Zeta Potentials and Isoelectric Points of Biomolecules: The Effects of Ion Types and Ionic Strengths

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A systematic study of the zeta potential and isoelectric point of biomolecules such as BSA, amylase, invertase and phenylalanine has been performed in various salt solutions for 0.001 M and 0.1 M ionic strenght. Chloride salts; KCl, NaCl, $CaCl_2$, and $MgCl_2$ and potassium salts; KCl, KNO_3, K_2CO_3 and K_2SO_4 were used to test the effects of cations and anions on the zeta potential of biomolecules, respectively. The absolute zeta potential of biomolecules decreased with increasing ionic strenght; divalent ions had a profound influence on reducing zeta potential than monovalent ions. The cations were less effective at low pH and became more effective at high pH for both of ionic strength. Anions possessed a more potent effect on the zeta potential at high and low pH except for 0.1 M salts concentration at high pH. In general, isoelectric point of biomolecules changed with ionic environment. In addition, the effects of ions on the zeta potential of biomolecules were also interpreted for Hofmeister series.

1. INTRODUCTION

The adsorption of biomolecules to a surface includes complex mechanisms that comprise electrostatic, van der Waals, hydrophobic and steric interactions etc. One of the most important adsorption mechanisms is electrostatic interactions. Zeta potential is a measure of the magnitude of electrostatic interactions between charged surfaces. It forms at the interface of a solid and a surrounding liquid. Its measurement brings detailed insight into the dispersion mechanism and colloidal stability of biomolecules. The zeta potential represents the surface charge which occurs in the presence of an aqueous solution when functional groups dissociate on surface or ions adsorb onto surfaces from the solution. Varying the pH value of the aqueous phase influences two mechanisms;

functional groups dissociation and ions adsorption. In addition to the solution pH, concentration and type of salt present in the solution affect electrical charge of the biomolecules [1, 2].

The development of a net charge at particle surface affects the distribution of ions in the surrounding interfacial region, resulting in an increased concentration of counter ions close to surface. The liquid layer surrounding the particle consists of an inner region called the Stern layer and an outer region called the diffuse layer. Electrical double layer consists of Stern layer and diffuse layer. In the Stern layer the ions are strongly bound to particle surface, in the diffuse layer the ions are less firmly attached. Within the diffuse layer there is a notional boundary and any ions within this boundary will move with particle when it moves in the liquid; but any ions outside the boundary will stay where they are- this boundary is called the slipping plane. The potential that exists at this boundary is known as the zeta potential [3, 4].

Knowing the zeta potential is important for the characterization of electrochemical surface properties. The zeta potential is a key parameter for a number of applications including characterization of biomedical polymers, electrokinetic transport of particles or blood cells, biocompatibility tests for medical devices or the characterization of clothing material properties in the textile industry, pharmaceuticals, membrane separation, mineral processing, water treatment, protein separation and purification [5, 6].

The isoelectric point (IEP) is the pH of a protein solution at which the net charge or zeta potential of protein is zero. At the IEP of protein, its structure is more hydrophobic, more compact and less stable due to absence of inter-particle repulsive forces [7, 8]. Hence, proteins can easily aggregate and precipitate at their IEPs. The difference in the IEP values of biomolecules is obviously caused by different ionic environment such as ionic strength, pH and ion type. In addition, the used experimental methods can be changed the value of the isoelectric point due to the measurement techniques. Therefore, there are several different values of IEP for the same protein in the literature. For example, it was found that IEP of *Candida Antarctica,* A-type lipase, is either 4 or 7.5 if the electrophoretic mobility or the isoelectric focusing technique is used, respectively [3]. Especially, the knowledge of zeta potential and IEP values of proteins can be invaluable in identifying the pH and ionic strength of solution that will give the successful separation process of protein solutions.

Schultz et al. [4] investigated the influence of the zeta potential on the adsorption process of immobilized lipase from *C. antarctica* type-A on a type of micro magnetic particles. They demonstrated the important role of zeta potential in enzyme immobilization and evaluated zeta potential as a potential tool for selecting the optimum matrix for maximal binding in addition to optimizing the reaction conditions. Sabaté and Estelrich [9] studied the interaction of α-amylase with *n*-alkylammonium bromides surfactants above and below their critical micellar concentrations in phosphate buffer. The authors determined that the IEP of α-amylase in water was 3.9 and reported that the size of the protein-surfactant complex was maximal when proteins were at their point of zero charge. Lee et al. [10] investigated the effect of anions on the zeta potentials of lysozyme crystals suspended in aqueous 0.05 M sodium acetate buffer at 10°C. The authors observed that the mobility decreased with increasing salt concentration due to anion adsorption by the lysozyme crystal. Boström et al. [11] examined how the dispersion force between a protein and the surrounding ion cloud affects the nature of this cloud, the protein charge, and the Debye length of the solution. The authors presented

model calculations, performed within a modified ion-specific double-layer theory, which demonstrated the large effect of including these ionic dispersion potentials. Blanco et al. [12] investigated the binding of different surfactants with globular proteins such as myoglobin, ovalbumin, and catalase by means of the electrophoretic mobility of the protein–surfactant complexes. The authors determined the zeta potential, number of binding site and Gibbs energies binding of surfactants onto protein. Salis et al. [13] studied the shifts of isoionic and IEP of BSA protein in different concentrations of NaCl. They informed that the salt concentration effects in terms of ion-protein nonelectrostatic potentials and a modified Poisson-Boltzmann equation for a charge regulated spherical colloidal particle in NaCl salt solutions. Rezwan et al.[14] investigated the change of zeta potential as a function of the amount of protein adsorbed on the surface of colloidal alumina particles. The authors determined the number of charges involved in the adsorption using titration experiments and proposed a new adsorption model based on the results derived from these experiments.

As in scientific examples given above, the research activities related to the zeta potential of biomolecules have been done in order to identify interaction mechanism between biomolecules and solid or liquid surfaces. The solubility, aggregation and hydration behavior of proteins in various aqueous salt solutions at different pH conditions have been also extensively studied in the literature. These studies generally focused on the effects of Hofmeister series on protein stability [15-19].

In this study, the influences of various salts and their ionic strength and pH of solution on the zeta potential and IEP of biomolecules were investigated, thus generating a set of crucial information for their application as food, pharmaceutical and cosmetic, membrane separation industries. In addition, the effects of salts on the zeta potential of biomolecules were also interpreted for Hofmeister series. Bovine serum albumin, amylase, invertase and phenylalanine were especially chosen as model biomolecules because of their industrial importance.

2. MATERIALS AND METHODS

2.1. Materials

The salts used in the experiments; KCl, $MgCl_2$, $NaCl$, $CaCl_2$, KNO_3, K_2SO_4 and K_2CO_3 were of analytical grade (obtained from Merck; Darmstadt, Germany). The biomolecules; bovine serum albumin (BSA; A 9647), α-amylase (AMY; *Bacillus sp*, type IIA, A 6380), (D, L)-phenylalanine (PHE; P 1876) and invertase (INV; I 9253) were obtained from Sigma-Aldrich (Dorset, UK). All solutions were prepared from deionized water (Milli Q system, Millipore, Gradient model) with a resistivity of 18.2 MΩ cm. KOH and HCl (Merck, Darmstadt, Germany) were used to adjust pH of solutions.

2.2. Determination of the Zeta Potential

The zeta potential of biomolecules was performed using Zetasizer NanoZS instrument (Malvern Instruments Ltd., United Kingdom) with automatic titrator unit (MPT-2). The titrator unit was equipped with a sample container, which was connected through a capillary system, and with a

peristaltic pump with a folded capillary cell. The protein solutions were titrated from pH value 7.5 to 2.5 using 0.1 M HCl and 0.1 M KOH under constant stirring. Before the automatic titration, freshly prepared protein solutions were filtered using 0.45 µm PVDF filter. All measurements were made using protein solutions with a concentration of 0.5 g/L. The zeta potential of proteins was measured by phase analysis light scattering method for the 0.001 M and 0.1 M salt concentrations. Chloride salts; KCl, NaCl, CaCl$_2$, and MgCl$_2$ were used to test the effect of cations on zeta potential of biomolecules and potassium salts; KCl, KNO$_3$, K$_2$CO$_3$ and K$_2$SO$_4$ were used to test the effect of anions on zeta potential of biomolecules.

The instrument software programme (Dispersion Technology Software) calculated the zeta potential through the electrophoretic mobility using the Henry equation (Eq. 1).

$$\mu_E = \frac{2 \cdot \varepsilon \cdot \zeta \cdot f(\kappa a)}{3 \cdot \eta} \tag{1}$$

Where μ_E is the electrophoretic mobility, ε; dielectric constant, ζ; zeta potential, κ; the reciprocal electrical double layer which depends on ionic strength of the solution, a; the radius of the biomolecules, $f(\kappa a)$; Henry's corrective term, η; viscosity of solution. Assuming the double layer thickness is much less than the particle size, the Smoluchowski approximation was used in calculations [3, 20]. The Debye screening length or electrical double layer thickness, κ^{-1} (nm), was calculated by using Eq. (2) [10].

$$\kappa^{-1} = \left(\frac{\varepsilon_o \cdot \varepsilon_r \cdot k_B \cdot T}{2000 \cdot e^2 I.N} \right)^{0.5} \tag{2}$$

In Eq.(2), ε_o is the dielectric constant of free space (8.854x10^{-12} C.V^{-1}.m^{-1}), ε_r is the dielectric constant of water (78.5), k_B is Boltzmann's constant (1.38x10^{-23} J.K^{-1}), T is the absolute temperature (K), e is the magnitude of the electron charge (1.6022x10^{-19} C), N is Avagadro's number (6.02x10^{23}mol^{-1}), and I is the ionic strength of the salt solution (M). The ionic strength was calculated by using Eq.(3) [21].

$$I = \left(\frac{\sum z^2{}_i \cdot C_i}{2} \right) \tag{3}$$

In Eq. (3), C_i is the ion concentrations and z_i is the ion valency.

3. RESULTS AND DISCUSSION

3.1. Cation Effects on Zeta Potential and IEP Values of Biomolecules

To investigate the effect of various kinds of cations and their ionic strengths on the zeta potential of biomolecules, the chloride salts; KCl, NaCl, CaCl$_2$ and MgCl$_2$ were used in the

experiments. In Figs. 1-4, there are plotted zeta potential changes of BSA, AMY, INV and PHE proteins in 0.001 M and 0.1 M salt solutions as a function of pH. In all cases, the values of measured zeta potentials of biomolecules became significantly smaller as the salt concentration increased due to decreasing the thickness of electrical double layer, which was equal to the inverse of the parameter κ. For the 1:1 salts, the electrical double layer thicknesses calculated from Eq. (2) were 9.62 nm and 0.962 nm in 0.001 M and 0.1 M salt concentrations, respectively. For 1:2 electrolytes, electrical double layer thicknesses were 6.08 nm and 0.608 nm in 0.001 M and 0.1 M salt concentrations, respectively.

At low pH, biomolecules generally presented positive zeta potentials which decreased when pH was raised. The values of IEP were determined from the Figs.1-4 at which the zeta potential of biomolecules was zero. All of the biomolecules had IEP in 0.001 M and 0.1 M salt concentrations except for PHE in $MgCl_2$ solutions (Figs. 4 a, b). The IEP values of the biomolecules are given in the Table 1. The IEP values of the biomolecules changed with increasing salt concentration except for BSA in KCl solutions. The IEP of BSA was indicated at pH=4.68 for 0.001 M and 0.1 M KCl concentrations, so IEP of BSA was found to be independent of KCl concentration.
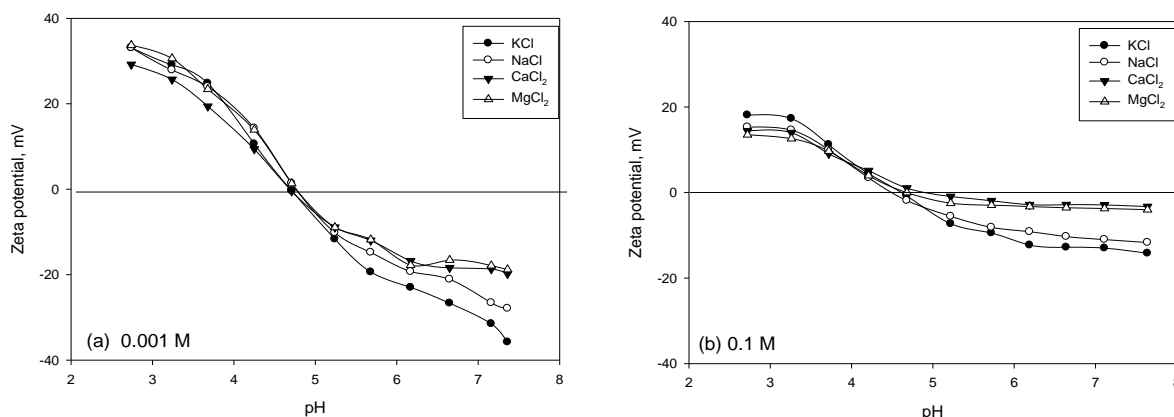

Figure 1. Cation effects: variation of zeta potential of BSA as a function of pH; 0.001 M salt concentrations (a), 0.1 M salt concentrations (b)
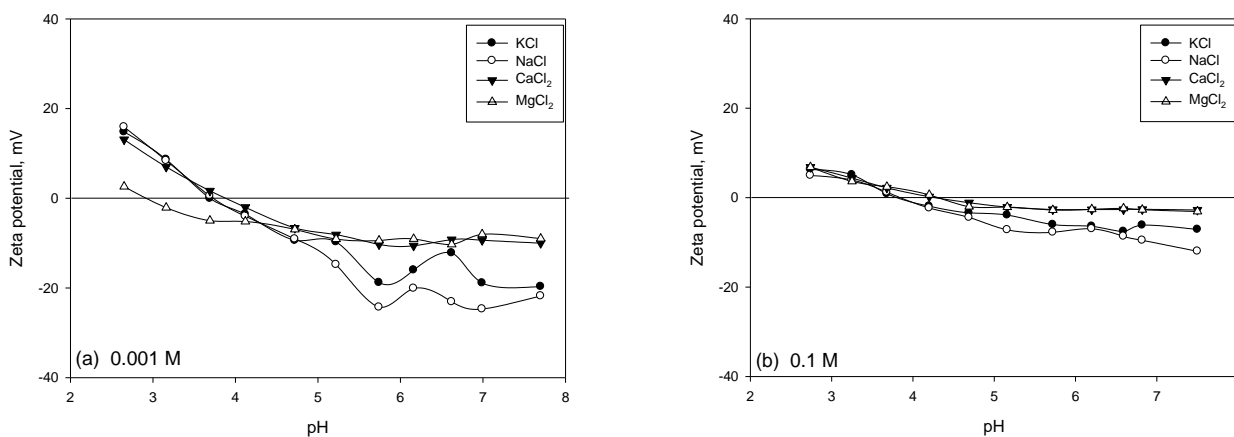

Figure 2. Cation effects: variation of zeta potential of AMY as a function of pH; 0.001 M salt concentrations (a), 0.1 M salt concentrations (b)
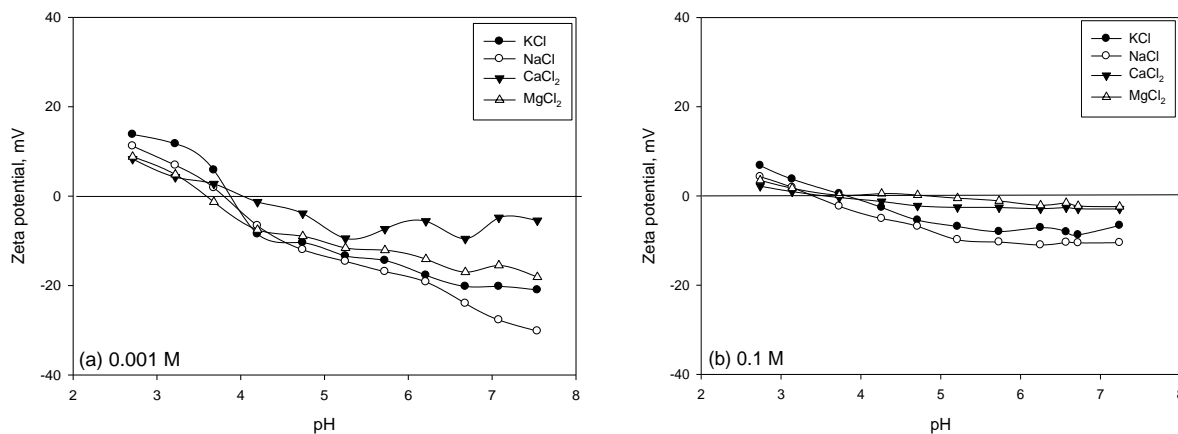
Figure 3. Cation effects: variation of zeta potential of INV as a function of pH; 0.001 M salt concentrations (a), 0.1 M salt concentrations (b)
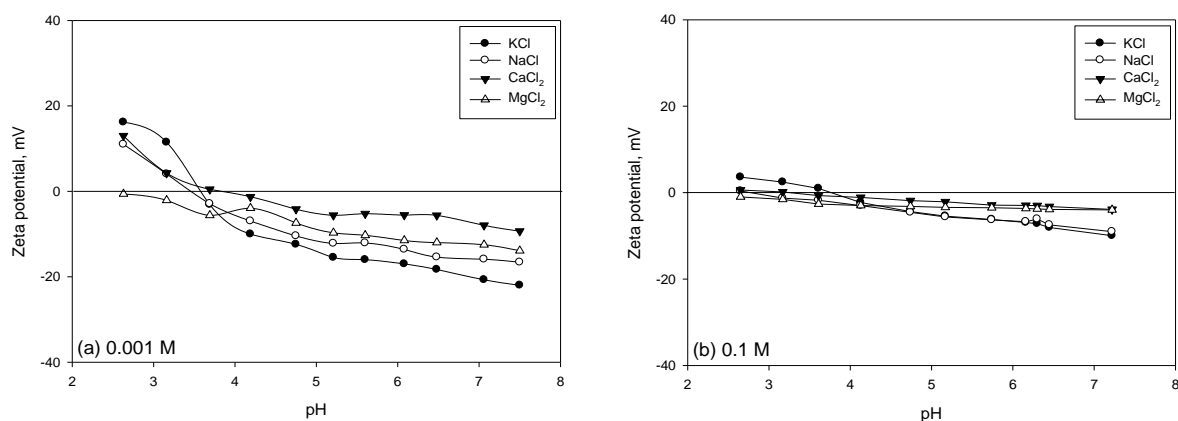

Figure 4. Cation effects: variation of zeta potential of PHE as a function of pH; 0.001 M salt concentrations (a), 0.1 M salt concentrations (b)

This showed that neither K^+ nor Cl^- ions specifically adsorbed on the BSA surface suggesting that KCl was an indifferent electrolyte for BSA. Johnson et al. [22] informed that concerning the zeta potential versus pH data, KCl was indifferent electrolytes for the α-alumina surface. Kulmyrzaev and Schubert [23] also reported that the IEP of whey protein was found to be independent of KCl concentration. The discrepancies in values of IEP of other biomolecules were due to the specific ions adsorption and/or charge screening (see Table 1) [22, 23].

Table 1. Cation effects on IEP of biomolecules in different ionic strengths

Kind of cation	IEP of BSA		IEP of AMY		IEP of INV		IEP of PHE	
	0.001 M	0.1 M	0.001 M	0.1 M	0.001 M	0.1 M	0.001 M	0.1 M
KCl	4.68	4.68	3.66	3.79	3.82	3.78	3.59	3.75
NaCl	4.76	4.51	3.72	3.85	3.78	3.29	3.46	2.74
CaCl₂	4.69	4.90	4.00	4.25	4.03	3.53	3.78	3.21
MgCl₂	4.76	4.68	2.91	4.30	3.59	4.68	-	-

Above the IEPs of biomolecules at the range of pH=5.0-7.5 values, monovalent cations increased the values of absolute zeta potential and divalent cations reduced the values of absolute zeta potential. The absolute values of zeta potential followed the order: $K^+ > Na^+ > Ca^{2+} \approx Mg^{2+}$ for BSA and $Na^+ > K^+ > Ca^{2+} \approx Mg^{2+}$ for AMY in 0.001 M and 0.1 M salt solutions. The order of the cations did not change with increasing ionic strength for BSA and AMY. For INV, the orders of cations were $Na^+ > K^+ > Mg^{2+} > Ca^{2+}$ and $Na^+ > K^+ > Ca^{2+} > Mg^{++}$ in 0.001 M and 0.1 M salts concentration, respectively. The absolute zeta potential decrease was in this order: $K^+ > Na^+ > Mg^{2+} > Ca^{2+}$ and $K^+ \approx Na^+ > Mg^{2+} \approx Ca^{2+}$ for PHE in 0.001 M and 0.1 M salts concentration, respectively.

Below the IEPs of biomolecules at the range of pH=2.5-3.5, the absolute values of zeta potential decrease followed this order: $K^+ \approx Na^+ \approx Mg^{2+} > Ca^{2+}$ for BSA in 0.001 M salt concentration and $K^+ > Na^+ \approx Mg^{2+} \approx Ca^{2+}$ in 0.1 M salt concentration. For AMY, the orders of cations were $Na^+ \approx K^+ > Ca^{2+} > Mg^{2+}$ and $Na^+ \approx K^+ \approx Ca^{2+} \approx Mg^{2+}$ in 0.001 M and 0.1 M salt concentrations, respectively. The orders of cations for absolute zeta potential decrease of INV were $K^+ > Na^+ > Mg^{2+} \approx Ca^{2+}$ and $K^+ > Na^+ \approx Mg^{2+} \approx Ca^{2+}$ in 0.001 M and 0.1 M salts concentration, respectively. For PHE, the absolute zeta potential decrease was in this order: $K^+ > Na^+ \approx Ca^{2+} > Mg^{2+}$ and $K^+ > Na^+ \approx Ca^{2+} \approx Mg^{2+}$ for 0.001 M and 0.1 M salts concentration, respectively.

The range of the zeta potential measurements decreased as the ionic strength was increased due to the compression of double layer. The results indicated that monovalent cations (K^+ and Na^+) were more effective than divalent cations (Mg^{2+} and Ca^{2+}) on the increasing of absolute zeta potential of biomolecules at pH lower and higher than their IEPs.

3.2. Anion Effects on Zeta Potential and IEP Values of Biomolecules

The zeta potential changes of BSA, AMY, INV and PHE biomolecules in 0.001 M and 0.1 M KCl, KNO_3, K_2SO_4 and K_2CO_3 salt solutions as a function of pH are shown in Figs. 5-8, respectively. As the concentration of salt was increased, the value of absolute zeta potential decreased due to charge screening in electrical double layer. The values of IEP of biomolecules were determined from the Figures 5-8 at which the zeta potential of biomolecules was zero. IEP values were given in Table 2. Whereas BSA and INV had IEP for all kind of salts in 0.001 M and 0.1 M concentrations, AMY and PHE did not have IEP for K_2SO_4 and K_2CO_3 for both of two salt concentrations. The IEP values of the biomolecules changed with increasing salt concentration except for BSA in KCl solutions and INV in K_2CO_3 solutions. In addition, IEP values of biomolecules showed changes in other salt solutions. The changes in IEP resulted from charge screening and/or specific ion adsorption from the salts solution.

Above the IEPs of biomolecules at the range of pH=5.0-7.5 values, the absolute values of zeta potential decrease followed the anion order: $Cl^- > NO_3^- > SO_4^{2-} > CO_3^{2-}$ for BSA, AMY, PHE and INV in 0.001 M salt concentration. The effect of anions on the zeta potential of biomolecules was not observed at high salt concentration. The absolute zeta potential of biomolecules was in the same order for all salt solutions in 0.1 M concentration; $Cl^- \approx NO_3^- \approx SO_4^{2-} \approx CO_3^{2-}$.

Below the IEPs of biomolecules at the range of pH=2.5-3.5, the absolute zeta potential decrease was in this order: $Cl^- > NO_3^- > CO_3^{2-} > SO_4^{2-}$ for all biomolecules in 0.001 M and 0.1 M salt

concentrations except for INV and PHE in 0.1 M salt concentration. The order of anions for INV and PHE in 0.1 M salt concentration were $Cl^- > NO_3^- \approx CO_3^{2-} \approx SO_4^{2-}$ and $Cl^- \approx NO_3^- > CO_3^{2-} \approx SO_4^{2-}$, respectively.
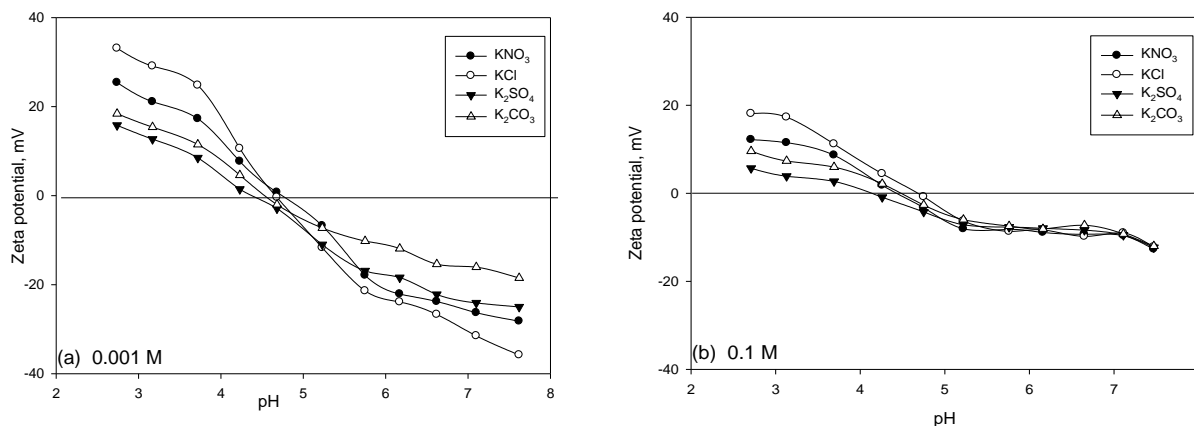

Figure 5. Anion effects: variation of zeta potential of BSA as a function of pH; 0.001 M salt concentrations (a), 0.1 M salt concentrations (b)
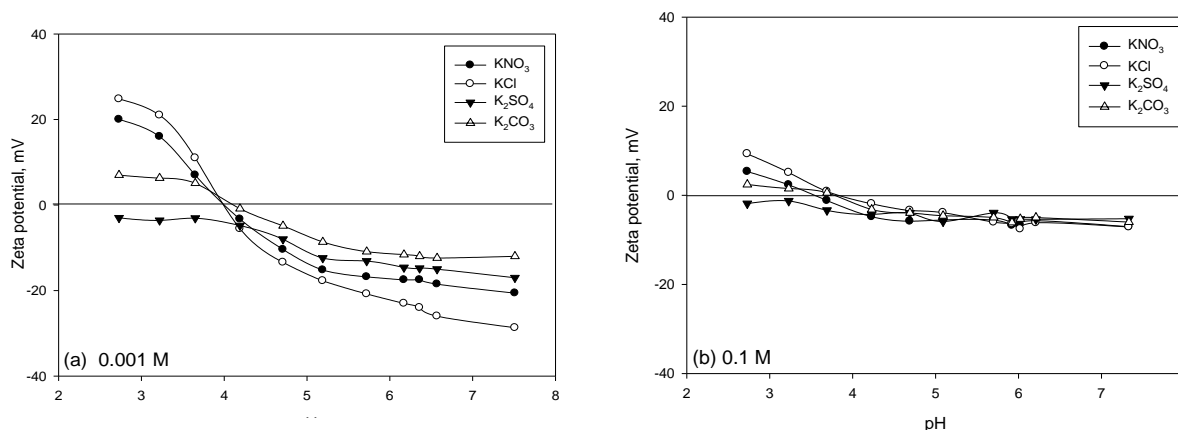

Figure 6. Anion effects: variation of zeta potential of AMY as a function of pH; 0.001 M salt concentrations (a), 0.1 M salt concentrations (b)
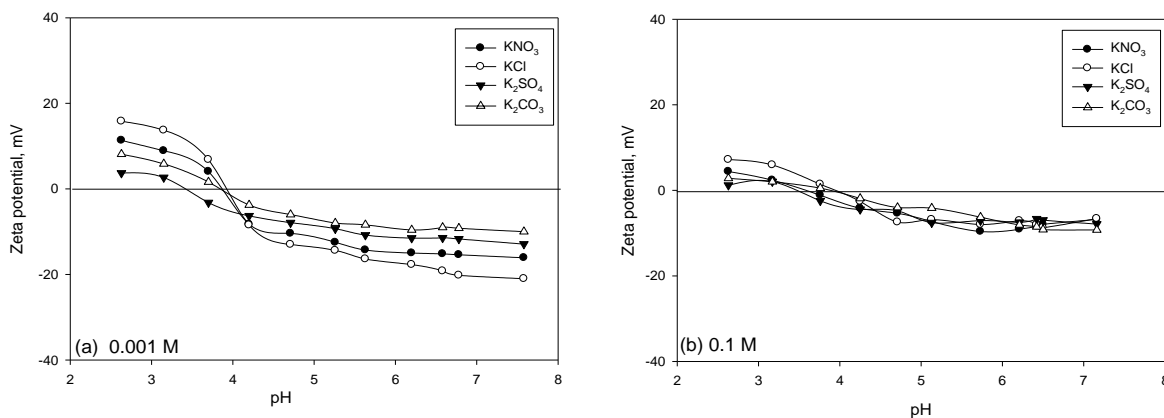

Figure 7. Anion effects: variation of zeta potential of INV as a function of pH; 0.001 M salt concentrations (a), 0.1 M salt concentrations (b)
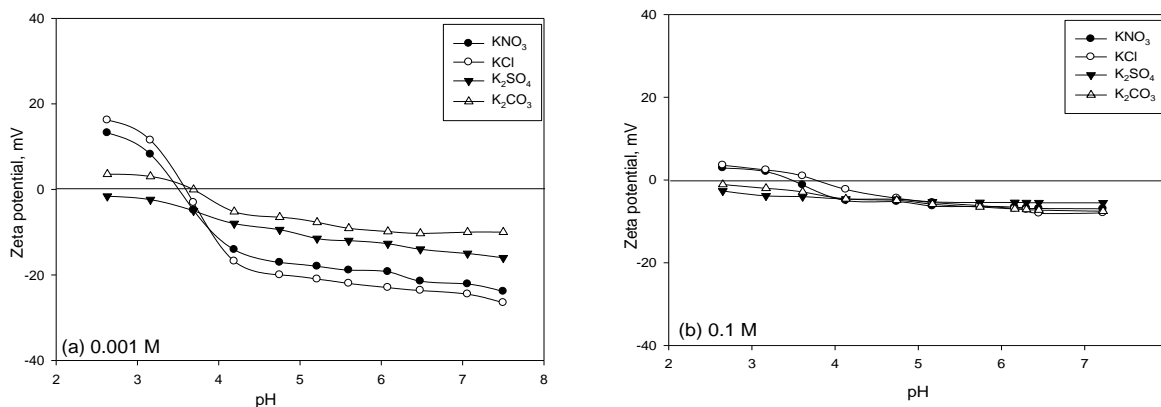
Figure 8. Anion effects: variation of zeta potential of PHE as a function of pH; 0.001 M salt concentrations (a), 0.1 M salt concentrations (b)

Table 2. Anion effects on IEP of biomolecules at different ionic strengths

Kind of anion	IEP of BSA		IEP of AMY		IEP of INV		IEP of PHE	
	0.001 M	0.1 M	0.001 M	0.1 M	0.001 M	0.1 M	0.001 M	0.1 M
KCl	4.68	4.68	3.98	3.78	3.90	3.95	3.59	3.74
KNO$_3$	4.75	4.40	3.98	3.54	3.86	3.59	3.51	3.48
K$_2$SO$_4$	4.36	4.13	-	-	3.40	3.47	-	-
K$_2$CO$_3$	4.54	4.47	4.20	3.78	3.87	3.87	3.67	-

The range of the zeta potential measurements decreased as the salt concentration was increased due to the compression of electrical double layer. The results indicated that the screening of surface charge by divalent anions (SO_4^{2-} and CO_3^{2-}) caused a reduction of zeta potential comparing the monovalent anions (Cl^- and NO_3^-) at pH higher and lower than their IEPs for 0.001 M salt concentration. In the high salt concentration, no difference was observed between the effects of monovalent and divalent anions on biomolecules zeta potential at pH higher than IEP of biomolecules.

4. CONCLUSIONS

Hofmeister series show the solubility of many proteins in salt solutions. For anions these series read $CO_3^{2-} > SO_4^{2-} > HPO_4^{2-} > OH^- > F^- > HCOO^- > CH_3COO^- > Cl^- > Br^- > NO_3^- > ClO_3^- > I^- > ClO_4^- > SCN^-$. For cations, the order is $Ca^{2+} > Mg^{2+} > Li^+ > Na^+ > K^+ > Rb^+ > Cs^+ > NH_4^+$. The above series are the so-called direct Hofmeister series [24-26]. In this study, the reverse Hofmeister series were observed for the effects of kosmotropic and chaotropic ions on zeta potential of biomolecules. Anions and cations acting as co-ion or counter-ion, the obtained results demonstrated that decrease in absolute zeta potential of biomolecules followed the reverse Hofmeister series, more or less. It can be concluded that both cations and anions interact with biomolecules in both co-ion and courter-ion cases. For example, despite the increase in electrostatic repulsion at higher pH for anions acting as co-ions,

anions can be bound with biomolecules via ion-spesific interactions. The order of anion and cation related to zeta potential changed depending on biomolecules, ionic strength and pH of solution. The Hofmeister effects for anions were not observed in 0.1 M salt concentrations for pH above IEPs (Figs. 5b-8b). It was observed that the cations were less effective at low pH range and were more effective at high pH range for both of ionic strength (Figs.1-4). Anions also possessed a more potent effect on the zeta potential at high and low pH range except for 0.1 M salts concentration at high pH range (Figs. 5-8). Clarke and Lüpfert [27] informed that cations were found to be capable of reducing the dipole potential of phosphatidylcholine vesicles, although much less efficiently than can anions. Ninham [28] examined the efficiency of a standard restriction enzyme in cutting DNA as a function of salt and salt type and reported that anions rather than cations showed the greatest variation. Depending on investigated parameters, the effect of anions and cations on biomolecules can be different. In this study, it was also observed that the anions and cations had different effect on zeta potential and IEP of biomolecules depending on ionic environment.

In general, divalent ions are more effective at salting out than monovalent ions as reported by Baldwin [29]. A similar conclusion about the effect of divalent ions on the zeta potential of biomolecules can be made. In this study, the results indicated that regardless of kind of ions *(kosmotropic or chaotropic)*, divalent ions were more effective than monovalent ions on the reducing the absolute zeta potential of biomolecules. This finding of divalent ions is consistent with the previous electrokinetics findings of several researchers [29-32].

The pH of solution is one of the most important parameters affecting the zeta potential of biomolecules. Apart from the pH of solution, zeta potential is a function of concentration and kind of salt. A zeta potential value on its own without specifying pH, concentration and kind of salt, is a nearly unimportant number. The availability of detailed data on zeta potential of biomolecules at different ionic environment (type and concentration of salt and pH of solution) will lead to better process control especially for the biomolecules separation process.

This work includes a systematic study on effect of cations and anions on the zeta potential of biomolecules. Biomolecules used in this study are used as model proteins in numerous studies of protein adsorption and the obtained results bring some insights into the importance electrostatic interactions in adsorption and separation processes of biomolecules.

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