Original Research Article

TITLE: Effect of interacting organic co – solutes with enzyme substrate complex on the hydrolysis of raw soluble starch with α-amylase: Theory and Experimentation.

ABSTRACT

Aims: The objectives of the *in vitro* study were to examine the applicability of thermodynamic models for the interaction of reaction mixture components to enzyme catalyzed reaction, and to determine the effect of co – solutes on the velocity of hydrolysis of a substrate with alpha amylase.

Design: Experimental

Place and Duration of Study: Chemistry & Biochemistry Department, Research Division of Ude International Concepts limited (RC: 862217) and Department of Biochemistry, Ambrose Alli University, Ekpoma. This study is part of a series of research that lasted for about 4.5years between February, 2011 and June, 2015.

Methodology: Bernfeld method of enzyme assay was used to generate data on catalytic activity of the enzymes. Reaction mixture with co – solutes was the test while the control was without any co – solute.

Results: Human salivary alpha amylase (HSAA) had Gibbs free energy ($\Delta\Delta G$) of interaction ranging from $4.49\times10^{+5}$ to $8.34\times10^{+5}$J kg /mol^2 while porcine alpha amylase (PAA) had values ranging from $-4.83\times10^{+5}$ to $-6.73\times10^{+5}$ J kg /mol^2 due to aspirin – sucrose treatment. Treatment with a mixture of ethanol and sucrose yielded values which ranged from $-2.27\times10^{+2}$ to $-1.51\times10^{+2}$J kg / mol^2 and from $-1.16\times10^{+3}$ to $-0.86\times10^{+3}$Jkg / mol^2 for HSAA and PAA respectively. HSAA and PAA exhibited *m* – values (the capacity of additives to force unfolding or refolding of protein,) equal to –1.09±0.02 kJ/mol and –3.29±0.02 kJ/mol respectively in the

presence of a mixture of milk and ethanol. In the absence of milk the free energy of native to destabilized (unfolded) transition ($\Delta G_{N\rightarrow U}$) were – 0.29±0.08 and 14.17±0.07kJ/mol for HSAA and PAA respectively.

Conclusion: The free energy of co – solute interaction with reactants is very much applicable to the enzyme catalyzed reaction. The presence of aspirin caused higher activities of the enzymes than control. The presence of sucrose caused higher activity of HSAA than control. Unlike HSAA, the presence of milk (extra calcium salt content) enhanced the activity of PAA

Keywords: Enzyme-substrate complex, aspirin, ethanol, sucrose, milk.

1. Introduction

Human transit across the Indian Ocean through the right route during winter in particular may be perfectively aided with hot tea highly fortified with milk in tea cups instead of "Lord's dry gin" but with the understanding that no human system is perfect; this may be the "gospel"!

It is known that food additives improve taste and shelf life of food made available on the table in private and hospitality industries but serious consideration is hardly given to effect of additives and drugs on alpha amylase function. Industries may have their standard under strict regulation, but local use of additive such as colourant (as an example) may not take into cognizance the effect on digestive enzyme in particular. Ingestion of alcohol during meal or shortly after meal can also affect the rate of digestion. The presence of ethanol in gastrointestinal tract is known [1]. The implication is that in both *in vitro* and *in vivo* environment the activity of an enzyme such as alpha amylase can be reduced. In this regard, Blakeney and Stone [2] have shown that there was a decreasing trend in the activity of *Bacillus Licheniformis* alpha amylase with increase in the concentration of ethanol. It has been shown that alpha amylase from saliva and plasma of habitual alcohol drinkers is significant [3]. This raises the question as to whether the effect of any osmolyte on tissue is the same as the effect on the molecule such as enzyme. Kharkrang and Ambasht [4] reported increase in plant (pearl millet – *Pennisetum glaucum*) alpha amylase activity following treatment with aspirin. Although aspirin is not an additive, its reported effect *in vivo* and *in vitro* has attracted interest. After treatment with aspirin, significant alterations in the activities

of intestinal disaccharide hydrolases in both homogenate and intestinal brush border membrane (BBM) preparations were reported [5, 6].

Stabilizers, the organic type, in particular, are sucrose, glucose, tri – methylamine N – oxide (TMAO) etc are most often object of intense investigation [7]. Sucrose is implicated in shifting the equilibrium between protein conformational states towards the more compact conformation [8] just as ethanol and dimethyl sulphoxide oppose each other in their effect on the temperature dependence of the conformational stability of Brain (Na^+ K^+) ATPases [9]. Sucrose is part of alcoholic beverage known as beer, and with known effect of aspirin and ethanol, it has become the object of this research to investigate the effect of both compounds and as a mixture with sucrose on the activity of alpha amylase.

Milk is a multi – component ($\gg$2 components) and multifunctional and cooperate with other factors to promote and modulate growth and development of not only neonates [10] ingesting breast milk, but adolescence ingesting other processed cow milk, for instance. Organic substances including protein, lactose, and inorganic constituents like calcium salt in milk are potential stabilizers also. The effect of emulsified milk alone and as a mixture with ethanol is also object of investigation.

Very stable ES is helpful in biomass conversion, production of molasses, de – sizing of textile materials [11], most importantly digestion in human situation *etc*. Therefore, the aims of the research were: (i) to show that the theory of pair wise Gibbs free energy of interaction between reaction mixture components is very much applicable to enzyme catalyzed reaction, (ii) to determine Gibbs energy of interaction which influences encounter complex and enzyme substrate complex ([ES]) formation in the presence of ethanol, aspirin and a mixture of each of the former and sucrose in the formation of ES, (iii) to verify the effect of milk – ethanol mixture on the activity of the enzymes and characterize the effect in terms of free energy of folded to unfolded transition and the *m* – values (*i.e.* the capacity of an osmolyte to force (un)folding of a protein).

2. Theoretical section

The formation of enzyme-substrate complex (ES) is seen to proceed from bimolecular catalytic reaction assumed to occur through reactive encounter complexes defined as the subset of reactant state

species able to proceed directly to low lying energy levels [12]. In order to exceed the limit imposed on catalytic efficiency by failure to form ES, the complexes need to be stabilized [13, 14]. The encounter complexes including ES, is one in which two molecules are held together by fluctuating short – range interactions and contacts that stabilize the fully bound state [15].

Meanwhile the thermodynamic model for interaction between a reacting molecule and inert hydrophobic co – solute is known [16]. There are hydrophobic-hydrophobic and hydrophilic-hydrophilic interactions [17]. The equation [16] employed in the quantitative determination of pair-wise solute-solute interaction parameter is as follows:

$$\ln [k_{(mc)} / k_{(mc = 0)}] = 2[g_{cx} - g_c^{\#}]\, m_c / RTm_o^2 - N\, \varphi M_1\, m_c \qquad (1)$$

where $k_{(mc)}$ is the (pseudo –) first – order rate constant in a reaction mixture containing co – solute whose concentration is m_c and $k_{(mc = 0)}$ is the rate constant in the absence of the co – solute; R and T are the molar gas constant and thermodynamic temperature; m_o is the (hypothetical) ideal reference state and it is equal to 1mol/kg**;** $g_{cx} - g_c^{\#}$ is the difference in interaction Gibbs free energies between the co – solute c and the reactants β (and by extension substrate and a biochemical catalyst) on one hand and the activated complex # on the other hand; M_1, φ, N and m_c are the molar mass of water, practical osmotic coefficient for the aqueous solution, the number of water molecules, and the molality of the added co-solute respectively [16]. Equation 1 is derived by combining thermodynamics and transition state theory [16].

The number of water molecules involved in the rate-determining step is perhaps just one activated water molecule [18]. It is not clear why two should be part of Eq 1. Reaction in aqueous medium entails proper orientation of water in the activated complex but the hydrolytic role of water could be inhibited if the encounter complex of reactant and added solute results in the blocking of the reaction centre from attack by water [16]: This situation increases the entropic cost of fixing water to its site on the complex for its action [19]. Unlike, less polar and non – polar solvents, water stabilizes partial charges in complexes thereby stabilizing the encounter complex or transforms them into low energy state species [12, 13] Thus the interpretation of rate retardations is in terms of the effects of added co-solute on the activity coefficients of initial and transition states of the esters undergoing hydrolysis [16] Extension of this interpretation to biological level should clearly relate to the active site which may likely

be blocked by the added co – solute [16]. Alkaline solutions and acidic solution under special condition can reverse ester (otherwise known as alkyl alkanoate) formation thereby suggesting reversibility of the reaction in line with Le Chatelier's principle. Though non-biological, the hydrolysis of ester is similar in principle to the formation and eventual hydrolysis of soluble potato starch by alpha amylase. In this regard Buurma *et al*.[16] recognized the biological significant of the medium effect on the reactions taking place at the active site. The issue is that (pseudo –) first order rate constant is applicable thereby, rigidly imposing limitation to the applicability of the theory to ES such as alpha amylase – starch complex. The following, except unforeseen exception, shows that initial theory (modified) can be applied to the interpretation of the stability of ES.

$$\ln\{(1/K_{m\,(mc)})/(1/K_{m\,(mc=0)})\} \equiv \ln(K_{m\,(mc=0)}/K_{m\,(mc)})$$

$$= \ln(k_{-1\,(mc=0)}/k_{1\,(mc=0)}) - \ln(k_{-1\,(mc)}/k_{1\,(mc)}) \qquad (2)$$

where $K_{m\,(mc)}$ and $K_{m\,(mc=0)}$ are the Michaelis – Menten constant in the presence and in the absence of the co – solute respectively. The right hand side is construed from the fact that in the equation [E] + [S] $\rightleftharpoons$ [ES] where E and S are free enzyme and substrate respectively, the 2nd order rate constant for the forward reaction is expressed as: $k_1 = k_{-1}/K_m$ where k_1 and k_{-1} are the rate constants for the forward and backward reaction respectively; K_m remains the Michaelis – Menten constant (dissociation constant). The parameter k_1 (= k_{-1}/K_m) is based on Henri – Michealis – Menten approach which assumes that a rapid equilibrium is established between the reactants (E + S) and the ES complex, followed by slower conversion of ES complex back to free enzyme (E) and product (P). Therefore, the model assumes that $k_2 \ll k_{-1}$; so, K_s (K_m) $\cong k_{-1}/k_1$ [20] where K_s is the equilibrium dissociation constant. It is also postulated that $K_s = k_{-1}/k_1$ at high enzyme concentration and thermodynamic equilibrium is possible under such situation [21]. It should be understood that at the initial stage, the so – called transient phase, there is almost perfect linearity in the relationship between velocity of hydrolysis of substrate (v) and [S] with very high coefficient of determination that approaches unity (> 0.99). This very probable when [S] is less than K_m. It is unlikely therefore, that K_s should be equal to K_m when [S] $\gg K_m$. None the less K_m is used just for

the purpose of this experiment but it is not intended to imply that $K_m = K_s$. Michaelis – Menten constant is attainable when $[S] \gg K_m$. With native starch suspension in water or buffer, most of the starch molecules are not in the bulk as may be attested to by the observation that native starch suspension is not largely digestible unlike gelatinized starch [22]. This claim is backed with the observation that 1.6 units of alpha amylase in dissolved starch digest yielded twice the percentage hydrolysis of starch granules with 12units in starch granule digest [22]. Thus most of the undissolved starch remained undigested just as very large part of the enzyme is free as substantiated by the observation that the fraction of enzyme molecules bound productively with starch granules is small compared with the total amount in the system [23].

Meanwhile a generalized 2nd order rate constant k for an enzyme catalyzed reaction can be expressed as:

$$k_1 = \{([S] - [E])\, t\}^{-1} \ln \{[E]\,([S] - \delta) / [S]\,([E] - \delta)\} \quad (3)$$

where δ is the molar concentration of the substrate transformed or the molar concentration of that part of the total enzyme's molar concentration and t is the duration of assay. It should be emphatically realized that the spectrophotometer measures only the concentration of maltose (if maltose is the only reducing sugar) yielded from hydrolyzed starch. The rearrangement of second order equation as can be found in most general (bio) chemistry text books produces "a pseudo-first order rate constant" k_{DPR}.

$$k_{DPR} = k_1([S] - [E]) = \ln \{[E]\,([S] - \delta)/[S]([E] - \delta)\}/t \quad (4)$$

where k_1 is a 2nd order rate constant while the product of it and simple arithmetic difference between the concentrations of substrate and enzyme yields another constant that has the unit of 1st order rate constant. If $\ln \{[E]\,([S] - \delta)/[S]\,([E] - \delta)\}$ is plotted versus t, the resulting slope should be equal to $k_1([S] - [E])$. Therefore, k_1 should be equal to slope/([S] – [E]). Mean while if the right hand side of Eq (2) is rearranged, the equation becomes:

$$\ln (K_{m(mc=0)} / K_{m\,(mc)}) = \ln(k_{-1(mc=0)} / \ln k_{-1(mc)}) + \ln(k_1(m_c)/k_{1(mc=0)}) \quad (5)$$

In Eq (5), $\ln (k_{-1(mc=0)} / \ln k_{-1(mc)})$ is $(\Delta G_{-1}^{\#}{}_{(mc=0)} - \Delta G_{-1}^{\#}{}_{(mc)})/RT$; If $\ln(k_{1\,(mc)} / k_{1\,(mc=0)})$ is replaced directly with Eq (3), the difference between the initial concentrations of substrate and enzyme ([S] – [E]) cancels out, because [S] – [E] appears as the nominator and denominator, where k_1 is defined in generalized form in Eq (3). Cancellation of [S] – [E] leaves a ratio $\ln\{[E]([S] - \delta_{(mc)})/[S]([E] -$

$\delta_{(mc)}$)}/In{[E]([S] – $\delta_{(mc\,=\,0)}$)/[S]([*E*] – $\delta_{(mc\,=\,0)}$)}. Expectedly, the value of δ may not be same in the presence and absence of any additive, the subject matter of this investigation. Also, In ($k_{1(mc)}$ /$k_{1(mc\,=\,0)}$) is ($\Delta G_1^{\#}{}_{(mc)}$ – $\Delta G_1^{\#}{}_{(mc\,=\,0)}$)/*RT* (16,17). Therefore, it ought not to be over emphasized to speak in favour of the general applicability of the theory of pair – wise Gibbs free energy of interaction at the stage of ES formation for enzymes. Thus Eq (5) provides direct link between thermodynamics and transition state theory [16, 17] ($\Delta G_{-1}^{\#}{}_{(mc\,=\,0)}$ – $\Delta G_{-1}^{\#}{}_{(mc)}$)/*RT* and ($\Delta G_1^{\#}{}_{(mc)}$ – $\Delta G_1^{\#}{}_{(mc\,=\,0)}$)/*RT* are similar to report in the past [17]**.** This position is similar to the equation elsewhere (24):

$$\Delta\Delta G^{\#}_{(T)} = \Delta G^{\#}_{cat} - \Delta G^{\#}_{aq} \qquad (6)$$

where $\Delta G^{\#}_{cat}$ and $\Delta G^{\#}_{aq}$ are, respectively, the quasi – thermodynamic free energy of activation for the enzymatic and the uncatalyzed reaction. However, what seems to be unclear is the claim that Eq (6) is "justified when [S] is high such that the enzyme is saturated, and the reaction is unimolecular with rate constant, k_2" [24]. Does $\Delta\Delta G^{\#}_{(T)}$ require large [S] to be valid? So long as there is substrate, the active or native enzyme can accelerate the transformation or conversion of substrate whereas the totally or partially unfolded aqueous solution of the enzyme will either totally or partially transform/convert the substrate. The presence of totally unfolded enzyme which has lost its catalytically active three dimensional forms in a reaction mixture notwithstanding, such reaction mixture which undergo any form of reaction is as good as uncatalyzed reaction. Moreover, it should be noted that *RT* InK_m is indeed the Gibbs free energy of ES formation [18]. Detailed derivation of the equation in the form similar to Eq (1) but with minor modification is in the appendix section.

The influence of solvent and mixed solvents had been an important issue [25 – 28]. The main issue is that, there is either preferential binding on or exclusion of co – solutes otherwise called osmolytes, from the enzyme surface domain. Binding and exclusion have opposite effects. In non-biological reaction the formation of charge transfer complex (CT) is influenced by the polarity of the solvents. Thus the association constants of CT with co – solutes in solution were known to increase with the decrease in polarity of the solvent [12]. Be it binding, association, or exclusion, the magnitude of any of the interaction parameter is quantified in terms the *m* – value, defined as the capacity of an osmolyte (co – solute) to either force folding or unfolding of a protein. It is the slope of the plot of free energy of

folded to unfolded transition versus osmolyte molar concentration. The equations are spelt out in the method subsection.

3. Material and Methods

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

The chemicals used were: Sucrose (St Lious France); soluble potato starch (Sigma Chemicals Co, USA); ethanol, hydrochloric acid and sodium chloride (BDH Chemical Ltd, Poole England); 3,5-dinitrosalicylic acid (DNA) (Lab Tech Chemicals, India**);** Tris (Kiran Light Laboratories, USA**)**; porcine pancreatic alpha amylase (PAA) (Sigma, Adrich, USA); human salivary alpha amylase (HSAA) in its crude form direct from a donor; all other chemicals were of analytical grade and solutions were made in distilled water. Strong commercial detergent was purchased from Procter and Gamble, Ibadan, Nigeria.

A mass equal to 0.01g of PAA was dissolved in 20mL of distilled water to give 500µg/mL while soluble starch solution was prepared by dissolving 1g in tris – HCl(aq) buffer (90mL), 5mL 6% (W/W) NaCl(aq), and 5mL distilled water to give 1g/100mL. Appropriate dilutions carried out were for the determination of K_m and V_{max} at 37^{o}C and *p*H 7.4. The detergent being very alkaline and its solution mixed with milk had to be diluted and neutralized and had the *p*H adjusted to 7.4 using 0.1m hydrochloric acid. The final concentration of emulsified milk was 1/161th of stock milk.

Centrifuged saliva diluted with a mixture of tris – HCl buffer, NaCl(aq) and distilled water gave a final solution whose concentration is ½ the concentration of stock saliva solution. Centrifugation was at approximately 3000rpm (or at 1343 g). The control reaction mixture was free from appropriate osmolyte. The test reaction mixture contained osmolyte(s) at 37^{o}C. In testing for the effect of one or a mixture of co-solute(s) otherwise called osmolytes, ethanol/aspirin was first added to the enzyme solution and if the second co-solute was required it was then added before 1mL of the substrate (native soluble starch without heat treatment) was added and the duration of assay was 5 minutes. In testing for the effect of a mixture of milk and ethanol, 0.5mL of each, 1mL each of substrate and enzyme were mixed; but if milk or ethanol alone is tested for as control, 0.5mL of distilled water, 0.5mL of either ethanol or milk, 1mL of substrate, and 1mL of enzyme were mixed.

The activity of 1mL of the enzyme was measured by the 3, 5 – dinitrosalicylic acid method [29]. Spectrophotometer readings for the determination of amount of maltose yielded were taken at a wavelength, 540nm, and the extinction coefficient was 181.1/M.cm. But further centrifugation (at a rate stated earlier) of the reaction mixture after termination of reaction was carried out in order to sediment suspended undigested starch granules and consequently prevent interference with transmittance thereby achieving stable absorbance. Activity of enzyme was measured as units/mL. 1U **=** molarity of product $\times$ 1 mL of substrate /1000mL)/5min.1mL of enzyme. In all, 0.5mL of ethanol, 0.25mL of sucrose, and 0.5mL of aspirin were used as the case may be. K_m values for the calculation of In ($K_{m(mc=0)}$ /$K_{m\ (mc)}$) were determined according to the method of Lineweaver – Burk [30]. The values of G(c) (the pair wise Gibbs free energy of interaction) were derived from an indispensible principle reported in the paper by Engberts and Blandamer [17] as follows:

$$\text{In}\,(k_{1(mc)}/k_{1(mc=0)}) - \text{In}\,(k_{-1(mc)}/k_{-1(mc=0)})$$

$$= \text{In}(1/K_{m(mc)}) - \text{In}(1/K_{m(mc=0)})$$

$$= (\Delta\Delta G(\text{c})\, m_c/RT) - \Delta n\varphi M_1 m_c \qquad (7)$$

$\Delta\Delta G$(c) is determined by plotting In ($1/K_{m(mc)}$) – In($1/K_{m(mc=0)}$) against m_c. The slope from such plot is equal to $\Delta\Delta G$(c)/RT. The final formulation is shown in appendix A. Here, $\Delta\Delta G$(c) is for the purpose of simplicity referred to as the Gibbs energy of co – solute interaction otherwise, it is, as defined earlier in the text as the difference between the Gibbs energy of interaction between (i) the added co – solute and the initial state (IS) of the reactants including the enzyme and (ii) the added co – solute and the activated complex (AC); the double change ($\Delta\Delta$) in Gibbs free energy is due to what could be clearly seen at the left hand side of Eq (1); k_1 and k_{-1} are the rate constant of the forward and backward directions of the equilibrium E + S $\rightleftharpoons$ ES; (m_c) and (m_c = 0) represent in the presence and absence of the osmolyte respectively.

The m – values described as the slope of the protein folding stability with osmolyte concentration [27] is determined by plotting free energy of protein (un)folding (ΔG^o) against co – solute concentration. This approach has been described innovatively elsewhere [7], but briefly restated as follows for quick and easy reference: According to Rösgen *et al* [27], m – value for the protecting osmolyte (or a kosmotrope) is

positive while the *m* – value for destabilizing osmolyte (or a chaotrope) is negative. The equation linking ΔG^{o} and *m* – values is, as often cited in literature, in terms of the presence of minus sign [31].

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

Other scholars [32] use the equation in which the plus sign is the case:

$$\Delta G^{o} = \Delta G^{o}_{N\rightarrow D} + m[\text{co} - \text{solute}] \quad (9)$$

where $\Delta G^{o}_{N\rightarrow D}$ is the Gibbs free energy of unfolding, native to denatured state transition (N→D) in the absence of co – solute. “Round dining/hospitality – table disagreement as to the choice of model, either Eq (8) or Eq (9), to be used must not, however, overturn cups of tea fortified with milk”.

$$U = (\text{SA} - \text{SA}_{obs})/(\text{SA} - \text{SA}_{min}) \quad (10)$$

where SA, SA_{obs}, and SA_{min} are specific activity of the native enzyme, observed specific activity under the influence of additives, and minimum specific activity resulting from the effect of destabilizer.

Equation (10) follows original Pace’s equation [33] that depends on fluorescence data. The equation is:

$$U = (A_N - A_{obs})/(A_N - A_D) \quad (11)$$

were A_{obs} is the observed absorbance used to follow unfolding in the transition region, and A_N and A_D are the values of absorbance of the native and denatured conformation of the protein, respectively, and *U* is the fraction of the unfolded enzyme. The fraction of folded is 1–*U*. Thus according to Pace [33],

$$K_{eq} = U/(1 - U) \quad (12)$$

Based on the assumption of two state models, K_{eq} is the equilibrium constant for the process N⇌U. Meanwhile,

$$\Delta G^{o} = -\ln K_{eq} \quad (13)$$

Substituting Eq (12) into Eq (13) gives,

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

3.1. Statistical analysis.

Except otherwise stated, data are expressed as Mean±S.E.M., where S.E.M is the standard error of the mean. All calculations were manually carried out with electronic calculator.

4. Results

4.1 Gibbs free energy change for co – solute interaction with reaction mixture components.

In Table (1) are thermodynamic parameters (namely Gibbs free energy ($\Delta\Delta G$(c)) values) of co – solute interaction with solution components in the presence of single osmolyte such as aspirin and a mixture of aspirin and sucrose. In a reaction mixture in which aspirin is the only osmolyte, the $\Delta\Delta G$(c) values for PAA were negative unlike the values for HSAA (Table 1). The *r*-value for HSAA was larger than the value for PAA.

Table 1. Gibbs free energy of interaction of co-solute in a mixture of aspirin and sucrose.

[Sucrose]	**HSAA**		**PAA**	
(mmol/kg)	$\Delta\Delta G$(c)	*r*	$\Delta\Delta G$(c)	*r*
	(10^5Jkg/mol^2)		(10^5Jkg/mol^2)	
0.00	4.48±0.783	0.99	– 6.49±1.166	0.77
3.60	4.49±0.932	0.87	– 4.83±0.338	0.82
7.19	6.96±0.579	0.94	–5.73±0.184	0.87
14.38	8.34±0.262	0.97	– 6.73±0.37	0.85

HSAA and PAA are human salivary and porcine alpha amylase respectively. $\Delta\Delta G$(c) is the difference between the Gibbs energy of interaction between (i) the added co - solute and the initial state (IS) of the reactants including the enzyme and (ii) the added co - solute and the activated complex (AC). This leads to final state of enzyme-substrate complex; r is the correlation coefficient; [Sucrose] is the concentration of sucrose in mmolKg; df =1 in the assay of HSAA in aspirin-sucrose system while it is 2 in other system

and results obtained are presented as: Mean±SEM. Assay of enzymes was at 310.13K while $K_{m(mc)}$ values were determined by Lineweaver-Burk plot following the assay of the enzymes in the presence of a mixture of aspirin and sucrose at different fixed concentration of sucrose.

In a mixture of aspirin and sucrose (Table 1), there was difference in magnitude and sign of $\Delta\Delta G$(c) values between PAA and HSAA: For instance while the magnitude of $\Delta\Delta G$(c) in both enzymes were similar in the presence of 3.60mM sucrose, the values are however, different at higher concentrations of sucrose; the sign for PAA were all negative unlike the sign for HSAA. The *r*-values were comparable.

Table 2. Gibbs free energy of interaction of co-solute in mixture of ethanol and sucrose

[Sucrose]	**HSAA**		**PAA**	
(mmol/kg)	$\Delta\Delta G$(c) (10^2Jkg/mol^2)	*r*	$\Delta\Delta G$(c) (10^3Jkg/mol^2)	*r*
0.00	– 3.60±1.3	0.96	0.31±0.005	0.91
3.60	– 2.27±0.619	0.97	– 1.16±0.023	0.91
7.19	– 3.88±0.438	0.94	– 1.04±0.010	0.90
14.38	– 1.51±0.361	0.98	– 0.86±0.010	0.90

HSAA and PAA are human salivary and porcine alpha amylase respectively. ΔΔG(c) is the difference between the Gibbs energy of interaction between (i) the added co - solute and the initial state (IS) of the reactants including the enzyme and (ii) the added co - solute and the activated complex (AC); r is the correlation coefficient; [Sucrose] is the concentration of sucrose in mmol/kg; df = 2 in the assay of HSAA in aspirin-sucrose system. Results obtained are presented as: Mean±SEM. Assay of enzymes was at 310.13K while $K_{m\ (mc)}$ values were determined by Lineweaver-Burk plot after the assay of the enzymes in the presence of a mixture of ethanol and sucrose at different fixed concentration of sucrose. ΔΔG(c) is obtained by multiplying the slope (gradient) of the line from the plot of $\ln K_{m\ (mc\ =\ 0)} / K_{m\ (mc)}$ versus molal concentration of the co-solute by RT.

In Table 2 are thermodynamic parameters for interaction of co – solute(s) solution components in the presence of single osmolyte such as ethanol only and in the presence of a mixture of ethanol and sucrose. The enzymes differed in the sign of $\Delta\Delta G$(c) values in the presence of ethanol only. However, both enzymes had high *r*-values.

4.2. Effect of aspirin and a mixture of it and sucrose on the velocity of hydrolysis of soluble potato starch

The results in Table 3 show that the activities of the enzymes in the presence of aspirin with and without sucrose were higher than control values. In reaction mixture containing aspirin as the only osmolyte, there was an increasing trend in the activities of HSAA and they were several folds higher than the activities of PAA in similar reaction mixture. PAA showed decreasing trend. Except at different fixed concentration of sucrose equal to 3.57mmol/L and 7.14mmol/L, the activity of HSAA in mixed osmolytes of aspirin and sucrose was to some extent lower than the activity in sucrose free reaction mixture (the control). The activity of PAA in sucrose free reaction mixture (control) was lower than in all sucrose containing reaction mixture. There was irregular incremental trend in the activity of PAA with increasing concentration of aspirin at different concentration of sucrose. This was unlike the activity of HSAA except at 0.76mmol/kg of aspirin, due perhaps, to fluctuation in temperature. Activities of HSAA in a mixture of osmolytes, and in the presence of aspirin only, in the reaction mixture, were higher than activities in osmolyte free reaction mixture and, there was incremental trend in the activities (Table 3).

Table 3. Activities of alpha amylase in a mixed osmolyte of sucrose and aspirin at different fixed concentration of sucrose

	HSAA					
[Aspirin] (mmol/kg)	$v/10^2$ mUmL^{-1}					
	[Sucrose]/mmol/kg					
	0.00	3.60	7.19	14.38	28.76	57.75
0.763	1.39±0.116	1.79±0.069	1.67±0.021	1.76±0.196	1.80±0.001	1.54±0.081
1.526	1.50±0.013	1.93±0.053	1.34±0.033	1.17±0.029	1.20±0.004	1.15±0. 004
3.052	1.68±0.024	2.06±0.007	1.37±0.0.12	1.39±0.035	1.56±0.035	1.71±0.084

4.578	2.05±0.061	2.26±0.162	1.95±0.122	1.83±0.263	2.00±0.089	1.74±0.015
6.104	2.46±0.878	2.83±0.878	2.79±0.087	2.76±0.237	2.95±0.204	3.04±0.047

PAA

[Aspirin] (mmol/kg)	$v/10^2$mUmL^{-1} [Sucrose]/mmol/kg					
	0.00	3.57	7.14	14.29	28.57	57.14
0.763	0.74±0.032	1.18±0.017	2.58±0.072	3.09±0.124	4.61±0.017	4.85±0.09
1.526	0.73±0.159	1.86±0.033	1.68±0.026	2.04±0.067	1.80±0.071	1.64±0.115
3.052	0.69±0.010	1.32±0.044	1.04±0.031	1.06±0.087	1.32±0.038	1.40±0.046
4.578	0.40±0.064	0.61±0.100	0.83±0.015	0.92±0.058	1.06±0.055	1.15±0.023
6.104	0.21±0.066	0.61±0.100	0.83±0.100	0.92±0.058	1.05±0.055	1.15±0.023

The activities of untreated (control) HSAA and PAA are 111.32mU/mL and 109.98mU/mL respectively. HSAA and PAA are human salivary and porcine pancreatic alpha amylase respectively. Raw starch was the substrate. The original unit of activity was expressed in mol/dm^3 /mL.min. The number of moles of product maltose in 1mL is: the molarity of product $\times$ *1 mL of substrate /1000mL. Therefore, 1unit* **=** *(molarity of product* $\times$ *1 mL of substrate /1000mL)/5min.1mL of enzyme. This is intended to avoid confusion.*

4.3 Effect of ethanol and a mixture of it and sucrose.

All the activities of HSAA in a mixture of ethanol and sucrose were higher than control containing non-consumable ethanol only; but none is up to control without non-consumable ethanol. The activity of HSAA and PAA in a reaction mixture containing only aqueous non-consumable ethanol (industrial ethanol) as the only osmolyte was lower than control values. However, the activity in a reaction mixture containing non-consumable ethanol and sucrose was higher than control values (Table 4).

In both HSAA and PAA containing reaction mixtures, the activities were less than control at all dilution factors. However, there was increasing trend in activity of PAA unlike HSAA. Between 0.89 mol/L and 4.33mol/L non-consumable ethanol, the range of activity of PAA was (38.3 - 61.1) U/mL in a total

reaction volume of 2.75 mL. In similar circumstance, it was (57.9 - 31.8) U /mL for HSAA. These can be found in Table 4

Table 4. Activities of alpha amylase in a reaction mixture containing sucrose and ethanol at different fixed concentration of sucrose.

HSAA						
[ETH] (mol/L)	$V/10^2$ mUmL^{-1}					
	[Sucrose]/mmol/L					
	0.00	3.57	7.14	14.29	28.57	57.14
0.866	0.58±0.718	1.14±0.118	0.95±0.614	0.77±0.109	0.7±0.03	0.69±0.045
1.734	0.49±0.651	0.9±0.03	0.66±0.614	0.70±0.238	0.66±0.343	0.65±0.096
2.406	0.40±0.578	0.82±0.126	0.59±0.126	0.65±0.241	0.61±0.446	0.66±0.042
3.367	0.36±0.579	0.74±0.403	0.49±0.358	0.61±0.446	0.60±0.403	0.65±0.300
4.331	0.32±0.578	0.65±0.387	0.40±0.224	0.60±0.432	0.51±0.519	0.62±0.134
PAA						
[ETH] (mol/L)	$V/10^2$ mUmL^{-1}					
	[Sucrose]/mmol/L					
	0.00	3.57	7.14	14.29	28.57	57.14
0.866	0.38±0.389	2.85±0.951	2.67±0.135	2.23±0.122	2.2±0.140	1.96±0.178
1.734	0.40±0.420	1.54±0.003	1.53±0.003	1.35±0.013	1.35±0.013	1.34±0.013

2.406	0.54±0.578	1.2±0.03	1.34±0.039	1.42±0.047	1.42±0.047	1.50±0.000
3.367	0.59±0.133	0.40±0.009	0.45±0.004	0.49±0.011	0.49±0.011	0.69±0.019
4.331	0.61±0.373	0.54±0.054	0.62±0.039	0.66±0.035	0.66±0.035	0.93±0.027

One unit (1U) of enzyme activity is 1×10^{-6} mol of maltose produced per minute when the substrate, 1mL of raw starch, is hydrolyzed by 1mL of the enzyme in 5 minutes. HSAA is crude human salivary amylase; PAA is porcine alpha amylase; v is activity (mU/mL) at 37^{o}C; ETH is non-consumable ethanol (that is ~100% ethanol that should not be ingested). The activities of untreated (control) HSAA and PAA are 111.32mU/mL and 109.98mU/mL respectively.

In a mixture of non-consumable ethanol and sucrose, there was decreasing trend in activity for both enzymes. But the activity of PAA is higher than control between 0.89 - 1.73mol/L of non-consumable ethanol. There higher activity of PAA at each fixed concentration of sucrose in the presence of 4.33mol/L of non-consumable ethanol than in the absence of sucrose. All the activities of HSAA were lower than control reaction mixture containing zero concentration of any osmolyte – both non – consumable ethanol and sucrose free reaction mixtures. The activities of PAA in a mixture of osmolytes containing molar concentration of ethanol ranging from 0.89 to 2.41mol/L and sucrose were higher than control containing only non – consumable ethanol.

The effect of emulsified milk was tested. The activity of HSAA reported as Mean±SD is 0.21±0.01 U/mL in the presence of milk only. This was found to be lower than the activity 0.304±0.003 U/mL of control without milk or any osmolyte whatsoever. There is no doubt that milk contains minerals like calcium and magnesium *etc* as may be accounted for by the observation that these minerals are not altered by the stage of lactation [34, 35]. This being a general case implies that, the presence of the calcium salt in particular may have accounted for the diminution in the activity of milk treated HSAA when compared with control and the activity (0.49±0.64U/mL; n = 3) of milk treated PAA similar to report elsewhere [36] including 3days postpartum (colostrums), 1.3week, and 6 weeks lactation activities equal to 8.97±0.70, 0.004±0.001, and 3.55±0.89U/mL respectively [37]. As claimed elsewhere [38], under similar condition free from additives, the control activities of HSAA and PAA are similar, 0.304±0.003 and

0.304±0.002U/mL respectively. The relative activities of the enzymes expressed as percentage of control and plotted versus molar concentration of ethanol is illustrated in Figs 1and 2 for HSAA and PAA respectively. Figure 1 clearly shows that in the presence of a mixture of milk and ethanol, there is a decreasing trend in the activity of HSAA. This is unlike PAA (Fig. 2)

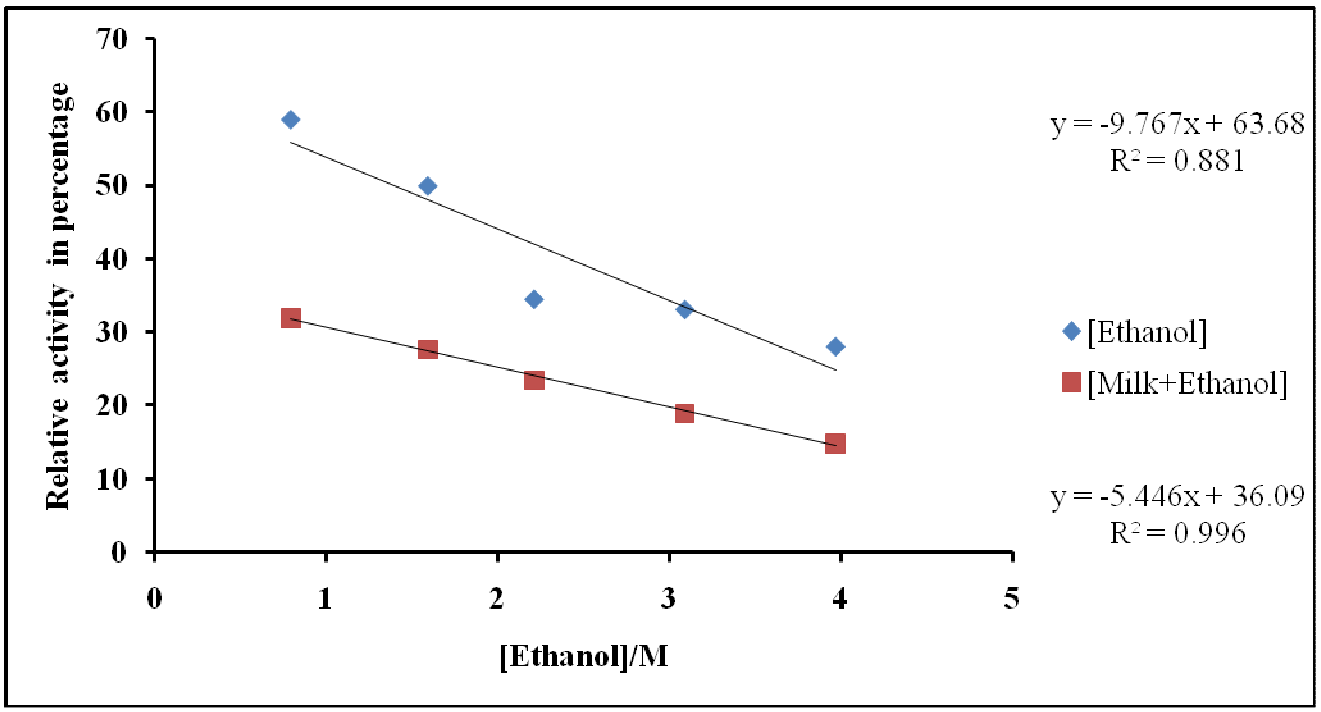
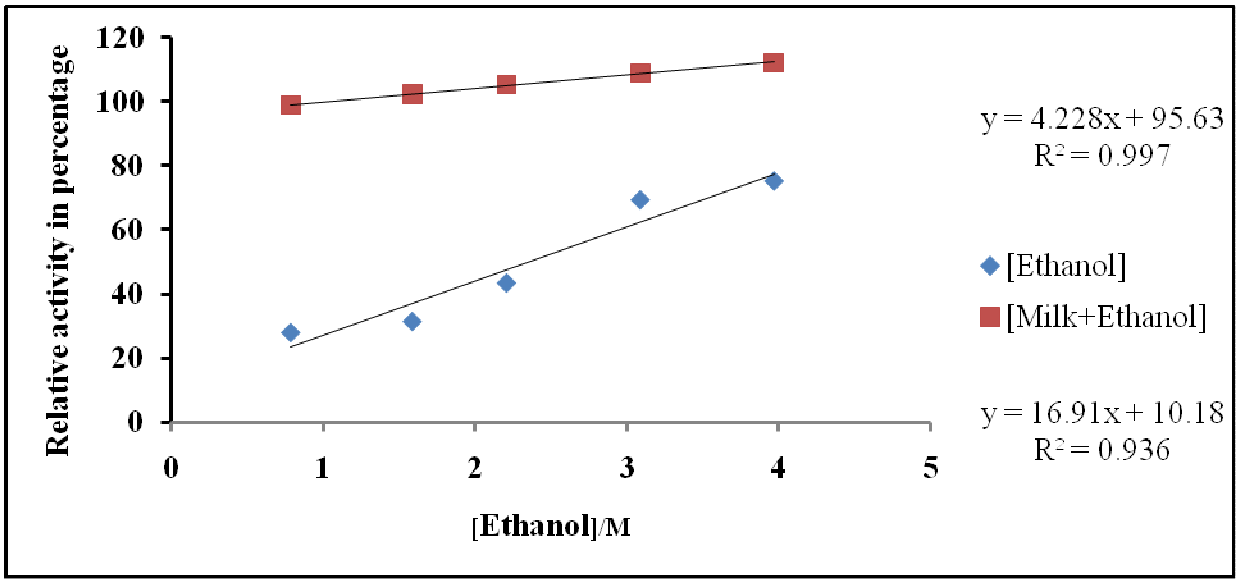


Fig. 1 Variation of relative activity of human salivary alpha amylase (HSAA) as percentage of control without any additive. (◆): *is the assay of HSAA in the presence of ethanol only and* (■)*: refer to assay in the presence of milk – ethanol mixture.*



348
349
350 **Fig. 2 Variation of relative activity porcine pancreatic alpha amylase (PAA), as percentage of**
351 **control without any additive.** (◆)*: is the assay of PAA in the presence of ethanol only and* (■)*: refer to*
352 *assay in the presence of milk – ethanol mixture.*

353
354 Investigation of the effect of additive to either force folding or unfolding, the *m* – value has its
355 result presented graphically in Fig 3. The *m* – values for HSAA and PAA were negative though the
356 magnitude for PAA is higher than for HSAA. These values are –1.09±0.02 and – 3.29±0.02kJ/mol for
357 HSAA and PAA respectively. The free energies ($\Delta G_{N\rightarrow U}$) of folding to unfolding transition in the absence
358 of stabilizing agent are – 0.29±0.08 and + 14.17±0.07kJ/mol for HSAA and PAA respectively.

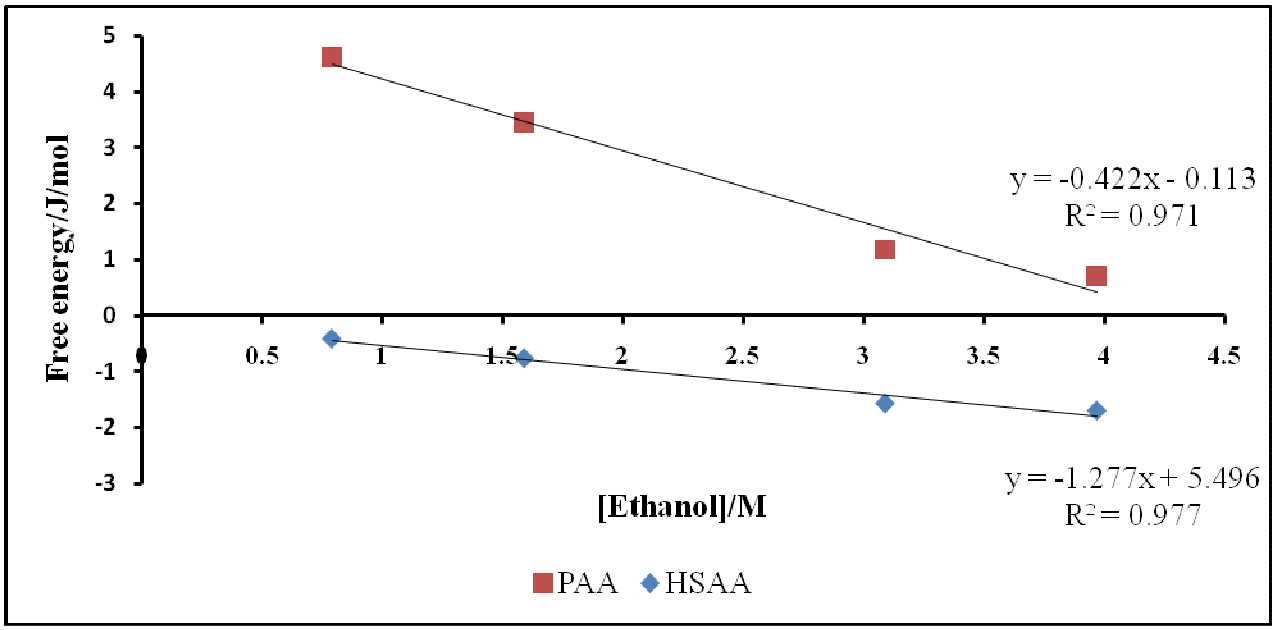


Fig. 3. Determination of *m* - value and free energy of folding - unfolding transition. *The m – values are –1.09±0.02 and –3.29±0.02kJ/mol for HSAA and PAA respectively while ($\Delta G^{o}_{N\rightarrow U}$) for HSAA and PAA in this study are –0.29±0.08 and +14.17±0.02kJ/mol, respectively.* (■)*: is the assay of PPA in the presence of ethanol-milk mixture while* (◆)*: refer to assay HSAA in ethanol-milk mixture.*

5. Discussion

The effect of the presence of aspirin, ethanol and a mixture of each and sucrose on the stability of enzyme substrate complex was investigated. Looking at the data one can easily say that the plot of natural logarithm of relevant parameter earlier stated versus the molal concentration of co – solute otherwise called osmolyte may either yield a positive or negative slope at this level of investigation. The important issue is that the magnitude of $\Delta\Delta G$ shows the likelihood of interaction between solution components. However, inhibition is likely to be less with very dilute destabilizing or interacting (preferential binding) osmolyte, and according to the nature of inhibition, the K_m may be lower so that low In ($k_{m(mc=o)}$ /$K_{m(mc)}$ may be compensated for by low [osmolyte] in the relation ∂In($k_{m(mc=o)}$ /$K_{m(mc)}$/∂[osmolyte] – the slope. If the concentration of osmolyte alone or in combination with other osmolyte is very low and stabilizing, $K_{m(mc=0)}$ /$K_{m(mc)}$ > 1, the value of $\Delta\Delta G$ will be large. If $K_{m(mc=0)}$ /$K_{m(mc)}$ < 1 in the presence of low destabilizing osmolyte, the value of $\Delta\Delta G$ will also be large. This is clearly based on simple mathematical principle. This is clearly in agreement with the assertion that “the effects of the changing environment on

polarity and chemical and enzyme reactivity have been assessed as a function of solute concentration" [39]. This is clearly evidenced in Tables 1 and 2 where in the absence of sucrose, in the presence of aspirin and ethanol respectively ([Aspirin] $\ll$ [Ethanol]) the values of $\Delta\Delta G$ for HSAA and PAA in the presence of aspirin only is ~ $10^3 \times$ the values in the presence of ethanol only. All negative $\Delta\Delta G$(c) values pointed to the stabilization of the IS of both substrate and enzyme and consequently a destabilization of AC/ES [16, 17]. The implication is that the enzymes role as a modulator/stabilizer of a transition-state ensemble [14, 26] might have been inhibited. Thus, the so-called diffusional encounter complex of two components (described as a transient state) cannot be held together by fluctuating short-range interaction in contrast to report elsewhere [15]. The implication is that the activity of the enzyme can be negatively affected as the case may be. Thus as Table 1 show, the presence of aspirin and sucrose appeared to have partially inhibited the activity of PAA due perhaps, to the stabilization of the initial state of the enzyme at the expense of the ES.

The adducible reason, from known effects of the polarity of solvent on the stability of complexes is the blocking by the co-solute of the reaction centre on the ES from attack by water [16]. Also, if bimolecular association kinetics can be represented by a two – step process with an intermediate state (AB)* known as a transient (or encounter complex) according to the scheme (40), A + B $\leftrightarrow$ (AB)* $\rightarrow$C, it becomes apparent that any agent or factor that can disrupt the process of encounter complex formation and ultimately the activated complex, including the ES, would inhibit or retard the rate of hydrolysis of the substrate. It is worthy of note that the values of $\Delta\Delta G$(c), ranging from – 227 to – 102Jkg/mol^2 in the presence of ethanol in a mixture of it and sucrose reported for HSAA in particular are similar to those reported for the neutral hydrolysis of esters: Those past values are –120 and –231 Jkgmol^2 in the presence of ethanol and propan – 2 – ol respectively as well as values such as – 142, –201, and –227 Jkgmol^2 in the presence of D – galactose, D – glucose, and D – mannose respectively [17]. The value reported for sucrose is – 541Jkg/mol^2 [17]. These values may concern non – biological reactions but they share a general principle with more complex biological reactions. It can be deduced from this finding that the OH-groups which are stabilizing agents and much more available in sugars, disaccharides in

particular, are not in the right concentration to overcome the destabilizing effect of – CH_2 – rich hydrophobic co – solutes.

The presence of ethanol disrupts the spatial structure of water around the macromolecules like proteins (41) which affects the 3 – D (3 - dimensional) structure of the enzyme. Furthermore, since polar solvent is known to strip water off protein core and external domain [42] there may have been insufficient water molecules to stabilize partial charges in protein / substrate and ultimately the encounter complex / (ES) contrary to expectation [12, 13]. Additional support to those reasons is the high entropic cost (entropic cost is only for the purpose of explanation otherwise it is not covered by the scope of the research) of fixing water to its reactive site on the complex for its action [19]. The effect of ethanol is greater for HSAA than PAA while sucrose which generally has opposite effect to ethanol seemed to affect PAA more than it does for HSAA. This may be as a result of greater 3 – D structure for PAA than for HSAA. The $\Delta\Delta G$(c) due to the presence of ethanol in the hydrolysis of esters and amides reported in the past (17) is negative in sign and similar in sign to current finding as applicable to HSAA; both are comparable in magnitude (Table 2). This was not the case in respect of PAA in which $\Delta\Delta G$(c) was positive and almost thrice in size. This may have to do with greater rigidity of PAA which achieved greater conformational flexibility due to effect of ethanol similar to past report in different condition such as requirement for improved flexibility or plasticity of protein molecule among psychrophiles [43 – 45] and as it is the case of bad solvent being a good solvent for protein (PAA) [46]. On the contrary, all positive $\Delta\Delta G$(c) parameters associated with induced rate accelerations due to added co - solutes were indicative of stabilization of the AC relative to IS perhaps through favourable polar interactions with the co-solute and increased hydrophobicity of the components of encounter complex [16, 17]. This was well reflected in the effect of aspirin only and ethanol only on HSAA and PAA respectively. Thus, the presence of sucrose in different fixed concentration in a mixture of aspirin and sucrose exhibited positive $\Delta\Delta G$(c) parameters for HSAA and negative $\Delta\Delta G$(c) parameters for PAA.

The theory of savage-wood additivity of group interactions (SWAG) also described as pair – wise group interaction parameter offers source of explanation [16, 17]: The observed negative $\Delta\Delta G$(c) parameter which implied rate retardation is explained on the basis of a rate-decreasing contribution of -

CH_2- groups while positive $\Delta\Delta G$(c) parameter can be analyzed and explained in terms of rate-enhancing contribution from OH groups [17]. The question that needs to be asked is whether there is upper limit to the stability of ES/encounter complex above which it becomes unfavorable to transformation to product? This is against the backdrop of further increase in stability in aqueous solution upon an increase in the hydrophobic nature of the encounter complex constituents [16]. However, -CH_2- group is hydrophobic and its hydrophobic nature increases with the size of it in terms of $n(CH_2)$ where n »1. Yet it has been reported to possess two opposing effects. Increase in favourable interaction upon increasing the hydrophobic nature of the reactant (ester for instance) and co – solute conformed to an increase in the stability of the encounter complex by hydrophobic interaction [16]. Therefore, stability should increase with large n. But if -CH_2- is rate decreasing implied in SWAG then, the purported stabilization due to increasing n, may be as a result of its effect on ES. This situation is relatively more favourable to PAA, whose activities showed incremental trend with increasing concentration of ethanol, but were less than control value without ethanol. This implies that the encounter complex preceding the formation of ES formation was partially stabilized due the interaction between the complex and ethanol. Cognate to this is the issue of concentration of added co – solute to the reaction mixture such that a plot of In K_m (m_c = 0)/K_m (m_c) versus such concentration would produce a slope that is either high or low in accordance with the degree of dilution of the co-solute.

What is obvious is that at higher degree of dilution (low concentration of co-solute), the effect of destabilizing co-solute will be reduced because free energy cost for interacting with the substrate, enzyme, and ES *etc* should be unfavourable. The same issue is applicable to stabilizing co – solute. It is not certain therefore, how figure 2 appears in the model according to Buurma *et al* [16]. Stability of ES is also, said to be promoted by translational entropy of departing water of hydration [47]. This seemed to be against preferential hydration of protein following exclusion of protecting osmolyte, sucrose for instance, as in this work, from the vicinity of protein surface domain [26, 28, 48].

Upon careful examination of the data (Tables 3 and 4) , one can see that while aspirin has stabilizing effect on HSAA, ethanol had opposite effect but such effect of ethanol in particular was less pronounced on PAA. With respect to HSAA, the effect of aspirin is similar to its effect on pearl millet alpha amylase (4) and on rat intestinal alpha amylase [49]. The effect on PAA is the same as the effect on

458 hydrolases in both homogenates and brush border membrane preparations in which there was decrease
459 in the activity of the enzyme following treatment with aspirin [6]. The effect on HSAA is also similar to the
460 effect on rat pancreatic alpha amylase whose activity increased (49). Like the effect of a mixture of aspirin
461 and gum on rat intestinal alpha amylase [49], a mixture of aspirin and sucrose caused a rise in the activity
462 of HSAA with increase in the concentration of aspirin. This was unlike PAA similar to the observed
463 decrease in the activity of rat pancreatic alpha amylase [49]. Also the decrease in the activity of HSAA
464 and PAA below control (though there was increasing activity of PAA unlike HSAA with increasing
465 concentration of ethanol) is similar to the effect of ethanol on *B. Licheniformis* whose activity decreased
466 after treatment with ethanol [2]. However, Onyeson and Erude [3] observed increase in the activity of the
467 salivary and plasma enzyme in alcoholics. But it is not certain whether the assay was conducted *in vitro* in
468 the presence of ethanol.

469 The need for conformational flexibility confirms the claim regarding the effect of ethanol on PAA.
470 Although the activities of PAA in the presence of different concentrations of ethanol were lower than
471 control activities, there were increasing trend in activities with increasing concentration of ethanol. This
472 suggests that there was increasing conformational flexibility that could not totally inhibit the activity of the
473 enzyme, pointing to the fact that there must be optimum conformational flexibility as against structural
474 rigidity for function, an issue mostly applicable to cold adapted enzymes, otherwise called psychrophiles
475 [44]. The effect of ethanol is similar to the view that a bad solvent can become useful [45, 46] in manner
476 dependent on the nature of the enzyme such as greater rigidity of PAA [28] but against the known
477 destabilizing role of ethanol on most enzymes. In other words the effect of ethanol on PAA, in particular,
478 in reducing rigidity (or global compact state) is in line with the view that many enzymatic reactions cannot
479 be understood from the rigid – protein viewpoint since conformational changes or flexibility provides a
480 mechanism for achieving enzyme specificity [40]. Thus, the structural and functional characteristic of the
481 enzyme must be sustained by a mechanism which brings a balance between compact state structure and
482 conformational flexibility. Extreme ends of the structure may not enhance the function of the enzyme.

483 The ring structure of aspirin is a major source of hydrophobic properties while the size of ethanol
484 makes it less hydrophobic. In their capacity as single co-solute, they presented different thermodynamic
485 effects: While HSAA showed positive $\Delta\Delta G$(c), PAA showed the opposite sign in the presence of aspirin

only. In the presence of ethanol, the enzymes showed differences in the sign of the parameter. The positive sign of $\Delta\Delta G$(c) in the presence of ethanol implied that there was at least partial rate enhancement as opposed to total rate inhibition of PAA unlike HSAA in agreement with theory [16, 17]. This is therefore, applicable to the situation where IS species is stabilized at the expense of AC (*e.g.* ES) as applicable to HSAA. This is therefore, a confirmation of the implication of negative $\Delta\Delta G$(c) [16] which is evidence of rate inhibition. Nonetheless, in this investigation the presence of ethanol and sucrose has rate retarding effect on HSAA and PAA respectively. It is certain, therefore, that PAA has greater conformational stability than HSAA, hence presence of sucrose in a mixture of it and aspirin may have rigidified PAA similar to observation elsewhere [28] and to a greater extent than HSAA, while presence of ethanol resulted in significant unfolding (decrease in activity) above optimum degree of conformational flexibility needed for function. Hence in respect of HSAA, there is need to stabilize the ES which may need higher concentration of a stabilizer such as sucrose. There is need because, the ES may undergo dissociation let alone the encounter complex in the presence of ethanol for instance in agreement with the view that an encounter complex will not always proceed toward the final complex [50]. Since encounter complex formation precedes the formation of active complex, ES, for instance, which is said to be stabilized by both hydrophobic and electrostatic interaction [50], the presence of co – solute may either alter the dielectric environment to an extent not compatible with the minimum required for functional structure formation even though as have been reported [16], that its hydrophobic effect also enhances hydrophobic interaction in the complex.

Alpha amylase from various sources presents different homologues with different dependences on calcium salt for activity – stability complementarity sustenance. Thus some homologues may show independence on calcium ion [51, 52]. This present study showed that the presence of calcium ion in milk seemed to have retarded the activity of HSAA; otherwise one should have expected a strong protective effect against destabilizing effect of ethanol that should have led to higher activity. A plot of relative activity (as a percentage of control) versus molar concentration of ethanol, in the presence of ethanol alone (Fig. 1) shows decreasing trend with higher “declivity” than similar trend in the presence of ethanol – milk mixture. The emulsified fat content using strong commercial detergent may not have been responsible otherwise the higher slope could not have been the case. The probable reason may be as a

result of the failure of the chloride ion (from sodium chloride) content of the reaction mixture to oppose the inhibiting effect of calcium ion whose binding to the protonated state of Glu – 233 of the enzyme should have been weakened by the presence of chloride ion so as to make the opposition effective [53]. This may be justified if cognizance is taken of the fact that saliva contains not just proteins but calcium salt and combined with extra calcium salt in milk, it is obvious that there may be imbalance in the ratio [Calcium ion]:2[Chloride ion].

The issue of the effect of excess calcium ion had been observed elsewhere in the presence of excess extracellular calcium chloride at temperatures ranging from 25 – 60^{o}C [36] and loss of stability and cognate activity by *Bacillus hamapalus* alpha amylase at much higher concentration of calcium chloride and temperature > 70^{o}C (54). On the other hand PAA like most other homologue like HSAA, has calcium binding site in which calcium ion creates an ionic bridge between two β-structures which promotes the three dimensional form for function and stability [55]. Thus the fact that PAA is exposed to extra calcium salt in the milk is not sufficient to cause inhibition of the commercial enzyme, PAA (purchased enzyme in the highest state of purity) that may not have been fortified with extra calcium leaving only perhaps, the intrinsic calcium ion unlike saliva from mammalian source, without exception, whose alpha amylase content, including minerals such as calcium, sodium, potassium and phosphate, is part of well known composite fluid milieu [56]. The reaction mixture which contained sodium chloride may have been the source of chloride ion that have been implicated to be required for full activity [57] and whose removal leads to significant decrease in activity [53].

The sign of the *m* – value determine whether a compound stabilizes or destabilizes a protein [58]; there is experimental evidence that with urea as a denaturant the *m* – value obtained from linear extrapolation method of protein is constant and negative and invariant to the concentration of urea [59, 60]. This is in line with Eq (9) [58] The *m* – values for protecting (stabilizing) osmolytes are found to be positive in sign, and are commonly assumed to be constant. This assumption was found to be true experimentally for trimethylamine-N-oxide [61] and glycine – betaine [60]. The reason as to the choice of either Eq (8) or Eq (9) as in literature is not obvious or clear. Nonetheless, the outcome of assay in the presence of denaturant or stabilizer alone or a mixture of them should reveal the sign of *m* – value as to whether or not there was folding (native – like activity (7)) and unfolding (loss of activity (7)). In the light of

this is the observation that stabilizers namely, TMAO, proline, sorbital *etc* showed +*m*–values, 1.57±0.31, 2.33±0.47, 1.22±0.75kcal/mol/M respectively for N – terminal activation domain (AF1) of the glucocorticoid receptor [62]. But in this present report based on the sign of the slope of the plot of free energy versus molar concentration of ethanol mixed with milk, the *m* – values for HSAA and PAA are –1.09±0.09kJ/mol and – 3.29±0.02kJ/mol; *ab initio*, PAA surprising showed increasing trend in relative activity with increasing concentration of ethanol (Fig. 2) and coupled with stabilizing effect of milk content, calcium salt in particular, one would have expected a total reversal of the effect of ethanol to achieve much less negative *m* – value and activity much higher than controls without any additive including milk and with milk only. This is to say that the *m* – value should have been positive. However, the observed *m* – value may be as it is because just as the presence of sucrose with increasing concentration of ethanol and aspirin lead to decreasing activity of PAA, a situation observed also for HSAA in the presence of increasing concentration of ethanol only, there is also the presence of a disaccharide, lactose, in milk.

Since "the slope, *m*, obtained from the LEM analysis represents the cooperativity of the transition and is a measure of the efficacy of the osmolyte in forcing a protein to either fold or unfold" [63] the negative *m* – values obtained for both enzymes suggest that there was obviously inhibition of activity with increasing concentration of ethanol. The equilibrium constant (K_{eq}) for native to unfolded transition has its implication such that values of it less than 1 implies that the fraction of native protein (*N*) is > unfolded protein (*U*). Therefore, increasing value of K_{eq} implies that *U* is increasing as should be expected from Eq (12). The higher activity (high *N* in line with Baskakov and Bolen (7) verified postulation) with milk only than without milk, *i.e.* the control, shows that PAA is favourably depended on calcium content of milk, being stabilized by it as observed in the presence of extra calcium chloride in previous investigation [36]. This is unlike HSAA in this investigation and in the past [36]. The paradox however, is the observation that sucrose and proline have negative *m* – values, – 0.2 and – 0.1cal/mol/M respectively for cold shock protein (CspTm) while guanidinium chloride and urea, well known denaturants have positive *m* – values, 2.4 and 0.9 cal/mol/M, respectively. The urea *m* – value, 1.84±0.02kcal/mol/M is reported for Barnase [61].

On the other hand, the free energy ($\Delta G^{o}_{N\rightarrow U}$) of transition from native to unfolded, for HSAA and PAA in this study are –0.29±0.08 and +14.17±0.02kJ/mol, respectively. It may not require any unknown skill to obtain conclusive facts about these $\Delta G^{o}_{N\rightarrow U}$ values but all that may be needed is just a careful examination of those values of $\Delta G^{o}_{N\rightarrow U}$ and correlate with the activities of the enzymes in the presence of milk only. In line with Rösgen *et al* [58] and Auton *et al*. [63], the negative value of $\Delta G^{o}_{N\rightarrow U}$ for HSAA testifies to the fact that the presence of milk and its content, calcium salt in particular, was inhibiting the activity of the enzyme in the absence and presence of ethanol; $A_N > A_{Milk} > A_{[Ethanol + Milk]}$ where A_N, A_{Milk}, and $A_{[Ethanol + Milk]}$ are activities of native enzyme in buffer only, milk only and ethanol – milk mixture. Figure 1 gives additional illustration to this position. This is unlike PAA in which $A_{Milk} > A_{[Ethanol + Milk]} > A_N$ with supportive illustration in Fig. 2. Thus the much higher magnitude of $\Delta G^{o}_{N\rightarrow U}$ with positive sign shows that in the absence of ethanol, the calcium salt content of milk stabilized and enhanced the activity of PAA which is much in agreement with the high activity in milk only. Negative $\Delta G^{o}_{N\rightarrow U}$ implies spontaneity of folding - unfolding transition. Positive $\Delta G^{o}_{N\rightarrow U}$ as applicable to PAA only means that such transition is less spontaneous. $\Delta G^{o}_{N\rightarrow U}$ values in the absence of urea, GdmCl, sucrose, and proline had negative sign viz: – 6.1, – 5.8, – 6.3, and – 6.3kcal/mol respectively for cold shock protein Tm [64].

6. Conclusion

Unlike PAA, the presence of aspirin only enhanced the activity of HSAA. Both enzyme showed decreasing trend in activity with increasing ethanol in the presence of sucrose. The observed sign of the Gibbs free energy of encounter complex formation remains evidence of either rate enhancement (positive $\Delta\Delta G$(c)) or rate retardation (negative $\Delta\Delta G$(c)). The sign of $\Delta\Delta G$(c) seemed to be a function of the nature of the enzyme as can be seen in the differences in the sign of $\Delta\Delta G$(c) between PAA and HSAA. These scenarios seemed to validate the model. From activity measurements, extrapolated $\Delta G^{o}_{N\rightarrow U}$, and m – values, it is very obvious that while ethanol retards the rate of hydrolysis of raw starch, it is also a fact that the presence of calcium salt in milk enhanced the activity of PAA unlike HSAA. Higher concentration of milk calcium salt/sucrose may be needed to fortify milk for HSAA so as to oppose higher concentration of ethanol. It is very important to ensure that food additives (or drugs) do not have adverse effect on ES.

595 **REFERENCES**

596 1 Halsted CH, Robles EA, Mezey E, Distribution of ethanol in the human gastro intestinal tract,
597 Am. J. Clin. Nutr., 1973; 26: 831 – 834. doi: 10.1016/j.mehy.2015.04.021

598 2. Blakeney AB, Stone BA, Activity and action pattern of *Bacillus Licheniformis* alpha
599 amylase in aqueous ethanol, FEBS Letters. 1985; 186 (2): 229 – 232. doi: 10.1016/0014-
600 5793(85)80714-6

601 3. Onyesom I, Erude HO, α - Amylase activity in the saliva and plasma of habitual alcohol drinkers
602 Biokemistri 2004; 16 (1): 11 – 14. doi*:* 10.1111/j.1360-0443.1991.tb02627.x.

603 4. Kharkrang K, Ambash PK, Purification and characterization of alpha amylase from seeds of pearl
604 millet *(Pennisetum typhoides*). Journal of Proteins and Proteomics 2012; 3(1): 47 – 60.

605 5. Sanyal NS, Kaushal N, Effect of two non – steroidal anti – inflammatory drugs, aspirin and
606 nmesulide on the G – glucose transport and disaccharide hydrolases in the intestinal brush
607 border membrane. Pharmacological reports, 2005; 57**:** 833 – 838. doi:
608 10.1080/15376520600640040

609 6. Sood N, Kaushal N, Sanyal SN, Effect of different non – steroidal ant – inflammatory drugs,
610 aspirin, nimesulide and celecoxib on the disaccharide hydrolases and histoarchitecture of the rat
611 intestinal brush border membrane. Nutr Hosp. 2008; 23: 326 – 331. doi: 10.1007/s00204-008-
612 0311-0.

613 7. Baskakov I, Bolen DW, Forcing thermodynamically unfolded proteins to fold
614 (communication). J. Biol. Chem. 1998; 273(9): 1 – 5. doi: 10.1074/jbc.273.9.4831

615 8. Kendrick BS, chang BS, Arakawa T, Peterson B, Randalph TW, Manning MC, Carpenter JF,
616 Preferential exclusion of sucrose from recombinant interleukin – receptor antagonist; Role in
617 restricted conformational mobility and compaction of native state. Proc. Natl. Acad. Sci. U.S.A.
618 1997; 94: 11017–11920. doi: 10.1073/pnas.94.22.11917

619 9. Swann AC, Opposite effects of ethanol and dimethyl sulphoxide on temperature dependence of
620 enzyme conformational and univalent cation binding. J. Biol. Chem., 1983; 10: 11780 – 11786.

10. Sobolevaa SE, Dmitrenok PS, Verkhovoda TD, Bunevaa VN, Sedykha SE, Nevinskya GA, Very stable high molecular mass multiprotein complex with DNase and amylase activities in human milk. J. Mol. Recognit. 2015; 28**:** 20–34. doi: 10.1002/jmr.2409.

11. Souza PM, Pérola, Magalhães PDO, Application of microbial alpha amylase industry – A review. Brazilian Journal of Microbiology 2010; 41: 850 – 861. doi*:* 10.1590/S1517-83822010000400004

12. Subhani MS, Aslam S, Qureshi R, Rahman A, UV spectroscopic studies of charge transfer complexes of 2-hydroxy-1,4-naphthoquinone. J. Chem. Soc. Pak. 2008; 30 (2): 232 – 236.

13. Pascal R, Do enzymes bind their substrates in the ground state because of a physico – chemical requirement? Bioorg. Chem. 2003; 31 (6): 485 – 493. doi: 10.1016/S0045-2068(03)00102-0

14. Ma B, Kumar S, Tsai C – J, Hu Z, Nussinov R, Transition-state ensemble in enzyme catalysis: Possibility, reality or necessity? J. Theor. Biol. 2007; 4 (21): 383 – 397. doi:10.1006/jtbi.2000.1097

15. Sugase K, Dyson V, Wright PE, Mechanism of coupled folding and binding of an intrinsically disordered protein. Nature 2007; 447(21): 1021–1024. doi:10.1038/nature05858;

16. Buurma NJ, Pastorello L, Blandermer MJ, Engberts JBFN, Kinetic evidence for hydrophobically stabilized encounter complexes formed by hydrophobic esters in aqueous solutions containing monohydric alcohols. J. Am. Chem. Soc. 2001; 123: 11848 – 11853, doi: 10.1021/ja010617w

17. Engberts JBFN, Blandamer MJ, Reactant-solute encounters in aqueous solutions studied by Kinetic methods: hydration co-sphere overlap and camouflage effects (Review commentary). J. Phys. Org. Chem, 1998; 11: 841 – 866.

18. Tanaka A, Hoshino E, Thermodynamic and inactivation parameters for the hydrolysis of amylase with *Bacillus* α - amylases in a diluted anionic surfactant solution. J**.** Biosci. Bioeng, 2002; 93 (5): 485 – 489. doi: 10.1021/bp060057a.

19. Petukhov M, Rychkov G, Firsov L, Serrano L, H-bonding in protein hydration revisited. Protein Sci. 2004; 13(8): 2212 – 2129. doi: 10.1110/ps.04748404

20. Copeland RA, Enzymes: A practical introduction to structure, mechanism, and data analysis, 2nd edn, John Wiley and Sons Inc Publication, New York, 2000, 113 – 119. doi/10.1002/0471220639.ch8.

21. Schnell S, Maini PK, Enzyme kinetics at high enzyme concentrations. Bull. Math. Comput. Model. 2002; 35: 137 – 144. doi:10.1006/bulm.1999.0163.

22. Walker GJ, Hope PP, The action of some alpha amylases on starch granules. Biochem. J. 1963; 86**:** 452 – 462.

23. Slaughter SL, Ellis PR, Butterworth PJ, An investigation of the action of porcine pancreatic alpha amylase on native and gelatinized starches. Biochim. Biophys. Acta. 2001; 1525: 29 – 36. doi*:* 10.1021/bm101070r

24. Gao J, Ma S, Major DT, Nam K, Pu J, Truhlar DG, Mechanisms and free energies of enzymatic reactions Chem. Rev. 2006; 106: 3188 – 3209. doi: 10;1021/cr050293

25. Baskakov I, Wang A, Bolen DW, Trimethlamine – N – Oxide counteracts urea effects on rabbit muscle lactate dehydrogenase function: A test of the counteraction hypothesis. Biophys. J. 1998; 74: 2666 – 2673. doi:10.1016/S0006-3495(98)77972-X

26. Timasheff SN, Protein solvent preferential interacting, protein hydration, and the modulation of biochemical reactions by solvent components. Biochemistry. 2002; 99 (15): 9721 – 9726. doi: 10.1073/pnas.121171498

27. Rösgen J, Pettitt BM. Bolen DW. An analysis of the molecular origin of osmolyte – dependent protein stability Protein Sci. 2007; 16: 733 – 743. doi*:* 10.1110/ps.062671607

28 Anuradha SN, Prakash V, Structural stabilization of bovine *β*-Lactoglobuline in presence of polyhydric alcohols. Ind. J. Biotechnol**.** 2008; 437 – 447. doi: 10.1016/S0076-6879(73)27030-1

29. Bernfeld P. Amylases, alpha and beta. Methods. Enzymol 1955**;** 1: 149 – 152. doi: 10.12691/jaem-2-3-2

30. Lineweaver H, Burk D, The determination of Enzyme Dissociation constants, J. Am. Chem. Soc. 1934; 56: 658 – 666. doi:10.1021/ja01318a036

673 31. Pace CN, Shaw KL, Linear extrapolation method of analyzing solvent denaturation curves
674 PROTEINS: Structure, Function, and Genetics Suppl, 2000; 4, 1–7. doi:
675 10.1002/1097013432(2000)41.
676 Harries D, Rösgen J, A practical guide on how osmolytes modulate macromolecular properties.
677 Methods Cell Biol. 2008; 84: 679 – 735. doi: 10.1016/S0091-679X(07)84022-2
678 33. Pace CN. Measuring and increasing protein stability. Trends Biotechnol, 1990; 8: 93 – 98. doi:
679 10.1016/0167-7799(90)146-O
680 34. Guy EJ, Jenness R, Separation, concentration, and properties of alpha amylase from cows' milk.
681 J. Diary Sci. 1958; 4 (1): 13 – 27. doi: 10.3168/jds.s0022-0302(58)90861-0
682 35**.** Kirksey A, Ernst JA, Roepke JL, Tsai TL, Influence of oral contraceptives before pregnancy on
683 the mineral content of human colostrums and of more mature milk. Am. J. Clin. Nutri. 1979; 32:
684 30 – 39. doi: 10.1007/BF02748404
685 36. Udema II, The effect of additives and temperature on the velocity of hydrolysis of raw starch with
686 human salivary α - amylase. IJBcRR. 2015; 10(1): (In press).
687 37 Jones JB, Mehta NR, Hamosh M, Alpha amylase in preterm milk human milk**,** J. Pediatr
688 Gastroenterol. Nutr. 1982; 1(1): 43 – 48*.*
689 38. Butterworth JP, Warren, FW, Ellis PR Human alpha amylase and starch digestion: An
690 interesting marriage. Starch/Stärke 2011; 63: 395 – 405. doi: 10.1002/star.201000150
691 39. Assad N, den Otter JM, Engberts Jan BFN, Aqueous solutions that model the cytosol: studies on
692 polarity, chemical reactivity and enzyme kinetics.Org. Biomol. Chem. 2004**;** 2**:**1404 – 1412. doi:
693 10.1039/b402886d
694 40. Mereghetti R, Kokh D, McCammon A, Wade RC, Diffusion and association processes in
695 biological system: theory, computation and experiment, BMC Biophysics 2011; 4(2): 2 – 6.
696 41. Hackl EV, Blagoi YP, Effect of ethanol on structural transitions of DNA and polyphosphates under
697 Ca^{2+} ions action in mixed solutions. Acta Biochimica Acta Polonica 2000; 47(1): 103 – 112.
698 42. Yang L, Dordick S, Shekhar G, Hydration of enzyme in non-aqueous media is consistent with
699 solvent dependence of its activity, Biophys. J. 2004; 87: 812 – 821. doi:
700 10.1529/biophysj.104.041269

43. D'Amico S, Gerday C, Feller G, Structural determination of cold adaptation and stability in a psychrophilic α-amylase, Biologia, Bratislavia.57/suppl 2002**;** 11: 213 – 219. doi:10.1529./biophysj.104.041269

44. D'Amico S, Marx JC, Gerday C, Feller G, Activity-stability relationship in extremophilic enzymes, J. Biol. Chem 2003; 278 (10): 7891 – 7896. doi*:* 10.1021/jp901921r

45. Ovando S, Waliszeuski SN, Pardio VT, The effect of hydration time and ethanol concentration on the rate of hydrolysis of extracted vanilla beans by commercial cellulose preparations (A), Int. J. Food Sci. Technol. 2005; 40 (9): 1011 – 1018. doi: 10.1111/j.1365-2621.2005.01035.x

46. Mitchell DC, Lawrence JTR, Litman BJ, Primary alcohols modulate the activation of the G. protein-coupled receptor rhodopson by a lipid-mediated mechanism. J. Biol. Chem. 1996; 271 (32): 19033 – 19036. doi*:* 10.1074/jbc.271.32.19033.

47. Harano Y, Kinoshita M, Translational – entropy gain of the solvent upon protein folding. Biophys. J. 2005; 89: 2701 – 2710. doi*:* 10.1529/biophysj.104.057604.

48. Arakawa T, Prestrelski SJ, Kenney WC, Carpenter JF, Factors affecting short – term and long-term stabilities of protein, Adv. Drug Delivery Rev 1992; 10: 1 – 28. doi*:* 10.1002/jps.21409.

49. Nasif WA, Lotfy M, Mahmoud MR, Protective effect of gum acacia against the aspirin induced intestinal and pancreatic alterations, Biochemistry 2011; 20: 3062 – 3067. doi:10.1016/j.arcmed.2012.09.010

50. Scanu S, Foerster JM, Ullmann GM, Ubbink M, Role of Hydrophobic Interactions in the Encounter complex formation of the plastocyanin and cytochrome f complex revealed by paramagnetic NMR spectroscopy, J. Am. Chem. Soc. 2013; 135: 7681−7692, doi: 10.1021/ja4015452.

51. Chakraborty S, Raut G, Khopade A, Mahadik K, Kokare C, Study on calcium ion independent alpha amylase from *Streptomyces Strain A3*, In J. Biotech 2012**;** 11: 427 – 437, (2012).

52. Atsbha TW, Haki GD, Abera S, Gezmu TB, Thermo-stable, calcium independent alpha amylase from two Bacillus species in afar, Ethiopia, IRJPAC 2015; 6(1): 9 – 18, 53.Feller G, Bussey Ole, Houssien C, Gerday C. Structural and functional aspects of chloride binding to *Alteromonas haloplanctis* α - amylase J. Biol. Chem. 1996; 271(39): 23836 – 23841. doi: 10.1074/jbc.271.39.23836

54. Nielsen AD, Fuglsang CC, Westh P, Effect of calcium ions on the irreversible denaturation of a recombinant *Bacillus halmapalus* alpha amylase: a calorimetric investigation. Biochem J. 2003; 373: 337 – 343. doi: 10.1042/BJ20030220

55. Buisson G, Dué e E, Haser R, and Payon F, Three dimensional structure of porcine pancreatic alpha amylase at 2.9Å resolution role of calcium in structure and activity, EMBO J. 1987; 6(3): 3909 – 3916.

56. Lavy E, Goldberger D, Friedman M, Steinberg D, *p*H Values and Mineral content of saliva in different breeds of dogs *Israel Journal of Veterinary Medicine* 2012; 67(4): 244 – 248. doi:10.1016/j.chemosphere.2013.07.075

57. Levitzki A, Steer ML, The allosteric activation of mammalian alpha amylase by chloride. Eur. J. Biochem 1974; 41: 171 – 180. doi: 10.1111/j.1432-1033.1974.tb03257.

58. Rösgen J, Pettitt BM, Bolen DW, Structure of solution of aqueous biochemical compounds – pair correlations. Biophys J. 2005; 89: 2988 – 2997.

59. Timasheff SN, Xie G, Preferential interactions of urea with lysozyme and their linkage to protein denaturation. Biophys. Chem. 2003; 105: 421 – 448. doi: 10.1016/S0301-4622(03)00106-6

60. Ferreon AC, Bolen DW, Thermodynamics of denaturant induced unfolding of a protein that exhibits variable two-state denaturation. Biochemistry 2004; 43: 13357 – 13369. doi:10.1021/bi048666j

61. Mello CC, Barrick D, Measuring the stability of partly folded proteins using TMAO, Protein Sci. 2003; 12**:** 1522–1529. doi*:* 10.1002/pro.52.

62. Kumar R, Serrette HM, Khan SH, Miller AL, Thompson EB, Effects of different osmolytes on the induced folding of the N – terminal activation domain (AF1) of the glucocorticoid receptor, Arch Biochem Biophys. 2007; 465 (2): 452 – 460. doi*:* 10.1016/j.abb.2007.06.019

63. Auton M, Ferreon ACM, Bolen DW. Metrics that differentiate the origin of osmolyte effects on protein stability: a test of the surface tension proposal, J. Mol. Biol. 2006; 361: 983 – 992. doi*:* 10.1016/j.jmb.2006.07.003.

64. O' Brien EP, Ziv G, Haran G, Broks BR, Thirumalai D, Effects of denaturants and osmolytes on proteins are accurately predicted by the molecular transfer model, Proc. Natl. Acad. Sci. U.S.A., 2008; 105(36): 3403 – 1340. doi: 10.1073/pnas.0802113105

Appendix A

Formulation of equation for the determination of the Gibbs energy of encounter complex formation E-S complex

From [E] + [S] ⇌ [ES] in which the rate constant for forward reaction and backward reaction are k_1 and k_{-1} respectively,

$$K_m = k_{-1}/k_1 \quad (A.1)$$

$$k_1 = k_{-1}/K_m \quad (A.2)$$

$$k_{1\,(mc)} = k_{-1}(m_c)/K_{m\,(mc)} \quad (A.3)$$

$$k_{1\,(mc\,=\,0)} = k_{-1(mc\,=\,0)}/K_{m\,(mc\,=\,0)} \quad (A.4)$$

By dividing A.3 by A.4 the following was obtained:

$$k_1\,(m_c)/k_{1\,(mc\,=\,0)} = k_{-1(mc)}\,K_{m\,(mc\,=\,0)}/K_{m\,(mc)}\,k_{-1(mc\,=\,0)} \quad (A.5)$$

In line with principle enunciated by Engberts and Blandamer [17] and Buurma *et al* [16],

$$\ln k_{-1(mc)}/\,k_{-1(mc\,=\,0)} = \Delta G_{-1mc}/RT - \phi_{-1}n_{-1}M_{mc} \quad (A.6)$$

$$\ln\, k_{1\,(mc)}/k_{1\,(mc\,=\,0)} = \Delta G_{\,(mc)}/RT - \phi\, nM_{mc} \quad (A.7)$$

By taking the natural log of Eq (A.5), the Gibbs free energy of interaction in the forward reaction is:

$$\ln K_{m\,(mc\,=\,0)}/K_{m\,(mc)} + \ln k_{-1(mc)}/k_{-1(mc\,=\,0)} = \Delta G\, m_c/RT - \phi\, nM_{mc} \quad (A.8)$$

Equation (A.8) contains $\ln k_{-1(mc)}/\,k_{-1(mc\,=\,0)}$ defined in Eq (A.6); therefore, substituting it for Eq (A.6) yields after rearrangement the equation:

$$\ln K_{m\,(mc\,=\,0)}/K_{m\,(mc)} = (\Delta G_{mc} - \Delta G_{-1mc})/RT + Mm_c(\phi_{-1}n_{-1} - \phi n)$$

$$= (\Delta\Delta G m_c/RT) - Mm_c\phi\Delta n \quad (A.9)$$