

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 ± 7.07 J/mol) for unfolding, was higher at lower temperature and higher salt concentration. The magnitude of entropy of activation (*e.g.*, -279 ± 0.06 J/mol.K) and free energy of activation (89.09 ± 0.01 kJ/mol) for unfolding was higher at higher temperature and lower salt concentration. **Activation energy (2.98 ± 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 ($P < 0.05$).

5
6 *Keywords: Porcine pancreatic alpha amylase, activation and apparent thermodynamic parameters,*
7 *calcium binding constant, folding first order rate constant, folding equilibrium constant, conformational*
8 *stability.*

9 10 **1. INTRODUCTION**

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

32 between a β -strand and a α -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 β -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 β 4, A β 6, and A β 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 > NO₃⁻¹ > I⁻¹ ClO₄⁻¹ > 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_{den} is the irreversible rate constant for the denaturation of the calcium-depleted enzyme, k_{den}^* is
85 that for the calcium-bound enzyme, k_{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

92 transition, and the depleted form contributions in Eq.(1) should be very infinitesimal compared to the
 93 bound form. **If this assumption or postulation is reasonable and applied to the experimental data,**
 94 **expected from this investigation**, the linear relationship between the values of reciprocal of apparent
 95 first-order rate constant and the reciprocal of calcium ion concentrations can be determined: At this
 96 juncture, it is important to point out that Tanaka and Hoshino did not subject the test enzyme to
 97 chelating agents (though calcium free reaction mixture containing enzyme was tested) as may be
 98 testified as follows: "The reaction mixture consisting of 1.3110⁻⁸M BAA, 25 mM Tris acetate buffer (pH
 99 7.0) and 0.002-0.02 mM CaCl₂ was incubated at 45-65°C for the appropriate time intervals"[10]. Thus
 100 there is no question of the use of apoenzyme. In the experiment by the authors [10], residual activity
 101 measured as percentage of control implied that there was decrease in activity even with increasing
 102 concentration of the salt at different temperatures. Then, the question is, what model may be suitable
 103 if despite increasing temperature in the presence of increasing concentration of the salt, the activity of
 104 the enzyme is increasing?

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

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

$$115 \quad k_{obs} = (K_b k_{den}^* [Ca^{2+}_{(aq)}]) / (1 + K_b [Ca^{2+}_{(aq)}]) \quad (2)$$

116 For the purpose of comprehension, a stepwise approach beginning from Eq. (2) leads to the 2nd step
 117 after rearrangement:

$$118 \quad 1 + K_b [Ca^{2+}_{(aq)}] = K_b k_{den}^* [Ca^{2+}_{(aq)}] / k_{obs} \quad (3)$$

$$119 \quad 1/k_{obs} = 1/K_b k_{den}^* [Ca^{2+}_{(aq)}] + 1/k_{den}^* \quad (4)$$

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

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

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

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

$$155 \quad 1/k_{\text{obs}} = K_b [\text{Ca}^{2+}_{(\text{aq})}]/k_{\text{den}} + 1/k_{\text{den}} \quad (5)$$

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

$$165 \quad K_{\text{eq}} = U/N \gg 1 \quad (6)$$

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

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

$$172 \quad v_U = \partial U/\partial t = - \partial N/\partial t = k_{\text{den}} N \quad (7)$$

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

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

$$177 \quad k_{\text{den}} = \ln (N_0/N_1)/t \quad (8)$$

178 Equation (8) can be translated to:

$$179 \quad k_{\text{den}} \sim \ln (v_0/v_1)/t \quad (9)$$

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

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

$$185 \quad N_1/N_0 > 1 \quad (10)$$

186 where N_1 and N_0 are respectively new population of the folded and initial native population taken as
 187 100%. This is so because, $N_0 \rightarrow N_1$ which simply imply that the native enzyme assumes greater
 188 catalytic power with the possibility too that extremely small population of unfolded may have become
 189 folded in the presence of the stabilizer which perhaps may have opposed partially unfavourable
 190 ambient aqueous solution of unsteady pH. As observed elsewhere [6], extra calcium salt could also
 191 retard the activity of a holoenzyme. Therefore, the following may hold:

$$192 \quad v_N = \partial N / \partial t = k_{den}^* N \quad (11)$$

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

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

$$196 \quad k_{den}^* = \ln(N_1/N_0)/t \quad (12)$$

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

$$198 \quad k_{den}^* \sim \ln(v_1/v_0)/t \quad (13)$$

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

201 It should be pointed out however, that neither Eqs (9) nor (12) would hold if $U = N$ and $\partial N = 0$.
 202 Indeed Eq. (12) can only hold when for whatever reason, final form of the native enzyme is
 203 catalytically better than original native form which implies that there may have been increase in the
 204 native form population. Under such circumstance, there cannot be any unfolded state. What is
 205 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
 206 conclusion.

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

$$210 \quad k_{obs} = \ln(v_1)/t \quad (14)$$

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

214 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
 215 $[\text{Ca}^{2+}_{(\text{aq})}]$ when $v_1 < v_0$, the value of N_1 can then be determined from the intercepts. The following may
 216 be useful for the determination of N_1 .

$$217 \quad 1/k_{\text{den}}^* = l_{\text{NT}} \quad (15a)$$

218 where l_{NT} is the intercept.

$$219 \quad k_{\text{den}}^* = 1/l_{\text{NT}} \quad (15b)$$

220 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,

$$221 \quad N_1 = N_0 \exp(t/l_{\text{NT}}) \quad (16)$$

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

$$223 \quad N_1 = N_0 \exp(-t/l_{\text{NT}}) \quad (17)$$

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

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

230 In this case, the fraction of unfolded is:

$$231 \quad U = N_0 \exp(t/l_{\text{NT}}) - N_0 \quad (18)$$

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

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

241 $/(1-\alpha)$ where intuitively α is the fraction of the folded protein while $1-\alpha$ is the unfolded fraction.

242 However, this current approach takes the form:

$$243 \quad K_F = N_0 \exp(-\Delta H_{NT}) / (N_0 \exp(-\Delta H_{NT}) - N_0) \quad (19a)$$

244 Simplification of Eq. (19a) gives:

$$245 \quad K_F = \exp(-\Delta H_{NT}) / (\exp(-\Delta H_{NT}) - 1) \quad (19b)$$

246 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 $[N]/[U] >$

247 1 when $k_{den} \rightarrow$ zero. If conformational flexibility increases with increase in temperature due to entropic

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

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

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

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

252 \rightarrow zero such that:

$$253 \quad K_U = (1 - \exp(-\Delta H_{NT})) / \exp(-\Delta H_{NT}) \quad (20)$$

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

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

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

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

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

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

260 the presence of calcium chloride.

261 **3. MATERIALS AND METHODS**

262 **3.1 Materials**

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

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

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

266 97% pure, was purchased from Lab Tech Chemicals, India; 3, 5 – sodium potassium tartrate

267 tetrahydrate which is 97% pure was purchased from Kermel, China; Hydrochloric acid, sodium

268 hydroxide, and sodium chloride were purchased from BDH Chemical Ltd, Poole England; Tris was from

269 Kiran Light Laboratories, USA; calcium chloride was from Lab Tech Chemicals, India; other chemicals

270 were of analytical grade as indicated by manufacturer and solutions were made in distilled water.

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

274 **3.2 Methods**

275 A solution of enzyme was prepared by dissolving 0.01g in 20 mL tris – HCl_(aq) buffer as stock
276 at pH 7.4 as a matter of choice as in previous research elsewhere [16]. The enzyme was used as
277 purchased without further purification. The choice of concentration was at my discretion so as to
278 enhance the detection of the occurrence of activity of the enzyme which has lower activity with raw
279 starch [16]. One gram of raw soluble potato starch was mixed in 100mL of tris – buffer at pH 7.4 to
280 give 10g/L; potato starch in aqueous buffer is not a true solution [16]. Various molar solution of
281 calcium chloride ranging from 1 – 5 mM were prepared in distilled water; but the hydrated salt was
282 first thermally dehydrated to constant mass before measurement of mass was made. Assay was
283 carried out with and without (as control) calcium chloride at various temperature ranging from 37 –
284 60°C (310.15 – 333.15K). Two types of blanks were prepared. Blank containing only substrate and
285 blank containing only the enzyme were used. The sum of the absorbance at 540 nm was subtracted
286 from the test absorbance to give corrected absorbance.

287 **3.2.1 Assay of porcine pancreatic alpha amylase for the determination of velocity of** 288 **amylolysis of raw soluble potato starch, apparent rate constant, rate constant for** 289 **conditional irreversible folding and calcium ion binding constant.**

290 An *in vitro* assay of alpha-amylase was according to Bernfeld method [19]. The holoenzyme,
291 porcine pancreatic alpha amylase (PPA), was assayed for a short duration of 5 minutes and the final
292 activity is obtained according to Beer – Lambert law as follows: corrected absorbance at wavelength,
293 540nm, \times dilution factor (*i.e.* 3)/ ϵl , where ϵ ($181.1\text{M}^{-1}\text{cm}^{-1}$), C, and l are molar absorption coefficient,
294 molar concentration of product, and path length respectively. Details on the assay of the enzyme were
295 as described elsewhere [16].

296 “The initial apparent unit of activity is M/mL.min (number moles of reducing sugar yielded per
297 litre (L) of substrate per mL of enzyme per minute). Since 1 mL of substrate was hydrolyzed, the
298 number of moles of reducing sugar yielded per minute in 1mL using 1 mL of enzyme and maltose as
299 standard is $x\text{mmol/mL.min}$. Therefore, 1UI = micromoles maltose released/mL enzyme in the reaction
300 mixture/5 min” [16].

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

312 **3.2.2. The determination of activation parameters for unfolding**

313 Thermodynamic activation parameters, activation energy (E_a) enthalpy of activation (ΔH^\ddagger),
 314 and entropy of activation (ΔS^\ddagger), were obtained using apparent rate constant (K_{obs})

$$315 \ln k_{obs} = \ln A - E_a/RT. \quad (21)$$

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

$$320 \ln k_x = \ln A - E_a/RT. \quad (22)$$

$$321 \Delta H^\ddagger = E_a - RT \quad (23)$$

$$322 \Delta S^\ddagger = \Delta G^\ddagger - \Delta H^\ddagger \quad (24)$$

$$323 \Delta G^\ddagger = \ln (k_B T/hk_x) \quad (25)$$

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

327 **3.2.3. The determination of apparent thermodynamic parameters for calcium ion** 328 **binding**

329 The apparent thermodynamic parameters namely Gibbs free energy (ΔG°), enthalpy (ΔH°),
330 and entropy (ΔS°) for the binding of calcium ion were determined according to the following equations
331 adopted by Tanaka and Hoshino [10].

$$332 \quad \Delta G^\circ = -RT \ln K_b \quad (26)$$

$$333 \quad \partial \ln K_b / \partial T = \Delta H^\circ / RT^2 \quad (27)$$

334 Equation (27), being van't Hoff equation is used to determine ΔH° by plotting $\ln K_b$ versus $1/T$.

$$335 \quad \Delta S^\circ = (-\Delta G^\circ + \Delta H^\circ) / T \quad (28)$$

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

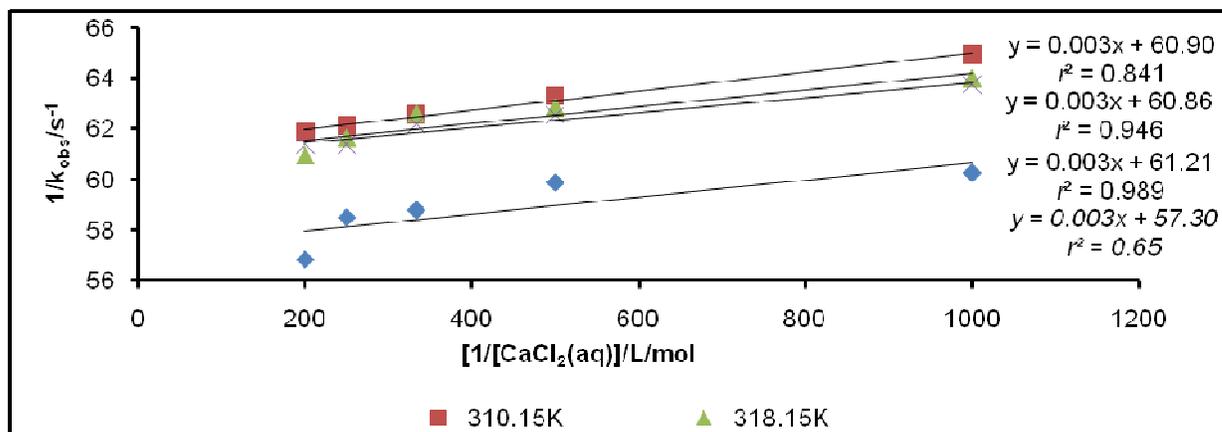
338 **3.2.4. Statistical analysis**

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

343 **4.0 RESULTS**

344 In the analysis of result, it is necessary to bear in mind the implication of the exposure of
345 holoenzyme to extra-load of calcium chloride. This should be in sharp contrast to calcium ion or salt-
346 depleted enzyme exposed to increasing temperature alone and apoenzyme exposed to increasing
347 temperature in the presence of increasing molar concentration of the salt. In order to determine rate
348 constant in the absence of unfolding, the reciprocal of apparent rate constant was plotted versus
349 reciprocal of molar concentration of the salt, calcium chloride (Fig. 1). There was the need to show the
350 effect of temperature in the presence of calcium salt as indicted in Fig. 2. Figure 2 shows that, the
351 apparent rate constant showed increasing trend with increase in temperature. The *t*-test carried out to
352 verify whether or not the presence of calcium salt caused significant change in the velocity of
353 amylolysis showed that the calculated *t* which was higher at 310.15K than at 318.15K, is > critical *t* (*t*
354 $_{0.05 (2),4}$). Thus there was significant change in the velocity of amylolysis ($P < 0.05$).

355



356

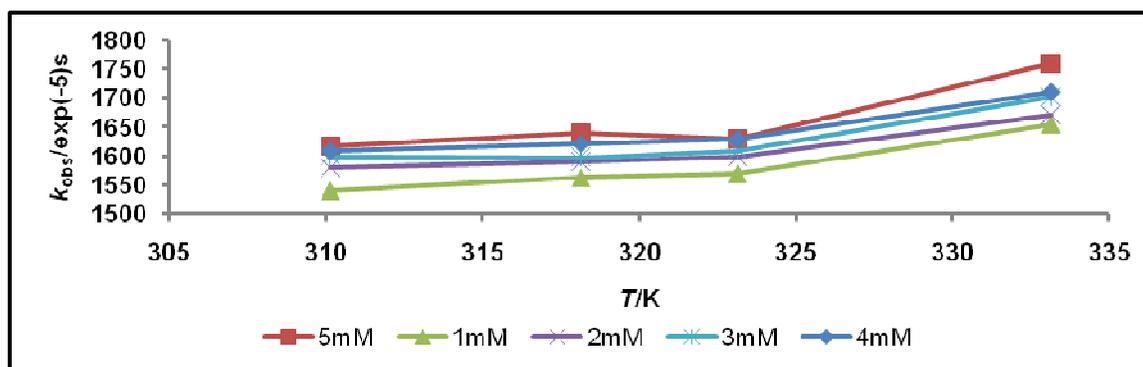
357 **Fig.1. Plot of reciprocal of apparent first-order rate constant k_{obs} versus reciprocal of $[CaCl_2(aq)]$**

358 **for the determination of rate constant for refolding of the enzyme and calcium ion binding**

359 **constant at 310.15 K and 318.15 K. (■), (▲), (x), and (◆) are assays and plots at 310.15 K, 318.15**

360 **K, 323.15 K, and 333.15 K respectively.**

361



362

363 **Fig. 2. Plot of apparent rate constant (k_{obs}) versus absolute temperature (T) showing the trend**

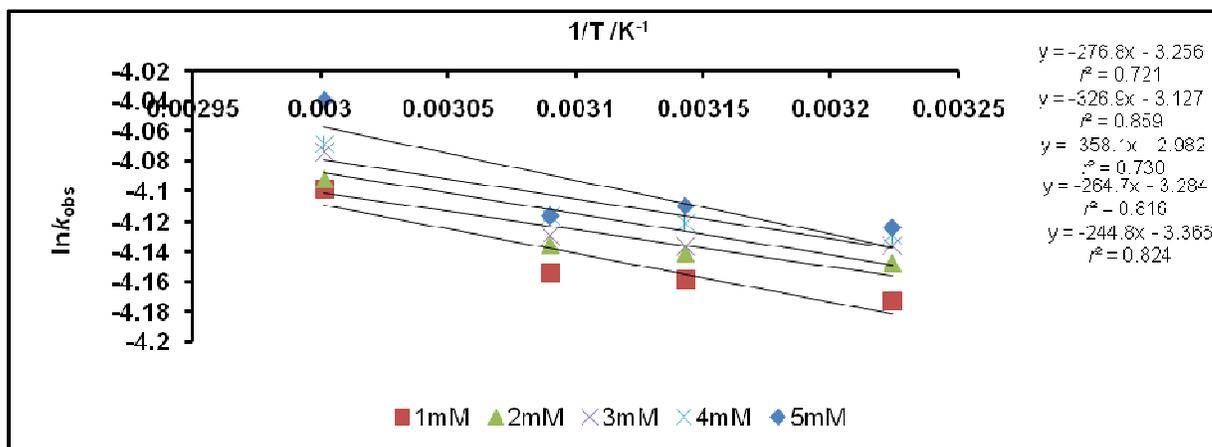
364 **of k_{obs} with increasing T at different molar concentration of calcium chloride. (■), (▲), (x), (*),**

365 **and (◆) are assays and plots at 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM respectively.**

366

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

372



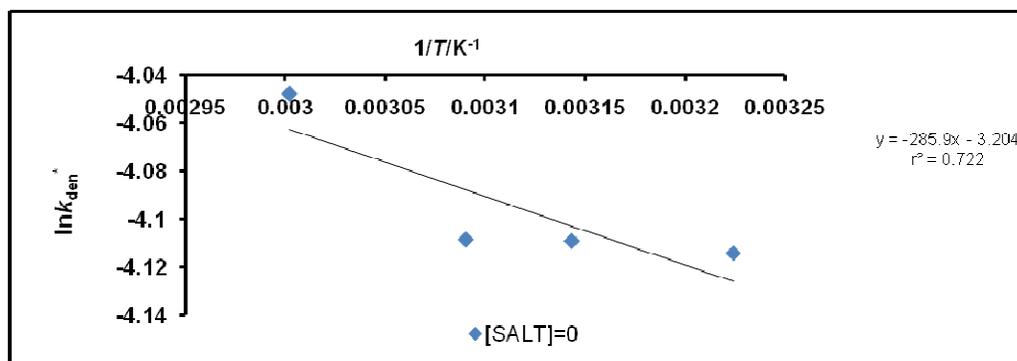
373

374 **Fig. 3. Arrhenius plot for the determination of activation energy for unfolding of enzyme at**

375 **different salt molar concentrations. (■), (▲), (×), (*), and (◆) are assays and plots at 1 mM, 2 mM,**

376 **3 mM, 4 mM, and 5 mM respectively.**

377



378

379 **Fig. 4. Arrhenius plot for the determination of activation energy for the refolding of the enzyme**

380 **at different salt molar concentrations.**

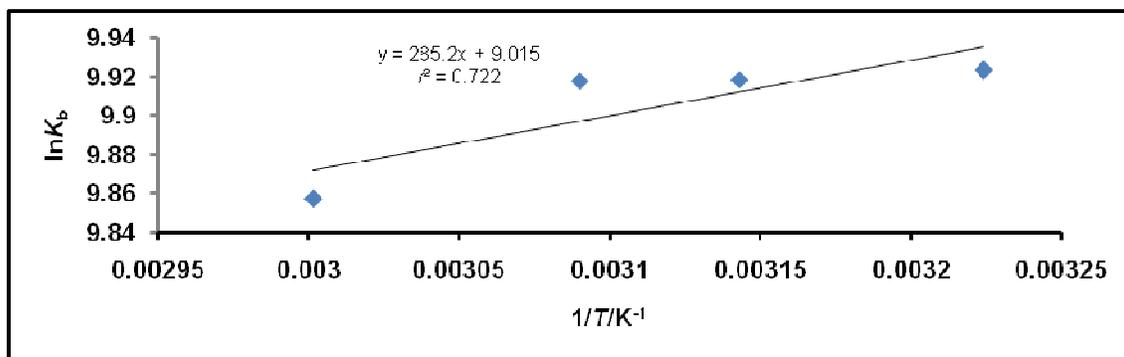
381 **The determination of enthalpy of calcium ion binding to sites other than active site domain**

382 **was carried out according to van't Hoff method by plotting natural logarithm of binding constant versus**

383 **reciprocal of thermodynamic temperature (Fig. 5). In the same vein, the enthalpy of folding was**

384 **determined (Fig.6).**

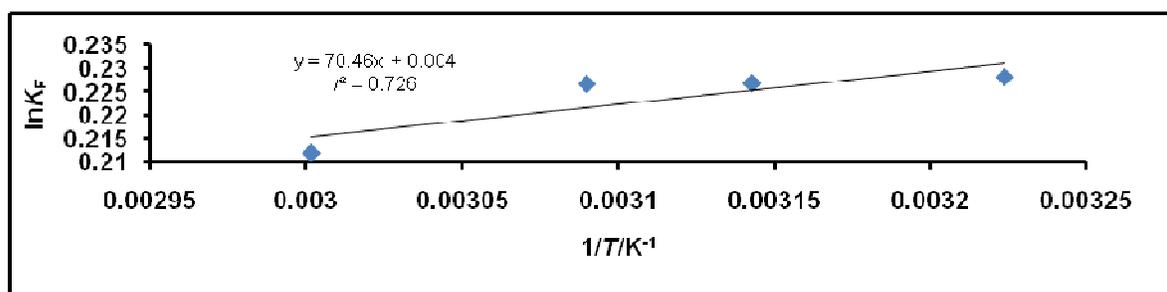
385



386

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

391



392

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

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

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

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

411 **Table 1. Activation parameters for the binding of calcium ions and folding of the**
 412 **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			

413 $\Delta H^\ddagger = \Sigma(Ea - RT)/2 \pm SD$; $\Delta S^\ddagger = \Sigma(\Sigma(\Delta G^\ddagger)/2 - \Delta H^\ddagger)/2 \pm SD$; The activation parameters Ea , ΔH^\ddagger , ΔS^\ddagger ,

414 ΔG^\ddagger , and A are activation energy, enthalpy of activation, entropy of activation, free energy of

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

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

427 **Table 2. Calcium ion binding constant and thermodynamic parameters for calcium ion**
 428 **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
ΔG_b (kJ/mol)	-25.59±0.00	-26.24±0.00	-26.65±0.00	-27.31±0.01
ΔH_b (kJ/mol)	-2.37±0.01			
ΔS_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
ΔG_f (J/mol)	-588.49±0.15	-600.19±0.15	-609.19±0.00	-586.96±0.22
ΔH_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

429 $\Delta S_b = \Sigma(\Delta G_b - \Sigma\Delta H_b/2)/2 \pm \text{SD}$; K_b , and k_{den} , are calcium ion binding constant and rate constant for the
 430 folding; ΔS_b , ΔG_b , and ΔH_b are entropy, free energy, and enthalpy of calcium ion binding respectively.
 431 ΔG_f , ΔH_f , and ΔS_f are free energy, enthalpy, and entropy of folding of the enzyme respectively.
 432

433 5. DISCUSSION

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

456 The Gibbs free energy of activation for the unfolding of the protein following the binding of
457 calcium ion appears to be higher at higher temperatures than lower temperature (Table 1). This
458 seems to suggest that the energy barrier for unfolding of the enzyme is higher at higher temperatures
459 due to the opposing effect of calcium ions by their rigidifying or stabilizing effect on the 3-D structure
460 [23]. It seems too that the free energy of activation at higher salt concentration is lower than at lower
461 salt concentration; this was unlike the report for *Bacillus amyloliquefaciens* [10] which, unlike PPA in
462 this study showed higher enthalpy of activation for unfolding at higher salt concentration. This may be

463 as a result of the effect of higher salt concentration in promoting greater conformational flexibility due
464 to increasing chloride ion component, because chloride ion opposes the effect of calcium ion; a
465 protonated state of Glu – 233 in the presence of chloride ion weakens the strength of calcium ion
466 binding and concomitantly its inhibitory effect also [3]. Being endothermic, higher thermal environment
467 may be needed for calcium ion binding such that with higher temperatures the binding of the ion
468 should be more spontaneous. This may therefore, promote higher rate of folding in opposition to
469 unfolding with increase in temperature, that may be imposed by the presence of extra calcium ion
470 without insinuating indefinite process as shown in Table 1.

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

477 Forge *et al.* [22] reported folding rate constant equal to 20.2/s at pH, 7.2 and 20°C in 10 mM
478 calcium chloride while Deitcher *et al* [26] reported folding rate constant ranging from 0.05 to 0.1/min.
479 This range is about 10-20 fold lower than values recorded in this investigation. Enhanced folding
480 which implies stability promotes catalytic activity of the enzyme similar to the observation that the
481 presence of calcium ion increases the activity and stability of the enzyme at high temperatures [27].
482 Current values (Table 1) of folding rate constant at different temperatures are however, closer to the
483 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
484 respectively [18]. Before proceeding further into the discussion, it is imperative to state that the
485 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
486 other favourable and practicable condition, that the calcium ion component can resist total unfolding
487 so long as increasing velocity of hydrolysis of substrate is observed with increasing temperature. This
488 is exactly opposite to what is expected when there is decrease in activity, such that Eq. (5) becomes
489 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
490 in the activity of the enzyme due to unfavourable unfolding due to the presence of the salt either with
491 increasing or decreasing temperature. Most reactions become more spontaneous with increase in
492 temperature. The implication is that binding of cation and folding of the enzyme cannot be extricated

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

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

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

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

534 Looking at Table 2 one observes that the calcium ion binding constant showed lower values
535 at higher temperatures similar to the observation by Nielsen *et al.* [9] and Tanaka and Hoshino [10]
536 with respect to *B. halmapalus* alpha amylase (BHA) and *Bacillus amyloliquefaciens* alpha amylase
537 (BAA) respectively. The values with respect to BAA were similar to current report for PPA at higher
538 temperature, 60°C and 65°C but the values at lower temperatures were much higher than values for
539 PPA. This is most likely considering the fact that the binding constant at temperature lower than 37°C
540 (specifically 22°C) as originally reported for human salivary alpha amylase elsewhere [31] was very
541 much higher than values at 37°C and above. Values reported for BHA were very much higher than
542 values for PPA in this report at all temperatures. The expression for the stability of the product of any
543 biochemical reaction such as calcium ion binding was exothermic though much less than the report
544 for BAA at pH 7.5 [10] and value for BAA as high as **-149 kJ/mol** at pH 7 [32]; but all observations
545 **point** to stable calcium-enzyme complex at sites other than active site. While the entropic term, $T\Delta S$,
546 of calcium ion binding is **-109.13±0.61 kJ/mol**, there is proposition that calcium binding may consist
547 both of contributions from calcium binding itself and of protein folding; thus there may be need to
548 subtract the contribution of calcium binding enthalpy (**- 8 kJ/mol**) and entropic term (**- 42 kJ/mol**) so
549 as to yield folding thermodynamic parameters such as **-157 kJ/mol** for enthalpic term and **-151**
550 **kJ/mol** for entropic term ($T\Delta S$) and ultimately, the value of the free energy of folding in terms of $\Delta G =$
551 $\Delta H - T\Delta S$, should be **- 6 kJ/mol** [10]. **However, the result presented in Table 2 shows some similarities**
552 **and differences** in comparison with the above observation in literature. The magnitude of the enthalpy

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

562 In the same Table 2, it can be observed that folding equilibrium constant is slightly greater
563 than unity and the values appeared to decrease with temperature and consequently the free energies
564 though express spontaneity are nevertheless much lower than literature value of -39.87 kJ/mol at
565 303.15K reported for BAA [10]. On the whole one can suggest that calcium ion binding and folding of
566 PPA in the face of extra calcium salt are enthalpically driven and spontaneous as observed for other
567 homologues. But entropic term seemed to show greater contribution being greater than enthalpic term
568 ($T\Delta S > \Delta H$). However, there is clear-cut difference in the entropic term between literature information
569 for BAA and PPA in this study. While the entropies of calcium ion binding and for folding reported for
570 PPA were positive, the case reported for BAA is negative [10, 32]. Naturally folding results in
571 decrease in conformational entropy coupled with hydration at higher temperature as it is the case with
572 BAA. But due to dehydration, as calcium ions bind (Tanaka and Hoshino, 2002) what may be called
573 translational entropy gain (Harano and Kinoshita, 2005) may occur as applicable to PPA in this study.

574 6. CONCLUSION

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

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

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