*Biomacromol. J. Vol. 1, No. 1, 58-68, July 2015.*

**Biomacromolecular Journal**
www.bmmj.org

# The Effect of Hoffmeister Salts on the Chaperoning Action of β-Casein in Preventing Aggregation of Reduced α-Lactalbumin

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*(Received 23 June 2014, Accepted 21 January 2015)*

## ABSTRACT

Protein aggregation and precipitation is associated with many debilitating diseases including Alzheimer's, Parkinson's, and light-chain amyloidosis. β-Casein, a member of the casein family, has been demonstrated to exhibit chaperone-like activity to protect protein form aggregation. Hofmeister salts (lyotropice series) are a class of ions which have an effect on the solubility and also the stability of proteins. In this study, using a range of Hofmeister salts ($Na_2SO_4$, NaCl and KSCN) altered the rate of aggregation and precipitation of α-lactalbumin. The rate of aggregation of α-lactalbumin increased in the presence of all the added salts. However, $Na_2SO_4$ had the greatest effect on the rate of aggregation of α-lactalbumin. β-Casein effectively prevented the aggregation of α-lactalbumin but not as well as in the presence of the salt. Interestingly, in the presence of $Na_2SO_4$, β-casein was the poorer chaperone toward aggregation of α-lactalbumin compare to in the presence of NaCl and KSCN. Our result showed that all salts had structural effects on the β-casein which affects its chaperone ability. In summary, structural change and kinetic factors maybe be determinant the poorer chaperone ability of β-casein in the presence of salts.

**Abbreviations:** ANS: 1-Anilino-8-naphthalene sulfonic acid, DTT: 1,4-dithiothreitol, α-LA: α-lactalbumin, β-CN: β-casein

**Keywords:** Chaperon, β-Casein, Hoffmeister salts, α-Lactalbumin

## INTRODUCTION

The ability of proteins to fold to their functional states is an astonishing example of the power of biological evolution at the molecular level. Proteins are known to reach their correct conformation from many different unfolded states. The process of folding can be described in terms of a universal mechanism that appears to be based on the generation of the correct overall topology through interactions involving a relatively small number of residues. Protein misfolding is an intrinsic aspect of normal folding within the complex cellular environment, and its effects are minimized in living systems by the action of a range of protective mechanisms including molecular chaperones and quality control systems. Unfolded and misfolded proteins have a tendency to aggregate to form a variety of species including the highly organized and kinetically stable amyloid fibrils [1]. So Protein aggregates have been implicated in a wide variety of diseases known as amyloidoses, including Alzheimer's, Parkinson's, and Prion disease [2].

The folding and translocation of many newly synthesized proteins in the cell is kinetically assisted by specialized proteins known as molecular chaperones. In the absence of chaperones, the unfolded proteins could mutually associate *via* exposed hydrophobic regions and precipitate out of the solution. Chaperones recognize these unfolded proteins and prevent their aggregation by formation of a complex with proteins. In other word, they recognize unfolded proteins and prevent their aggregation by forming a complex with the proteins [3].

Bovine α-lactalbumin, with molecular mass of 14,200

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KDa [4] (Acharya *et al*. 1991), is a globular protein found in milk. It contains four disulfide bonds and chelates calcium and zinc ions, thus modulating their absorption [5]. A partly folded state of α-lactalbumin can be induced by removing the calcium ion at neutral pH [6-7], which makes the disulfides prone to reduction and leads to protein unfolding [8]. This form of α-lactalbumin, called the 'molten globule' state, has little tertiary structure but retains most elements of its secondary structure [9]. The molten globule state of α-lactalbumin is thought to be a target for interacting with sHsps [8,10-11]. Being an unstable state, it can aggregate and precipitate from solution. This aggregation is suppressed by molecular chaperones such as sHsps [8,11]. Caseins are a family of phospholipid proteins in milk contains αS1, αS2, β, κ-casein. These proteins are commonly found in mammalian milk, making up 80% of the proteins in cow milk and between 20% and 45% of the proteins in human milk [4]. The β-casein molecule is a single polypeptide consisting of 209 amino acids with a molecular mass of ~24 kDa. The basic charge of the molecule is focused on the polar hydrophilic N-terminal region in contrast to the almost neutral hydrophobic C-region, so that the β-casein molecule has pronounced amphiphilic properties. Only short fragments of the protein polypeptide chain form small regions of the secondary structure makes the molecule more flexible. Due to these characteristics, β-casein in water solution exhibits surfactant properties and a strong tendency to self-association forming spheroidal micelles [5].

The Hofmeister series (lyotropice series) represents an ordering of ions that widely influence chemical and physical processes in biological and colloidal systems. The series was discovered in 1888 by Franz Hofmeister whereby it was shown that ions had consistent effects on the solubility of proteins [6]. In general, protein aggregation and precipitation can be modified or regulated by the addition of salts from the Hofmeister series, which `ranks' salts according to their effectiveness in disrupting the structure of water, *i.e.* $SO_4^{2-}$ ($HPO_4^{2-}$) > $F^-$ > $Cl^-$ > $NO_3^-$ > $Br^-$ > $I^-$ (=$ClO_4^-$) > $SCN^-$. The first ions in the series (*e.g.* $SO_4^{2-}$) increase the surface tension of water and the structure of water molecules around hydrophobic groups, thereby decreasing the solubility of non-polar molecules. In contrast, the last ions in the series decrease the surface tension of water, thereby increasing the solubility of nonpolar molecules. In other word the anions on the left side are increasingly kosmotropic. Moving to the right, the anions become increasingly chaotropic. Chloride is often considered to be the dividing line between chaotropic and kosmotropic behavior [6,7].

## MATERIALS AND METHODS

### Materials

Bovine α-lactalbumin (14 kDa), β-casein (24 kDa), 1,4-dithiothreitol (DTT), $NaN_3$, 1-anilino-8-naphthalene sulfonic acid (ANS), Hoffmeister salts ($Na_2SO_4$, NaCl and KSCN) were obtained from Sigma-Aldrich (St. Louis, USA).

### Visible Absorption Spectroscopy

The ability of β-casein to prevent the aggregation of α-lactalbumin in the presence and absence of Hoffmeister salts ($Na_2SO_4$, NaCl and KSCN) was measured *via* visible absorption spectroscopy.

α-Lactalbumine (in a 50 mM sodium phosphate buffer, 1 mM EDTA, pH 7.4, 0.05% $NaN_3$) was incubated at 37 °C in the presence and absence of 0.1 M salt and β-casein (1:1 molar ratio). The unfolding and aggregation of proteins was induced by adding DTT to a final concentration of 20 mM. Light scattering was then observed at 340 nm at 37 °C using an ELISA plate reader (Biotek E808, USA).

The rate constant of the aggregation of the target protein was determined by fitting an exponential function $F(t) = A_1 + A_2 (1 - e^{-kt})$ to the light scattering data using Sigmaplot software (version 8.0). A1 and A2 are constants such that F(0) = A1 + A2, where κ is the rate constant and t is the time (in minute). Each experiment was done at least three times, and the results are shown as means ± SEM.

### Fluorescence Spectroscopy

Intrinsic fluorescence intensity was monitored on samples containing α-lactalbumin (10 μm), in the presence and absence of β-casein (10 μM) and Hofmeister salt (0.1 M) to investigate the effect of salt on the environment of the tryptophan (Trp) residues of α-lactalbumin and β-casein. Samples were incubated in a 50 mM sodium phosphate buffer, 0.05% (w/v) $NaN_3$, at pH 7.4 for 3 h. Fluorescence

intensity was measured on a varian fluorescence spectrofluorimeter. Tryptophan residues were excited at 295 nm using a 2.5-nm slit width, and emission spectra were recorded from 300-400 nm with a 5-nm slit width. Samples were placed in a 10-mm pathlength quartz cuvette. The spectrofluorimeter was set to 700 V with a scan speed of 240 nm $min^{-1}$.

An ANS binding assay was used to assess changes in the exposure of hydrophobic pocket of α-lactalbumin upon interaction of target protein with β-casein in the presence and absence of Hofmeister salt. α-Lactalbumin (10 μm), was incubated in the presence and absence of β-casein (in 1:1 molar ratio) and salt (0.1 M). The experiments were done in a 50 mM sodium phosphate buffer, 0.05% (w/v) $NaN_3$, and pH 7.4 with 20 mM DTT. The ANS fluorescence was monitored on a Varian spectrofluorimeter. The excitation and emission wavelengths were set to 400 and 600 nm, with 2.5- and 5-snm slit widths. The fluorescence emission intensity was studied in a 10-mm pathlength quartz cuvette with a volume of 350 μl titrated with 1 μl of a 10-mM ANS solution in a 50 mM sodium phosphate buffer, 0.05% (w/v) $NaN_3$, pH 7.4, with 1 min of stirring after each addition. Titration was continued until the fluorescence intensity reached a plateau.

### Circular Dichroism Spectroscopy

The overall tertiary and secondary structure of β-casein in the presence and absence of Hofmeister salts ($Na_2SO_4$ , NaCl and KSCN) was examined using far- (190-250) and near-UV circular dichroism (CD) spectroscopy (250-350 nm). The experiments were done in a 10 mM phosphate buffer (pH 7.4). The measurements were taken in a 1 cm pathlength cuvette using a JASCO J-810 spectropolarimeter (Jasco, Corporation, Japan) connected to a Desaga water bath. Spectra were recorded with a data interval of 1 nm, response time of 4 S and a scan rate of 100 nm $min^{-1}$. Each spectrum was an average of four scans, with a baseline scan subtracted.

## RESULTS

### The Effect of $Na_2SO_4$, NaCl and KSCN on β-Casein Chaperone Activity by Visible Aabsorption Spectroscopy

Visible absorption spectroscopy has been done to research the effect of $Na_2SO_4$, NaCl and KSCN on the chaperoning activity of β-casein in preventing the amorphous aggregation of α-lactalbumin. α-Lactalbumin, as a target protein was used to determine the effects of mentioned salts on its aggregation. After adding DTT, reduction of disulfide bonds of α-lactalbumin was accrued and the unfolding, aggregation and precipitation of this protein were induced. This is apparent from the increase in light scattering at 340 nm that means the aggregation of reduced α-lactalbumin occurred (Fig. 1a). After addition of $Na_2SO_4$ the increase in the light scattering was observed which indicates that $Na_2SO_4$ increased the rate of aggregation and precipitation of reduced α-lactalbumin. This is also apparent from the first rate constant such that in the absence of $Na_2SO_4$, the rate constant of α-lactalbumin is $2.1 \pm 0.02$ $min^{-1}$ while in the presence of $Na_2SO_4$ it increased to $(7.9 \pm 0.03)$ $10^{-1}$ $min^{-1}$ (Table 1).

In the presence of β-casein at a 1:1 molar ratio of α-lactalbumin: β-casein, the aggregation and precipitation of α-lactalbumin decreased. However in the presence of $Na_2SO_4$ the ability of β-casein to prevent this aggregation was markedly decreased (Fig. 1a). The rate constants for α-lactalbumin in spectroscopy also clearly approved the effect of $Na_2SO_4$ on decreasing chaperone activity of β-casein. (Table 1).

In the case of NaCl, this salt also increased the rate of aggregation and precipitation of reduced α-lactalbumin but with a lower effect compare to $Na_2SO_4$. As shown in the Fig. 1b and Table 2 in the present of this salt the chaperon ability of β-casein also decreased. Although, NaCl showed a less effect on the chaperone ability of β-casein compare to $Na_2SO_4$. The calculated rate constants approved that (Table 2).

KSCN was also increased the aggregation and precipitation of α-lactalbumin but with the lowest effect compare to $Na_2SO_4$ and NaCl. In other word, aggregation and precipitation of reduced α-lactalbumin in the presence of KSCN have not so much differ compare with the absence of this salt (Fig. 1c). However in the presence of KSCN, like other salts, the chaperon activity of β-casein was also decreased (Table 3).

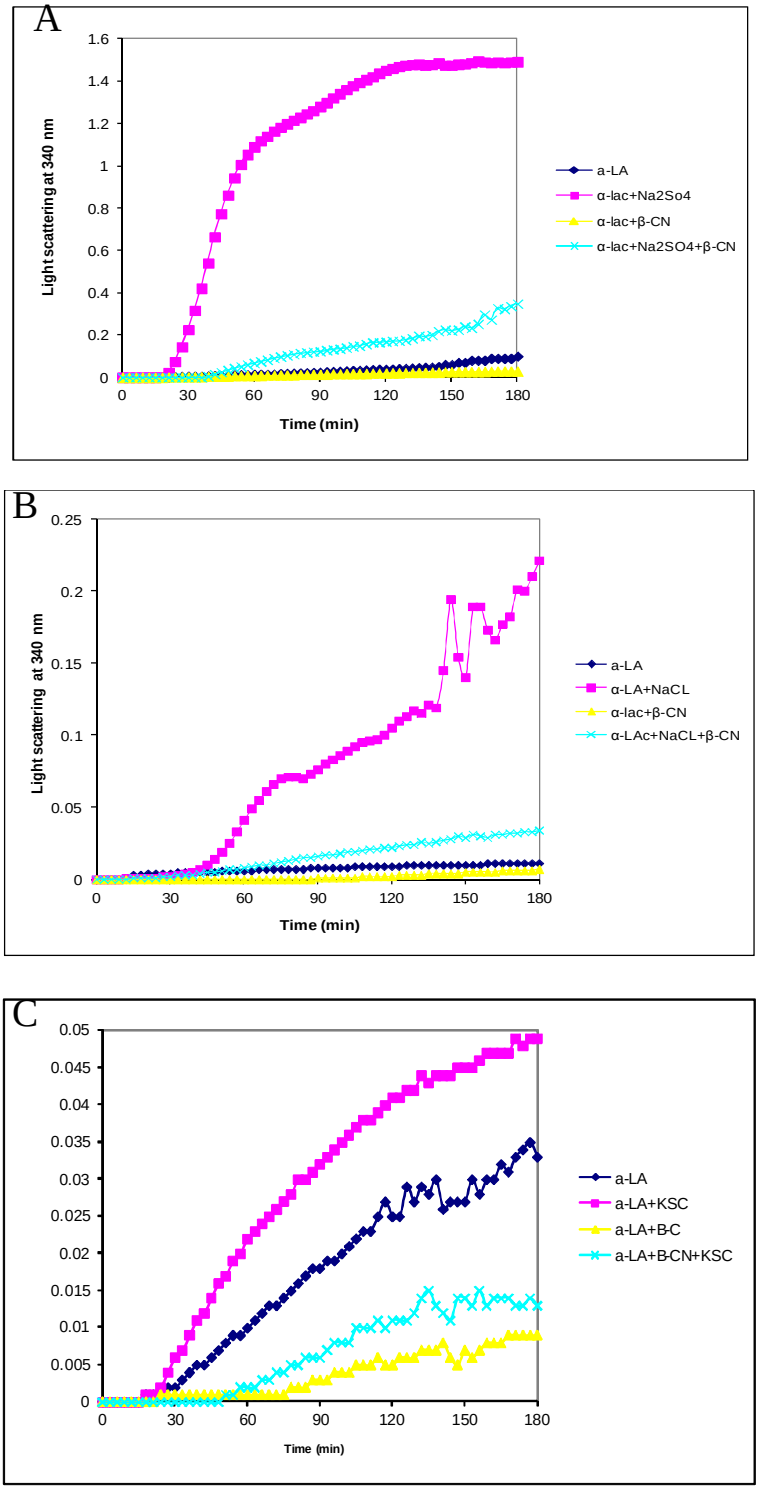


**Fig. 1.** Visible absorption spectroscopy at 340 nm of α-lactalbumin (2.5 mg. $ml^{-1}$) aggregation in the presence and absence of β-casein (in 1:1 molar ratio) and Hofmeister salts (0.1 M): $Na_2SO_4$ (A), NaCl (B) and KSCN (C). All experiments were conducted in a 50 mM sodium phosphate buffer, 0.05% $NaN_3$, pH 7.4 With the addition of 1 mM EDTA at 37 °C. Note that all the experiments were done three times.

**Table 1.** The Rate Constants for α-Lactalbumin in Spectroscopy Results in the Presence and Absence of β-Casein and $Na_2SO_4$.The Rate Constants were Calculated by Fitting Exponential Function toL light Scattering Data Using Sigmaplot Software

| Samples components | Rate constant× $10^{-1}$ ($min^{-1}$) |
|---|---|
| α-LA | 2.1 ± 0.02 |
| α-LA+ $Na_2SO_4$ | 7.9 ± 0.30 |
| α-LA+ β-CN | 1.6 ± 0.01 |
| α-LA+ $Na_2SO_4$+ β-CN | 2.4 ± 0.02 |

**Table 2.** The Rate Constants for α-Lactalbumin in Spectroscopy Results in the Presence and Absence of β-Casein and NaCl. The Rate Constants were Calculated by Fitting Exponential Function to Light Scattering Data Using Sigmaplot Software

| Samples components | Rate constant× $10^{-1}$ ($min^{-1}$) |
|---|---|
| α-LA | 3.1 ± 0.1 |
| α-LA+ NaCl | 4.0 ± 0.3 |
| α-LA+ β-CN | 2.8 ± 0.2 |
| α-LA+ NaCl+ β-CN | 3.4 ± 0.1 |

**Table 3.** The Rate Constants for α-Lactalbumin in Spectroscopy Results in the Presence and Absence of β-Casein and KSCN. The Rate Constants were Calculated by Fitting Exponential Function to Light Scattering Data Using Sigmaplot Software

| Sample components | Rate constant× $10^{-1}$ ($min^{-1}$) |
|---|---|
| α-Lac | 3.9 ± 0.02 |
| α-LA+ KSCN | 4.2 ± 0.30 |
| α-LA+ β-CN | 2.7 ± 0.01 |
| α-LA+ KSCN+ β-CN | 3.2 ± 0.10 |

## Intrinsic Fluorescence Spectroscopy of α-Lactalbumin in the Presence and Absence of β-casein and Hoffmeister Salts ($Na_2SO_4$ , NaCl and KSCN)

For further research about the effect of $Na_2SO_4$, NaCl and KSCN on the aggregation of α-lactalbumin and the chaperon activity of β-casein, intrinsic fluorescence spectroscopy was done. It has been shown that the intrinsic fluorescence of tryptophan residues is altered during the process of protein unfolding [8]. Thus, changes in fluorescence intensity can indicate alterations in the polarity of the environment of the tryptophan residues during interaction between the chaperone and the target protein [9].

α-Lactalbumin showed high fluorescence intensity due to the Trp residue. Upon addition of $Na_2SO_4$ to reduced α-lactalbumin the intensity of maximum fluorescence intensity increased (Fig. 2A). This indicate the structural change around the Trp residue of reduced α-lactalbumin and exposure the tryptophan residues of this protein. Adding β-casein to reduced α-lactalbumin led to decrease the maximum fluorescence intensity such that the fluorescence intensity of sum of the β-casein and α-lactalbumin (α-LA+β-CN) was lower compared to the sum of them alone (α-LA)+(β-CN) (Fig. 2A). This means that the β-casein protected α-lactalbumin from unfolding upon the formation of a complex with α-lactalbumin [12]. By adding $Na_2SO_4$ to the mixture of α-lactalbumin and β-casein, the fluorescence intensity was increased compare with the absence this salt. This shows the effect of $Na_2SO_4$ either on the chaperone ability of β-casein or on the unfolding of α-lactalbumin (Fig. 2A).

Adding NaCl to reduced α-lactalbumin increased the maximum fluorescence intensity. So this salt also changed the structure of the reduced α-lactalbumin and consequence exposure of the tryptophan residues (Fig. 2b). In addition, NaCl increased the fluorescence intensity of sum of the two target and chaperon proteins, so this salt also caused the decrease in the chaperoning ability of β-casein (Fig. 2B).

KSCN like other salts ($Na_2SO_4$ and NaCl) also showed the same trends (Fig. 2C).

## ANS Binding to the Mixtures of β-Casein and Hoffmeister Salts with the Reduced α-Lactalbumin

ANS is a hydrophobic fluorescent molecular probe that has been used for examining the non-polar character of proteins and membranes. In other word this dye attaches to the exposured hydrophobic surfaces, so it could be used to determine the structural changes of proteins [11,12].

The effect of Hoffmeister salts on ANS binding by β-casein and α-lactalbumin was examined to investigate on the exposure of hydrophobic pocket and the conformation changes of these proteins in the presence and absence of $Na_2SO_4$, NaCl and KSCN. As revealed in Fig. 3A, reduced α-lactalbumin showed high ANS binding which is indicative of the exposed hydrophobic regions on the surface of protein. Upon the addition of $Na_2SO_4$, the ANS fluorescence intensity increased about 54%, which is indicative of the more exposure of hydrophobic regions to the solution. By addition of β-casein to the reduced α-lactalbumin, the fluorescence intensity decreased 41% compare to the sum of those of the individual component proteins, implying that there was interaction between them. In the presence of $Na_2SO_4$, however, the fluorescence intensity of α-lactalbumin decreased only 19% by addition of β-casein. Control experiment showed that upon addition of $Na_2SO_4$ the fluorescence intensity of β-casein decreased by 70%. In addition as we mentioned $Na_2SO_4$ increased the fluorescence intensity in α-lactalbumin. It may be concluded that the decrease in ANS fluorescence for the reduced α-lactalbumin and β-casein mixture in the presence of $Na_2SO_4$ most likely arises from the increase and decrease in the exposure of the hydrophobic groups of the reduced α-lactalbumin and the β-casein respectively.

The other salts (NaCl and KSCN), exhibited very similar trends implying that all salts have effect on the structure of both α-lactalbumin and β-casein (Figs. 3B & C).

## Circular Dichroism Spectroscopy of β-Casein in the Presence and Absence of Hoffmeister Salts

Our result proved that in the presence of $Na_2SO_4$, NaCl and KSCN the chaperone activity of β-casein decreased. Now in order to investigate the effect of Hoffmeister salts on the structure of β-casein the circular dichroism spectroscopy as a supplementary technique has been done.

CD spectroscopy in the near-UV of β-casein showed negative ellepticity at 261-270 nm, 280-290 nm and 298 nm due to the eight phenylalanine, three tyrosine and one tryptophan residues [14]. Adding $Na_2SO_4$ to β-casein shifted

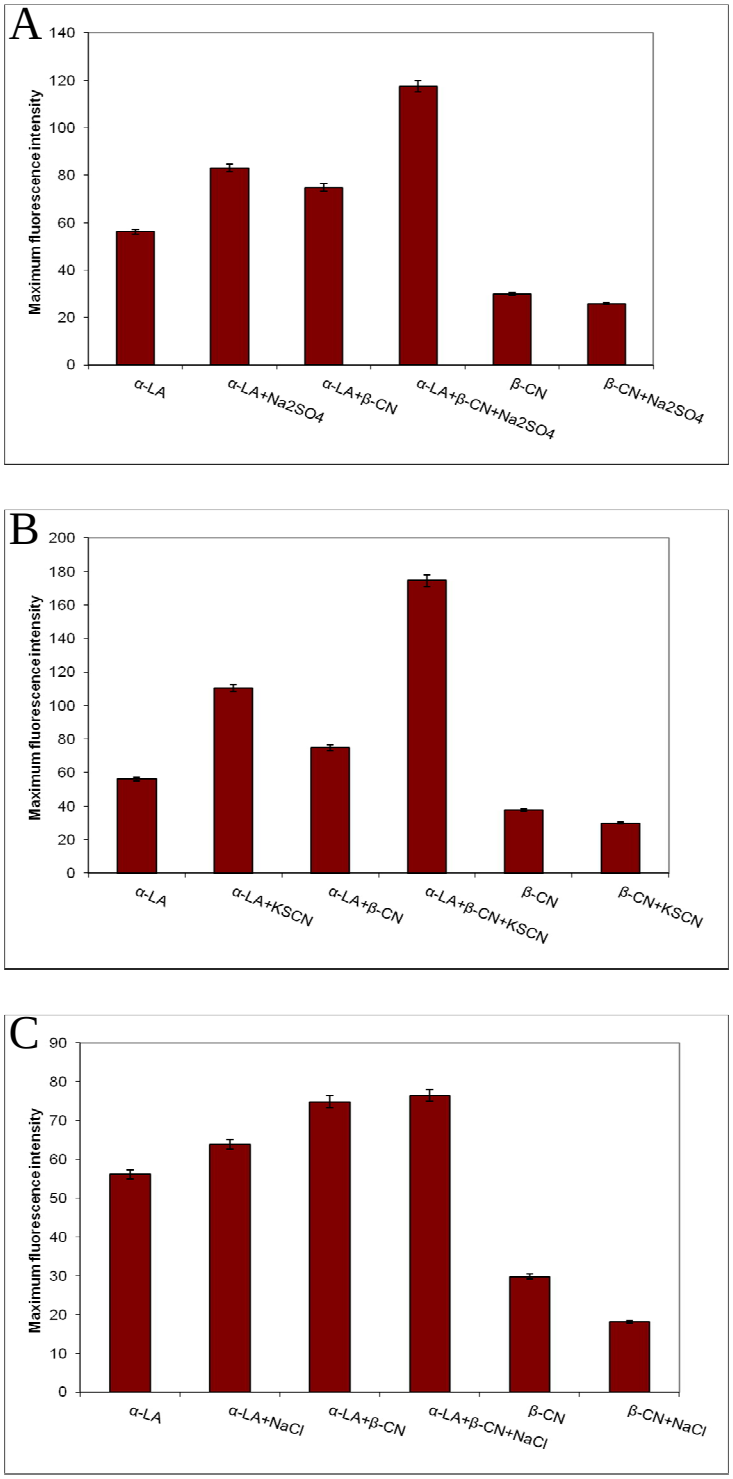


**Fig. 2.** The maximum intrinsic fluorescence measurement of α-lactalbumin (10 μM) in the presence and absence of β-casein (10 μM) and Hoffmeister salts includes ($Na_2SO_4$ (A), NaCl (B) and KSCN (C) (0.1 M). All experiments were done in a 50 mM sodium phosphate buffer, 0.05% $NaN_3$, and pH 7.4 at 37 ° in 3 h incubation.

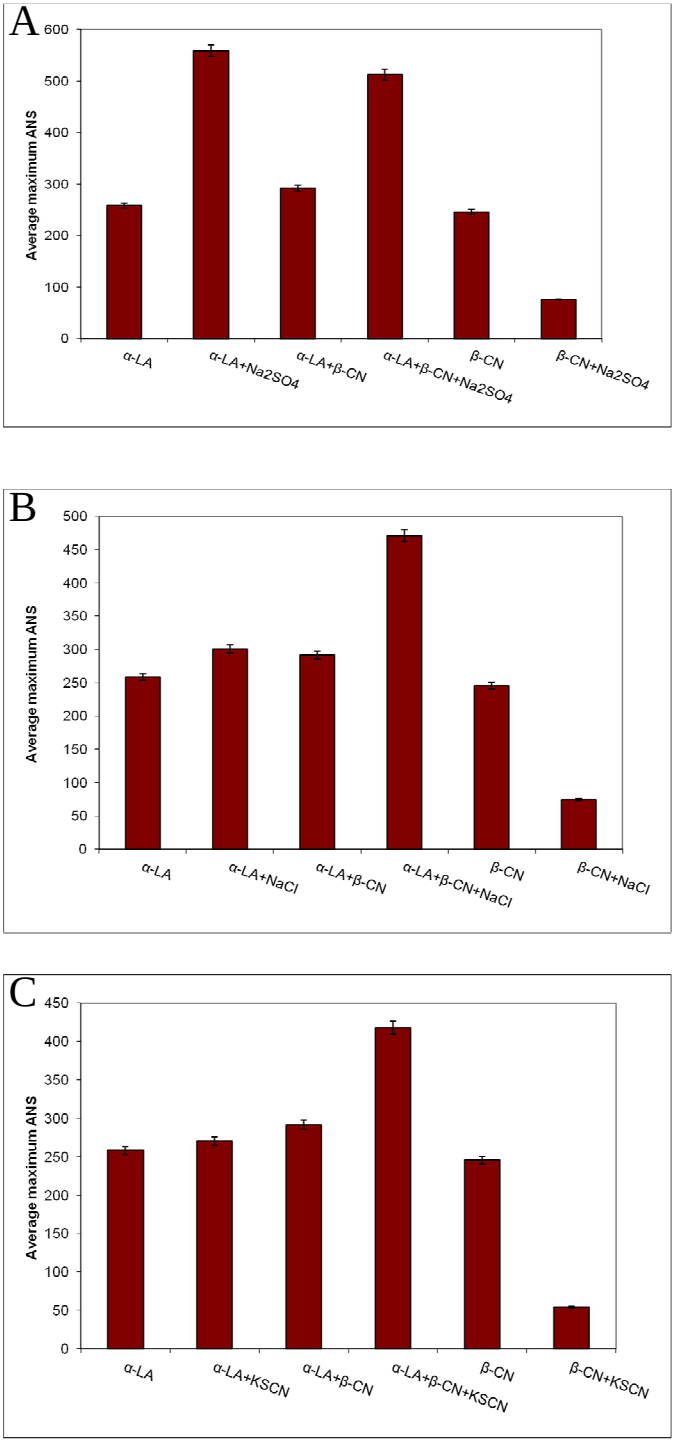


**Fig. 3.** Average maximum ANS fluorescence bound of α-lactalbumin (10 μM) and β-casein (10 μM) in the presence and absence of $Na_2SO_4$ (a), NaCl (b) and KSCN (c) (0.1 M). All experiments were conducted in a 50 mM sodium phosphate buffer, 0.05% $NaN_3$ and pH 7.4 at 37 °C

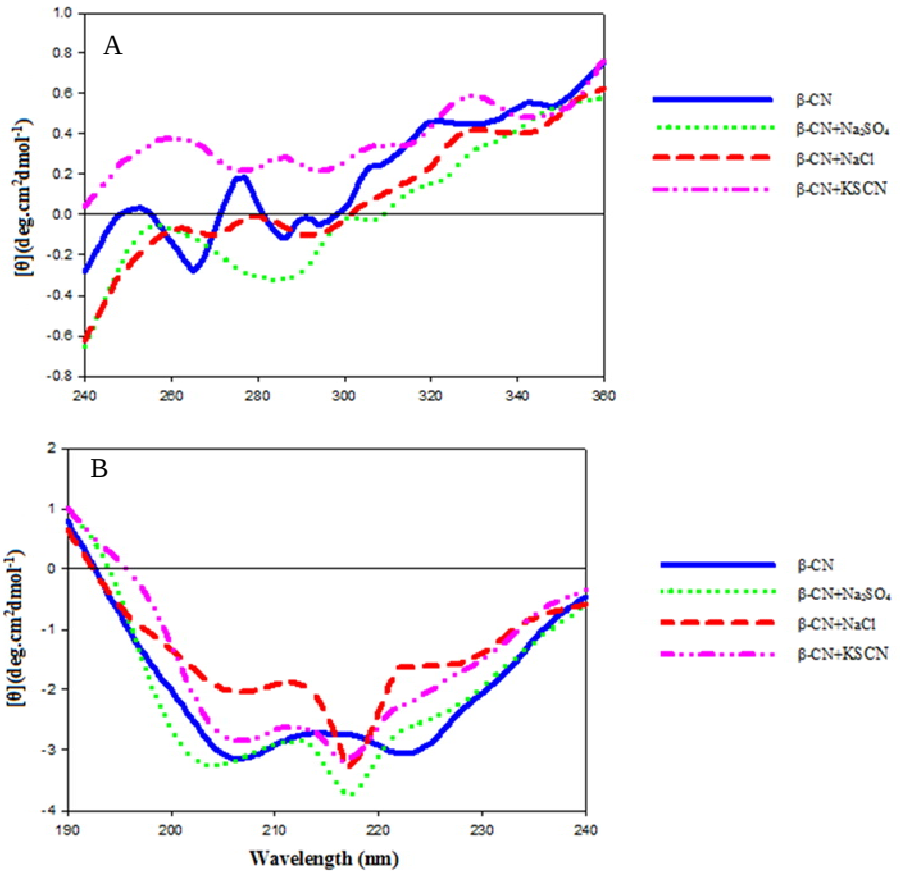


**Fig. 4.** Near-UV (a) and Far-UV (b) CD spectra of β-casein alone and in the presence of $Na_2SO_4$, NaCl and KSCN. Protein concentration was 0.4 and 0.2 $mg.ml^{-1}$ for near and far-UV spectroscopy, respectively, in 10 mM phosphate buffer, pH 7.2 and 37 °C in a J.810 spectropolarimeter with a 1 cm pathlength cell.

the signal of the Phe residue from 261-270 nm to the more positive and shorter wavelengths, indicating some change in the environment of the phenylalanine residue. In addition, $Na_2SO_4$ shifted the signal around the 280-290 nm wavelengths and the 298 nm toward the longer wavelengths that means the effect of $Na_2SO_4$ in the environments around of the tyrosine and tryptophan. NaCl and KSCN showed the same effects (Fig. 4A). NaCl and KSCN showed the same effect indicating Hoffmeister salts had an effect on the tertiary structure of β-casein.

To investigate the effect of Hoffmeister salts on the secondary structure of β-casein, Far-UV spectroscopy was done. β-Casein alone showed a negative ellipticity at around 208 and 222 nm indicating α-helixes structure. Far-UV CD measurements of β-casein in presence of all salts showed an ellipticity at 217 nm that demonstrated the β-sheets structure (Fig. 4B). In addition as shown in (Fig. 4B) the ellipticity of β-casein related to the $Na_2SO_4$ showed more decrease in the ellipticity compared to the other salts. Thus $Na_2SO_4$, had the most effect on the structure of β-casein.

## DISCUSSIONS

Protein misfolding is phenomenon leading to aggregation and precipitation. During the aggregation, proteins lose their natural configurations and accumulate together and finally precipitate. Chaperons such as β-casein decrease the aggregation of protein *via* the formation of a

complex with intermediate protein. Hoffmeister salts include $Na_2SO_4$, NaCl and KSCN have been shown to have an effect on protein aggregation and precipitation [7]. In this study, the effects of Hoffmeister salts on the chaperoning actions of β-casein to prevent the aggregation of target protein investigated.

Visible absorption spectroscopy illustrated that the aggregation and precipitation of reduced α-lactalbumin in the presence of all used Hoffmeister salts increased. But depending on the kind of salt, the effect on the protein was different *i.e.* $Na_2SO_4$ had the most effect on the rate of aggregation and precipitation compare to the NaCl and KSCN respectively. In fact, the effect of these salts on the rate of protein aggregation is a rough order of $Na_2SO_4$ > NaCl > KSCN. This is agreement with the result of the lindner *et al*. [7] that they showed the same result.

$Na_2SO_4$ is the first salt in Hoffmeister series and has kosmotropic behavior. It increases the surface tension of water and the structure of water molecules around the hydrophobic groups that causes the decreasing in solubility of non-polar molecules. As a consequence enhances the hydrophobic interactions and increase aggregation. In contrast, KSCN is the last salt in Hoffmeister series and has strongly chaotrope behavior. It decreases the surface tension of water around the non-polar groups and increases the solubility of them thereby debilitates the hydrophobic interactions. Thus, this leads to decrease the rate of aggregation and precipitation of protein. NaCl in terms of above features is between the $Na_2SO_4$ and KSCN [15].

The chaperone action of β-casein in preventing the aggregation of a variety of target proteins has been previously demonstrated [16-19]. Light scattering results also indicated that β-casein effectively prevented the aggregation of reduced α-lactalbumin. Although in the presence of salts used in this study, chaperoning ability of β-casein has been decreased such that in the presence of $Na_2SO_4$, β-casein showed the less chaperone ability.

There are two possible causes for this observation: (a) Increasing in the rate of aggregation and precipitation of α-lactalbumin in the presence of salts (b) The effect of these salts on the structure and as a consequence chaperone activity of β-casein.

Two possible mechanisms by which salts interfere with chaperone action of β-casein were investigated by intrinsic fluorescence spectroscopy and ANS binding assay.

With regards to point a the intrinsic fluorescence of reduced α-lactalbumin showed a large structural change occurred around the environmental polarity of Trp residues in the presence of all salts. In confirmation of intrinsic results, ANS binding assay also showed that the presence of salts resulted in the exposure of hydrophobic region in the α-lactalbumin. In relation to point b intrinsic fluorescence indicated that salts caused some structural change in β-casein in its C-terminal domain which affects its chaperone ability. Hoffmeister salts induced the polarity of the environment of Trp residues in β-casein. Thereby β-casein in the presence of these salts was not able to do its chaperon activity as well as in the absence of salts. ANS binding assay also showed that the presence of salts the exposure of hydrophobic region in the α- in β-casein decreased. Circular dichroism spectroscopy also showed that the effect of $Na_2SO_4$, NaCl and KSCN on the tertiary structure of β-casein and the environment of aromatic residues was significant. Similarly, Far spectra showed that all Hofmeister salts used in this study, especially $Na_2SO_4$, had an effect on the secondary structure of β-casein.

## CONCLUSIONS

In overall, our results indicated that all of salts have been used, had an effect both on the destabilizing of α-lactalbumin and conformation of β-casein. β-Casein suppressed aggregation of α-lactalbumin but not as well as in the absence of salts. β-Casein interact with stressed target protein by its C-terminal to preventing aggregation [17]. Salts had effect on the conformation and chaperoning action of β-casein probably by effect on the C-terminal region. Since the effect of salts on the aggregation of α-lactalbumin and structural change of β-casein were different, so chaperone ability of β-casein in the presence of different salt was different. Thus kinetic factor and structural change in β-casein is determinant the reduce efficiency of β-casein as a molecular chaperone in the presence of salts.

## ACKNOWLEDGMENTS

Financial assistance from the Research Council of University of Sistan and Baluchestan is gratefully

acknowledged.

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