

# **Original Research Article**

**Title: Calcium ion binding characteristics of porcine pancreatic alpha amylase outside active site domain and implications: Theory and experimentation.**

## **ABSTRACT**

**Aims:** To: i) show that unfolding and folding of an enzyme can be investigated or interpreted using different aspects of the same model ii) determine the folding rate constant; iii) determine the equilibrium constant for folding; iv) determine activation parameters for unfolding and folding and apparent thermodynamic parameters for calcium ion binding and folding of the enzyme.

**Study Design:** Experimental.

**Place and Duration:** Department of Biochemistry, Ambrose Alli University and Research Division of Ude International Concepts Limited (RC 862217) B. B. Agbor Delta, Nigeria. The research spanned between 2013 and 2016.

**Methodology:** Bernfeld method of enzyme assay was used. Controls were free from calcium chloride. Porcine alpha amylase was assayed at different thermodynamic temperatures for duration of 5 minutes.

**Results:** The enthalpy of activation (e.g.,  $401.32 \pm 7.07$  J/mol) for unfolding, was higher at lower temperature and higher salt concentration. The magnitude of entropy of activation (e.g.,  $-279 \pm 0.06$  J/mol.K) and free energy of activation ( $89.09 \pm 0.01$  kJ/mol) for unfolding was higher at higher temperature and lower salt concentration. **Activation energy ( $2.98 \pm 0.01$  kJ/mol) was higher at higher concentration of calcium chloride.** Free energy of activation, exothermic enthalpy and negative entropy for folding were higher at higher temperatures except at 333.15 K with respect to entropy change. Calcium ion binding constant decreased with increase in temperature and binding was spontaneous, exothermic and positive in entropic term. Folding equilibrium constant values were lower at higher temperatures. Free energy of folding though spontaneous showed irregular trend while the positive entropy increased but decreased at 333.15 K. The exothermic enthalpy for folding was low.

**Conclusion:** The data generated fitted well to the first order equation for calcium ion treated enzyme. The presence of calcium salt stabilized the enzyme against unfolding hence the high free energy and negative entropy of activation (an expression of increased order of the complex) of

unfolding. Calcium ion binding and folding of porcine pancreatic alpha amylase, though exothermic, are entropically driven (entropic term > enthalpic term) and spontaneous. The presence of calcium ion resulted in significant change in the velocity of amylolysis ( $P < 0.05$ ).

*Keywords: Porcine pancreatic alpha amylase, activation and apparent thermodynamic parameters, calcium binding constant, folding first order rate constant, folding equilibrium constant, conformational stability.*

## 1. INTRODUCTION

Porcine pancreatic alpha amylase ( $\alpha$  – 1, 4 – glucan – 4 – glucano – hydrolase E.C. 3.2.1.1) is one of several homologues of starch hydrolases and can be described as a mesophile being moderately dependent on thermal environment for its optimal catalytic activity. Although it is not widely implicated in industrial applications unlike bacterial and fungal homologues [1], it is nevertheless, a very good example of a digestive enzyme needed to study the effect of binding of extra biologically important cofactors on the structure and function of the enzyme via kinetic methods. The importance of folding or unfolding cannot be overemphasized because a careful analysis of protein unfolding and refolding mechanisms may yield important insights into the thermodynamic origins of protein folding, which, may help in elucidating the molecular basis of degenerative disease such as Alzheimer disease and Parkinson disease or diabetes [2]. Stability in the course of industrial application is generally very vital to optimization of the objectives of industrial establishment. Moreover, there is what may be termed physiological unfolding of mesophiles and thermophiles in particular to achieve conformational flexibility which is needed for catalytic function [3]. The presence of what is described as extra calcium ion binding sites as observed elsewhere to the effect that “in porcine pancreatic amylase (PPA), initial heavy-atom substitution experiments seemed to indicate the presence of more than one calcium ion-binding site” [4]; this suggests that the enzyme has intrinsic cation and anion as expected of calcium ion and chloride ion dependent amylases [5, 6]. Thus structurally porcine alpha amylase (PPA) consists of three domains viz: The larger, in the N-terminal part, which consists of 330 amino acid residues is the central domain which has the typical parallel-stranded alpha beta barrel structure ( $\alpha\beta$ )<sub>8</sub>, the C-terminal domain which forms a distinct globular unit where the chain folds into an eight-stranded antiparallel,  $\beta$ -barrel, and the third domain which lies

32 between a  $\beta$ -strand and a  $\alpha$ -helix of the central domain [6]. A pair of aspartic acids has been  
33 implicated to be involved in catalytic activity while a number of other residues which surround the  
34 substrate seem to participate in its binding via hydrogen bonds and hydrophobic interactions [6]. "The  
35 'essential' calcium ion has been located near the active site region and between two domains, each of  
36 them providing two calcium ligands. On the basis of sequence comparison, the calcium binding site is  
37 suggested to be a common structural feature of all alpha amylases"[6]; the chloride binding site lies  
38 on the axis of the domain, A $\beta$ -barrel, near its carboxyl-end: The nearest amino acids forming contacts  
39 with the chloride ion are positively charged residues, such as Arg. 195, Lys. 257, and Arg. 337, that  
40 extend from strands A $\beta$  4, A $\beta$  6, and A $\beta$  8 respectively [6].

41 The implication is that extra calcium and chloride may bind to other sites which may not be  
42 catalytic but may influence the catalytic function as a result of the structural change that may occur  
43 due to the interacting extra salt [7, 8]. The binding of a 2nd calcium ion (when in excess) to the  
44 carboxyl group of Glu – 233, in a bidentate mode, and of Asp – 197, in a unidentate mode, in the  
45 enzyme, leads to inhibition [3]. On the other hand a protonated state of Glu – 233 in the presence of  
46 chloride ion is purported to weaken the strength of calcium ion binding and concomitantly its inhibitory  
47 effect also [3]. These effects are therefore, as a result of the effect of excess salt on three dimensional  
48 structure of the enzyme.

49 Meanwhile, Nielsen *et al.* [9] and Tanaka and Hoshino [10] showed remarkable interest in  
50 calcium ion binding to alpha amylase homologues. They seem to show similar interest in irreversible  
51 unfolding of calcium chloride treated alpha amylase. But the question is: must it always be irreversible  
52 unfolding? Could there be irreversible or at least continuous folding? However, irreversible folding  
53 where applicable cannot be *ad infinitum*. In line with this immediate issue is the observation that  
54 addition of excess EDTA to *Bacillus subtilis* alpha amylases resulted in a 40% decrease in the activity  
55 in 2h at 25°C; but there was complete reactivation after 2 h on addition of excess calcium ions (which  
56 is expected to follow first order rate mechanism) while a similar decrease in activity, though observed  
57 at mere 37°C, could not be reversed completely on addition of calcium ions [11]. It must also be  
58 pointed out that the effect of the presence of calcium chloride as in the work of Tanaka and Hoshino  
59 [10] may not be the same, in its totality, with the presence of calcium sulphate as in the work of  
60 Nielsen *et al.* [9] because chloride and sulphate (VI) ions may exhibit different degree of physico-  
61 biochemical effect on the enzymes in line with Hofmeister series viz:  $\text{H}_2\text{PO}_4^{-1} > \text{SO}_4^{-1} > \text{F}^{-1} > \text{Cl}^{-1} > \text{Br}^{-1}$

62  $> \text{NO}_3^{-1} > \text{I}^{-1} \text{ClO}_4^{-1} > \text{SCN}^{-1}$  [12]; cosolutes can influence both the surfaces of the solid bodies and the  
 63 structure of the liquid (water)[13] which can influence the activity of the enzyme. Therefore, the  
 64 chloride and sulphate (VI) ions may not possess the same effect to the same degree on the solvent  
 65 and solute – the enzyme. Thus it seems unfolding or refolding is a function of the environment to  
 66 which the enzyme is intolerant or tolerantly exposed respectively. If irreversible unfolding can be  
 67 amenable to first order kinetics under a given circumstance, then it may not be inconceivable to  
 68 advocate for the amenability of irreversible refolding to first order kinetics given the absence of  
 69 circumstance that promotes the opposite.

70 Thus, based on the observation of the fact that there is residual activity or even activity higher  
 71 than control, there must be subpopulation of folded and at least partially unfolded molecules of the  
 72 enzyme such that the equilibrium position is either to the left or to the right of equilibrium until the shift  
 73 in equilibrium reaches the far end in the presence of enabling factor. Hence the objectives of this  
 74 investigation are to: i) Show that unfolding and folding can be investigated or interpreted using  
 75 different aspects of the same model ii) determine the folding rate constant; iii) determine the  
 76 equilibrium constant for folding; iv) determine activation parameters for (un)folding and apparent  
 77 thermodynamic parameters for calcium ion binding and folding of the enzyme.

## 78 2. FURTHER THEORETICAL DEVELOPMENT

79 The formulation of model is based on a blend of principles emanating from the works of  
 80 Tanaka and Hoshino [10], and Tabassum *et al.* [14]. *Ab initio*, Tanaka and Hoshino [10] proposed and  
 81 adopted the equation below:

$$82 \quad k_{\text{obs}} = (k_{\text{den}} + K_b k_{\text{den}}^* [\text{Ca}^{2+}_{(\text{aq})}]) / (1 + K_b [\text{Ca}^{2+}_{(\text{aq})}]) \quad (1)$$

83  
 84 where  $k_{\text{den}}$  is the irreversible rate constant for the denaturation of the calcium-depleted enzyme,  $k_{\text{den}}^*$  is  
 85 that for the calcium-bound enzyme,  $k_{\text{obs}}$  is the apparent first-order rate constant, and  $K_b$  is the binding  
 86 constant of the calcium ion to the enzyme. It must be made clear that so long as there is residual or  
 87 higher activity than control, there are likely initial subpopulations of enzymes partially or totally  
 88 inactivated and subpopulation that is activated such that, overtime, there may be net activation or  
 89 inactivation as the case may be. Thus in the presence of the salt there may be net activation with the  
 90 result that the calcium-bound rate constant should be much greater than that of the calcium-depleted  
 91 form; therefore, one assumes that only the bound form contributes mainly to the unfolding-folding

transition, and the depleted form contributions in Eq.(1) should be very infinitesimal compared to the bound form. If this assumption or postulation is reasonable and applied to the experimental data, expected from this investigation, the linear relationship between the values of reciprocal of apparent first-order rate constant and the reciprocal of calcium ion concentrations can be determined: At this juncture, it is important to point out that Tanaka and Hoshino did not subject the test enzyme to chelating agents (though calcium free reaction mixture containing enzyme was tested) as may be testified as follows: “The reaction mixture consisting of  $1.3110^{-8}$  M BAA, 25 mM Tris acetate buffer (pH 7.0) and 0.002-0.02 mM  $\text{CaCl}_2$  was incubated at 45-65°C for the appropriate time intervals”[10]. Thus there is no question of the use of apoenzyme. In the experiment by the authors [10], residual activity measured as percentage of control implied that there was decrease in activity even with increasing concentration of the salt at different temperatures. Then, the question is, what model may be suitable if despite increasing temperature in the presence of increasing concentration of the salt, the activity of the enzyme is increasing?

However, this investigation focuses, in part, on the binding of extra calcium salt and as such binding constant, and cognate apparent thermodynamic parameters and activation parameters of the binding process which become inevitable part of the investigation. Moreover, extra calcium ion binding must be at other sites other than the intrinsic binding sites within the active site domain. In other words unfolding kinetics is not a major part of the investigation even though conformational flexibility occasioned by thermally induced unfolding arising from the break-up of some of the weak binding interaction leading to increase in conformational entropy of activation is the usual characteristics of mainly thermophiles and moderate thermophiles to a lesser extent [15].

In the light of the scope of this investigation in which  $k_{\text{den}} \sim 0$ , the rate equation in the absence of major unfolding for calcium ion treated enzyme is:

$$k_{\text{obs}} = (K_b k_{\text{den}}^* [\text{Ca}^{2+}_{(\text{aq})}]) / (1 + K_b [\text{Ca}^{2+}_{(\text{aq})}]) \quad (2)$$

For the purpose of comprehension, a stepwise approach beginning from Eq. (2) leads to the 2<sup>nd</sup> step after rearrangement:

$$1 + K_b [\text{Ca}^{2+}_{(\text{aq})}] = K_b k_{\text{den}}^* [\text{Ca}^{2+}_{(\text{aq})}] / k_{\text{obs}} \quad (3)$$

$$1/k_{\text{obs}} = 1/K_b k_{\text{den}}^* [\text{Ca}^{2+}_{(\text{aq})}] + 1/k_{\text{den}}^* \quad (4)$$

However, like Tanaka and Hoshino relative activity is adopted herein as applied in a thesis [16] though it has been realized that the rate equation for unfolding leading to decrease in activity under

condition similar to current study, was mistakenly applied with the result that this research offers opportunity for amendment.

However, a serious reappraisal of Eqs (1), (2), (3), and (4) reveals vital issues that may attract contrasting opinion from scholars: For instance one may wish to know why the equations should be strictly for irreversible unfolding kinetics? Calcium chloride treated enzyme ought to show stability with increasing concentration of the salt but not *ad infinitum* as it has been shown that when calcium ions are in excess, the enzyme, *B. halmapalus* alpha amylase unfolds irreversibly at high temperatures [9]. How would the equations apply should there be increasing activity above the activity of the native holoenzyme **with** extra load of calcium salt, with increasing temperature? The same authors [9] observed that at low temperature the same enzyme undergoes irreversible denaturation when depleted of the salt let alone at high temperatures. "Perhaps scientifically naïve, otherwise one would have speculated that unfolding under any circumstance should be governed by first order rate constant,  $k_{\text{den}}$  and should there be refolding,  $k_{\text{den}}^*$  may be folding first order constant in the presence of calcium chloride if there was increasing velocity of hydrolysis (activity that can be expressed as relative activity), a speculation that may prompt recrimination". "But it may not be scientifically naïve to suggest that, when the enzyme mixed with the salt and undergoing unfolding at higher temperature is removed and placed in lower temperature thermal environment containing the same concentration of salt, the enzyme may refold continuously until all molecules are fully refolded". In this regard, Nielsen *et al.* [9] opined that at temperature below 65°C, the de naturation of calcium-bound *Bacillus halmapalus* alpha amylase (BHA) is negligible, since inactivation proceeds almost exclusively as denaturation of calcium-depleted BHA. Thus according to Tanaka and Hoshino [10] the unfolding rate constant,  $k_{\text{den}}$  for calcium-depleted enzyme may be applicable because this was applied to calcium-bound enzyme whose activity decreased with increasing temperature. But this may contradict the fact that calcium-treated or calcium-bound enzyme is the case whose implied first order rate constant for unfolding is different. "This does not lead to a jungle of confusion without a way out of invisibility". The model as shown in Eq (1) seem to suggest that there are two aspect to it, one being that unfolding could assume preeminence due to the effect of destabilizing forces, overcoming in the process the stabilizing forces, the multiple binding interactions intrinsic to the enzyme; hence, Eq (5) below becomes inevitable such that  $k_{\text{den}}^*$  tends to zero. In this case there is irreversible unfolding. But irreversible unfolding is not automatic because it begins at a point in which the energy is sufficient to

cause total unfolding of the total number of molecules. Therefore, Eq. (5) below remains relevant to the end of unfolding at which all binding interactions is nonexistence as the three dimensional (3-D) structure **may have** totally collapsed.

$$1/k_{\text{obs}} = K_b [\text{Ca}^{2+}_{(\text{aq})}]/k_{\text{den}} + 1/k_{\text{den}} \quad (5)$$

At the beginning of unfolding at the melting point, there is a mixture of folded and unfolded hence the absorbance is not the same at any temperature. The absorbance continues to rise with increase in temperature above the melting point until all the molecules unfold or denatures such that further increase in temperature does not lead to increase in absorbance, hence the curve becomes horizontal to the temperature axis of the plot. The same scenario is expected with increasing concentration of denaturants. It seems top-level scholars have no time to reflect deeply into this issue. Thus before maximum absorbance or total unfolding, the equilibrium merely shifts toward the unfolded state with higher concentration in the presence of enabling factor. Hence, an equilibrium constant such as the following is expected:

$$K_{\text{eq}} = U/N \gg 1 \quad (6)$$

where  $U$  and  $N$  are the fraction of unfolded and folded enzyme respectively.

Now the question is can  $N$  tend to zero? If such situation is applicable it would imply that the velocity of hydrolysis (or activity ( $v$ )) of substrate would tend to zero. Meanwhile any residual activity is a function of the presence of folded enzyme and categorically speaking it is not a function of the unfolded enzyme hence, the value of  $v$  for the native enzyme in its entirety is much greater than when there is a subpopulation of the unfolded enzyme. Therefore, one should expect that:

$$v_U = \partial U / \partial t = - \partial N / \partial t = k_{\text{den}} N \quad (7)$$

where  $v_U$  and  $t$  are the rate of unfolding with increasing temperature before final and total unfolding of all enzyme molecules and duration of assay respectively.

From Eq (7), the result (similar to the case elsewhere [17]) obtainable upon integration and rearrangement is:

$$k_{\text{den}} = \ln (N_0 / N_1) / t \quad (8)$$

Equation (8) can be translated to:

$$k_{\text{den}} \sim \ln (v_0 / v_1) / t \quad (9)$$

where  $v_0$  and  $v_1$  ( $v_0 > v_1$ ) are respectively, the activity of native enzyme without salt and the enzyme with salt. As a matter of fact, activity of the enzyme due to the effect of the salt is best described as

residual activity. On the other hand, the activity of the native enzyme could increase with temperature in the presence of stabilizing agents well above what it could have been in the absence of the stabilizer. Thus,

$$N_1/N_0 > 1 \quad (10)$$

where  $N_1$  and  $N_0$  are respectively new population of the folded and initial native population taken as 100%. This is so because,  $N_0 \rightarrow N_1$  which simply imply that the native enzyme assumes greater catalytic power with the possibility too that extremely small population of unfolded may have become folded in the presence of the stabilizer which perhaps may have opposed partially unfavourable ambient aqueous solution of unsteady pH. As observed elsewhere [6], extra calcium salt could also retard the activity of a holoenzyme. Therefore, the following may hold:

$$v_N = \partial N / \partial t = k_{den}^* N \quad (11)$$

where  $v_N$  is the rate of refolding. The modeling is anchored on the fact that there is either overwhelming unfolding or refolding leading to net unfolding or refolding rate constant.

The result after integration of Eq. (11) and rearrangement is:

$$k_{den}^* = \ln(N_1/N_0)/t \quad (12)$$

As it was the case with  $N \rightarrow U$ , Eq.(12) can be transformed to:

$$k_{den}^* \sim \ln(v_1/v_0)/t \quad (13)$$

where indeed,  $v_1 > v_0$ . Equations (9) and (13) represent a simplification of the final forms which may not fall within the immediate scope of these derivations.

It should be pointed out however, that neither Eqs (9) nor (12) would hold if  $U = N$  and  $\partial N = 0$ . Indeed Eq. (12) can only hold when for whatever reason, final form of the native enzyme is catalytically better than original native form which implies that there may have been increase in the native form population. Under such circumstance, there cannot be any unfolded state. What is peculiar to Eqs (8) and (12) is that  $N_0/N_1$  in Eq. (8) and  $N_1/N_0$  in Eq. (12)  $> 1$ . Yet they lead to different conclusion.

The 2<sup>nd</sup> aspect of the model development follows from Tabassum *et al* [14] method which is slightly different from the method of Tanaka and Hoshino who used relative activity. Thus according to Tabassum *et al* [14],

$$k_{obs} = \ln(v_1)/t \quad (14)$$



where  $v_1$  is the residual activity if there is decrease in activity and if there was increase in activity it can best be described as higher activity above initial native activity. However, relative rate of hydrolysis of starch is adopted for the purpose of this research as was the case elsewhere [10].

What may be clear is that from the plot of  $1/k_{\text{obs}}$  versus  $1/[\text{Ca}^{2+}_{(\text{aq})}]$  when  $v_1 > v_0$ , and versus  $[\text{Ca}^{2+}_{(\text{aq})}]$  when  $v_1 < v_0$ , the value of  $N_1$  can then be determined from the intercepts. The following may be useful for the determination of  $N_1$ .

$$1/k_{\text{den}}^* = l_{\text{NT}} \quad (15a)$$

where  $l_{\text{NT}}$  is the intercept.

$$k_{\text{den}}^* = 1/l_{\text{NT}} \quad (15b)$$

But  $k_{\text{den}}^* = \ln(N_1/N_0)/t$ , therefore, with Eq. (15b),  $1/l_{\text{NT}} = \ln(N_1/N_0)/t$  and ultimately,

$$N_1 = N_0 \exp(t/l_{\text{NT}}) \quad (16)$$

By similar derivational steps, the equation for  $N_1$  when  $v_1 < v_0$  can be expressed as:

$$N_1 = N_0 \exp(-t/l_{\text{NT}}) \quad (17)$$

Equations (16) and (17) show that folding or gain in native structure is opposite to unfolding or loss of native structure.

In order to determine Gibbs free energy of unfolding and folding, simple cognate models need to be derived as follows. The folding process entails the transition of what may be seen as “minor fraction” of unfolded in the native initial state population to a greater folded population in the presence of calcium salt. Thus the final folded fraction is as expressed in Eq. (12).

In this case, the fraction of unfolded is:

$$U = N_0 \exp(t/l_{\text{NT}}) - N_0 \quad (18)$$

Since  $K_{\text{eq}} = U/N$ , when there is folding (N)-unfolding (U) transition ( $N \rightarrow U$ ) on the basis of the equilibrium equation ( $N \rightleftharpoons U$ ) characteristic of two state model then  $1/K_{\text{eq}}$  should portray the opposite direction ( $U \rightarrow N$ ). Thus  $1/K_{\text{eq}}$  should be unmistakably,  $N/U$ .

Nonetheless consideration is given to transition from what may be called “native to greater native state” on account of which the term, constant of folding ( $K_F$ ) according to Greenfield [18] is now to be used instead of  $1/K_{\text{eq}}$ . Most importantly is the fact, according to Greenfield [18], that  $K_F = [N]/[U]^n$  where  $n$  is the number of chains that may associate upon folding of polymeric proteins. Nevertheless  $n$  is one in this research. Closer to the needs of this research is the equation [18] for the folding constant (otherwise called equilibrium constant for folding) for a monomeric protein which is  $K_F = \alpha$

/(1- $\alpha$ ) where intuitively  $\alpha$  is the fraction of the folded protein while 1- $\alpha$  is the unfolded fraction.

However, this current approach takes the form:

$$K_F = N_0 \exp(t/l_{NT}) / (N_0 \exp(t/l_{NT}) - N_0) \quad (19a)$$

Simplification of Eq. (19a) gives:

$$K_F = \exp(t/l_{NT}) / (\exp(t/l_{NT}) - 1) \quad (19b)$$

$K_F$  can only be > 1 if  $\alpha > 0.5$  but with Eq. (19b),  $K_F$  may always be >1 in line with the fact that  $[N]/[U] >$

1 when  $k_{den} \rightarrow$  zero. If conformational flexibility increases with increase in temperature due to entropic

term even in the presence of calcium chloride, then at lower temperature say, 37°C, the entropic term

cannot dominate because weak interacting forces are less heat labile at lower temperature; thus

favourable folding of “minor” subpopulation of the enzyme may occur in the presence of calcium salt.

But this is not necessarily a general case for all amylases. The situation may be different when  $k_{den}^*$

$\rightarrow$  zero such that:

$$K_U = (1 - \exp(-t/l_{NT})) / \exp(-t/l_{NT}) \quad (20)$$

In this case unfolding equilibrium constant  $K_U = [U]/[N] > 1$

It is obvious that there is uncertainty as to the applicability of Tanaka and Hoshino model with

respect to Eq. (4) for calcium bound and Eq. (5) for calcium depleted enzyme. Since no depletion is

the case and increasing activity above control is the case, it seems Eq. (4) may be useful in the

determination of binding parameters and cognate thermodynamic parameters. On account of this

uncertainty, alternative models may be formulated in the future to accommodate non-inactivation in

the presence of calcium chloride.

### 3. MATERIALS AND METHODS

#### 3.1 Materials

The chemicals: Alpha amylase (EC 3.2.1.1; 1,4- $\alpha$ -D-glucan glucanohydrolase (porcine pancreatic alpha

amylase (PPA)) was purchased from Sigma, Aldrich, USA; potato starch (contains 0.2% glucose and it

is about 99% pure) was purchased from Sigma Chemicals Co, USA; dinitrosalicylic acid (DSA) which is

97% pure, was purchased from Lab Tech Chemicals, India; 3, 5 – sodium potassium tartrate

tetrahydrate which is 97% pure was purchased from Kermel, China; Hydrochloric acid, 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 as indicated by manufacturer and solutions were made in distilled water.

Equipment: pH 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.

## 3.2 Methods

A solution of enzyme was prepared by dissolving 0.01g in 20 mL tris – HCl<sub>(aq)</sub> buffer as stock at pH 7.4 as a matter of choice as in previous research elsewhere [16]. The enzyme was used as purchased without further purification. 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 [16]. One gram of raw soluble potato starch was mixed in 100mL of tris – buffer at pH 7.4 to give 10g/L; potato starch in aqueous buffer is not a true solution [16]. Various molar solution of calcium chloride ranging from 1 – 5 mM were prepared in distilled water; but the hydrated salt was first thermally dehydrated to constant mass before measurement of mass was made. Assay was carried out with and without (as control) calcium chloride at various temperature ranging from 37 – 60°C (310.15 – 333.15K). Two types of blanks were prepared. Blank containing only substrate and blank containing only the enzyme were used. The sum of the absorbance at 540 nm was subtracted from the test absorbance to give corrected absorbance.

### 3.2.1 Assay of porcine pancreatic alpha amylase for the determination of velocity of amylolysis of raw soluble potato starch, apparent rate constant, rate constant for conditional irreversible folding and calcium ion binding constant.

An *in vitro* assay of alpha-amylase was according to Bernfeld method [19]. The holoenzyme, porcine pancreatic alpha amylase (PPA), was assayed for a short duration of 5 minutes and the final activity is obtained according to Beer – Lambert law as follows: corrected absorbance at wavelength, 540nm,  $\times$  dilution factor (*i.e.* 3)/ $\epsilon l$ , where  $\epsilon$  ( $181.1\text{M}^{-1}\text{cm}^{-1}$ ), C, and  $l$  are molar absorption coefficient, molar concentration of product, and path length respectively. Details on the assay of the enzyme were as described elsewhere [16].

“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, 1UI = micromoles maltose released/mL enzyme in the reaction mixture/5 min” [16].

In order to determine the rate constant for folding the reciprocal of apparent rate constant was plotted versus reciprocal of molar concentration of the salt. By combining the slope and intercept the calcium ion binding constant is calculated. Most importantly, assay was carried out at different temperatures ranging from 37-60°C in the presence and absence of calcium chloride unlike approach by Tanaka and Hoshino [10] who brought the solution of enzyme with and without the salt to lower temperature by “ice-cooling” the mixture without specifying the final temperature. This is to say that at each temperature, duplicate assays were conducted in the presence of different concentration of calcium chloride ranging from 1mM to 5mM giving a total of 48 assays, covering test and control. The work of the previous authors [10] however, provides without losing major scientific quality, a good background for the examination of the calcium salt binding characteristics of the enzyme using raw starch in this study.

### **3.2.2. The determination of activation parameters for unfolding**

Thermodynamic activation parameters, activation energy ( $E_a$ ) enthalpy of activation ( $\Delta H^\ddagger$ ), and entropy of activation ( $\Delta S^\ddagger$ ), were obtained using apparent rate constant ( $K_{obs}$ )

$$\ln k_{obs} = \ln A - E_a/RT. \quad (21)$$

where  $A$ ,  $R$ , and  $T$  are pre – exponential factor, gas constant, and absolute (thermodynamic) temperature respectively. In realization of the fact that  $k_{den}$  and  $k_{den}^*$  are first order rate constants, it is not out of place to determine the thermodynamic activation parameters using them, whether or not it is the first time to do so according to well known Arrhenius method. Hence,

$$\ln k_x = \ln A - E_a/RT. \quad (22)$$

$$\Delta H^\ddagger = E_a - RT \quad (23)$$

$$\Delta S^\ddagger = \Delta G^\ddagger - \Delta H^\ddagger \quad (24)$$

$$\Delta G^\ddagger = \ln (k_B T/hk_x) \quad (25)$$

where  $k_x$  for the purpose of this investigation and in line with the trend in the data generated is either  $k_{den}^*$ , or  $k_{obs}$  as the case may be; and  $h$  and  $k_B$  are the Planck’s constant and Boltzmann constant respectively.

### **3.2.3. The determination of apparent thermodynamic parameters for calcium ion binding**

The apparent thermodynamic parameters namely Gibbs free energy ( $\Delta G^\circ$ ), enthalpy ( $\Delta H^\circ$ ), and entropy ( $\Delta S^\circ$ ) for the binding of calcium ion were determined according to the following equations adopted by Tanaka and Hoshino [10].

$$\Delta G^\circ = -RT \ln K_b \quad (26)$$

$$\partial \ln K_b / \partial T = \Delta H^\circ / RT^2 \quad (27)$$

Equation (27), being van't Hoff equation is used to determine  $\Delta H^\circ$  by plotting  $\ln K_b$  versus  $1/T$ .

$$\Delta S^\circ = (-\Delta G^\circ + \Delta H^\circ) / T \quad (28)$$

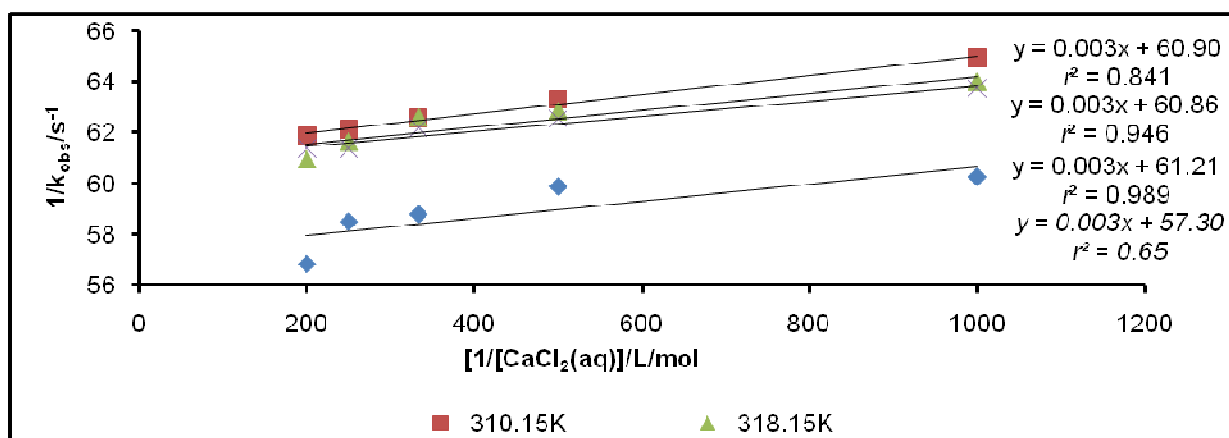
The free energy of folding and enthalpy of folding can be determined by replacing  $K_b$  with  $K_f$  in Eq. (26) and Eq. (27) respectively.

### 3.2.4. Statistical analysis

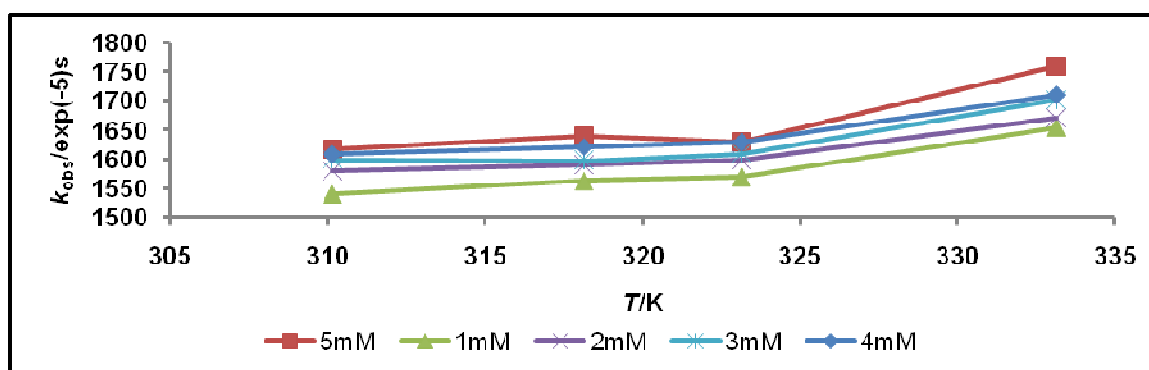
Except otherwise stated, data are expressed as Mean $\pm$ SD, where SD is the standard deviation. All calculations including *t*-test for significant difference between control and test except SD (determined using Microsoft Excel) were manually carried out with electronic calculator. Assays were carried out in duplicates.

## 4.0 RESULTS

In the analysis of result, it is necessary to bear in mind the implication of the exposure of holoenzyme to extra-load of calcium chloride. This should be in sharp contrast to calcium ion or salt-depleted enzyme exposed to increasing temperature alone and apoenzyme exposed to increasing temperature in the presence of increasing molar concentration of the salt. In order to determine rate constant in the absence of unfolding, the reciprocal of apparent rate constant was plotted versus reciprocal of molar concentration of the salt, calcium chloride (Fig. 1). There was the need to show the effect of temperature in the presence of calcium salt as indicted in Fig. 2. Figure 2 shows that, the apparent rate constant showed increasing trend with increase in temperature. The *t*-test carried out to verify whether or not the presence of calcium salt caused significant change in the velocity of amylolysis showed that the calculated *t* which was higher at 310.15K than at 318.15K, is > critical *t* (*t*<sub>0.05 (2),4</sub>). Thus there was significant change in the velocity of amylolysis (*P* < 0.05).

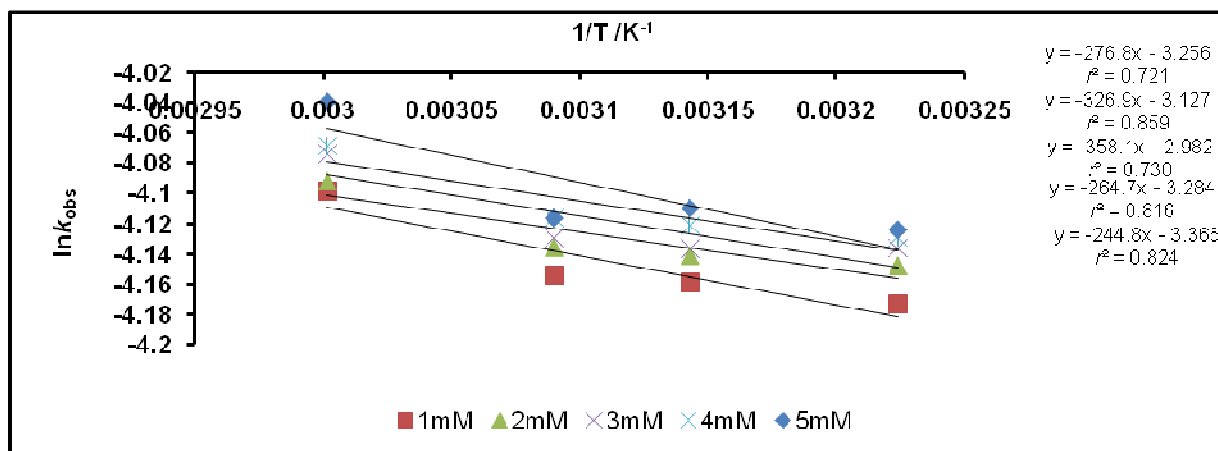


**Fig.1. Plot of reciprocal of apparent first-order rate constant  $k_{obs}$  versus reciprocal of  $[CaCl_2(aq)]$  for the determination of rate constant for refolding of the enzyme and calcium ion binding constant at 310.15 K and 318.15 K. (■), (▲), (×), and (◆) are assays and plots at 310.15 K, 318.15 K, 323.15 K, and 333.15 K respectively.**

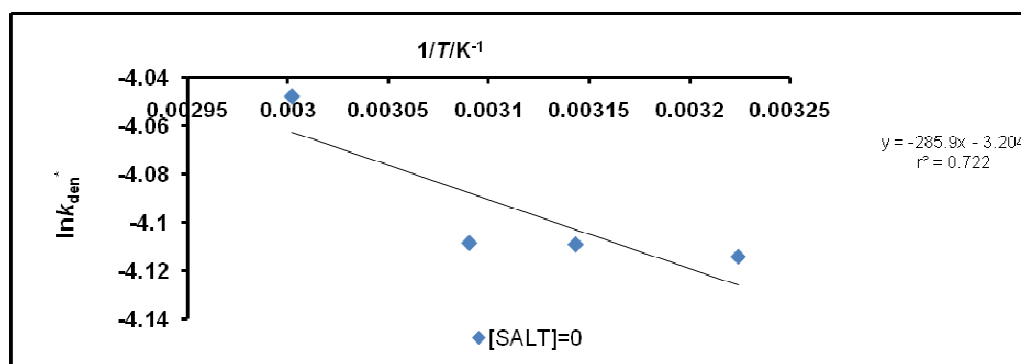


**Fig. 2. Plot of apparent rate constant ( $k_{obs}$ ) versus absolute temperature ( $T$ ) showing the trend of  $k_{obs}$  with increasing  $T$  at different molar concentration of calcium chloride. (■), (▲), (×), (\*), and (◆) are assays and plots at 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM respectively.**

Thermodynamic activation parameters and apparent thermodynamic parameters for calcium ion binding on sites other than catalytically functional site were determined and the results are shown in Table 1. In Table 1 are results for first order rate constant and the activation energies for (un)folding while in Table 2 are results for calcium ion binding constant and apparent thermodynamic parameters for folding. The usual Arrhenius plots, Figs 3 and 4 for unfolding and folding respectively were used to obtain the activation energies.

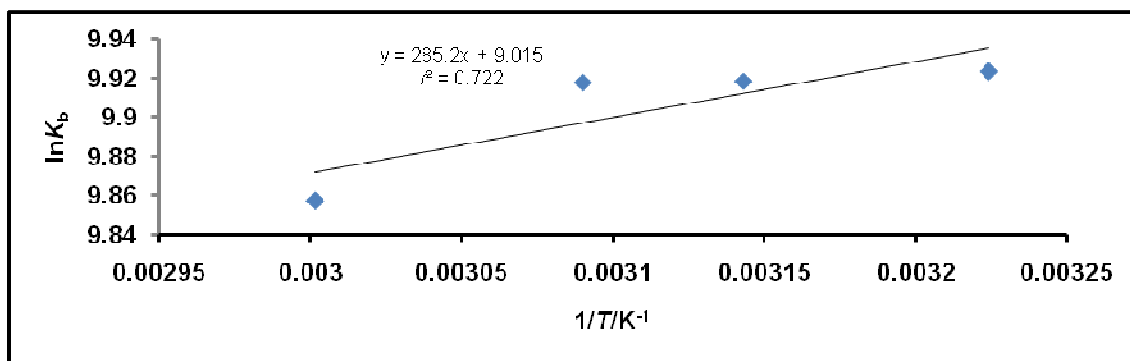


**Fig. 3. Arrhenius plot for the determination of activation energy for unfolding of enzyme at different salt molar concentrations. (■), (▲), (x), (\*), and (◆) are assays and plots at 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM respectively.**

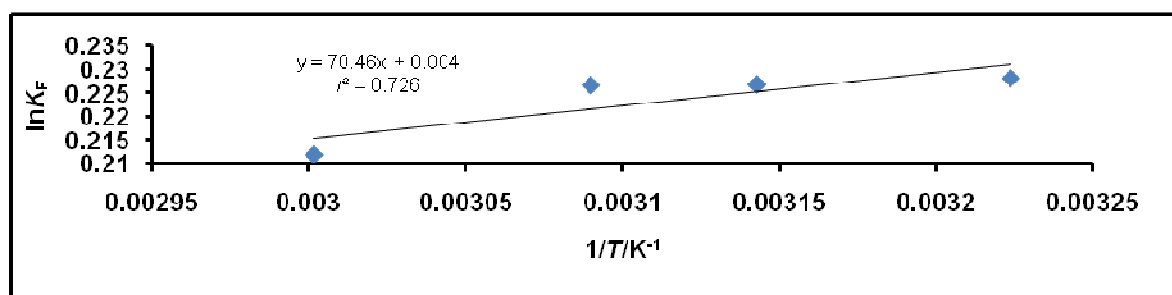


**Fig. 4. Arrhenius plot for the determination of activation energy for the refolding of the enzyme at different salt molar concentrations.**

The determination of enthalpy of calcium ion binding to sites other than active site domain was carried out according to van't Hoff method by plotting natural logarithm of binding constant versus reciprocal of thermodynamic temperature (Fig. 5). In the same vein, the enthalpy of folding was determined (Fig.6).



**Fig. 5. van'Hoff plot for the determination of enthalpy of calcium ion binding to sites apart from intrinsic sites.** Calcium ion binding constant ( $K_b$ ) is obtained from the combination of the intercept and slope of the plot of the reciprocal of apparent rate constant versus reciprocal of molar concentration of calcium chloride.



**Fig. 6. van't Hoff plot for the determination of enthalpy of folding in the presence of cation.**

As shown in Table 1, the Gibbs free energy of activation for unfolding exhibited incremental trend with increase in temperature at different molar concentration of the salt. Expectedly, the enthalpy of activation for unfolding, though endothermic, exhibited decreasing trend with temperature in line with implication of Eq. (23) but it is higher at higher concentration of the salt. Besides, the activation energy values appeared to be higher at higher salt concentration (Table 1). Looking at Table 1, it could be seen that there was large decrease in entropy. The difference in the magnitude of entropy change is not large but the values of the parameter are slightly higher at higher temperature but lower at higher salt concentration (Table 1).

In line with proposed folding rate constant, it is observed that there was increasing values of the parameter with increasing temperature and parallel to this, is also the increasing trend in the values of free energy of activation for folding with increasing temperature as may be found in Table 1. The enthalpy of activation for folding is exothermic, the magnitude of which showed increasing trend



with temperature just as the negative magnitude of entropy of activation for folding showed increasing trend though the value at 333.15K is only lower than value at 323.15K (Table 1). The Arrhenius pre-exponential factor for unfolding is higher at higher salt concentration (Table 1). It is also, higher than value reported for folding of the enzyme. Also in Table 1 is the activation energy for folding that is lower than  $RT$ .

**Table 1. Activation parameters for the binding of calcium ions and folding of the enzyme.**

1		5	
310.15	318.15	310.15	318.15
86.82±0.00	89.09±0.01	86.70±0.00	88.96±0.00
141.32±21.21	74.81±21.21	401.32±7.07	334.81±7.07
-279.47±0.07	-279.79±0.06	-278.25±0.02	-278.59±0.02
2.72±0.02		2.98±0.01	
43.85±0.35		50.69±0.14	
310.15	318.15	323.15	333.15
1633.70±0.35	1642.00±0.35	1643.00±0.00	1715.50±0.71
86.67±0.00	88.96±0.00	90.49±0.00	93.11±0.00
-201.68±67.601	-268.19±9.90	-309.77±9.90	-392.91±9.90
-280.11±0.04	-280.47±0.04	-280.71±0.04	-280.66±0.03
2.37±0.01			
40.50±0.71			

$\Delta H^\# = \Sigma(E_a - RT)/2 \pm SD$ ;  $\Delta S^\# = \Sigma(\Sigma(\Delta G^\#)/2 - \Delta H^\#)/2 \pm SD$ ; The activation parameters  $E_a$ ,  $\Delta H^\#$ ,  $\Delta S^\#$ ,  $\Delta G^\#$ , and  $A$  are activation energy, enthalpy of activation, entropy of activation, free energy of

activation and pre-exponential factor respectively. The corresponding activation parameter for folding is represented by  $F$  as subscript.

The calcium ion binding constant showed decreasing trend with temperature just as negative free energy for the binding of cation showed increasing trend in magnitude with increasing temperature (Table 2). As recorded in Table 2, the enthalpy of calcium ion binding is exothermic and the positive entropy showed decreasing trend except the value at 333.15K which is larger than all values at lower temperatures. The folding equilibrium constant showed decreasing trend with temperature while the negative free energy of folding exhibited increasing trend in magnitude except the value at 333.15K, which, is lower in magnitude than values at lower temperatures (Table 2). The enthalpy of folding is exothermic and the positive entropy of folding exhibited increasing trend. However, the value of the positive entropy at 333.15K was lower in magnitude than all values at lower temperatures (Table 2).

**Table 2. Calcium ion binding constant and thermodynamic parameters for calcium ion binding and folding.**

$T$ (K)	310.15	318.15	323.15	333.15
$K_b$	20403.55±4.72	20390.00±4.71	20286.67±7.07	19100±9.43
$\Delta G_b$ (kJ/mol)	-25.59±0.00	-26.24±0.00	-26.65±0.00	-27.31±0.01
$\Delta H_b$ (kJ/mol)	-2.37±0.01			
$\Delta S_b$ (J/mol.K)	74.86±0.00	75.02±0.00	75.12±7.07	74.84±0.02
$K_F/\text{Exp}(-4)$	12563.40±0.71	12547.00±0.71	12545.00±0.00	12360.30±0.99
$\Delta G_F$ (J/mol)	-588.49±0.15	-600.19±0.15	-609.19±0.00	-586.96±0.22
$\Delta H_F$ (J/mol)	- 585.83±1.41			
$\Delta S_F/\text{Exp}(-3)$ (J/mol.K)	8.56±0.47	47.00±2.10	72.00±0.00	3.39±0.70

$\Delta S_b = \Sigma(\Delta G_b - \Sigma\Delta H_b/2)/2 \pm \text{SD}$ ;  $K_b$ , and  $k_{\text{den}}$ , are calcium ion binding constant and rate constant for the folding;  $\Delta S_b$ ,  $\Delta G_b$ , and  $\Delta H_b$  are entropy, free energy, and enthalpy of calcium ion binding respectively.  $\Delta G_F$ ,  $\Delta H_F$ , and  $\Delta S_F$  are free energy, enthalpy, and entropy of folding of the enzyme respectively.

## 5. DISCUSSION

“The actual calcium binding is considered to include the folding of enzyme, because the calcium-depleted apoenzyme is known to be inactive and easily unfolded; thus the obtained parameters include the process of calcium binding and structural folding of the protein” [10]. This quotation is pregnant with simple yet vital interpretation viz: though it has been suggested that as folding proceeds, the equilibrium population partitions into two predominant species, unfolded and native, with only negligible population of intermediate species [20], under any given condition outside the optimal environment of the enzyme, presence of extra calcium salt for instance, with increasing temperature, there may be subpopulations of unfolded, partially folded, and folded, such that the final direction should therefore, depend on preponderance of any of the opposing forces either for folding or unfolding. This seems to be in agreement with the view that the folding of sufficiently small proteins is highly cooperative, akin to first order phase transition [21]. Also, the data obtained elsewhere show that folding in the presence of calcium ion is similar to that in its absence, although the rate is increased by more than two orders of magnitude [22]. There is the issue of calcium binding/unfolding, a process that demands activation process or input of activation energy. However, on account of this study in which the apparent rate constant, showed increasing trend (Fig. 2) as to imply that the enzyme is under optimal condition for its 3-D structure of its native state, it may seem that there is no form of unfolding. On the contrary there must be what can be described as functional unfolding needed for catalytic activity peculiar to mesophiles and thermophiles in particular [3]. The question is at what point does functional unfolding begin? “The answer may be at the point of highest stability under a given condition, the presence of extra salt for instance, to be proceeded with thermally induced unfolding backed with the effect of chloride component in the presence of the substrate prior to its transformation”.

The Gibbs free energy of activation for the unfolding of the protein following the binding of calcium ion appears to be higher at higher temperatures than lower temperature (Table 1). This seems to suggest that the energy barrier for unfolding of the enzyme is higher at higher temperatures due to the opposing effect of calcium ions by their rigidifying or stabilizing effect on the 3-D structure [23]. It seems too that the free energy of activation at higher salt concentration is lower than at lower salt concentration; this was unlike the report for *Bacillus amyloliquefaciens* [10] which, unlike PPA in this study showed higher enthalpy of activation for unfolding at higher salt concentration. This may be

as a result of the effect of higher salt concentration in promoting greater conformational flexibility due to increasing chloride ion component, because chloride ion opposes the effect of calcium ion; 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 [3]. Being endothermic, higher thermal environment may be needed for calcium ion binding such that with higher temperatures the binding of the ion should be more spontaneous. This may therefore, promote higher rate of folding in opposition to unfolding with increase in temperature, that may be imposed by the presence of extra calcium ion without insinuating indefinite process as shown in Table 1.

As can be observed in Table 1, there are negative entropies in opposition to what is expected of unfolding; this may be as a result of the calcium ions' action in promoting folding though the magnitude of negative entropy seem to be higher at lower salt concentration due perhaps to less effect of chloride component of the salt that is known to oppose the cation component [24]. The ultimate effect of calcium ion action is what has been described as rigidification and loss of conformational entropy following folding of the enzyme [25].

Forge *et al.* [22] reported folding rate constant equal to 20.2/s at pH, 7.2 and 20°C in 10 mM calcium chloride while Deitcher *et al* [26] reported folding rate constant ranging from 0.05 to 0.1/min. This range is about 10-20 fold lower than values recorded in this investigation. Enhanced folding which implies stability promotes catalytic activity of the enzyme similar to the observation that the presence of calcium ion increases the activity and stability of the enzyme at high temperatures [27]. Current values (Table 1) of folding rate constant at different temperatures are however, closer to the reported values of  $2.3 \pm 0.6 \times 10^{-2}$ /s and  $2.8 \pm 0.8 \times 10^{-2}$ /s for peptide WT and G14 peptide respectively [18]. Before proceeding further into the discussion, it is imperative to state that the implication of Eq. (4) is that when  $[\text{CaCl}_{2(aq)}] \rightarrow \infty$ ,  $1/[\text{CaCl}_{2(aq)}] \rightarrow \text{zero}$ ; this implies theoretically, given other favourable and practicable condition, that the calcium ion component can resist total unfolding so long as increasing velocity of hydrolysis of substrate is observed with increasing temperature. This is exactly opposite to what is expected when there is decrease in activity, such that Eq. (5) becomes the case in which as  $[\text{CaCl}_{2(aq)}] \rightarrow \text{zero}$ , the unfolding rate constant  $\rightarrow \text{zero}$  so long as there is decline in the activity of the enzyme due to unfavourable unfolding due to the presence of the salt either with increasing or decreasing temperature. Most reactions become more spontaneous with increase in temperature. The implication is that binding of cation and folding of the enzyme cannot be extricated

from each other. Hence, the proposition, that the calcium ion binding is parallel to the folding of the enzyme [10]. Conditional irreversible folding is intended to imply that continuous folding must be contingent on the availability of extraneous factors that can promote refolding until the entire protein population becomes folded. But this is not without input of free energy of activation [28]. The free energy of activation appeared to be larger at higher temperature. This may be as result of increasing conformational flexibility (this is needed for catalytic function) occasioned by the increasing thermal energy of the environment in addition to the known effect of the chloride component which is described as activating or stimulating (activating effector) on account of which it is able to increase so-called turnover number otherwise called rate constant for product formation ( $k_{cat}$ ) by 30-fold towards either starch or p-nitrophenylmaltoside [24] through enhanced conformational flexibility opposed to rigidification, a function of the cation component. Besides the protonated state of Glu – 233 in the presence of chloride ion weakens the strength of calcium ion binding and concomitantly its inhibitory effect also [3] and possibly its rigidifying effect.

Thus not just activation energy (free energy) for inactivation (unfolding) alone that occurs with increasing temperature but there could also be free energy of activation for folding, the balance of which determines the direction of equilibrium but not without the presence of stabilizer. There is a strong evidence that the direction of equilibrium is towards folding hence the increasing activity/apparent rate constant (but with a slight drop at some point (Fig. 2)), though not without conformational flexibility needed for enzyme function in the presence of the salt. Thus one may suggest that the increasing free energy of activation for unfolding with increasing temperature may have been opposed by the increasing calcium salt. In other words there may be increasing resistance to unfolding even with increasing temperature due to the presence of excess calcium salt. But the presence of substrate presents different scenario to the effect that part of the unfolding must be seen as a necessary part of conformational flexibility needed for catalytic function but within limits stabilized by the cation.

Low activation energy for any process is a pointer to the likelihood of the spontaneous nature of such process accounting for the fact that high thermal energy may not be necessary for commencement of such process. Hence the low activation energy for irreversible folding should necessarily lead to very low enthalpy of activation or as observed in this report, negative enthalpy of activation for folding. The negative enthalpy is higher at higher temperature as to suggest that

unfolding is slightly unfavourable or that the unfolded state or complex is transient at high salt concentration as shown in Table 1. As stated earlier the entropy of activation is negative suggesting loss of conformational entropy **in line with** stability or rigidification of the activated complex unlike the report for *Bacillus amyloliquefaciens* [10] or unlike what has been described as the positive entropy of activation which **implies increasing** local disorder in the transition state when compared with the ground state reported for tryptophan synthase [29]. The **reasons** advanced for the increase in entropy (positive entropy change) for *Bacillus halmapalus* alpha amylase, the release of water molecules from the calcium ion binding site and the dehydration of non-polar surfaces on calcium ion binding [9, 30] **are** a direct contrast with what may be the underlying **reasons** for entropy decrease as observed for PPA. But the large negative entropy should also arise from the fact that there was folding from relatively more flexible ground state to more ordered transition state as the cation binds [15].

Looking at Table 2 one observes that the calcium ion binding constant showed lower values at higher temperatures similar to the observation by Nielsen *et al.* [9] and Tanaka and Hoshino [10] with respect to *B. halmapalus* alpha amylase (BHA) and *Bacillus amyloliquefaciens* alpha amylase (BAA) respectively. The values with respect to BAA were similar to current report for PPA at higher temperature, 60°C and 65°C but the values at lower temperatures were much higher than values for PPA. This is most likely considering the fact that the binding constant at temperature lower than 37°C (specifically 22°C) as originally reported for human salivary alpha amylase elsewhere [31] was very much higher than values at 37°C and above. Values reported for BHA were very much higher than values for PPA in this report at all temperatures. The expression for the stability of the product of any biochemical reaction such as calcium ion binding was exothermic though much less than the report for BAA at pH 7.5 [10] and value for BAA as high as **-149 kJ/mol** at pH 7 [32]; but all observations **point** to stable calcium-enzyme complex at sites other than active site. While the entropic term,  $T\Delta S$ , of calcium ion binding is **-109.13±0.61 kJ/mol**, there is proposition that calcium binding may consist both of contributions from calcium binding itself and of protein folding; thus there may be need to subtract the contribution of calcium binding enthalpy (**- 8 kJ/mol**) and entropic term (**- 42 kJ/mol**) so as to yield folding thermodynamic parameters such as **-157 kJ/mol** for enthalpic term and **-151 kJ/mol** for entropic term ( $T\Delta S$ ) and ultimately, the value of the free energy of folding in terms of  $\Delta G = \Delta H - T\Delta S$ , should be **- 6 kJ/mol** [10]. However, the result presented in Table 2 shows some similarities and differences in comparison with the above observation in literature. The magnitude of the enthalpy

of calcium binding in this study is less than literature value but both are exothermic though for homologues from different sources. The entropy of calcium ion binding at 30°C and the free energy of calcium ion binding for BAA are  $-138.55 \text{ J/mol.K}$  (from  $-42 \text{ kJ/mol/303.15 K}$ ) and  $-34 \text{ kJ/mol}$  respectively [10]. This entropy value, though not very widely different from report in this investigation (Table 2) it is nevertheless opposite in sign to current result. The current result (entropy of calcium ion binding) being positive, may be attributable to the release of water molecules from the calcium-binding site and dehydration of non-polar surfaces on calcium ion binding [9]. The free energy of calcium ion binding to BAA is expectedly spontaneous similar to values presented herein (Table 2) in this investigation, but the literature value is higher.

In the same Table 2, it can be observed that folding equilibrium constant is slightly greater than unity and the values appeared to decrease with temperature and consequently the free energies though express spontaneity are nevertheless much lower than literature value of  $-39.87 \text{ kJ/mol}$  at 303.15K reported for BAA [10]. On the whole one can suggest that calcium ion binding and folding of PPA in the face of extra calcium salt are enthalpically driven and spontaneous as observed for other homologues. But entropic term seemed to show greater contribution being greater than enthalpic term ( $T\Delta S > \Delta H$ ). However, there is clear-cut difference in the entropic term between literature information for BAA and PPA in this study. While the entropies of calcium ion binding and for folding reported for PPA were positive, the case reported for BAA is negative [10, 32]. Naturally folding results in decrease in conformational entropy coupled with hydration at higher temperature as it is the case with BAA. But due to dehydration, as calcium ions bind (Tanaka and Hoshino, 2002) what may be called translational entropy gain (Harano and Kinoshita, 2005) may occur as applicable to PPA in this study.

## 6. CONCLUSION

The current result shows that unfolding and folding are antiparallel processes amenable to the different aspect of the same model and so can be qualitatively described and quantified in terms of various parameters. The free energy of activation for unfolding was high due to the presence of calcium ion. The enthalpy of activation for unfolding is positive while the value of activation energy for unfolding was low. Calcium ion binding constant and equilibrium constant for folding exhibited decreasing trend with increase in temperature. Calcium ion binding and folding of PPA in the face of extra calcium salt though exothermic, are entropically driven (entropic term,  $T\Delta S > \Delta H$ ) and spontaneous. The entropies of folding being positive may be a probable confirmation of

conformational flexibility needed for catalytic function. Ultimately, calcium salt stabilized the enzyme against increasing temperature leading to greater significant change ( $P < 0.05$ ) in the rate of amylolysis at 310.15K than at 318.15K.

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