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Effect of Salts and Organic Osmolytes is Due to Conservative Forces

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

Background: The impression given in the literature was that the net interaction potential energy, *V* is the difference between Coulombic-Coulombic and total weaker interaction energies. It is proposed however, that in aqueous solution all particles with full formal charges and partial charge (dipoles) contribute to the total interaction as applicable to conservative field but not to the exclusion of hydrophobic interaction if applicable.
Objectives: The objectives are 1) To theoretically elucidate the basis of the intermolecular interaction, 2) To show that effectiveness of an osmolyte which may include inorganic ion to force macromolecular, (un)folding, the *m*-value, is a function of the implicit mobility (or translational velocity) of the cosolute, 3) To link the *m*-value to conservative forces (or potential energies, *V*) and 4) Quantitate the values of *V*.
Methods: A major theoretical investigation and experimentation using Bernfeld method.
Results and Discussion: There were higher velocities of amylolysis with salt than without it in the presence of ethanol. The magnitude of the calculated *V* and energy equivalent of the entropic term were higher with higher concentration of ethanol unlike was the case with graphically determined values which were generally higher in magnitude than calculated values. The values of the calculated *V* and intermolecular distance were respectively higher in magnitude and longer with higher concentration of the salt.

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Conclusion: The attractive interaction between a macromolecule and a cationic counter ion is due to long ranged ion-ion interaction which ultimately enhances the effect of short ranged interaction. Higher salt concentration promotes long ranged interaction. The translational velocity of the solvent and cosolute has a role in the quantification of intermolecular distance. A mathematical relationship exists between *m*-value and $-V$ (or 2 *K.E.*). The values of *V* can be calculated based on the derived equations.

Keywords: Interaction potential energies; conservative field forces; m-values; porcine pancreatic alpha-amylase; ethanol; calcium chloride.

1. INTRODUCTION

The term osmolytes had been of remarkable interest to scientist in the past and in contemporary time. The interactions, binding mainly and exclusion are of interest. Thermodynamics of interaction attracts the interest of researchers [1]. But the issue of conservative forces which bring particles closer or set them apart in a way that alters protein structure seemed not to attract as much interest. Interaction potentials are combined with Coulombic force or its cognate energy in way that gave the impression that it does not include potential energy component as expected in conservative field. There are two types of osmolytes which are mainly organic and inorganic in nature. The organic osmolytes have been widely studied [2]. There is also a current shift towards the study of inorganic cations and anions due to the known effects of the ions at low and high concentrations [3]. The issue is the salting – in and salting – out effect of the salt at suitable concentration. These phenomena are encountered whenever separation or purification of macromolecules, proteins in particular, is of interest. Scholars had resulted to an age-long concept known as Hofmeister series. This is the ordering of ions according to their propensity to alter the hydrogen bonding network of water [3-5]. The presence of excess salt in the soil can affect water availability to the plants. This may have prompted interest in sodic soils [6]. Calcium ion is a constituent of bone and teeth, and a cofactor of some protein such as pancreatic and salivary alpha-amylase [7]. Apart from intrinsic calcium in some proteins, the mesophilic and thermophilic amylases and other calcium dependent enzymes were studied with the presence of the salt in the reaction mixtures at much higher temperature for the study of the stability of the enzyme [8-10]. The concern was that there may be a mechanism by which ions altered the stability of macromolecules in solution; these ions have either been described as kosmotropes or chaotropes which possess different ways of action [5]. The concern that had not been explicitly expressed and investigated theoretically and experimentally as much as had been done with respect to the known effects of the salt is the possible role of electrostatics of various kinds, as may be applicable, that can precipitate the interaction between inorganic ions and the macromolecules in solution under normal temperature and outside normal temperature range. Here the issue of conservative forces becomes very important. Although interactions between different proteins may have been described in the literature [11], interaction can also occur between the same macromolecules. Examples are interaction between the same proteins, interaction between substrate molecules (e. g. polysaccharide), *etc*, leading to what have been referred to as solvation and self solvation as the case may be [2,12]. The presence of osmolytes, inorganic and organic can alter the extent and strength of the different interaction. Thus the objectives of this research are 1) To theoretically elucidate the basis of the intermolecular interaction, 2) To show that effectiveness of an osmolyte which may include inorganic ion to force macromolecular, (un)folding the *m*-value, is a function of the implicit mobility (or translational velocity) of the cosolute, 3) To link the *m*-value to conservative forces (or potential energies, *V*), and 4) Quantitate the values of *V*.

2. THEORY

This key section offers a theoretical background for the mechanism of action of inorganic ions on the stability of biomolecules. Ion-ion interaction in which the ions in aqueous solution possess full charge is subjected to quantifying equation in which the principle of conservative forces or the cognate potential energy is ignored. The kinetic and potential energy components were ignored as it is the case in the literature [11]. A cation with charge number, *q* equal to $n+$ is equivalent to a net number of positron (positive electron) equal to *n* ($+e$) where $+e$ denotes well known

positron, while an anion with *q* equal to *m* – may be equivalent to a net number of electrons equal to *m* (– e) where – e denotes negative electron; *n* may or may not be equal to *m*. At infinite dilution the electrostatic potential energy and the electrostatic kinetic energy are equal to 0. Only the thermal energy ($k_B\theta$ where k_B and θ are the Boltzmann constant and thermodynamic temperature respectively) dominate at infinite dilution. This immediate issue will be revisited and applied subsequently.

The departure of kosmotropes from the surface domain of the protein increases the water activity around the protein; this may constitute hydration otherwise there is no way "reverse diffusion" the movement of water from bulk with higher concentration of kosmotrope to the protein surface domain with higher water activity can occur". Therefore, at some low concentration of the kosmotrope which is excluded, the concentration of the protein may be such that has its water activity equal to that in the vicinity of kosmotrope. But at very much higher concentration of the latter its water activity may become much less than water activity round the protein. The consequence is that an osmotic gradient is created leading to movement of water from the surface domain of the protein to the region containing the kosmotrope. This amount to significant dehydration reducing the tendency by the protein to form binding interaction with water molecules that culminates in precipitation – salting out (a protein binding association due to substantial absence of hydrogen bonding between water of hydration and bulk water).

"On the other hand, chaotropes ('water structure breakers') are known to destabilise folded proteins and give rise to salting-in behavior" [3,13]. Once again, the implication is that, chaotropes as 'structure breakers' and destabiliser of folded proteins (*i.e*. unfolding of enzyme to be specific) are able to solubilise enzymes and yet render them less catalytically active going by the definition of chaotropes as destabilisers. This seems to imply that unfolded proteins which results from destabilisation does not necessarily become less soluble; besides the unfolded is said to be more hydrated than the folded protein [14]. Simple thermodynamic equations illustrating the movement of water molecules to and fro the protein surface domain due respectively to preferential binding by chaotropes and preferential exclusion at constant temperature and pressure are respectively.

$$\theta\Delta S = -R\theta \ln (C_{1\text{-surf}}/C_{1-\text{bulk}}) \quad (1)$$

Where $C_{1-\text{surf}}$ and $C_{1-\text{bulk}}$ are the concentration of water on protein surface domain and in the bulk respectively; $C_{1-\text{bulk}} > C_{1-\text{surf}}$ as applicable to the effect of binding chaotropes; R and θ are the gas constant and ambient thermodynamic temperature respectively.

$$\theta\Delta S = -R\theta \ln (C_{1\text{-bulk}}/C_{1-\text{surf}}) \quad (2)$$

Where, $C_{1-\text{bulk}} < C_{1-\text{surf}}$, as applicable to the effect of the exclusion of kosmotropes. Equations (1) and (2) are intended to imply that there is always translational entropy gain whenever the departure of water occurs either to or from protein surface domain. With respect to Eq. (1), it is imperative to restate that when a chaotrope binds there is a displacement of protein hydration water [1] leading to instantaneous gain in translational entropy that can be defined as $\Delta S = -RT \ln(\rho_F/\rho_I)$. Here $\rho_I < \rho_F$ (where I and F designate initial and final regarding protein surface water density). This is with the assumption that despite the departing water of hydration the surface of the unfolded protein due to destabilising osmolyte is always more hydrated [14]. This aspect of departing water is then an addition to the displaced surface domain water by the binding ions or the preferentially binding osmolyte with destabilising potential.

The departure of water of hydration should lead to a decrease in the density of protein water of hydration. The density of water molecules close to the protein surface due to the effect of polar and non-polar group is as high as 1.25 g/cm^3 within 3-4.25 Å [15]. But as soon as the protein surface domain becomes enriched with the presence of the salt, the resulting concentration which, may be greater than bulk concentration, leads to osmotic potential gradient that promotes the diffusion of water from the bulk to the protein surface domain. Next, one may wish to known why some ions are excluded while others bind.

In the first place, preparation of high concentration protein is difficult to achieve for several protein pharmaceuticals due to low solubility [16]. But it is important to note that charged macromolecules, sometimes collectively referred to as polyelectrolyte possess multiple charge [11]. If the pH = pI, the net charge on protein is zero; at pH<pI, the net charge may be positive; and at pH>pI, the net charge is negative; and at the p*K* of a given ionisable group, the net charge will be −1⁄2 for Asp, Glu,

Tyr, and Cys and +1⁄2 for His, Lys and Arg [17]. This does not preclude the issue of partial charge as may be applicable to polar molecules.

The main issue is how the inorganic ions interact with the protein. When bovine serum albumin is mixed with water, it takes prolonged steering to dissolve it. The dissolved protein interacts with the aqueous medium yielding solution structure described according to Kirkwood Buff theory [12] and widely applied by researchers [2]. Hydration of the protein is followed by hydrogen bonding with water molecules in the bulk. The mechanism of interaction of the ions with the enzyme, porcine pancreatic alpha amylase (PPAA) is of interest in this research. The structural feature of the enzyme, in part, that can aid this research is given as follows. The amino acid residues involved in the catalytic reaction are a pair of aspartic acids. Other nearby residues which surround the substrate may participate in its binding via hydrogen bonds and hydrophobic interactions [18]. The 'essential' calcium ion has been located near the active site region and between two domains, each of them providing two calcium ligands [18]. This is to suggest that it is not just proteins molecules that may interact or associate.

In purely neutral solution, a protein is not substantially ionised. For purpose of this theoretical exposition a protein is composed of polar, neutral, hydrophobic, aliphatic type, hydrophobic, aromatic type, unique amino acid, proline and glycine, basic, and acidic amino acids. A neutral solution of protein presents protein molecules which may interact with each other via either basic or acidic side chains which respectively, binds the amino and carboxyl terminal of the protein molecules. There could also be interaction via polar side chains and either the basic or acidic side chains. All these notwithstanding are views expressed in literature: "The electrostatic interaction between two proteins will be dominated by the direct Coulomb interaction provided that the net charge, *Z*, is sufficiently different from zero. The induced interactions will only play an important role at pH values close to the isoelectric point of one of the proteins" [19]. One peculiar phenomenon which, seems to be a paradox, is that association can take place even when the protein and the polyelectrolyte carry the same charge [20]. Protein-protein association may not be an exception. The interpretation is based on the assumption that the ion-dipole interaction can overcome the repulsive ion-ion interaction [20]; but ion-ion interaction attractive or repulsive is between species that possess full or formal charge, and it is therefore, expected to be much stronger than ion-dipole interaction. However, it is imperative to state that protein association may not be beneficial because it leads to disease state [21,22]. But associations apart from other reasons are most likely in concentrated solution and with increasing temperature outside normal range. This is against the backdrop of the fact that most *in vitro* assays are carried out at very low concentration of the enzyme (nanomol-micromol scale). Nonetheless, the size of the enzyme makes association likely. Protein-protein contact compromises the chances the enzyme has to make contact with the substrate thereby pointing to the issue of stochasticity in catalysed reactions. The appropriate orientation of each molecule with respect to the nearest molecule should determine the likelihood of any association.

The concern of this research remains the choice of appropriate model that should describe the force of interaction and ultimately interaction energy. The potential of mean force ($\omega(R)$) between two such charge distributions as applicable to protein in a reaction mixture containing salt solution is evaluated in a multipole expansion to give interaction free energy as follows [11]:

$$\beta\omega(R) \approx E_{\mathrm{ii}} - E_{i-in} - E_{in} - E_{id} - E_{dd} \quad (3)$$

Where, E_{ii}, E_{i-in}, E_{in}, E_{id}, and E_{dd} are the ratio of ion-ion interaction energy (I_{E}) to $k_{\mathrm{B}}\theta$ (where k_{B} and θ are Boltzmann constant and thermodynamic temperature respectively; $\beta = 1/k_{\mathrm{B}}\theta$), ratio of ion-induced dipole I_{E} to $k_{\mathrm{B}}\theta$, the ratio of induced dipole-dipole I_{E} to $k_{\mathrm{B}}\theta$, ratio of ion-dipole I_{E} to $k_{\mathrm{B}}\theta$, and dipole-dipole I_{E} to $k_{\mathrm{B}}\theta$. The full equations of E_{ii}, E_{i-in}, E_{in}, E_{id}, and E_{dd} can be found in a thesis [11]. However, the I_{E}, or $\omega(R)$, appears to be a part of the total energy of the system, the solution of either a protein and a polyelectrolyte or the same protein going by the definition of conservative forces and cognate energy applicable to potential fields which may be electrical and gravitational in nature. For any association or preferential binding to occur there must be a translational motion that requires the kinetic energy. The minus sign in Eq. (3), suggests that the parameters indicated are potential energies of interaction if in line with convention the potential energy at ∞ is equal to 0. One may wish to know if the total energy of the molecule enables the

molecule to migrate towards another molecule. Besides, $\omega(R)$ is not defined as net energy equal to kinetic energy. When the potential energy in an electrolyte is unlocked and converted to kinetic energy the electrons migrate, generating current by so doing. Equation (3) is better discussed in chemical physics. Conversion of potential energy to kinetic energy enables work to be done; there is nothing to show that the potential energies implied in Eq. (3) are convertible to kinetic energies.

In a buffer, it is either pH < 7 or > 7 (it may be =7 by choice), such that the enzyme may acquire a net charge; in this case the following interaction energy equations are the case [11].

$$\text{Ion – ion interactions energy} = k_{\mathrm{B}}\theta.\frac{l_{\mathrm{B}}Z_{\mathrm{A}}Z_{\mathrm{B}}}{R}\frac{e^{-\kappa(R-a)}}{(1+\kappa a)} \quad (4)$$

Where, k_{B}, R, θ, Z, κ, and a are the Boltzmann constant, distance between centres of the particles, thermodynamic temperature, charge number (or valence), inverse Debye length [11], and the closest distance between the "central ion" (protein b) and surrounding ions which is approximately the protein radius [11]. The subscripts A and B denote different chemical species; they may be the same species possessing similar or different charges interacting with each other.

However, if conservative forces are considered, ion-ion interaction energy cannot be an exception; this implies that there should be total energy of the system being the sum of kinetic and potential energy [23]. This issue is handled shortly. But there may be other groups in the protein that merely exhibit polarity. Therefore, there should also be interaction between cationic chaotrope and one polar end describable by the interaction energy given as:

$$\text{Ion – dipole interaction energy} = -k_{\mathrm{B}}\theta\frac{\left(l_{\mathrm{B}}Z_{\mathrm{A}}\mu_{\mathrm{B}}\right)^2}{6R^4}\frac{\left(e^{-\kappa(R-a)}\right)^2}{(1+\kappa a)^2}(1+\kappa R)^2 \quad (5)$$

$$\text{Ion – induced ion interaction energy} = -k_{\mathrm{B}}\theta\frac{l_{\mathrm{B}}^2Z_{\mathrm{A}}^2C_{\mathrm{B}}}{2R^2}\frac{\left(e^{-\kappa(R-a)}\right)^2}{(1+\kappa a)^2} \quad (6)$$

Other interaction energies [11] are

$$\text{Induced-induced interaction energy} = -k_{\mathrm{B}}\theta\frac{l_{\mathrm{B}}^2C_{\mathrm{A}}C_{\mathrm{B}}}{2R^2}\,e^{-2\kappa\mathrm{R}} \quad (7)$$

$$\text{Dipole-dipole interaction energy} = -k_{\mathrm{B}}\theta\frac{\left(l_{\mathrm{B}}\mu_{\mathrm{A}}\mu_{\mathrm{B}}\right)^2}{3R^6} \quad (8)$$

Where, C is the protein charge capacitance (charge fluctuation) given as $\langle \mathrm{Z}^2\rangle_0 - \langle \mathrm{Z}\rangle_0^2$ [11] where $\langle\ldots\rangle_0$ denotes an average over all configurations in the unperturbed system. The parameters, μ_{A} and μ_{B} are respectively the electric dipole moment (whose unit is Cm) for species A and B. However, it appears that μ is equal to $\langle|\Sigma \mathrm{r_i}\ \mathrm{z_i}\ |\rangle$ where r_{i} is the vector from the centre-of-mass of a macromolecule to the charge and z_{i} is the charge number as may be implied in the work of Lund [11]. Equations (5), (6), (7), and (8) are for illustration and for qualitative analysis.

Again details of the steps leading to equations 3, 4, 5, 6, 7, and 8 are reserved for the chemical physicists. However, there is need to point out the observed differences in some equations as applicable to Eq. (8). The equation [23] $v_{\mathrm{d-d}}(r) = -\frac{2}{3k_{\mathrm{B}}\,\theta}\frac{\mu_1^2\mu_2^2}{4\pi\varepsilon_0 \mathrm{r}^6}$ is described as the orientation-averaged potential energy ($V_{\mathrm{d-d}}(r) = \omega(\mathrm{R})/k_{\mathrm{B}}\theta$) of 2 dipoles and ε_0 is the permittivity in free space. The negative sign is relevant to conservative energy in a field and it is as expected for potential energy. However, as long as the SI unit (CODATA) of dipole moment (μ) is c m then there is dimensional issue with the equation and cannot be applied to physical system let alone biological system. Perhaps, $V_{\mathrm{d-d}}(r) = -\frac{2}{3k_{\mathrm{B}}\,\theta}\frac{\mu_1\mu_2}{4\pi\varepsilon_0 \mathrm{r}^3}$ could be more appropriate where μ the dipole number has the dimension of length and by multiplying with the elementary charge one obtains the proper dipole moment, μe [24]. Hence with μ as dipole number with dimension of length, dimensional consistency is maintained. However, this remains a speculation intended to draw attention to the literature information.

There are also dipole-induced dipole interaction potential energy ($v_{\text{d-id}}$) and London or dispersion energy (V_{disp}). The equation of $V_{\text{d-id}}$ and V_{disp} can be found in the literature [23]. The net "long-range attractive potential energy (V_{net})" [23] for 2 neutral molecules is the sum

$$V_{\mathrm{net}} = V_{\mathrm{d-id}} + V_{\mathrm{d-d}} + V_{\mathrm{disp}} \quad (9)$$

Here potential energy V is according to the notation in the literature [23] which is hereby adopted without prejudice to any other notation earlier in the text. Approximating intermolecular

potential energy, V as the sum of the short-ranged and long-ranged potential $(V_{\mathrm{d-id}} + V_{\mathrm{d-d}} + V_{\mathrm{disp}})$ [23] gives an intermolecular potential, the Lennard-Jones potential,

$$V = 4\varepsilon\left[\left(\frac{\sigma}{\mathrm{r}}\right)^{12} - \left(\frac{\sigma}{\mathrm{r}}\right)^{6}\right] \quad (10)$$

Meanwhile it is not easy to determine individual type of interaction potential energy. In a solution of the protein and inorganic ions there may be all kinds of interaction potential energies. Besides, it is the attractive interaction that brings chemical species together for binding leading to any kind of transformation as may be applicable. However, there is need to invoke the principle of conservative energy. Therefore, the total energy of interaction in a mixture of inorganic ions (but they could be organic solutes) and protein taken as a conservative system, where conservative forces act, is given as

$$E = K.E. + V \quad (11)$$

Where E (or $\omega(R)$) is the total energy as suggested in this research, $K.E.$ and V are the electrostatic kinetic energy and potential energy respectively. $K.E.\,\mathrm{is\ equal\ to}(-V/2)$. This implies that $K.E.$ is half of V and opposite in sign but equal and similar in sign to E. Using what is called the primitive model [11], 2*E (mv²)* is equal to – *Z (*+*) Z (*–*) e*² /4πε₀ε_r*r*. Meanwhile, *mv*²/2 is equal to *Z (*+*) Z (*–*) e*² /8πε₀ε_r*r*. This is such that *E* is equal to – *Z (*+*) Z (*–*) e*² /8πε₀ε_r*r*. This simply means that the total energy is half of the potential energy and of the same sign while the kinetic energy is half of the potential energy and of opposite sign. One should therefore, consider taking the sum of all potential energies, divide by two and multiply by a minus sign.

Where electrostatic attraction or repulsion begins, the electrostatic potential energy is always generally equal to – *Z (*+*) Z (*–*) e*²/4πε₀ε_r*r*. A protein (Enzyme of interest in particular) may possess a net charge according to the preponderance of either basic or acidic functional groups with a given pH. This is due to the side chains that may possess amino or carboxylic groups that are ionisable. However, there are also non-ionisable but polar groups. Depending on the orientation of protein to another similar protein there may be repulsive or attractive interaction. Hence it is not certain why Chari et al. [22] referred to electrostatic charge-charge repulsion as the most common long-range potential governed by Coulomb law. This is against the backdrop of the occurrence of protein-protein interaction (PPI) promoted by attractive electrostatic potentials. This is not restricted to unfolded proteins but it is applicable to folded proteins, as to imply in Kirkwood-Buff (KB) notation [12], a case of self-correlation expressed in terms of KB integrals, G_{22}. Such situation compromises the function of the enzyme due to what had been termed sequestration phenomenon [25].

Therefore, potential energy of interaction (the attractive case) should not be restricted to interactions where permanent or temporary partial charges of species exist. Thus,

$$K.E. = \frac{-k_{\mathrm{B}}\theta\left(\begin{array}{c} -\frac{l_{\mathrm{B}}Z_{\mathrm{A}}Z_{\mathrm{B}}e^{-\kappa(R-a)}}{R}\frac{}{(1+\kappa a)} - \frac{l_{\mathrm{B}}^2Z_{\mathrm{A}}^2C_{\mathrm{B}}\left(e^{-\kappa(R-a)}\right)^2}{2R^2}\frac{}{(1+\kappa a)^2} - \\ \frac{(l_{\mathrm{B}}Z_{\mathrm{A}}\mu_{\mathrm{B}})^2\left(e^{-\kappa(R-a)}\right)^2}{6R^4}\frac{}{(1+\kappa a)^2}(1+\kappa R)^2 - \frac{l_{\mathrm{B}}^2C_{\mathrm{A}}C_{\mathrm{B}}}{2R^2}e^{-2\kappa \mathrm{R}} \\ -\frac{(l_{\mathrm{B}}\mu_{\mathrm{A}}\mu_{\mathrm{B}})^2}{3R^6} - \ldots \end{array}\right)}{2} \quad (12)$$

As an example, water-water correlation (which in KBT notation is G_{11}) is occasioned by hydrogen bond pregnant with potential energy as implied in dipole-dipole interaction expected in a polar molecule like water. Although Eq. (12) is intended for attractive case in which as may be applicable to preferential interaction by binding there is also exclusion in which the cosolute departs from the surface domain of the protein. In this case as the particle or cosolute departs as a consequence of exclusion, the potential energy of each particle with respect to each other increases, becoming less negative in magnitude; but ultimately it is the kinetic component that is the key driver of translational motion.

Before proceeding further, there is need to disclose that in a reaction mixture containing ions there are interactions between the substrate and enzyme some of which are not catalytically oriented, interactions between ions and the enzymes, the substrate, and the product. The focus is mainly on the interaction between the proteins and ions (but not restricted to protein-ion interaction) that may influence the binding of the enzyme with the substrate. Besides, the protein and substrate, the polysaccharide in particular, are the target molecules with respect to the advancing bullet species, the ions, in accordance with the net charge of the protein and the polarity of the substrate. It is known that cations including Ca^{2+} form strongly cation-π complexes with aromatic amino acid side chains [26]. Thus it is

not unlikely that ion-ion, ion-induced, and ion-dipole interaction cannot be restricted to between proteins or between protein and polysaccharide as substrate, rather interaction between the cation, Ca^{2+} and protein in slightly alkaline medium (pH = 7.4) may occur. It can also occur between the inorganic ion and substrate. Let it be known that the interaction between an inorganic cation such as calcium ion and side chain carboxylate ion is strictly electrostatic amenable to the so-called primitive model.

On the basis of hard shell model protein as a whole or any part of it that can interact attractively with oppositely charged species, be it organic or inorganic can be amenable to the equation of interaction potential energies. This can further be buttressed with the unavoidable fact that ion-dipole and dipole-dipole interactions occurs between dissolved partially ionised protein and water otherwise protein would be totally insoluble; hydration is not by covalent bond formation with dissolved solute. Another example is the miscibility of ethanol with water via dipole-dipole interaction leading to H-bond formation. This is applicable to simple sugars and some higher monohydric and polyhydric alkanols. Besides, soluble inorganic salts such as calcium chloride for instance ionise in water but do not remain as dry ions; so this is another strong case of ion-dipole interaction with water. Therefore, scholars may not give the expression that only macromolecule-macromolecule interact-tion should be amenable to such interaction free energy equations except on grounds of scope of research. Cation (inorganic) can always bind to polar groups in globular protein side chain. Hence the equation of interaction potential energy ($V_{\text{ion-2}}$) for protein-protein interaction may be

$$V_{\text{ion}-2} = k_{\text{B}}\theta\left(-\frac{l_{\text{B}}Z_{\text{A}}Z_{\text{B}}}{R}\frac{e^{-\kappa(R-a)}}{(1+\kappa a)} - \frac{-l_{\text{B}}^2Z_{\text{A}}^2C_{\text{B}}\left(e^{-\kappa(R-a)}\right)^2}{2R^2}\frac{}{(1+\kappa a)^2} - \frac{\left(l_{\text{B}}Z_{\text{A}}\mu_{\text{B}}\right)^2}{6R^4}\frac{\left(e^{-\kappa(R-a)}\right)^2}{(1+\kappa a)^2}\cdots\right) \quad (13)$$

Equation (13) which, is a general one, is applicable to any pair of macromolecule. In this case, the monovalent anion binds with charged amino acid moieties with formal positive charge and partial charge. The binding of the cation may for obvious reason be stronger than the anion. Binding of the cation to hydroxyl group of polysaccharide may not be impossible considering the fact that dipole exist in such group. This is often ignored in the consideration of the effect of bulk calcium chloride on holoamylase at high temperature assays [10].

The interaction potential energy ($V_{\text{i-3S}}$) between ion and substrate may therefore, be given as

$$V_{\text{i}-S} = -k_{\text{B}}\theta\left(\frac{\left(l_{\text{B}}Z_{\text{i}}\mu_{\text{S}}\right)^2}{6R^4} - \cdots\right) \quad (14a)$$

Where, i and *S* are inorganic ion and substrate respectively. The interaction potential energy between ethanol and the charged enzyme may be given as

$$V_{\text{2i}-\text{eth}} = -k_{\text{B}}\theta\frac{-\left(l_{\text{B}}Z_{\text{2i}}\mu_{\text{eth}}\right)^2}{6R^4} \quad (14b)$$

Where 2i and eth denote charged enzyme and ethanol respectively.

With the proteins only, partially or completely ionised, interaction potential energy may the sum

$$V_{\text{i}-2} = k_{\text{B}}\theta\left(-\frac{l_{\text{B}}Z_{\text{i}}Z_2}{R} - \frac{-\left(l_{\text{B}}Z_{\text{i}}\mu_2\right)^2}{6R^4} - \cdots\right) \quad (14c)$$

Where, the subscript 2 denotes protein. Summation may be necessary if there are many carboxylate groups and many polar groups.

$$V_{\text{i}-2}^{\text{sum}} = k_{\text{B}}\theta\left(-l_{\text{B}}\sum_1^{\infty}\frac{Z_{\text{i}}Z_2}{R} - l_{\text{B}}^2\sum_1^{\infty}\frac{\left(Z_{\text{i}}\mu_2\right)^2}{6R^4} - \cdots\right) \quad (14d)$$

Where, sum means the result of summation.

Since the presence of net charge in protein - porcine pancreatic alpha amylase (PPAA) - in slightly alkaline solution, presents a stronger electrostatic force than the partial charge on polysaccharide, the protein can easily interact with calcium ion. But binding of polysaccharide to the enzyme is needed for transformation to product. The strongly ionised protein-polysaccharide interaction energy ($V_{\text{2-3S}}$) may be given as

$$V_{2-3\text{S}} = k_{\text{B}}\theta\left(\frac{-\left(l_{\text{B}}Z_2\mu_{\text{S}}\right)^2}{6R^4}\frac{\left(e^{-\kappa(R-a)}\right)^2}{(1+\kappa a)^2} - \cdots\right) \quad (15)$$

Most enzymes are active at neutral pH. Under such condition there may be partial ionisation of the protein first via the side chain carboxylic groups in some cases while in other it may not be the case. Citing Hebert et al. [27], for instance, Shaw et al. [28] posit that the net charge of wild type ribonuclease Sa is − 7 at pH equal to 7. Therefore, interaction pattern between protein and other solution components may show slight complication. Different fractions of the enzyme may

possess interaction potential energies described by equations (13), (14), and (15). There may be complex formation between calcium ion and π-bond of the side chain carboxyl group as applicable to aromatic ring of some amino acids [26]. This is however, very speculative. Unionised enzyme in neutral pH can undergo a dipole-dipole interaction with the substrate. The dipole-dipole interaction potential energy with substrate is

$$V_{3S-2} = k_{\mathrm{B}}\theta\left(-\frac{\left(l_{\mathrm{B}}\mu_2\mu_{\mathrm{S}}\right)^2}{3R^6}\right) \quad (16)$$

Equation (16) has no screening factor as in literature [11] but no reason is available for now. At this juncture it is necessary to state that the appearance of screening factor in some equations imply the presence of salt in solution. Removal of the screening factor, though not shown separately implies the absence of any salt in solution.

The lower activity of enzyme at neutral pH may be explained based on the week interaction potential energies which dominate or constitute part of the total interaction energies. The strongest interaction energy is the ion-ion interaction energy made possible at optimal pH range, either acidic or alkaline. With the prevailing significant amount of weak binding interaction potential, thermal perturbation may dislodge the substrate from the active site leading to lower velocity of catalytic action; this is however, against the suggestion that substrate unbinding increases the rate of catalysis [29]. In the absence of calcium salt as control in solution whose pH is either < or > 7, the only ionised species is the protein. Therefore, if all the protein molecules are assumed to ionise, then ion-dipole interaction potential energy should be the case. The potential energy ($V_{2i-\mathrm{S}d}^{C=0}$) is given as

$$V_{2i-\mathrm{S}d}^{C=0} = k_{\mathrm{B}}\theta\left(-\frac{\left(l_{\mathrm{B}}Z_{2\mathrm{i}}\mu_{\mathrm{S}}\right)^2}{6R^4} - \cdots\right) \quad (17)$$

Where C = 0 is the absence of salt. The figure, 2, *S*, i and *d* are the protein, substrate, ion, and dipole respectively; the substrate is dipolar while the enzyme is ionic i.e. formally charged. This should be between the enzyme and polysaccharide or any smaller sugar and organic osmolyte that bind, if applicable. One can recall that a pair of Asp is involved in the catalytic action of PPAA; other nearby residues which surround the substrate may facilitate binding via H-bonding. The implication of the structure just stated is that within the domain of the active site at pH greater than 7 (=7.4) as in previous research [30] Eq. (17) is applicable; the H-bonding component entails dipole-dipole interaction with the substrate such that the binding interaction energy should be

$$V_{2d-\mathrm{S}d}^{C=0} = -k_{\mathrm{B}}\theta\frac{\left(l_{\mathrm{B}}\mu_2\mu_{\mathrm{S}}\right)^2}{3R^6} \quad (18)$$

Therefore, within the domain of the enzyme active site, the total potential energy (V_{T}) without salt is

$$V_{\mathrm{T-C}} = k_{\mathrm{B}}\theta\left(-\frac{l_{\mathrm{B}}Z_{\mathrm{i}}Z_{\mathrm{S}}}{R} - \frac{\left(l_{\mathrm{B}}Z_{2\mathrm{i}}\mu_{\mathrm{S}}\right)^2}{6R^4} - \frac{\left(l_{\mathrm{B}}\mu_2\mu_{\mathrm{S}}\right)^2}{3R^6}\right) \quad (19)$$

With salt, screening factor need to be introduced to give

$$V_{\mathrm{T+C}} = k_{\mathrm{B}}\theta\left(-\frac{l_{\mathrm{B}}Z_{\mathrm{i}}Z_{\mathrm{S}}}{R}\frac{e^{-\kappa(R-a)}}{(1+\kappa a)} - \frac{\left(l_{\mathrm{B}}Z_{\mathrm{i}}\mu_{\mathrm{S}}\right)^2}{6R^4}\frac{\left(e^{-\kappa(R-a)}\right)^2}{(1+\kappa a)^2} - \frac{\left(l_{\mathrm{B}}\mu_2\mu_{\mathrm{S}}\right)^2}{3R^6}\right) \quad (20a)$$

One major difference between preferential interaction by binding and exclusion in terms of potential energy is that in the former, the long range interaction energy brings the interacting solutes together [11] and enables the weaker short range interaction potential including hydrophobic interaction [31] to contribute to the total binding energy. On the other hand preferential exclusion diminishes the effect of the short-range potential energy of interaction since intermolecular distance tends to infinity. Meanwhile, it is not certain whether all the different potential energies can be measured at the same time. Thus as indicated elsewhere [32] one can see the total potential energies of interaction as being equal to $-e^2/4\pi\varepsilon_0\varepsilon_{\mathrm{r}}R$ multiplied by a factor given as Θ which may be greater than 1 if charge-charge (ion-ion) interaction is involved, otherwise, it may be less than 1 without ion-ion interaction. The resulting equations for the salt-free and salt-included cases are generally given respectively as

$$-\frac{\Theta e^2}{4\pi\varepsilon_0\varepsilon_{\mathrm{r}}R} = k_{\mathrm{B}}\theta\left(-\frac{l_{\mathrm{B}}Z_{\mathrm{A}}Z_{\mathrm{B}}}{R} - \frac{l_B^2Z_{\mathrm{A}}^2C_{\mathrm{B}}}{2R^2} - \frac{\left(l_{\mathrm{B}}Z_{\mathrm{A}}\mu_{\mathrm{B}}\right)^2}{6R^4}(1+\kappa R)^2 - \frac{l_{\mathrm{B}}^2C_{\mathrm{A}}C_{\mathrm{B}}}{2R^2} - \frac{\left(l_{\mathrm{B}}\mu_{\mathrm{A}}\mu_{\mathrm{B}}\right)^2}{3R^6} - \cdots\right) \quad (20b)$$

$$-\frac{\Theta e^2}{4\pi\varepsilon_0\varepsilon_r R} = k_B\theta\left(-\frac{l_B Z_A Z_B}{R}\frac{e^{-\kappa(R-a)}}{(1+\kappa a)} - \frac{l_B^2 Z_A^2 C_B}{2R^2}\frac{\left(e^{-\kappa(R-a)}\right)^2}{(1+\kappa a)^2} - \frac{\left(l_B Z_A \mu_B\right)^2}{6R^4}\frac{\left(e^{-\kappa(R-a)}\right)^2}{(1+\kappa a)^2}(1+\kappa R)^2 - \frac{l_B^2 C_A C_B}{2R^2}e^{-2\kappa R} - \frac{\left(l_B \mu_A \mu_B\right)^2}{3R^6} - \ldots\right) \quad (20c)$$

Equations (20b) and (20c) are respectively suitable for the interaction between two similar (or different macromolecule, polysaccharides, simple sugars, polypeptides, short polypeptides, proteins *etc* as substrates in particular, and enzymes) organic macromolecule without salt and with salt.

2.1 Alternative Potential Energy (P.E.) Expression for Interacting Solute with Macromolecule in terms of Displacement

As stated earlier, it is the kinetic component of the total energy that drives the enzyme, ion, substrate, and any other component towards each other. Movement is resisted by the solvent. Redirecting our mind to the main issue in question, interaction of ions with the macromolecule, brings one to various theories, Kirkwood-Buff theory in particular and the theory of work against solvent resistance. All electrostatic potential energies, as shown earlier in the text, are not equal in strength. The strength of ion-ion interaction should be greater than ion-dipole interaction. The exhibition of the effect(s) resulting from various interaction energies may occur at different rate. This explains in part while the *m*-values of different osmolytes and, inorganic ions differ. Invoking popular Newton's law requires for the purpose of this research that at temperature above absolute zero Kelvin, all macroscopic particles are in constant motion. Thus if there is either attractive or repulsive interaction, a transient acceleration may occur followed by a decrease, leading to terminal velocity, u_{i-t}. One can begin from the equations: $6\pi\eta r_{ia}u_{i-t}$ is equal to $\boldsymbol{\xi_{eff}}/R_0$ (where $\boldsymbol{\xi_{eff}}$ the effective energy of motion is equal to $-V/2$ i.e. half of the net effective potential energy) and $\frac{6\pi\eta r_{ia}D_\theta}{L} = k_B\theta/L$; common to both equations is $6\pi\eta r_i$ and *L* is equal to $\sqrt[3]{V_1}$ in m^3 (where V_1 is the molar volume of water × exp (– 6)). Meanwhile to be effective implies that the total potential energy is not available because of coercive force opposed to motion unlike in gas phase as explained elsewhere [33,34]: But $\frac{D_\theta}{L}$ is equal to $u_{\theta-t}$ as derivable from Einstein's original equation, and formulated elsewhere [33,34].However, u_{i-t} and $u_{\theta-t}$ represent terminal velocities resulting from solvent resistance. At the beginning of transit at a time approximately equal to zero, the velocities are given as $\phi_i u_{i-t}$ (u_i) and $\phi_\theta u_{\theta-t}$ (u_θ) where ϕ_i and ϕ_θ are much greater 1. Thus, the relation below may hold.

$$k_B\theta/Lu_\theta = \boldsymbol{\xi_{eff}}/R_0u_i \quad (21a)$$

Where, u_i, η and θ are the velocity of cation resulting from electrostatic attraction, viscosity constant, and thermodynamic temperature respectively while R_0 is the intermolecular distance of the particles; u_θ is equal to $\frac{D_\theta}{L}$ [33, 34]. Simplification and rearrangement give

$$\boldsymbol{\xi_{eff}} = \frac{u_i k_B\theta R_0}{u_\theta L} \quad (21b)$$

In Eq. (21b), $\boldsymbol{\xi_{eff}}$ is expressed in part in terms of u_i. It can be expressed partly in terms of u_θ, R_0 and ϑy given that y is very infinitesimal but important and, it is the distance covered between interfacial space; ϑ is equal to $\sqrt[2]{M_2/M_i}/\left(\sqrt[2]{M_2/M_i}+1\right)$, and it is a fraction of the total distance covered by the smaller particle, the cation; the symbols, M_2 and M_i are the molar masses of the macromolecule and inorganic cation respectively. Basic Newtonian mechanics shows that

$$u_i = \sqrt[2]{u_\theta^2 + \frac{2\boldsymbol{\xi_{eff}}\vartheta y}{m_i R_0}} \quad (22)$$

Where, m_i is the mass of the cation, calcium ion. Substitution of Eq. (22) into Eq. (21b) gives after rearrangement and making $\boldsymbol{\xi_{eff}}$ subject of the formula,

$$\boldsymbol{\xi_{eff}} = \frac{\left(\frac{\vartheta y}{m_i R_0} \pm \sqrt[2]{\left(\frac{\vartheta y}{m_i R_0}\right)^2 + \left(\frac{L}{R_0 k_B\theta}\right)^2 u_\theta^4}\right)(R_0 k_B\theta)^2}{(u_\theta L)^2} \quad (23)$$

Equation (23) shows that if the magnitude of R_0 is high the strength of the conservative force or energy could be high. If it is high, it means that species has a wide field of electrostatic influence. The contrary is the case if R_0 is short. If y tends to 0, Eq. (23) reduces to

$$\boldsymbol{\xi_{eff}} = \frac{R_0 k_B\theta}{L} \quad (24)$$

If this is the case and, bearing Eq. (21b) in mind, u_i should be equal to u_θ.

2.2 Linking Conservative Energies with *m*-value

Linking the conservative energies with *m*-values requires that the diffusion coefficient (D_{i}) of the solution components needs to be known. The radius (r_{iaq}) of the ion is as observed in the literature [35].

$$D_{\mathrm{i}} = \frac{k_{\mathrm{B}}\theta}{6\pi\eta r_{\mathrm{iaq}}} \tag{25}$$

Based on the definition of *m*-value, the capacity of a cosolute either to force unfolding or (re)folding [36], one should expect that there may be forces generated either upon binding or upon exclusion of osmolytes which respectively forces unfolding or refolding as the case may be. In this case, $\boldsymbol{\xi}_{\mathbf{eff}}$ the effective energy given below, therefore, represents the work done to force unfolding or folding. As explained in the literature [33, 34], the effective energy is given as

$$\boldsymbol{\xi}_{\mathbf{eff}} = \sqrt[3]{4m_{\mathrm{i}}} \cdot \sqrt[3]{(\boldsymbol{\xi}\, D_{\mathrm{i}}/L)^2} \tag{26}$$

The net binding energy, $\boldsymbol{\xi}$ either the attractive case or the repulsive case, which are respectively, the energy involved in preferential interaction by binding and exclusion is equivalent to either the energy of motion of the in-coming (binding) or the departing (excluded) osmolyte. However, the total energy equal to $\boldsymbol{\xi}$ is not available for driving the motion due to the coercive forces in the solution.

In line with the original work of Marcelo [36], Harries and Rösgen [37] defines the *m*-value as the slope ($m-\mathrm{value} = \partial\Delta G/\partial C_{\mathrm{os}}$) of the plot of the native to denatured free energy change as a function of the osmolyte concentration (C_{os}). Since the sign of a slope may indicate either a negative or a positive correlation, the slope remains the best descriptor of the capacity of an osmolyte to either unfold or fold a biopolymer. This is in line with the suggestion that the *m*-value is positive in sign where protecting osmolyte is the case, while it is negative for the destabilizing osmolyte [38].

For destabilising and stabilising osmolytes are respectively the following equations

$$-m-\mathrm{value}.C_{\mathrm{os}}\alpha\,\boldsymbol{\xi} \tag{27}$$

$$m-\mathrm{value}.C_{\mathrm{os}}\alpha\,\boldsymbol{\xi} \tag{28}$$

With these postulations in view (Eq. (27) and Eq. (28)), there is need to recall that all different kinds of interaction potential energy (*e.g*. ion-ion or charge-charge, ion-induced, ion-dipole *etc*), converted to kinetic energy via the conservative energy equation, Eq. (11), should determine the extent of unfolding or folding. Since the pH was 7.4, it is expected that PPAA should possess carboxylate groups mainly. However, the net charge that may be negative with magnitude > 1 may not be known without separate determination. The implication is that the cations will be attracted to the locations of the carboxylate groups while the chloride ions should be excluded. Ethanol is generally known to bind and unfold a protein and so it might be expected to add to the effect of calcium (but on the contrary given known effect of calcium ion as stabiliser while ethanol is a fluidiser) while being opposed to the effect of chloride at the prevailing alkaline pH. Therefore, Eq. (14b), may best serve the binding interaction potential of ethanol with the enzyme, while Eq. (14c) is the case for cation-protein interaction potential.

With a clear understanding that there is always a conformational entropy change, one can further postulate that the difference between the total electrostatic potential force and the thermal force is equal to the net force resulting from the effect of a cosolute in terms of energy (Eq. (26)) expended in (un) folding with concomitant conformational entropy change of the macromolecule. The relevant equation is therefore, given as:

$$-\frac{V}{\sqrt[3]{N_{\mathrm{A}}}.\dot{R}} - \frac{k_{\mathrm{B}}\theta}{L} = \frac{\boldsymbol{\xi}}{R_0\sqrt[3]{N_{\mathrm{A}}}} \tag{29a}$$

The equation for $\boldsymbol{\xi}$ in Eq. (26) and Eq. (29a) is $\boldsymbol{\xi} = k_{\mathrm{B}}\theta\,\mathrm{In}\,\frac{1}{K_{\mathrm{eq}}} + \theta\,\Delta S$. Where N_{A}, S, and $\dot{R}$ are the Avogadro's number, entropy, and $r_2 + r_{\mathrm{ia}}$ (r_2 and r_{ia} are the hydrodynamic radius of the enzyme and radius of inorganic ion). K_{eq} is the equilibrium constant for the folding – unfolding transition. Meanwhile, if N_{A} is not part of the equation, then instead of L, L / $\sqrt[3]{N_{\mathrm{A}}}$, *i.e*. approximately 3.103 exp (−10) m should be in place. Rearrangement of Eq. (29a) gives

$$\mathrm{In}\,\frac{1}{K_{\mathrm{eq}}} = \frac{\left(\frac{2K.E.}{\sqrt[3]{N_{\mathrm{A}}}.\dot{R}} - \frac{k_{\mathrm{B}}\theta}{L}\right)R_0\sqrt[3]{N_{\mathrm{A}}}}{k_{\mathrm{B}}\,\theta} - \frac{\theta\,\Delta S}{k_{\mathrm{B}}\,\theta} \tag{29b}$$

A plot of $\mathrm{In}\,\frac{1}{K_{\mathrm{eq}}}$ versus calculated R_0 gives a slope given as

$$S_{\mathrm{lope}} = \frac{\left(\frac{2\mathrm{K.E.}}{\sqrt[3]{N_{\mathrm{A}}}.\dot{R}} - \frac{k_{\mathrm{B}}\theta}{L}\right)\sqrt[3]{N_{\mathrm{A}}}}{k_{\mathrm{B}}\,\theta} \tag{29c}$$

Making 2*K.E.* (– *V*) subject of the formula gives

$$2K.E. = k_{\mathrm{B}}\,\theta\left(\frac{1}{L} + S_{\mathrm{lope}}/\sqrt[3]{N_{\mathrm{A}}}\right)\sqrt[3]{N_{\mathrm{A}}}.\dot{R} \qquad (29d)$$

Linking the *m*-value with the total kinetic energy or the potential energy as implied in the conservative field, requires the understanding that

$$\frac{2K.E.\,R_0}{\dot{\mathrm{R}}\,k_{\mathrm{B}}\,\theta} - \frac{\sqrt[3]{N_{\mathrm{A}}}.R_0}{L} - \frac{\theta\,\Delta S}{k_{\mathrm{B}}\,\theta} = \mathrm{I\,n}\,\frac{1}{K_{\mathrm{eq}}} = \frac{m\text{−value}.C_{\mathrm{os}}}{k_{\mathrm{B}}\,\theta} + \frac{\Delta G_{(C_{\mathrm{os}}=0)}}{k_{\mathrm{B}}\,\theta} \qquad (30a)$$

Where, C_{os} and $\Delta G_{(C_{\mathrm{os}}=0)}$ are the molar concentration of the osmolyte and the free energy of folding-unfolding transition as C_{os} tends to 0. Making the *m*-value subject of the formula gives

$$(+/-)m - \mathrm{value} = \left(\frac{2K.E.\,R_0}{\dot{R}} - \frac{\sqrt[3]{N_{\mathrm{A}}}.R_0k_{\mathrm{B}}\,\theta}{L} - \theta\,\Delta S - \Delta G_{(C_{\mathrm{os}}\to 0)}\right)/C_{\mathrm{os}} \qquad (30b)$$

Where (+/–) means that the *m*-value may either be positive or negative. It should be clearly seen that in Eq. (30b), the *m*-value is directly proportional to either the kinetic energy or half the potential energy multiplied by a negative unit integer.

There are events due to the presence of the osmolytes, either organic and / or inorganic as the case may be. When there is destabilisation *i.e.* unfolding there is an increase in conformational entropy describable as in the literature [39]. In this situation, the binding of the solute to the biomolecule reduces the bulk concentration such that

$$\Delta S_{\mathrm{CONF(U)}} = -R\,T\,\mathrm{I\,n}\,\frac{\mathrm{C_{os(F)}}}{\mathrm{C_{os(I)}}} \qquad (31)$$

Where, $C_{\mathrm{os(1)}}$ is greater $C_{\mathrm{os(F)}}$ (in the bulk) and $\Delta S_{\mathrm{CONF(U)}} = f(-m - \mathrm{value})$. I and F denote the initial and final respectively. It should be noted that $m - \mathrm{value}$ is not *m* minus value. If (re) folding is the case, there may be a conformational entropy decrease such that

$$\theta\Delta S_{\mathrm{CONF(F)}} = 3R\,\theta\,\mathrm{I\,n}\,\frac{r_{2\mathrm{N}}}{r_{2\mathrm{U}}} \qquad (32)$$

Equation (32) is however, with reservation because enzymes are always in a state of relative flexibility in aqueous medium at ambient temperatures except at freezing temperatures. The C_{os} in the bulk is greater than around the enzyme's surface domain due to exclusion. Thus,

$$\theta\Delta S_{\mathrm{CONF(F)}} = -R\,\theta\,\mathrm{I\,n}\,\frac{\mathrm{C_{os(F)}}}{\mathrm{C_{os(I)}}} \qquad (33)$$

Where, $C_{\mathrm{os(F)}}$ is greater than $C_{\mathrm{os(I)}}$ (in the bulk) and $-\Delta S_{\mathrm{CONF(F)}}$ is equal *f* (*m*-value). However, the equation chosen for the determination of the conformational entropy is described in method section with clear motivation.

2.3 The Interparticle Distance of Mutual Interaction: The Attractive (or binding) and Repulsive (or exclusion) Cases

Further progress can be made with the derivation of the interparticle distance of mutual interaction between species in solution. Here Newtonian formalism is applied. By this means and taken the meaning of *m*-value into consideration, one can relate the latter to Eq. (23). First, take the positive root of Eq. (23) and expand to give

$$\frac{\boldsymbol{\xi}_{\mathbf{eff}}^2(u_0L)^4}{4(R_0k_{\mathrm{B}}\theta)^4} - \frac{2\boldsymbol{\xi}_{\mathbf{eff}}(u_0L)^2\vartheta\psi}{(R_0k_{\mathrm{B}}\theta)^2R_0m_i} - \frac{u_0^4L^2}{R_0^2k_{\mathrm{B}}^2\theta^2} = 0 \qquad (34a)$$

Simplification and rearrangement gives

$$k_{\mathrm{B}}^2\theta^2u_0^4L^2R_0^2 + \frac{2\boldsymbol{\xi}_{\mathbf{eff}}(u_0L)^2k_{\mathrm{B}}^2\theta^2\vartheta\psi R_0}{m_i} - \frac{\boldsymbol{\xi}_{\mathbf{eff}}^2(u_0L)^4}{4} = 0 \qquad (34b)$$

Redefining ψ as $R_0 - (r_2 + r_{\mathrm{iaq}})$ where r_2 and r_{iaq}(r_{iaq} can be obtained from Eq. (25).) are the radii of the protein and the cation respectively and substituting into Eq. (34b) gives

$$\left(k_{\mathrm{B}}^2\theta^2u_0^4L^2 + \frac{2\boldsymbol{\xi}_{\mathbf{eff}}\vartheta(u_0L)^2k_{\mathrm{B}}^2\theta^2}{m_i}\right)R_0^2 - \frac{2\boldsymbol{\xi}_{\mathbf{eff}}(u_0L)^2k_{\mathrm{B}}^2\theta^2}{m_i}\vartheta(r_2 + r_{\mathrm{iaq}})R_0 - \boldsymbol{\xi}_{\mathbf{eff}}^2(u_0L)^4 = 0 \qquad (34c)$$

Therefore,

$$R_0 = \frac{\frac{2\boldsymbol{\xi}_{\mathbf{eff}}(u_0\,L)^2k_{\mathrm{B}}^2\theta^2}{m_i}\vartheta(r_2+r_{\mathrm{iaq}}) \pm \sqrt{\left(\frac{2\boldsymbol{\xi}_{\mathbf{eff}}\,(u_0L)^2k_{\mathrm{B}}^2\theta^2}{m_i}\vartheta(r_2+r_{\mathrm{iaq}})\right)^2 + 4\,\boldsymbol{\xi}_{\mathbf{eff}}^2(u_0L)^4\left(k_{\mathrm{B}}^2\theta^2u_0^4L^2 + \frac{2\vartheta\boldsymbol{\xi}_{\mathbf{eff}}\,(u_0\,L)^2k_{\mathrm{B}}^2\theta^2}{m_i}\right)}}{2\left(k_{\mathrm{B}}^2\theta^2u_0^4L^2 + \frac{2\vartheta\,\boldsymbol{\xi}_{\mathbf{eff}}\,(u_0\,L)^2k_{\mathrm{B}}^2\theta^2}{m_i}\right)} \qquad (35a)$$

$$R_0 = \frac{\frac{2\boldsymbol{\xi_{eff}}\, k_B\, \theta}{m_i}\vartheta\,(r_2+r_{iaq}) \pm \sqrt{k_B^2\theta^2\left(\frac{2\boldsymbol{\xi_{eff}}}{m_i}\vartheta\,(r_2+r_{iaq})\right)^2 + 4\,\boldsymbol{\xi}_{eff}^2\left(u_\theta^4 L^2 + \frac{2\vartheta\,\boldsymbol{\xi_{eff}}\,(u_\theta L)^2}{m_i}\right)}}{2k_B\theta\left(u_\theta^2 + \frac{2\vartheta\,\boldsymbol{\xi_{eff}}}{m_i}\right)} \quad (35b)$$

$$R_0 = \frac{\frac{2\boldsymbol{\xi_{eff}}\, k_B\, \theta}{m_i}\vartheta\,(r_2+r_{iaq}) + \sqrt{k_B^2\theta^2\left(\frac{2\boldsymbol{\xi_{eff}}}{m_i}\vartheta\,(r_2+r_{iaq})\right)^2 + 4\,\boldsymbol{\xi}_{eff}^2\left(u_\theta^4 L^2 + \frac{2\vartheta\,\boldsymbol{\xi_{eff}}\,(u_\theta L)^2}{m_i}\right)}}{2k_B\theta\left(u_\theta^2 + \frac{2\vartheta\,\boldsymbol{\xi_{eff}}}{m_i}\right)} \quad (36)$$

Where, in all cases in this research, the parameter u_θ^2 is given as $u_\theta^2 = \sqrt[3]{4m_i}\,.\sqrt[3]{(k_B\theta\, D_i/L)^2}\,/m_i$ [33].

3. MATERIALS AND METHODS

3.1 Materials

As in previous research [30] the chemicals used were: Insoluble potato starch from Sigma Chemicals Co, USA; ethanol, hydrochloric acid, and sodium chloride from BDH Chemical Ltd, Poole England; 3, 5-dinitrosalicyclic acid (DNA) from Lab Tech Chemicals India; Tris from Kiran Light Laboratories and BSA from Sigma USA; porcine pancreatic α – amylase (PPAA) (Alpha-amylases (α-1, 4-D-glucan glucanohydrolase; E.C. 3.2.1.1)) from Sigma, Aldrich, US. Calcium chloride was purchased from Lab Tech Chemicals, India. All other chemicals were of analytical grade and solutions were made in distilled water purchased from local market.

3.2 Equipment

A 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 Methods

As stated elsewhere [30], 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 90 mL of tris-HCl buffer, 5 mL of 6% (W/W) NaCl (aq.) and 5 mL of distilled water to give 1 g/100 mL. The assay at 37°C for the generation of the velocities of the hydrolysis of starch is according to Bernfeld method [40]; 1 mL solution of 3, 5-dihydrosalicylic acid is added to the reaction mixture to terminate the action of the enzyme at the end of the duration of assay. Then the reaction mixture is heated in a water bath for 5 minutes for colour development, cooled in cold water, and diluted with 9 mL of distilled water before taking spectrophotometric reading at 540 nm with extinction coefficient equal to 181.1/M/cm.

In order to calculate ΔS and K_{eq} needed for the calculation of first, $\boldsymbol{\xi}$ and $\boldsymbol{\xi_{eff}}$ and ultimately, R_0 the following equations are adopted.

$$\Delta S = k_B\, \mathrm{I\,n}\, \frac{1}{1-f_u} \quad (37)$$

Where, the fraction of the unfolded enzyme f_U is given as a modified form of Baskakov and Bolen [41] equation as follows.

$$f_U = \frac{v_N - v_{min}}{v_{max} - v_{min}} \quad (38)$$

$$K_{eq} = \frac{f_U}{1-f_U} \quad (39)$$

Where, v_N, v_{min}, and v_{max} are the velocities of amylolysis with native untreated enzyme, unfolded enzyme due to a destabiliser and a re-folded enzyme treated with a stabiliser respectively. The motivation in the use of Eq. (38) is that f_U should be decreasing as v_{max} increases with increasing concentration of the stabilising osmolyte with a fixed value of v_N and v_{min}. With the values of f_u (in Eq. (37)) and K_{eq}, the values of $\boldsymbol{\xi}$ at different concentration of the salt is calculated after substituting the values into the equation given as

$$\boldsymbol{\xi} = k_B\theta\, \mathrm{I\,n}\, \frac{1}{K_{eq}} + \theta\,\Delta S \quad (40)$$

The values of $\boldsymbol{\xi_{eff}}$ can then be calculated after substituting the calculated values of $\boldsymbol{\xi}$ into Eq. (26). Then the values of R_0 can be calculated after substituting the values of $\boldsymbol{\xi_{eff}}$ into Eq. (36). Finally a plot of $\mathrm{I\,n}\, \frac{1}{K_{eq}}$ versus R_0 gives a slope from where the total *K.E.* or $-V$ can be calculated according to Eq. (29d).

3.4 Statistical Analysis

The velocities of amylolysis were determined in triplicates. The mean values were used to determine the equilibrium constant and fraction of unfolded enzymes (Eq. (39) and Eq. (38)

respectively). The standard deviation (SD) in velocity of amylolysis was determined according to the method of Hozo et al. [42]. Velocity measurements were recorded as mean ± SD.

4. RESULTS AND DISCUSSION

The objectives of this research are realisable if velocities of the amylolysis of raw starch with the alpha-amylase are measured with and without additives, the cosolutes, ethanol, and calcium chloride in solution. Such velocities are shown in Table 1. The quality of the data generated at the prevailing temperature in an improvised water bath notwithstanding, they enabled the determination of various parameters, the equilibrium constants in particular and the fraction of the unfolded enzyme based on modified models in the literature [33]. There were increasing velocities with increasing concentration of both ethanol and the salt. The increase with higher concentration of ethanol may be due to error arising from improvised water bath if the solubilising effect of ethanol on the insoluble starch is precluded. But the increase in the velocity of amylolysis with higher concentration of salt is known in the literature [8]. This informed the choice of the equation for the determination of background parameters stated earlier. This is also with the understanding that the velocity of amylolysis without salt but with ethanol represents the minimum, v_{min}.

The determination of the intermolecular distance between interacting molecules required pieces of information about the net attractive and repulsive force or energy. Such intermolecular distance is where initial attractive or repulsive interaction commences. Theoretical researches seemed to indicate this but without its determination [43,44]. The determination of net interaction energies or the attractive forces is a possibility with Eq. (28a) and Eq. (40). The calculated values of the energies are shown in Table (2a). The calculated corresponding entropy changes are shown in Table (2b). The values of the calculated *K.E*. were increasing with higher concentration of both the salt and ethanol. The increasing values of the interaction energies are however, known in the literature [26]. The decrease in entropy with higher concentration of ethanol seemed paradoxical considering the known effect of ethanol. The same trend observed with higher concentration of the salt, the cation component in particular, may be as expected because it is a known stabiliser.

However, substituting calculated values of the effective net energies into Eq. (36) followed by calculations gives the intermolecular distance, R_0 (Table 3). It appeared that the intermolecular distance at the commencement of mutual perturbative effect, attraction in this case as applicable to calcium ion, was longer with higher concentration of the salt as well as with higher concentration of ethanol. The fact that much stronger electrostatic interaction occurs between formally charged molecules is known in the literature [11]. If the protein net charge is high, far from pI, it will in most cases dominate the interaction via the direct ion-ion term, $l_B Z_A Z_B/R$ [11]. The ion-induced and ion-dipole terms and other weaker interactions are more short range and will under these conditions usually only make a small contribution. However, when the stronger long ranged electrostatic forces narrow the inter-molecular distance, the weaker intermolecular short ranged forces become important as applicable to an enzyme and its substrate, starch for instance [11].

The focus of this research is mainly cation (inorganic)-enzyme's net charge interaction and other weaker interactions which are respectively best described by Eq. (4) (ion-ion or formal charge-formal charge) and Eq. (5) (inorganic cation-protein polar group interactions). The interaction between the enzyme and ethanol may be described by Eq. (8) and Eq. (16) (dipole-dipole interaction) and protein ion (with net charge)-ethanol dipole interactions (Eq. (17)). In a concluding remark, Chari et al. [22] posited that "at high concentrations and low ionic strength, long-range electrostatic interactions in the form of charge-charge repulsions are present. Short-range interactions such as dipole-dipole attractions are also significant. In contrast, at low concentrations where inter-protein distance is large, only electrostatic repulsions are significant". But this may refer to a full charge-charge case. This is very much applicable to enzyme-substrate, enzyme-ethanol, cation (calcium ion)-protein interaction in this research.

With information about the intermolecular distance at the beginning of attractive interaction, it is now possible to graphically determine the net maximum attractive interaction energy as to imply not just charge-charge interaction energy but all other weaker interaction energies which become important as the intermolecular distance shortens [11,22]. This issue is best described with Eq. (20c). As Table 4 shows, the net

attractive force and kinetic energy decreased with higher concentration of ethanol unlike the potential force and potential energy which as expected based on convention increased(that is, the variables $\rightarrow 0$). The energy equivalent of the entropic term also showed a decrease with higher concentration of ethanol. This seems surprising considering the effect of ethanol on proteins. This may be as a result of the higher velocity of amylolysis with higher concentration of ethanol, if artifact arising from improvised water bath is precluded.

Table 1. The experimentally determined velocity of amylolysis

[Ethanol]/mol/L	[Salt]/mmol/L	[Salt] /mmol/L	[Salt] /mmol/L	[Salt] /mmol/L
	0.25	0.75	1.00	0.00
	v	*v*	*v*	*v*
	(exp (– 4)M/ml.min)	(exp (– 4)M/ml.min)	(exp (– 4)M/ml.min)	(exp (– 4)M/ml.min)
2.4	1.09 ± 0.01	1.21 ± 0.19	1.24 ± 0.04	3.83 ± 0.39
3.23	1.16 ± 0.06	1.30 ± 0.02	1.32 ± 0.06	5.37 ± 0.38

Cal. means calculated. Other symbols are defined in the text

Table 2a. Calculated kinetic and potential energies of attraction at different salt concentrations

[Ethanol]/mol/L	[Salt]/mmol/L	[Salt] /mmol/L	[Salt] /mmol/L
	0.25	0.75	1.00
	Cal. *K.E.*& *V*	Cal. *K.E.*& *V*	Cal. *K.E.*& *V*
	(exp (–20) J)	(exp (–20) J)	(exp (–20) J)
2.4	~ 1.870	~ 1.877	~ 1.654
	~ – 3.741	– 3.754	~ – 3.759
3.23	~ 1.879	~ 1.884	~ 1.885
	~ – 3.759	~ – 3.768	– 3.770

Cal. means calculated. Other symbols are defined in the text. The negative values are the potential energies

Table 2b. Calculated change in energy equivalent to the entropic term following folding – unfolding transition

[Ethanol]/mol/L	[Salt]/mmol/L	[Salt] /mmol/L	[Salt] /mmol/L
	0.25	0.75	1.00
	Cal. $\theta\Delta S$ (–19)	Cal. $\theta\Delta S$ (–19)	Cal. $\theta\Delta S$ (–19)
	(exp (– 20) J)	(exp (– 20) J)	(exp (– 20) J)
2.4	~ 0.923	~ 0.604	~ 0.560
3.23	~ 0.604	~ 0.411	~ 0.394

Cal. means calculated. Other symbols are defined in the text

Table 3. Calculated intermolecular distance

[Ethanol]/mol/L	[Salt]/mmol/L	[Salt] /mmol/L	[Salt] /mmol/L
	0.25	0.75	1.00
	Cal. R_0 *I* nm	Cal. R_0 *I* nm	Cal. R_0 *I* nm
2.4	~ 4.930	~ 7.604	~ 8.076
3.23	~ 6.998	~ 9.921	~ 10.169

R_0 denotes intermolecular distance. Cal. means calculated. Other symbols are defined in the text

Table 4. Graphically determined net maximum electrostatic force, entropy, and kinetic energy

[Ethanol] (mol/L)	G.D. $K.E./\sqrt[3]{N_A}$ Ŕ (exp (–19) N)	*P.F.* (exp (–19) N)	G.D. $\theta\Delta S$ (exp (– 20)J)	G.D. *K.E.* (exp (– 20)J)	G.D. *V* (exp (– 20)J)
2.4	~ 0.901	~ –1.803	1.572	2.048	~ – 4.096
3.23	~ 0.874	~ –1.748	~ 1.160	1.986	~ – 3.972

G.D. means graphically determined net maximum kinetic energy, K.E. otherwise, given as – P.E. (potential energy, V). P.F. is the net total attractive potential force. Other symbols are defined in the text

General observations showed that while the calculated kinetic and potential energies increased in magnitude with higher concentration of ethanol (Table 2a), it was not so with graphically determined energies. This seems to defy possible interpretation. However, the magnitudes of the graphically determined parameters were higher than the calculated cases. While the focus of this research remains binding interactions, one may not totally ignore the role of chloride which by virtue of being a counter ion was likely to be excluded or repelled from the negatively charged enzyme. Nonetheless, it cannot be precluded from interacting with the some polar side chains where partial positive charge may occur. Besides it is a known activator of alpha amylase [45] and, as such, PPAA cannot be an exception. Although organic osmolyte was stated in some places in the text and title, the determination of its interaction potential with enzyme remains outside the scope of this research. While the strict concern of dieticians, medical, pharmaceutical, and other related scientist may be well known, the outcome of their efforts depends on interaction potential between the active ingredient and the target. This concern had been expressed elsewhere [22] with respect to the preparation of injectable drug in an appropriate concentration in a way that can minimise the effect of overall net attractive interaction potential. Furthermore, temporary dipoles (induced dipole) soon become repulsive after an initial transient attractive interaction. This is unlike interaction between permanent dipoles, charged particles, and hydrophobes. Thus, in the presence of formal charges, permanent dipoles, induced dipole-induced dipole, van der Waals interactions, applicable to the same biomolecule(s) *etc* may not present significant repulsive term effect within a shorter intermolecular distance.

5. CONCLUSION

The attractive interaction between a macromolecule and a cationic counter ion is due to long ranged ion-ion interaction which ultimately enhances the effect of short ranged interaction. Higher salt concentration promotes long ranged interaction. The translational velocity of the solvent and cosolute has a role in the quantification of intermolecular distance. A mathematical relationship exists between *m*-value and $-V$ (or 2 *K.E.*). Indeed, the *m*-value is directly proportional the intermolecular distance at the commencement of attractive (or repulsive/exclusion) interaction. The values of *V* can be calculated based on the derived equations.

DISCLAIMER

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 author and producers of the products because there is no intention 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 author.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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