Journal Pre-proof

Impact of heat treatment in whey proteins-soluble soybean polysaccharides electrostatic complexes in different pH and biopolymer mass ratio conditions

Daniela E. Igartúa , Fedra A. Platania , Agustina Balcone , Gonzalo G. Palazolo , Dario M. Cabezas

PII: S2772-5022(22)00144-5
DOI: https://doi.org/10.1016/j.afres.2022.100184
Reference: AFRES 100184

To appear in: *Applied Food Research*

Received date: 23 May 2022
Revised date: 21 July 2022
Accepted date: 2 August 2022

Please cite this article as: Daniela E. Igartúa , Fedra A. Platania , Agustina Balcone , Gonzalo G. Palazolo , Dario M. Cabezas , Impact of heat treatment in whey proteins-soluble soybean polysaccharides electrostatic complexes in different pH and biopolymer mass ratio conditions, *Applied Food Research* (2022), doi: https://doi.org/10.1016/j.afres.2022.100184

Impact of heat treatment in whey proteins-soluble soybean polysaccharides electrostatic complexes in different pH and biopolymer mass ratio conditions

Daniela E. Igartúa[1,2], Fedra A. Platania[1], Agustina Balcone[1], Gonzalo G. Palazolo[1,2], Dario M. Cabezas[1,2,*].

[1] Universidad Nacional de Quilmes, Departamento de Ciencia y Tecnología, Laboratorio de Investigación en Funcionalidad y Tecnología de Alimentos (LIFTA), Roque Sáenz Peña 352, B1876BXD, Bernal, Buenos Aires, Argentina.

[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290, C1425FQB, Ciudad Autónoma de Buenos Aires, Argentina.

[*] **Corresponding author:**

Dario M. Cabezas.

ORCID: 0000-0002-7920-4852

E-mail address: dario.cabezas@unq.edu.ar

Full address: Roque Sáenz Peña 352, (B1876BXD), Bernal, Buenos Aires, Argentina.

Tel. (+54 11) 4365 7100 ext. 5615

Authors:

Daniela E. Igartúa- E-mail address daniela.igartua@unq.edu.ar (ORCID: 0000-0003-2285-5539)

Fedra A. Platania- E-mail address: fedraplatania@gmail.com

Agustina Balcone- E-mail address: balconeagustina@gmail.com

Gonzalo G. Palazolo-E-mail address: gonzalo.palazolo@unq.edu.ar (ORCID: 0000-0002-1544-5745)

Declaration of Competing Interest

The authors declare that they do not have any conflict of interest.

Author contributions

Daniela E. Igartúa: Conceptualization, Data curation, Formal analysis, Supervision, Investigation, Methodology, Writing - original draft. Fedra A. Platania: Data curation, Formal analysis, Investigation, Methodology. Agustina Balcone: Data curation, Formal analysis, 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.

Acknowledgments

Fedra A. Platania and Agustina Balcone are grateful for the student fellowships granted by the Consejo Interuniversitario Nacional (CIN). Daniela E. Igartúa, Gonzalo G. Palazolo, and Dario M. Cabezas are members of the Scientific Research Program from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). The authors thank both Fuji Oil Co. Ltd (Osaka, Japan) and Arla Foods Ingredients Argentina, S.A. (Buenos Aires, Argentina) for providing soluble soybean polysaccharides and whey protein isolate, respectively.

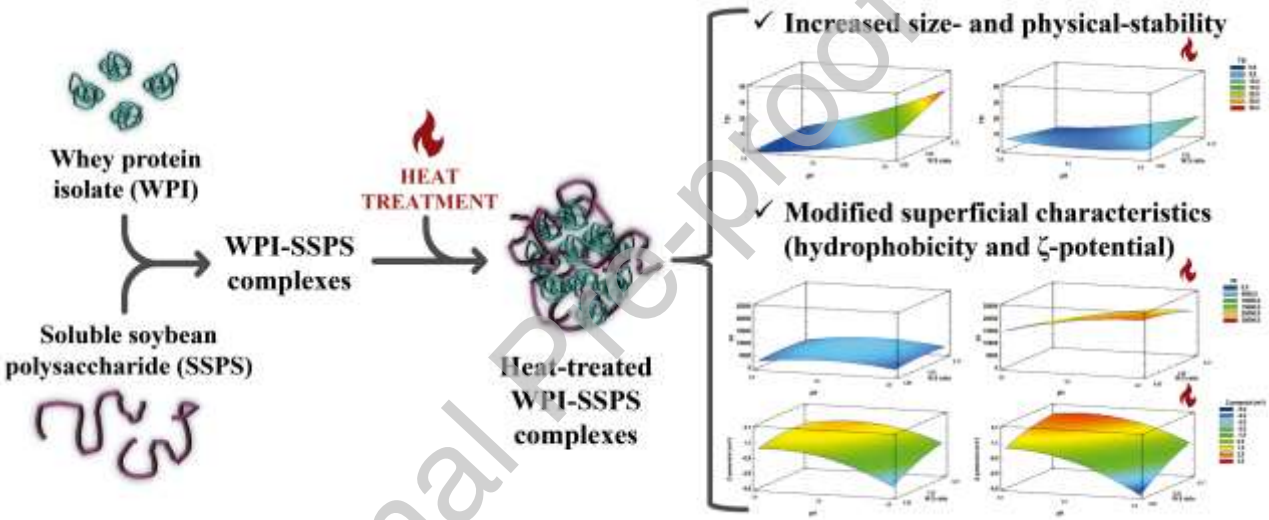
Funding

This research was supported by the Universidad Nacional de Quilmes (1300/19 I + D grant) and Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2021-2023, 11220200100354CO grant). The financial support is gratefully acknowledged.

Impact of heat treatment in whey proteins-soluble soybean polysaccharides electrostatic complexes in different pH and biopolymer mass ratio conditions

Highlights

- Whey protein isolate (WPI) and soluble soybean polysaccharides (SSPS) formed complexes
- Heat treatment modified the size, hydrophobicity and ζ-potential of complexes
- Heat treatment improved the size- and physical-stability of complexes
- Conditions of pH and WPI:SSPS mass ratio changed the impact of heat treatment
- Heat-treated WPI-SSPS complexes could be used as drug delivery system in acid foods



Abstract

The formation of electrostatic complexes between whey protein isolate (WPI) and soluble soybean polysaccharide (SSPS) was firstly studied to select the suitable conditions of pH and WPI:SSPS mass ratio. Then, the influence of a heat treatment (90 °C, 20 min) on the characteristics of WPI-SSPS complexes was studied in the selected conditions of pH (3.0, 3.5, and 4.0) and WPI:SSPS ratio (1:0.50 to 1:0.17) by ζ-potential, particle size distribution, surface hydrophobicity, and physical stability in a 3×2 design by statistical response surface methodology. This analysis showed that the heat treatment significantly increased the Z-average and surface hydrophobicity of complexes and increased their size and physical stability over time. This work provides new information on the impact of heat treatment on molecular interactions in WPI-SSPS systems. The obtained data is useful to design heat-treated WPI-SSPS complexes with specific characteristics of size, charge, hydrophobicity, and physical stability. Furthermore, this work provides an optimized WPI-SSPS

nanocomplex that could be implemented as a delivery system for hydrophobic bioactive compounds in food matrices.

Keywords

Whey protein; Soy polysaccharide; Electrostatic assembly; Protein-polysaccharide complexes; Heat treatment.

1. Introduction

Manufactured foods exist in a colloidal state as dispersions, emulsions, foams, gels, or their mixed variants. In recent years, the interaction of proteins and polysaccharides and the formation of protein-polysaccharide electrostatic complexes have gained considerable attention in the food industry since their application as modulators of these food matrices exponentially increased. Specifically, protein-polysaccharide complexes allow the construction of economically viable food systems with high biocompatibility and biodegradability, and new functionalities, like the encapsulation and delivery of bioactive molecules, fat replacement, emulsion and foam stabilization, formation of edible films, coatings, and food texturization (Li et al., 2022).

Different protein-polysaccharide systems have been studied up to now, but mixtures based on whey protein isolate (WPI) are the most attractive due to their nutritional value, functional properties, and low cost (Du et al., 2022). WPI is a heterogeneous mixture of whey proteins composed of ~80% β-lactoglobulin (MW: 18.3 kDa) and ~12% α-lactalbumin (MW: 14.2 kDa), and also of bovine serum albumin (MW: 66.3 kDa), bovine lactoferrin (MW: 80 kDa), lactoperoxidase (MW: 70 kDa), immunoglobulins (MW light chain: 25 kDa; MW heavy chain: 50 kDa), and other minor proteins (Madureira et al., 2007; Phillips and Williams, 2011).

Soluble soybean polysaccharides (SSPS) are promising polysaccharides for mixture with WPI due to their high water solubility, low bulk viscosity, high-temperature stability, and their uses as soluble dietary fiber and functional ingredient (Maeda and Nakamura, 2009; Xu and Liu, 2016). SSPS are composed of a backbone of rhamnogalacturonan and branches of β-1,4-galactan and α-1,3 or α-1,5-arabinan. Besides, the polysaccharide chains have associated protein moieties contributing with properties that could facilitate the complexation with proteins (Li et al., 2020; Nakamura et al., 2004). The three SSPS majority components had molecular weights of 550 kDa, 25 kDa, and 5 kDa,

respectively, and the average MW is estimated to be several hundred kDa (Phillips and Williams, 2009).

Nevertheless, WPI-SSPS mixtures are not still used in the food industry since there is a lack of fundamental understanding of the physical and chemical interactions between them. As far as we know, only two previous studies analyzed the interaction between WPI and SSPS (Cabezas et al., 2019; Zamani et al., 2020). In our previous work, the effect of heat treatment on the complex formation at pH 3.5 and 4:1 WPI:SSPS ratio was studied, comparing single- and two-step assembly approaches for application in emulsions subjected to freeze-thawing (Cabezas et al., 2019). Recently, WPI-SSPS mixtures obtained at pH 7.0 and 3.5 with or without heat treatment were employed for WPI stabilization in beverages (Zamani et al., 2020). However, the impact of heat treatment in other conditions of pH and WPI:SSPS ratio were not further characterized.

The protein-polysaccharide complexes formation is conditioned by several factors, such as biopolymer characteristics (molecular weight, molecular conformation, charge density, and rigidity), biopolymer ratio, biopolymer total concentration, environmental conditions (pH and ionic strength), and additional treatments (Behrouzain et al., 2020). Specifically, the performance of heat treatment may modify the formation and the stability of the complexes (Bédié et al., 2008; Gaber et al., 2018); even the formation of nano- or micro-particles was reported after heating to a temperature higher than the protein's thermal denaturation temperature (Jones et al., 2010; Jones and McClements, 2011). As a general rule, the heat-treated complexes have shown great stability against dissociation or aggregation than native proteins, native polysaccharides, or electrostatic complexes without thermal treatment (Gaber et al., 2018). From a practical point of view, it is important to control the characteristics of heat-treated complexes, i.e., their size, electrical properties, and environmental stability (Chanasattru et al., 2009). As heat treatment could induce the protein unfolding, the changes in their surface characteristics must be studied by analyzing the changes in ζ-potential (which is associated with the exposure of charged amino acidic residues) and surface hydrophobicity (which is associated with the exposure of hydrophobic residues). Also, analysis of ζ-potential is a useful tool for the understanding of biopolymers complexation since the electrical attractive forces between them determine the possibility and the strength of interaction (Ghadermazi et al., 2019). In addition, as these changes in surface characteristics could induce aggregation of proteins, the particle size distribution of protein and protein-polysaccharide complexes must be characterized. Finally, the physical stability of the system must be studied for potential applications. In this sense, the Turbiscan technique is a useful tool for monitoring the destabilization kinetics of a system and assessing the extent of eventual phase separation (Chevalier et al., 2019).

The present work hypothesizes that the heat treatment can differentially modify the characteristics of the WPI-SSPS complexes according to the conditions in which it is carried out (pH and biopolymer mass ratio). Therefore, the aim was to investigate the impact of heat treatment (90 °C, 20 min) on the characteristics and stability of WPI-SSPS electrostatic complexes prepared in selected pH and WPI:SSPS mas ratio conditions. For this, ζ-potential, particle size distribution, surface hydrophobicity, and physical stability were studied before and after heat treatment.

2. Materials and methods

2.1. Materials

Soluble soybean polysaccharides (SSPS, Soyafibe-SCA100) were donated by Fuji Oil Co. Ltd (Osaka, Japan). According to the supplier's datasheet, the SSPS chemical weight composition was 75.1% w/w total dietary fiber, 7.8% w/w crude protein (N×6.25), 7.8% w/w crude ash, and 5.8% w/w moisture. Whey protein isolate (WPI, Lacprodan® DI-9224) was donated by Arla Foods Ingredients Argentina S.A. (Buenos Aires, Argentina). According to the supplier's datasheet, the WPI chemical weight composition was 86.5% w/w crude protein (N×6.25), 1.25% w/w salts 0.1% w/w lactose, and 0.1% w/w total fat. SSPS and WPI powders were used with no further purification. Double-distilled water was used to prepare the solutions and suspensions. *N,N*-Dimethyl-6-propionyl-2-naphthylamine (PRODAN) was purchased from Santa Cruz Biotechnology (Texas, USA). All the other analytical-grade chemicals were purchased from local distributors (Buenos Aires, Argentina).

2.2 Optimization of conditions to obtain WPI-SSPS electrostatic complexes

2.2.1 WPI and SSPS dispersions

WPI and SSPS dispersions at 4.00 % w/w were prepared by mixing the solid powers into double-distilled water under constant mixing at 200 rpm for 2 h at room temperature (25 ± 2 °C). To prevent microbial growth, sodium azide at 0.02 % w/w final concentration was added to all dispersions. The pH of SSPS stock dispersion was 5.80 ± 0.04, while the pH of WPI stock dispersion was 7.00 ± 0.02. Both dispersions were stored at room temperature, protected from light, until use.

2.2.2 WPI-SSPS mixtures preparation

To make binary mixtures with desired WPI-SPSS mass ratios (1:1, 1:0.50, 1:0.25, 1:0.17, 1:0.125, and 1:0.1 W:S), appropriate proportions of the two stock dispersions were mixed with a magnetic

stirrer for 1 h at room temperature (25 ± 2 °C). Then, the pH of each mixture was adjusted with 1.0 M HCl to desired pH (from 2.0 to 7.0 to every 0.5 pH units). pH was measured using a C861 Consort pH/mV meter with a PY-P10-25 Sartorius electrode (resolution ±0.01), calibrated daily before use. Then, double-distilled water was added to complete the final mixture mass. In all the dispersion, the final concentration of WPI was maintained at 1.00 % w/w, and the concentration of SSPS was varied between 1.00 and 0.10 % w/w. The total biopolymer concentration varied between 1.10 and 2.00 % w/w. As control samples, individual 1.00 % w/w WPI and SSPS dispersions were used.

2.2.3 WPI-SSPS mixtures characterization

The turbidity of WPI, SSPS, and WPI-SSPS mixtures at different pH and W:S ratio was measured as the absorbance intensity at 600 nm (Lan et al., 2018) using a Biochrom Libra S4 visible spectrophotometer (Biochrom Instruments; UK). All measurements were conducted at room temperature (25 ± 2 °C).

From the parameterization of the turbidity results as a function of pH, four critical pH values were calculated: (1) pH_{opt}, the pH where occurs the maximum complex formation was determined at the maximum turbidity point; (2) pH_c, the pH where starts the formation of intramolecular soluble complexes was determined as a slight increase in the turbidity; (3) $pH_{\varphi 1}$, the pH where start the formation of intramolecular insoluble complexes was determined by extending tangent lines on both sides of the inflection point at pH higher than pH_{opt}; (4) $pH_{\varphi 2}$, the pH where start the disassemble of complexes was determined by extending tangent lines on both sides of the inflection point at pH lower than pH_{opt} (Zhong et al., 2021).

Finally, the ζ-potentials of WPI, SSPS, and WPI-SSPS mixture at 1:0.25 W:S ratio were determined with a Zetasizer Nano ZSP ZEN 5600 analyzer (Malvern Instrument, UK) at pH values from 2.5 to 7.0. For the assay, dispersions were diluted (1:3 v/v) with double-distilled water previously adjusted at each pH with HCl 1.0 M, to avoid multiple light scattering effects (Cabezas et al., 2019). The determinations were performed at room temperature (25 ± 2°C), using refractive index values of 1.54 and 1.33 for biopolymer (WPI, SSPS, and WPI-SSPS mixture) and dispersant, respectively.

2.3 Impact of heat treatment in WPI-SSPS complexes

2.3.1 WPI-SSPS complexes preparation

From the analysis of the results obtained so far, three W:S ratios (1:0.50, 1:0.25, and 1:0.17) and three pH conditions (3.0, 3.5, and 4.0) were chosen to obtain WPI-SSPS complexes. Hence, the WPI-SSPS complexes were prepared as in section 2.2.2, by mixing appropriate proportions of both

biopolymers dispersion and adjusting the pH to the desired value with 1.0 M HCl. Then, double distilled-water was added to ensure that final concentration of WPI was 1.00 % w/w in all the complexes, and the final concentration of SSPS was variable between 0.50 and 0.17% w/w. Besides, to study the effect of heat treatment, aliquots of resultant mixtures (35.00 g) were heated in a water bath at 90.0 (±1.0) °C for 20 min. The conditions of heat treatment were selected based on previous results obtained by differential scanning calorimetry (DSC) (Cabezas et al., 2019). After heat treatment, the samples were cooled in a bath with running tap water until reaching room temperature (25 ± 2 °C), a process that took approximately 15 minutes per sample. The mass of water lost during heating was adjusted by adding double-distilled water (to recover the initial 35.00 g). Individual WPI and SSPS dispersions with and without heat treatment were used as control samples.

2.3.2 WPI-SSPS complexes characterization

The ζ-potentials of WPI, SSPS, and WPI-SSPS complexes were determined as previously explained in section 2.2.3.

The hydrodynamic diameter (Z-average, intensity weighted, average diameter) and polydispersity index (PDI) of WPI, SSPS, and WPI-SSPS complexes were determined at room temperature (25 ± 2 °C) by dynamic light scattering using a Zetasizer Nano ZSP ZEN 5600 analyzer (Malvern Instrument, UK). For measurements, the dispersions were diluted (1:3 v/v) with double-distilled water previously adjusted at specific pH with HCl 1.0 M (Cabezas et al., 2019). The measurements were performed on days 0, 28, and 56.

The protein surface hydrophobicity values of WPI and WPI-SSPS mixtures were determined by fluorescence using the PRODAN probe, according to previously reported methods (Alizadeh-Pasdar and Li-Chan, 2000, 2001) with some modifications. The 1.4 mM PRODAN stock solution was prepared in methanol and stored at -20 °C in brown screw-capped vials. Each experiment day, PRODAN was diluted in methanol to 0.07 mM (1/20 v/v) and held in ice until use. WPI and WPI-SSPS complexes were serially diluted with double-distilled water previously adjusted to specific pH with 1.0 M HCl solution to obtain protein concentrations ranging from 0.5 to 0.032 % w/v. Later, each dilution was placed in a black 96-well plate (Greiner Bio-one, USA) at 200 µL/well. The fluorescence emission in each well was determined using a multi-mode plate reader Cytation 5 equipment (BioTek Instruments, USA). The excitation wavelength was 365 ± 9 nm, while the emission wavelength was 465 ± 20 nm. Then, 20 µl of diluted PRODAN were placed in each well, and the plate was agitated for 10 minutes 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 surface hydrophobicity (S_0).

The physical stability of WPI, SSPS, and WPI-SSPS complexes was monitored with a Turbiscan Lab® analyzer (Formulaction, France), according to the multiple light scattering theory. Dispersions were placed in cylindrical glass tubes and transmission (T) and backscattering (BS) were determined throughout the sample up to day 56 (Shang et al., 2021). The samples were statically stored at room temperature and protected from light during this period. The stability of the dispersions was analyzed based on their Turbiscan stability index (TSI), calculated by TurbiSoft software (Formulaction, France). TSI is a relative number without units that allows comparison of the relative stability of different samples based on the variations of T and BS profiles of the sample over time; the larger the TSI, the lower the stability of the sample (Chevalier et al., 2019).

2.4 Statistical analysis

All the preparations and characterization assays were conducted at least in triplicate and the results were expressed as mean ± standard deviation. The statistical analyses were performed by one-way ANOVA or two-way ANOVA followed by multiple comparisons post-test using the Graph Pad Prism v6.0 software. Significant differences were considered only when the p-value<0.05. Furthermore, the effects of the variables (pH, WPI:SSPS mass ratio, and heat treatment) on the ζ-potential, Z-average, surface hydrophobicity, and TSI of WPI-SSPS soluble complexes were examined by a 3×2 factorial design for the statistical response surface methodology by Statgraphics Centurion XVI v16.1.18 (StatPoint Technologies Inc., USA).

3. Results and discussion

3.1 Optimization of conditions to obtain WPI-SSPS electrostatic complexes

WPI, SSPS, and WPI-SSPS mixtures in a wide range of pH conditions (2.0 to 7.0) and W:S ratio (1:1 to 1:0.1) were analyzed by turbidimetry (Figure 1A), critical pH values (Figure 1B), ζ-potential (Figure 1C), and visual appearance (Figures 1D and SM1).

The WPI control presented a marked increase in its turbidity in pH close to 4.5 during acidification (Figure 1A). WPI precipitated and formed translucent dispersion in pH ranging from 4.0 to 5.0 that can be observed after 24 hours of preparation (Figure 1D). These results are consistent with the

formation of large-size protein aggregates with high light-scattering capacity, which becomes one of the major challenges to incorporating WPI in acidic foods. In agreement, the ζ-potential of WPI control varied from + 22.2±1.4 mV at pH 2.5 to - 27.9±0.9 mV at pH 7.0 (Figure 1C), with the isoelectric point (pI) occurring at 4.3. So, the high initial turbidity and the storage precipitation could be explained by the relatively weak electrostatic repulsion between protein molecules at pH close to pI (Behrouzain et al., 2020; Zhong et al., 2021).

The SSPS control generated translucent dispersions (Figure SM1) with low turbidity (Figure 1A) and negative ζ-potential (Figure 1C) in the entire pH range. These results are consistent with those previously reported (Zhao et al., 2018) and with the presence of galacturonic acid as a component sugar of this polysaccharide.

The WPI-SSPS mixtures presented less turbidity than the WPI control in the entire pH range (Figure 1A). This result could be explained by the formation of protein-polysaccharide particles with smaller size and light-scattering capacity than protein aggregates. Besides, as the W:S ratio increased (from 1:0.10 to 1:1), a shift in the maximum turbidity towards lower pH was observed (Figure 1A). This observation means that the pH value should be reduced to promote the optimal interaction between biopolymers. A similar trend for mixtures of pea protein isolate and alginate with 20:1 to 1:1 biopolymer mass ratio was previously reported (Klemmer et al., 2012).

Since the protein-polysaccharide complexes are formed by the electrostatic attraction of biopolymers with opposite net charges, WPI and SSPS were compatible at $2.5 < pH < 4.3$. This biopolymer interaction was confirmed by the change in the ζ-potential of the mixture at 1:0.25 W:S ratio concerning the WPI and SSPS controls (Figure 1C). It is assumed that the strongest WPI-SSPS interactions occurred when the electrical charge of the mixtures was nearly neutralized (Gorji et al., 2018). Moreover, the formation of stable electrostatic complexes is verified by the formation of dispersions that do not present precipitation in pH close to WPI pI (Figures 1D and SM1).

From the parameterization of the turbidity results, the critical pH values were calculated (Figure 1B). Independently of W:S ratio, the biopolymers interaction was characterized by four different states by decreasing pH. In the first state, where $pH > pH_c$, the WPI and SPPS presumably co-existed as individual molecules due to electrostatic repulsion between strongly negatively biopolymers and steric hindrance. Non-significant differences were obtained in the pHc for any W:S ratio ($p>0.05$). In the second state, where $pH_c > pH > pH_{\varphi 1}$, the WPI and SPPS interacted with each other forming intramolecular soluble complexes. This could happen above the pI of WPI since SSPS can bind to positive patches on the protein surface, even though both the protein and polysaccharide have a net

negative charge (Behrouzain et al., 2020; Jones et al., 2010). As well, non-significant differences were obtained in the $pH_{\varphi 1}$ for any W:S ratio ($p>0.05$). In the third state, where $pH_{\varphi 1} > pH > pH_{\varphi 2}$, going through the pH_{opt}, the WPI and SSPS interacted with each other forming insoluble complexes; they were stable in suspension, presumably by their small size (Gaber et al., 2018). The pH range in which electrostatic complexes are formed (range between pH_c and $pH_{\varphi 2}$) was widened as the W:S ratio increased from 1:0.10 to 1:1, because $pH_{\varphi 2}$ significantly decreased from 3.0 in the 1:0.10 mixture to 2.5 in the 1:1 mixture ($p<0.01$). Specifically, both pH_{opt} and $pH_{\varphi 2}$ presented significant differences ($p<0.05$) for mixtures obtained at 1:1, 1:0.50, 1:0.25, and 1:0.17 W:S ratio, whereas non-significant differences were observed for these parameters in 1:0.17, 1:0.125, and 1:0.10 W:S ratio. This phenomenon is probably associated with both a greater tendency to charge neutralization in WPI-SSPS mixtures and the formation of a stronger interconnected network when the protein assumes a more positive charge (lower pH) and a higher amount of SSPS was available (higher W:S ratio) (Zhong et al., 2021). The same trend has been previously reported for different biopolymers systems (Fioramonti et al., 2014; Lan et al., 2018). Finally, in the fourth state, at $pH < pH_{\varphi 2}$, the complexes disassembled probably due to the loss of charge of the SSPS molecules that reach the pKa of their acidic groups.

From the results obtained so far, the conditions to assure WPI-SSPS complexation were chosen. Specifically, pH conditions of 3.0, 3.5, and 4.0 were selected since attractive electrostatic interactions are ensured in that range, as can be seen in the results of ζ-potential (Figure 1C) and through turbidity and visual observation (Figures 1A and SM1). Besides, the 1:0.5, 1:0.25, and 1:0.17 W:S mass ratios conditions were chosen because significant changes in their critical pH values were observed (Figure 1B).

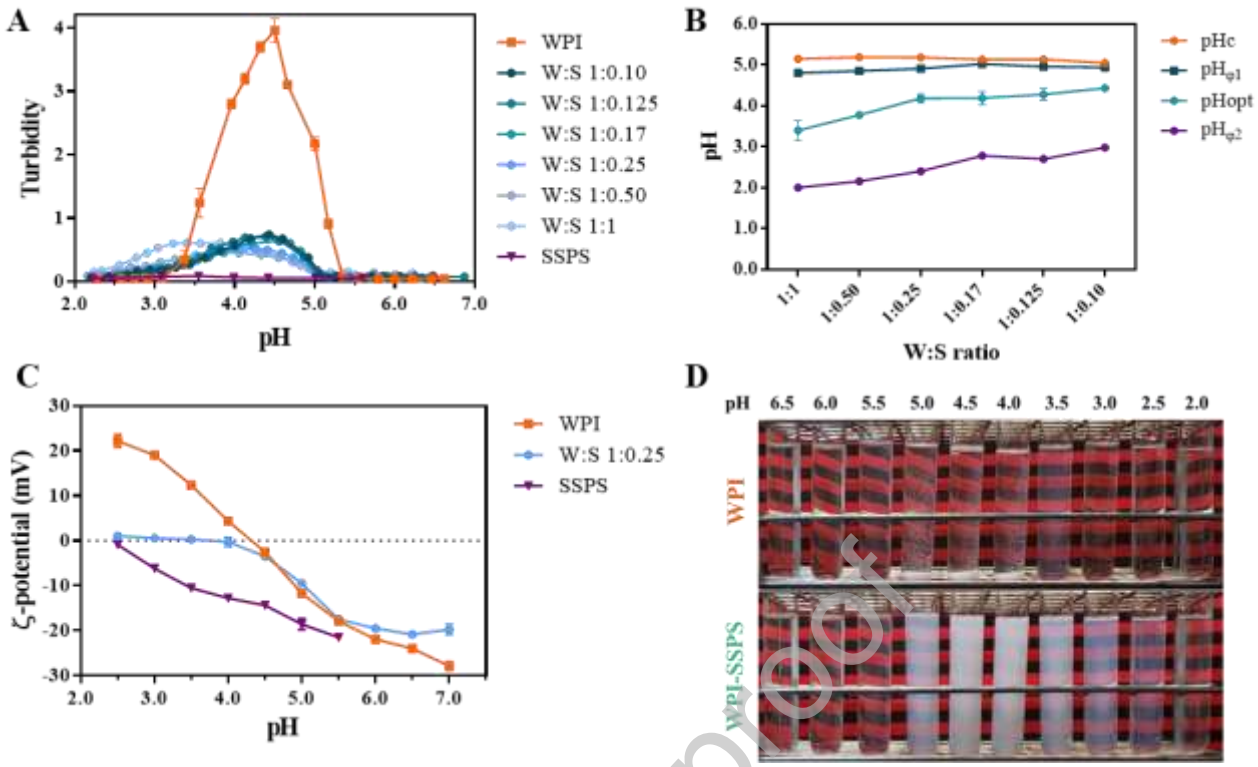


Figure 1 – WPI-SSPS mixtures characterization. (A) Turbidity of WPI, SSPS, and WPI-SSPS mixtures at different W:S ratios as a function of pH. **(B)** Critical pH values as a function of the W:S ratio. **(C)** ζ-potential of WPI, SSPS, and WPI-SSPS mixture at 1:0.25 W:S ratio as a function of pH. **(D)** Photographs of WPI and WPI-SSPS mixture at 1:0.25 W:S ratio as a function of pH after 24 hours of preparation.

3.2 Impact of heat treatment in WPI-SSPS complexes

To understand the impact of protein denaturalization on the characteristics of WPI-SSPS electrostatic complexes, the influence of heat treatment (90 °C, 20 min) was studied in the optimized conditions of pH and W:S ratio. WPI, SSPS, and WPI-SSPS complexes with and without heat treatment were characterized by ζ-potential (Figure 2), Z-average and PDI (Figure 3), surface hydrophobicity (Figure 4), and physical stability overtime followed by TSI (Figure 5).

According to ζ-potential measurements (Figure 2A), a net positive charge was observed in the WPI controls, whose module significantly increased ($p<0.01$) as the pH moved away from the pI. On the contrary, a net negative charge was observed in the SSPS control, whose module significantly reduced ($p<0.05$) as the pH moved close to the pKa. These complementary net charges allow the formation of WPI-SSPS complexes with ζ-potential values close to zero. In the case of SSPS, no

significant ζ-potential variation was observed after heat treatment ($p>0.05$), while in the case of WPI at pH 3.0 and 3.5, a significant increase in surface charge was observed after heat treatment ($p<0.05$). This variation is consistent with the protein unfolding caused by thermal treatment, which modifies the number of charged groups exposed on the surface of protein and protein-aggregates. Analogous results were reported for α-lactalbumin after treatment at 70 °C (Li & Zhao, 2018).

Respect the ζ-potentials of WPI-SSPS complexes, the performed statistical analysis (two-way ANOVA) reported that heat treatment had a significant effect ($p<0.05$). In particular, the ζ-potential module increased after the heat treatment (either positive or negative value), which could mean that more charged groups were exposed on the surface of complexes after the thermal treatment. According to the statistical response surfaces (Figures 2B and SM2A), the heat treatment did not significantly affect the ζ-potential of WPI-SSPS complexes. However, the pH, the W:S ratio, and the interaction between these parameters and heat treatment significantly affected the ζ-potential. As the pH decreased (from 4.0 to 3.0), the ζ-potential of WPI-SSPS complexes became more positive since proteins contribute with a higher amount of positive charge and polysaccharides contribute with a lesser amount of negative charge, so SSPS did not completely neutralize the WPI. A similar trend was previously reported for mixtures of β-lactoglobulin or α-lactalbumin with alginate, where the ζ-potential values increased with the decrease of pH (Gorji et al., 2018). In the same way, as the W:S ratio decreased (from 1:0.50 to 1:0.17), the complexes became more positive, which could be due to the lesser amount of SSPS available to neutralize the positive charge of the WPI. This result agrees with those previously obtained for WPI-hyaluronic acid complexes at different protein:polysaccharide ratios, where the ζ-potential becomes negative as the amount of hyaluronic acid increases since an increase in anionic groups provided by the acid polysaccharide neutralized the cationic groups of proteins (Zhong et al., 2021). In the same way, similar results were previously reported for WPI-pectin complexes, where ζ -potential changed from negative to positive values as the pectin:WPI ratio decreased from 1:2 to 1:30, i.e. as pectin concentration decreased (Zamani et al., 2020). The effects caused by the change in pH and W:S ratio were significantly enhanced in the samples that underwent heat treatment. These results show that the heat treatment has different impacts on the WPI-SSPS ζ-potential depending on the conditions in which it is carried out.

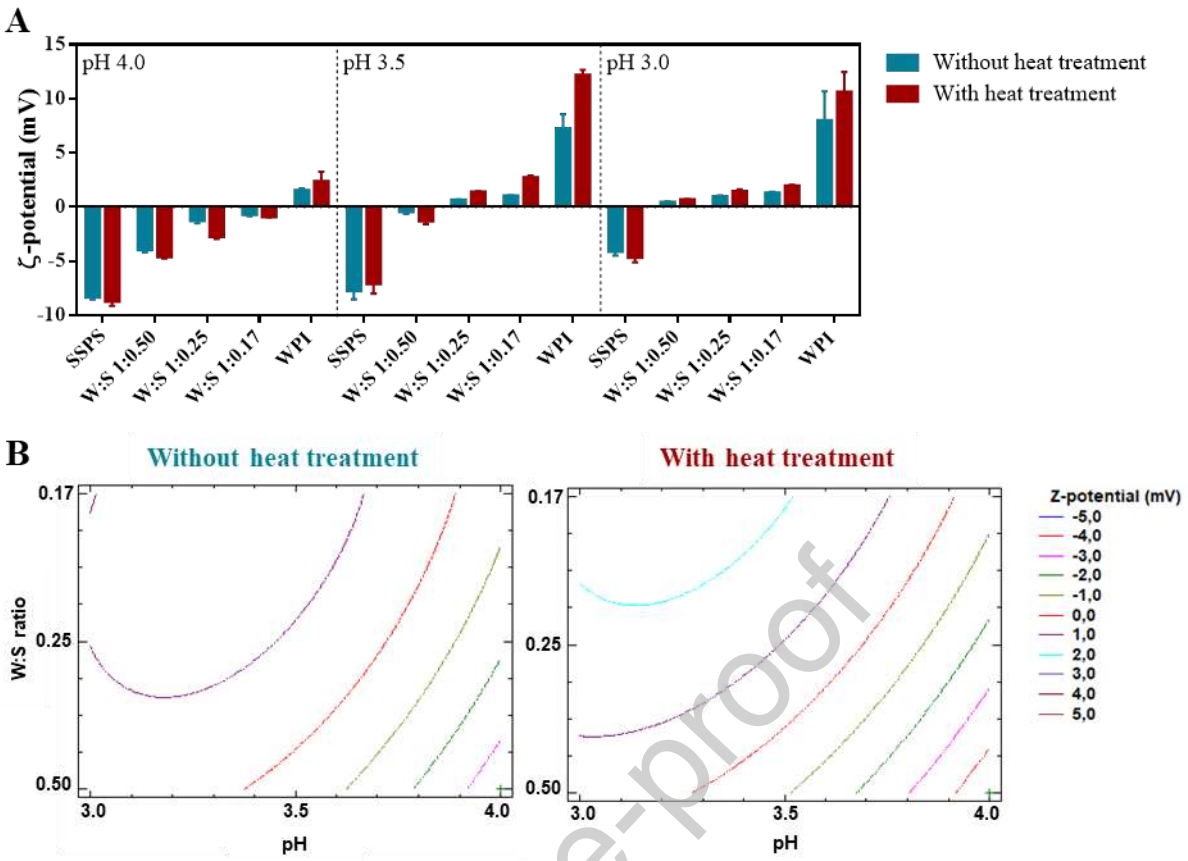


Figure 2 – Impact on ζ-potentials. (A) ζ-potential of WPI, SSPS, and WPI-SSPS complexes in different pH conditions. **(B)** Level curves representation of statistical surfaces for ζ-potential as a function of pH and W:S ratio.

According to Z-average and PDI measurements (Figure 3A), the WPI control formed larger protein aggregates at pH 4.0 than at pH 3.5 or 3.0 ($p<0.001$), which was expected due to the closest between pH and pI (Shang et al., 2021). As seen in ζ-potential measurement, WPI presented less superficial charges at pH 4.0 justifying the protein interaction into large aggregates. When the heat treatment was performed under these conditions, a significant increase in Z-average was observed ($p<0.05$), consistent with the exposure of hydrophobic regions of protein chains that interact with each other given extensive aggregation (Ahmed et al., 2018). Independently of the pH and the heat treatment, the PDI of the WPI controls was greater than >0.5, showing the polydispersity in the size of protein aggregates. On the other hand, the SSPS control presented a nanosized Z-average that did not modify with heat treatment or pH conditions ($p>0.05$). However, in all the SSPS samples, the PDI was greater than 0.6 and the particle size distributions were multimodal, showing the dispersity of these polysaccharide chains.

The WPI-SSPS complexes presented nanometric sizes (100 nm < Z-average < 200 nm) and low polydispersity (PDI < 0.2), independently of pH, W:S ratio, and the performance of heat treatment. According to the statistical analysis (two-way ANOVA), the heat treatment gave rise to a significant increase in Z-average in all the heat-treated complexes concerning the sample without treatment, except for the sample with a 1:0.5 W:S ratio at pH 4.0. Likewise, according to the statistical response surfaces (Figures 3B and SM2B), the heat treatment and the pH significantly affected the Z-average of complexes, whereas the W:S ratio did not. When the heat treatment was performed, the Z-averages of the complexes were increased, but remained nanosized (Z-average < 200 nm). Also, the particle size distributions remained monomodal and PDI maintained low values (PDI < 0.2). This increment in Z-averages could be due to diverse phenomena, including the aggregation process (of proteins or protein-polysaccharide complexes), swelling process, and rearrangement in the structure in the protein-polysaccharide complexes, among others. However, similar results were previously obtained for lactoferrin-pectin and β-lactoglobulin-pectin complexes during heating (Bengoechea et al., 2011; Jones et al., 2010). The authors hypothesized that pectin may sterically block some of the hydrophobic sites on the unfolded protein chains, restricting the interaction between them and inhibiting the protein aggregation. On the other hand, as the pH decreased (from 4.0 to 3.0), the Z-average of WPI-SSPS complexes was reduced. This could be due to the formation of a stronger interconnected network between protein and polysaccharides.

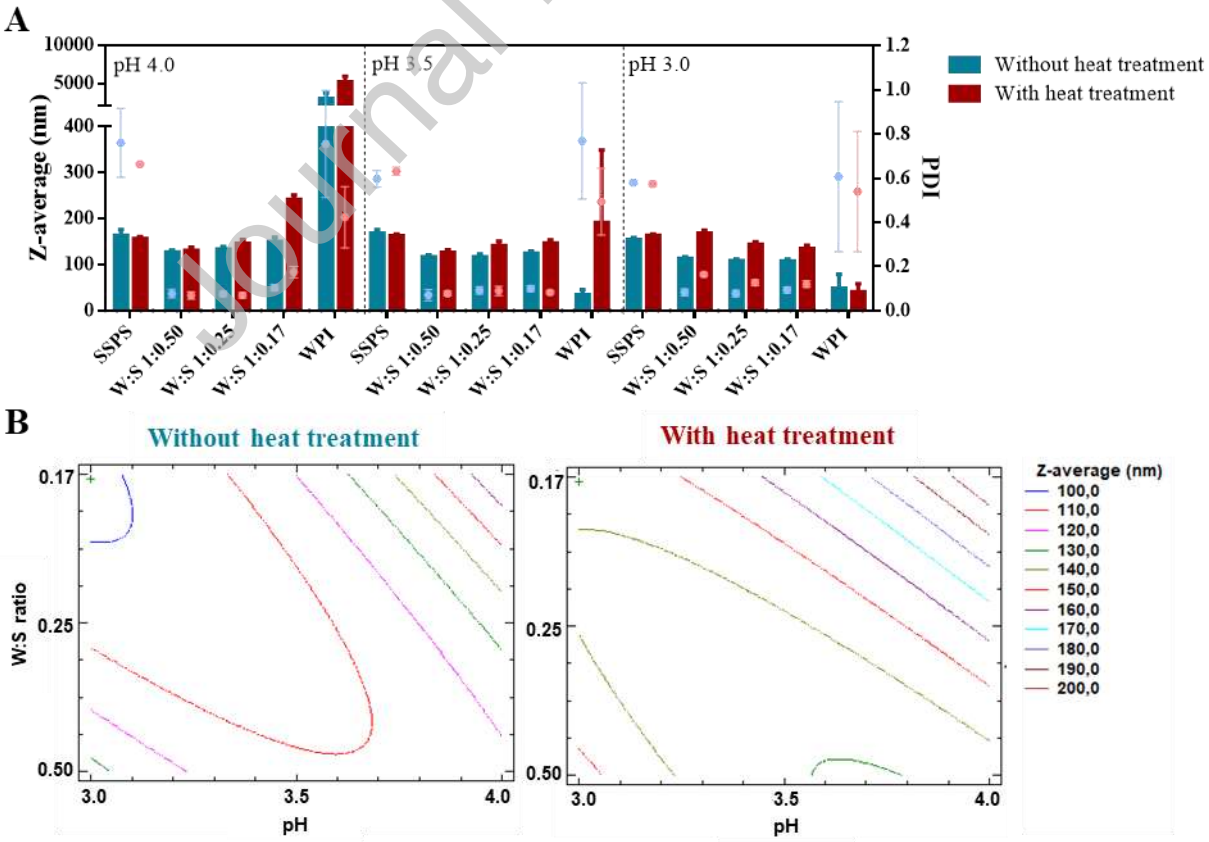


Figure 3 – Impact on particle size. (A) Z-average (left y-axis, bars) and polydispersity index (PDI, right y-axis, dots) of WPI, SSPS, and WPI-SSPS complexes in different pH conditions. **(B)** Level curves representation of statistical surfaces for Z-average as a function of pH and W:S ratio.

In addition to the measurements on the production day, the Z-average was monitored during storage on days 28 and 56 (Figure SM3). For the WPI control at pH 4.0, important variations were observed in the Z-average and the particle size distributions, all being multimodal and with high PDI values. For the complexes, Z-averages remained between 100 and 200 nm, without presenting significant changes at day 56 (concerning day 0), except in the samples without heat treatment at 1:0.25 and 1:0.17 W:S ratios and pH 4.0. In most cases, the heat treatment showed the capacity to stabilize the Z-average of the complexes over time.

According to surface hydrophobicity (S_0) measurements (Figure 4A), the heat treatment significantly increased the S_0 ($p<0.01$) of WPI and WPI-SSPS complexes, which is based on the alteration of protein secondary structure and exposure of hydrophobic regions to the surface of the protein aggregates or the complexes (Fioramonti et al., 2014; Liu et al., 2020). The interaction with SSPS did not inhibit the WPI unfolding process, leading to an increment of S_0 in the complexes. However, the interaction with SSPS reduced the aggregation process, stabilizing the particles in a nanometric size even until day 56 as we explained earlier. In the WPI-SSPS complexes without heat treatment, non-significant differences were observed for samples at different pH and W:S ratios ($p>0.05$). On the contrary, in the heat-treated WPI-SSPS complexes, a significant reduction of S_0 was observed in samples at pH 3.0 concerning those at pH 3.5 and 4.0. In the same line, the statistical response surfaces methodology (Figures 4B and SM2C) reported that the heat treatment and the pH had significant effects on the S_0 of complexes, whereas the W:S ratio did not. In samples without heat treatment, as the pH modified from 4.0 to 3.5 and from 3.5 to 3.0, the S_0 of complexes firstly increased and then decreased, respectively for each pH range. On the other hand, in samples with heat treatment, as the pH increased (from 3.0 to 4.0), the S_0 of complexes increased. Once again, these results confirm that the pH conditions in which the heat treatment is carried out modify the characteristics of the WPI-SSPS complexes.

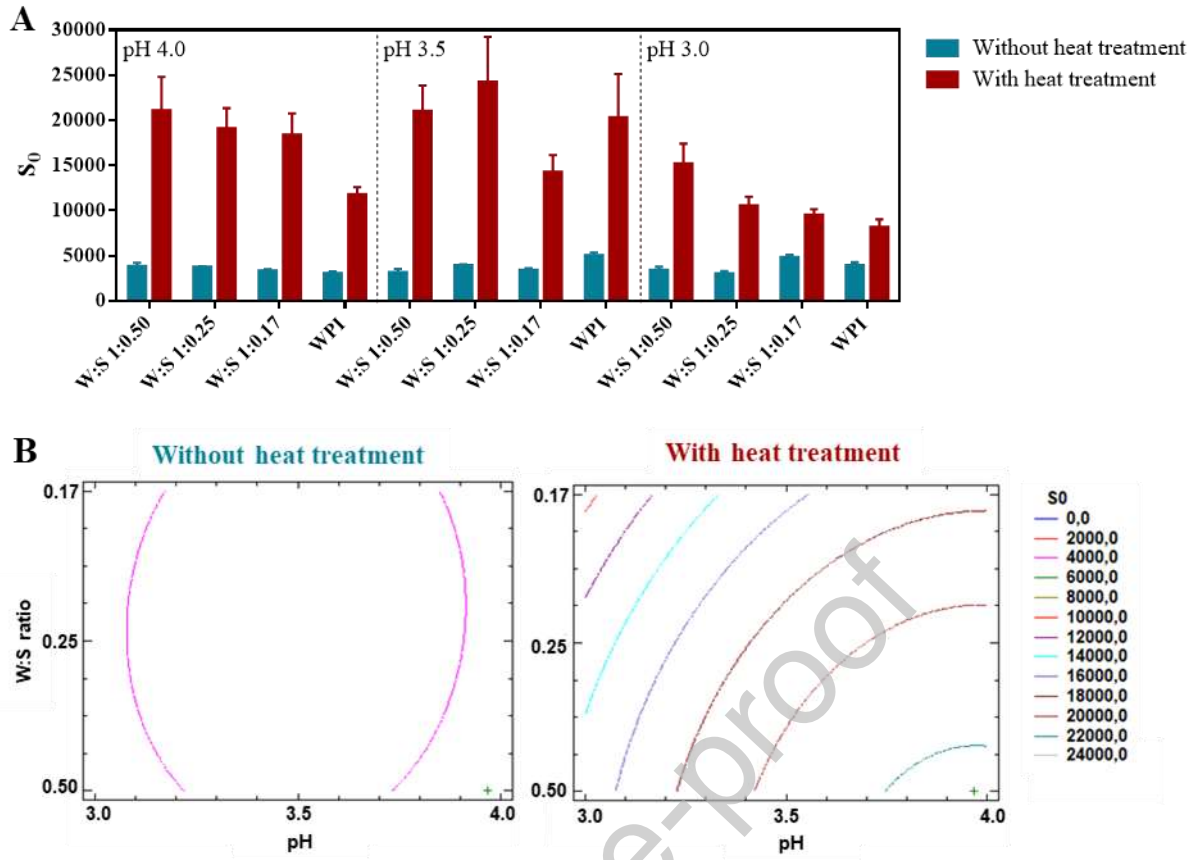


Figure 4 – Impact on surface hydrophobicity. (A) Surface hydrophobicity (S_0) of WPI and WPI-SSPS complexes in different pH conditions. **(B)** Level curves representation of statistical surfaces for S_0 as function of pH and W:S ratio.

According to TSI results (Figure 5A), WPI control at pH 4.0 presented a rapid destabilization even on the same day of preparation, which was reflected in the precipitation of the large particles. Precipitation was evidenced by the increase of BS at the bottom zone of the range of length and by the increase of T in the upper zone of the range of length. This trend was observed both in the samples with and without heat treatment. In contrast, WPI control at pH 3.5 without heat treatment was stable, while performing the heat treatment leads to the aggregation of proteins and the consequent destabilization of the system, showing the drawback of implementation of WPI in acid foods that have thermal treatments. Otherwise, WPI-SSPS complexes showed more stability than the WPI control, independent of the W:S ratio and heat treatment. These complexes exhibited less change in the T and BS profiles and lower TSI values (smaller than 20) over time, indicating better stability of WPI in the presence of SSPS than WPI alone. Similar results were previously reported for WPI-*Flammulina velutipes* polysaccharide complexes and pea protein isolate-propylene glycol

alginate complexes, which showed better Turbiscan stabilities than respective protein controls (Guo et al., 2019; Shang et al., 2021).

According to the statistical response surfaces (Figures 5B and SM2D), the pH and the interaction between pH and heat treatment significantly affected the TSI of complexes at day 56, while the W:S ratio and heat treatment did not. At pH 3.0 and 3.5, all complexes showed a stable trend with global TSI values below 10. At pH 4.0, without heat treatment, the samples with the lowest W:S ratio (1:0.17 and 1:0.25) presented higher TSI value, so less stability, than those obtained at a higher W:S ratio (1:0.50). This trend could be due to, as the concentration of SSPS increased, complexes with greater stability could be obtained since the presence of a polysaccharide interacting with the protein provided adequate electrostatic and steric repulsions due to the pectin-like structure with branches of SPPS (Z. Li et al., 2020). In addition, after heating, the samples with the lowest W:S ratio (1:0.17 and 1:0.25) presented a reduction in TSI values, indicating a stabilization effect of the heat treatment. Similar results were obtained for WPI-pectin complexes at pH 4.5 (Gentés et al., 2010).

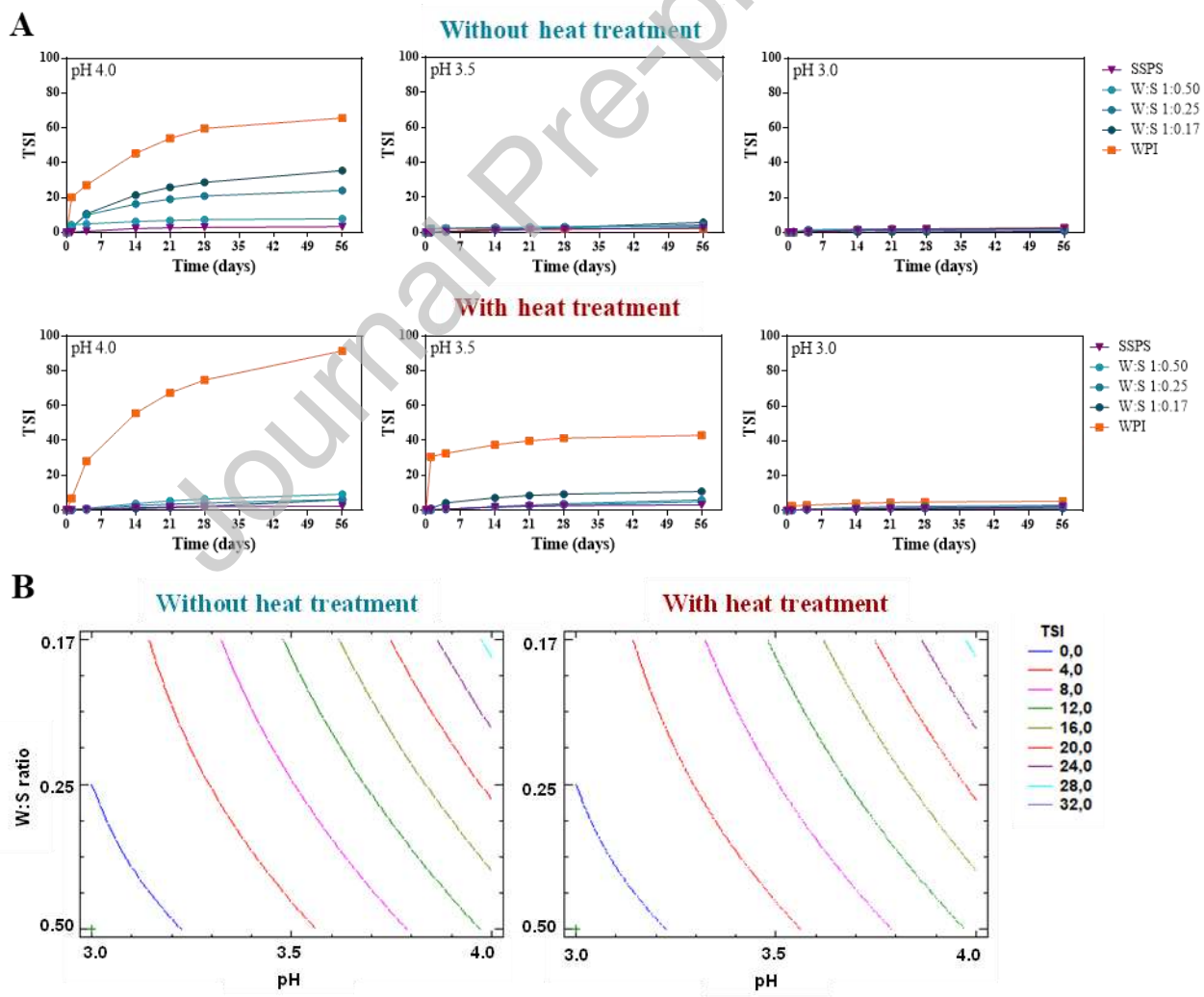


Figure 5 – Impact on physical stability. (A) Turbiscan stabilization index (TSI) of WPI, SSPS, and WPI-SSPS complexes in different pH conditions. **(B)** Level curves representation of statistical surfaces for TSI at day 56 post-production as a function of pH and W:S ratio without or with heat-treatment.

4. Conclusion

The present study demonstrates that the performance of heat treatment could modulate the characteristic of electrostatic complexes between WPI and SSPS under different conditions (pH or W:S ratio). The Z-average of complexes was incremented by heating. In addition, the S_0 and ζ-potential were modified due to the exposure of both hydrophobic and ionic residues to the surface of the complexes after protein unfolding. Even though the heat treatment induced rearrangement of the protein-polysaccharide-water interactions, the size of the complexes remained stable and nanosized for at least 56 days, and the physical stability was not negatively modified. According to the statistical optimization of responses, the WPI-SSPS complexes must be prepared at pH 3.5 and 1:0.5 W:S ratio, with heat treatment, to present lower Z-average (126 nm), high hydrophobicity (20990), greater stability (lower TSI, 3.6), and a ζ-potential close to zero (-1.26 mV). Taken together, these results provide further insight into the impact of heat treatment on the molecular interactions in protein-polysaccharide systems. Moreover, the knowledge generated in this work is useful to design heat-treated WPI-SSPS nanocomplexes with specific characteristics of size, charge, hydrophobicity, and physical stability. The possibility of modifying their characteristics would allow the incorporation and use of these complexes in food matrices for different purposes. For instance, the optimized heat-treated WPI-SSPS complexes could be employed for encapsulation and delivery of hydrophobic bioactive compounds, for the stabilization of emulsions and foams, or for the modification of optical and rheological properties of the food matrix. However, the novel and improved functionality of these heat-treated WPI-SSPS nanocomplexes concerning non-heated complexes and WPI control must still be studied. The perspective of our group is to implement the heat-treated WPI-SSPS complexes to encapsulate and stabilize curcumin in acidic beverages, studying the functionality and stability against simulated gastrointestinal digestion.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Statement

This work does not involve the use of human subjects or animal experiments.

References

Ahmed, K. F., Aschi, A., Nicolai, T. , 2018. Formation and characterization of chitosan-protein particles with fractal whey protein aggregates. Colloids Surfaces B Biointerfaces, 169, 257–264. https://doi.org/10.1016/j.colsurfb.2018.05.030

Alizadeh-Pasdar, N., Li-Chan, E. C. Y. , 2000. Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. J. Agric. Food Chem., 48(2), 328–334. https://doi.org/10.1021/jf990393p

Alizadeh-Pasdar, N., Li-Chan, E. C. Y. , 2001. Application of PRODAN fluorescent probe to measure surface hydrophobicity of proteins interacting with κ-carrageenan. Food Hydrocoll., 15(3), 285–294. https://doi.org/10.1016/S0268-005X(01)00027-3

Bédié, G. K., Turgeon, S. L., Makhlouf, J. , 2008. Formation of native whey protein isolate-low methoxyl pectin complexes as a matrix for hydro-soluble food ingredient entrapment in acidic foods. Food Hydrocoll., 22(5), 836–844. https://doi.org/10.1016/j.foodhyd.2007.03.010

Behrouzain, F., Razavi, S. M. A., Joyner, H. , 2020. Mechanisms of whey protein isolate interaction with basil seed gum: Influence of pH and protein-polysaccharide ratio. Carbohydr. Polym., 232, 115775. https://doi.org/10.1016/j.carbpol.2019.115775

Bengoechea, C., Jones, O. G., Guerrero, A., McClements, D. J. , 2011. Formation and characterization of lactoferrin/pectin electrostatic complexes: Impact of composition, pH and thermal treatment. Food Hydrocoll., 25(5), 1227–1232. https://doi.org/10.1016/j.foodhyd.2010.11.010

Cabezas, D. M., Pascual, G. N., Wagner, J. R., Palazolo, G. G. , 2019. Nanoparticles assembled from mixtures of whey protein isolate and soluble soybean polysaccharides. Structure, interfacial

behavior and application on emulsions subjected to freeze-thawing. Food Hydrocoll., 95, 445–453. https://doi.org/10.1016/j.foodhyd.2019.04.040

Chanasattru, W., Jones, O. G., Decker, E. A., McClements, D. J. , 2009. Impact of cosolvents on formation and properties of biopolymer nanoparticles formed by heat treatment of β-lactoglobulin–Pectin complexes. Food Hydrocoll., 23(8), 2450–2457. https://doi.org/10.1016/J.FOODHYD.2009.07.003

Chevalier, L. M., Rioux, L. E., Angers, P., Turgeon, S. L. , 2019. Study of the interactions between pectin in a blueberry puree and whey proteins: Functionality and application. Food Hydrocoll., 87, 61–70. https://doi.org/10.1016/J.FOODHYD.2018.07.038

Du, Q., Zhou, L., Lyu, F., Liu, J., Ding, Y. , 2022. The complex of whey protein and pectin: Interactions, functional properties and applications in food colloidal systems – A review. Colloids Surfaces B Biointerfaces, 210, 112253. https://doi.org/10.1016/j.colsurfb.2021.112253

Fioramonti, S. A., Perez, A. A., Aríngoli, E. E., Rubiolo, A. C., Santiago, L. G. , 2014. Design and characterization of soluble biopolymer complexes produced by electrostatic self-assembly of a whey protein isolate andsodium alginate. Food Hydrocoll., 35, 129–136. https://doi.org/10.1016/j.foodhyd.2013.05.001

Gaber, M., Mabrouk, M. T., Freag, M. S., Khiste, S. K., Fang, J. Y., Elkhodairy, K. A., Elzoghby, A. O. , 2018. Protein-polysaccharide nanohybrids: Hybridization techniques and drug delivery applications. Eur. J. Pharm. Biopharm., 133, 42–62. https://doi.org/10.1016/j.ejpb.2018.10.001

Gentés, M. C., St-Gelais, D., Turgeon, S. L. , 2010. Stabilization of whey protein Isolate-pectin complexes by heat. J. Agric. Food Chem., 58(11), 7051–7058. https://doi.org/10.1021/jf100957b

Ghadermazi, R., Khosrowshahi Asl, A., Tamjidi, F. , 2019. Optimization of whey protein isolate-quince seed mucilage complex coacervation. Int. J. Biol. Macromol., 131, 368–377. https://doi.org/10.1016/j.ijbiomac.2019.03.026

Gorji, E. G., Waheed, A., Ludwig, R., Toca-Herrera, J. L., Schleining, G., Gorji, S. G. , 2018. Complex Coacervation of Milk Proteins with Sodium Alginate. J. Agric. Food Chem., 66(12), 3210–3220. https://doi.org/10.1021/acs.jafc.7b03915

Guo, Q., Su, J., Yuan, F., Mao, L., Gao, Y. , 2019. Preparation, characterization and stability of pea protein isolate and propylene glycol alginate soluble complexes. Lwt, 101, 476–482.

https://doi.org/10.1016/j.lwt.2018.11.057

Jones, O., Decker, E. A., McClements, D. J. , 2010. Thermal analysis of β-lactoglobulin complexes with pectins or carrageenan for production of stable biopolymer particles. Food Hydrocoll., 24(2–3), 239–248. https://doi.org/10.1016/j.foodhyd.2009.10.001

Jones, O. G., McClements, D. J. , 2011. Recent progress in biopolymer nanoparticle and microparticle formation by heat-treating electrostatic protein-polysaccharide complexes. In *Advances in Colloid and Interface Science* (Vol. 167, Issues 1–2, pp. 49–62). Elsevier. https://doi.org/10.1016/j.cis.2010.10.006

Klemmer, K. J., Waldner, L., Stone, A., Low, N. H., Nickerson, M. T. , 2012. Complex coacervation of pea protein isolate and alginate polysaccharides. Food Chem., 130(3), 710–715. https://doi.org/10.1016/j.foodchem.2011.07.114

Lan, Y., Chen, B., Rao, J. , 2018. Pea protein isolate–high methoxyl pectin soluble complexes for improving pea protein functionality: Effect of pH, biopolymer ratio and concentrations. Food Hydrocoll., 80, 245–253. https://doi.org/10.1016/j.foodhyd.2018.02.021

Li, H., Wang, T., Hu, Y., Wu, J., Van der Meeren, P. , 2022. Designing delivery systems for functional ingredients by protein/polysaccharide interactions. Trends Food Sci. Technol., 119, 272–287. https://doi.org/10.1016/j.tifs.2021.12.007

Li, H., Yuan, Y., Zhu, J., Wang, T., Wang, D., Xu, Y. , 2020. Zein/soluble soybean polysaccharide composite nanoparticles for encapsulation and oral delivery of lutein. Food Hydrocoll., 103, 105715. https://doi.org/10.1016/j.foodhyd.2020.105715

Li, Q., Zhao, Z. , 2018. Characterization of the Structural and Colloidal Properties of α-Lactalbumin/Chitosan Complexes as a Function of Heating. J. Agric. Food Chem., 66(4), 972–978. https://doi.org/10.1021/acs.jafc.7b04628

Li, Z., Kuang, H., Yang, J., Hu, J., Ding, B., Sun, W., Luo, Y. , 2020. Improving emulsion stability based on ovalbumin-carboxymethyl cellulose complexes with thermal treatment near ovalbumin isoelectric point. Sci. Rep., 10(1). https://doi.org/10.1038/s41598-020-60455-y

Liu, J., Yang, J., Abliz, A., Mao, L., Yuan, F., Gao, Y. , 2020. Influence of thermal treatment on physical, structural characteristics and stability of lactoferrin, EGCG and high methoxylated pectin aggregates. Lwt, 125, 109221. https://doi.org/10.1016/j.lwt.2020.109221

Madureira, A. R., Pereira, C. I., Gomes, A. M. P., Pintado, M. E., Xavier Malcata, F. , 2007. Bovine

whey proteins - Overview on their main biological properties. Food Res. Int., 40(10), 1197–1211. https://doi.org/10.1016/j.foodres.2007.07.005

Maeda, H., Nakamura, A. , 2009. Soluble soybean polysaccharide. In *Handbook of Hydrocolloids: Second Edition* (pp. 693–709). Elsevier. https://doi.org/10.1533/9781845695873.693

Nakamura, A., Yoshida, R., Maeda, H., Furuta, H., Corredig, M. , 2004. Study of the role of the carbohydrate and protein moieties of soy soluble polysaccharides in their emulsifying properties. J. Agric. Food Chem., 52(17), 5506–5512.

Phillips, G. O., Williams, P. A. , 2009. Handbook of Hydrocolloids: Second Edition. In *Handbook of Hydrocolloids: Second Edition*. Elsevier. https://doi.org/10.1533/9781845695873

Phillips, G. O., Williams, P. A. , 2011. Handbook of Food Proteins. In *Handbook of Food Proteins*. Elsevier. https://doi.org/10.1533/9780857093639

Shang, J., Liao, M., Jin, R., Teng, X., Li, H., Xu, Y., Zhang, L., Liu, N. , 2021. Molecular properties of flammulina velutipes polysaccharide–whey protein isolate (Wpi) complexes via noncovalent interactions. In *Foods* (Vol. 10, Issue 1). https://doi.org/10.3390/foods10010001

Xu, Y. T., Liu, L. L. , 2016. Structural and Functional Properties of Soy Protein Isolates Modified by Soy Soluble Polysaccharides. J. Agric. Food Chem., 64(38), 7275–7284. https://doi.org/10.1021/acs.jafc.6b02737

Zamani, H., Zamani, S., Zhang, Z., Abbaspourrad, A. , 2020. Exceptional colloidal stability of acidified whey protein beverages stabilized by soybean soluble polysaccharide. J. Food Sci., 85(4), 989–997. https://doi.org/10.1111/1750-3841.15041

Zhao, R. X., Qi, J. R., Liu, Q. R., Zeng, W. Q., Yang, X. Q. , 2018. Fractionation and characterization of soluble soybean polysaccharide esterified of octenyl succinic anhydride and its effect as a stabilizer in acidified milk drinks. Food Hydrocoll., 85, 215–221. https://doi.org/10.1016/j.foodhyd.2018.07.023

Zhong, W., Li, C., Diao, M., Yan, M., Wang, C., Zhang, T. , 2021. Characterization of interactions between whey protein isolate and hyaluronic acid in aqueous solution: Effects of pH and mixing ratio. Colloids Surfaces B Biointerfaces, 203, 111758. https://doi.org/10.1016/j.colsurfb.2021.111758