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Rice proteins – gum arabic coacervates: Effect of pH and polysaccharide concentration in oil-in-water emulsion stability

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Abstract

In the context of replacing animal proteins in food matrices, rice proteins (RP) become promised because they come from an abundant plant source, are hypoallergenic, and have high digestibility and nutritional value. However, commercial protein isolates obtained by spray drying have low solubility and poor functionality, especially in their isoelectric point. One way to modify these properties is through interaction with polysaccharides, such as gum arabic (GA). Therefore, this work aims to evaluate the effects of pH and GA concentration on the interaction and emulsifying activity of RP:GA coacervates. First, the effects of pH (2.5 to 7.0) and GA concentrations (0.2 to 1.0 wt.%, giving rise to RP:GA mass ratios of 1:0.2 to 1:1.0) in RP:GA blends were evaluated. The results demonstrated that biopolymers present opposite net charges at pH between 2.5 and 4.0. At pH 3.0, insoluble coacervates with complete charge neutralization were formed by electrostatic interactions, while at pH 5.0 it was observed that the presence of GA prevented the RP massive aggregation. Second, selected blends with 0.4 or 1.0 wt.% of GA (RP:GA mass ratios of 1:0.4 or 1:1.0) at pH 3.0 or 5.0 were tested for their ability to stabilize oil-in-water emulsions. The emulsions were characterized for 21 days. It was observed that the GA increased the stability of RP emulsions, regardless of the pH and polysaccharide concentration. Taken together, our results show that it is possible to combine RP and GA to improve the emulsifying properties of these plant proteins at pH conditions close to their isoelectric point, expanding the possibility of implementation in food systems.

Keywords: Coacervates; plant proteins; natural gum; functional properties; emulsification; acid emulsions.

1. INTRODUCTION

In recent years, attention has been directed to vegetable proteins due to the advantages they bring from the point of view of human and environmental health, production costs, and functionality. In addition, vegetable proteins are considered a renewable and sustainable alternative to animal proteins (Nikbakht Nasrabadi et al., 2021). Thus their incorporation into food responds to a demand for completely vegetable foods by a part of society in constant growth (Hassoun et al., 2022). In particular, rice proteins (RP) have aroused increasing interest in the food industry since they are hypoallergenic, and several studies have suggested that RP (in many cases, RP hydrolysates and specific peptide fractions therefrom) have antioxidant, hypocholesterolemic, and anticancer

activities (Amagliani et al., 2017; G. H. Li et al., 2007; Pantoa et al., 2020; Singh et al., 2021). As well, it has been reported that RP present an essential amino acid composition close to the ideal model recommended by FAO/WHO (Zheng et al., 2023) and have higher digestibility and biological value than other cereals such as wheat, corn, and barley (Ghanghas et al., 2022; Han et al., 2015). Therefore, the development of RP isolates and their use as food ingredients have become a major trend in recent years.

RP isolates are composed of albumin (~1%-5%), prolamin (~2%-8%), globulin (~4%-15%), and glutelin (~75%-80%) fractions (Hoogenkamp et al., 2017). Glutelin is presented as extensively aggregated, disulfide-bonded, highly hydrophobic, and glycosylated proteins (Takaiwa et al., 1999). Since the global behavior of protein isolates is largely determined by the behavior of its major protein fraction, RP isolates are highly insoluble and show poor surface activity properties (Amagliani et al., 2017; Dai et al., 2022). In addition, the spray drying process, which is used to obtain commercial protein powders, tends to increase aggregation and decrease solubility (Li et al., 2022). To the best of our knowledge, only a few published works used commercial RP isolates as emulsifying agents due to these limitations. In particular, the commercial RP isolate utilized in the current work presented large aggregates (mean diameter: 19.8 ± 0.6 μm) with very low solubility (2.7 ± 1.0 %) at pH 7.0 (Igartúa et al., 2024). Therefore, improving the solubility of RP is a crucial challenge to fully take advantage of these high-quality plant proteins. In a previous paper, the potential of pH-shifting, ultrasound, and/or heat treatments performed alone or in combination to increase RP solubility was studied (Igartúa et al., 2024). The obtained results showed that the combination of pH-shifting, heat, and ultrasound treatments performed in that order increased the solubility (91.8 ± 6.4 %) and induced the formation of less compact and more dispersed protein aggregates. The modified RP were able to form oil-in-water (O/W) emulsions with smaller particle size and enhanced stability at pH 7.0 than the untreated RP (Igartúa et al., 2024). However, the ability of these modified RP derivates to stabilize O/W emulsions under acidic pH conditions has not been studied yet. It is expected that proteins exhibit poor emulsifying properties at acid pH due to the proximity of their isoelectric point. One strategy to overcome these limitations is combine the proteins with polysaccharides to increase the electrostatic and steric repulsions between the droplet surfaces (Zang et al., 2019). The hypothesis of the present work is that the combination of modified rice protein with gum arabic can increase the O/W emulsifying activity of rice proteins in acid pH-conditions.

Gum arabic (GA) is an exudate of *Acacia Senegal* and *Acacia Seyal* trees that is composed of a mixture of highly-branched arabinogalactan-type polysaccharides, with an average molecular weight range of 260–1160 kDa, and less than 2 wt.% of proteins (Dror et al., 2006). The serine and threonine residues of these proteins are covalently bound to carbohydrates. GA is negatively charged at pH values above 2.2, due to the carboxylic groups on its glucuronic acid residues (Zang et al., 2019), and is highly soluble in water but does not significantly increase the viscosity up to 50 wt.% concentration (X. Liu et al., 2023).

Particularly, this work aims to evaluate the effects of pH and polysaccharide concentration on the RP:GA blend behavior and emulsifying ability. In this sense, derivatives of commercial rice protein isolates -obtained by a combination of pH-shifting, heating, and ultrasound treatments- are combined with GA to obtain coacervates and said coacervates are employed as emulsifying agents at acidic pH conditions.

2. MATERIALS AND METHODS

2.1. Materials

The rice protein (RP) isolate was donated by Grupo Saporiti S.A. (Buenos Aires, Argentina). According to the supplier, the isolate was obtained from *Oryza sativa,* and solid powder was obtained by spray drying. Also, the powder was composed of 83.6 wt.% of protein (N×6.25), 6.3 wt.% of carbohydrate, 6.1 wt.% of fat, 1.5 wt.% of ash, and 2.3 wt.% of moisture. The crude protein concentration was confirmed by the Kjeldahl method, using the same nitrogen-to-protein conversion factor. The gum arabic (GA, CAS 9000-01-5) was purchased to Biopack (Buenos Aires, Argentina). According to the supplier´s datasheet, the GA powder presented 83.7% purity, with 4.2 wt.% of ash, and 12.1 wt.% of moisture. Both RP and GA powders were used without further purification. The bovine serum albumin (BSA, CAS 9048-46-8, purity > 99.0%, fatty-acid free), bicinchoninic acid (BCA, 2,2′-Biquinoline-4,4′-dicarboxylic acid disodium salt, CAS 979-88-4), and sodium azide (CAS 26628-22-8) were obtained from Sigma Aldrich (Missouri, USA). The PRODAN fluorescent probe (*N,N*-Dimethyl-6-propionyl-2-naphthylamine, CAS 70504-01-7) was provided by Santa Cruz Biotechnology (Texas, USA). Refined sunflower oil was purchased from a local supermarket (Molino Cañuelas, Buenos Aires, Argentina). All the other chemicals were of analytical grade and purchased from local distributors (Buenos Aires, Argentina). All pH determinations were done using a C861 Consort pH/mV meter with a PY-P10-25 Sartorius electrode (resolution ±0.01), calibrated daily before use. Weight determinations were performed using a calibrated Ohaus® Pioneer® PA214

analytical balance (resolution ±0.0001 g). Finally, dilutions were performed using calibrated Gilson® automatic pipettes.

2.2 RP modification and dispersion

Commercial protein isolates obtained by spray drying tend to be difficult to disperse since large protein aggregates are formed during drying. Hence, to obtain a dispersion of rice proteins (RP), a combination of pH-shifting, heat, and ultrasound treatments was performed according to Igartúa et al. (2024). Briefly, 2.50 g of the RP isolate was weighted, and deionized water was added to obtain 95.00 g of dispersion. The pH was adjusted to 12.0 by adding NaOH 1 M, and the dispersion was mixed for 1 h at room temperature (25 ± 2°C). Then, the dispersions (95.00 g in a glass beaker with 6.5 cm of diameter) were placed in a hot-water bath until it reached 90°C for 30 min, followed by placing the samples in an ice-water bath to cold them. After that, the dispersions (95.00 g in a glass beaker with 6.5 cm of diameter) were sonicated for 5 min with a 30/30 sec on/off cycle and 100% amplitude using a Sonics Vibra Cell VCX750 ultrasound homogenizer (Sonics & Materials, Inc.; USA) with the 28 mm diameter tip immersed 1/3 in a beaker. The temperature increase during sonication was avoided by putting the beaker in an ice-water bath. Next, the pH of the dispersions was adjusted to 7.0 by adding HCl 1 M. Finally, the necessary quantity of deionized water was added to obtain 100.00 g of dispersions (2.50 wt.% modified RP dispersion), followed by overnight standing for full hydration. For storage, sodium azide at 0.02 wt.% was added to prevent microbial growth. The RP dispersion was stored at 4°C protected from light until use.

2.3 GA dispersion

To prepare GA dispersion, 4.00 g of GA solid powder was weighted, and deionized water was added to obtain 100.00 g of dispersion. The dispersion was mixed at room temperature (25 ± 2°C) until complete dissolution. For storage, sodium azide at 0.02 wt.% was added. The GA dispersion was stored at 4°C until use.

2.4 Preparation and characterization of RP:GA blends

2.4.1 Preparation of RP:GA blends

RP:GA blends were obtained by weighing appropriate proportions of RP and GA dispersions, adjusting the pH from 7.0 to 2.5 -every 0.5 pH-units- using 1 M HCl solution, and mixing for 1 h at room temperature. The evaluated RP:GA mass ratios were 1:0.2, 1:0.4, 1:0.6, 1:0.8, and 1:1.0. In all the blends, the RP final concentration was maintained at 1.0 wt.%, and the GA concentration was varied from 0.2 to 1.0 wt.%, for every

0.2 concentration-units. Therefore, the total biopolymer concentration in RP:GA blends varied from 1.2 to 2.0 wt.%. Individual RP and GA dispersions at 1.0 wt.% were used as control samples.

2.4.2 ζ-potential

The ζ-potential determinations were performed at room temperature (25 ± 2°C) using a Zetasizer Nano ZSP ZEN 5600 analyzer (Malvern Instruments, UK). The ζ-potential was measured in RP and GA control dispersions at pH values from 7.0 to 2.5, every 0.5 pH-units, and in RP:GA blends at pH 7.0, 5.0, and 3.0. To avoid multiple light scattering effects, samples were diluted tenfold (1/10 v/v) with deionized water previously adjusted at each pH. The refractive index values were 1.33 for the dispersant and 1.54 for RP, GA, and RP:GA blends. For elucidation of the type and the magnitude of the interactions as a function of pH, the strength of electrostatic interaction (SEI) between RP and GA was calculated as the multiplication of ζ-potential values of individual biopolymer at each specific pH, according to Equation (1):

$$\mathrm{SEI}\ (\mathrm{mV}^2) = \zeta\ \mathrm{potential}_{\ \mathrm{RP}}\ (\mathrm{mV}) \times \zeta\ \mathrm{potential}_{\ \mathrm{GA}} (\mathrm{mV}) \quad (1)$$

Negative SEI values indicate attractive forces between biopolymer molecules, while positive SEI values indicate repulsive forces between them (J. Liu et al., 2021).

2.4.3 Protein solubility

The protein solubility was measured in RP control and RP:GA blends at pH values from 7.0 to 2.5, every 0.5 pH unit. For determination, samples were centrifuged for 10 min at 7200g and 20°C using a Hermle Z 200 A centrifuge (LaborTechnik, Germany), and the supernatants were collected. The protein concentrations in supernatants were determined using the bicinchoninic acid (BCA) method first described by Smith et al. (1985) with minor modifications (Igartúa et al., 2024). On the day of the experiment, 50 parts of reagent A (BCA 11.0 wv%, Na_2CO_3 2.0 wv%, NaOH 0.4 wv%, sodium tartrate 0.16 wv %, and $NaHCO_3$ 0.95 wv %, pH 11.25) were mixed with 1 part of reagent B ($CuSO_4$-$5H_2O$ 4.0 wv%) to obtain reagent C. Then, 25 µL of each supernatant was mixed with 200 µL of reagent C in each well of a 96-well plate. These mixtures were incubated at room temperature for 20 min and then the absorbance at 562 nm was determined using the Cytation 5 microplate reader (BioTek Instruments Inc., USA). The protein concentration was determined by comparison with a standard calibration curve performed with bovine serum albumin (BSA) in deionized water, which exhibited linearity from 0.025 to 2.0 mg/mL (R^2=0.999). The protein solubility was calculated as the ratio between the protein

concentration in the supernatant after centrifugation with respect to the initial protein concentration in samples before centrifugation.

2.4.4 Visual appearance and construction of Phase Diagram

The appearance of RP and GA controls and RP:GA blends at pH-values from 7.0 to 2.5 was recorded after 24 h of standing at room temperature. All images were taken using the same digital camera under the same background. Then, the samples were classified into four groups based on the turbidity of the suspension and the presence of precipitate as translucent suspension, precipitate with translucent suspension, precipitate with cloudy suspension, or precipitate with very cloudy suspension.

2.4.5 Protein surface hydrophobicity

The protein surface hydrophobicity was determined in freshly prepared RP and RP:GA blends at pH 7.0, 5.0, and 3.0 using the PRODAN probe according to a previously reported method (Alizadeh-Pasdar & Li-Chan, 2000) with minor modifications (Igartúa et al., 2022). A 1.4 mM PRODAN stock solution in methanol was prepared and stored at −20°C in brown screw-capped vials. On each experimental day, PRODAN was diluted to 0.07 mM in methanol (1/20 v/v) and held on ice until use. For RP and RP:GA blends, adequate agitation was carried out before taking the samples. These samples were serially diluted in deionized water to obtain protein concentrations ranging from 0.2 to 0.00625 wt.%. Later, each dilution was placed at 200 µL/well in a black 96-well plate (Greiner Bio-one, USA). The fluorescence emission in each well was determined using a multi-mode plate reader Cytation 5 (BioTek Instruments, USA). The excitation wavelength was 365 ± 9 nm, while the emission wavelength was 465 ± 20 nm. Then, the diluted PRODAN was placed at 20 µl/well, and the plate was agitated for 10 min in darkness. The fluorescence emission after PRODAN incorporation was determined as previously described. The difference between the fluorescence emission intensity after and before PRODAN incorporation was plotted against the protein concentration. The slope was determined by linear regression analysis and used as an index for protein surface hydrophobicity.

2.4.6 Particle size distribution

The particle size distributions of freshly prepared RP and RP:GA blends at pH 7.0, 5.0, and 3.0 were determined by static light scattering using a Mastersizer 2000E analyzer associated with a Hydro 2000MU wet dispersion unit (Malvern Instruments Ltd., UK). The pump speed in the dispersion unit was set at 2000 rpm. The refractive index values were set at 1.33 and 1.54 for the dispersant and biopolymers (RP or RP:GA blends), respectively. In

addition to particle size distributions, the volume- and surface-weighted average diameters (d43 and d32, respectively) were analyzed since the first is more sensitive to the presence of large particles while the second is more sensitive to the presence of small particles (McClements, 2004).

2.4.7 Fluorescence microscopy

The RP and RP:GA blends at pH 7.0, 5.0, and 3.0 were observed using the fluorescence microscope Cytation 5 (BioTek Instruments Inc., USA) after staining with rhodamine B. The samples for visualization were taken after proper shaking of freshly prepared dispersions. For staining, 10 µL of ethanolic rhodamine B solution (0.05 wt.%) was added to 1 mL of each sample. Then, the stained dispersions were put in a glass slide and covered with a coverslip for observation at 200 X magnification (20 X ocular magnification plus 10 X digital detector magnification).

2.5 Preparation and characterization of RP:GA coacervates stabilized emulsions

2.5.1 Preparation of O/W emulsions

Based on the results obtained with the previously described methods (Section 2.4), the RP:GA coacervates with 1:0.4 and 1:1.0 mass ratios at pH 5.0 and 3.0 were selected to evaluate their emulsification properties. A two-step homogenization process was used to prepare the O/W emulsions following the methodology described by Cabezas et al. (2019). In short, pre-emulsions were obtained by mixing 90.00 g of each sample with 10.00 g of refined sunflower oil at 20000 rpm for 2 min in a plastic beaker with a 4.0 cm diameter using an Ultraturrax T25 homogenizer with an S25N-8G dispersion tool (IKA Labortechnik, Germany). Then, the pre-emulsions were sonicated for 2 min (20/20 sec on/off cycle, 70% amplitude) using a Sonics Vibra Cell VCX750 ultrasound homogenizer (Sonics & Materials, Inc.; USA), with the 28 mm diameter tip immersed 1/3 in the plastic beaker. The temperature increase during sonication was avoided by putting the beaker in an ice-water bath.

2.5.2 Particle size distribution

The particle size distributions of emulsions were determined using a Mastersizer 2000E analyzer associated with a Hydro 2000MU wet dispersion unit (Malvern Instruments Ltd., UK). For measurements, emulsions were previously diluted (1:4 v/v) with deionized water or SDS 1.0 wt.% solution. SDS is used as a deflocculating agent (Zhi et al., 2022). The pump speed in the dispersion unit was set at 2000 rpm. The refractive index values for the oil droplets and dispersant were set at 1.47 and 1.33, respectively; d43 was used to express the particle size. For

storage stability, the emulsions were placed in sealed plastic tubes and statically stored at 4°C for 21 days. The particle sizes without and with SDS were determined on days 1 and 21 post-production.

2.5.3 Conventional microscopy

The emulsions were observed by conventional optical microscopy using a microscope Cytation 5 (BioTek Instruments, Inc., USA). For this, emulsions on days 1 and 21 were diluted (1:4 v/v) with deionized water or SDS 1.0 wt% solution, and 10 µL of each dilution was put in a glass slide and covered with a coverslip for observation at 200 X magnification (20 X ocular magnification plus 10 X digital detector magnification).

2.5.4 Visual appearance

The appearance of emulsions without dilution placed in cylindrical glass tubes was recorded by taking images during quiescent storage. The images were taken using the same digital camera under the same background.

2.5.5 Physical stability

The physical stability of emulsions was monitored using a Turbiscan Lab® analyzer (Formulaction, France), according to the multiple light scattering theory. For this, emulsions without dilution were placed in cylindrical glass tubes, and transmission (%T) and backscattering (%BS) profiles were determined throughout the samples up to day 21 of quiescent storage at 4°C (Shang et al., 2021).

2.6 Statistical analysis

All the preparations and characterization assays were conducted at least in triplicate. The acquired data were grafted and statistically analyzed using the Graph Pad Prism v8.0 software. The results were expressed as average ± standard deviation. In graphs, different letters were used to represent significant differences ($p<0.05$) according to One-way or Two-way ANOVA followed by Tukey's multiple comparisons post-test.

3. RESULTS AND DISCUSSION

3.1 Characterization of RP:GA blends

3.1.1 Effect of pH

In protein-polysaccharide blends, different phase behaviors can be observed depending on biopolymers' characteristics, environmental conditions, protein:polysaccharide mixing ratio, and total biopolymer concentration (Carpentier et al., 2021). Complex coacervation can occur when biopolymers present opposite net charges and undergo the physicochemical mechanism of associative separation (Lan et al., 2020b). First, to understand the

driving force for complex coacervation, the ζ-potentials of RP and GA control dispersions at pH values between 7.0 and 2.5 were determined and the strength of electrostatic interaction (SEI) was calculated (Fig. 1A). Then, RP, GA, and RP:GA blends in a wide range of pH conditions (7.0 to 2.5) and RP:GA mixing ratios (1:0.2 to 1:1.0) were analyzed by protein solubility (Fig. 1B), phase diagram (Fig. 1C), and visual appearance (Fig. 1D).

Figure 1

The RP control presented ζ-potential varying between +27.3 ± 1.3 mV at pH 2.5 and –29.5 ± 0.8 mV at pH 7.0, with the isoelectric point close to 4.0 (Fig. 1A). In addition to changes in ζ-potential values, a reduction in protein solubility (Fig. 1B) and phase separation by precipitation (Figs. 1C and 1D) were observed at pH ranging from 5.5 to 3.5. These results are justified by the lack of electrostatic repulsion between protein particles at pH close to isoelectric point which leads to extensive protein aggregation.

The GA control presented negative ζ-potentials in the entire pH range, varying between –11.0 ± 0.4 mV at pH 2.5 and –28.3 ± 1.3 mV at pH 7.0 (Fig. 1A). This result agrees with those previously reported by Luo et al. (2021) and Chen et al. (2023). Also, the presence of glucuronic acid as a main sugar of this polysaccharide justified the obtained results. The GA formed stable and translucent dispersions throughout the studied pH range (Figs. 1C and 1D).

From the RP and GA ζ-potentials, the SEI values were calculated (Fig. 1A). According to these results, RP and GA presented attractive interactions at pH < 4.0. However, the best neutralization condition was observed at pH 3.0, where RP and GA presented +18.8 ± 0.8 mV and -13.2 ± 0.5 mV, respectively. Similar results were previously reported for the interaction of purified rice glutelin with GA (Luo et al., 2021).

The RP:GA blends presented different protein solubility and phase behavior depending on the pH and RP:GA mixing ratio (Figs. 1B, 1C, and 1D). At pH 7.0 and 6.5, RP:GA blends formed turbid dispersions with similar protein solubility to RP. At pH 6.0, RP:GA blends showed precipitation with turbid suspensions, but the turbidity of these suspensions was reduced as the GA concentration increased from 0.2 to 1.0 wt.% (Fig. 1C). Also, the protein solubility was significantly reduced as the GA concentration increased. Although both RP and GA displayed a net negative charge at pH 6.0 (Fig. 1A), proteins and protein aggregates may begin to display positively charged patches on their surface as pH approaches the isoelectric point, allowing interaction with negatively charged polysaccharides. The incipient interaction of RP and GA may explain the precipitation of the proteins and their low solubility at this pH condition. In this sense, the existence of positively charged patches on

the protein surface is more important than the total net charge for complex coacervation (Salminen et al., 2022). At pH from 5.5 to 3.5, RP:GA blends showed precipitation with translucent suspensions, as RP control. On the one hand, the lowest GA concentrations (0.2 and 0.4 wt.%) significantly increased the protein solubility with respect to RP. These results reveal the interaction of RP and GA at pH conditions even higher than pI. This interaction managed to increase the protein solubility possibly due to the formation of soluble complexes. On the other hand, the highest GA concentrations (0.6, 0.8, and 1.0 wt.%) did not modify the protein solubility with respect to RP. However, as the concentration of GA in the supernatant was not determined, it is not possible to confirm at this point whether the precipitate is formed by proteins alone or by RP:GA insoluble complexes (coacervates). Similar results were reported by Hasanvand and Rafe (2018) for rice bran protein–flaxseed gum blends with different gum concentrations. Finally, at pH 3.0 and 2.5, RP:GA blends showed precipitation with translucent suspensions instead of turbid suspensions as was observed for RP (Fig. 1C). Also, as GA concentration increased from 0.2 to 1.0 wt.%, the protein solubility was significantly reduced with respect to RP (Fig. 1B). This result confirms the formation of RP:GA complex coacervates at pH conditions below the protein isoelectric point. In addition, these results agree with the fact that, at pH 3.0, charge neutralization occurs between the biopolymers (Fig. 1A). In agreement, a molecular docking study performed by Luo et al. (2021) revealed that hydrogen bonding, hydrophobic interaction, and electrostatic interactions were the main force driving the non-modified rice glutelin and GA complex coacervation at this pH-condition. Finally, the greater turbidity for the mixtures at pH 2.5 compared to pH 3.0 can be explained by the protonation of the carboxyl groups in the polysaccharide at pH values close to pKa, which results in the reduction of negative charges. The latter also leads to a decrease in the coacervation capacity due to the loss of attractive electrostatic forces between the biopolymers (Salminen et al., 2022).

Based on the results obtained so far, it was decided to continue working with RP, GA, and RP:GA blends at certain pH values. Firstly, pH 7.0 was selected as a pH value where RP and GA presented great electrostatic repulsion (highest SEI, Fig. 1A) and the protein solubility and phase behavior of RP were not affected by the presence of GA (Figs. 1B, 1C, and 1D). Second, pH 5.0 was selected as a pH value close to protein isoelectric point where GA might interact with positively charged patches of the RP aggregates surface although they both presented negative net charge (Fig. 1A). Third, pH 3.0 was selected since it corresponds to the pH value where

biopolymers present opposite net charges and occur the charge neutralization (Fig. 1A). In addition, at pH 3.0, differences in protein solubility were observed as a function of the RP:GA mass ratio (Fig. 1B).

3.1.2 Effect of RP:GA mass ratio

The RP, GA, and RP:GA blends at pH 7.0, 5.0, and 3.0 were further characterized by studying their ζ-potentials (Fig. 2A), protein surface hydrophobicity (Fig. 2B), volume-based and surface-based particle size distributions (Figs. 2C and 2D, respectively), and volume- and surface-weighted average diameters (d43 and d32, Figs. 2E and 2F, respectively). Also, the aggregation state was observed by fluorescence microscopy (Fig. 3).

Figure 2

Figure 3

According to ζ-potentials (Fig. 2A), RP control was negatively charged at pH 7.0 and 5.0, and positively charged at pH 3.0. Otherwise, GA control was negatively charged at all pH conditions, but the ζ-potential modules significantly decreased as the pH reduced. At pH 7.0 and 5.0, all RP:GA blends presented negative ζ-potential values as RP and GA controls. At pH 3.0, RP and GA opposite charges allow the biopolymer electrostatic attraction and complex coacervation, forming RP:GA coacervates with intermediate ζ-potential. The ζ-potential values of RP:GA blends became more positive as the GA concentration reduced (from 1.0 to 0.2 wt.%) since a lesser amount of negatively charged polysaccharide chains were available to neutralize positively charged proteins. The RP:GA blends with 0.4 and 0.6 wt.% of GA presented the ζ-potential values closest to zero, i.e., achieve the best neutralization mass-ratio conditions. Similar trends were reported by Lan et al. (2020a) for pea proteins-sugar beet pectin interaction in different mixing ratios.

Concerning the protein surface hydrophobicity (Fig. 2B), an increase from pH 7.0 to 5.0 and a reduction from pH 5.0 to 3.0 were observed for RP control. These results could be explained by the changes in the ζ-potential values as the pH moved close (to pH 5.0) and then away (to pH 3.0) from the protein isoelectric point. At pH 5.0, the ζ-potential module of RP was lower than in the other conditions. In this situation, the proteins present minor repulsion, which allows the formation of large protein aggregates (as will be discussed in the particle size results). These protein aggregates can present surfaces with greater hydrophobicity than proteins under pH conditions far from the isoelectric point. On their part, the RP:GA blends presented surface hydrophobicity values similar to RP in all the pH conditions. At pH 3.0, where electrostatic binding between RP and GA is confirmed, the reported

values for blends were statistically identical to those obtained for RP, indicating that electrostatic interactions do not affect the surface hydrophobicity of proteins. Only the samples with a RP:GA 1:0.4 ratio presented a significant increase or a significant decrease in surface hydrophobicity values respect to RP at pH 5.0 or pH 7.0, respectively. However, this sample did not present significant differences concerning the other RP:GA blends, demonstrating that the RP:GA ratio does not modify the surface hydrophobicity of proteins.

Regarding the particle size characterization, both RP and RP:GA blends had lower d43 and d32 diameters at pH 7.0 than at pH 5.0 and 3.0 (Figs. 2E and 2F). In agreement, the micrographs (Fig. 3) showed that proteins and protein aggregates are smaller at this pH condition. At pH 7.0, the volume-based particle size distributions showed the presence of two populations with similar volume-fraction (Fig. 2C), while the surface-based particle size distributions showed that the main population is the smaller one (Fig. 2D). These results were consistent with the micrographs (Fig. 3) and the protein solubility (Fig. 1B) results. At pH 5.0, RP formed large protein aggregates that can be visualized in micrographs (Fig. 3). The latter results can be explained by the proximity between pH and protein isoelectric point, which induced less superficial charge and more hydrophobicity in RP (Figs. 2A and 2B) leading to extensive protein aggregation and reduction of protein solubility (Fig. 1B). At pH 5.0, the d43 and d32 of RP:GA blends was reduced as the GA concentration increased from 0.2 to 1.0 wt.% (Figs. 2E and 2F), showing that the presence of GA decreased the RP aggregation process. So, even at a pH higher than the protein isoelectric point, the proteins-polysaccharides interactions gave rise to the formation of smaller and more dispersed coacervates than protein aggregates alone. Similarly, Salminen et al. (2022) reported that apple pectin interacted with pea proteins and formed particles with d43 between 25 and 81 μm depending on protein:pectin ratio; the increase in pectin concentration allowed the formation of complexes with reduced size. Finally, at pH 3.0, RP presented smaller aggregates compared to pH 5.0, but larger than at pH 7.0 (Fig. 3). In addition, the presence of GA in the RP:GA blends gave rise to lower values of d43 and d32 than those obtained for RP (Figs. 2E and 2F). Also, the RP:GA particles showed narrowed surface-based particle size distributions than the RP control (Fig. 2D). These results agree with the observation of more compact and uniform protein aggregates and protein-gum coacervates (Fig. 3).

Based on the obtained results, it was decided to continue working with blends with 1:0.4 and 1:1.0 RP:GA mixing ratios. The sample with 0.4 wt.% GA was selected since presented neutralization of charge at pH 3.0 (Fig. 2A) and higher surface hydrophobicity at pH 5.0 (Fig. 2B). The sample with 1.0 wt.% GA was selected because

presented a significant reduction of protein solubility at pH 3.0 (Fig. 1B) and the lowest particle sizes at pH 5.0 (Fig. 2C).

3.2 Characterization of RP and RP:GA stabilized emulsions

Proteins are commonly used as food emulsifiers due to their ability to absorb at the oil-water interface and form interfacial films (McClements, 2004). However, protein emulsifying properties could be affected by the protein aggregation state, which also depends on the pH condition (Xu et al., 2016). Particularly, protein aggregates (protein particles) can produce Pickering emulsions driven by the adsorption and high deformation resistance of colloidal particles at the oil-water interface (Niroula et al., 2021; Tavasoli et al., 2022). Hence, the RP and RP:GA coacervates emulsifying ability at pH 5.0 and 3.0 was studied. The O/W emulsions were macroscopically (Fig. 4) and microscopically (Fig. 5) observed up to day 21 post-preparation. Moreover, the d43 was characterized without and with the addition of SDS (Fig. 6), which acts as a deflocculating agent displacing the proteins from the droplet surface (Guldiken et al., 2023). Finally, the physical stability of emulsions without dilution was studied by their backscattering (%BS) profiles (Fig. 7) for 21 days.

Figure 4

Figure 5

Figure 6

Figure 7

The GA emulsions, regardless of the pH condition, presented rapid separation into oil and aqueous phases (oiling off), as can be observed macroscopically from day 1 post-preparation (Fig. 4) and in micrographs (Fig. 5). Therefore, the results of particle size and physical stability for GA emulsions are not presented.

The RP emulsions at both pH conditions did not undergo oiling-off for at least 21 days of storage. In addition, the RP emulsions did not show a tendency to coalesce, as can be deducted from comparing their d43 at days 1 and 21 of storage (Fig. 6). As explained in section 3.1.2, RP dispersions presented soluble proteins and protein aggregates of various sizes. While soluble proteins could stabilize the oil-water interface by adsorption and unfolding, forming a film stable to coalescence, protein particles could stabilize the interface by Pickering-type phenomena, which also provide resistance to coalescence. Altogether, the results demonstrate that the proteins and protein aggregates present in the RP dispersions allowed the formation and stabilization of oil droplets for at

least 21 days of storage. Still, the RP emulsions presented flocculation, which can be deduced by comparing d43 without and with SDS (Fig. 6) and examining the micrographs (Fig. 5). Also, the RP emulsions suffered creaming, as can be seen in their visual appearance (Fig. 4) and the %BS profiles (Fig. 7). The RP emulsions at pH 5.0 showed a quick separation of a cream layer, presenting an increment of %BS at the middle and upper zones of the tube and a reduction of %BS at the bottom zone. The rapid separation of a cream phase is justified since the highest d43 of flocs (without SDS, Fig.6A) and individual droplets (with SDS, Fig. 6B) were obtained at this pH condition. At pH 5.0, the electrostatic repulsion between the droplets may not be sufficiently large to overcome attractive interactions, such as van der Waals and hydrophobic attraction (Zang et al., 2019). The RP emulsions at pH 3.0 also presented creaming, but the serum layer remained turbid, presenting droplets and flocs. This result could be explained by the lower sizes of both flocs and individual droplets with respect to the observed at pH 5 (Figs. 5 and 6). Similar results were obtained by Xu et al. (2017) for emulsions stabilized with hydrolyzed rice glutelin, which presented flocculation and creaming due to the aggregation of droplets near their isoelectric point. Altogether, these results demonstrate the difficulty of including rice proteins in acid emulsions.

The RP:GA emulsions did not suffer oiling off or coalescence for at least 21 days of storage, as can be observed macroscopically (Fig. 4) and by comparing their d43 through storage time (Fig. 6). Again, the RP:GA dispersions presented soluble proteins, protein aggregates, and principally protein-polysaccharide coacervates (RP:GA particles) of various sizes. So, in RP:GA emulsions, the oil-water interface could be stabilized by both soluble proteins and aggregates, producing droplets that do not undergo coalescence for at least 21 days of storage. However, these emulsions presented flocculation, which can be deduced by comparing the d43 with and without SDS (Fig. 6) and observing in the micrograph (Fig. 5). Notably, RP:GA emulsions had higher creaming stability than RP emulsions at both pH condition. These results were highlighted through the visual appearance (Fig. 4) and the %BS profiles (Fig. 7). In RP:GA emulsions the separation of a cream phase was significantly slower (Fig. 7), and the volume of serum obtained was lower than in RP emulsions at both pH conditions (Figs. 4 and 7). On the one hand, at pH 5.0, the increased stability obtained by using RP:GA coacervates could be explained since the individual droplets and flocs had significantly smaller sizes than those of RP emulsions (Figs. 5 and 6). Thus, the improved emulsifying properties of RP:GA coacervates could be related to the smaller particle size of protein aggregates in these samples (Fig. 3), which might accelerate the protein and RP:GA particles absorption to the oil-water interface and play a key role in the resistance to rupture of the interfacial film. In Pickering emulsions, it

has been previously described that smaller particles are more effective in preventing droplet coalescence and stabilizing emulsions as compared to the larger particle sizes, because of low diffusivity resulting in slow adsorption kinetics of the larger particles and less efficient packing at the interface (Niroula et al., 2021; Tu et al., 2023). On the other hand, at pH 3.0, the increased stability of RP:GA emulsions could be related to the flocculation. Although the individual droplets presented the same or smaller sizes than in RP emulsions (with SDS, Fig. 6B), the flocs presented higher sizes (without SDS, Fig. 6A). Since GA presents a low radius of hydration due to their high degree of branching, they cannot cause depletion flocculation (Zang et al., 2019), but they can induce bridging flocculation. The flocs might stabilize the emulsion by increasing the viscosity of the system, thus reducing the creaming rate. In accordance with those results, the emulsifying properties of oak and hemp proteins were improved after their complex coacervation with GA (X. Liu et al., 2023; Naderi et al., 2020).

4. CONCLUSION

The present study showed that coacervates composed of modified rice proteins and gum arabic can be formed by associative complexation under acidic conditions. These coacervates extended the range of pH values where modified rice protein could be successfully used as emulsifiers. Moreover, emulsions prepared with RP:GA coacervates showed smaller droplet sizes and greater stability than those prepared with RP alone at pH 5.0 and 3.0. This work indicates that RP:GA coacervates may be employed as a natural emulsifier in the development of acid-emulsion-based food products. Still, further studies are needed to evaluate the emulsion stability against thermal treatments and other environmental stresses.

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Author contributions

Daniela E. Igartúa: Conceptualization, Data curation, Formal analysis, Supervision, Investigation, Funding acquisition, Project administration, Methodology, Writing - original draft, Writing - review & editing. María Celeste Dichano: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. Maxwell N. Morales Huanca: Investigation, Methodology. Gonzalo G. Palazolo: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. Dario M. Cabezas: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

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539 **FIGURE CAPTIONS**

540 **Fig. 1 – Characterization of RP, GA, and RP:GA blends as a function of pH. (A)** ζ-potentials (mV) of RP and
541 GA dispersions and strength of electrostatic interaction (SEI). **(B)** Protein solubility (%) in RP and RP:GA blends.
542 **(C)** Phase diagram after 24 h of preparation, classified according to translucent suspension (♦), precipitate with
543 translucent suspension (●), precipitate with cloudy suspension (●), or precipitate with very cloudy suspension (■).
544 **(D)** Visual appearance of RP, GA, and RP:GA blends after 24 h of preparation.

545

546 **Fig. 2 – Characterization of RP, GA, and RP:GA blends as function of mixing ratios. (A)** ζ-potentials (mV).
547 **(B)** Protein surface hydrophobicity. **(C)** Volume-based (%) particle size distributions. **(D)** Surface-based (%)
548 particle size distributions. **(E)** Volume-weighted average particle diameters, d43 (μm). **(F)** Surface-weighted
549 average particle diameters, d32 (μm). Different lowercase letters represent significant differences between
550 samples according to One-way ANOVA followed by Tukey's multiple comparisons post-test.

551

552 **Fig. 3 – Aggregation state of RP and RP:GA blends at pH 7.0, 5.0, and 3.0.** Fluorescence microscopy of RP
553 and RP:GA blends at pH 7.0, 5.0, or 3.0 after staining with rhodamine B (scale bar: 100 μm).

554

555 **Fig. 4 – Visual appearance of RP, GA, and RP:GA emulsions** Representative images of RP, GA, and RP:GA
556 stabilized emulsion on days 0, 1, and 21 post-preparation.

557

558 **Fig. 5 – Microscopical observation of RP, GA, and RP:GA emulsions**. Representative micrograph of RP, GA,
559 and RP:GA stabilized emulsion on days 1 and 21 post-preparation without and with the addition of SDS (scale
560 bar: 100 μm).

561

562 **Fig. 6 – Particle size characterization of RP, GA, and RP:GA emulsions. (A)** Volume-weighted average
563 particle diameters (d43) of RP and RP:GA stabilized emulsions without the addition of SDS on day 1 and 21 post-
564 preparation at each pH condition. **(B)** d43 of RP and RP:GA stabilized emulsions in presence of SDS on days 1
565 and 21 post-preparation at each pH condition. Different lowercase letters represent significant differences
566 between samples on the same day of storage (RP, 1:0.4, or 1:1.0), while different uppercase letters represent

567 significant differences between the same sample on different days of storage (1 or 21) according to Two-way
568 ANOVA followed by Dunnett's multiple comparisons post-test.
569
570 **Fig. 7 – Physical stability RP, GA, and RP:GA of emulsions. (A)** Backscattering (%BS) profiles of RP
571 emulsions during quiescent storage for 21 days (504 h). **(B)** %BS profiles of emulsions prepared with RP:GA
572 1:0.4 during quiescent storage for 21 days (504 h). **(C)** %BS profiles of emulsions prepared with RP:GA 1:1.0
573 during quiescent storage for 21 days (504 h).