1 ## *Original Research Article*

2 **Title: Calcium ion binding characteristics of porcine pancreatic alpha amylase outside**

3 **active site domain and implications: Theory and experimentation.**

4 **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 a 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 while activation energy was low and higher at higher salt concentration. Free energy of activation, exothermic enthalpy and negative entropy for folding were higher at higher temperatures except at 333.15K 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 333.15K. 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 PPA though exothermic, are entropically driven (entropic

term > enthalpic term) and spontaneous.

1. INTRODUCTION

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

34 binding via hydrogen bonds and hydrophobic interactions [6]. "The 'essential' calcium ion has been

35 located near the active site region and between two domains, each of them providing two calcium

36 ligands. On the basis of sequence comparison, the calcium binding site is suggested to be a common

37 structural feature of all alpha amylases". [6]. The chloride binding site lies on the axis of the domain

38 Aβ-barrel near its carboxyl-end: The nearest amino acids forming contacts with the chloride ion are

39 positively charged residues, Arg. 195, Lys. 257 and Arg. 337 extending from strands Aβ4, Aβ6 and

40 Aβ8 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 enzyme,

45 leads to inhibition [3]. On the other hand a protonated state of Glu – 233 in the presence of chloride

46 ion is purported to weaken the strength of calcium ion binding and concomitantly its inhibitory effect

47 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: $H_2PO_4^{-1} > SO_4^{-1} > F^{-1} > Cl^{-1} > Br^{-1}$

62 $> NO_3^{-1} > I^{-1} ClO_4^{-1} > SCN^{-1}$ [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

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 unfolding and folding and

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

83   where k_{den} is the irreversible rate constant for the denaturation of the calcium-depleted enzyme, $k_{den}{}^*$ is

84   that for the calcium-bound enzyme, k_{obs} is the apparent first-order rate constant, and K_b is the binding

85   constant of the calcium ion to the enzyme. It must be made clear that so long as there is residual or

86   higher activity than control, there are likely initial subpopulations of enzymes partially or totally

87   inactivated and subpopulation that is activated such that overtime there may be net activation or

88   inactivation as the case may be. Thus in the presence of the salt there may be net activation with the

89   result that the calcium-bound rate constant should be much greater than that of the calcium-depleted

90   form; therefore, one assumes that only the bound form contributes mainly to the unfolding-folding

91   transition, and the depleted form contributions in Eq.(1) should be very infinitesimal compared to the

92   bound form. If this assumption or postulation is reasonable and applied to the experimental data, the

93   linear relationship between the values of reciprocal of apparent first-order rate constant and the

94 reciprocal of calcium ion concentrations can be determined: At this juncture, it is important to point out

95 that Tanaka and Hoshino did not subject the test enzyme to chelating agents (though calcium free

96 reaction mixture containing enzyme was tested) as may be testified as follows: "The reaction mixture

97 consisting of 1.3110^{-8}M BAA, 25 mM Tris acetate buffer (pH 7.0) and 0.002-0.02 mM $CaCl_2$ was

98 incubated at 45-65°C for the appropriate time intervals"[10]. Thus there is no question of the use of

99 apoenzyme. In the experiment by the authors [10], residual activity measured as percentage of

100 control implied that there was decrease in activity even with increasing concentration of the salt at

101 different temperatures. Then, the question is, what model may be suitable if despite increasing

102 temperature in the presence of increasing concentration of the salt, the activity of the enzyme is

103 increasing?

104 However, this investigation focuses in part, on the binding of extra calcium salt and as such

105 binding constant, and cognate apparent thermodynamic parameters and activation parameters of the

106 binding process which become inevitable part of the investigation. Moreover, extra calcium ion

107 binding must be at other sites other than the intrinsic binding sites within the active site domain. In

108 other words unfolding kinetics is not a major part of the investigation even though conformational

109 flexibility occasioned by thermally induced unfolding arising from the break-up of some of the weak

110 binding interaction leading to increase in conformational entropy of activation is the usual

111 characteristics of mainly thermophiles and moderate thermophiles to a lesser extent [15].

112 In the light of the scope of this investigation in which $k_{den} \sim 0$, the rate equation in the absence

113 of major unfolding for calcium ion treated enzyme is:

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

115 For the purpose of comprehension, a stepwise approach beginning from Eq. (2) leads to the 2nd step

116 after rearrangement:

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

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

119 However, like Tanaka and Hoshino relative activity is adopted herein as applied in a thesis [16]

120 though it has been realized that the rate equation for unfolding leading to decrease in activity under

121 condition similar to current study, was mistakenly applied with the result that this research offers

122 opportunity for amendment.

123   However, a serious reappraisal of Eqs (1), (2), (3), and (4) reveals vital issues that may attract

124   contrasting opinion from scholars: For instance one may wish to know why the equations should be

125   strictly for irreversible unfolding kinetics? Calcium chloride treated enzyme ought to show stability with

126   increasing concentration of the salt but not *ad infinitum* as it has been shown that when calcium ions

127   are in excess, the enzyme, *B. halmapalus* alpha amylase unfolds irreversibly at high temperatures [9].

128   How would the equations apply should there be increasing activity above the activity of the native

129   holoenzyme without extra load of calcium salt, with increasing temperature? The same authors [9]

130   observed that at low temperature the same enzyme undergoes irreversible denaturation when

131   depleted of the salt let alone at high temperatures. "Perhaps scientifically naïve, otherwise one would

132   have speculated that unfolding under any circumstance should be governed by first order rate

133   constant, k_{den} and should there be refolding, k_{den}^* may be folding first order constant in the presence of

134   calcium chloride if there was increasing velocity of hydrolysis (activity that can be expressed as

135   relative activity), a speculation that may prompt recrimination**. "**But it may not be scientifically naïve

136   to suggest that, when the enzyme mixed with the salt and undergoing unfolding at higher temperature

137   is removed and placed in lower temperature thermal environment containing the same concentration

138   of salt, the enzyme may refold continuously until all molecules are fully refolded". In this regard,

139   Nielsen *et al.* [9] opined that at temperature below 65°C, the denaturation of calcium-bound *Bacillus*

140   *halmapalus* alpha amylase (BHA) is negligible, since inactivation proceeds almost exclusively as

141   denaturation of calcium-depleted BHA. Thus according to Tanaka and Hoshino [10] the unfolding rate

142   constant, k_{den} for calcium-depleted enzyme may be applicable because this was applied to calcium-

143   bound enzyme whose activity decreased with increasing temperature. But this may contradict the fact

144   that calcium-treated or calcium-bound enzyme is the case whose implied first order rate constant for

145   unfolding is different. "This does not lead to a jungle of confusion without a way out of invisibility". The

146   model as shown in Eq (1) seem to suggest that there are two aspect to it, one being that unfolding

147   could assume preeminence due to the effect of destabilizing forces, overcoming in the process the

148   stabilizing forces, the multiple binding interactions intrinsic to the enzyme; hence, Eq (5) below

149   becomes inevitable such that k_{den}^* tends to zero. In this case there is irreversible unfolding. But

150   irreversible unfolding is not automatic because it begins at a point in which the energy is sufficient to

151   cause total unfolding of the total number of molecules. Therefore, Eq. (5) below remains relevant to

152   the end of unfolding at which all binding interactions is nonexistence as the three dimensional (3-D)

153   structure has totally collapsed.

154                                 $1/k_{obs} = K_b [Ca^{2+}_{(aq)}]/k_{den} + 1/k_{den}$ (5)

155   At the beginning of unfolding at the melting point, there is a mixture of folded and unfolded

156   hence the absorbance is not the same at any temperature. The absorbance continues to rise with

157   increase in temperature above the melting point until all the molecules unfold or denatures such that

158   further increase in temperature does not lead to increase in absorbance, hence the curve becomes

159   horizontal to the temperature axis of the plot. The same scenario is expected with increasing

160   concentration of denaturants. It seems top-level scholars have no time to reflect deeply into this issue.

161   Thus before maximum absorbance or total unfolding, the equilibrium merely shifts toward the unfolded

162   state with higher concentration in the presence of enabling factor. Hence, an equilibrium constant

163   such as the following is expected:

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

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

166   Now the question is can N tend to zero? If such situation is applicable it would imply that the

167   velocity of hydrolysis (or activity (v)) of substrate would tend to zero. Meanwhile any residual activity is

168   a function of the presence of folded enzyme and categorically speaking it is not a function of the

169   unfolded enzyme hence, the value of v for the native enzyme in its entirety is much greater than when

170   there is a subpopulation of the unfolded enzyme. Therefore, one should expect that:

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

172   where v_U and t are the rate of unfolding with increasing temperature before final and total unfolding of

173   all enzyme molecules and duration of assay respectively.

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

175   rearrangement is:

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

177   Equation (8) can be translated to:

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

179   where v_0 and v_1 are respectively ($v_0 > v_1$), the activity of native enzyme without salt and the enzyme

180   with salt. As a matter of fact, activity of the enzyme due to the effect of the salt is best described as

181   residual activity. On the other hand, the activity of the native enzyme could increase with temperature

182   in the presence of stabilizing agents well above what it could have been in the absence of the

183   stabilizer. Thus,

184 $N_1/N_0 > 1$ (10)

185   where N_1 and N_0 are respectively new population of the folded and initial native population taken as

186   100%. This is so because, $N_0 \rightarrow N_1$ which simply imply that the native enzyme assumes greater

187   catalytic power with the possibility too that extremely small population of unfolded may have become

188   folded in the presence of the stabilizer which perhaps may have opposed partially unfavourable

189   ambient aqueous solution of unsteady pH. As observed elsewhere [6], extra calcium salt could also

190   retard the activity of a holoenzyme. Therefore, the following may hold:

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

192   where v_N is the rate of refolding. The modeling is anchored on the fact that there is either

193   overwhelming unfolding or refolding leading to net unfolding or refolding rate constant.

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

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

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

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

198   where indeed, $v_1 > v_0$. Equations (9) and (13) represent a simplification of the final forms which may

199   not fall within the immediate scope of these derivations.

200   It should be pointed out however, that neither Eqs (9) nor (12) would hold if $U = N$; $\partial N = 0$.

201   Indeed Eq. (12) can only hold when for whatever reason, final form of the native enzyme is

202   catalytically better than original native form which implies that there may have been increase in the

203   native form population. Under such circumstance, there cannot be any unfolded state. What is

204   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

205   conclusion.

206   The 2nd aspect of the model development follows from Tabassum et al [14] method which is

207   slightly different from the method of Tanaka and Hoshino who used relative activity. Thus according to

208   Tabassum et al [14],

209 $k_{obs} = \ln(v_1)/t$ (14)

210   where v_1 is the residual activity if there is decrease in activity and if there was increase in activity it

211   can best be described as higher activity above initial native activity. However, relative rate of

212   hydrolysis of starch is adopted for the purpose of this research as was the case elsewhere [10].

213   What may be clear is that from the plot of $1/k_{obs}$ versus $1/[Ca^{2+}_{(aq)}]$ when $v_1 > v_0$ and versus

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

215   be useful for the determination of N_1.

$$1/k_{den}^* = I_{NT} \qquad \qquad (15a)$$

217   where I_{NT} is the intercept.

$$k_{den}^* = 1/I_{NT} \qquad \qquad (15b)$$

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

$$N_1 = N_0 \exp (t/I_{NT}) \qquad \qquad (16)$$

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

$$N_1 = N_0 \exp (-t/I_{NT}) \qquad \qquad (17)$$

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

224   native structure.

225   In order to determine Gibbs free energy of unfolding and folding, simple cognate models need

226   to be derived as follows. The folding process entails the transition of what may be seen as "minor

227   fraction" of unfolded in the native initial state population to a greater folded population in the presence

228   of calcium salt. Thus the final folded fraction is as expressed in Eq. (12).

229   In this case, the fraction of unfolded is:

$$U = N_0 \exp (t/I_{NT}) - N_0 \qquad \qquad (18)$$

231   Since $K_{eq} = U/N$, when there is folding (N)-unfolding (U) transition (N→U) on the basis of the

232   equilibrium equation (N⇌U) characteristic of two state model then $1/K_{eq}$ should portray the opposite

233   direction (U→N). Thus $1/K_{eq}$ should be unmistakably, N/U.

234   Nonetheless consideration is given to transition from what may be called "native to greater

235   native state" on account of which the term, constant of folding (K_F) according to Greenfield [18] is now

236   to be used instead of $1/K_{eq}$. Most importantly is the fact, according to Greenfield [18], that $K_F = [N]/[U]^n$

237   where n is the number of chains that may associate upon folding of polymeric proteins. Nevertheless

238   n is one in this research. Closer to the needs of this research is the equation [18] for the folding

239   constant (otherwise called equilibrium constant for folding) for a monomeric protein which is $K_F = \alpha$

240    $/(1-\alpha)$ where intuitively α is the fraction of the folded protein while $1-\alpha$ is the unfolded fraction.

241   However, this current approach takes the form:

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

243   Simplification of Eq. (19a) gives:

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

245   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] $>$

246   1 when $k_{den} \rightarrow$ zero. If conformational flexibility increases with increase in temperature due to entropic

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

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

249   favourable folding of "minor" subpopulation of the enzyme may occur in the presence of calcium salt.

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

251    $\rightarrow$ zero such that:

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

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

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

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

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

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

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

259   the presence of calcium chloride.

260   **3. MATERIALS AND METHODS**

261   **3.1 Materials**

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

263   amylase (PPA)) was purchased from Sigma, Aldrich, USA; so-called soluble potato starch was

264   purchased from Sigma Chemicals Co, USA; dinitrosalycilic acid (DSA) was purchased from Lab Tech

265   Chemicals, India; 3, 5 – sodium potassium tartrate tetrahydrate was purchased from Kermel, China;

266   Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd,

267   Poole England; Tris was from Kiran Light Laboratories, USA; calcium chloride was from Lab Tech

268   Chemicals, India; other chemicals were of analytical grade and solutions were made in distilled water.

269   Equipment: pH meter (tester) was from Hanna Instruments, Italy; electronic weighing machine was

270   from Wensar Weighing Scale Ltd, Chennai; Centrifuge, 800D model was from China; 721/722 visible

271   spectrophotometer was from Spectrum Instruments Co Ltd, China.

272   **3.2   Methods**

273   A solution of enzyme was prepared by dissolving 0.01g in 20 mL tris – HCl$_{(aq)}$ buffer as stock

274   at pH 7.4 as a matter of choice as in previous research elsewhere [16]. The choice of concentration

275   was at my discretion so as to enhance the detection of the occurrence of activity of the enzyme which

276   has lower activity with raw starch [16]. One gram of raw soluble potato starch was mixed in 100mL of

277   tris – buffer at pH 7.4 to give 10g/L; so-called soluble starch in aqueous buffer is not a true solution

278   [16]. Various molar solution of calcium chloride ranging from 1 – 5 mM were prepared in distilled

279   water; but the hydrated salt was first thermally dehydrated to constant mass before measurement of

280   mass was made. Assay was carried out with and without (as control) calcium chloride at various

281   temperature ranging from 37 – 60°C (310.15 – 333.15K).

282   <u>**3.2.1   Assay of porcine pancreatic alpha amylase for the determination of velocity of**</u>

283   <u>**amylolysis of raw soluble potato starch, apparent rate constant, rate constant for**</u>

284   <u>**conditional irreversible folding and calcium ion binding constant.**</u>

285   An *in vitro* assay of alpha-amylase was according to Bernfeld method [19]. The holoenzyme,

286   porcine pancreatic alpha amylase (PPA), was assayed for a short duration of 5 minutes and the final

287   activity is obtained according to Beer – Lambert law as follows: corrected absorbance at wavelength,

288   540nm, $\times$ dilution factor (*i.e.* 3)/ε l, where ε (181.1M^{-1}cm^{-1}), C, and l are molar absorption coefficient,

289   molar concentration of product, and path length respectively. Details on the assay of the enzyme were

290   as described elsewhere [16].

291   "The initial apparent unit of activity is M/mL.min (number moles of reducing sugar yielded per

292   litre (L) of substrate per mL of enzyme per minute). Since 1 mL of substrate was hydrolyzed, the

293   number of moles of reducing sugar yielded per minute in 1mL using 1 mL of enzyme and maltose as

294   standard is xmmol/mL.min. Therefore, 1U = micromoles maltose released/mL enzyme in the reaction

295   mixture/5 min" [16].

296   In order to determine the rate constant for folding the reciprocal of apparent rate constant was

297   plotted versus reciprocal of molar concentration of the salt. By combining the slope and intercept the

298   calcium ion binding constant is calculated. Most importantly, assay was carried out at different

299   temperatures ranging from 37-60 °C in the presence and absence of calcium chloride unlike approach

300   by Tanaka and Hoshino [10] who brought the solution of enzyme with and without the salt to lower

301   temperature by "ice-cooling" the mixture without specifying the final temperature. The work of the

302   authors however, provides without losing major scientific quality, a good background for the

303   examination of the calcium salt binding characteristics of the enzyme using raw starch in this study.

304   **3.2.2. The determination of activation parameters for unfolding**

305   Thermodynamic activation parameters, activation energy (Ea) enthalpy of activation ($\Delta H^{\#}$),

306   entropy of activation ($\Delta S^{\#}$), were obtained using apparent rate constant (K_{obs})

307    $$\ln k_{obs} = \ln A - E a / RT. \tag{21}$$

308   where A, R, and T are pre – exponential factor, gas constant, and absolute (thermodynamic)

309   temperature respectively. In realization of the fact that k_{den} and k_{den}^{*} are first order rate constants, it is

310   not out of place to determine the thermodynamic activation parameters using them, whether or not it

311   is the first time to do so according to well known Arrhenius method. Hence,

312    $$\ln k_{x} = \ln A - E a / RT. \tag{22}$$

313    $$\Delta H^{\#} = E a - RT \tag{23}$$

314    $$\Delta S^{\#} = \Delta G^{\#} - \Delta H^{\#} \tag{24}$$

315    $$\Delta G^{\#} = \ln (k_{B} T / h k_{x}) \tag{25}$$

316   where k_{x} for the purpose of this investigation and in line with the trend in the data generated is either

317   k_{den}^{*}, or k_{obs} as the case may be; and h and k_{B} are the Planck's constant and Boltzmann constant

318   respectively.

319   **3.2.3. The determination of apparent thermodynamic parameters for calcium ion**

320   **binding**

321   The apparent thermodynamic parameters namely Gibbs free energy (ΔG^{o}), enthalpy (ΔH^{o}),

322   and entropy (ΔS^{o}) for the binding of calcium ion were determined according to the following equations

323   adopted by Tanaka and Hoshino [10].

324    $$\Delta G^{o} = -RT \ln K_{b} \tag{26}$$

325    $$\partial \ln K_{b} / \partial T = \Delta H^{o} / RT^{2} \tag{27}$$

326   Equation (27), being van't Hoff equation is used to determine ΔH^{o} by plotting $\ln K_{b}$ versus $1/T$.

327    $$\Delta S^{o} = (-\Delta G^{o} + \Delta H^{o})/T \tag{28}$$

328   The free energy of folding and enthalpy of folding can be determined by replacing K_b with K_F in Eq.

329   (26) and Eq. (27) respectively.

330   **3.2.4. Statistical analysis**

331   **4.0   RESULTS**

332   In the analysis of result, it is necessary to bear in mind the implication of the exposure of

333   holoenzyme to extra-load of calcium chloride. This should be in sharp contrast to calcium ion or salt-

334   depleted enzyme exposed to increasing temperature alone and apoenzyme exposed to increasing

335   temperature in the presence of increasing molar concentration of the salt. In order to determine rate

336   constant in the absence of unfolding, the reciprocal of apparent rate constant was plotted versus

337   reciprocal of molar concentration of the salt, calcium chloride (Fig. 1). There was the need to show the

338   effect of temperature in the presence of calcium salt as indicted in Fig. 2. Figure 2 shows that, the

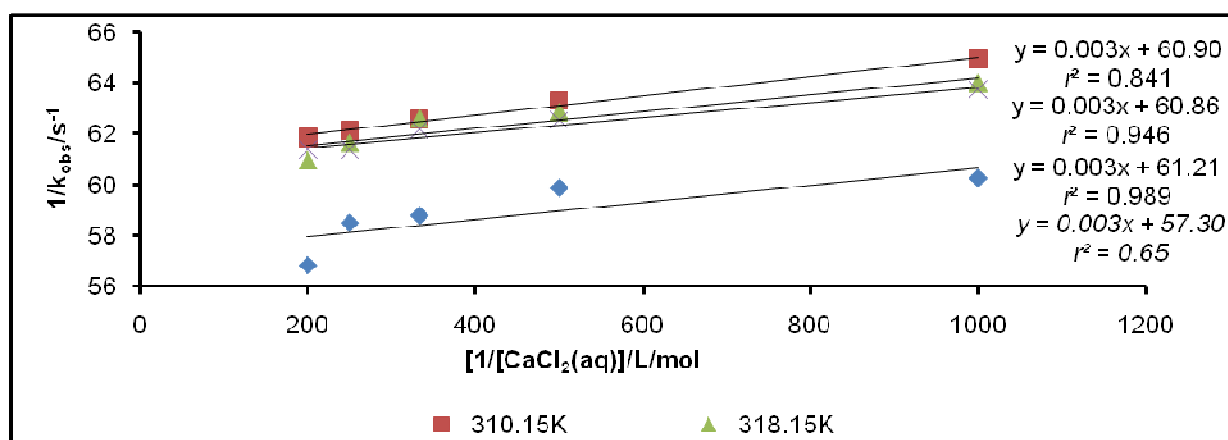339   apparent rate constant showed increasing trend with increase in temperature.

340

341

342   **Fig.1. Plot of reciprocal of apparent first-order rate constant k_{obs} versus reciprocal of [CaCl$_{2(aq)}$]**

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

344   **constant at 310.15K and 318.15K**. (■), (▲), (×), and (♦) are assays and plots at 310.15K, 318.15K,

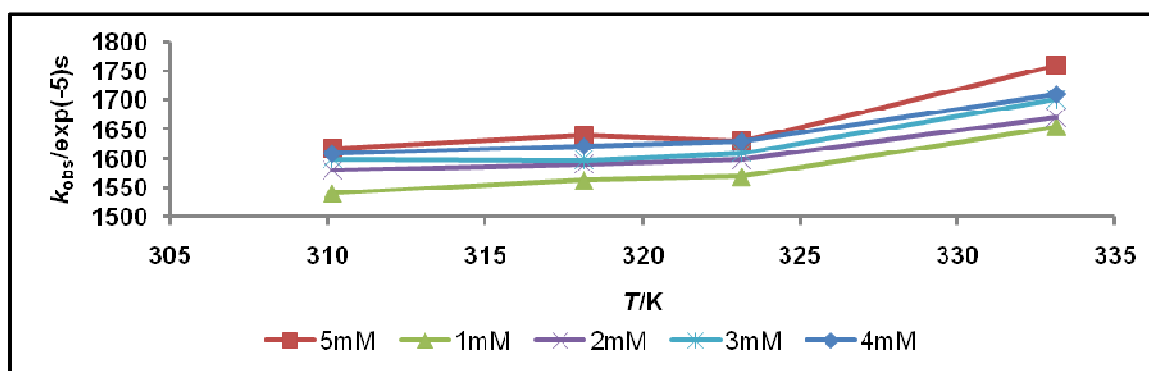345   323.15k, and 333.15K respectively.

346

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

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

The determination of enthalpy of calcium ion binding to sites other than active site domain was carried out according to van't Hoff method by plotting natural logarithm of binding constant versus reciprocal of thermodynamic temperature (Fig. 5). In the same vein the enthalpy of folding was determined (Fig.6).
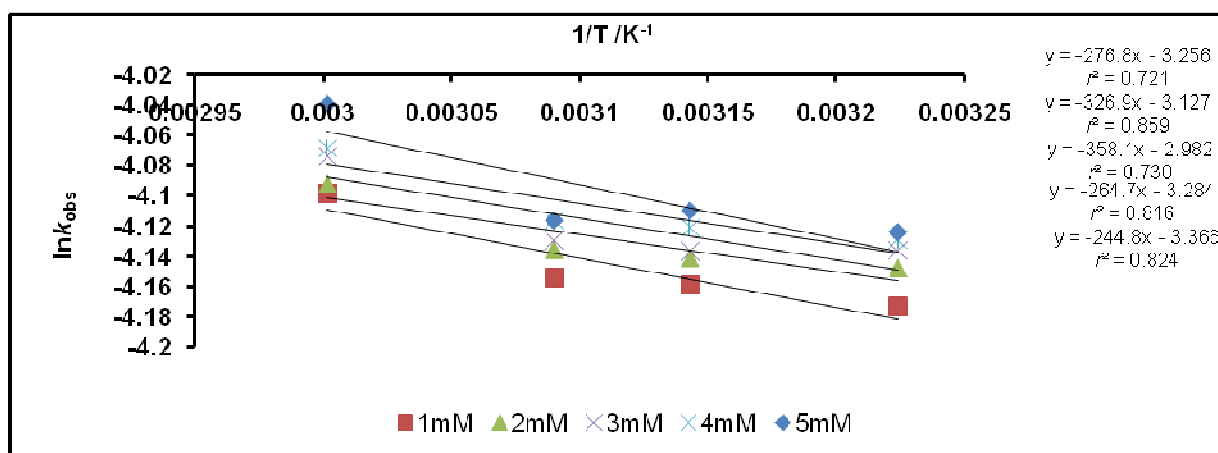
363   **Fig. 3. Arrhenius plot for the determination of activation energy for unfolding of enzyme at**

364   **different salt molar concentrations.** (■), (▲), (×), (∗), and (♦) are assays and plots at 1mM, 2mM,
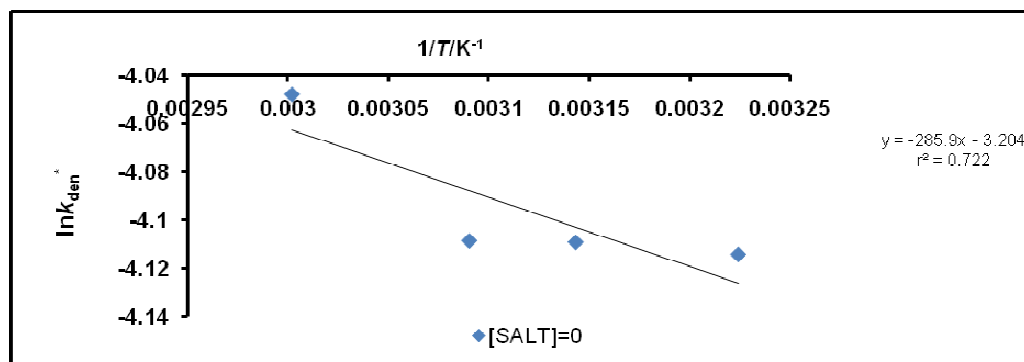
365   3mM, 4mM, and 5mM respectively.

366

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

368   **at different salt molar concentrations.**

369   The enthalpy of calcium ion binding was determined by plotting natural logarithm of binding

370   constant versus reciprocal of thermodynamic temperature (Fig. 5). In the same vein, the enthalpy of

371   folding was determined by plotting the natural logarithm of folding equilibrium constant versus
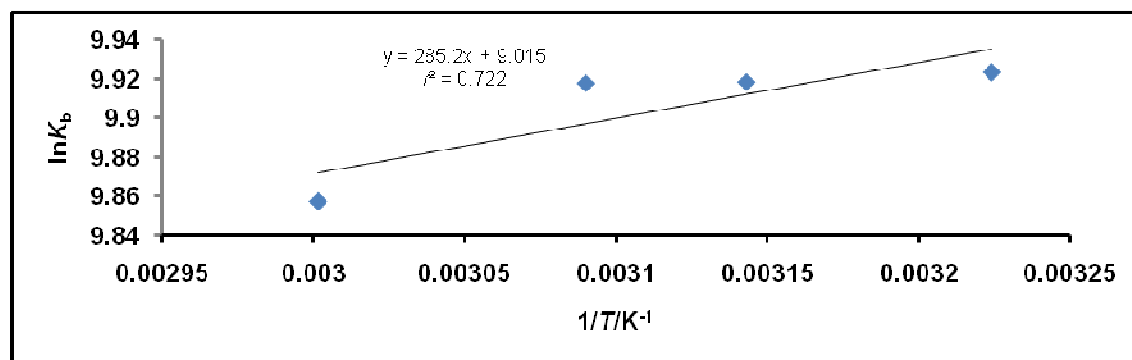
372   reciprocal of thermodynamic temperature (Fig.6).

373

374

375   **Fig. 5. van'Hoff plot for the determination of enthalpy of calcium ion binding to sites apart from**

376   **intrinsic sites.** Calcium ion binding constant (K_b) is obtained from the combination of the intercept

377   and slope of the plot of the reciprocal of apparent rate constant versus reciprocal of molar

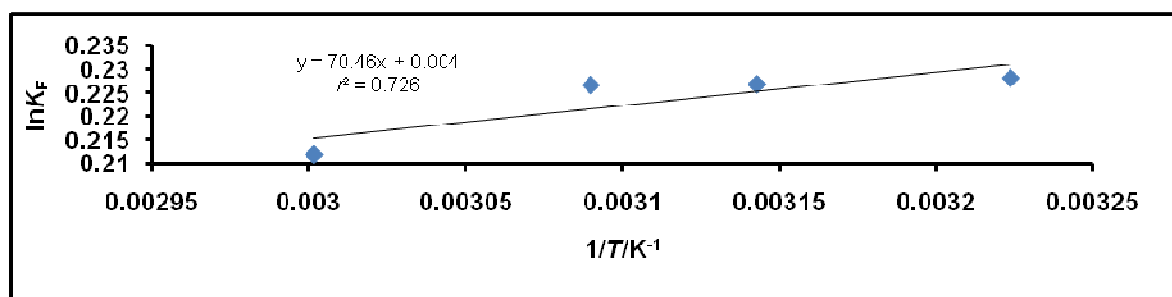378   concentration of calcium chloride.

379

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

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

In line with proposed folding rate constant it is observed that there was increasing values of the parameter with increasing temperature and parallel to this is also the increasing trend in the values of free energy of folding with increasing temperature as may be found in Table 1. The enthalpy of activation for folding is exothermic, the magnitude of which showed increasing trend with temperature just as the negative magnitude of entropy of folding showed increasing trend though the value at 333.15K is only lower than value at 323.15K (Table 1). Also in Table 1 is the activation energy for folding that is lower than RT.

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

406 **enzyme.**

[CaCl$_2$(aq)] (mM)	1		5	
T (K)	310.15	318.15	310.15	318.15
$\Delta G^{\#}$ (k J/mol)	86.82±0.00	89.09±0.01	86.70±0.00	88.96±0.00
$\Delta H^{\#}$ (J/mol)	141.32±21.21	74.81±21.21	401.32±7.07	334.81±7.07
$\Delta S^{\#}$ (J/mol.K)	-279.47±0.07	-279.79±0.06	-278.25±0.02	-278.59±0.02
EA (k J/mol)	2.72±0.02		2.98±0.01	
T (K)	310.15	318.15	323.15	333.15
K_{den} / Exp(-5) (1/S)	1633.70±0.35	1642.00±0.35	1643.00±0.00	1715.50±0.71
$\Delta G_F^{\#}$ (k J/mol)	86.67±0.00	88.96±0.00	90.49±0.00	93.11±0.00
$\Delta H_F^{\#}$ (J/mol)	-201.68±67.601	-268.19±9.90	-309.77±9.90	-392.91±9.90
$\Delta S_F^{\#}$ (J/mol.K)	-280.11±0.04	-280.47±0.04	-280.71±0.04	-280.66±0.03
Ea$_F$ (kJ/mol)	2.37±0.01			

407 $\Delta H^{\#} = \Sigma(Ea - RT)/2 \pm SD$; $\Delta S^{\#} = \Sigma(\Sigma(\Delta G^{\#})/2 - \Delta H^{\#})/2 \pm SD$; *The activation parameters Ea, $\Delta H^{\#}$, $\Delta S^{\#}$,*

408 *and $\Delta G^{\#}$ are activation energy, enthalpy of activation, entropy of activation, and free energy of*

409 *activation respectively. The corresponding activation parameter, for folding, is represented by*

410 *subscript,* F.

411 The calcium ion binding constant showed decreasing trend with temperature just as the free

412 energy of the cation binding was negative with increasing magnitude with increasing temperature

413 (Table 2). As recorded in Table 2, the enthalpy of calcium ion binding is exothermic and the positive

414 entropy showed decreasing trend except the value at 333.15K which is larger than all values at lower

415 temperatures. The folding equilibrium constant showed decreasing trend with temperature while the

416 negative free energy of folding exhibited increasing trend in magnitude except the value at 333.15K,

417 which, is lower in magnitude than values at lower temperatures (Table 2). The enthalpy of folding is

418 exothermic and the positive entropy of folding exhibited increasing trend but the value at 333.15K was

419 lower in magnitude than all values at lower temperatures (Table 2).

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

421 **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/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			
ΔS_F/Exp(-3) (J/mol.K)	8.56±0.47	47.00±2.10	72.00±0.00	3.39±0.70

422 $\Delta S_b = \Sigma(\Delta G_b - \Sigma\Delta H_b/2)/2 \pm SD$; K_b, and $k_{den}{}^*$, are calcium ion binding constant and rate constant for the
423 folding; ΔS_b, ΔG_b, and ΔH_b are entropy, free energy, and enthalpy of calcium ion binding respectively.
424 ΔG_F, ΔH_F, and ΔS_F are free energy, enthalpy, and entropy of folding of the enzyme respectively.
425

426 **5. DISCUSSION**

427 "The actual calcium binding is considered to include the folding of enzyme, because the

428 calcium-depleted apoenzyme is known to be inactive and easily unfolded; thus the obtained

429 parameters include the process of calcium binding and structural folding of the protein" [10]. This

430 quotation is pregnant with simple yet vital interpretation viz: though it has been suggested that as

431 folding proceeds, the equilibrium population partitions into two predominant species, unfolded and

432 native, with only negligible population of intermediate species [20], under any given condition outside

433   the optimal environment of the enzyme presence of extra calcium salt for instance with increasing

434   temperature, there may be subpopulations of unfolded, partially folded and folded, such that the final

435   direction should therefore, depend on preponderance of any of the opposing forces either for folding

436   or unfolding. This seems to be in agreement to the view that the folding of sufficiently small proteins is

437   highly cooperative, akin to first order phase transition [21]. Also, the data obtained elsewhere show

438   that folding in the presence of calcium ion is similar to that in its absence, although the rate is

439   increased by more than two orders of magnitude [22]. There is the issue of calcium binding/unfolding,

440   a process that demands activation process or input of activation energy. However, on account of this

441   study in which the apparent rate constant, showed increasing trend (Fig. 2) as to imply that the

442   enzyme is under optimal condition for its 3-D structure of its native state, it may seem that there is no

443   form of unfolding. On the contrary there must be what can be described as functional unfolding

444   needed for catalytic activity peculiar to mesophiles and thermophiles in particular [3]. The question is

445   at what point does functional unfolding begin? "The answer may be at the point of highest stability

446   under a given condition, the presence of extra salt for instance, to be proceeded with thermally

447   induced unfolding backed with the effect of chloride component in the presence of the substrate prior

448   to its transformation".

449   The Gibbs free energy of activation for the unfolding of the protein following the binding of

450   calcium ion appears to be higher at higher temperatures than lower temperature (Table 1). This

451   seems to suggest that the energy barrier for unfolding of the enzyme is higher at higher temperatures

452   due to the opposing effect of calcium ions by their rigidifying or stabilizing effect on the 3-D structure

453   [23]. It seems too that the free energy at higher salt concentration is lower than at lower salt

454   concentration; this was unlike the report for *Bacillus amyloliquefaciens* [10] which also unlike PPA in

455   this study showed higher enthalpy of unfolding at higher salt concentration. This may be as a result of

456   the effect of higher salt concentration in promoting greater conformational flexibility due to increasing

457   chloride ion component, because chloride ion opposes the effect of calcium ion; a protonated state of

458   Glu – 233 in the presence of chloride ion weakens the strength of calcium ion binding and

459   concomitantly its inhibitory effect also [3]. Being endothermic, higher thermal environment may be

460   needed for calcium ion binding such that with higher temperatures the binding of the ion should be

461   more spontaneous. This may therefore, promote higher rate of folding in opposition to unfolding with

462   increase in temperature, that may be imposed by the presence of extra calcium ion without

463   insinuating indefinite process as shown in Table 1.

464   As can be observed in Table 1, there are negative entropies in opposition to what is expected

465   of unfolding; this may be as a result of the calcium ions' action in promoting folding though the

466   magnitude of negative entropy seem to be higher at lower salt concentration due perhaps to less

467   effect of chloride component of the salt that is known to oppose the cation component [24]. The

468   ultimate effect of calcium ion action is what has been described as rigidification and loss of

469   conformational entropy following folding of the enzyme [25].

470   Forge *et al.* [22] reported folding rate constant equal to 20.2/s at pH, 7.2 and 20°C in 10mM

471   calcium chloride while Deitcher *et al* [26] reported folding rate constant ranging from 0.05-0.1/min.

472   This range is about 10-20 fold lower than values recorded in this investigation. Enhanced folding

473   which implies stability promotes catalytic activity of the enzyme similar to the observation that the

474   presence of calcium ion increases the activity and stability of the enzyme at high temperatures [27].

475   Current values (Table 1) of folding rate constant at different temperatures are however, closer to the

476   reported values of 2.3±0.6×10 exp (-2)/s and 2.8±0.8×10 exp (-2)/s for peptide WT and G14 peptide

477   respectively [18]. Before proceeding further into the discussion, it is imperative to state that the

478   implication of Eq. (4) is that when $[CaCl_{2(aq)}] \to \infty$, $1/[CaCl_{2(aq)}] \to$ zero; this implies that theoretically

479   and given other favourable and practicable condition, the calcium ion component can resist total

480   unfolding so long as increasing velocity of hydrolysis of substrate is observed with increasing

481   temperature. This is exactly opposite to what is expected when there is decrease in activity, such that

482   Eq. (4) becomes the case in which as $[CaCl_{2(aq)}] \to$ zero, the unfolding rate constant $\to$ zero so long

483   as there is decline in the activity of the enzyme due to unfavourable unfolding due to the presence of

484   the salt either with increasing or decreasing temperature.   Most   reactions   become   more

485   spontaneous with increase in temperature. The implication is that binding of cation and folding of the

486   enzyme cannot be extricated from each other hence the proposition that the calcium ion binding is

487   parallel to the folding of the enzyme [10]. Conditional irreversible folding is intended to imply that

488   continuous folding must be contingent on the availability of extraneous factors that can promote

489   refolding until the entire protein population becomes folded. But this is not without input of free energy

490   of activation [28]. The free energy of activation appeared to be larger at higher temperature. This may

491   be as result of increasing conformational flexibility (this is needed for catalytic function) occasioned by

492   the increasing thermal energy of the environment in addition to the known effect of the chloride

493   component which is described as activating or stimulating (activating effector) on account of which it is

494   able to increase so-called turnover number otherwise called rate constant for product formation (k_{cat})

495   by 30-fold towards either starch or p-nitrophenylmaltoside [24] through enhanced conformational

496   flexibility opposed to rigidification, a function of the cation component. Besides the protonated state of

497   Glu – 233 in the presence of chloride ion weakens the strength of calcium ion binding and

498   concomitantly its inhibitory effect also [3] and possibly its rigidifying effect.

499   Thus not just activation energy (free energy) for inactivation (unfolding) alone that occurs with

500   increasing temperature but there could also be free energy of activation for folding, the balance of

501   which determines the direction of equilibrium but not without the presence of stabilizer; there is a

502   strong evidence that the direction of equilibrium is towards folding hence the increasing

503   activity/apparent rate constant (but with a slight drop at some point (Fig. 2)), though not without

504   conformational flexibility needed for enzyme function in the presence of the salt. Thus one may

505   suggest that the increasing free energy of activation for unfolding with increasing temperature may

506   have been opposed by the increasing calcium salt; in other words there may be increasing resistance

507   to unfolding even with increasing temperature due to the presence of excess calcium salt. But the

508   presence of substrate presents different scenario to the effect that part of the unfolding must be seen

509   as a necessary part of conformational flexibility needed for catalytic function but within limits stabilized

510   by the cation.

511   Low activation energy for any process is a pointer to the likelihood of the spontaneous nature

512   of such process accounting for the fact that high thermal energy may not be necessary for

513   commencement of such process. Hence the low activation energy for irreversible folding should

514   necessarily lead to very low enthalpy of activation or as observed in this report, negative enthalpy of

515   activation for folding. The negative enthalpy is higher at higher temperature as to suggest that

516   unfolding is slightly unfavourable or that the unfolded state or complex is transient at high salt

517   concentration. The entropy of activation is negative suggesting loss of conformational entropy as

518   opposed to stability or rigidification of the activated complex unlike the report for *Bacillus*

519   *amyloliquefaciens* [10] or unlike what has been described as the positive entropy of activation which

520   implies that increasing local disorder in the transition state when compared with the ground state reported for

521   tryptophan synthase [29]. The reason advanced for the increase in entropy (positive entropy change) for

522   Bacillus halmapalus alpha amylase, is the release of water molecules from the calcium ion binding

523   site and the dehydration of non-polar surfaces on calcium ion binding [9, 30] is a direct contrast with

524   what may be the underlying reason for entropy decrease as observed for PPA. But the large negative

525   entropy should also arise from the fact that there was folding from relatively more flexible ground state

526   to more ordered transition state as the cation binds [15].

527   Looking at Table 2 one observes that the calcium ion binding constant showed lower values

528   at higher temperatures similar to the observation by Nielsen *et al.* [9] and Tanaka and Hoshino [10]

529   with respect to *B. halmapalus* alpha amylase (BHA) and *Bacillus amyloliquefaciens* alpha amylase

530   (BAA) respectively. The values with respect to BAA were similar to current report for PPA at higher

531   temperature, 60 °C and 65 °C but the values at lower temperatures were much higher than values for

532   PPA. This is most likely considering the fact that the binding constant at temperature lower than 37 °C

533   (specifically 22 °C) as originally reported for human salivary alpha amylase elsewhere [31] was very

534   much higher than values at 37 °C and above. Values reported for BHA were very much higher than

535   values for PPA in this report at all temperatures. The expression for the stability of the product of any

536   biochemical reaction such as calcium ion binding was exothermic though much less than the report

537   for BAA at *p*H 7.5 [10] and value for BAA as high as −149kJ/mol at *p*H 7 [32]; but all observations

538   points to stable calcium-enzyme complex at sites other than active site. While the entropic term, TΔS,

539   of calcium ion binding is −109.13±0.61 kJ/mol there is proposition that calcium binding may consist

540   both of contributions from calcium binding itself and of protein folding; thus there may be need to

541   subtract the contribution of calcium binding enthalpy (− 8kJ/mol) and entropic term (− 42kJ/mol) so as

542   to yield folding thermodynamic parameters such as −157kJ/mol for enthalpic term and −151kJ/mol for

543   entropic term and ultimately, the value of the free energy of folding in terms of $\Delta G = \Delta H - T\Delta S$, should

544   be − 6kJ/mol [10]. However, the result presented in Table 2 shows some similarities and differences in

545   comparison with the above observation in literature. The magnitude of the enthalpy of calcium binding

546   in this study is less than literature value but both are exothermic though for homologues from different

547   sources. The entropy of calcium ion binding at 30 °C and the free energy of calcium ion binding for

548   BAA are −138.55J/mol.K (from − 42kJ/mol/303.15K) and − 34kJ/mol respectively [10]. This entropy

549   value, though not very widely different from report in this investigation (Table 2) it is nevertheless

550   opposite in sign to current result. The current result (entropy of calcium ion binding) being positive,

551   may be attributable to the release of water molecules from the calcium-binding site and dehydration of

552   non-polar surfaces on calcium ion binding[9].The free energy of calcium ion binding to BAA is

553   expectedly spontaneous similar to values (Table 2) in this investigation, but the literature value is

554   higher.

555   In the same Table 2, it can be observed that folding equilibrium constant is slightly greater

556   than unity and the values appeared to decrease with temperature and consequently the free energies

557   though express spontaneity are nevertheless much lower than literature value of − 39.87kJ/mol at

558   303.15K reported for BAA [10]. On the whole one can suggest that calcium ion binding and folding of

559   PPA in the face of extra calcium salt are enthalpically driven and spontaneous as observed for other

560   homologues. But entropic term seemed to show greater contribution being greater than enthalpic term

561   ($\Delta S > \Delta H$). However, there is clear-cut difference in the entropic term between literature information for

562   BAA and PPA in this study. While the entropies of calcium ion binding and for folding were positive

563   the case reported for BAA is negative [10, 32]. Naturally folding results in decrease in conformational

564   entropy coupled with hydration at higher temperature as it is the case with BAA. But due to

565   dehydration, as calcium ions bind (Tanaka and Hoshino, 2002) what may be called translational

566   entropy gain (Harano and Kinoshita, 2005) may occur as applicable to PPA in this study.

567   **6. CONCLUSION**

568   The current result shows that unfolding and folding are antiparallel processes amenable to the

569   different aspect of the same model and so can be qualitatively described and quantified in terms of

570   various parameters. Unfolding usually expected with increasing temperature may have been opposed

571   by higher salt concentration, hence the high free energy of activation for unfolding. The enthalpy of

572   activation which is positive and low expresses moderately unstable unfolded state while the low value

573   of activation energy for unfolding expresses low barrier against unfolding which may be as a result of

574   high thermal environment. Calcium ion binding and folding of PPA in the face of extra calcium salt

575   though exothermic, are entropically (entropic term, $T\Delta S > \Delta H$) driven and spontaneous. The entropies

576   of folding being positive may be a probable confirmation of conformational flexibility needed for

577   catalytic function. Ultimately, calcium salt stabilized the enzyme against increasing temperature.

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