Heteroprotein complex formation of bovine serum albumin and lysozyme: Structure and thermal stability

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The formation of a heteroprotein complex obtained by the interaction of bovine serum albumin (BSA) and lysozyme (Lys) was investigated by pH variation using turbidimetric analysis and zeta potential (ζ) at different protein ratios and NaCl concentrations. The complexes were formed in a pH range between 8.0 and 11.0, with the ratio r = 0.5 at pH 9.0 presenting the highest complexation. The addition of NaCl decreased the interaction at concentrations of 10 mM. The complex formation occurred between the isoelectric points (pI) of the proteins, close to a balance of charges, mainly by electrostatic interactions with some participation of hydrogen bonds. Differential scanning calorimetry suggested that the interaction gave rise to a new biopolymer due to the formation of a single denaturation point at 67 °C. The structures formed had an average size of ~1.7 μm, well above that of the isolated proteins, and microscopic analysis revealed that the complexes had a globular structure. BSA/Lys complexes may be a potential bioactive encapsulating agent and may be used as a food ingredient.

1. Introduction

Coacervation is defined as the colloidal separation of two-phase liquid systems (IUPAC, 1997), and complex coacervation, known as an associative phase separation, is motivated by the attraction of oppositely charged biopolymers (Souza & Garcia-Rojas, 2017; Tolstoguzov, 1991; Turgeon, Schmitt, & Sanchez, 2007; Zhang, Zhang, Abbas, & Karangwa, 2013). Coacervate complexes are formed mainly by electrostatic interactions, and for this reason, their formation has been influenced by variations in pH and ionic strength because they can change the surface charge density of the molecules (Li, Shim, Wang & Reaney, 2012). Four pH values can be identified during the formation of complexes: pH2 represents the formation of soluble complexes between biopolymers and can be observed with a slight increase in turbidity during the titration; pH1 marks the formation of insoluble complexes (coacervates) and is observed as the rapid increase in turbidity; pHmax marks the maximum point of turbidity and represents the point of electrical equivalence; and pHβ occurs shortly after reaching the maximum and is observed as the reduction in turbidity, indicating the end of the complex formation (Kruij & Tuinier, 2001; Liu, Shim, Wang, & Reaney, 2015; Souza & Garcia-Rojas, 2017; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003).

A number of studies have reported the formation and potential technological application of the coacervate complexes between polysaccharides and proteins (Souza & Garcia-Rojas, 2015; Water et al., 2014; Yuan, Kong, Sun, Zeng, & Yang, 2017). More recently, studies have been proposed to understand protein-protein complex coacervation (Anema & de Kruij, 2014; Anema & Kruij, 2013; Pathak, Rawat, Aswal, & Bohidar, 2016). A heteroprotein complex between two animal proteins, lactoferrin and β-lactoglobulin, has been well studied (Yan et al., 2013; Flanagan et al., 2015). However, Adal et al. (2017) recently proposed a study of heteroprotein complexes between lactoferrin and pea proteins. Despite a limited number of studies, these heteroprotein complexes have already been shown to be a versatile agent for the protection and transport
of bioactive foods, as reported by Diarrassouba et al. (2015), when proposing the encapsulation of vitamin D3 using β-lactoglobulin and lysozyme, and Chapeau et al. (2016), when using lactoferrin and β-lactoglobulin for the encapsulation of vitamin B9.

Among food ingredients, proteins are one of the most indispensible due to their functional and technological properties (Howell, Yeboah & Lewis, 1995). Eggs, especially egg whites, are one of the most consumed high-protein foods with high nutritional and functional value (Kovacs-Nolan, Phillips, & Mine, 2005; Stadelman & Cotterill, 1990). Lysozyme is a globular glycoprotein with enzymatic action presenting in high concentrations in egg whites (3.4%). It has a molar mass of 14 kDa, an isoelectric point (pI) of 10.7 and numerous functional properties, which are mainly antimicrobial (Sgarbieri, 1996).

When, mainly obtained from cheese production, is another source rich in proteins of high nutritional value but has been mostly discarded in small dairy properties (Pelegrine & Carrasqueira, 2008). Bovine serum albumin is one of the protein constituents of whey, consisting of approximately 583 amino acid residues, with a molar mass of 66 kDa and a pI of approximately 5.0. It is one of the most studied proteins of this group, particularly because of its structural similarity with human serum albumin (HSA) (Zhao, Li, Harris, 2009).

The objective of this work was to study the influence of pH, NaCl concentration, and total mass ratio of proteins in the process of heteroprotein complex formation resulting from the interaction between bovine serum albumin and lysozyme and to characterize these complexes by Fourier Transform Infrared Spectroscopy (FTIR), microscopy (SEM and Optical) and Differential Scanning Calorimetry (DSC).

2. Materials and methods

2.1. Materials

Lysozyme (Lys, purity > 90%) and bovine serum albumin (BSA, Purity > 96%) were obtained from Sigma-Aldrich (St. Louis, USA). Sodium chloride (NaCl, purity > 99%), hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from VETEC Ltd. (Rio de Janeiro, Brazil). Ultrapure water with a conductivity of 0.05 μS/cm was used (Gehaka, Master-P & D, Brazil).

2.2. Formation of complexes

2.2.1. Sample preparation

The concentrations of Lys and BSA were set at 0.1% w/w in the system. BSA and Lys solutions were used as controls and seven ratios (r) of BSA:Lys were studied: 8 (8:1); 4 (4:1); 2 (2:1); 1 (1:1); 0.5 (1:2); 0.25 (1:4); and 0.125 (1:8). To evaluate the influence of NaCl on the complex formation, five different NaCl concentrations (0; 10, 50, 100 and 300 mM) were studied for the BSA:Lys ratio, which resulted in greater turbidity. For the preparation of the solutions, the proteins were weighed using an analytical balance (Shimadzu, AY 220, Philippines) and agitated with a magnetic stirrer (Novatecnica, model NT101, Brazil) for 30 min. The pH of the solutions was pre-adjusted to 2.0 and measured using a benchtop pH meter (mPA-210, Tecnopan, Brazil) for subsequent turbidimetric titration.

2.2.2. Turbidimetric titration

The sample transmittance was measured at a wavelength of 400 nm in a 1-cm quartz cuvette using a spectrophotometer (Biochrom mod. Libra S12, England). The equipment was calibrated for 100% transmittance (T) with ultrapure water. The turbidity was defined as $\tau$ (cm$^{-1}$), given by Equation (1), where I is the incident light intensity and I$_0$ is the light intensity after traversing the sample (Gulão, de Souza, Andrade & Garcia-Rojas, 2016). Solutions containing a defined BSA:Lys ratio and the respective NaCl concentration had their pH adjusted (2.0–12.0) with HCl and NaOH with the aid of a bench pH meter and a magnetic stirrer. The pH of the solutions was monitored, and a 1-mL aliquot was sampled to measure the transmittance value. All titrations were performed at room temperature (−25 °C) with four replicates, and the interval between measurements was approximately 1 min (Adapted from Gulão, Souza, Silva, Coimbra, & Garcia-Rojas, 2014).

$$\tau = -\ln \left(\frac{I}{I_0}\right)$$

2.3. $\zeta$ potential

The $\zeta$ potential of the isolated proteins and the ratios studied were determined by Zetasizer (Malvern Instruments, Nano-ZS, UK). Samples (10 mL) at 0.1% (w/w) were placed on the titrater (Malvern Instruments, MPT-2, UK). The pH of the solutions were adjusted with the aid of 500 mM HCl, 250 mM NaOH, and 25 mM NaOH, and the analyses were conducted as a function of pH (2.0–12.0) in the range of 0.25 ± 0.1. The $\zeta$ potential was calculated based on the mathematical model of Smoluchowski, and each sample was measured in triplicate at 25 °C.

2.4. Particle size

The hydrodynamic diameter (d, nm) of the BSA/Lys complexes, in addition to the BSA and Lys standard solutions, were determined at a fixed pH by the DLS (Dynamic Light Scattering) technique. This was done using a Zetasizer (Malvern Instruments, Nano-ZS, UK) equipped with a He-Ne laser at a wavelength of 632.8 nm and a fixed detection angle of 90°. Samples were placed in glass cuvettes and measured in triplicate at 25 °C.

2.5. Characterization of heteroprotein complexes

For the characterization, was chosen the complexes in the ratio, pH and NaCl concentration according to the item 2.2.2. After preparation, the solution had its pH adjusted and was allowed to stand at 4 °C overnight. Then, the solution was centrifuged (Ortoalresa, digest 21 R, Spain) at 500×g for 10 min at a temperature of 20 °C (Anema & de Kruif, 2014). The supernatant was then discarded and the precipitate was placed in the ultra-freezer (Terroni, COLD 120, Brazil) at −40 °C for approximately 24 h. Soon after, it was placed in the benchtop freeze dryer (Terroni, Enterprise I, Brazil) for drying and stored in a desiccator with silica gel until use.

2.5.1. Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectra were obtained for the BSA and Lys standard samples and the lyophilized complexes of the defined BSA:Lys ratios. The analyses were performed with a FTIR spectrometer (Bruker, Vertex 70, Germany) using KBr (potassium bromide) tablets and were read in the range of 4000–500 cm$^{-1}$.

2.5.2. Optical microscopy

An aliquot of the coacervate complex in 0.1% (w/w) solution, r = 0.5, and pH 9.0, was placed between a lamina and coverslip, transported to the optical microscope Axioplan (Zeiss, Gottingen, Germany) coupled to an AxioCam MRc camera (Zeiss, Gottingen, Germany) and viewed at 100× with oil immersion.
2.5.3. Scanning electron microscopy (SEM)

The micrographs of the lyophilized complexes for the defined BSA/Lys ratios (r = 0.5 and pH 9.0) were placed under the Scanning Electron Microscope (Hitachi, TM-3000, Japan) with a tungsten filament and detected in electron mode with an acceleration voltage of 15 kV.

2.5.4. Differential Scanning Calorimetry (DSC)

DSC analysis was performed using a calorimeter (TA Instruments, Q200, USA). Indium (In) standards were used to calibrate the energy and temperature of the equipment, and nitrogen was used as the purge gas. Lyophilized samples (~3 mg) were weighed in hermetic aluminum crucibles with the aid of a precision scale (Mettler Toledo, Mx5, USA), and the samples were dosed with at least twice the amount of water required to maintain hydration. Prior to analysis, the crucibles were sealed and kept at room temperature for at least 2 h to equilibrate. Samples were analyzed over a temperature range of 25–120 °C at the rate of 5 °C/min, and an empty, sealed crucible was used as a reference (Yuan, Wan, Yang, & Yin, 2014). The determination of the peak transition temperature as well as the enthalpy change were analyzed by the Universal V4.5A® software (TA Instruments, USA). The denaturation temperature (Td) was defined as the maximum peak value, and the denaturation enthalpy change (ΔHd) was obtained by the integral of the peak area. All experiments were performed in duplicate.

3. Results and discussion

3.1. Effect of pH and BSA/Lys ratio on the formation of heteroprotein complexes

Fig. 1 shows the effect of pH variation on the formation of heteroprotein complexes. Fig. 1a shows the effect of pH (2.0–12.0) on the variation of turbidity of isolated protein solutions of BSA and Lys. During the turbidimetric analysis of Lys, a slight increase in turbidity was observed from pH 9.5 to 11.5. This occurs in most systems containing a single protein near its pI and is due to the decrease in solubility because of the decrease in repulsion. Therefore, self-aggregation occurs with a consequent increase of turbidity (Qin et al., 1998). However, for BSA, it was not possible to verify any turbidity change during the pH variation, suggesting that the BSA was not able to form self-aggregates of sufficient size to be detected by the spectrophotometer at the studied wavelength in this case. This is due to the large proportion of hydrophilic groups on the surface of the molecule (Fennema, 2009).

In Fig. 1b, the turbidity variation is represented by different BSA/Lys ratios under the same conditions. In this figure, it is observed that near pH 5.0, there is a slight increase in turbidity with rapid elevation around pH 7.0, where it remains until it rapidly decreases before pH 11.0. It is noted that higher turbidity values were recorded between pH 8.0 and 10.0 for practically all ratios studied. Among the ratios studied, the ratio r = 0.5 was highlighted because it presented the highest turbidity, probably because there was a complete neutralization of the loads at this ratio. For the other ratios, the decrease in turbidity values occurred naturally due to the excess of either Lys or BSA in the system. In this case, the molecules that are not associated become more soluble in the solution than the associated (complex) molecules (Gulao et al., 2014). Therefore, the turbidity intensity and the consequent formation of heteroprotein complexes are also influenced by the ratio of this alteration in the system load balances, an important factor for interaction between the biopolymers (Ye, Flanagan, & Singh, 2006).

When the heteroprotein complex from the BSA/Lys ratio r = 0.5 in the absence of NaCl was formed during pH variation, three sequential processes could be identified, as shown in Fig. 1c. The first point observed is pHc, which shows the beginning of the formation of soluble complexes, indicating a discrete increase in turbidity was observed even before pH 4.0. At this stage, the...
interactions begin to occur but in a weaker manner due to the low density of soluble complexes, which due to their small size, are not detected by the wavelength used. The pHₚᵢ of the stage was observed as a rapid increase in turbidity and occurred around pH 7.5 and is due to the formation of insoluble complexes (heteroprotein complexes), which are larger and more detectable than the soluble complexes. The pHₚᵢ occurred shortly after the turbidity peak and before pH 11.0, corresponding to the dissociation of the complexes due to electrostatic repulsion (Vinayahan, Williams, & Phillips, 2010; Sgarbieri, 1996). The formation of coacervates in the pH range between pHₚᵢ and pHₚᵢ₂ is due to Lys having a higher density of positive charges at a pH below its pI and the BSA having a higher density of negative charges at a pH above its pI, which allows for interaction by electrostatic attraction in this range. Diarrassouba et al. (2015) also observed this interaction with a consequent increase in turbidity for a 2:1 ratio of β-lactoglobulin and lysozyme, which occurred between pH 6.8 and 10 but did not occur close to either pH of 5 or 11.

Fig. 1d clearly illustrates the influence of pH (5.0–12.0) on turbidity variation at the r = 0.5 ratio of BSA/Lys. Here, it is possible to see a sudden increase in turbidity between pH 8.0–10.0, indicating the formation of insoluble complexes. Electrostatic attraction is the main force involved in the formation of BSA/Lys coacervated complexes. For this reason, the study of the effect of pH on the interaction between proteins is a relevant factor because in addition to influencing the density of biopolymer loads, it also determines the range of stability of the coacervate and the best conditions for its formation (Diarrassouba et al., 2015; Li et al., 2012; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). However, a slight increase in turbidity observed even before reaching the pl of Lys may be explained by the fact that during the coacervation process, non-electrostatic interactions may be involved in addition to electrostatic interactions, mainly consisting of hydrogen bonds occurring at a pH above the pl of the protein and which are favored by a low density of charges, as reported in the literature (Vries & Cohen Stuart, 2006; Jones & Mc Clements, 2010; Schmitt & Turgeon, 2011; Girard, Turgeon, & Gauthier, 2002; Yoshida, Sokhakian, & Dubin, 1998). Liu et al. (2015) studied the influence of urea, which is capable of breaking hydrogen bonds in the complex formation between BSA and a flaxseed polysaccharide. Their results showed that the addition of urea reduced the turbidity, indicating that the complex formation between the studied biopolymers was influenced not only by electrostatic interactions but also by hydrogen bonds.

Based on the pHₚᵢ, pHₚᵢ₁, and pHₚᵢ₂ data derived from Fig. 1b, a phase diagram of BSA/Lys containing systems was constructed as a function of the total mass ratio and pH, as shown in Fig. 2. The diagram corresponds to the complex formation phases based on turbidimetric analysis during the NaOH titration of the BSA/Lys blends. The pHₚᵢ₁ values have been shown to be essentially unchanged as a function of the variation of the BSA/Lys ratio. This is because the dissociation of the complexes occurs by the protonation of BSA and not by the influence of the ratio (Li et al., 2012). However, because pHₚᵢ₁ and pHₚᵢ₂ were ratio dependent, they showed a slight decrease as the ratios were increased.

Between pHₚᵢ₁ and pHₚᵢ₂, an extensive area corresponding to the formation of heteroprotein complexes was observed for all the reasons studied and because the two proteins are oppositely charged in that range. Soluble complexes were formed in the pH range between 5.0 and 6.0 due to the low density of charges near the BSA pl. Above pH 12.0 and below pH 2.0, there was no formation of complexes because the proteins were soluble in the medium.

3.2. ζ potential

The variation of the ζ – potential and pl in the absence of NaCl are shown in Fig. 3. To verify the charge density of the isolated proteins (BSA and Lys) and the complex formed at the defined ratio (r = 0.5), the ζ potential (mV) as a function of pH (2.0–11.0) was determined, as shown in Fig. 3a. The BSA ζ–potential (mV) ranged from +35.5 to –23.5 mV at pH 2.0 to pH 8.0, with a zero-electrical charge (pl) at 5.2. According to the literature, the pl of BSA (0.05%, w/w) is close to 5.0 (Vinayahan et al., 2010). Liu et al. (2015) found similar results, where the load variation occurred in the range of −17.5 mV at a pH of approximately 6.0 to +32.8 mV at a pH close to 1.5. The authors mentioned that this occurs due to the protonation of amines (–NH₂) and carboxylic groups (–COO⁻) of BSA. The ζ– potential of Lys had a variation in the range of 10 to −20 mV between pH 8 and 10.5, with a pl around pH 9.5, which has already been reported in the literature by Anema and Kruijff (2013). These pl values are close to those obtained in this study. The ζ– potential of the coacervate formed at the ratio r = 0.5 ranged from +8.7 to −19.3 mV during the titration from pH 5.0 to 10.0, indicating pH 7.55 as the pl. It is interesting to note that the pl of the complex occurred exactly between the pl of the two proteins. This is possible because BSA is above its pl (5.22) while negatively charged, and Lys is below its pl (9.5) while positively charged, thus allowing interaction between the opposing charges of the two proteins with the consequent formation of the coacervate.

Fig. 3b shows the pl values of the isolated proteins and all other ratios studied. It was observed that ratios containing more BSA than Lys (r = 2, 4, and 8) had a pl closer to that of BSA. This is due to the presence of a greater amount of negative charge due to the excess of BSA in the system that was not neutralized by the interaction with Lys. In the ratios containing more Lys than BSA (r = 0.5, 0.25, and 0.125), the pl was closer to that of Lys, which was in excess in the system and did not interact with BSA.

When comparing the pl of the BSA/Lys ratios in Fig. 3b with the values of pHₚᵢ₂ determined by the turbidimetric analysis, and while composing the phase diagram for Fig. 2, it was verified that there is a certain similarity in the pH values. This indicates that the interaction was more intense when the electric charge of the mixtures was neutral (pl). However, when the pl of 7.5 for the r = 0.5 ratio was compared with Fig. 1b, it was observed that the highest turbidity intensity occurred at pH 9.0 and 10.0. In other words, it was above the referenced pl but between the pls of the two isolated proteins.
3.3. Effect of NaCl on the formation of heteroprotein complexes

The influence of NaCl on the formation of coacervated BSA/Lys complexes at the ratio \( r = 0.5 \) was studied by turbidimetric analysis, as shown in **Fig. 4**. In this figure, it was observed that the addition of NaCl influenced the formation of the coacervates in a negative way, even in small concentrations near 10 mM and up to 50 mM. In addition, a change in the pHc, pHq1, and pHq2 values can be observed at the 10 mM concentration, which not only resulted in a reduction in the turbidity intensity but also in the formation range of the complexes.

These results are in accordance with those reported in the literature, such as by Li et al. (2012), who studied different concentrations of NaCl and their influence on the formation of complexes between BSA and beet pectin. They observed that the addition of NaCl at very small concentrations (from 1 to 200 mM) favored the formation of complexes. However, there was a reduction in turbidity when the NaCl concentration was increased to 500 mM, indicating that the complexation had been suppressed.

Liu et al. (2015) evaluated the influence of NaCl between BSA and flaxseed gum (*Linum usitatissimum* L.). They observed that the turbidity decreased and that the values of pHc, pHq1, and pHq2 were altered with a consequent reduction in the range between pHq1 and pHq2 at the two concentrations of NaCl studied (50 mM and 100 mM) compared with no addition of NaCl. A reduction in complexation can be explained by the addition of NaCl due to both the competition of Na\(^+\) ions binding to the negative charge sites and by the competition of Cl\(^-/\) ions binding to those of positive charge. As a result, there is a reduction in the electrostatic interaction between the protein molecules (Klassen, Elmer, & Nickerson, 2011; Liu et al., 2015; Seyrek, Dubin, Tribet, & Gamble, 2003).

3.4. Particle size

The particle size distribution of BSA and Lys during the formation of heteroprotein complexes was monitored by DLS to elucidate the interactions between biopolymers. The intensity profiles as a function of size (d.nm) for BSA and Lys solutions (0.1% w/w), as well as for the \( r = 0.5 \) ratio mixture, were determined at pH 9.0 without the addition of NaCl and are shown in **Table 1**.

It was observed that the BSA presented three different sizes: one of greater intensity (67.0\% ± 1.3) with an average size of 5.2 d nm ± 0.13, a second (29.5\% ± 1.0) with an average size of 192.1 d nm ± 10.3, and a third (3.5\% ± 1.2) with an average size of 5360 d nm ± 81.9. The second and third average sizes are attributed to the presence of impurities or self-aggregation (Liu et al., 2015). The first observed average size (~5.0 d nm) of BSA is consistent with that reported in the literature (Vinayahan et al., 2010; Hiroshi, Kikuchi, Ogawa, & Kokufuta, 2007).

In the Lys size distribution, an average size of greater intensity (91.8\% ± 0.5) was found in the range of 279.5 d nm ± 38.4, along with one of lower intensity (81.8\% ± 0.5) with an average size of 1.99 d nm ± 0.16. Considering that the first size observed is due to self-aggregation occurring at this pH (Fig. 1a), the average size of the first one is in line with that reported in the literature (Parmar & Muschol, 2009; Antonov, Zhuravleva, Cardinaels, & moldenaers, 2015).

When analyzing the solution containing BSA/Lys at pH 9.0, where the greatest formation of heteroprotein complexes occurred,
the principal values of the protein monomers were not observed but have an average size of 1755 d nm ± 167.7 (-100%) with a polydispersity index (PDI) of 0.54 ± 0.18.

It should be noted that the mean particle size observed in the BSA/Lys solution is much higher than those found for the solutions containing the isolated proteins, suggesting that these conditions resulted in an interaction between positive and negative charges of the BSA and Lys proteins in the formation of heteroprotein complexes. Diarrassouba et al. (2015) studied the interaction between β-lactoglobulin and lysozyme and observed particle sizes of approximately 2 μm, which are similar to those found in our study.

3.5. Chemical and morphological characterization of heteroprotein complexes

Infrared spectra of the BSA and Lys proteins and the BSA/Lys complex containing 0.1% (total mass) and r = 0.5 ratio are shown in Fig. 5. The most sensitive regions of the FTIR spectra associated with the protein structure are amides I, II, and III. Amide I is between the bands 1625 and 1750 cm⁻¹ and is formed by stretching the C=O group (free carboxyl). Amide II is located between the bands 1475 and 1575 cm⁻¹ and refers to stretching of N-H groups. Finally, amide III is located between the bands 1225 and 1425 cm⁻¹, which corresponds to the stretching of the C-N and N-H groups (Dong, Huang, & Caughey, 1990; Huang, Balakrishnan, & Spiro, 2006; Schmidt, Giacomelli, & Soldi, 2005; Stuart, 2006). In addition to the amides, the stretching of the N-H and O-H groups of the free amino acids (Barth & Zscherp, 2002) can be identified between the bands 3300 and 3170 cm⁻¹.

The major FTIR spectra observed for BSA and Lys are between the bands 1300 cm⁻¹ and 1700 cm⁻¹, representing amides I, II, and III and the stretching of the N-H and O-H groups near the 3300 cm⁻¹ range. In the FTIR spectra for the complexes formed from BSA and Lys, similar structures were observed but with lower intensities. The formation of complexes through electrostatic interactions between the -COO⁻ (C = O) clusters of one protein and the -NH₃⁺ (NH) clusters of the other justifies the reduction verified in the bands corresponding with the amides (1400 cm⁻¹ to 1700 cm⁻¹). In addition, a reduction in intensity for the 3300 cm⁻¹ band observed in the BSA/Lys complex would indicate the

![Fig. 5. FTIR spectra of BSA, Lys, and BSA/Lys ratio r = 0.5. The complexes were prepared at pH 9 without addition of NaCl.](image)

![Fig. 6. Optical microscopy (a) and SEM (b and c) of the coacervated BSA/Lys complexes r = 0.5 at pH 9.0.](image)
formation of complexes not only by electrostatic interactions but also by hydrogen-like bonds (Barth & Zscherp, 2002; Huang, Sun, Xiao, & Yang, 2012).

The micrographs of the heteroprotein complexes are shown in Fig. 6. Optical microscopy was used to better understand the structure of the heteroprotein complex formed in the natural state (in solution). Images of the complexes at pH 9.0 and ratio $r = 0.5$ are shown in Fig. 6a. Optical microscopy $(100\times)$ revealed the presence of spherical structures, similar to those observed by Desfougères, Croguennec, Lechevalier, Bouhallab, and Nau (2010) and are described as having a homogeneous internal content. Turgeon and Laneuville (2009) described coacervates as vesicles that could be used in the microencapsulation process. Although the identification of biopolymers is still not well understood, especially in coacervate complexes formed by proteins, it is believed that there is a uniform distribution of proteins inside the spheres (Diarrassouba et al., 2015).

The images provided by SEM (Fig. 6b and c) show that the lyophilized samples containing the heteroprotein complexes obtained from the BSA/Lys ratio $r = 0.5$ at pH 9.0 have similar spherical structures. Diarrassouba et al. (2015) studied complexes between β-lactoglobulin and Lysozyme, observed similar structures and described them as having a regular and spherical structure.

3.6. Differential Scanning Calorimetry (DSC)

Fig. 7 shows the thermograms of the isolated proteins, Lys and BSA, as well as for the heteroprotein complex formed at pH 9.0 and ratio $r = 0.5$. All samples analyzed showed an endothermic peak characteristic of the denaturation process (Yuan et al., 2014). The mean denaturation temperatures $(T_d)$ of the isolated proteins were $72.3^\circ C \pm 0.07$ and $59.7^\circ C \pm 0.09$ for Lys (Fig. 7a) and BSA (Fig. 7b), respectively, and were in accordance with values reported in the literature (Bye & Falconer, 2013; Michnik, 2003). In the thermogram of the complex (Fig. 7c), only one single endothermic peak characteristic of the denaturation process was verified. It is interesting to note that no other peak relative to the denaturation point of the isolated proteins was observed, probably indicating that a new biopolymer was formed with thermal characteristics different from those of the two original proteins. The mean $T_d$ verified for the complex was $67.0^\circ C \pm 0.07$, i.e., between the average $T_d$ values of the two proteins. This increase in stability is probably due to the electrostatic interactions between proteins and was also observed in FTIR (Fig. 5), which led to coacervation and reduction in protein mobility without a change in protein structure (Water et al., 2014). The mean denaturation enthalpy change $(\Delta H_d)$ of $9.2 \pm 0.80$ for the complexes was also between the values observed for Lys ($35.8 \pm 2.71$) and BSA ($6.4 \pm 2.03$). The increase of $\Delta H_d$ compared to the BSA values indicates that the interaction between Lys and BSA was induced by electrostatic interactions, as observed by Yuan et al. (2014) when evaluating the thermal stability of soy protein with chitosan. The results obtained, referring to the thermal characteristic of the complex formed, are very relevant regarding the future applicability of these complexes in food matrices.

4. Conclusion

Insoluble complexes were formed between the pI values of proteins and near the pI characterizing that the interaction occurred by opposite charges, reaching a maximum when there was equilibrium of these charges. These results, together with the FTIR, showed that electrostatic interactions predominated the formation of complexes, but hydrogen bonds also participated in the interaction. The addition of NaCl at the concentrations studied had no positive effect and visibly reduced the interaction at concentrations of 10 mM. Under the conditions studied, spherical structures with an average size of 1.7 μm were observed and would allow use of the complex as a microencapsulating agent. The DSC suggested that the interaction gave rise to a new biopolymer having a denaturation temperature between that of the two proteins individually. This study contributed to the future applications of heteroprotein complexes of BSA/Lys for the microencapsulation process of food bioactives.