Distinct Roles of Salt Cations and Anions upon the Salting-out of Electro-Positive Albumin

Pedro P. Madeira,* Mara G. Freire, João A. P. Coutinho

CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

*Corresponding author: Tel: +351 234401507; Fax: +351-234370084; E-mail address: p.madeira@ua.pt

Abstract

Precipitation experiments of electro-positive albumin by the action of a wide number of salts, and at different concentrations, were performed at a constant temperature (25 ºC). The pH range studied covered extreme acidic conditions up to hydronium concentrations where the dissociation of the protein carboxyl groups become noticeable. The time required for the clouding phenomenon to occur and the quantity of salted-out protein were also ascertained. The results here reported show that the salt anion is the main salting-out species for the positively charged protein, where their efficacy in salting-out albumin from aqueous solution increases in the order: $F^- < Cl^- < Br^- < NO_3^- < I^- < SCN^- \sim ClO_4^- < SO_4^{2-}$. Although at extreme pH conditions the salt cation has no significant influence on the protein salting-out, experiments performed at higher pH values, where the carboxyl groups starts to dissociate, revealed a non-monotonic effect of the salt upon protein precipitation. We interpret this observation as a result of the presence of different protein forms, with which the salt cation participates in chemical equilibrium. Overall, the proteins salting-out phenomenon induced by salt can be rationalized by a general mechanism driven by electrostatic interactions and chemical equilibrium concepts.

Introduction

Salts, like sodium or potassium chlorides, must play a role in the life phenomena. Experiments have shown that they are essential components for the maintenance of life[1]. It goes without saying that their ubiquitous presence in the protoplasm, where many biological events occur, cannot be attributed to a fortuitous incident. Salts action on life phenomena depend to a great extent on their character and valence[1], with outstanding similarities with their effects on proteins[1]. Loeb[1-2], based on these observations, suggested that salts effect on life phenomena is the result of their action on some proteins.

Innumerous concepts have been advanced to explain the effect of salts upon proteins properties: The "water-attracting power" of salts, first advanced by Hofmeiter[3-4]; the idea that it is a consequence of the effects of the various ions on the structure of the solvent[5-7]; or that is the outcome of specific interactions of the ions along the polymer chain[8],[9-15]. All these theoretical attempts to explain the effect of salts on proteins have failed, inasmuch they are unable to provide a framework of compelling acceptance to reconcile their ideas with the Hofmeister precipitation experiments[3-4, 16]. Hofmeister established, more than one century ago, the ions relative effectiveness, expressed as a series of anions and cations, in precipitating a protein out of solution[3-4, 16]. The Hofmeister approach to the problem has been criticized over the years[2, 10], but the series, or at least similar ones, have been observed in many instances[7].

Proteins have multiple charged groups, most of which are "active" at neutral pH, and these sites are certainly the preferential locus of interaction with ions. We considered the hypothesis that the macroscopic observations of the effects of ions upon proteins properties at neutral pH could be the outcome of multiple events occurring simultaneously, and consequently difficult to disentangle. Our approach consist on a

gradual study of the precipitation phenomena. First by "eliminating" some of these charged sites, i.e. the negative ones, and then gradually letting them to be active. It is hoped in this manner to have a better understanding of the separate effect of ions over protein properties. Accordingly, we studied here the effect of various salts on the precipitation of electro-positive bovine serum albumin (BSA) at a constant temperature. The pH range studied covered extreme acidic conditions up to hydronium concentrations where the dissociation of the protein carboxyl groups become noticeable[17]. BSA is a prototype protein for physicochemical studies[18]. It is commercially available in an adequate degree of purity and at large quantities. It has been studied from many different perspectives[18], which could be advantageous for our analysis. Following the Hofmeister experiments and assumptions, we determined the quantity of salt required for the first clouding of the protein solution to occur. The time required for the clouding phenomenon to happen and the quantity of salted-out protein were also addressed.

Experimental

Materials:

Salts: The salts used were LiCl (from Merck, > 99 %), NaCl (from Panreac, 99.5 %), KCl (from Chem-Lab, 99.5 %), NH_4Cl (from Merck, 99.8 %), $CaCl_2$ (from Panreac, 95 %), $MgCl_2$ (from Sigma, > 99 %), NaBr (from Fluka, 99 %), NH_4Br (Riedel-de Haën, 99.8 %), KI (Normapur, 99.7 %), NaSCN (Merck, > 98.5 %), $NaClO_4.H_2O$ (Panreac, > 98 %), KNO_3 (Panreac, 98 %), $Li_2SO_4.H_2O$ (Merck, 99 %), Na_2SO_4 (Sigma-Aldrich, > 99 %), and K_2SO_4 (Sigma, 99 %). In a preliminary set of experiments the salts were dried under vacuum for at least 48 h. The concentration of stock solutions as measured by atomic adsorption and ion-selective electrodes, prepared from these dried salts, were compared

with stock solutions prepared with salts without additional purification. The drying process proved to be unnecessary, and in all experiments reported here the salts were used without additional purification.

BSA: The bovine serum albumin used was fatty acid free (< 0.02 %) from Fisher Scientific, lot 66-1375, with purity > 98 %, ash content below 3 % (heavy metals < 10 ppm) and an isoelectric point (pI) of ~ 4.7. In preliminary tests, the protein was further purified by dialysis over one week by replacing water every 24 h. The progress of dialysis was checked by conductivity. The protein was afterwards lyophilized. In another set of tests the protein was dried under vacuum from 4 up to 48 h. Several precipitation studies were made and compared using the additionally purified protein or as commercially acquired. The purification procedure proved to be unnecessary. Accordingly, the results reported correspond to the use of BSA without additional purification steps.

Others: Sulfuric acid, 95 %, and hydrochloric acid, 37 %, both from Sigma-Aldrich, were used as received. Stock solutions were prepared in volumetric flasks and titrated against sodium hydroxide using phenolphthalein as indicator. Sodium hydroxide from Panreac, 98 %, was used. Stock solutions were prepared in volumetric flasks and titrated against potassium hydrogen phthalate using phenolphthalein as indicator. Potassium hydrogen phthalate from Panreac, 99.8 %, was dried at 100 ºC overnight. The water used was ultra-pure water, double distilled, passed by a reverse osmosis system and further treated with a Mili-Q plus 185 water purification apparatus.

Methods:

Precipitation experiments: In a first set of experiments it was determined the approximate quantity of salt required to the first clouding of albumin to occurs. Having this estimative, the precipitation curves were then be obtained by measuring the absorbance of protein

solutions differing in the concentration of salts at an adequate wavelength. In a preliminary series of experiments quartz cuvettes of different path lengths, as well as different solution volumes on microplates, were tested. Various wavelengths, namely 350 nm, 450 nm, 600 nm and 720 nm were tested. All tests gave similar results.

The precipitation curves reported here, unless otherwise indicated, were obtained in the following manner: 12 solutions were prepared in eppendorf tubes, all containing the same quantity of protein, acid, and increasing concentrations of a given salt. The final volume (500 μL) was completed by the addition of water. The required quantities of stock solutions of all components and water were dispensed with a Multipette Xstream pipette (Eppendorf, Hamburg, Germany). After the addition of all solutions, with the exception of the protein stock solution, the mixture was homogenized. During the addition of the protein stock solution, eppendorf tubes were gently mixed. The eppendorf tubes were then inverted 5 times, and maintained in an incubator (protected from light) at 25 ºC for 24 h. After this period, aliquots of 150 μL from each tube were transferred with a Multipette Xstream pipette into UV micro plates. The plates, protected from light, were shaken at 250 rpm for 15 min, at 25 ºC, in an incubating microplate shaker with temperature control (VWR, Pennsylvania, USA). The absorbance at 350 nm was then read on a BioTeck Synergy HT microplate reader with temperature control. For the experiments over time the microplates were covered with an appropriate sealing to prevent evaporation. Control tests with time were performed. No signs of eventual evaporation or contamination were detected.

pH measurements: The pH was measured with a Metter Toledo Seven Excellence pH meter with temperature control. To obtain the combination of BSA with acid the following method was used: Solutions with the same concentration of acid without and with protein at a concentration of 10 $g.L^{-1}$ were prepared and left to equilibrate for 1 h at

25 ºC. After calibration according to manufacturer instructions, the electrode was inserted in a sample for at least 5 min at constant temperature (25 ± 0.2 ºC), after which the pH was measured (at least three measurements were performed). The pH values with and without protein were plotted graphically (given in Figure S1 in the Supplementary Information). This type of curve is extremely useful for the precipitation studies, since it provides direct information on the quantity of acid necessary to add to a protein solution to bring it to a required pH value. Additional information can be found in the Supplementary Information.

Results and Discussion

Bovine serum albumin is composed of many dissociable groups[17, 19-20]. In particular, it contains one α- and ninety-nine β,γ-carboxyl groups, whose pk_a values amounts to 3.75 and ~ 3.95[17], respectively. Our study covers the extreme acidic conditions, up to hydronium concentrations corresponding to pH values slightly below the pK_a of the carboxyl groups, where the dissociation of the same are expected to become noticeable. The precipitation results for BSA (pH 2.44) by the action of six chloride salts, sodium bromide and sodium nitrate are illustrated in Figure 1.

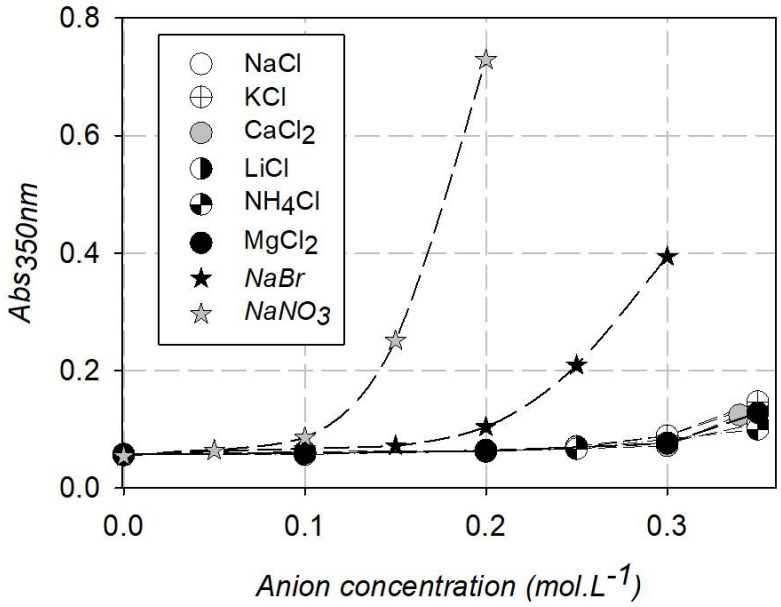

Figure 1. Salting-out of BSA (10 $g.L^{-1}$) by the action of LiCl, NaCl, KCl, NH_4Cl, $MgCl_2$, $CaCl_2$, NaBr and $NaNO_3$ (pH 2.44), after incubation for 12 h at 25 ºC. Here and in the remaining figures, the lines shown have no physical meaning and are provided to aid on the visualization of the data.

152

153 The most relevant information inferred from Figure 1 lays on the behaviour exhibited by
154 the six chloride-based salts. Within the experimental uncertainty, the quantity of salts
155 required to precipitate BSA, expressed in mol per L, is the same for LiCl, NaCl, KCl, and
156 NH_4Cl, which are twice the values required with $MgCl_2$ or $CaCl_2$ to induce the same
157 effect. The outcome revealed by the chloride salts on the salting-out of electro-positive
158 albumin can be rationalized if the protein is assumed to behave like a common salt at this
159 pH range. More specifically, electro-positive albumin reacts with the chloride anion as
160 follows:

161 $$PH_m^{m+} + mCl^- \leftrightarrow P(HCl)_m \qquad (1)$$

162 where PH_m^{m+} represents the electro-positive albumin, P the protein and m the number of
163 hydrogen ions with which the protein combines. Therefore, the presence of Cl^- ions in
164 excess shift the reaction to the right, repressing the ionization of the albumin-salt like
165 compound, leading to its precipitation. It should be remarked that the suggestion that the

albumin behavior at low pH resembles that of common salts is not new[21-23]. Our explanation of the observed phenomenon is in agreement with the interpretation of results obtained by viscosity, freezing point depression, precipitation with alcohols, and electrometric measurements made by others[21-23].

Before going into further discussion of these results, we shall introduce two additional parameters, namely the quantity of salted-out protein and the time required for the clouding phenomenon to occur, two parameters that were never considered together. The full precipitation curve for BSA by the action of NaCl, KCl and $CaCl_2$ is illustrated in Figure 2. The results obtained confirm the previous observations taken from the analysis of Figure 1. It should be remarked that the quantity of the chloride anion added, above which no further precipitation is observed (ca. 0.70 $mol.L^{-1}$) is the same, regardless of the cation with which it is combined. The quantities of salted-out protein can be appraised by the full precipitation curves obtained for different protein concentrations, whose results are depicted in Figure 3. It is evident that the required quantity of NaCl to bring BSA out of solution varies considerably with protein concentration, being in agreement with the literature[3-4, 16, 24]. If a comparison is made at the beginning of precipitation, these values vary from ca. 0.42 M, 0.63 M and 0.75 M NaCl for 10.0, 5.0 and 2.5 $g.L^{-1}$ of BSA, respectively.

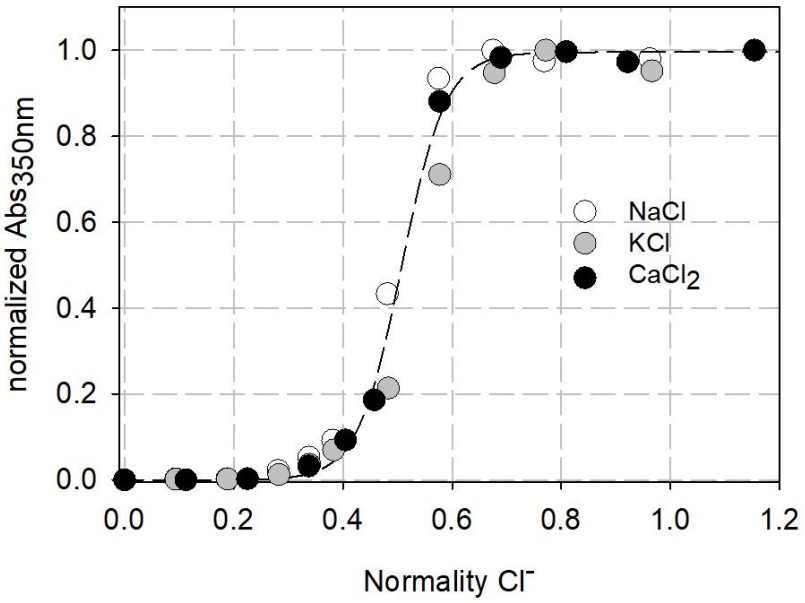

Figure 2. Full precipitation curves of BSA (10 $g.L^{-1}$) by the action of NaCl, KCl and $CaCl_2$ (pH 2.44), after incubation for 12 h at 25 ºC.

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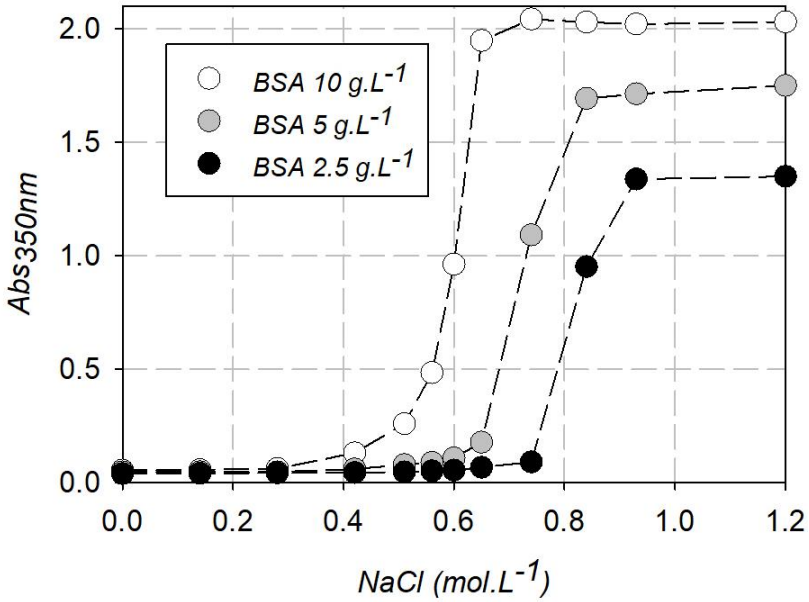

Figure 3. Precipitation curves of BSA at three concentrations (2.5, 5.0 and 10.0 $g.L^{-1}$) by the action of NaCl (pH 2.6), after incubation for 24 h at 25 ºC.

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187 The other relevant parameter is the control of the time required for the clouding to be
188 detected. Its relevance can be gauged from the results depicted in Figure 4. The required
189 quantity of NaCl to precipitate BSA varies between 0.65 M, if immediate clouding is
190 measured, 0.55 M if the measurement is performed after 12 h, down to 0.3 M if
191 precipitation is addressed only after 48 h. Therefore, it is evident that a comparison of the
192 efficacy of different salts without considering the time may lead to wrong conclusions.
193

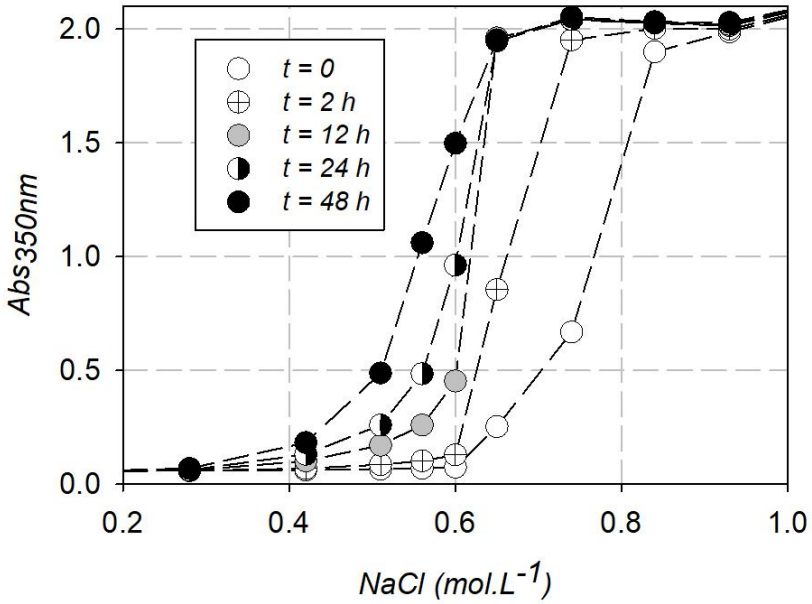

Figure 4. Effect of the time required to salt out BSA (10.0 $g.L^{-1}$) of solution by the action of NaCl. T = 25 ºC, pH 2.6.

194 The behaviour exhibited by albumin at pH 2.44 in the presence of six chloride salts,
195 shown in Figure 1, was tested at other pH values and in the presence of different anions.
196 The reaction of albumin with three different anions, namely Cl^-, Br^- and SO_4^{2-}, each of
197 which combined with three different cations, including Li^+, Na^+, K^+, NH_4^+, Mg^{2+} and
198 Ca^{2+}, was studied at the HCl concentration of 0.0175 M corresponding to a final pH of
199 2.6. The precipitation curves obtained are illustrated in Figure 5. These results obtained
200 are in agreement with those previously described, according to which the anion is the

main species inducing the precipitation of albumin. Alkali metals such as Li^+, Na^+, and K^+, alkaline earth metals such as Mg^{2+} and Ca^{2+}, as well as NH_4^+, have apparently no effect on the salting-out of electro-positive albumin, at least at this pH range. These results agree with those shown before, according to which BSA at extreme acidic conditions seems to behave like a salt ion, whose precipitation can be induced by the excess of anions.

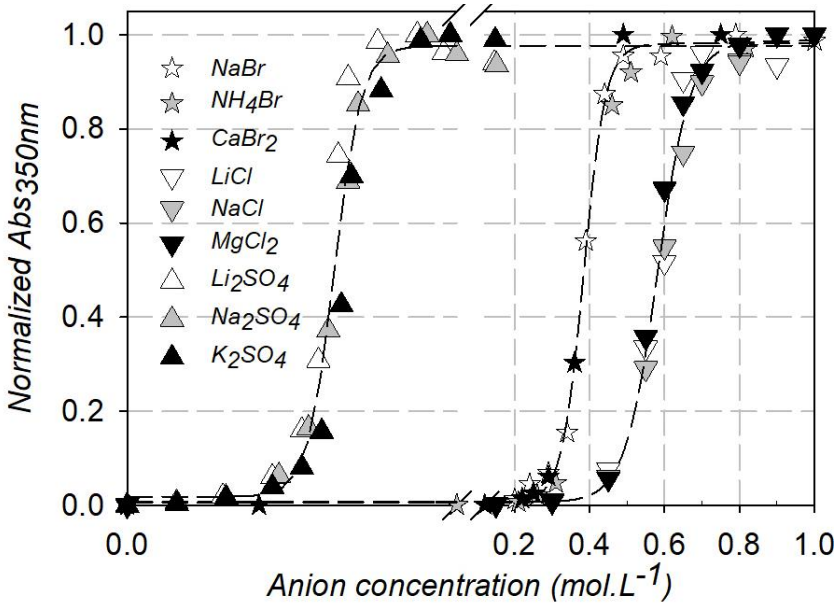

Figure 5. Full precipitation curves of BSA (10.0 $g.L^{-1}$) by the action of nine salts (pH 2.6), after standing for 24 h at 25 ºC.

If the previous assumption is correct, the efficacy of different anions in precipitating a protein can be established at these experimental conditions, i.e. at pH 2.6. The results obtained for seven anions, all combined with Na^+ or K^+, are plotted in Figure 6. The fluoride anion was also studied (NaF), but no clouding was observed up to a salt concentration of 0.8 M, even after 3 days. No higher concentrations could be tested due to the solubility limit of sodium fluoride. The results obtained reveal that the anions

promote the precipitation of the electro-positive form of albumin in the following order: $Cl^- < Br^- < NO_3^- < I^- < SCN^- \sim ClO_4^- < SO_4^{2-}$, where the fluoride anion should be the first in the series.

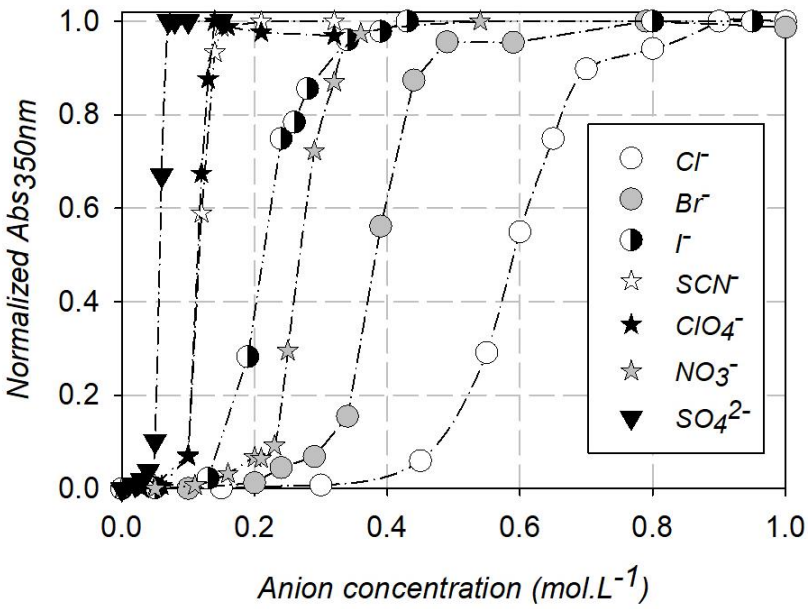

Figure 6. Anions ability to precipitate BSA. The results were obtained at protein concentration of 10 $g.L^{-1}$ at pH 2.6, after incubation for 24 h at 25 ºC.

Our premise, which is based on the formation of albumin-salt like compounds through the interaction of the salt anion with the positive charges on the backbone of the protein, if correct, should harmonize with chemical concepts. According to these, the valence of the ion is of primary relevance. Divalent anions should be more effective in this regard than monovalent ones; the results obtained with sulfate, which is the strongest precipitating agent, are in agreement with this premise. For the monovalent anions, according to traditional chemical concepts [2], the further is the electron away from the nucleus the more easily it combines with positive charges. Therefore, the anions size in aqueous solution should be a good proxy to confirm this hypothesis. Y. Marcus[25] obtained the intrinsic ionic molar volumes for electrolytes in aqueous solutions at 25 °C, reporting

229 the following values: 14.3, 18.1, 27.8, 29.1, 36.0, 46.6, and 47.1 $cm^3.mol^{-1}$ (± 2.0 $cm^3.mol^{-1}$) for F^-, Cl^-, Br^-, NO_3^-, I^-, SCN^- and ClO_4^-, respectively. This trend agrees with our observations and supports our arguments.

232 Since proteins are all composed by the same amino acids, this series for the anions would be of a general character. The effect of salts on different properties of six proteins[26-32], at pH values below the pI, are summarized in Table S1 in the Supplementary Information. The results obtained seems to support the precipitation mechanism here proposed.

236 We additionally performed experiments at higher pH values, where the dissociation of the carboxyl groups was expected to become noticeable. The precipitation results for BSA by the action of six chloride salts are shown in Figure 7.

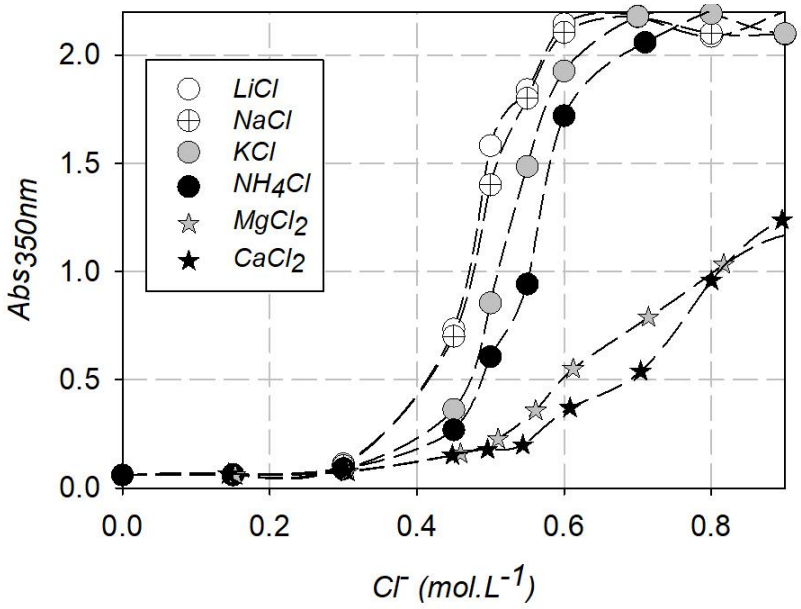

Figure 7. Precipitation curves of BSA (10 $g.L^{-1}$) by the action of six chloride salts (pH 3.08), after incubation for 24 h at 25 ºC.

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240 Two relevant aspects appear from the analysis of Figure 7. First, unlike in the previous set of results, the effect of the cation upon protein precipitation becomes noticeable at this pH 3. The second aspect rests on the quantity of protein salted out, which is higher for

the chloride salts composed of monovalent cations when compared with calcium and magnesium chloride. Cations are traditionally ordered according to their salting-out ability, first by combining them with the same anion, and then comparing the effect of the salts upon the phenomenon. According to this rationalization, the salting-out ability of the cations reported above increases in the following order: $Li^+ \sim Na^+ > K^+ > NH_4^+ > Mg^{2+} \sim Ca^{2+}$. The parallelism between these results and those reported by Hofmeister[3-4] and others[33-34] should be emphasized. The data are insufficient to draw a general conclusion about the relative effectiveness of cations in the salting-out phenomenon. However, we would like to emphasize that the observations pertain to the formation of salt-like compounds, and as such ion association.[35-37] Accordingly, contact, solvent-shared, or solvent-separated ion-pair should be considered as playing a possible role on the phenomenon taking place. Nonetheless, the fact that the salts composed of divalent cations precipitate less quantity of protein than salts formed by monovalent cations cannot be ignored, and a rational for this effect is demanded.

Numerous experimental techniques show that at pH 3 the dissociation of the carboxyl groups present in the protein [38] starts to occur. To address a rational for the observations reported in Figure 7, we hypothesize that different protein forms are present in solution in chemical equilibria between them. We shall represent the equilibrium between them as follows:

$$[P(COOH)_m]^{x+} \leftrightarrow [P(COO^-)_m]^{(x-m)+} + mH^+ \qquad (2)$$

When converting the $P(COOH)_m$ to the $P(COO^-)_m$ form, with a number of x positive charges, m carboxyl groups dissociate, conferring to the $P(COO^-)_m$ -form a (x-m) positive charge. It should be however emphasized that the simplification in the general chemistry

of proteins implied by this formalism is considerable. Nevertheless, the equations used will be helpful for the discussion and to emphasize some aspects relevant in our analysis. Due to the presence of some negative charges at the backbone of the protein, the free cations present in solution will most likely compete with the hydrogen ions for these sites. This concept can be formalized according the following equation, where C_{at}^{+} stands for the cation:

$$[P(COO^{-})_m]^{(x-m)+} + mC_{at}^{+} \leftrightarrow [P(COOC_{at})_m]^{(x)+} \quad (3)$$

Again, for the sake of simplicity, we opted to represent the combination of *m* monovalent cations with the $P(COO^{-})_m$-form. As a result, different protein forms will coexist in equilibrium. The initial protein quantity is subdivided between different forms, the concentrations of which will be lower than the initial one and depend on the equilibrium constants for the speciation to take place.

If our interpretation is correct, the stronger the interactions of the cation with negatively charged sites, the more noticeable this effect will be. These arguments explain the observations made from Figure 7, and in particular that the divalent Ca^{2+} and Mg^{2+} cations interact more strongly than monovalent cations with the negative charge, in coherence with chemical principles. The outcome thus translates in less protein being salted out and more salt required to induce this effect.

According to the general mechanism presented here, and since all protein forms at these pH values are positively charged, generally represented below by P^{n+}, can combine with anions (A^{-}), and the precipitation of all the protein-salt like compounds, below represented by PA_n, can be represented by the following general and simplified equation:

293 $$P^{n+} + nA^{-} \leftrightarrow PA_n \quad (4)$$

294

295 There are at least two important inferences that can be drawn from equations 2 to 4. In
296 the first place, they suggest that at least three different forms of the protein should coexist
297 around these pH values. It is beyond the scope of the present work to analyze in further
298 detail the coexistence of more than two protein forms. However, it is important to stress
299 out that experimental evidence for such phenomena has been given elsewhere and by
300 different analytical techniques (see [38-39] and references cited therein), in support of our
301 proposed mechanism. The other relevant consideration is that under the correct
302 experimental conditions it should be possible to separately precipitate different protein
303 forms. A confirmation of this hypothesis is illustrated in Figure 8. In this experiment, five
304 test tubes, each containing the same quantity of protein (10 $g.L^{-1}$) and hydrochloric acid
305 (ca. 0.0125 N) for a final pH value of 3.05, were prepared. The tubes also contained
306 increasing concentrations of $CaCl_2$, from 0 up to 1M.
307 Just after adding the salt, clouding was observed in presence of $CaCl_2$ at 1M (Figure 8, t
308 = 0 h), whereas in the remaining solutions no immediate clouding was perceived.
309 However, after 24 h, some clouding (Figure 8, t = 24 h) in all solutions containing $CaCl_2$
310 was observed. Furthermore, as the salt concentration raises, an increase in the salting-out
311 is observed up to a $CaCl_2$ concentration of 0.5 M, after which the further addition of salt
312 (up to 0.75 M) induces the protein salting-in, and if still more salt is added (up to 1 M) a
313 salting-out of the protein is again observed. Similar results are observed after days 2 up
314 to six (data not shown), with clouding in all tubes slightly more intense, but with relative
315 intensities similar to those observed after 24 h.

316

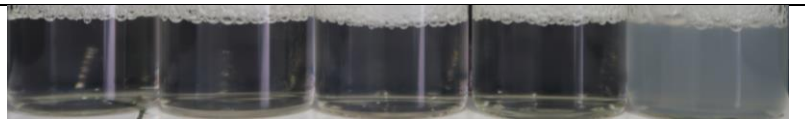
$CaCl_2$ (M)	0	0.25	0.5	0.75	1.0
t = 0 h					
t = 24 h					

Figure 8. Precipitation of BSA at (10 $g.L^{-1}$) with $CaCl_2$ (pH 3.05), at room temperature (~ 25ºC).

317

318 To interpret the results illustrated in Figure 8 it is important to realize that the quantity of
319 salt, or rather salt anion, to induce the salting-out phenomena depends both on the time
320 (see Figure 4) and on the protein concentration (see Figure 3). Also relevant is that the
321 concentration of the various protein forms depends on the quantity and strength of the
322 cation present (see equations 2 and 3 above). According to the results depicted in Figure
323 4, circa 0.65 M of Cl^- are required to produce immediate clouding of 10 $g.L^{-1}$ of the
324 albumin $P(COOH)_m$ form. To induce the same effect, i.e., immediate clouding, for a
325 protein concentration of 5 $g.L^{-1}$ and 2.5 $g.L^{-1}$, 0.8 M and 1.0 M of the chloride anion are
326 required, respectively (see Figure S2 in the Supplementary Information). Thus, the results
327 at t = 0 h in Figure 8, suggest that various forms of albumin coexist in equilibrium, whose
328 concentrations are expected to be lower (probably much lower) than 10 $g.L^{-1}$. The fact
329 that 2.0 M of Cl^- ($CaCl_2$ = 1 M) is required to produce immediate clouding supports this
330 idea.
331 The results at t = 24 h further introduce equilibrium kinetics effects and can be interpreted
332 in the following way. Up to a concentration of $CaCl_2$ 0.5 M, the results in Figure 8 can
333 be rationalized as a "general" salting-out phenomenon, where a protein form
334 predominates. It is premature at this point to speculate about which form predominates,
335 and we shall address this question on a forthcoming work. For the present discussion, we

assume that the $P(COOH)_m$ predominates, though a similar reasoning would holds if the $P(COO^-)_m$-form is the one present at higher concentration. Overall, an increase in the precipitating agent (Cl^-) translates into a higher precipitation of the protein. Above a certain salt concentration, another phenomenon starts to take place since the presence of other protein forms are no longer negligible. The higher the concentration of other protein forms, promoted by the presence of the calcium cations, the lower the concentration of the $P(COOH)_m$-form (or $P(COO^-)_m$-form) (see equations 2 and 3). The lower the concentration of the $P(COOH)_m$-form is, the higher the required quantity of salt to induce its precipitation. Therefore, the salting-in observed at a concentration of 0.75 M $CaCl_2$, is most likely due to the lower quantity of the $P(COOH)_m$-form available. In the last sample, $CaCl_2$ at a concentration of 1 M, there is the precipitation of another protein form, which should now be predominant in solution. It is relevant to stress out that the lack of observation of immediate clouding e.g. at $CaCl_2$ 0.5 M, which is clearly visible after 24 h, does not necessarily means that the equilibrium kinetics between the different protein forms is slow. The slow kinetics may be instead attributed to the precipitation mechanism itself. The results depicted in Figure 4 illustrate our point of view. Here, where a single protein form predominates, it can be seen that for a Cl^- concentration of 0.5M, clouding is only noticeable after 12 h.

In summary, the role of salt ions on the salting-out phenomena when a protein is positively charged can be described as follows:

(i) Anions are the major active species in salting albumin out of solution below its isoelectric point. Their effect seems to be controlled by chemical concepts. The higher the anion valence, the more effective it is in salting-out the protein. For anions with the same valence, the further away are the outer electrons from the nucleus the more effective it is in inducing the protein salting-out.

(ii) The role of the salt cations on the salting-out phenomenon below the protein pI is of a different type. The cations effect only becomes noticeable at pH values where the protein carboxyl groups start to dissociate, whereby different protein forms coexist. According to our interpretation, the cation main role is to participate in chemical equilibrium with protein forms, thus changing the concentration ratio of the protein forms present. The stronger the cation interacts with negative sites of the protein, the more noticeable becomes its effect.

According to our results, salts, which are always present in natural environments where proteins are active, can be agents that promote the equilibrium between different protein forms. Although our studies were restricted to a limited range of pH values, from extreme acidic conditions up to pH values where the carboxyl groups start to dissociate, the mechanism proposed here might be of general validity. More specifically, other amino acid side chain groups are known to dissociate at other pH values[17, 20], inducing the presence of distinct positive/negative charges at the backbone of the protein. Ions might combine with these sites according to a similar mechanism proposed here, thought their roles might interchange when protein's net charge changes from positive to negative, promoting the presence of other protein forms. These ideas explain the occurrence of known distinct BSA conformational transitions at different pH values[18, 40].

The mechanism of action of salts upon and between the proteins forms here proposed might also be a simplified version of reality. It is possible that the equilibrium reactions used above to explain the obtained results, and giving a rational for the coexistence of the known conformational transition of BSA, are indeed global chemical reactions of multiple elementary reactions taking place. Thus, the major reported conformational

isoforms could themselves be composed of different closed-related sub-forms. It is well known that chemical entities differing slightly in chemical structure or charge have drastically distinct biological activity or chemical properties[41]. This explains the extreme adaptability of proteins to stressful conditions, their vast biological activity and chemical reactivity, among many other unique protein properties.

From these considerations, we are inclined to put forward the rather speculative suggestion that the mechanism proposed above for the salting-out phenomena is of general validity and finds correspondence with the manifold protein's manifestations in aqueous solutions, such as enzymatic activity, protein misfolding, denaturation, aggregation, crystallization, protein-protein interactions, among many others. Although there are aspects that remain speculative or for which no conclusive evidence was obtained, it is hoped that the observations reported and interpretations proposed foster additional studies on this field under a new light, and eventually find support by new results.

Conclusions

A general mechanism for the effects of salt ions upon the salting-out of electro-positive albumin is outlined. Ions display different roles and have different impacts. When the protein is positively charged, it forms salt like albumin-anion compounds. The precipitation of the protein-anion compound can thus be rationalized on the suppression of the ionization of the albumin ion in the presence of excess of a common anion. On the other hand, according to our interpretation, the cation participates in chemical equilibria with the different protein forms, promoting the presence of additional proteinaceous-compounds. These ion roles are electrostatic in nature, and the relative effectiveness of

410 the ions on these effects can be rationalized by electric forces between charged bodies.
411 Since proteins are all formed by the same building blocks, the depicted mechanism is of
412 general validity for different proteins, which was also appraised with results taken from
413 the literature.
414

415 Conflicts of interest

416 There are no conflicts of interest to declare.
417

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