Original Research Article

Determination of calcium ion binding parameter of human salivary alpha-amylase by partial inactivation kinetics.

ABSTRACT

Aims: The aims were: i) to ascertain the applicability of a model for the determination of the effect of calcium depletion, to the effect of the presence of the calcium chloride, ii) quantify the thermodynamic activation parameters for unfolding of the enzyme with increasing temperature at different concentration of the salt, iii) to determine calcium binding parameters of the human salivary alpha amylase (HSαA) and cognate apparent thermodynamic parameters.
Study design: Experimental.
Place and Duration of Study: 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. Crude human salivary alpha amylase was assayed at different thermodynamic temperatures for duration of 5 minutes.
Results: The Gibbs free energies of activation ($\Delta G^{\#}$) at 4mM and 1mM $CaCl_{2\,(aq)}$ at 318.15K were 89.62 ± 0.00 and 89.35 ± 0.03 kJ/mol respectively. The corresponding enthalpies of activation were 6.82 ± 0.038 and 3.07 ± 0.09 kJ/mol and the entropies were -260.00±1.17 and -271.21 ± 0.29 J/mol.K respectively. The apparent unfolding rate constant, ranged from 138.50 ± 0.07 – 154.20 ± 0.11 × $1/10^4$/s. The $\Delta G^{\#}$ of unfolding as $[CaCl_{2(aq)}]\rightarrow$zero at 318.15 K is 89.22 ± 0.01 kJ/mol. The entropy of activation was -279.12 ± 19.20 J/mol.K. Calcium ion binding constant ranged from 42.39 ± 2.47 – 46.81 ± 1.31 1/M. The Gibbs free energy and entropy of calcium ion binding at 318.15K were -9.94 ± 0.08 kJ/mol and 55.08 ± 0.25 J/mol.K respectively. The unfolding equilibrium constant ranged from 62.83 ± 1.35 – 135.45 ± 5.88; the enthalpy and entropy of unfolding were 33.96 ± 0.13 kJ/mol and 143.96 ± 0.09 J/mol.K respectively at 318.15K
Conclusion: The model for the calculation of apparent inactivation rate of the enzyme is applicable to calcium treated enzyme. High Gibbs free energy of activation is due to increased "barrier" to unfolding. Large negative entropy of activation was a reflection of a more ordered transition state. Calcium ion binding and unfolding as $[CaCl_{2(aq)}]\rightarrow$zero were spontaneous and entropically driven.

Keywords: Crude human salivary alpha amylase, activation and apparent thermodynamic parameters, calcium binding constant, unfolding first order rate constant, unfolding equilibrium constant.

1. INTRODUCTION

Human salivary α-amylase (α – 1, 4 – glucan – 4 – glucano – hydrolase E.C. 3.2.1.1) is one of several calcium ion and chloride ion dependent hydrolases. Human salivary alpha amylase (HSαA) has a very important biological function of being involved in the first-pass digestion of polysaccharides, starch or glycogen. It is also useful in medicine and research. According to Rohledar and Nater and references therein [1], in this regard, a review report has shown that lower HSαA concentration is associated with asthma and atopic dermatitis in affected children, juvenile idiopathic arthritis patient, adolescents with cerebral palsy *etc*. On account of increased or attenuated HSαA in clinical populations, HSαA levels may

be used to measure the effects of psychotherapy, and in particular, it may be a useful marker in the context of pain or sleep research [2]. The list is by no means exhausted.

HSαA consists of 496 amino acids and it exists as a glycosylated isoform with higher molar mass than the non-glycosylated form [3]. The molecular structure adapted by HSαA is similar to the other mammalian alpha-amylases, including the human pancreatic alpha amylase with which it shares a very high degree of sequence identity (~97%) [4]. Most importantly according to Ramasubbu *et al*. [4], is the fact that HSαA is a monomeric calcium binding protein with a single polypeptide chain whose amino acid composition is distributed among three domains of the protein: domain A (residues 1-99; 170-494); domain B (residues 100-169); domain C (residues 405-496). Domain A bears the catalytic residues Asp 197, Glu 233, and Asp 300; domain B contains one calcium ion binding site; the role of domain is speculated to be the stabilization of domain A as it seems to shield hydrophobic residues from bulk solvent [5]. Structural determinations have shown that chloride-dependent alpha amylases, contain conserved chloride ion binding site located in domain A consisting of 3 residues, Arg 195, Asn 298, and Arg 337(sometimes Lys) [6]. Also conserved are Phe 256 and Phe 295 without any role in chloride binding but Phe 256 helps orient a water molecule chain that may be involved in the starch hydrolysis [4].

Studies on the effect of increasing temperature and adaptation of enzymes to their thermal environment had been studied in the past [7-10]. Also, there had been studies on calcium ion binding characteristic of enzyme, alpha amylase in particular under different conditions and with different methods [11-14]. Nielsen *et al*. [13] and references therein, observed that calcium ion dependent amylases are known to diminish in hydrolytic activity when exposed to temperature as low as 25 ^{o}C let alone at higher temperature. But complete reactivation after 2 h was achieved on addition of excess calcium ions. What needs to be born in mind is that there are extra calcium ion binding sites other than the intrinsic or conserved binding sites for calcium ion and chloride ion dependent enzymes. Presence of extra calcium ion may help in the stabilization of alpha amylase within the short period of assay as was the case in recent study [14].

Unfolding of enzyme beyond physiological limit for function leads to enzyme dysfunction resulting in lower catalytic activity, and somatic and psychopathological diseases. Unfolding and aggregation are features of several age related diseases [15]. Fortunately nature has put in place unfolded protein

response mechanism that can mitigate the effect of protein unfolding and sustain proteostasis [16, 17]. Therefore, studies of unfolding of proteins or enzymes in particular is important for understanding the energetic landscape leading to the active native conformations of the enzyme molecules [18], though attention in this *in vitro* investigation seem to focus on the decline in activity of crude HSαA with increasing concentration of calcium chloride in the face of increasing temperature within a short duration of assay. Thus the objectives of this research were: i) to reassert the applicability of a model initially intended for the effect of calcium depletion, that is, decreasing activity associated with conformational transition, folded-unfolded, to the effect of the presence of the extra calcium chloride, ii) quantify the thermodynamic activation parameters for unfolding of the enzyme with increasing temperature at different concentration of the salt, iii) to determine calcium binding parameters of the human salivary alpha amylase (HSαA) and cognate apparent thermodynamic parameters.

2. SUMMARY OF THEORETICAL DEVELOPMENT

Ab initio, Tanaka and Hoshino [12] proposed and adopted the equation below:

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

where k_{den} is the irreversible rate constant for the denaturation of the calcium-depleted enzyme, k^*_{den} is that for the calcium-bound enzyme, k_{obs} is the apparent first-order rate constant, and K_b is the binding constant of the calcium ion to the enzyme protein. The model presents two aspects viz: model for the determination of unfolding rate constant for calcium bound enzyme (case “a”) and a model for calcium depleted enzyme (case “b”). The impression however, is that unfolding beyond functional limit as applicable to case “a” must be at a first order rate constant different from case “b”. Thus in line with theory, if there is loss of activity even in the presence of bulk salt, there should be no question of calcium depleted enzyme, in the absence of chelating agent in particular. Therefore, case “a” should be applicable; but a plot of reciprocal of decreasing apparent rate constant for unfolding versus reciprocal of salt concentration may give negative slope so that other parameters such as calcium binding constant may assume negative sign which cannot be in agreement with the model in which, *ab initio*, as implied in the first principle (Eq. 1), there is no provision for negative slope let alone intercept. This leaves one with the conclusion that whenever there is loss of activity, in the presence of the salt, case “b” may be applicable. On the other hand, if there is increasing activity with increasing temperature in the presence of increasing

concentration of the salt, case "a" may be applicable as reported in article in the press [14]; in all cases the slope and intercept should be positive in line with original model which may therefore, serve two different purposes.

As a result of the data obtained from the determination of velocities of hydrolysis of raw potato at different temperatures, diminution in the activity of human salivary alpha amylase (HSαA) in the presence of extra load of calcium chloride, one aspect implicit in Eq. (1) is adopted. Thus Eq. (2) as explained earlier [14] based on the work of Tanaka and Hoshino [12] as shown below are employed.

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

However, there is another view by other authors [13] to the effect that at low temperature, the contribution from denaturation of calcium-bound *Bacillus halmapalus* alpha amylase (BHA) is insignificant, because inactivation proceeds almost exclusively as denaturation of calcium-depleted BHA and based on differential scanning calorimetry (DSC), the denaturation of calcium-bound BHA is negligible below 65°C which seems to be in line with temperature range of 25 – 60°C (298.15 – 333.15K) in this investigation. Like Tanaka and Hoshino relative activity is adopted herein as applied in a thesis [19] similar to approach by Tabassum *et al.* [20] who however, used the velocity directly instead of relative activity.

Before maximum absorbance or total unfolding, the equilibrium merely shifts toward the unfolded state with higher concentration. Consequently, the residual activity (v_1) should be much less than the initial activity (v_0) in the absence of the salt. Hence, an equilibrium constant, such as the following, is expected:

$$K_{eq} = U/N \gg 1 \quad (3)$$

where U and N are the fraction of unfolded and folded enzyme respectively. As shown in earlier report [14]

$$v_U = \partial U/\partial t = -\partial N/\partial t = k_{den} N \quad (4)$$

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.

$$k_{den} = \ln (N_0/N_1)/t \quad (5)$$

In the Eq. (5) N_0, I_{NT}, and t, are the initial native population of enzyme molecules, intercept ($1/k_{den}$ in Eq. (2)) and duration of assay respectively. "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^{o}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" [14]. But this is not necessarily a general case for all amylases. The situation may be different when $k_{den}^{*} \rightarrow$zero [12] such that:

$$K_U = (1\text{-}\exp(-t/I_{NT}))/\exp(-t/I_{NT}) \quad (6)$$

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

3.0. MATERIALS AND METHODS

3.1. Materials

The chemicals: Alpha amylase (EC 3.2.1.1;1,4-α-D-glucan glucanohydrolase (crude human salivary alpha amylase (HSαA)); as in previous report [14] potato starch (contains 0.2% glucose and it is about 99% pure) was purchased from Sigma Chemicals Co, USA; dinitrosalycilic 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: *p*H meter (tester) was from Hanna Instruments, Italy; electronic weighing machine was from Wensar Weighing Scale Ltd, Chennai; Centrifuge, 800D model was from China; 721/722 visible spectrophotometer was from Spectrum Instruments Co Ltd, China.

3.2. Methods

The methods are as described in article in the press [14] but re-enacted herein as follows: A solution of enzyme was prepared by subjecting saliva to centrifugation for 5 minutes at 3000 rpm (or at 1343 × g) using ordinary laboratory centrifuge (model 800D) subjected to 1:2 dilution in tris – $HCl_{(aq)}$ buffer at *p*H 7.4 as a matter of choice as in previous research elsewhere [19]. 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 [19]. One gram of raw soluble potato starch was mixed in 100mL of tris – buffer at *p*H 7.4 to give 10g/L; the starch in aqueous buffer is not a true solution [19]. Various molar solution of calcium chloride ranging from 1 – 4 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 40 – 60^{o}C (313.15 – 333.15K).

3.2.1 Assay of crude human salivary alpha amylase for the determination of velocity of amylolysis of raw soluble potato starch.

An *in vitro* assay of alpha-amylase was according to Bernfeld method [21]. The holoenzyme, crude human salivary alpha amylase (HSαA), was assayed as described in in article in the press [14] but re-enacted herein: The duration of assay was 5 minutes and the final activity is obtained according to Beer – Lambert law as follows: corrected absorbance at wavelength, 540nm, $\times$ dilution factor (*i.e.* 3)/ε *l*, where ε (181.1M^{-1}cm^{-1}), C, and *l* are molar absorption coefficient, molar concentration of product, and path length respectively. An assay of the enzyme was done in a total reaction mixture of 3 mL composed of 1 mL of substrate (raw soluble potato starch), 1 mL of enzyme, 0.5 mL of calcium chloride and 0.5 mL of distilled water (or 1 mL of distilled water where calcium chloride is not included in the reaction mixture). Measurement of absorbance was taken after 5 minutes of centrifugation at 3000rpm (or at 1343 $\times$ g) using centrifuge (model 800D). As in previous report [14, 19] centrifugation was needed to sediment coarse particles or fibers so as to prevent interference with spectrophotometric transmittance that could otherwise yield high false absorbance. Two blanks, one containing only substrate and the other containing only crude enzyme extract were prepared, the absorbance from both blanks were summed up and subtracted from test absorbance to give corrected absorbance. The relative activity is expressed as 100 $\times$ $v_{[Salt]}/v_{[Salt]=0}$ where $v_{[Salt]}$ and $v_{[Salt]=0}$ are velocity of hydrolysis of starch with and without salt respectively.

"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" [22].

3.2.2. The determination of activation parameters for calcium ion binding

Activation energy (Ea) for unfolding and binding of cation was obtained by plotting natural logarithm apparent rate constant (K_{obs}) against reciprocal of absolute temperature while other activation parameters were obtained according to equations that follow.

$$\ln k_{obs} = \ln A - Ea/RT. \quad (7)$$

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

$$\ln k_x = \ln A - Ea/RT. \quad (8)$$

$$\Delta H^{\#} = Ea - RT \quad (9)$$

$$\Delta S^{\#} = \Delta G^{\#} - \Delta H^{\#} \quad (10)$$

$$\Delta G^{\#} = \ln (k_B T/h k_x) \quad (11)$$

where k_x values may be k_{den}, k_{den}^{*}, and k_{obs} while, 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 (ΔG^{o}), enthalpy (ΔH^{o}), and entropy (ΔS^{o}) for the binding of calcium ion were determined according to the following equations adopted by Tanaka and Hoshino [12].

$$\Delta G^{o} = - RT \ln K_b \quad (12)$$

$$\partial \ln K_b = - \Delta H^{o} \partial T/RT^2 \quad (13)$$

Equation (13), being van't Hoff equation is used to determine ΔH^{o} by plotting $\ln K_b$ versus $1/T$.

$$\Delta S^{o} = (-\Delta G^{o} + \Delta H^{o})/T \quad (14)$$

3.2.4. The determination of apparent thermodynamic parameters for unfolding and folding

$$\Delta G_F = -RT \ln K_F \quad (15)$$

$$\Delta G_U = -RT \ln K_U \quad (16)$$

Determination of enthalpy of unfolding is by van't Hoff plot.

3.2.5. STATISTICAL ANALYSIS

Except otherwise stated, data are expressed as Mean±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 carried out with electronic calculator. Assays were carried out in duplicates.

4. RESULTS

The test for the effect of extra loads of calcium ions on the function and stability/structure of HSαA required control without salt. The relative rates as percentage of control showed decreasing trend with increasing concentration of the salt (the actual values were not shown in any Table). The natural logarithm of the percentage decrease in velocity of hydrolysis at higher salt concentration is lower than at lower salt concentration. However, the presence of the salt resulted in significant decrease ($P < 0.05$; $t_{cal} > t_{0.05(1)4}$) in the velocities of hydrolysis of the substrate by the salt treated enzyme. Since the result presented was the consequence of the effect of the presence and absence of externally applied calcium salt it implied that, *ab initio*, holoenzyme was investigated apart from the fact that the source of the crude enzyme, saliva, has its mineral content which includes calcium ions. The effect of increasing temperature to which the enzyme was exposed within a short duration (5 min) of assay at different molar concentration of the salt was investigated and displayed by plotting apparent rates versus temperature (Fig. 1). The plot, at different molar concentration of the salt ranging from 1 – 4 mM, Fig. 1, shows increasing trend with increasing temperature that seemed not to be very regular. The apparent rate constant when $[CaCl_{2(aq)}] \rightarrow$ zero, was determined by extrapolation from the intercept of the plot of $1/k_{obs}$ versus $[CaCl_{2(aq)}]$ (Fig. 2). But for the value (~ 0.7) at 333.15 K, the plots showed high coefficient of determination (r^2) ranging from 0.94-0.99 at lower temperatures ranging from 313.15 - 323.15 K. 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.

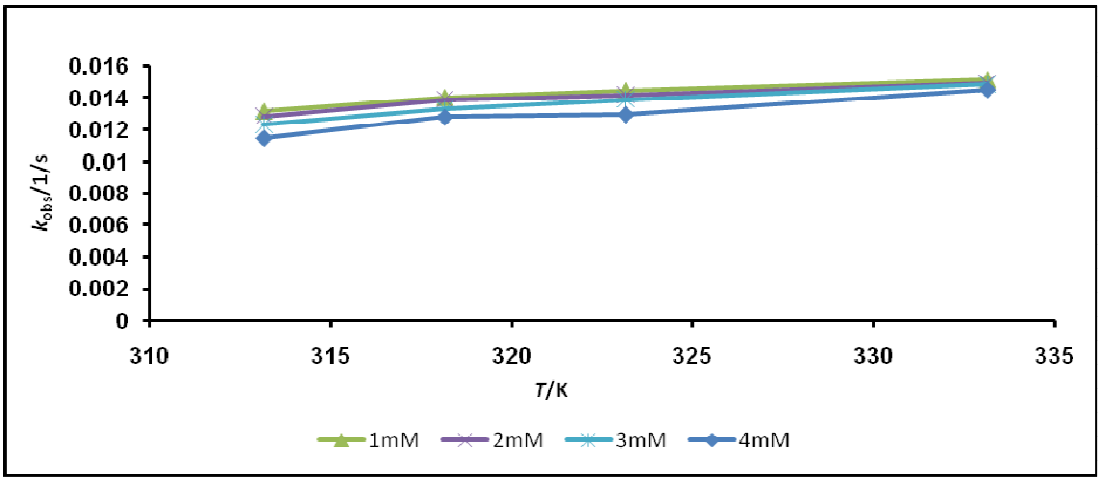

Fig. 1. Plot of apparent rate (k_{obs}) versus absolute temperature (T) showing the trend of k_{obs} with increasing T at different molar concentration of calcium chloride. (▲), (×), (∗), *and* (◆) *are assays and plots at 1 mM, 2 mM, 3 mM, and 4 mM respectively*.

In order to determine rate constant in the absence of unfolding, the reciprocal of apparent rate was plotted versus reciprocal of molar concentration of the salt, calcium chloride (Fig. 2).

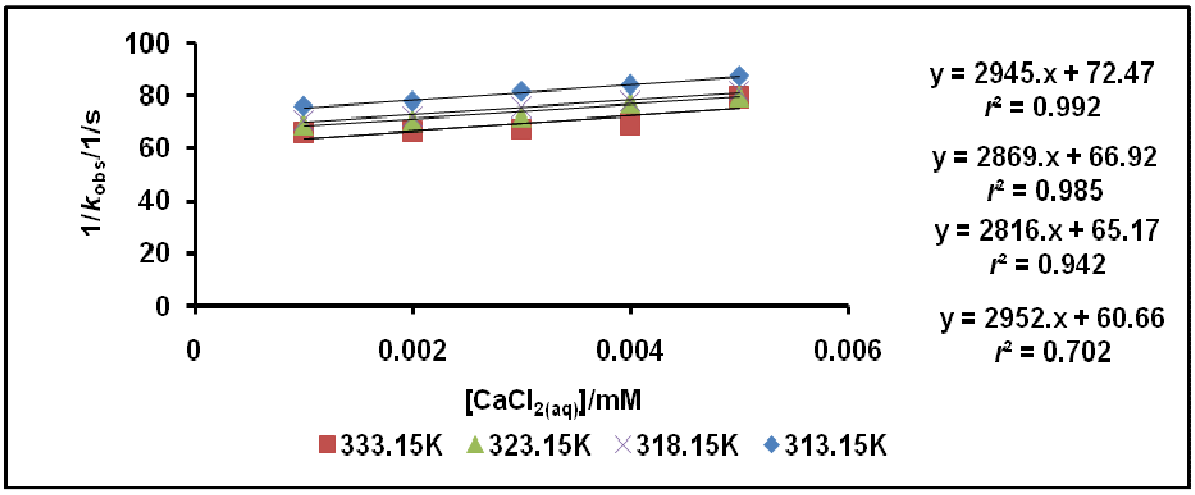

Fig. 2. Plot of reciprocal of calculated apparent rate k_{obs} versus reciprocal of $[CaCl_{2(aq)}]$ for the determination of rate constant for unfolding of the enzyme at different temperatures as $[CaCl_{2(aq)}]$ →zero. (■): *Assay at 333.15K*; (▲): *Assay at 318.15K*; (×): *Assay at 323.15K*; (◆): *Assay at 313.15K.*

In other to examine the important issue of activation parameters, natural logarithm of apparent rates in the presence of different concentrations of the salt were plotted versus $1/T$ (Fig. 3) according to Arrhenius theory. The plots showed high values of r^2 ranging from 0.91 - 0.97. Similar plots (Fig. 4) were carried out where [$CaCl_{2(aq)}$] → zero, with r^2 value of about 0.95. The apparent enthalpy of calcium ion binding was determined according to van't Hoff method by plotting natural logarithm of calcium binding constant determined by combining the intercept and the slope from the plot of $1/k_{obs}$ versus [$CaCl_{2(aq)}$] as explained in Eq. (2) versus $1/T$ (Fig. 5). The r^2 value is 0.94 (Fig. 5). In the same vein, enthalpy of unfolding was determined by plotting natural logarithm of folding equilibrium constant versus $1/T$ (Fig. 6).

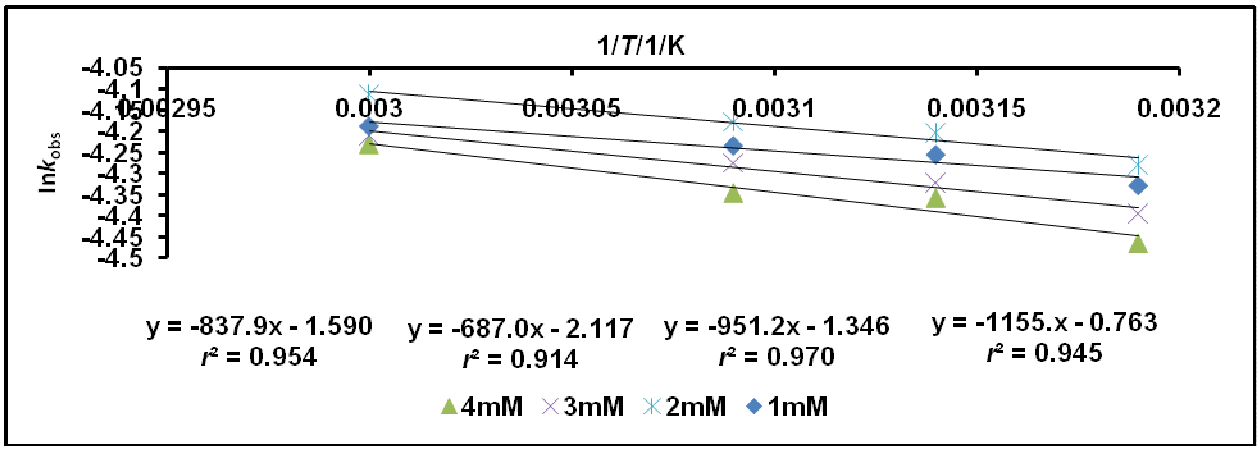

Fig. 3. Arrhenius plots for the determination of activation energy for unfolding of the enzyme at different salt molar concentrations. (▲), (×), (∗), (◆), *are assays and plots at 4mM 3mM, 2mM, and 1mM respectively*.

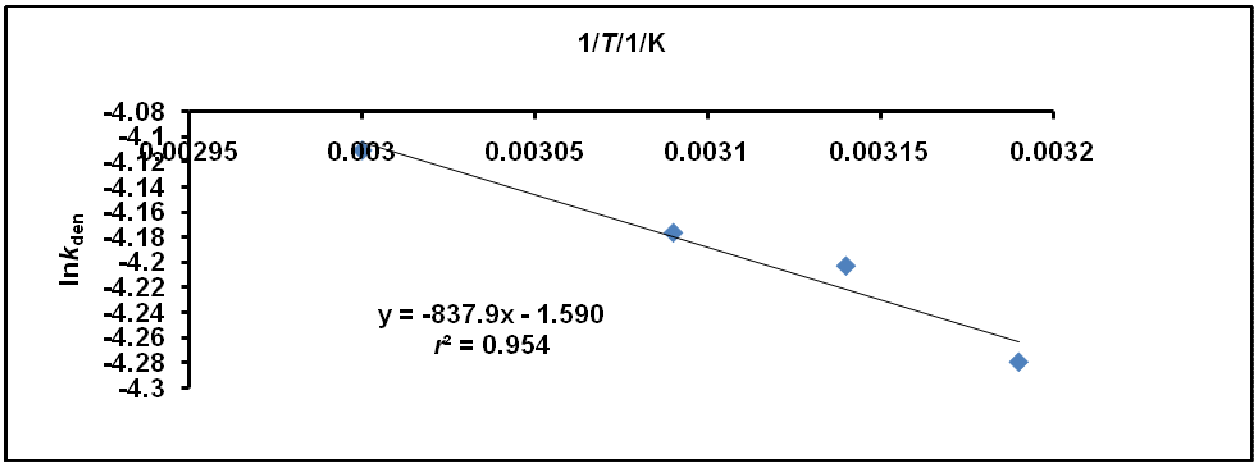

Fig. 4. Arrhenius plots for the determination of activation energy for the unfolding of the enzyme as $[CaCl_{2(aq)}] \rightarrow 0$.

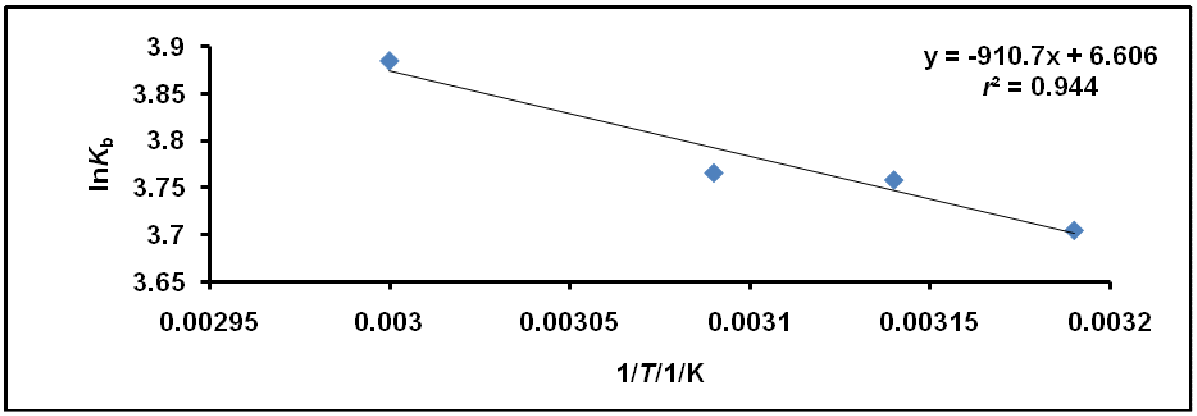

Fig. 5. van't Hoff plot for the determination of enthalpy of calcium ion binding to sites apart from intrinsic sites. *Calcium ion binding constant (K_b) is obtained from the combination of the intercept and slope of the plot of the reciprocal of calculated apparent rate versus reciprocal of molar concentration of calcium chloride.*

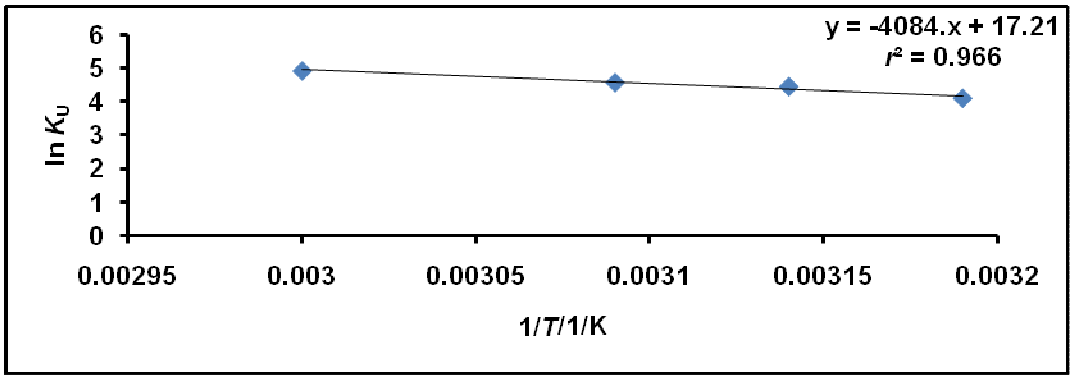

Fig. 6. van't Hoff plots for the determination of enthalpy of unfolding of the enzyme.

As shown in Table 1 the Gibbs free energy activation for unfolding showed increasing trend with increase in absolute temperature at different concentration of the salt. The values of the parameter were higher at higher salt concentration. Although the enthalpies of activation values were much higher at higher salt concentration, the values at higher temperature were nevertheless lower than values at lower temperature. The magnitude of the negative entropies of activation is higher at lower salt concentration than at higher salt concentration. The values were higher at lower temperatures. The activation energy for unfolding was much higher at higher salt concentration. The pre-exponential factor at higher salt concentration was more than three-fold higher than the value at lower concentration.

The unfolding rate constant as $[CaCl_{2(aq)}] \rightarrow$ zero, showed increasing trend with increase in temperature. Expectedly, the magnitude of the Gibbs free energy of activation showed increasing trend with increase in temperature. The negative entropy of activation showed irregularity in trend in which the least value is at 323.15K. However, the enthalpy of activation for unfolding as $[CaCl_{2(aq)}] \rightarrow$ zero were higher at lower temperatures. The activation energy as $[CaCl_{2(aq)}] \rightarrow$ zero lies between values observed in the presence of 1mM and 4mM of calcium chloride. The pre-exponential factor was ~ two-fold higher and slightly more than two-fold less than value observed in the presence of 1mM and 4mM of calcium chloride respectively.

Table 1. Activation parameters for the unfolding of the enzyme.

[$CaCl_2$(aq)] (mM)	1		4	
T (K)	313.15	318.15	313.15	318.15
$\Delta G^{\#}$ (kJ/mol)	88.10±0.01	89.35±0.03	88.46±0.00	89.62±0.00
$\Delta H^{\#}$ (kJ/mol)	3.12±0.09	3.07±0.09	6.86±0.0.37	6.82±0.38
$\Delta S^{\#}$ (J/mol.K)	-271.38±0.30	-271.21±0.29	-260.58±1.17	-260.08±1.17
Ea (kJ/mol)	5.72±0.09		9.47±0.38	
A (10^{-3})/s)	120.00±4.24		446.00±60.10	
T (K)	313.15	318.15	323.15	333.15
k_{den} (10^{-4})/s)	138.50±0.07	151.80±0.04	152.86±0.28	154.20±0.11
$\Delta G_u^{\#}$ (kJ/mol)	87.97±0.01	89.22±0.01	90.58±0.03	93.29±0.03
$\Delta H_u^{\#}$ (J/mol)	4.37±0.59	4.32±0.59	4.28±0.59	4.20±0.59
$\Delta S_u^{\#}$ (J/mol.K)	-266.96±1.90	-266.86±19.20	-267.06±1.82	-267.42±1.78
Ea_u (kJ/mol)	6.97±0.59			
A (10^{-3})/s)	207.00±45.96			

$\Delta H^{\#} = \Sigma(Ea - RT)/2 \pm SD$; $\Delta S^{\#} = \Sigma(\Sigma(\Delta G^{\#})/2 - \Delta H^{\#})/2 \pm SD$; *The activation parameters* Ea, $\Delta H^{\#}$, $\Delta S^{\#}$, *and* $\Delta G^{\#}$ *are activation energy, enthalpy of activation, entropy of activation, and free energy of activation respectively for the binding of calcium ion; the corresponding activation parameters for unfolding is represented by subscript* u.

Looking at Table 2, it will be observed that the values of calcium ion binding constant and the negative Gibbs free energies of calcium ion binding showed increasing trend in magnitude with increase in temperature. Like the positive entropies which showed decreasing trend with temperature, the enthalpy of calcium ion binding is also, positive.

The values of equilibrium constant for unfolding were increasing with increase in temperature, and the corresponding negative Gibbs free energies showed similar trend. The enthalpy and entropies of unfolding were positive. However, the entropies were not regular in trend, being highest at 318.15K and least at 333.15K (Table 2).

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

T **(K)**	**313.15**	**318.15**	**323.15**	**333.15**
K_b **(1/M)**	**42.39±2.47**	**42.89±1.26**	**43.23±2.57**	**46.81±1.31**
ΔG_b **(kJ/mol)**	**-9.78±0.15**	**-9.94±0.08**	**-10.12±0.16**	**-10.65±0.15**
ΔH_b **(kJ/mol)**	**7.58±0.05**			
ΔS_b **(J/mol.K)**	**55.34±0.64**	**55.08±0.25**	**54.77±0.48**	**54.72±0.47**
K_U	**62.83±1.36**	**87.89±0.94**	**98.83±4.68**	**135.45±5.88**
ΔG_U **(kJ/mol)**	**-10.78±0.06**	**-11.84±0.03**	**-12.34±0.13**	**-13.60±0.12**
ΔH_U **(kJ/mol)**	**33.96±0.13**			
ΔS_U **(J/mol.K)**	**142.87±0.18**	**143.96±0.09**	**143.28±0.40**	**142.75±0.36**

$\Delta S_b = \Sigma(\Delta G_b - \Sigma\Delta H_b/2)/2 \pm SD$; K_b, k_{den}^*, and k_{den} *are the calcium ion binding constant, rate constant for folding, and rate constant for unfolding respectively*; ΔS_b, ΔG_b, *and* ΔH_b *are entropy, free energy, and*

enthalpy of calcium ion binding respectively; ΔG_U, ΔH_U, *and* ΔS_U *are free energy, enthalpy, and entropy of folding of the enzyme respectively.*

5. DISCUSSION

So far the effects of extra load of calcium chloride have been investigated via assay of enzyme's hydrolytic activity. 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 [14]. The issue of heterogeneity of population of enzyme composed of partially folded (molten globule), folded, and negligible intermediate species had been advanced [23]. Thus the fact that there were residual activities expressed as percentage of control without calcium salt should not be unexpected. However, the significant decline ($P < 0.05$) in activity with increase in the concentration of the salt vis – à – vis increasing temperature implied that the presence of extra salt had inhibiting effect on the enzyme. Then the questions are: is the observation due to increase of conformational entropy beyond functional physiological limits needed for function? Is the observation due to decrease in conformational entropy below minimum limit needed for function? The answers are not farfetched. Meanwhile temperature adaptation of enzymes, alpha amylases from different phyla, the psychrophiles, mesophiles, and thermophiles in particular have been studied extensively [7-10; 22] but not necessarily exhaustively. The need for conformational flexibility expressed as positive conformational entropy has been emphasized [7-10; 22]. Thus it has been observed that cold adapted enzyme exhibit high ground state conformational entropy thereby reducing the need for higher thermal environment as it is the case with moderate thermophiles (mesophiles), and thermophiles [13, 22]. Therefore, the answers to the questions may be as follows: since calcium ions and chloride ions have different effects, rigidification and activation/stimulation respectively, then it seems the expected thermally induced conformational entropy increase in addition to the effect of the chloride component in the promotion of flexibility, may have been substantially opposed by the calcium ion component. Consequently there may have been a reduction in positive conformation entropy well below minimum needed for effective performance of catalytic function.

But here we are with so much emphasis on the role of calcium ion without similar attention to the chloride component. It would have been very proper to consider the effect of calcium ions alone if the anionic component has insignificant or no known effect on structure-function complementarity of the enzyme; Tanaka and Hoshino [12] investigated the effect of calcium chloride but with little or no concern for the effect of chloride component. On the other hand Nielsen and Westh [13] invested the effect of calcium ions using calcium sulphate. While the sulphate component is higher than chloride, in the Hofmeister series, it is not certain whether the sulphate ion has the same effect on the enzyme as chloride ion. However, little concern was expressed in an earlier paper in the press [14]. The second answer as follows suggests that, the enzyme being amenable to increased conformational entropy or flexibility with increasing temperature, it may likely exceed the optimal physiological limit of conformational entropy, which is further orchestrated by the chloride ion component that promotes flexibility. This is tantamount to inactivation or dysfunctional unfolding of the calcium chloride - dependent enzyme. This may call for the use of instrumentation that can monitor the direction of shift in conformational entropy. Nonetheless, there may have been either excess conformational entropy or excess rigidification leading to decline in relative rates with increasing concentration of the salt as reflected in Fig. 2 where it should be understood that $1/k_{obs}$ at lower concentration of the salt is lower than at higher concentration of the salt.

It is important to state that calcium ions seemed to have been implicated in the competitive inhibition of *Bacillus amyloliquefaciens* alpha amylase (BAA) [24]. Thus calcium ion is required for the stabilization of α-amylase because of primary binding (essential binding), but has been shown to inhibit hydrolytic catalysis due to secondary binding at the catalytic site in the enzyme as observed in this investigation but unlike recent report for porcine pancreatic alpha amylase (PPA)[14]. The enzymatic hydrolysis was inhibited by a relatively high concentration of calcium ions ($[Ca^{2+}]$ ≥2.0 mM) [24]. As in this investigation also, Nielsen *et al* [13] observed that *B. halmapalus* alpha amylase (BHA) unfolded at higher temperature in the presence of excess calcium ion. Meanwhile the concentration of calcium salt used in this investigation ranges from 1-4mM. This inhibiting effect of calcium ions had been explained on account of 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 [25]. But there is also a claim that 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 [26]. This position seems to suggest that the chloride component seemed to have failed to totally oppose the effect of calcium in this regard. This is, notwithstanding the well known fact that calcium ion enhances the activity of alpha amylase, *Haloarcula hispanica* amylase [27] and as observed recently in respect of PPA using raw starch as substrate [14], just as chloride activation was encountered in halophilic amylase of *Marinobacter* sp EMB8 [28, 29].

The activation parameters recorded in Table 1 show that the Gibbs free energies of activation for unfolding were high and compare with values reported at 50^{o}C for *Bacillus amyloliquefaciens* α-amylase (BAA)[12] and they were similar to the report for PPA [14]. The results are expression of the fact that the presence of calcium ions promotes rigidifying effect on the 3-D structure of the enzyme in the face of increasing temperature and in the presence of chloride ion component of the salt. This is similar to the view that the free energy change between the native and the transition state which characterized the unfolding barrier height was found to be proportional to the number of calcium ions bound to the protein structures [30]. The enthalpy of activation for unfolding was lower at higher temperature. It is however higher at higher salt concentration similar to the report for PPA recently [14]. The value of the parameter reported for PPA [14] was lower than current report for HSαA in this investigation. Just as the values ($\Delta H^{\#}$) reported for BHA were exceedingly greater than those reported for BAA so the values for the latter were much greater than values reported for HSαA in this investigation. From the point of view of stability, high enthalpic values are suggestive of unstable process or product. Therefore, it may be inferred that the unfolding of the enzyme, HSαA is next to PAA in terms of being enthalpically favourable in the presence of calcium salt when compared with other homologues with exceedingly higher $\Delta H^{\#}$ reported in literature. The entropies of activation for unfolding reported herein for HSαA though negative in sign similar to those of PPA [14], they are however, less than the values for PPA. Moreover, while those for PPA showed incremental trend with increase in temperature those reported herein for HSαA, decreased with increase in temperature. It may imply therefore, that the transition state complex for HSαA is less ordered than those of PPA just as both HSαA and PPA are exceedingly more ordered than BAA whose entropy of activation for unfolding was large and positive [12]. It need to be mentioned that there had been report to

the effect that thermally induced unfolding for calcium deleted enzyme gives expanded 3-D structure of the enzyme but with loss in secondary structure, a situation that indicates increase in conformational entropy [31] as may be applicable to BAA [12]. There is also the claim that thermally induced unfolded protein may possess compact 3-D conformation leading to lowered conformational entropy which may account for the negative entropy of unfolding as observed for HSαA in this study and PAA in the past. This assumption however, remains speculative even if calcium treated enzymes were the case as opposed to salt depleted enzyme reported in literature stated earlier.

One should not lose sight of the occurrence of partially unfolded proteins referred to as molten globule which possesses native like compact 3-D structure which may likely be enhanced with the presence of calcium ions. While activation Gibbs free energies $\Delta G^{\#}$ which are measures of the spontaneity of the inactivation processes, were lower than the $\Delta H^{\#}$ values, which, may be due to the positive entropic contribution during the inactivation process [12] there is a contrary situation to the effect that $\Delta G^{\#}$ being higher than $\Delta H^{\#}$ as reported for HSαA in this study and for PPA in the previous report, may be due to large negative entropy. There are issues of kosmotrophs and chaotrophs to the effect that activity and stability usually follows the Hofmeister series, and that an enzyme solution is normally stabilized by kosmotropic anions and chaotropic cations but destabilized by chaotropic anions and kosmotropic cations [32]. However, the chloride ions are activators or stimulators because it is known to promote conformational flexibility or physiologically needed partial unfolding/destabilization needed for function. As such it is difficult to label the chloride component of the salt as kosmotropic ion which stabilizes the salt depended enzyme which ought to be the function of chaotropic cations such as calcium ion. The activation energy (*E*a), an expression of temperature dependent, reported for HSαA in this study was higher than report for PPA [14]. Both enzymes however, possess higher *E*a at higher salt concentration; this point to the resistance offered to unfolding, due to the effect of the cations. It has also, been shown that, *E*a values for BHA [13], were higher at higher salt ($CaSO_4$) concentration, just as Violet and Meunier [33] observed very high *E*a in the presence of 5mM calcium chloride.

Unlike BHA [13] and HSαA in this study whose rate constant for unfolding was low, the values for BAA [12] were very high and ranges between 9.35-67.57/s. The value for HSαA in this study is ~ six-fold

higher than the value reported for BHA at 323.15K. Research has also shown that, the 1st order rate constant of unfolding are 3.6±1.2 × 10^{-3}/s and 2.9±0.7 × 10^{-2}/s for triple helical WT peptide and single stranded peptide containing mutations respectively [34]. The 1st order rate reported for HSαA is closer in magnitude to value reported for the single stranded peptide. The activation parameters as $[CaCl_{2(aq)}]$→zero, are very similar to the situation in the presence of the salt. The free energies of activation ($\Delta G_U^{\#}$) were much higher than the enthalpies of activation ($\Delta H_U^{\#}$) due to large negative entropies of activation. This (as with the case for the presence of the salt), is suggestive of a lowered conformational entropy of the transition complex. Although the extra salt is inhibiting or unfold the enzyme when in excess, one would have expected much lower $\Delta G_U^{\#}$ values as $[CaCl_{2(aq)}]$→zero. But it seemed there were even higher values at higher temperatures. This might be as a result of the holoenzyme in its crude form with calcium ions in saliva being, in principle, more stable as should be expected if the externally added cations and anions were not added as implied when $[CaCl_{2(aq)}]$→zero. Since binding of Ca^{2+} stabilizes native-like contacts in the partially folded species and reduces the barriers for the conversion of the protein to its native state [35] the corollary is that the presence of intrinsic and extra calcium ions in saliva should increase the barrier for unfolding. This scenario is further accentuated by the value of the activation energy of unfolding being within the range of the values reported in the presence of 1 and 4mM of salt.

Unlike reports for other homologues of alpha amylases [12-14] HSαA exhibited very low calcium ion binding constant (K_b). While the concentration regime of calcium salt used in respect of HSαA is the same as that used for PPA [14], those used for other homologues were very much lower in concentration. It must be reemphasized that K_b reported for BAA ranges between tens-hundreds of thousands and those reported for BHA are in hundreds of thousands while those of PPA and HSαA were in few tens of thousands and in mere double digits figures respectively. Besides while the K_b values showed decreasing trend with increase in temperature as observed for BHA [13], BAA [12], and PPA [14], the situation with HSαA in this study is different, showing instead, increasing trend with increasing temperature. It seems the concentration regime applied may be a contributing factor considering the fact that if $[Ca^{2+}]_1 \ll [Ca^{2+}]_2$ then $1/[Ca^{2+}]_1 \gg 1/[Ca^{2+}]_2$. The implication is that a plot of $1/k_{obs}$ versus $[Ca^{2+}]$ would give large slope or

424 small slope if plotted versus 1/[Ca^{2+}], if [Ca^{2+}] is very low. Both situations can influence the outcome of
425 any relevant calculations.

426 The Gibbs free energies for calcium ion binding for HSαA showed increasing negative trend, an
427 expression of spontaneity, similar to reports for BHA [13] and PPA [14]. The magnitude of this parameter
428 differed among these homologues in the following order: BHA>PPA>HSαA. The enthalpy (ΔH) of calcium
429 ion binding for HSαA in this study is endothermic as was the case for BAA [24] with much higher
430 magnitude. Previously Tanaka and Hoshino (2002) reported positive ΔH = 149 kJ/mol. The positive value
431 of ΔH for HSαA is unlike the negative ΔH reported for PPA [14], with smaller magnitude than HSαA, and
432 BHA [13] in particular which was more exothermic and much larger in magnitude. The positive enthalpy
433 suggests that the binding of calcium ion to HSαA is less stable than the binding on other homologues.
434 The entropies of calcium ion binding for HSαA in this report, though positive as was the case for BHA [13]
435 and PPA [14], were however, smaller in magnitude. However, since the entropic term $T\Delta S$ » ΔH, the
436 entropic term should be seen to contribute more to the calcium ion binding. In other words the binding
437 process is largely entropy driven. I must not fail to mention however, that previous report of entropy for
438 PPA is very similar to the report for BHA, and the report for HSαA is about 1.3-fold less than the values
439 for PPA and BHA previously cited, and about three-fold smaller than the positive entropy reported for
440 BAA [24]. Previously Tanaka and Hoshino [12] reported positive ΔS = 360 J/mol/K for BAA. The positive
441 entropies are accounted for in terms of the release of water molecules from the calcium-binding sites and
442 dehydration of non-polar surfaces on calcium binding, either at the binding site, or as a result of coupled
443 conformational changes in the protein [13]. It is difficult to state categorically what the effect of sulphate
444 ions could be when compared with the effect of chloride ions which are lower in the Hofmeister series;
445 what is obvious however, is that chloride has opposite effect to calcium ions. The chloride opposes
446 calcium ion and promotes conformational flexibility which implies that there could be increase in
447 conformational entropy coupled with effect of increasing thermal energy.

448 None of these authors cited has anything to do with thermodynamics of unfolding based on the
449 model in this research. Nonetheless, PPA in the past report [14] and HSαA in this research report showed
450 opposite conformational transformations, folding and unfolding respectively. The magnitudes of the

451 folding equilibrium constant for PPA were much less than the unfolding equilibrium constant of unfolding
452 for HSαA. The corresponding equilibrium constant for HSαA and PPA showed increasing trend and
453 decreasing trend respectively with increase in temperature. The corresponding Gibbs free energy (ΔG) for
454 both homologues were negative pointing to spontaneity of folding and unfolding for PAA and HSαA
455 respectively. Such spontaneity was more pronounced for the folding of HSαA. The ΔG values for HSαA
456 showed regular trend, increasing with increase in temperature unlike report for PPA that had a break in
457 trend with the least value at 333.15 K. Also, similar trend in entropy (ΔS) of unfolding and folding of HSαA
458 and PPA [14] respectively, occurred with the least value at 333.15 K. However, ΔS values for unfolding of
459 HSαA were higher than for folding of PPA [14]. Both PAA and HSαA showed positive ΔS for folding and
460 unfolding respectively. The enthalpy of unfolding of HSαA is positive and ~ 58-fold larger than the
461 negative ΔH reported for PPA [14]. From the data in Table 2, it is clear that the entropic term $T\Delta S > \Delta H$
462 and, therefore, unfolding of HSαA in this report is entropically driven as $[CaCl_{2(aq)}] \rightarrow$ zero. This is totally
463 different from the folding of PPA which is enthalpically driven [14].

464 **6. CONCLUSION**

465 The original model can be used for the qualitative and quantitative description of calcium ion
466 binding characteristics of the enzyme. The high free energy of activation for the unfolding in the presence
467 of calcium salt and as $[CaCl_{2\ (aq)}] \rightarrow$ zero suggests that the presence of added calcium ion as well as the
468 existing cation in saliva enhanced the capacity of the holoenzyme to resist total unfolding with increasing
469 temperature. The large negative entropy and low enthalpy of activation parameters are pointers to a more
470 ordered and stable transition state of the holoenzyme. Low calcium binding constant may be due to the
471 presence of salivary calcium ions. Binding of calcium ions and unfolding were spontaneous and
472 entropically driven. Calcium ion may be used to control the stability and activity of the enzyme. Feature
473 research may require the use of gelatinized starch.

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