Original Research Article

Theoretical Investigation into the Change in the Number of Water Molecules in Solvent Inaccessible Region of an Enzyme and Enzyme-Substrate Complex

ABSTRACT

Background: There may be dry enzymes, but water remains indispensable for the catalytic action of enzymes. There is not as much interest in how the presence of a drug such as aspirin and a psychoactive compound such as ethanol may affect the water-mediated role of the enzyme.

Objectives: The objectives of this research are: 1) To assess the changes in the number of water molecules interacting with the enzyme-substrate complex and the solvent inaccessible region of a protein, 2) to determine the free energy difference due to preferential solvation and hydration, and 3) to re-examine theoretical issues in literature and relate them to the interpretation of the results.

Methods: A major theoretical research and minor experimentation using Bernfeld method.

Results and discussion: The presence of ethanol/aspirin alone yielded only dehydration of the osmolyte inaccessible region and the enzyme substrate complex (*ES*). There was positive free energy difference ($\Delta\Delta G$) if the equilibrium constant for hydration change ($K_{eq}(1)$)> the equilibrium constant for folding-unfolding transition ($K_{eq}(3)$); it is negative where $K_{eq}(3) > K_{eq}(1)$. Analysis of various models made them valuable for the interpretation

of result for feature application.

Conclusion

The change in the number of water molecules in an osmolyte inaccessible region of the enzyme and those interacting with the *ES* may be either positive or negative due respectively to sucrose and ethanol/aspirin. The spontaneity of two processes, hydration and folding-unfolding transition, the free energy difference, differs. The model for water stripping, preferential interaction concept, and the KBI for osmolation and hydration can guide the interpretation of the effects of any cosolute.

Keywords: Porcine pancreatic alpha amylase; change in Gibbs free energy; change in the number of water molecules; enzyme-substrate complex; osmolyte-inaccessible region of enzyme; cosolutes; Kirkwood-Buff Integrals(KBI).

1. INTRODUCTION

There are quite a lot of controversies surrounding the hydration of biomolecules. The hydration of biomolecules is not in doubt but the effect of such hydration on internal dynamics of the biomolecules is commonly of general interest to scientists [1]. However, this does not exclude intermolecular dynamics needed for contact with each other or with other solution components otherwise the needed contact for whatever transformation may not occur; hence the proposition that enzymes most diffuse towards the substrate to align itself with it to achieve a catalytic orientation [2]. This is notwithstanding current trend in the development of immobilised enzymes, from amylase family. It must however, be made clear that it is very impossible to digest polysaccharide without hydration of both substrate and enzyme. A lot of interest has been shown in immobilised enzymes [3] for different reasons. In those studies concern has been shown for the need for hydration, its purpose and effect on the kinetic and thermodynamic stability of the enzyme. But there seems not to be much interest on how the presence of drug such as aspirin and psychoactive compound such as ethanol may affect the role of all kinds of hydration of the enzyme.

Some enzymes are known to possess conserved water molecules as part of the structure of the enzyme's active site suggesting they play an important function in the active site stability, flexibility, ligand coordination and residue positioning, hence their guided evolutionary conservation [4]. Nuclear Magnetic resonance spectroscopy (NMR) analysis of the hydration process indicates that the onset of catalytic activity is a direct consequence of an increase in enzyme's (lysozyme's) conformational flexibility; it has been suggested that this increased flexibility may be due, in part, to the reduced interaction of charged and / or polar amino acid residues within the enzyme molecule caused by water's ability to effect dielectric screening [5]. Yet there is objection against total reliance on flexibility for function considering that an enzyme activity can occur at very low hydration levels, coupled with reduction in protein's flexibility; this according to [6] calls for a rethink regarding the dynamic requirement for an enzyme activity and stability.

In this research, the changes in the number of water molecules interacting with the enzyme via its enzyme-substrate complex due to the presence of the additives that appears not to feature very prominently in literature have become the concern of this research. In this regard the view by Damien is relevant. Citing other workers, Laage *et al* [7] posits that water strongly influences the structure and function of biomolecules within it. According to them [7] the most relevant interactions are hydrogen bonds, a mainly local type of weak bonding among water molecules which also exist between water and the polar or ionic groups of the biomolecule; this is apart from other long-range Coulomb forces between formally charged groups of the biomolecule. Other forces are hydrophobic forces; the latter is relevant for the aggregation of hydrophobic moieties; it can also enhance protein folding. It is known elsewhere [8] that hydrogen bonding occurs in binary mixtures of organic solutes such as ethanol and sucrose in this research. There could be altered dielectric property of the primary solvent, water that can influence changes in the conformational stability of the enzyme. It is obvious that the relevance of water is accomplished through various forms of

interaction that cannot preclude interaction energy and solution structure in the presence of additives in particular.

It should be realised that the presence of cosolvent or cosolute can alter the effect of aqueous solvent on the structure and function of the enzyme. The thermodynamic and activation parameters in terms of energy associated with *ES* may not remain the same in the presence of cosolvents, otherwise called osmolytes. The description of the interaction requires mathematical models that will be briefly addressed in theoretical section while a detailed qualitative aspect of theory is to be addressed in the discussion section as part of interpretational goal. The objectives of this research are 1) To assess the changes in the number of water molecules interacting with enzyme-substrate complex and solvent inaccessible region of a protein, 2) determine the free energy difference due to preferential solvation and hydration and 3) reexamine theoretical issues in literature and relate same to the interpretation of results.

2. THEORY

To begin with there is need to state that the major motivation of this section is the need to establish a justifiable theoretical background that can enhance the quality and perhaps, the validity and serve as a basis for the generation and possibly the interpretation of result. This section has two parts viz: The review of the derived equation related to difference in interaction free energy and the changes in the number of water molecules interacting with the enzyme substrate complex ([*ES*]); the second part is concerned with the change in the number of water molecules in osmolyte-inaccessible regions. The equation [9] adopted as in previous publication [10] in the quantitative determination of pair-wise solute-solute interaction parameter is as follows:

$$\ln[k_{(m_3)}/k_{(m_3=0)}] = \frac{2[g_{cx}-g_c^{\#}]m_3}{RTm_0^2} - N\varphi M_1 m_3 \qquad (1)$$

where $k_{(m_3)}$ is the (pseudo –) first – order rate constant in a reaction mixture containing co – solute whose concentration is m_3 and $K_{(m_3=0)}$ 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 1 mol/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_3 are the molar mass of water, practical osmotic coefficient for the aqueous solution, the number of water molecules, and the molarity of the added cosolute respectively [9]. The equation seems to represents another way of expressing preferential interaction, a thermodynamic phenomenon applicable to multi-component solution. In the original equation by Buurma *et al* [9],

$$RT\ln\left(k_{(m_3)}/k_{(m_3=0)}\right) = \Delta G(c)m_3 - N\varphi RTM_1m_3 \qquad (2)$$

Where, *R* is the universal gas constant.

Thus,

$$G(c)_{m_3} = \frac{2[g_{cx}-g_c^{\#}]m_3}{m_0^2} \qquad (3)$$

The most important function of the enzyme is the lowering of activation energy and free energy of activation. Enzyme – substrate cannot proceed to product without initial activation which however occurs at a lower energy cost. Previous research attempted to apply this concept of pair-wise solution component interaction to biological system such as enzyme catalysed reaction in the presence of cosolute [10]. Here a more straight forward approach is further adopted to achieve similar result. If assay is at very high enzyme concentration, and if the substrate is not soluble, and if the raw insoluble starch was the substrate as in this research, a situation that satisfies the condition for reverse quasi steady state approximation (rQSSA) [11], then the equilibrium dissociation constant of the substrate

from the complex given as $K_s = k_{-1}/k_1$ where k_{-1} and k_1 are the rate constant for the dissociation of enzyme-substrate complex (*ES*) and the 2nd order rate constant for the formation of the *ES* respectively, should be the case. But the concept is also applicable to a situation where the substrate concentration is very high such that $\frac{[E_0]}{K_M+[S_0]} \ll 1$ ($[E_0]$, K_M, and $[S_0]$) are the total concentration of enzyme, the Michaelis-Menten constant, and concentration of the substrate) as to satisfy the condition for standard QSSA (sQSSA) [11]. This takes the form $K_M = \frac{k_{-1}+k_2}{k_1}$ where k_2 is the rate constant for product formation and release. However, the key issue is that the rate constants for the dissociation of *ES* can be expressed respectively as

$$k_{-1} = K_s\, k_1 \quad (4)$$

$$k_{-1} = K_M\, k_1 - k_2 \quad (5)$$

In this research Eq. (4) unlike Eq. (5) does not present any issue because $\mathrm{In}[k_{-1(m_3)}/$

$$k_1 = -\frac{\partial \mathrm{In}([S](t)/[S]_0)}{\partial t[E_0]} \quad (6)$$

Where the concentration of enzyme ($[E_0]$) assayed is held constant or fixed while the concentration of the substrate in time $t = 0$ is $[S_0]$, and $[S](t)$ is the concentration of the substrate in time, t.

$$\mathrm{In}(k_{s(m_3=0)}/(k_{s(m_3)}) = (\Delta G m_3 - \Delta G_{-1} m_3)/RT + M m_3(\varphi_{-1} n_{-1} - n\varphi) \quad (7)$$

$$= \frac{\Delta\Delta G m_3}{RT} + M m_3 \varphi \Delta n \quad (8)$$

It is not in doubt that Michaelis-Menten (MM) constant is a sum of equilibrium constants. This is to say that it is given as $K_M = \frac{k_{-1}}{k_1} + \frac{k_2}{k_1}$; this implies that

$$S + E \rightleftharpoons ES \rightleftharpoons P + S_{FR} + E \quad (9)$$

The variable, S_{FR} as explained elsewhere [12], is the fragment of the polysaccharide left after a given catalytic cycle; no single polysaccharide is totally hydrolysed by an appropriate hydrolase.

The change in the number of water molecules in osmolyte-inaccessible regions, ΔN_w, is given by the slope of line relating $\ln(K_{eq})$ and the osmolyte concentration as follows [13].

$$\ln K_{eq} = -\frac{\Delta N_W [\text{Osmolal}]}{55.56} \quad (9b)$$

Leading to Eq. (9b) from the perspective of osmolyte-inaccessible regions is simply reaffirmation of the principle of preferential exclusion anchored on Kirkwood-Buff theory [KBT] of solution structure that has been popularised in recent papers [14, 15]. The theoretical interest arises from what appears to be a common ground for Eq. (2), Eq. (8), and Eq. (9) in that the number of water molecules for different purposes can be calculated from all equations, one from the slope (Eq. (9)) and the other from intercept of either Eq. (2) or Eq. (8). Equation (8) which arises from theoretical exposition of Buurma *et al* [8] represented the first time observation was made of the appearance of variable - concentration of cosolute - in two places as an independent variable in an equation.

Soluble polar organic substances called osmolytes may be excluded from the protein surface domain on account of their inability to penetrate protein's inner region. This issue is important in the light of the fact that solvent accessibility change plays a critical role in protein misfolding and aggregation, the culprit for several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) [16]. Furthermore, solvent accessibility may be part of the structural environment of amino acids in the protein that might influence the function-structural (mechanical) and catalytic in nature-of any of such amino acids [17]. Directly or indirectly, this important issues may have prompted research in this issue of

inaccessible core of the protein as exemplified in very recent research outcome which showed that the solvent-inaccessible cores of the three classes of proteins are equally densely packed [18]; this constitute steric hindrance to the penetration of relatively large organic osmolytes. This may have promoted excess flexibility that caused increasing velocity of hydrolysis with higher concentration of ethanol. One must not fail to point out that osmophobic concept [19] has been advanced as basis for the action that compels a protein to fold due to exclusion of such osmolyte which exist in nature from protein back bone.

On account of the issues raised in the text, there is need to recall that preferential osmolation, either negative or positive, can yield (re) folding and unfolding as the case may be leading to equilibrium state if a two-state model is assumed. Hence, the equilibrium constants (K_{eq}) defined mathematically and given below are of paramount relevance to a system in near dynamic equilibrium.

$$K_{\mathrm{eq}(3)} = \frac{[U]}{[N]} \tag{10}$$

Equation (10) is adapted from the work by Pace [20] which the author restate as $K_{eq(3)} = U/(1-U)$ where in this case U and $1-U$ denotes fraction of unfolded protein molecular population and fraction of folded protein respectively. Equation (10) or its alternative is expressible in two ways in accordance to whether or not the observed catalytic activity of the enzyme in the presence of osmolyte is greater than the same activity in the absence of the osmolyte. The equations which are applicable to the effects arising from the presence of an osmolyte are to be stated in method subsection. But there is also preferential hydration and dehydration due to preferential exclusion and binding of appropriate osmolyte respectively. This creates directional aqueous molecular motion to and from the protein's surface domain leading to an equilibrium system described by the second equation of equilibrium constant given elsewhere [21] as

$$K_{\mathrm{eq}(1)} = \exp\left(-\frac{\mathrm{In}a_1 C_1}{C_3}\Delta\Gamma_{23}\right) \tag{11}$$

Where $\Delta\Gamma_{23}$ is the change in preferential interaction by either binding or relative exclusion of an osmolyte; C_1 and C_3 are molar concentrations of water and osmolyte respectively; a_1 is the activity of water in aqueous solution of osmolyte. Meanwhile the equation of preferential interaction [15] is given as

$$\Delta\Gamma_{23} = \frac{\ln K_{eq(3)}}{\ln a_3} \quad (12)$$

The emergence of Eq. (12), as in previous publication (15), is as a result of the proposition that a parameter cannot be a devise-based measurable quantity (without definite or finite magnitude) as well as a constant quantity. A measurable quantity is an extensive thermodynamic parameter and, if a given ratio is always constant regardless of the magnitudes of the compared parameters, it becomes an intensive thermodynamic quantity. The report at the web site, en.Wikipedia.org (https//www.en.Wikipedia.org) shows that Richard C. Tolman was the author who first introduced the concept of extensive and intensive quantities.

3. MATERIALS AND METHODS

3.1 Material

As stated elsewhere[10], the materials are The chemicals used were: The chemicals used were: Sucrose (St Lious France); raw (native) 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 (EC 3.2.1.1) (Sigma, Adrich, USA); all other chemicals were of analytical grade and solutions were made in distilled water. Aspirin was purchased from CP Pharmaceuticals Ltd, Ash road North, Wrexham, LL 13 9UF, and U.K

3.2 Equipment

pH meter (tester) from Hanna Instruments, Mauritius; electronic weighing machine from Wensar Weighing Scale Ltd, Chennai; Centrifuge, 300D model from China; 721/722 visible spectrophotometer from Spectrum Instruments Co Ltd, China.

3.3 Method

As stated elsewhere [21], 0.01 g of PPA was dissolved in 20 mL of distilled water to give 500 μg/L while potato starch was prepared by dissolving 1 g in tris-HCl buffer (aq.) buffer (90 mL), 5 mL, 6% (W/W), NaCl (aq.) and 5 mL distilled water to give 1 g/100 mL. Approximate dilutions were carried out for the determination of K_M and v_{max} at 37^0C and pH of 7.4 by Lineweaver-Burk plot [22]. As in previous investigation [14] a method adopted for the determination of velocity (v) where $C_3 \rightarrow 0$ is a re-modification of the same equation found in literature [23]. It may appear theoretical but that is the essence of this research, a combination of a major theory and minor experimentation. Increasing v with increasing C_3, demands a plot of $\log v$ versus C_3 which gives an intercept, being an extrapolated velocity $(v_{C_{3\rightarrow 0}})$ of hydrolysis as $C_3 \rightarrow 0$.

$$\log v = \log v_{C_{3\rightarrow\infty}} - \beta[C_3]. \quad (13)$$

Equation (13) is for the increasing v, while for the decreasing case, it is given as

$$\log v = \log v_{C_{3\rightarrow\infty}} - \beta/[C_3]. \quad (14)$$

However, in order to obviate the effect of outliers, linear regression line was allowed to link the lowest point with the highest point for the determination of the minimum v as $C_3 \rightarrow 0$. Assay for the generation of velocities of the hydrolysis of starch is according to Bernfeld method [24].

The equation (Eq. (15)) below is adopted for the purpose of comparing the transition state energies of two different equilibrium systems dehydration/hydration and osmolation/exclusion equilibria due to the presence of osmolytes or cosolvents; it is therefore, restated as

$$\Delta\Delta G^{\#}_{\mathrm{ES}} = -RT\mathrm{In}\frac{K_{\mathrm{eq}(1)}}{K_{\mathrm{eq}(3)}} \quad (15)$$

The equilibrium constant $K_{eq(1)}$ is determined by substituting relevant parameters into Eq. (11); $K_{eq(2)}$ is determined by exploring either Eq. (16) or Eq. (17) below.

$$K_{eq(3)} = \frac{V_N - V_{OBS}}{V_{OBS} - V_{MIN}} \quad (16)$$

Where $V_N > V_{OBS} > V_{MIN}$ and the subscripts, N, OBS, MIN are respectively, catalytic activity of native enzyme, observed activity of treated enzyme and minimum activity of treated enzyme.

$$K_{eq(3)} = \frac{V_N - V_{MIN}}{V_{MAX} - V_N} \quad (17)$$

The issues that led to the emergence of Eq. (16) and Eq. (17) were addressed in part elsewhere [25]. Further details are currently in manuscript under preparation. Microsoft Excel (2007) was used to plot the dependent variable versus independent variable.

3.4 STATISTICAL ANALYSIS

The velocities of hydrolysis were determined in triplicates. The mean values were used to determine the equilibrium constant.

4. RESULTS AND DISCUSSION

Before analysis and discussion of results, there is a need to review Eq. (2) and Eq. (8). A careful examination of the equations reveals that the slope and intercept may not be positive or negative; the values depend on the magnitude of the ratio given as $K_{(m_3)}/K_{(m_3=0)}$. If $K_{(m_3=0)} < K_{(m_3)}$, with increasing values of $K_{(m_3)}$, the value of the ratio should be decreasing from smaller negative values to larger negative values, such that a plot of $\ln\left(K_{(m_3)}/K_{(m_3=0)}\right)$ versus m_3 should give a negative slope and definitely a negative intercept. It is also probable that $\frac{K_{(m_3)}}{K_{(m_3=0)}} > 1$ such that any plot may give a positive slope or correlation and either a positive intercept or intercept which is negative but much smaller in magnitude. Therefore, characteristics such as the magnitudes and signs of the slope and

intercept of a straight line from the plot express the type of change in the number of water molecules, which is either net hydration (positive) or net dehydration (negative). These account for the shapes of various curves shown in Figure 1 through 6. To show the direction of shift in the hydration process in the equilibrium, $E + S \rightleftharpoons ES$, a plot of Δn versus m_3 was carried out. The result (Fig. 1) shows that there was a decreasing trend in the change in the number of water molecules interacting with the ES. The observed trend is due to the effect of aspirin. The decreasing trend along the positive axis suggests that there was a decrease in hydration due to the effect of aspirin alone (Fig. 1). It is a loss-dehydration- the magnitude of which showed a decreasing trend, progressing towards net hydration (Fig. 2) due to the effect of the second cosolute, sucrose. In this case, the variation of the change in the number of water molecules with the molar concentrations of sucrose showed mixed trend. With a lower concentration of the cosolute (1.55 mmol/L and 0.73 mmol/L) – aspirin – there was an increasing trend unlike with higher concentration of the same cosolute, due perhaps, to the effect of the 2nd cosolute (sucrose) in the reaction mixture (Fig. 3).

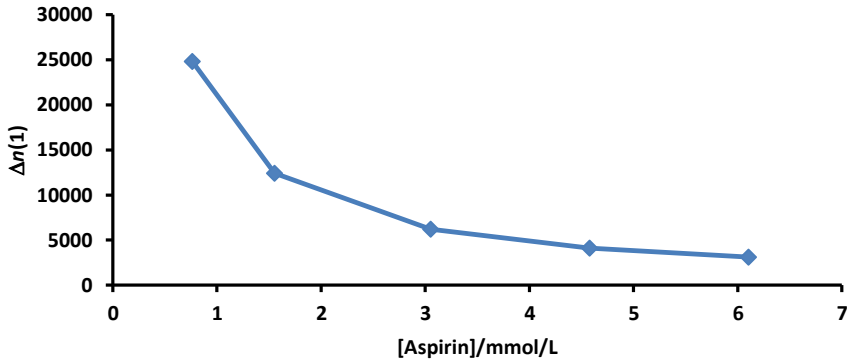

Fig. 1. **Variation of the change in the number of water molecules interacting with the enzyme- substrate complex with different concentration of aspirin.** *n is the number of water molecules.*

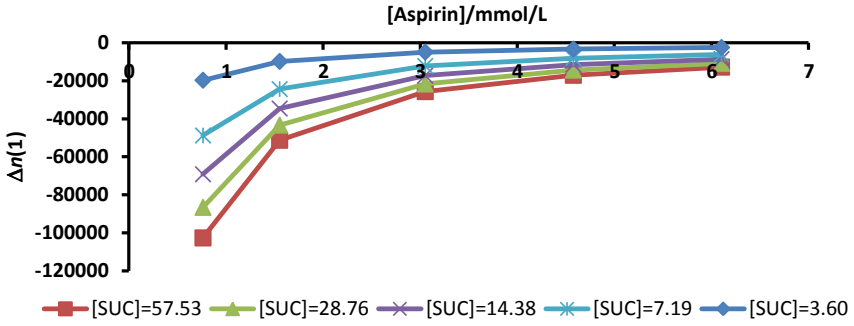

Fig. 2. **Variation of the change in the number of water molecules interacting with enzyme substrate complex with different [Aspirin] at different fixed concentration of sucrose whose concentration ranges between 3.60-57.53 mmol/L.** *n and SUC denote the number of water molecules and sucrose respectively.*

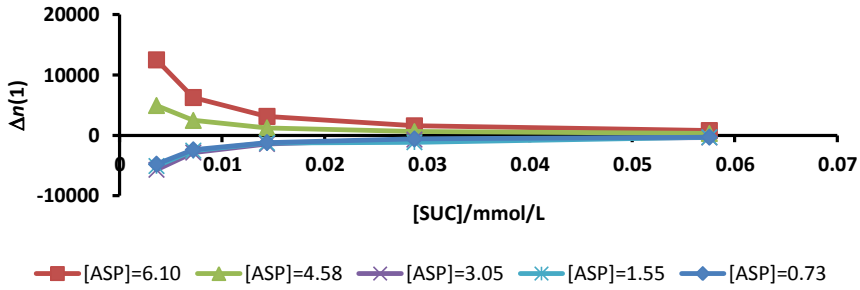

Fig. 3. ***Variation of the change in the number of water molecules interacting with the enzyme-substrate complex with different concentration of sucrose (SUC) at different fixed concentration of aspirin (mmol/L).*** *n~~(1)~~ and ASP denote the number of water molecules and aspirin respectively.*

Like the trend observed with the effect of aspirin there is also a decreasing trend in the positive values of Δn with increasing concentration of ethanol (Fig.4). Variation with different concentrations of ethanol exhibited similar trend observed for the variation of Δn with molar concentration of aspirin (Fig.5). With a mixture of ethanol and sucrose, there was, as was the case with the effect of a mixture of aspirin and sucrose, a mixed trend in the variation of Δn with molar concentrations of sucrose (Fig. 6). All these observation

notwithstanding, it is rather difficult to suggest why such observations cannot be mere coincidence taking into account the effect of high degree of improvisation in the conduct of the experiment. It is not an overemphasis to opine that ethanol is totally different from aspirin; while the former is essentially psychoactive, the latter is a well known non-steroidal anti-inflammatory drug [26, 27], and both have adverse effects on intestinal brush border membranes that could compromise the biological function of brush border membrane enzymes and transporters respectively.

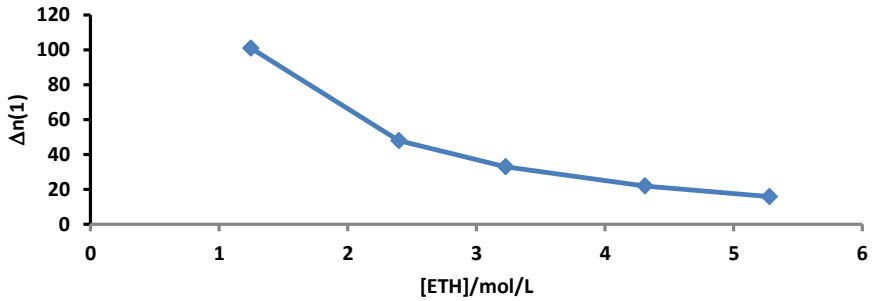

Fig. 4. ***Variation of the change in the number of water molecules interacting with enzyme substrate complex with different concentration of ethanol.*** *n~~(1)~~ and ETH denote the number of water molecules and ethanol respectively.*

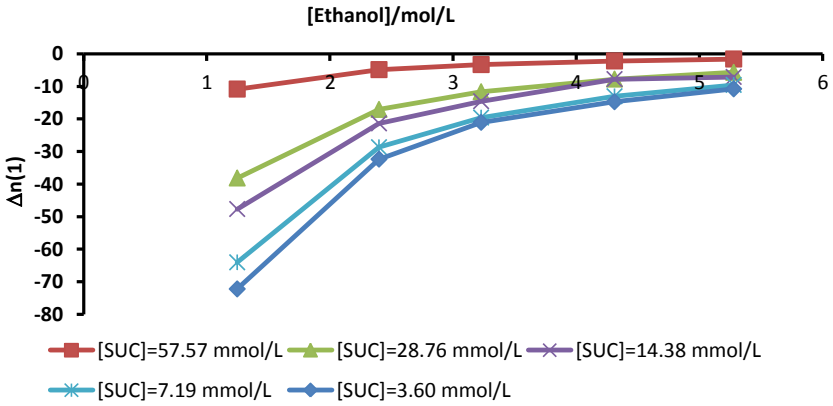

Fig. 5. Variation of the change in the number of water molecules interacting with enzyme- substrate complex with different ~~[Ethanol]~~ concentration of ethanol (mmol/L) at different fixed concentration of sucrose (mmol/L). ETH, *SUC and n~~(1)~~ denote ethanol, sucrose and number of water molecules respectively.*

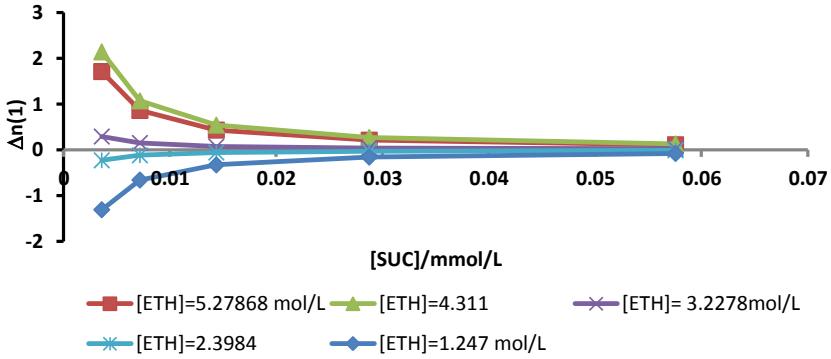

Fig. 6. Variation of the change in the number of water molecules interacting with enzyme-substrate complex with different ~~[Sucrose]~~ concentration of sucrose (mmol/L) at different fixed concentration of ethanol. SUC, *ETH and n~~(1)~~ denote sucrose, ethanol and number of water molecules respectively.*

Table 1. Change in the number of water molecules in osmolyte-inaccessible regions due to ethanol-sucrose mixture

[Ethanol]/(mol/L)		1.25		3.23	5.28	
ΔN_W		-601.67		615.050	3809.19	
r^2		0.82		0.53	0.77	
As function of ethanol concentration						
[Sucrose]/(mmol/L)	0.00	3.57	7.14	14.29	28.57	57.14
ΔN_W	-34.56	-50.06	-69.26	-36.00	-9.06	-21.11
r^2	0.86	1 (2dpts)	0.95	0.94	0.95	0.60

Changes in the number of water molecules (ΔN_W) are calculated as the product of slope and 55.56; the slope may be obtained from the plot of lnK_{eq} versus [cosolute] at a fixed concentration of the 2nd cosolute; dpts mean data points.

Comment [UW1]: Please indicate sucrose concentration

Comment [UW2]: Please indicate ethanol concentration

The change in the number of water molecules (ΔN_W) on osmolyte inaccessible region as a function of sucrose concentration is similar to the exclusion of aqueous solvent or dehydration with lower concentration of ethanol unlike with higher concentration ethanol in which there was hydration (Table 1). But as function of the concentration of ethanol, there was irregular trend couple with a case of dehydration similar to result obtained in only ethanol treated enzyme (sucrose concentration = 0) (Table 1). This is not unexpected considering ethanol as a fluidising and water-stripping agent.

Table 2. Change in the number of water molecules in osmolyte-inaccessible regions due to aspirin-sucrose mixture

[Aspirin]/(mol/L)	0.76	3.05	6.10
ΔN_W	2042.86	4380.01	6261.61
r^2	0.98	0.97	1 (2dpts)

As function of aspirin concentration

[Sucrose]/(mmol/L)	0.00	7.19	14.38	28.76	57.75
ΔN_W/exp(3)	-40.63	-80.84	-83.90	-52.85	-44.93
r^2	0.87	1(2dpts)	1(2dpts)	0.99	0.98

Changes in the number of water molecules (ΔN_W) are calculated as the product of slope and 55.56; the slope may be obtained from the plot of lnK_{eq} versus [cosolute] at a fixed concentration of the 2nd cosolute; dpts mean data points.

The change in the number of water molecules as a function of sucrose concentration showed increasing trend unlike such change as a function of aspirin concentration in which there was irregular trend and negative in sign as to imply dehydration (Table 2) similar to the result obtained due to the effect of aspirin alone. This may imply that aspirin like ethanol has water-stripping properties.

The difference in free energies between two thermodynamic processes dehydration/hydration and osmolation/exclusion arising from the effect of cosolutes and water are recorded in Tables (3a), (3b), (4a), and (4b). There is need to state that the data

Comment [UW3]: Please indicate sucrose concentration

Comment [UW4]: Please indicate aspirin concentration

generated is not an outcome of high precision measurement as a result of improvisation. This leaves room for further research using state-of-the-act facilities while the current data remains purely illustrative of the fact and principle enunciated in this research. Usually, a spontaneous process is one in which the free energy is relatively large and negative in sign. The effect of ethanol and aspirin separately alone, yielded a mixed result of negative and positive free energies as shown in Tables (3a) and (4a) respectively. The negative difference in free energy occurred with higher concentration of the cosolutes. With a mixture of ethanol and sucrose (Table 3b) and a mixture of aspirin and sucrose (Table 4b), the negative values occurred with higher concentration of ethanol and aspirin. What one can deduce is that positive $\Delta\Delta G$ occurs if $K_{eq}(1) > K_{eq}(3)$ and as such (de) hydration is more spontaneous. On the other hand if $K_{eq}(1) < K_{eq}(3)$, a negative $\Delta\Delta G$ may be given with the result that, osmolation/exclusion is more spontaneous.

Table 3a. Difference in free energies between dehydration/hydration and osmolation/exclusion with only ethanol.

[Ethanol]/mol/L	1.247	2.398	3.228	4.311	5.279
$\Delta\Delta G$ /kJ/mol	18.680	0.960	-0.380	-0.360	-0.280

$\Delta\Delta G$ *is the difference in free energy*.

Table 3b. Difference in free energies between (de)hydration and (de) osmolation with a mixture of ethanol and sucrose

	[Sucrose]/mmol/L				
	3.57	7.14	14.29	28.57	57.14
[Ethanol]/mol/L	$\Delta\Delta G$ /kJ/mol				
1.247	3.53	4.22	2.43	2.42	1.81
3.228	-1.92	-0.49	0.12	0.28	0.64
5.279	-	-11.35	-4.84	-0.95	4.44

$\Delta\Delta G$ *is the difference in free energy*.

Table 4a. Difference in free energies between dehydration/dehydration and osmolation/exclusion with only aspirin.

[Aspirin]/mmol/L	0.76	1.53	3.05	4.58	6.10
$\Delta\Delta G$ /kJ/mol	2.63	2.49	1.84	– 2.85	– 9.55

$\Delta\Delta G$ *is the difference in free energy*.

Table 4b. Difference in free energies between dehydration/hydration and osmolation/exclusion with a mixture of aspirin and sucrose

	[Sucrose]/mmol/L			
	7.19	14.38	28.57	57.14
	$\Delta\Delta G$			
[Aspirin]/mmol/L				
0.76	6.03	7.39	9.11	980
3.05	– 4.29	– 3.61	0.74	1.49
6.10	–	–	– 7.76	– 5.50

$\Delta\Delta G$ *is the difference in free energy*.

The results obtained so far are significant because of the biological role of water, and, there are a lot of theoretical basis for them. Such theoretical foundation or basis broadens the scope for further research. Beginning from what is known is the fact that proteins are strongly hydrated in aqueous medium. The density of water molecules close to

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the protein surface due to effect of polar and non-polar groups is as high as 1.25 g/mL within 3-4.25 Á of protein surface, mainly as result of large number of water molecules that are 3.75 Á from non-polar atoms; within 2.5 Á of the protein surface there is a small increase in density of water molecules due to electrostriction around the polar groups; but 3-4.5 Á from the surface, there is a slight decrease in density [28]. Water molecules are clustered perpendicular to the protein surface while in the parallel direction to the protein surface the water molecules are more disperse [28]. This means that given suitable pH, an enzyme exhibits a level of hydration needed for function. For a particular group, the fraction of time when a water protein hydrogen bond is formed otherwise called hydrogen bond probability (P_{hb}) is strongly dependent on protein accessible surface area (ASA). The lower the latter, the higher the entropic barrier (cost) that should be paid to significantly reduce the flux of water molecules on the protein surface hydration site where H-bond is expected [29]. In the same vein, Ooi & Oobatake [30] also posited that each atomic group interacts with water in proportion to its water-ASA. The effect of the presence of chaotropes is of major concern as it has been observed that more polar organic solvents (tetrahydrofuran and acetonitrile) replace mobile and weakly bound water molecules in the active site and leave primarily the tightly bound water in that region [31].

In this research, aspirin and ethanol decreased the velocities of amylolysis of raw potato starch. These velocities under the influence of aspirin and ethanol are respectively 21-74 U/mL and 38 – 61 U/mL, all being < the velocity of amylolysis (97 U/mL) by the untreated enzyme. This means that the entropic cost of fixing water of hydration increased as a consequence. This can be elucidated via the equation made popular by Petukhov [29]. The equation is

$$\Delta G_{\mathrm{hb}} = -RT\ \mathrm{In}\frac{P_{\mathrm{hb}}}{1-P_{\mathrm{hb}}} \qquad (18)$$

Equation (18) suggests that as $P_{hb} \rightarrow 1$ the value of the free energy of hydration tends toward higher negative magnitude as an expression of feasibility or spontaneity of hydration.

$$P_{\mathrm{hb}} = \frac{\exp\left(-\frac{\Delta G_{\mathrm{hb}}}{RT}\right)}{1+\exp\left(-\frac{\Delta G_{\mathrm{hb}}}{RT}\right)} \quad (19)$$

Hence if $P_{hb} \rightarrow 1$ (or if it's equivalent 100 $P_{hb} \rightarrow 100\%$), the entropic cost should tend to minimum. It is important to point out that the value of P_{hb} may be a function of the fraction of water population that can form H-bond with 4 water molecules and 3 water molecules both of which are a function of the prevailing temperature in a equation given according to Petukhov [29] as

$$P_{hb} = 100(4X_1+3X_2)/4 \quad (20)$$

Where X_1 and X_2 are respectively the fraction of water that can H-bond with 4 and 3 molecules of water. As this research shows, the presence of sucrose seems to have opposed the effect of aspirin and ethanol. As such it is expected that P_{hb} may have increased as a result of the effect of sucrose.

The role of water, or the effect of hydration, has its theoretical foundation that enhances the interpretation of results. It has been reported that "the hydration environment of a protein significantly affects its dynamics. This is why changes in the number of water in cosolvent inaccessible site of the protein have become very important because such can affect enzyme function. A positive change indicates that there may have been hydration and negative change means the opposite. Such changes may not have been possible if there was no initial hydration and preferential interaction with molecules of water. Although the method adopted by Mitchell and Litman [13] and Buurma [9] are different they have a common ground for addressing the issue of hydration changes. This is the case because osmolyte inaccessible region of the protein may accommodate the active site. The active site is either located within the protein's inner part or at locations close to the surface domain of the enzyme. Hence changes in the number of water molecules in an osmolyte inaccessible region cannot totally exclude the active site. This is the case, if one recalls that ES complex

is the result of complex formation between substrate and active site of the enzyme. Therefore, there could be changes in the number of water molecules interacting with ES.

In literature, following the application of osmotic stress, is the observation that protein-DNA complex can be hydrated with measurable volume changes. The sign of the changes of the number of water molecules interacting with the protein and ES as well as osmolyte inaccessible region indicates the occurrence of either hydration or dehydration. As in this research such change occurs when a cosolute is introduced into the medium. It has been observed that the catalytic activity of lyophilised oxidative enzyme was lower when directly suspended in organic solvents containing little water than when they are introduced into the same largely nonaqueous media by first dissolving them in water and then diluting with anhydrous solvents [32]. Despite the need for water for maximum catalytic activity of enzymes, an obvious paradox exists to the effect that, some enzymes (substilisin and alpha-chymotrypsin) showed a 100 billion-fold enhancement in nonpolar solvent like octane with just an amount of water much less than needed to form a monolayer [33]. This is attributed to an increase in the kinetic barrier (activation energy) needed to be overcome in order to transform from native to unfolded conformation [33]. This should not be surprising because unlike polar solvents, *e.g*. ethanol and polar solute, *e.g*. aspirin in this research, that have water-stripping power, octane does not being hydrophobic.

It is quite obvious that infinitesimal amount of water is needed to trigger catalytic action as to imply that water may be described as a *prima facie* example of an inorganic catalyst. Highly structured water molecules are needed around the protein surface as part of efficient chemistry of the protein by which they promote the protein's three dimensional (3-D) structures [34]. According to Csermely [35], water molecules within the region of perturbative influence of the enzyme provides the environment by which fluctuating changes in hydrogen bond could occur as a necessary requirement for protein flexibility, structural rearrangements leading to conformational transitions needed for catalytic functions. This very much in agreement with the observed hydration induced conformation and dynamic

changes which are completed just before the onset of enzyme biological function [36]. It goes to confirm that an increased rigidity in the protein at low hydration can be reversed when water is added to the dry enzyme leading to a “loosening up” or increase in flexibility [36]. Protein flexibility means inter-domain and catalytic site mobility made possible waters of hydration. The deduction one can make is that polar solvent like ethanol as in this research displaces the weakly bound structural water molecules and preferential water of hydration leading to alteration and distortion in the catalytic conformational transition needed for function that culminate in lower velocity of amylolysis.

However, the hydration effects are strongly dependent on both temperature and hydration. At cryogenic temperatures, hydration stiffens protein structure because of the hydrogen-bond interaction, whereas at physiological temperatures, hydration softens the structure through the activation of anharmonic motion”[1]. The hydration water dynamics and their dynamical coupling with the protein are presumed to be essential for protein dynamics and biological function [1]. The protein dynamics in question is actually intra-molecular dynamics needed for conformational flexibility for function. According to Chaplin (www1.lsbu.ac.uk), proteins are characterized by conformational flexibility, which entails a wide range of hydration states, in a state of dynamic equilibrium, facilitated by the ease of hydration. The ease of hydration is dependent on the activity of the surrounding water molecules. The enzymatic function of the enzyme is dependent on the position of the equilibrium, es⇌cs (where es and cs mean the expanded state and compact state of water respectively) around the protein; the es is also called the Ih-type with lower density-the low density water (LDW) while cs is called Il-type with higher density-the high density water (HDW). The LDW and HDW are respectively more ordered and less ordered. Thus an intermediate mixture of nonionic kosmotropes and nonionic chaotropes such as sucrose and aspirin/ethanol respectively as in this research can enhance biological activity of the

enzyme: It is neither an excessive rigidity nor an over flexibility of the structure of the protein that can enhance the function of an enzyme.

The effect of a mixture of protecting and destabilising cosolute as observed in this research has its theoretical foundation. Beginning with aspirin and ethanol, the theory is described as preferential interaction by osmolation or by binding and by exclusion. The former, according to Timasheff [37], leads to water stripping according to the equation:

$$E.nH_2O + L \rightleftharpoons P.L + nH_2O \quad (21)$$

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Where, L is the ligand otherwise called cosolute. Citing his previous paper, Timasheff [36] posits that "the reference state is the protein dissolved in water, in which it is fully hydrated. Therefore, in a binary solvent, the binding of the nonaqueous solvent component to any locus must displace water, *i.e.*, binding is an exchange reaction" [37]. Unfortunately there is no equation for exclusion of ligand as at this moment. Nonetheless, the following equation may serve this purpose.

$$E.nH_2O \rightleftharpoons E.(n-\beta)H_2O + \beta H_2O \quad (22a)$$

Equation (22) (which reflects only the expulsion of water) symbolically shows that when an aqueous solution of a protein is introduced into a stabilising cosolute, the macromolecule will not be totally free from the molecules of the cosolute. Hence β is the small portion of L (stabilising osmolyte in this case) that binds while $L-\beta$ is the vast amount of L that is excluded given that β is equal to the amount of water displaced. This could have given rise to

$$L + E.nH_2O \rightleftharpoons E.(n-\beta)H_2O.\beta + \beta H_2O + L-\beta. \quad (22b)$$

However, one must not overlook the effect of osmotic stress that might be created when any cosolute is excluded leaving the surrounding of the protein more concentrated as to create concentration gradient. This can compel loosely bound water molecules to depart the protein

into the bulk; this may also be interpreted as a translational entropy gain of the aqueous solvent that drives re-folding [38]. This is in agreement with the view that osmotic pressure controls the activity of water in an aqueous compartment inaccessible to neutral solutes (osmolytes). The osmotic stress created then induces the release of bound water from macromolecules into bulk solvent. Macromolecular conformations are thus shifted toward the state with the smallest volume, which is the state with the least amount of bound water [39, 40]. The folded state promoted by stabilisers such as sucrose in this research has smaller volume. This is another evidence of the importance of hydration, be it water of hydration or water of preferential hydration. Osmotic stress will always occur when there is the presence of a stabiliser in particular.

Further evidence is the observation about individual, internal water molecules that may be reactants in a catalyzed reaction and/or may be integral parts of a protein structure, providing stereospecific interactions; the correlation between hydration and increased activity means that it is likely that the observed hydration-induced dynamical changes may facilitate activity [6]. However, the presence of amylolytic activities, in the absence of intra molecular motion, indicates that the motions are not an absolute requirement; this seems to imply that if enzyme activity can occur at very low hydration levels, and if at these levels protein flexibility is reduced, then the dynamic requirement for enzyme activity and stability may be questionable [6]. Here one must strongly disagree on account of this research outcome and on the basis of common sense. The lower amylolytic activity of ethanol/aspirin treated-enzyme suggest that the structural water within the protein core and active site in particular may be weakly intact, but other catalytically supportive mobile water molecules may have been stripped off. In other words the ubiquitous surface hydration shell has influence on protein dynamics and function such that if adversely affected by the chaotropes [6], a reduction in amylolytic activity becomes inevitable. This clearly explains the decrease in the velocity of amylolysis for ethanol/aspirin treated enzyme. Scholars who are only interested in balanced diet may consume the usual diet thrice without water for two days but

with implication of being inflicted with indigestion and constipation despite the fact that ab initio, the gastrointestinal tract is not dry. No matter the length of time no reaction can be noticed in a dry mixture of enzyme and its substrate.

This can be accounted for in terms of Kirkwood-Buff theory (KBT) of solution structure which states that the average structure of all solutions [41] is given by radial distribution function $(g_{\alpha\beta}(r))$ between two species, namely, α and β. The term, radial distribution function, is a measure of the deviation from the random distribution of particles of type β from a central particle of type α as a function of the distance (r) from the central particle [41]. A positive or negative deviation of $g_{\alpha\beta}(r)$ (also known as pair correlation function) from unity, at a certain distance corresponds to excess or deficit of β at the indicated distance from the particle designated as α. The issue remains the combined effect of aspirin/ethanol and sucrose.

According to Bolen and Baskakov [42], the osmophobic effect of osmolyte is a vital property that is beneficial to life, being the capacity for an unfavourable interaction between the osmolyte/cosolute and peptide backbone. In the same vein, Baskakov and Bolen [43] opined that the osmophobic effect of stabilisers on the peptide back bone made the unfolded state of protein in osmolyte solution very unfavourable relative to the folded state; therefore, it was the strongly destabilising effect of stabilisers such as sucrose on the unfolded state as in this research, that forces the enzyme to refold. From the perspective of thermodynamic stability, Bolen and Baskakov [42], see solvophobic action which Schellman [44] and Rösgen *et al* [41] called excluded volume action, as a factor which raised the free energy of the denatured state, shifting the equilibrium in favour of the native state. In this research sucrose is a well known stabiliser which acts by preferential exclusion. On account of this sucrose was able to enhance the amylolytic velocities of sucrose treated-enzyme in a reaction mixture containing aspirin (3.052 mmol/L) and ethanol (3.228 mol/L): The velocities ranges from 132-140 and 116-136 U/mL respectively. These values were higher than values

obtained for the untreated native enzyme (97 U/mL), only ethanol-treated (102 U/mL) and only aspirin treated (69 U/mL) enzyme.

The phenomena of solvophobic and solvophilic effect are the root cause of the change of biological function-either an increase or decrease in the velocity of catalytic action for instance as noted in this research. Osmolytes (as cosolvents/cosolute) may be solvophobic or solvophilic (preferential exclusion or osmolation *i.e*. preferential interaction by binding) which causes respectively refolding and unfolding; this presupposes changes in the volume or 3-D structure of the macromolecule. This needs interpretational analysis based on what Rösgen *et al* called inverse KBT. It is usually a context between solvation and hydration change expressed via the KB integrals (KBIs). From the point of view of preferential hydration integral, the following equation is inevitable. The partial molar volume of the protein is in contention. Thus, the change in G_{pw} due to folding to unfolding transition is given as

$$\Delta_N^D(G_{pw}) = -\Delta_N^D V_{prot} + \frac{m\phi_{os}}{RT} \qquad (23)$$

Where $\Delta_N^D V_{prot}$ is the partial molar volume of the protein, ϕ_{os} is the volume fraction of the osmolyte, G_{pw} is the KBI for hydration, m is the short form of m-value, the capacity of osmolyte to cause conformational change and Δ_N^D means folded to unfolded transition. For the ideal case as may be applicable to dilute solution of sucrose, positive m-value for the protecting osmolyte, should be such that $\Delta_N^D(G_{pw})$ may be positive in sign as to imply an increase in the number of water molecules around the protein. This view is premised on the fact that the (re)folded state has smaller hydrodynamic radius than the unfolded which is also more hydrated [40]. The implication of this premise is that $\Delta_N^D V_{prot}$ being small, implies that it's negative magnitude $(-\Delta_N^D V_{prot})$ may be small. The outcome is that the right hand side (RHS) may be large and positive. It must be made clear that $\Delta_N^D(G_{pw})$ needs to be

649 determined but it remains outside the scope of this research. This view explains the effect of
650 sucrose which promotes initial preferential hydration of the enzyme before other
651 physicochemical events such osmotic stress effect due to concentration gradient created by
652 the excluded osmolyte.

653 The equation for the osmolation case is given as

654 $$\Delta_{\mathrm{N}}^{\mathrm{D}}\left(G_{\mathrm{po}}\right) = -\Delta_{\mathrm{N}}^{\mathrm{D}}V_{\mathrm{prot}} - \frac{m(1-\phi_{\mathrm{os}})}{RT} \quad (24)$$

655 In this research, aspirin was noticed to have reduced the velocity of amylolysis as to imply
656 that the enzyme was destabilised. In this case, the m-value may be large and negative;
657 there may be a positive value of $\Delta_{\mathrm{N}}^{\mathrm{D}}\left(G_{\mathrm{po}}\right)$. This is to say the number of water molecules
658 around the protein decreases while there is a relative excess of the osmolyte around the
659 protein surface domain including some molecule that may have penetrated into the protein
660 3-D structure.

661 A very important deduction one can make is that hydration and dehydration are
662 merely precedent to the initial events, preferential exclusion and binding respectively.
663 Otherwise, upon unfolding due to osmolation, maximum hydration may occur as expected
664 for the unfolded protein [41]; this is clearly the case because if there is excess of the
665 osmolyte on the protein surface, osmotic gradient should be created towards the protein.
666 Diffusion of water towards the unfolded should occur, making available enough water
667 molecules for the hydration of exposed polar groups. This is without prejudice to the initial
668 displacement of weakly bound water by the binding of the osmolyte. On the other hand
669 translational entropy gain of departing water from hitherto hydrated protein due to excluded
670 osmolyte compels the protein to (re)fold. As presented in literature [41] the first order case
671 which seem to be applicable to highly concentrated osmolyte/cosolute, requires the
672 introduction of apparent hydrated molar volume of the former as follows: Equation (23), by
673 so doing, is transformed to

$$\Delta_{\mathrm{N}}^{\mathrm{D}}\left(G_{\mathrm{pw}}\right) = -\Delta_{\mathrm{N}}^{\mathrm{D}}V_{\mathrm{prot}} + \frac{m(1-C_3V_1)\phi_{\mathrm{os}}}{RT} \quad (25)$$

Equation (25) enables the determination of the integral for hydration at none destabilising concentration of the stabilising osmolyte as long as $1>C_3V_1$ and $V_1«1$. This equation is reserved for feature investigation in which the concentration range of sucrose may be 0.25 - 1.25 mol/L. For the purpose of discussion Eq. (25) reminds one of the high molar concentrations of ethanol explored in this research whose effect requires another equation slightly different from Eq. (25). The equation is

$$\Delta_{\mathrm{N}}^{\mathrm{D}}\left(G_{\mathrm{po}}\right) = -\Delta_{\mathrm{N}}^{\mathrm{D}}V_{\mathrm{prot}} - \frac{m(1-\phi_{\mathrm{os}})(1-C_3V_1)}{RT} \quad (26)$$

Since ϕ_{os} is the volume fraction of cosolvent (or rather mole fraction which covers non-solvent and solvents, *e.g*. sucrose and ethanol respectively), and its value being < 1 means that $1 - \phi_{\mathrm{os}}$ is always > 0. The implication is that for the osmolation (positive preferential interaction parameter) case $1–C_3V_1$ should also be > 0. This explains the osmolation (and its effect) whereby $\Delta_{\mathrm{N}}^{\mathrm{D}}\left(G_{\mathrm{po}}\right)$ needs to be positive due to the binding of ethanol alone and only aspirin in separate assays. Osmolation leads to unfolding and consequently, a decrease in the amylolytic action of the enzyme as observed. The question that needs to be answered is, what means can be applied for the determination of V_1? The issue of interest is always the hydration changes linked either to the ES or cosolvent inaccessible region of the protein. This is despite objection against total reliance on flexibility for function considering that some enzyme activity can occur at very low hydration levels, coupled with a reduction in protein's flexibility. On the contrary, Poole [36] observed that hydration induced conformation and dynamic changes are completed just before the onset of enzyme activity which occurs before all polar groups are hydrated. There was confirmatory evidence via increased alpha – helicity that leads to increased rigidity in the protein at low hydration (dry); this led to the deduction that when water is added to the dry enzyme a "loosening up" or increase in

flexibility occurs around a threshold of hydration [36]. It appears therefore, that it is an excessive flexibility that leads to total unfolding due to the action of destabilisers that reduces the biological function of the protein as observed in this research.

In summary there may be changes in the number of water interacting with *ES*, be it negative or positive. The change in the number of water molecules interacting with the *ES* as a function of ethanol/aspirin concentration indicates dehydration more so with a lower concentration of sucrose. Thus ethanol is destabilising. The change as a function of sucrose concentration with different concentration of ethanol/aspirin shows mixed trend, increasing hydration with lower fixed concentration of ethanol/aspirin and decreasing with higher fixed concentration of ethanol/aspirin. Thus sucrose promotes hydration being a protecting osmolyte.

Generally, the change in the number of water molecules (ΔN_W) in an osmolyte inaccessible region of the enzyme as a function of sucrose concentration with different fixed concentration of aspirin/ethanol is positive as to imply hydration. Perhaps, the increasing solubility of raw starch in increasing concentration of ethanol may presumably account for the negative ΔN_W with lower concentration of ethanol. The values of ΔN_W as a function of aspirin/ethanol concentration with different fixed concentration of sucrose are negative as to imply dehydration peculiar to osmolation by destabilising cosolute.

The spontaneity of the processes, folding to unfolding transition and accompanying hydration changes, has been illustrated with the quantification of the free energy difference; in line with the approach, the results shows that *ab initio* the equilibrium constant for hydration change ($K_{eq}(1)$) may be < or > equilibrium constant ($K_{eq}(3)$) for folding to unfolding transition. A positive free energy difference means that hydration change is more spontaneous than folding transition which may be attributable to the effect of sucrose. The converse is the case with ethanol/aspirin in which the free energy difference is negative ($K_{eq}(3) > K_{eq}(1)$).

For the purpose of interpretation, theories in literature were adopted for the elucidation of results. The model for water stripping effect of aspirin/ethanol, preferential interaction concept and the KBT for KBI for osmolation and hydration guided the interpretation of the root basis of the effects of the cosolutes.

5. CONCLUSION

The change in the number of water molecules in an osmolyte inaccessible region of the enzyme and those interacting with the *ES* may be either positive or negative due respectively to sucrose and ethanol/aspirin. The spontaneity of two processes, hydration and folding-unfolding transition, the free energy difference, differs. The mathematical model for water stripping, preferential interaction concept, and the KBI for osmolation and hydration can guide the interpretation of the effects of any cosolute.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

There is no competing interest.

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