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Acacia gum as modifier of thermal stability, solubility and emulsifying properties of α-lactalbumin

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ABSTRACT

Protein–polysaccharide conjugates often display improved techno-functional properties when compared to their individual involved biomolecules. α-Lactalbumin:acacia gum (α-la:AG) conjugates were prepared via Maillard reaction by the dry-heating method. Conjugate formation was confirmed using results of absorbance, *o*-phthalaldehyde test, sodium dodecyl sulfate–polyacrilamide gel electrophoresis (SDS–PAGE) and size exclusion chromatography. Techno-functional properties (emulsifying characteristics, solubility, and thermal stability) were evaluated for α-la, α-la/AG mixtures and α-la:AG conjugates. Conjugate thermal stability was improved compared to pure α-la treated at the same conditions of conjugate formation. Response surface methodology was used to establish models to predict solubility and emulsifying activity as functions of the salt concentration, pH and reaction time. α-la:AG conjugate solubility is affected in a complex manner by the three factors analyzed. Emulsifying activity index (EAI) of α-la is significantly affected by pH, while the α-la:AG EAI is affected by the three analyzed factors. Both solubility and EAI are maximized with pH 8.0, NaCl concentration of 0.3 mol L^{-1} and two days of Maillard reaction.

1. Introduction

As the world population increases food intake will rise, requiring an increased supply of protein that plays an important role in the formulation of food products (Sun-Waterhouse, Zhao, & Waterhouse, 2014). However, this increase cannot come from animal production alone (Townsend & Howarth, 2010). One solution is to improve food processability through protein modifications, e.g. protein/polysaccharide interactions (Jing, Yap, Wong, & Kitts, 2011). This interaction may increase protein heat stability, a necessary function in the food industry and influence the techno-functional properties of food proteins such as solubility, emulsifying and foaming properties (Yadav, Parris, Johnston, Onwulata, & Hicks, 2010).

Among the physical, chemical and enzymatic methods of modifying and extending protein applications, the Maillard reaction (MR) has been emphasized as a way to obtain proteins with new characteristics (Kato, 2002; Corzo-Martínez, Sánchez, Moreno, Patino, & Villamiel, 2012; Kasran, Cui, & Goff, 2013; Li et al., 2013). The reaction occurs spontaneously during food processing and when it is well-controlled, it can be a good method for protein modification in the food industry (Yadav et al., 2010; Corzo-Martínez et al., 2012; Li et al., 2013; Kasran et al., 2013).

Whey proteins are widely used in food products as a kind of functional additive due to their high nutritional value and excellent techno-functional properties (Panaras, Moatsou, Yanniotis, & Mandala, 2011). Alpha-lactalbumin (α-la) is the second most abundant protein in bovine whey after β-lactoglobulin. The stability of α-la depends on many factors, such as pH, the presence of salts and the source, purity and concentration of the protein. The techno-functional properties of whey proteins must be improved for their use to be expanded in foodstuff (Gu, Matsumura, Yamaguchi, & Mori, 2001).

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Acacia gum (AG) is a heteropolysaccharide usable in protein conjugation. AG is extracted from the stems and branches of acacia trees and has a highly branched structure. AG displays good emulsifying properties and has low viscosity compared to other polysaccharides of similar molar mass (Sanchez et al., 2008; Wang, Wang, Li, Adhikari, & Shi, 2011; Renard, Garnier, Lapp, Schmitt, & Sanchez, 2012).

The combination of these two biopolymers, α-lactalbumin and acacia gum, can induce a beneficial synergistic effect on the techno-functional properties of the protein. This study aimed to (i) produce and characterize conjugates obtained via Maillard reaction between α-lactalbumin and acacia gum; (ii) evaluate the functional properties, solubility, thermal stability and emulsifying properties of the conjugates compared to the pure protein; (iii) apply response surface methodology to evaluate the influence of pH, NaCl concentration and reaction time on the solubility and emulsifying properties of conjugates.

2. Materials and methods

2.1. Materials

Acacia gum (AG; purity >85%); was purchased from Vetec Química Fina (RJ, Brazil). Protein content of AG, which was further assesses through classical Kjeldahl method, was 2.2%. Powder α-lactalbumin (α-la; 95% protein, 90% of which was α-la) was kindly donated by Davisco Food International, Inc. (Eden Prairie, MN, USA). Both α-la and AG were used in the experiments without further purification. Standard molecular weight for SDS–PAGE and *o*-phthalaldehyde (OPA) were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical reagent grade. Deionized water obtained from the Milli-Q purification system (Millipore Co., MA, USA) was used in all experiments.

2.2. Preparation of α-lactalbumin–acacia gum conjugates

Conjugates of α-lactalbumin–acacia gum (α-la:AG) were prepared by Maillard reaction using the dry heating method (Kato, 2002). Protein and acacia gum (mass ratio 1:1) were dissolved in water. The mixture was stirred (Magnetic stirrer Fisatom, São Paulo, Brazil) for 1 h at 25 °C. This solution was frozen at −40 °C in an ultra freezer (Terroni, Brazil), lyophilized (Lyophilizer LS 3000, Terroni, Brazil) and incubated (Quimis Q 316 M, Brazil) at 60 °C at 79% relative humidity in a desiccator containing saturated KBr solution at different times (1, 2, 3, 5, 7, 9 and 11 days). The products (denoted as α-la:AG conjugate) were kept refrigerated until time of analysis. Pure α-la, used as control, was incubated under the same heating conditions.

2.3. Measurement of browning

The intensity of browning, an appropriate and simple indicator of the Maillard reaction progress, was measured according to Sun et al. (2011). α-la:AG conjugates were dissolved in water (5 mg mL^{-1}) and absorbance was measured at 420 nm (Spectrophotometer Care 50 Probe, Varian, USA). Measurements were taken three times.

2.4. Measuring the extent of reaction (OPA)

The conjugation extent of α-la with AG was indirectly measured using a colorimetric assay based on the reaction between *o*-phthalaldehyde (OPA) and free $-NH_2$ groups in proteins (Vigo, Malec, Gomez, & Llosa, 1992). Two hundred μL aliquots of the protein solution (2 mg mL^{-1}) were added to 4 mL of OPA reagent (1.40 mg mL^{-1}) in the tubes. The solutions were mixed and after 3 min of incubation at 25 °C, the absorbance was measured at 340 nm (Spectrophotometer Care 50 Probe, Varian, USA) (Xu, Yu, & Yang, 2010). A blank was prepared with water. The amount of available lysine (or NH_2) was determined using lysine as a standard. The assays were done in triplicate.

2.5. Sodium dodecyl sulfate–polyacrilamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to Laemmli (1970) with modifications, using a 15% acrylamide separating gel and a 5% acrylamide stacking gel containing 0.1% SDS. Samples of 0.25% w/v final protein concentration were dissolved in a denaturing buffer containing 2% SDS, 10% glycerol, 60 mmol L^{-1} Tris–HCl pH 6.8, 5% β-mercaptoethanol and 0.025% of bromophenol blue. The mixture was heated to 90 °C for 5 min and 10 μL was loaded onto the gel. A molecular weight calibration marker (Sigma) with protein molar mass ranging from 6.5 to 200 kDa was applied to the gel. The electrophoretic system was performed, at 30 mA, in vertical equipment (LCV 10 × 10, Loccus Biotechnology, Brazil), using Tris–HCl (25 mmol L^{-1}, pH 8.3) containing 0.1% SDS. The gels were stained for 30 min with coomassie brilliant blue (CBB, R-250) (0.125% CBB, 50% methanol containing 10% acetic acid) and destained in a solution containing 25% methanol and 7% acetic acid.

2.6. Size exclusion chromatography

Size-exclusion chromatography (SEC) was conducted using an AKTA chromatograph Purifier® System (10/100, Amersham Pharmacia Biotech, Sweden). SEC was used to evaluate the formation of conjugates, which present higher molar mass than the pure protein. The elution profiles were monitored by UV absorption (UV-900, Amersham Pharmacia Biotech, Sweden) at 280 nm. Samples (50 μL) were injected into a dextran/agarose column (Superdex® 75 HR 10/30, Pharmacia Biotech, Sweden) and run at a flow rate of 1.0 mL min^{-1} in a sodium phosphate buffer (pH 7.0), with an NaCl concentration of 0.15 mol L^{-1}. The maximum pressure in the column was 1.78 MPa. Conjugate concentration was 10 mg mL^{-1}. Molar mass estimation was based on the elution profile of pure protein.

2.7. Techno-functional properties of α-lactalbumin–acacia gum conjugates

2.7.1. Thermal stability of α-la:AG and pure α-la

Thermal stability as described in this study refers to the residual solubility after heat and was determined following Kato, Aoki, Kato, Nakamura, and Matsuda (1995) with modifications. Samples were dissolved in phosphate-citric acid buffer (10 mmol L^{-1} at pH 7.0) to reach the protein concentration of 2 mg mL^{-1} and heated (to 60, 70 or 90 °C) in a thermostatic bath (Tecnal TE-184, Brazil) for 20 min. The samples were cooled to 25 °C and centrifuged (model 5404, Eppendorf Germany) at 15,000 × *g* for 20 min. Supernatant absorbance was measured at 280 nm. The protein concentration of the solution was obtained using an analytical curve of pure α-la. Soluble protein was determined and expressed as a percentage of total protein in the initial solution. Thermal stability experiments were performed twice.

2.7.2. Solubility

Solubility was studied for solutions of pure α-la, an α-la/AG mixture and α-la:AG conjugate. Solutions containing 2 mg mL^{-1} of proteins were prepared in buffer solutions for which the pH and NaCl concentration were defined using a statistical design. pH ranged from 2 to 8 and NaCl concentration from 0 to 0.3 mol L^{-1}. Systems were stirred for 1 h at 25 °C, using a device to simulate an

Table 1
Uncoded levels of controllable factors analyzed on central composite rotatable design for solubility and EAI α-la and α-la:AG study.

Salt concentration (mol L^{-1})	pH	Time of Maillard reaction (T)[a]
0	2	0
0.044	2.8	1
0.15	5	2[b]
0.256	7.12	3
0.3	8	5

[a] Studied only for α-la:AG.
[b] Not included on EAI study.

agitated tank (Bonomo et al., 2003) and immediately centrifuged (Eppendorf 5404, Germany) for 15 min at 15,000 × *g*. Protein concentration was determined by measuring the absorbance at 280 nm (Spectrophotometer Care 50 Probe, Varian, USA) using an analytical curve of α-la. The solubility was calculated as a percentage by the following equation:

$$\text{Solubility}(\%) = \left(\frac{P_S}{P_I}\right) \times 100 \quad (1)$$

where P_s is the content of soluble protein in the supernatant (g), and P_I is the initial amount of protein in the sample (g).

2.7.3. Emulsifying properties

The emulsifying activity index (EAI) of pure α-la, α-la/AG mixture, and α-la:AG conjugate was determined according to Pearce and Kinsella (1978), with adaptations proposed by Cameron, Weber, Idziak, Neufeld, and Cooper (1991). Solutions of pure protein, α-la/AG mixture and α-la:AG conjugate were prepared in buffer solutions at predetermined pH and NaCl concentrations according to the experimental design. To form an emulsion, 5.0 mL of corn oil and 15.0 mL of solution (0.1%) were shaken together and subjected to homogenization (Ultra Turrax DI 25 Basic, IKA, Germany) at 20,500 rpm for 1 min at 20 °C. One hundred microliter of emulsion was taken from the bottom of the tube at different times (0, 5, 10, 15, 20 and 30 min) after homogenization, diluted in 10.0 mL of 0.1% w/v SDS solution and the absorbance of diluted emulsion was measured at 500 nm. EAI was calculated according to the following equation:

$$\text{EAI}\left(\frac{m^2}{g}\right) = \frac{2 \times 2.303 \times A_0 \times \text{DF}}{c \times (1-\theta) \times 10,000} \quad (2)$$

where A_0 is the absorbance of the diluted emulsions at time zero, DF is the dilution factor, c is the initial protein concentration (g mL^{-1}) and θ is the oil fraction to form the emulsion (0.25).

2.8. Statistical analysis

The influence of reaction time on thermal stability of α-la and α-la:AG at different temperatures was studied using a complete factorial design with two repetitions. The controlled factors were protein (α-la and α-la:AG), temperature (60, 70, and 90 °C), and reaction time (0 and 3 days). The thermal stability values obtained were analyzed by analysis of variance (ANOVA) followed by a *t*-test for effects ($p < 0.05$).

Response surface methodology was used to study the influence of the independent variables pH, salt concentration ([NaCl]), and Maillard reaction time (T) on the response variables solubility (S) and EAI using rotational central composite designs (CCD). CCD with two controllable factors (pH and salt concentration) and five central points was carried out for the α-la study ($2^2 + 2 \times 2 + 5 = 13$ trials) and for each Maillard reaction time tested on the α-la:AG study ($13 \times 5 = 65$ runs). The levels of controllable factors analyzed by CCD for solubility and EAI study of α-la and α-la:AG are shown in Table 1.

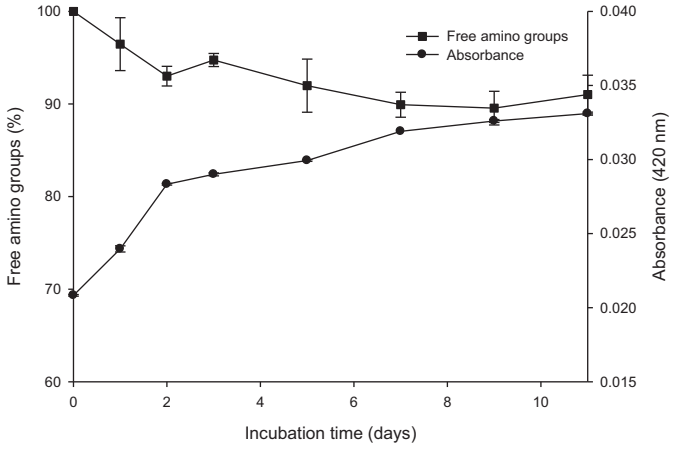

Fig. 1. Measurement of browning (absorbance 420 nm) and free amino groups conjugate content at different incubation times.

Initially, data were fitted to a second-order polynomial model:

$$Y = \beta_0 + \sum_{i=1}^{a} \beta_j X_i + \sum_{i=1}^{a} \beta_{jj} X_i^2 + \sum_{ij=1(i \neq j)}^{a} \beta_{ij} X_i X_j \quad (3)$$

where Y is the response value predicted by the model (solubility or EAI), β_0, β_i, β_{ii} and β_{ij} are the model coefficients; and X_i and X_j are levels of the independent variables. Response surfaces models were adjusted by ANOVA of regression followed by a *t*-test for the coefficients of the models. Coefficients were considered significant if $p < 0.05$ and the non-significant coefficients were removed from the initial model. Model quality was evaluated using R^2 and the *p*-values for regression and lack-of-fit from ANOVA regression of each model. A response optimizer was used to assess the levels of pH, NaCl concentration and reaction time that would simultaneously maximize solubility and EAI. In the composite desirability calculations, responses were treated with weight and relative importance value equal to 1.

3. Results and discussion

3.1. Formation of α-la:AG conjugates

Brown color development is often used as an indicator of Maillard reaction progress in foods and symbolizes an advanced stage of reaction (Sun et al., 2011). After the mild thermal treatment, a light brown color was observed in all conjugates (Supplementary data, Fig. S1). These results were corroborated by absorbance measurements of the mixtures at 420 nm. Fig. 1 shows a smooth increase in absorbance with incubation time. Browning increased more quickly in the first days. Gu et al. (2009) described the possible changes in chemical structure during heating of casein–glucose MR at high pH. These researchers showed that the slight increases in browning intensities might be due to the consumption of the amino group and acid production by MR. The chemical changes accompanying the MR in different systems leads to several changes as result of the consumption of some functional groups and the appearance of others (Gu et al., 2010). Similar results were obtained by Li et al. (2009) for the formation rice protein conjugate with different saccharides (glucose, lactose, maltodextrtrin, or dextran).

Maillard product formation occurs primarily at ε-amino group of lysine residues. As the reaction conditions.

The OPA test was applied to assess the percentage of free amino groups after different incubation times, which gives an indication of occurrence of Maillard reaction between α-la and AG. As the reaction conditions were mild (dry media, without extreme

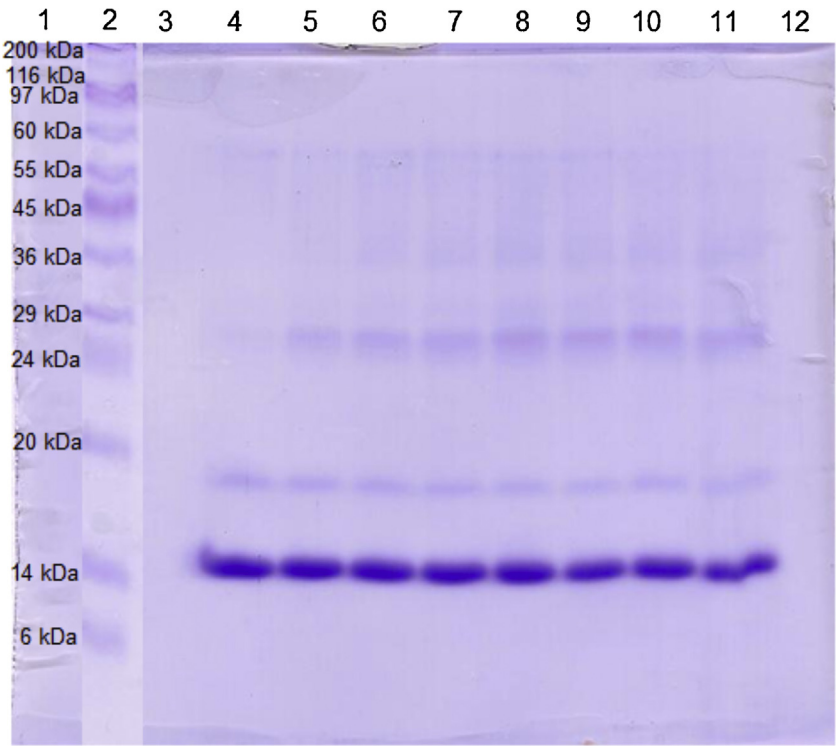

Fig. 2. Electrophoretic pattern of α-lactalbumin:acacia gum (initial concentration of 2 mg mL^{-1}; injection 10 μL). Line 2: molecular weight marker; Line 4: mixture of α-la/AG, line 5: conjugate 1 day, line 6: conjugate 2 days; line 7: conjugate 3 days, line 8: conjugate 5 days, line 9: conjugate 7 days; line 10: conjugate 9 days; line 11: conjugate 11 days.

temperatures and pH), it is believed that protein denaturation is limited and, thus, only ε-amino groups of surface-exposed amino acid residues took part in the reactions. According to data in Fig. 1, there was a decrease of ≈12% in the content of free amino groups, after 6–8 days of incubation, suggesting the occurrence of reaction between these groups of α-la and AG, thus forming conjugates. Compared to previous reports, involving whey protein isolate/dextran (Sun et al., 2011) and α-lactalbumin/D-allose (Sun, Hayakawa, Puangmanee, & Izumori, 2006), the behavior observed in the present study points to a somewhat limited degree of α-la:AG conjugation.

Niu, Jiang, Pan, and Zhai (2011) reported the glycation of wheat germ protein with different sacharides. The degree of glycosylation increased with reaction time and decreased with increased saccharide size. According to Kato (2002) the number of polysaccharides that bind with the protein is limited due to macromolecule steric hindrance. Thus, the complex structure of the acacia gum probably hindered the extent of the Maillard reaction.

3.2. SDS–PAGE electrophoresis

SDS–PAGE electrophoresis is not used for the analysis of AG gum, therefore we will analyze the results in terms of α-la (Motlagh, Ravines, Karamallah, & Ma, 2006). Fig. 2 shows the electrophoretic pattern of α-la/AG mixture without heating (line 4) and after different times of dry-heating at 60 °C (lines 5–11). A band corresponding to chemical species with molar mass ≈25 kDa appears in the conjugate patterns, and is not present in the mixture without heating (line 4), suggesting an interaction between α-lactalbumin and acacia gum to form conjugates. However, the literature reports that bands with molar masses between 24 and 36 kDa can be dimers of α-La (He et al., 2013). The amount of AG was insufficient to react with all α-la molecules. Thus, the intensity of α-la bands did not change after the dry-heating treatment. Diffused bands in the glycoproteins stains of the α-la:AG conjugate indicate polydispersive molar mass distribution of the reaction products. Similar results were reported by Kim, Choi, Shin, and Moon (2003) for BSA-galactomannan conjugate formation.

3.3. Size exclusion chromatography

SEC of the pure α-la, α-la/AG mixture and α-la:AG conjugate was performed to confirm conjugation occurrence. The main peak of α-la occurred at elution time of 13 min (Fig. 3). α-la:AG at different reaction times produces patterns of elution distinct from the pattern of pure α-la. Glycated protein molecules show a slightly lower elution volume compared to pure α-la, as a result of the increase in the hydrodynamic diameter upon conjugation. Likewise, as observed in the electrophoresis results, the conjugate elution profile showed unreacted α-la, evidenced by the high intensity of α-la peaks at the same time of pure protein elution. Similar results were reported by Haar, Westphal, Wierenga, Schols, and Gruppen (2011) for the glycosilation of α-la in dry-heating, with several mono- and oligosaccharides. O'Regan and Mulvihill (2009) also confirmed the sodium caseinate-maltodextrin conjugation using gel permeation chromatography.

3.4. Thermal stability of α-la:AG and pure α-la

Thermal stability was studied at pH 7.0, the usual condition in the food industry, using a complete factorial design to analyze the effect of reaction time (RT) and conjugation of α-la with AG at 60, 70 and 90 °C. Both main and interaction effects adjusted for the analyzed factors and the respective *p*-values are shown on Table 2. The significant interaction conjugation$^{*}t_H$ is represented in Fig. 4.

We observed that conjugation of α-la with AG had a significant and positive effect on thermal stability. The thermal stability of α-la:AG increased compared to pure α-la heated under the

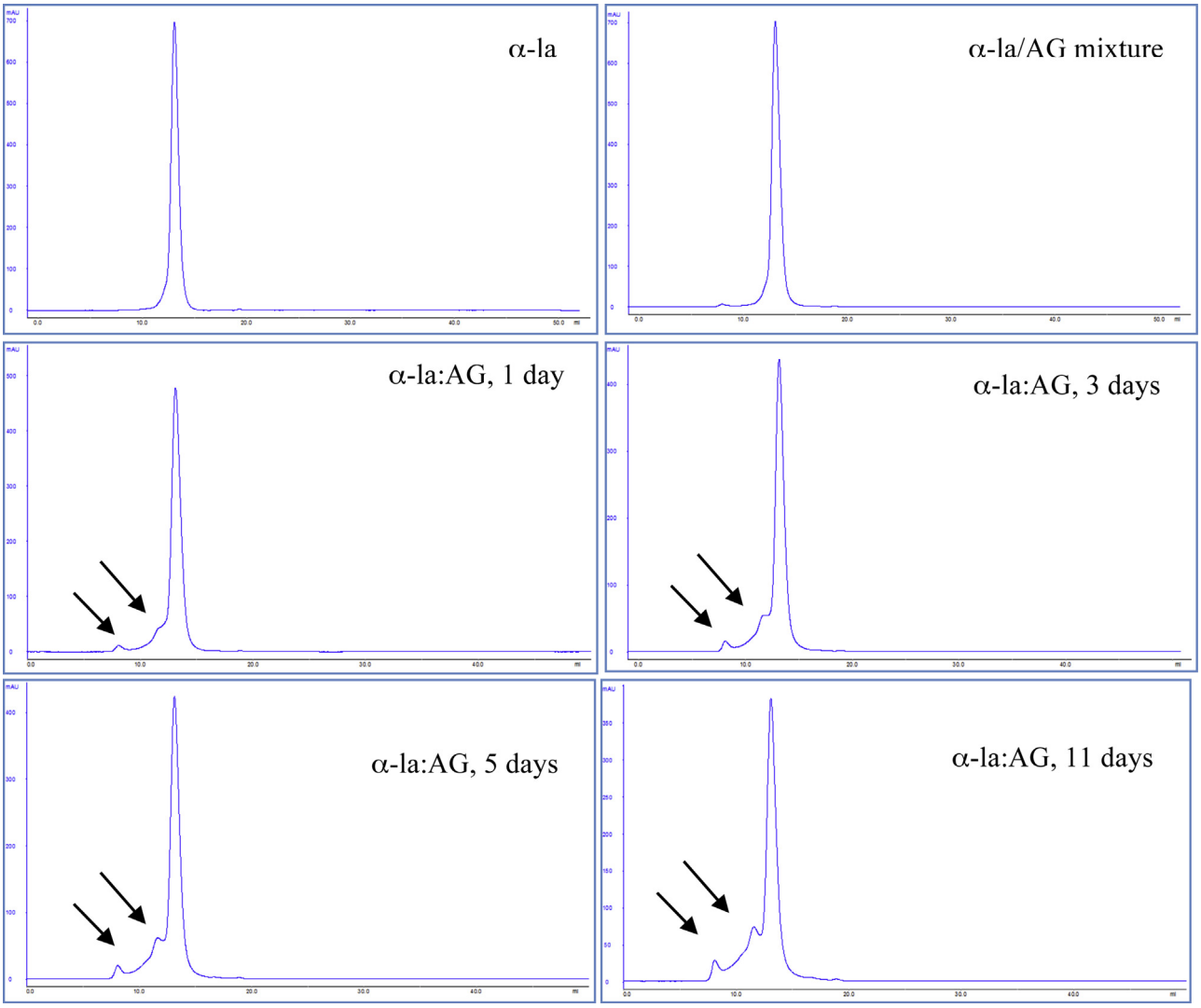

Fig. 3. Size exclusion chromatograms of α-la, α-la/AG mixture and α-la:AG conjugate.

same conditions. Significant interaction conjugation*RT shows that prior to the reaction the thermal stability of α-la is higher than α-la/AG mixture. When pure α-la and α-la:AG are heated for three days, however, the thermal stability of the α-la:AG conjugate is higher than pure protein. Álvarez, García, Rendueles, and Díaz (2012) reported that thermal stability of porcine blood protein isolated with dextran conjugate increases when the thermal treatment is applied. According to the authors, dextran obstructs protein–protein interactions, maintaining the native structure of the protein and avoiding the aggregation. Thus, the conjugate shows greater resistance to aggregation and subsequent precipitation. In this study, the acacia gum may have had the same effect as dextran.

Table 2
Main and interaction effects of temperature, conjugation and reaction time (RT) on thermal stability of α-lactoalbumin.

Term	Effect	*p*-Value
Temperature	1.281	0.022
Conjugation	3.197	0.000
RT	−2.647	0.000
Conjugation*RT	4.992	0.000

R^2 = 92.5%.

3.5. Solubility

Surface models were adjusted to evaluate solubility (*S*) of the pure α-la and α-la:AG conjugate at different pH and [NaCl] concentrations. This was done for conjugates obtained after different

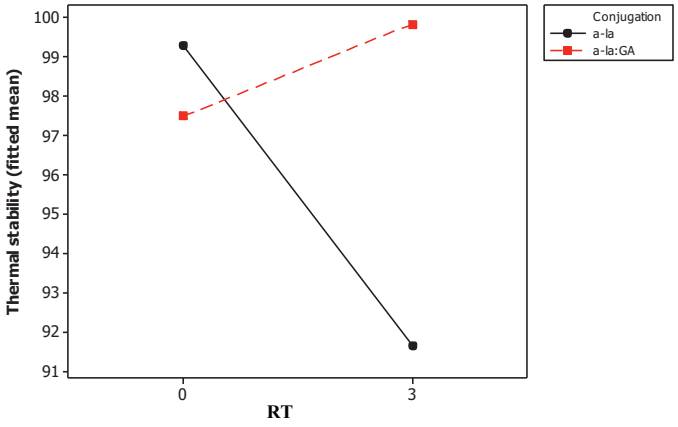

Fig. 4. Interaction plot for conjugation and reaction time (RT) on α-lactalbumin thermal stability.

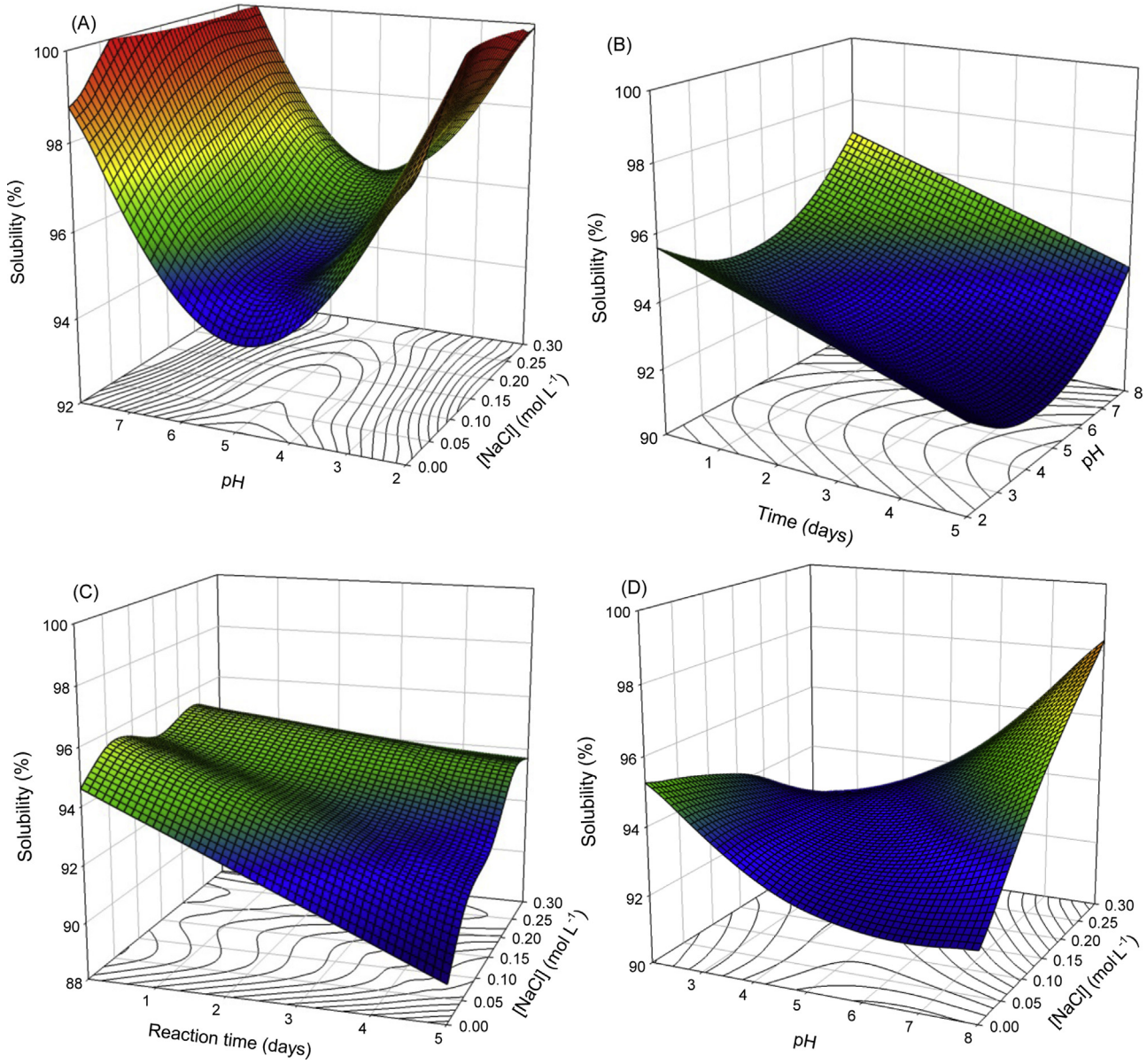

Fig. 5. Response surface plots describing the effect of pH and salt concentration (S) on the solubility of α-la (A), and the effect of pH, [NaCl] and reaction time (T) on the solubility of α-la:AG conjugate (B–D).

reaction time (T) using the experimentally obtained solubility values (Supplementary data, Table S1).

Eqs. (4) and (5) were derived from α-la and α-la:AG conjugates. The following models were obtained, with the significant coefficients ($p < 0.05$) denoted with (*):

$$S_{\alpha\text{-la}} = 109.277 - 6.261^*\text{pH} + 6.832\,[\text{NaCl}] + 0.641^*\text{pH}^2$$
$$R^2 = 82.0\% \quad (4)$$

$$S_{\alpha\text{-la:AG}} = 102.484 - 1.052^*T - 2.572^*\text{pH} - 27.739^*\,[\text{NaCl}] + 0.199^*\text{pH}^2 + 3.071T \times [\text{NaCl}] + 5.343^*P \times [\text{NaCl}] \quad R^2 = 42.5\% \quad (5)$$

The estimated coefficients and the respective p-values are listed in Table 3. The fitted values of α-la and of α-la:AG conjugate solubility, as well as their corresponding residuals, are presented in Supplementary data (Table S1).

According to Eqs. (4) and (5), the solubility of α-la is significantly affected only by pH, while the α-la:AG solubility is affected in a complex manner by the three factors analyzed. Moreover, low R^2 (42.5%) of α-la:AG solubility model and low residual square values indicate that other factors not analyzed in the present study, such as the proportion of protein:polysaccharide, reaction temperature and water activity of reaction rates, could affect α-la:AG solubility. These factors should be investigated in future studies to find best way to predict α-la:AG solubility. Surface plots of α-la and α-la:AG are shown in Fig. 5.

Eq. (4) and Fig. 5A show pH as the unique factor that exerts significant linear and quadratic effects on α-la solubility. As expected, pH ≈ 5 caused lower solubility, because this pH is near the isoelectric point (pI) of α-la. At pI, the net charge of the protein is near zero and the asymmetric charge of the protein results in electrostatic interactions, causing them to aggregate (Mu, Zhao, Zhao, Cui, & Liu, 2011).

Comparing the models showed lower solubility values were obtained with the presence of AG, even without occurrence of MR,

Table 3
Adjusted models for solubility and EAI of α-la and α-la:AG conjugate.

Response	Term	Coefficient	*p*-Value
α-la solubility	Constant	109.277	0.000
	pH	−6.261	0.000
	[NaCl]	6.832	0.134
	pH^2	0.641	0.000
	R^2 = 82.0%		
α-la:AG solubility	Constant	102.484	0.000
	T	−1.052	0.001
	pH	−2.572	0.002
	[NaCl]	−27.739	0.012
	pH^2	0.199	0.008
	T^*[NaCl]	3.071	0.072
	pH^*[NaCl]	5.343	0.007
	R^2 = 42.5%		
α-la EAI	Constant	57.3021	0.000
	pH	−4.8194	0.015
	[NaCl]	2.0378	0.758
	pH^2	0.4922	0.013
	R^2 = 51.9%		
α-la:AG EAI	Constant	62.312	0.000
	T	5.449	0.000
	pH	−8.132	0.000
	[NaCl]	−39.868	0.038
	T^2	−0.496	0.011
	pH^2	0.855	0.000
	$[NaCl]^2$	107.415	0.075
	T^*pH	−0.417	0.008
	R^2 = 60.7%		

at $T = 0$ (Fig. 5A and B). This behavior was verified for all reaction times and was more intense at lower pH values (pH < 5). The non-significance of the T^*pH interaction supported this observation. RT exerts significant, both linear and negative, effects on α-la:AG solubility (Fig. 5B and C). The negative effect of RT on the solubility was observed by other authors. Al-Hakkak and Al-Hakkak (2010) reported reduced solubility of egg white protein–pectin conjugate with reaction time.

Fig. 5C shows that solubility increases with increasing salt concentration for the same RT. This behavior can be confirmed by the significant T^*[NaCl] interaction.

Thus, conjugate solubility could be kept high by controlling the salt concentration. Higher values of solubility are needed to intensify techno-functional properties of proteins.pH exerts a quadratic effect on α-la:AG solubility, as observed for α-la. Fig. 5D suggests an increase in conjugate solubility at high pH values and high salt concentrations, as well as at low pH values and low salt concentration. This behavior can be confirmed by the significant and positive pH^*[NaCl] interaction.

Additionally, solubility data of pure α-la, heated α-la, α-la/AG mixture and α-la:AG conjugate were obtained and evaluated using ANOVA. Significant differences between means ($p < 0.05$) were identified using the Duncan test. The solubility of the samples was determined at pH 5.0 and an NaCl concentration of 0.15 mol L^{-1}.

Fig. 6 shows that the solubility of pure α-la, α-la/AG mixture and α-la:AG conjugate at 1 and 3 days were statistically the same ($p < 0.05$), and differed from the averages of the heated α-la at 1 and 3 days, which also differed from each other. According to these results, the solubility of α-la heated without the presence of AG is lower than the solubility of α-la heated with AG. Thus, at pH 5, the heating of the protein with gum, to form conjugates, did not alter the solubility of the protein, while when the protein was heated in the absence of the gum, solubility decreased.

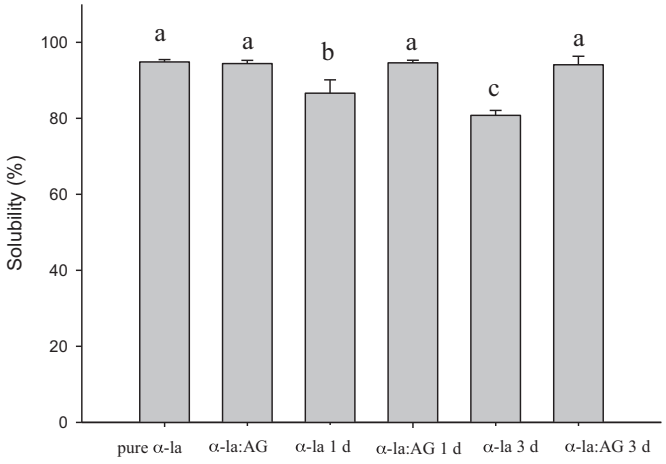

Fig. 6. Solubility of the different samples at pH 5.0 and NaCl concentration 0.15 mol L^{-1} (mean of five repetitions).

3.6. Emulsifying properties

Emulsifying properties of α-la and the α-la:AG conjugate were evaluated in terms of EAI, which is proportional to the oil–water interface area that can be covered per gram of protein. Surface models were adjusted to evaluate the EAI behavior of α-la and α-la:AG conjugates at different pH and [NaCl] values. This was done for conjugates obtained after different reaction times (T) using the experimentally obtained EAI values (Supplementary data, Table S2). Eqs. (6) and (7) were derived from the α-la and α-la:AG conjugates. The following models were obtained, with the significant coefficients ($p < 0.05$) denoted with (*):

$$EAI_{\alpha\text{-la}} = 57.302 - 4.819^*pH + 2.038\,[NaCl] + 0.492^*pH^2 \quad R^2 = 51.9\% \tag{6}$$

$$EAI_{\alpha\text{-la:AG}} = 62.312 + 5.449^*T - 8.132^*pH - 39.868^*\,[NaCl] - 0.496^*T^2 + 0.855^*pH^2 + 107.415[NaCl]^2 - 0.417^*T \times pH \quad R^2 = 60.7\% \tag{7}$$

The estimated coefficients and the *p*-values are listed in Table 3. The fitted EAI values of the α-la and of α-la:AG conjugate and its corresponding residuals are presented in Supplementary data (Table S2).

According to these models, the EAI of α-la is significantly affected by pH, while the α-la:AG EAI is complexly affected by the three analyzed factors. Moreover, low R^2 of α-la and α-la:AG and low residual square values indicate that other factors, not analyzed in the present study, could affect the EAI of the α-la and α-la:AG conjugate. Gu et al. (2009) showed that in addition to heating time, temperature and pH of the conjugates had a significant effect on the emulsifying properties of casein–glucose conjugates. These other factors should be investigated in future studies to find the best way to predict α-la:AG EAI.

Surface plots of α-la and α-la:AG EAI are shown in Fig. 7. In accordance with Eq. (6), Fig. 7A showed that pH was the unique factor that exerts significant linear and quadratic effects on the EAI of α-la response, as observed in α-la solubility (Fig. 5A). The pH exerts a very similar effect on α-la and α-la:AG, however higher values of EAI are obtained for the same pH values when the protein is conjugated with gum (Fig. 7A–D). The significant interaction T^*pH (Fig. 7B) shows that the effect of pH changes with the reaction time, becoming more pronounced with increased reaction time.

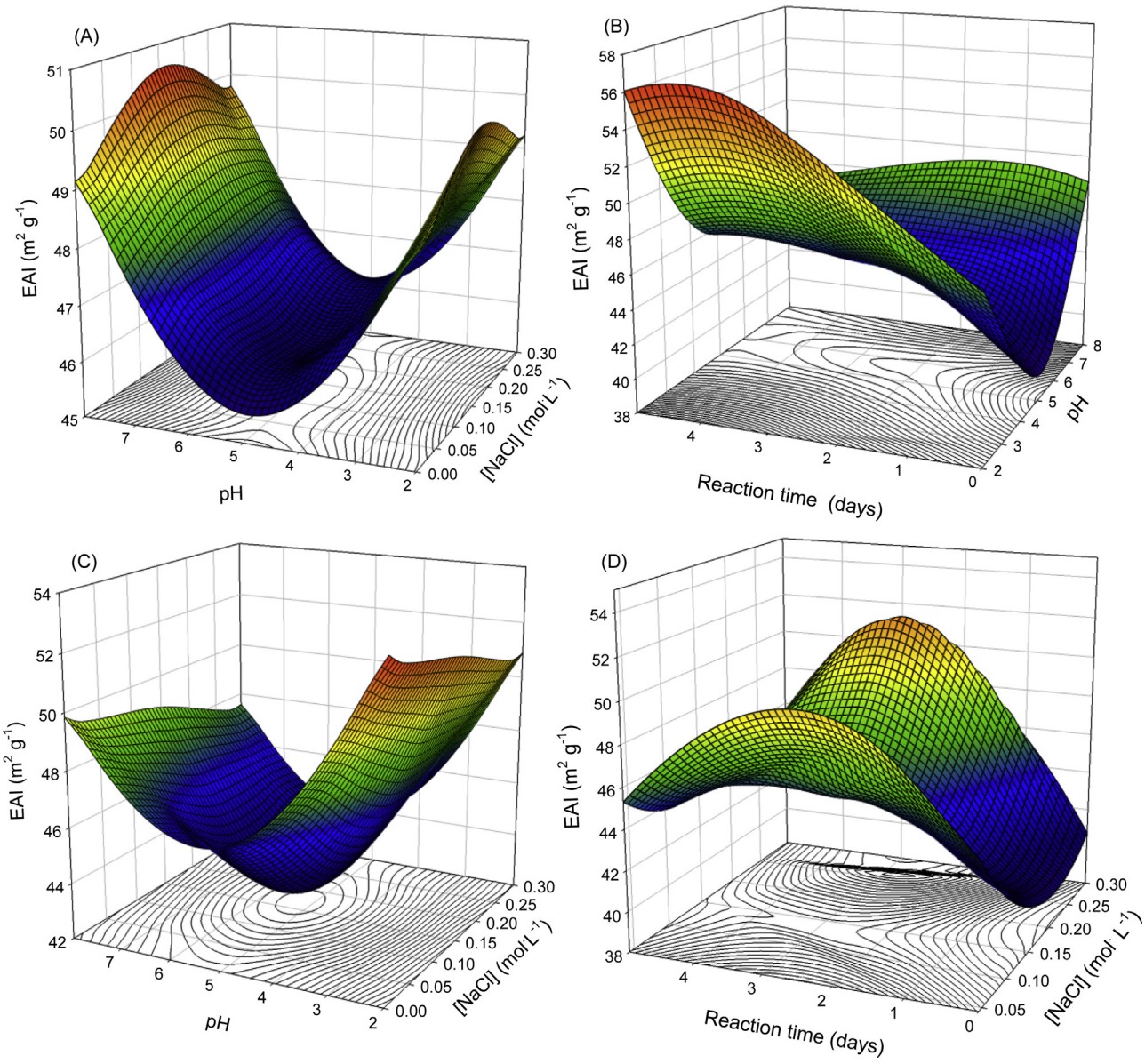

Fig. 7. Response surface plots describing the effect of pH and [NaCl] on the EAI of α-la (A), and the effect of pH, [NaCl] and reaction time (*T*) on the EAI of conjugate of α-la:AG (B–D).

This result suggests that the reaction of α-la and AG is necessary to change the conjugate EAI behavior in relation to pH. The reaction time can be adjusted to maximize the EAI according to the pH desired by industry.

The reaction time and [NaCl] concentration exerted significant and quadratic effects on α-la:AG conjugate, with the quadratic effect of time being stronger (Fig. 7D). This result, combined with the α-la EAI adjusted model (Fig. 7A) indicates that salt concentration exerts an effect on α-la EAI only in acacia gum mixture. Additionally, the salt concentration effect is independent of the time of Maillard reaction, as shown by the non-significance of T^*[NaCl] interaction. These results suggest that extreme values of pH and [NaCl] on the studied interval should be used to maximize the EAI of α-la:AG, while intermediate reaction time values, e.g. 3 days, should be used to maximize the EAI response. Chanamai and McClements (2002) obtained different result for the pure gum heated. These authors found that calcium chloride concentration, pH or temperature have no effect on emulsions stabilized by gum Arabic. Furthermore, previous reports showed that mild heating of AG caused only minor effects on its emulsification properties (Chikamai, Banks, Anderson, & Wang, 1996; Islam, Phillips, Sljivo, Snowden, & Williams, 1997).

The maximum composite desirability ($D = 0.804$) calculated using the adjusted models for solubility and EAI was obtained at pH 8.0, NaCl concentration 0.3 mol L^{-1} and two days of Maillard reaction, suggesting these levels be used for applications where maximum solubility and EAI are required simultaneously.

4. Conclusions

α-la:AG conjugates were formed in the conditions specified in this study. Measurement of browning, the OPA test, SDS–PAGE and SEC confirmed conjugate formation. Conjugates showed higher thermal stability than the heated protein without AG under the same conditions. Conjugates had lower solubility than pure protein. Reaction time decreased conjugate solubility. However, when compared to the heated protein without the gum,conjugate

solubility was higher. Conjugate emulsifying activity was significantly affected by pH, salt concentration and reaction time. The maximum emulsifying activity was achieved at intermediate reaction time values and extreme pH and salt concentrations.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.11.060.

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