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Original article

Characterisation of beta-lactoglobulin/sodium alginate dry films

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Summary Optimum beta-lactoglobulin/sodium alginate dry films (β-LG/SA(S)) formed from casting solutions containing β-LG 1.25% (w/v) and SA 1% (w/v) showed appropriate characteristics to be easily handled. SEM micrographs showed a less homogeneous microstructure of this films respect to sodium alginate dry films used as control (SA(F)). Tensile strength of 18 MPa and an elongation value of 5% were obtained for β-LG/SA(S). DSC thermograms did not demonstrate changes in thermal properties of SA in presence of β-LG. Studies based on fluorescence quenching by acrylamide did not show variations in the tertiary structure of β-LG when β-LG/SA(S) were dissolved in an aqueous environment. In addition, binding properties of the protein were similar to the native one when an alquilsulfonate compound was used as ligand. The maintenance of native binding properties of β-LG after the films' dissolution would allow the development of new carriers for food bioactive compounds based on β-LG/SA(S) dry films.

Keywords Beta-lactoglobulin, dry film, film characteristics, protein properties, sodium alginate.

Introduction

Proteins and polysaccharides are generally used to contribute to food structure, texture and stability (Bastos *et al*., 2018). However, in many systems, the mutual presence of both types of ingredients modifies their individual properties (Harnsilawat *et al*., 2006). The concern in the study of protein-polysaccharide interactions has increased in recent years. The knowledge about the formation of protein-polysaccharide complexes is interesting for the development of new food products, such as edible films; a solid matrix whose mechanical properties depend on film compounds, their concentration and compatibility (Falguera *et al*., 2011).

β-lactoglobulin (β-LG) is the main whey protein constituent, which is used as a food additive due to its functional properties, antioxidant activity and high nutritional value (Abd El-Maksoud *et al*., 2018). The ability of native β-LG to bind food bioactive compounds, such as polyphenols and hydrophobic vitamins, provides encapsulation, protection and delivery functions to this protein (Busti *et al*., 1998; von Staszewski *et al*., 2014; Zhang *et al*., 2014; Abd El-Maksoud *et al*., 2018; Berino *et al*., 2019). Different β-LG binding sites to hydrophobic and amphiphilic compounds have been studied: a main binding site in the hydrophobic pocket, a hydrophobic patch on the surface of the protein and a site at the interface between monomers when protein dimers are present (Zhang *et al*., 2014; Berino *et al*., 2019).

Sodium alginate (SA) is an anionic hydrophilic polysaccharide principally extracted from seaweeds (Yemenicioğlu *et al*., 2020), which is widely used in the food industry as a thickening, gelling and stabilising agent (Qomarudin *et al*., 2015). In addition, SA is one of the most commonly used biopolymers as film-forming material (Soazo *et al*., 2015; Báez *et al*., 2017; Comaposada *et al*., 2018). The application of these films in agriculture, medicine and food packaging fields is being widely investigated (Ma *et al*., 2019).

The interaction between β-LG and SA has been extensively studied in aqueous solution (Harnsilawat *et al*., 2006; Qomarudin *et al*., 2015; Stender *et al*.,

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2018). Harnsilawat *et al.* (2006) have demonstrated that β-LG forms soluble or insoluble complexes with SA depending on the pH of the medium. The isoelectric point of β-LG is estimated to be around pH 4.8, while SA is negatively charged in the pH range between 2.0 and 8.0. Insoluble complexes are formed at pH values in which both the protein and the polysaccharide have opposite electric charges (pH 3.0 and 4.0) due to strong electrostatic attraction between these two biopolymers. At pH values where the polysaccharide is negatively charged but the protein has little net charge (~pH 5.0), fairly soluble complexes are formed promoted by the binding of anionic SA to cationic patches on the surface of β-LG. At pH values where β-LG and SA have similar electric charges (pH 6.0 and 7.0) and at low protein concentrations, complexes are not formed due to the relatively strong electrostatic repulsion between them (Harnsilawat *et al.*, 2006).

On the other hand, little is known about the interaction between β-LG and SA in the dry state. The aim of this work was to complete the study of molecular interactions between β-LG and SA to obtain dry films that conserve the binding protein ability. This fact would allow the development of new carriers for food bioactive compounds. These studies were conducted considering (1) selection of casting solutions, (2) characterisation of dry films and (3) analysis of binding properties of the protein after film re-dissolution.

Materials and methods

Materials

β-LG from bovine milk and SA from brown algae (medium viscosity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

β-LG/SA mixtures

Stock solutions of β-LG 20% (w/v) and SA 2% (w/v) were prepared in phosphate buffer (20 mM, pH 7.0). Biopolymer mixtures containing SA 1% (w/v) and β-LG (0, 0.625, 1.25, 2.5, 5.0 and 10% (w/v)) were prepared by mixing different ratios of stock solutions with buffer (casting solutions). Protein concentration was determined by measurement light absorption at 280 nm with a Jasco V-550 spectrophotometer (Tokyo, Japan).

Turbidity measurements

Turbidity was determined to study the possible existence of aggregation or precipitation in all the systems prepared in Section 2.2. Turbidity (τ) was defined as:

$$\tau = 2.303\ \mathrm{Abs}_{600} \quad (1)$$

where Abs_{600} is the absorbance at 600 nm against a blank of phosphate buffer (20 mM, pH 7.0). The equipment used was a Jasco V-550 spectrophotometer (Tokyo, Japan). Triplicate measurements were taken for all samples, at a controlled temperature of 25 °C.

Preparation of β-LG/SA dry films

It should be noted that SA final concentration of 1% (w/v) was selected in all the casting solutions assayed in accordance with results obtained in previous works (Soazo *et al.*, 2015; Báez *et al.*, 2017). Aliquots of 2.7 mL of the different β-LG/SA mixtures prepared according to Section 2.2. were poured into rectangular plastic moulds (20 × 27 × 20 mm). The filled moulds were introduced into an oven (Tecno, Dalvo, Santa Fe, Argentina) for 3 h at 40 °C. Optimum films (β-LG/SA(S)) were selected based on the lack of physical defects, such as crack, bubbles and holes, on their high manageability and on their protein content. β-LG/SA (S) were physicochemically characterised by using scanning electron microscopy (SEM), mechanical assays, Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). A control film was prepared from SA 1% (w/v) casting solution without β-LG. This dry film was named as SA(F).

SEM studies of β-LG/SA(S)

SEM experiments were carried out in order to study the microstructures of β-LG/SA(S) and SA(F). Dry films were cryo-fractured, fixed at an angle of 90° to the surface and gold coated. Micrographs of films' cross-section were obtained with a scanning electron microscope (AMR 1000, Leitz, Wetzlar, Germany) at an accelerating voltage of 10 kV and at a magnification of 1000×.

Film thickness and mechanical properties of β-LG/SA(S)

β-LG/SA(S) and SA(F) were stored in darkness at 25 °C and 55% RH for 24 h. The thickness of three replicates of the films assayed was measured at nine locations of each film, using a micrometre (Schwyz TM, China). Mechanical tests of films were obtained using a texturometer (Mecmesin Multitest 2.5d, Mecmesin, Sterling, Va., U.S.A.) following the methodology employed by Báez *et al.* (2017). The mechanical parameters determined from stress-strain plots were tensile strength (TS) and elongation (E(%)) (Silva *et al.*, 2009). The measurements were made at 25 °C.

FTIR studies of β-LG/SA(S)

To record the FTIR spectra of β-LG/SA(S) and SA(F), an IR-Prestige-21 spectrophotometer (Shimadzu,

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Kyoto, Japan) was employed under attenuated total reflectance (ATR) mode (Soazo *et al*., 2015). The FTIR spectra were recorded in absorbance mode from 500 to 4000 cm^{-1} with a resolution of 4 cm^{-1} using 20 scans. Each one of the samples was run in duplicate at 25 °C.

DSC studies of β-LG/SA(S)

Thermal properties of β-LG/SA(S) and SA(F) were characterised using a differential scanning calorimeter (DSC-60, Shimadzu, Kyoto, Japan). Approximately, 10 mg of dry films were sealed into aluminium pans and scanned over a range between 30 and 350 °C with a heating rate of 10 °C min^{-1}. Each one of the samples was run in duplicate.

Dissolution of β-LG/SA(S) and β-LG fluorescence studies

The whole β-LG/SA(S) was immersed in 2.7 mL of phosphate buffer (20 mM, pH 7.0). Dissolution of the samples was achieved by stirring each one in a tube rotator at 10 rpm. for 1 h at 25 °C. Two types of protein solution were assayed in fluorescence studies. One type was obtained from dissolved films where β-LG concentration was adjusted to 20 μM in phosphate buffer (20 mM, pH 7.0). It should be noted that SA was present in the reconstituted solution. The other type of protein solution used as control was made using native β-LG (20 μM in phosphate buffer, at pH 7.0).

Intrinsic fluorescence measurements of β-LG were carried out with a Jasco FP-770 spectrofluorometer (Tokyo, Japan). A quartz cuvette filled with three millilitres of each of the samples was used. The measurements were made at 25 °C.

Surface hydrophobicity of β-LG

Surface hydrophobicity was estimated by quenching the intrinsic fluorescence of β-LG with acrylamide (Báez *et al*., 2013). The protein concentration used was 20 μM and the final acrylamide concentration ranged from 0 to 2 mM. The fluorescence intensity of β-LG at 337 nm was measured using an excitation wavelength set at 295 nm.

The results obtained were represented in a Stern-Volmer plot. The Stern-Volmer equation can be expressed as:

$$F_0/F = 1 + K_{SV}[\text{acrylamide}] \quad (2)$$

where F_0 is the fluorescence intensity of β-LG and F is the fluorescence intensity of the protein at each acrylamide concentration. The slope of the Stern-Volmer plot, K_{SV}, is an index of protein hydrophobicity. Each one of the samples was run in triplicate at 25 °C.

Binding of alkylsulfonate ligand (AL) to β-LG

The fluorometric titration with 1-tetradecanesulfonic acid (AL_{14}) 5 mM was used to study β-LG binding capacity (Busti *et al*., 2005). The values of binding parameters reported in this work were calculated according to the procedure of Cogan *et al*. (1976). Apparent dissociation constants (K′d) and apparent molar ratios of ligand/β-LG at saturation (*n*) were determined from ($P_0\alpha$) vs. B[α/(1 − α)] plots. P_0 is the total protein concentration (6.6 μM) and B is the total ligand concentration (AL_{14} concentration ranged from 0 to 33 μM). α is defined as the fraction of unoccupied binding sites on the protein molecules. The values of α were calculated using the following equation:

$$\alpha = (F_{max} - F)/(F_{max} - F_0), \quad (3)$$

where F_0 is the initial fluorescence intensity, F is the fluorescence intensity at a certain ligand concentration and F_{max} is the fluorescence intensity upon saturation of protein molecules. A straight line was obtained with an intercept of K′d/*n* and a slope of 1/*n*, Each one of the samples was run in triplicate at 25 °C.

Statistical analysis

Statistical analysis was performed using Statgraphics Plus for Windows (Manugistics Inc, Rockville, MA, USA). The analysis of variance (ANOVA) was used and when the effect of the factors was significant ($P < 0.05$), the test of multiple ranks honestly significant difference (HSD) of Tukey was applied (95% of confidence level).

Results and discussion

Characterisation of β-LG/SA mixtures

Different amounts of β-LG stock solution (pH 7.0) were added to SA 1% (w/v) solution in order to study the possible existence of aggregation or precipitation in casting solutions prior to drying treatment. Harnsilawat *et al*. (2006) reported that between pH 6.0 and 7.0, there was no complex formation in β-LG/SA aqueous solutions, due to the strong electrostatic repulsion between these two negatively charged biopolymers. These authors worked with biopolymer mixtures containing β-LG 0.1% (w/v) and SA in the range from 0 to 0.1% (w/v).

Turbidity was widely used to monitor protein/polyelectrolyte aggregation and precipitation in aqueous environments (Fioramonti *et al*., 2014). Turbidity does not change when biopolymers remain co-soluble and increases when soluble complexes were formed. Coacervation or associative phase separation promotes heterogeneous systems. Turbidity of β-LG solutions were negligible for the all the concentrations used in this work (results not shown). Table 1 shows that up to β-LG 1.25% (w/v) and SA 1% (w/v) no significant changes in the turbidity of β-LG/SA mixtures were

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Table 1 Turbidity of different β-LG/SA mixtures

β-LG concentration	SA concentration	Turbidity
0	1	0.101 ± 0.009[a]
0.625	1	0.114 ± 0.006[a]
1.25	1	0.112 ± 0.005[a]
2.5	1	0.126 ± 0.002[b]
5.0	1	0.132 ± 0.004[bc]
10.0	1	0.144 ± 0.005[c]

All concentrations are expressed in % (w/v). Data represents means ± standard deviations. Different letters indicate significant statistical differences ($P < 0.05$).

observed, indicating that both biopolymers formed a co-solution. On the contrary, turbidity measurements significantly increased as the concentration of β-LG was higher than 1.25% (w/v). These results demonstrated the presence of soluble protein/polysaccharide complexes even at pH 7.0. In this case, the high protein concentrations used became an important factor in the formation of soluble aggregates of higher size.

β-LG/SA dry films

Figure 1a shows the results obtained when β-LG/SA mixtures were dried. The resulting β-LG/SA films were continuous and uniform up to a concentration of 1.25% (w/v) β-LG in the casting solution. The dry films elaborated using higher protein concentrations broke during the drying process, presenting opaque and white solid residues. This fact may be due to physical exclusion and phase separation at relative high biopolymers concentrations (Olivas & Barbosa-Cánovas, 2008). Taking into account the selection criteria presented in Section 2.4, dry films obtained from casting solution containing final concentrations of β-LG 1.25% (w/v) and SA 1% (w/v) were selected as β-LG/SA(S).

SEM microstructural characterisation of β-LG/SA(S)

The surface and cross-section morphology of dry films were investigated by SEM. While SA(F) presented a continuous, smooth and homogeneous microstructure (Fig. 1b), β-LG/SA(S) microstructure was less homogeneous and more rough (Fig. 1c). A similar result was observed in alginate films in which cottonseed protein hydrolysates were incorporated (Oliveira Filho *et al.*, 2019), and also with the incorporation of collagen and pea proteins (Comaposada *et al.*, 2018).

Thickness and mechanical properties of β-LG/SA(S)

Table 2 shows that the thickness of β-LG/SA(S) is higher than the value corresponding to SA(F). This change was caused by the presence of a higher content of biopolymers in the casting solutions.

TS and E(%) values for SA(F) (Table 2) were similar to the values reported for cellophane films (Péroval *et al.*, 2002). The presence of β-LG decreased TS and did not modify significantly E(%) of SA(F). The protein possibly weakened relative strong interactions among hydrocolloid molecules decreasing the TS of the dry network (Pérez-Gago *et al.*, 1999). The loss of SA(F) compact microstructure promoted by the

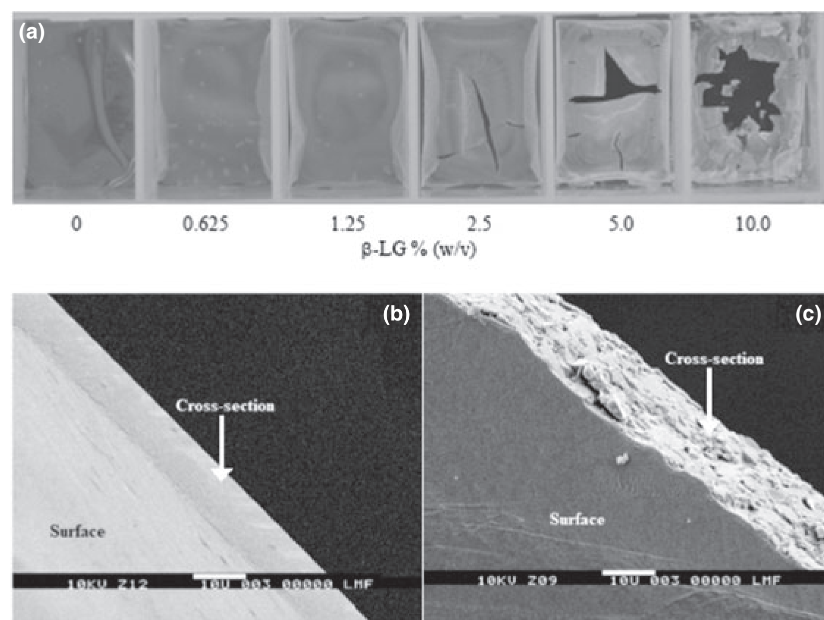

Figure 1 Visual aspect of β-LG/SA dry films (upper photograph): β-LG concentration in the casting solution varied in the range of 0-10% (w/v), while SA concentration was constant (1% (w/v)). SEM micrographs (lower photographs): (b) SA(F) and (c) β-LG/SA (S).

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Table 2 Thickness and mechanical properties of SA(F) and β-LG/SA(S)

Film sample	Thickness (μm)	TS (MPa)	E (%)
SA(F)	48 ± 2[a]	47 ± 4[b]	6 ± 1[a]
β-LG/SA(S)	108 ± 1[b]	18 ± 2[a]	5 ± 1[a]

Data represents means ± standard deviations of three samples of each system. Different letters indicate significant statistical differences ($P < 0.05$).

presence of protein molecules was evidenced by SEM studies shown in Section 3.2.1 (Fig. 1a, b).

FTIR and DSC studies of β-LG/SA(S)

Figure 2a shows FTIR spectra of SA(F) and β-LG/SA (S). The most important change promoted by protein-polysaccharide interaction in the dry state is the appearance of a new band in the vicinity of 1537 cm^{-1} in the FTIR spectrum of β-LG/SA(S). This variation can be attributed to the interaction between the

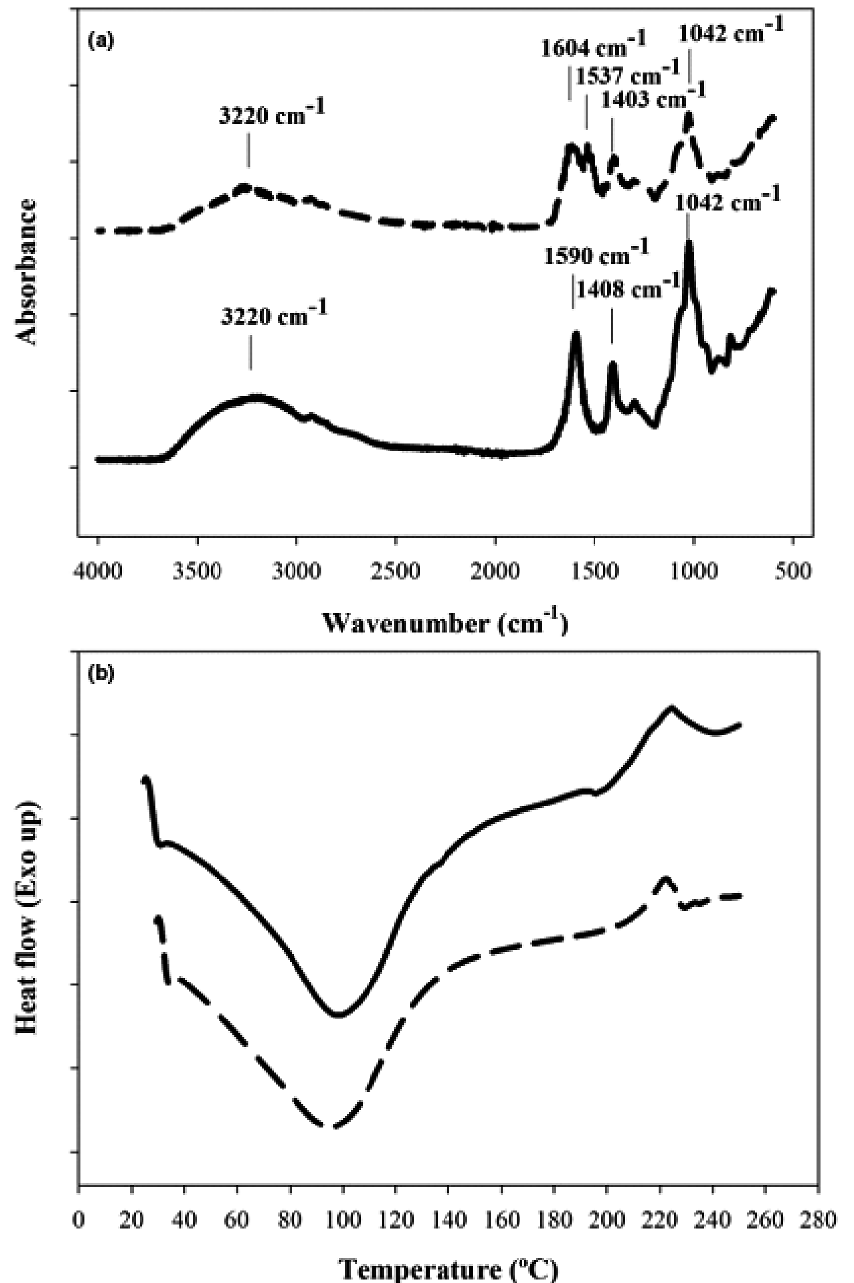

Figure 2 (a) FTIR spectra of SA(F) (———) and β-LG/SA(S) (------). (b) DSC thermograms of SA(F) (———) and β-LG/SA(S) (------).

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carboxyl group of SA (asymmetric stretching of the C-O bond of COO-) with the amide II of β-LG (N-H bending and C-N stretching) (Souza *et al.*, 2019).

Figure 2b shows DSC thermograms of the different samples assayed. In SA(F) and β-LG/SA(S) thermograms a wide and intense endothermic transition was found with peaks at 99.5 °C and 95.3 °C, respectively. These peaks belong to dehydration of the cross-linked network of SA dry films (Sarmento *et al.*, 2006; Soazo *et al.*, 2015). The exothermic peaks observed at temperatures between 220 and 275 °C resulted from degradation of alginate due to dehydration and depolymerisation of the protonated carboxylic groups and oxidation reactions of carbohydrate macromolecules (Sarmento *et al.*, 2006). The presence of β-LG did not affect the DSC pattern of SA(F).

Changes in β-LG after β-LG/SA(S) dissolution

Dissolution of β-LG/SA(S) was observed when it was immersed in an aqueous environment at pH 7.0. The reconstituted system presented a turbidity value similar to that obtained with the corresponding casting solution. This fact indicated that the resulting sample is a co-solution of individual macromolecules (Harnsilawat *et al.*, 2006).

It has to be noted that in fluorescence quenching and AL binding assays, protein concentration was brought to 20 μM (~0.04% (w/v)). This implied that titrations experiments were made in presence of SA concentration of ~0.03% (w/v). In order to establish if the drying and dissolution process modified the structure of β-LG, surface hydrophobicity of the protein was determined. Table 3 shows that K_{SV} values for fluorescence quenching of β-LG by acrylamide in different systems assayed in this work did not vary significantly. In addition, K_{SV} values obtained in all the cases were similar to the values reported in the literature for native β-LG at pH 7.0 (Báez *et al.*, 2017). Therefore, surface hydrophobicity indicated that no differences in the accessibility of acrylamide to tryptophanyl residues were observed. In conclusion, there were not macroscopic structural variations in the tertiary structure of the β-LG in all the systems studied in this work (Moro *et al.*, 2011).

Table 3 Fluorescence quenching and AL binding assays

System	K_{SV} (M^{-1})	K′d × 10^6 (M)	*n*
β-LG/SA(S)	1.65 ± 0.05^a	1.34 ± 0.33^a	2.05 ± 0.11^a
β-LG	1.76 ± 0.06^a	1.34 ± 0.65^a	2.24 ± 0.15^a
β-LG/SA	1.80 ± 0.06^a	NA	NA

Data represents means ± standard deviations of three samples of each system. Different letters indicate significant statistical differences ($P < 0.05$). NA, Not applied.

On the other hand, to determine if the drying and dissolution processes modified the binding properties of β-LG, the interaction between β-LG and AL_{14} was studied using the procedure of Cogan *et al.* (1976) (Table 3). The results indicated that there were not significant changes in the binding capacity of β-LG after β-LG/SA(S) dissolution in comparison with the native protein. The values of K′d and *n* informed in this work were of the same order of the values reported for native β-LG (Busti *et al.*, 1999).

Conclusions

This study provides information about β-LG and SA interactions in the dry state. Optimum dry films obtained in this work were continuous, uniform and presented high manageability. The films' protein content and the maintenance of β-LG binding properties after films re-dissolution would allow the development of new carriers for food bioactive compounds. Nevertheless, further studies are required before using these edible films for application in the food industry.

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Author Contributions

Germán D. Báez: Investigation (equal); writing–original draft (equal). **Emilce E. Llopart:** Investigation (equal); methodology (equal). **Romina P. Berino:** Investigation (equal); methodology (equal). **Andrea Moro:** Conceptualization (equal). **Roxana A. Verdini:** Conceptualization (equal); supervision (equal). **Pablo A. Busti:** Conceptualization (equal); writing–original draft (equal). **Néstor J. Delorenzi:** Writing–review and editing (equal).

Ethics approval

Ethics approval was not required for this research.

Conflict of interest

All authors declare that they have not any type of conflict of interest.

Peer review

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Data availability statement

Research data are not shared.

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