ethanol]) of ethanol can inhibit the function of the enzyme in the following order, low [Ethanol] < moderate [Ethanol] < high [Ethanol].

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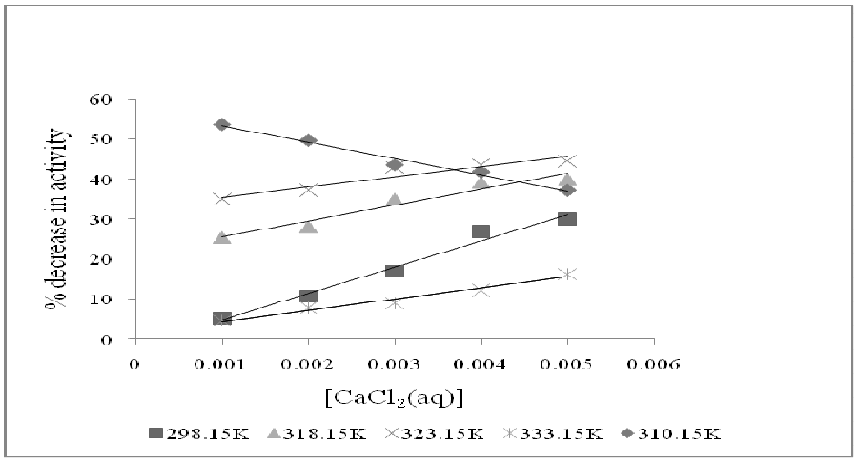

Fig. 1. Variation of percentage (%) decrease of the activity of human salivary alpha amylase (HSαA) with different concentrations of calcium chloride at different temperatures. The concentration of calcium chloride ranges from 1–5mM.

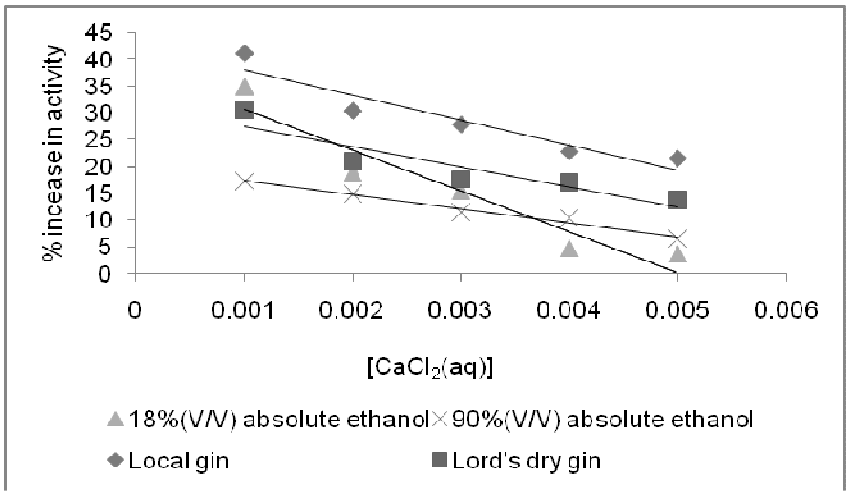

Fig. 2. Variation of % increase in the activity of HSαA in aqueous 18% and 90% absolute ethanol (95% (*V*/*V*)), local gin (LG) and Lord's dry gin (BG) mixed with different concentration

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of calcium chloride at 37°C. % decrease in activity of the enzyme is reported as mean±SD from duplicate assays (n = 2): The values in the presence of 18% and 90% absolute ethanol free from calcium chloride are 12.97±1.78% and 53.07±1.53% respectively. Also % decrease (or inhibition) in activity of the enzyme in LG and BG free from calcium chloride is 16.38±2.38 and 10.16±2.25% respectively.

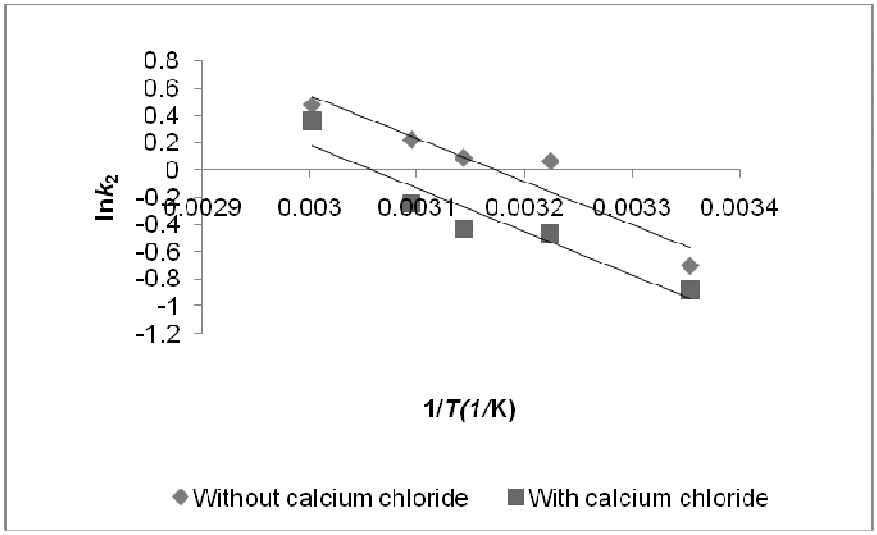

Fig. 3. Arrhenius plot: determination of activation energy for the enzymatic hydrolysis of raw soluble potato starch. Activation energy, *E*a, is presented as mean±SD from duplicate assay (n = 2). *E*a in the absence of $CaCl_2$ (aq) = 26.18±3.52kJ/mol and *E*a in the presence of $CaCl_2$(aq) = 26.56±1.07kJ/mol.

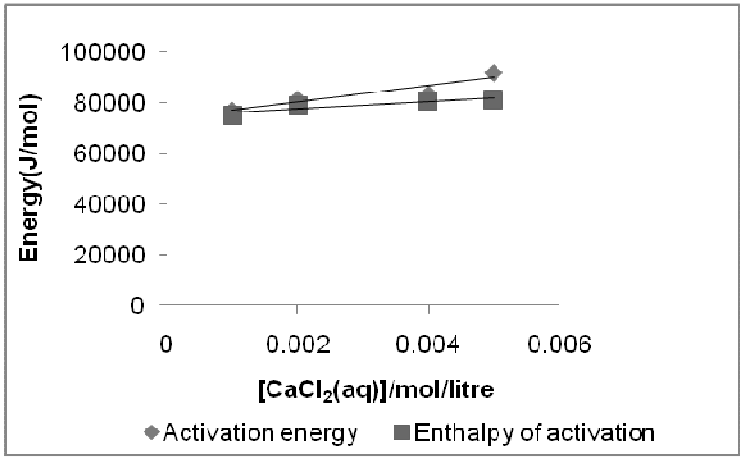

Fig.4. Variation of enthalpy of activation ($\Delta H^{\#}$) and activation energy (*E*a) obtained from the plot of ln*v*/*T* and ln*v* respectively versus 1/*T* with molar concentration of calcium chloride.

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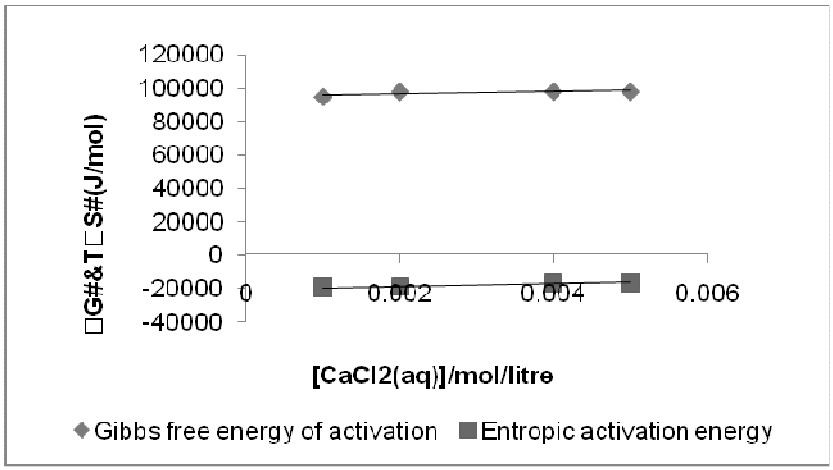

Fig.5. Variation of Gibbs free energy of activation ($\Delta G^{\#}$) and entropic energy ($T\Delta S^{\#}$) of activation with molar concentration of calcium chloride.

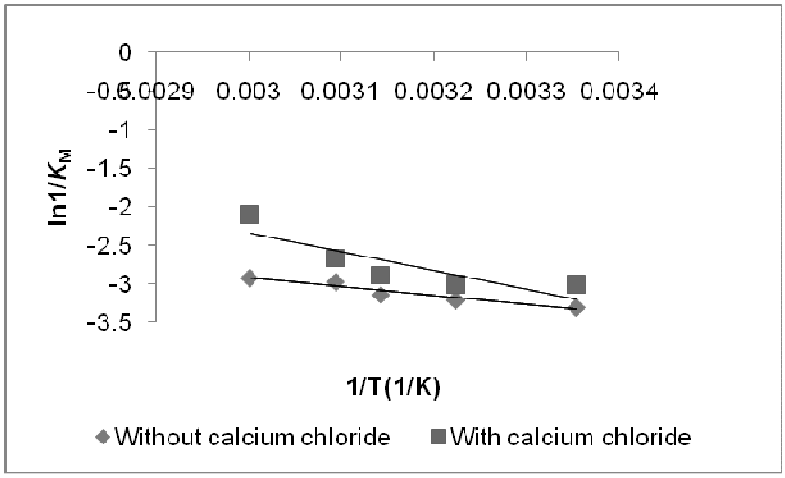

Fig. 6. van't **Hoff plot for the determination of Enthalpy (ΔH^{o}) of hydrolysis of raw soluble potato starch with human salivary alpha amylase.** ΔH^{o} values obtained without and with $CaCl_2$ (aq) in the reaction mixture are, 9.58±0.35kJ/mol and 20.18±0.27kJ/mol respectively.

Table 1a. Kinetic parameters for human salivary alpha amylase hydrolyzing native soluble potato starch at pH, 7.4.

T/(K)	**298.15**	**310.15**	**318.15**	**323.1**	**333.15**
$V_{max(c=0)}$/mU/mL	103±18	222±16	228±16	260±5	336±11
$K_{m(c=0)}$/g/L	27±0	24.9±0.3	23.2±1.4	19.6±0.4	18.7±0.3
$k_{2(c=0)}$/min	29.61±5.04	63.84±4.67	65.4±4.7	74.74±1.58	96.54±3.11
$k_2/K_{m(c=0)}$/L/min.g	1.08±0.18	2.56±0.19	2.8±0.23	3.81±0.08	5.16±0.17
$V_{max(c)}$ /mU/mL	87±5	130±7	135±16	163±11	298±12
$K_{m(c)}$ /g/L	20.4±2.0	20.3±0.7	18.1±1.5	14.59±0.52	8.2±0.1

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$k_{2(c)}$ /min	24.89±1.75	37.37±2.1	38.93±4.62	46.73±3.15	85.64±3.57
$k_2/K_{m(c)}$ /L/min.g	1.22±0.09	1.8±0.1	2.15±0.26	3.29±0.22	10.44±0.44

$V_{max(c=0)}$, $K_{m(c=0)}$, $k_{2(c=0)}$, and $k_2/K_{m(c=0)}$ are maximum velocity of hydrolysis of starch, Michaelis – Menten constant, rate constant for the formation of product, and specificity constant respectively in the absence of calcium chloride; $V_{max(c)}$, $K_{m(c)}$, $k_{2(c)}$, and $k_2/K_{m(c)}$ are maximum velocity of hydrolysis of starch, Michaelis – Menten constant, rate constant for the formation of product, and "specificity constant" respectively in the presence of calcium chloride. 1U = micromoles maltose released/mL enzyme in reaction mixture/5min. Using example at 310.15K: 222U ≡ 2.22×10^{-4} M/mL.min (2.22×10^{-4} mmol /mL.min). This is intended to avoid confusion.

Table 1b. Activation parameters for the ES complex formation and the hydrolysis of native soluble potato starch with human salivary alpha amylase.

T/(K)	**298.15**	**310.15**	**318.15**	**323.15**	**333.15**
$\Delta G_{(c=0)}^{\#}$/ kJ/mol	74.8±0.1	75.9±0.1	77.9±0.3	78.8±0.2	80.6±0.1
$\Delta H_{(c=0)}^{\#}$/ kJ/mol	23.7±3.8	23.6±3.5	23.5±3.5	23.5±3.8	23.4±3.3
$\Delta S_{(c=0)}^{\#}$/ J/mol.K	–171.3±12.6	–168.6±11.4	–170.8±11.1	–171±11	–171.6±10.6
$\Delta G_{(c)}^{\#}$ /kJ/mol	75.2±0.2	77.3±0.2	79.2±0.1	80.0±0.1	80.9±0.1
$\Delta H_{(c)}^{\#}$/ kJ/mol	24±2	24±1	23.9±2.4	23.9±1.1	23.8±1.1
$\Delta S_{(c)}^{\#}$/J/mol.K	–171.5±3.6	–171.9±3.5	–173.9±3.3	–173.8±3.2	–171.5±3.2
$\Delta(\Delta G)$/J/mol	–719.8±1.5	–525.4±0.1	–656.6±0.3	–793.1±0.1	–2283.5±0.4
$\Delta(\Delta S)$/J/mol.K	37.97±0.45	35.9±1.2	35.4±0.6	35.3±0.4	38.7±1.3

Ethanol	**BG**	**LG**	**18%(V/V)**	**90%(V/V)**
$\Delta G^{o}_{N\rightarrow D}$ /kJ/mol	–2.64±0.22	– 2.04±0.93	– 2.23±0.53	– 3.58±0.12
m – values (10^4J/mol.M)	108±33	113±3	58±8	66.0±0.3

$\Delta G_{(c=0)}^{\#}$, $\Delta H_{(c=0)}^{\#}$, and $\Delta S_{(c=0)}^{\#}$ are the Gibbs free energy, enthalpy, and entropy of activation respectively in the absence of calcium chloride; $\Delta G_{(c)}^{\#}$, $\Delta H_{(c)}^{\#}$, and $\Delta S_{(c)}^{\#}$ are the Gibbs free energy, enthalpy, and entropy of activation respectively in the presence of calcium chloride. BG and LG are

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branded gin (Lord's dry gin) and local gin popularly called *ogogoro* respectively. *E*a (activation energy) values in the presence and absence of calcium chloride are: 26.56±1.07 and 26.18±3.52kJ/mol respectively.