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Utilization of plant-based protein-polyphenol complexes to form and stabilize emulsions: Pea proteins and grape seed proanthocyanidins

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ABSTRACT

Plant-based proteins and polyphenols are increasingly being explored as functional food ingredients. Colloidal complexes were prepared from pea protein (PP) and grape seed proanthocyanidin (GSP) and the ability of the PP/GSP complexes to form and stabilize oil-in-water emulsions were investigated. The main interactions between PP and GSP were hydrogen bonding. The stability of PP-GSP complexes to environmental changes were studied: pH (2–9); ion strength (0–0.3 M); and temperature (30–90 °C). Emulsions produced using PP-GSP complexes as emulsifiers had small mean droplet diameters (~200 nm) and strongly negative surface potentials (~−60 mV). Compared to PP alone, PP-GSP complexes slightly decreased the isoelectric point, thermostability, and salt stability of the emulsions, but increased their storage stability. The presence of GSP gave the emulsions a strong salmon (red-yellow) color, which may be beneficial for some specific applications. These results may assist in the creation of more efficacious food-based strategies for delivering proanthocyanidins.

1. Introduction

Many food companies are replacing animal-based proteins, such as those derived from muscle, eggs or milk, in their products with plant-based ones. This trend is mainly driven by the perceived health and environmental benefits of plant-based proteins by consumers. Pea protein is a commonly studied plant protein because of its high abundance, low cost, low allergenicity, and good amino acid profile, as well as the fact that it is typically not genetically modified (Lam, Karaca, Tyler, & Nickerson, 2018; Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015). Commercial pea protein ingredients actually consist of an assortment of different proteins with albumins (18–25%) and globulins (55–80%) being the most common. The dominant globulin fraction is primarily composed of legumin (11S, 300–400 kDa) and vicilin (7S, 150–180 kDa) (Lam et al., 2018; Stone et al., 2015). Pea protein is a surface-active protein that can be used as a natural emulsifier to prepare emulsion-based delivery systems (Lam et al., 2018). However, it only has limited potential to inhibit lipid oxidation in emulsions. Previous research has indicated that plant-based polyphenols can act as effective antioxidants, but they are not typically located at the oil droplet surfaces where lipid oxidation usually occurs (Huang, Li, Qiu, Teng, & Wang, 2017; Liu, Zhang, Li, McClements, & Liu, 2018). Consequently, we hypothesized that the ability of pea proteins to inhibit oxidation in emulsions could be improved by forming complexes with antioxidant polyphenols.

Proanthocyanidins are natural plant-based polyphenols belonging to the flavonoid family, which are oligomers and polymers built from flavan-3-ol elements, such as catechin, epicatechin, afzelechin, epiafzelechin, gallocatechin, and epigallocatechin (Serrano, Puupponen-Pimia, Dauer, Aura, & Saura-Calixto, 2009). These phytochemicals are present at appreciable levels in a range of foods derived from plants including chocolate, cocoa, grapes, green tea, and red wine (Blade et al., 2016). Medical and nutritional scientists have focused on the potential health benefits of consuming proanthocyandin-rich foods, which are attributed to their diverse range of biological activities, including antioxidant, anticancer, antidiabetic, and anti-heart disease properties (Blade et al., 2016; Prior & Gu, 2005). Nevertheless, the application of proanthocyandins as nutraceuticals in foods is often restricted because of their susceptibility to breakdown when exposed to heat, oxygen, and light (Zhou et al., 2017). Furthermore, proanthocyanidins have an extremely low oral bioavailability, which is mainly a result of their chemical instability and poor solubility in gastrointestinal fluids (Zou, Li, Percival, Bonard, & Gu, 2012).

Proanthocyanidins have been shown to have high binding affinities

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for various types of protein, including salivary proteins, digestive enzymes, lysozyme, gelatin, wheat proteins, rice protein, and milk protein (Dai, Chen, et al., 2019; Girard, Bean, Tilley, Adrianos, & Awika, 2018; Jakobek, 2015). The colloidal protein–polyphenol complexes formed are typically held together by physical interactions, such as hydrogen bonds, hydrophobic attraction, and electrostatic interactions (Foegeding, Plundrich, Schneider, Campbell, & Lila, 2017; Liu et al., 2018). Nutraceuticals encapsulated within colloidal complexes often have increased bioavailability and bioactivity because they are protected against degradation within the gastrointestinal (GI) tract (McClements, 2014; Roger, Lagarce, Garcion, & Benoit, 2010). Consequently, it may be possible to enhance the functional attributes of proanthocyanidins by binding them to proteins to form protective colloidal carriers.

A number of researchers have focused on understanding the interactions of pea proteins with various kinds of polyphenols, including tannic acid, curcumin, phenolic acids, and polyphenol extracts (Guo et al., 2020; Hoskin, Xiong, & Lila, 2019; Li, Dai, et al., 2020; Song & Yoo, 2017; Tsai & She, 2006). However, to our knowledge, there has been little research on the interactions of pea proteins with proanthocyanidin. Furthermore, the potential for using pea protein-proanthocyanidin complexes as plant-based emulsifiers in oil-in-water emulsions is currently unknown. A recent study reported that rice bran protein-catechin complexes were effective emulsifiers, being capable of forming small oil droplets during high-pressure homogenization (Li, Zhao, et al., 2020). Other researchers have demonstrated that gelatin-proanthocyanidin complexes are able to improve the oxidative stability and reduce the lipid digestion rate of emulsions, but gelatin is an animal-based protein (Huang et al., 2017; Su et al., 2015). At present, the effectiveness of pea protein–polyphenol complexes as emulsifiers is unclear. Our hypothesis is that using grape proanthocyanidins in combination with pea protein will provide better functional properties in the emulsions than using pea proteins alone. In particular, we hypothesized that protein–polyphenol complexes could act as antioxidant emulsifiers, which would stabilize emulsions from lipid oxidation. For this reason, we characterized the nature of the colloidal complexes formed from pea proteins and grape seed proanthocyanidins, as well as their potential to produce stable oil-in-water emulsions.

2. Materials and methods

2.1. Materials

Pea protein (Vitessence™, 1803, PP) with a minimum of 80% protein content on a dry matter basis was kindly donated by Ingredion Inc. (Bridgewater, NJ). Bovine serum albumin (BSA), which was used as a standard, was purchased from Sigma-Aldrich Co. (St. Louis, MO). Grape seed proanthocyanidin (GSP) was purchased from DaXingAnLing Lingonberry Boreal Biotech Co., Ltd. (China, Heilongjiang). This GSP ingredient consisted of 95.0% proanthocyanidins according to the data sheet provided by the manufacturer. Flaxseed oil was obtained from a supermarket. Sodium chloride (NaCl) was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Double-distilled/deionized water (Milli-Q®) was utilized in the preparation of all aqueous solutions and emulsions. All pH adjustments were made using NaOH or HCl solutions.

2.2. Preparation of pea protein and procyanidin solution

Initially, the purity of the pea protein was enhanced using a selective precipitation approach developed previously (Lan, Chen, & Rao, 2018). In brief, powdered pea protein was dispersed in double distilled water (1:10 w/v), and then adjusted to pH 8.0. The pea protein solution was stirred overnight at 500 rpm using a magnetic stirrer at 4 °C. Insoluble matter was removed by centrifugation (8000 g, 30 min, 4 °C) using a bench top centrifuge. The supernatant was collected and then adjusted to pH 4.5, which is near the isoelectric point of the pea proteins, which caused the proteins to become insoluble and precipitate. The resulting suspension was centrifuged again (8000 g, 30 min, 4 °C) to separate the insoluble proteins. These proteins were then collected, dissolved in double distilled water, and the solution was adjusted to pH 8.0. The pea protein solution was then stirred using a magnetic stirrer at 500 rpm overnight at 4 °C, and then adjusted to pH 7.0. Finally, the protein concentration in the solution was determined by measuring the absorption at 280 nm using a UV–visible spectrometer (Genesys 150, Thermo Scientific, Waltham, MA). The protein concentration was reported as a BSA equivalent determined from a calibration curve prepared using known quantities of BSA. Powdered GSP was dispersed in double distilled water to obtain a GSP stock solution (0.5% wt/wt) and the system was then adjusted to pH 7.0 using sodium hydroxide solution at room temperature.

2.3. Preparation and characterization of PP/GSP colloidal complexes

2.3.1. Preparation

PP/GSP complexes were fabricated at room temperature using an approach described previously (Huang et al., 2017). Initially, the pea protein and GSP solutions were adjusted to pH 7.0 prior to being mixed together. A pipette was then used to add different amounts of the GSP solution into the PP solution, whilst stirring at 500 rpm for 5 min with a magnetic stirrer. Three mixed systems were created with the same final pea protein concentration (0.3 wt%) but different final GSP concentrations (0.1, 0.2, or 0.3 wt%).

2.3.2. Extinction coefficient of PP and PP/GSP systems

The extinction coefficient of the samples was measured at 600 nm using the same UV–visible spectrometer described above. The extinction coefficient is the sum of the turbidity (light scattering) and absorbance (light absorption). For some measurements, the turbidity was calculated by subtracting the measured absorbance of the GSP solution from the measured extinction coefficient of the PP/GSP suspensions. This procedure was carried to determine whether colloidal particles were formed after complexation.

2.3.3. PP/GSP complex characteristics

The dimensions (mean particle diameter), polydispersity index (PDI), and surface potential (ζ-potential) of the PP/GSP complexes were determined using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). Particle size and surface potential measurements were based on the principle of dynamic light scattering and microelectrophoresis, respectively. The refractive indices of the protein and water used in the calculations were 1.45 and 1.33, respectively.

2.4. Impact of environmental stresses on complex properties

Initially, aqueous solutions containing PP (0.3%) and PP-GSP (0.3–0.3%) complexes were prepared. The pH, temperature, and ionic strength of these solutions were then varied to simulate the range of environmental conditions that foods or beverages may experience during their lifetime. In particular, the pH-stability (pH 2–9, 0 mM NaCl, 25 °C), salt-stability (0 to 0.3 M NaCl, pH 7.0, 25 °C), and thermal-stability (30 to 90 °C at 1.0 °C/min, pH 7.0, 0 mM NaCl) of the PP and PP-GSP solutions were determined using methods described in detail elsewhere (Li et al., 2019).

2.5. Isothermal titration calorimetry analysis

PP-GSP interactions were analyzed by recording the changes in enthalpy that occurred when their solutions were combined in the reaction cell of an isothermal titration calorimeter (VP-ITC, Microcal, Inc., Northampton, MA) at 25.0 ± 0.1 °C according to a method described previously with some modification (Zhou et al., 2019). GSP

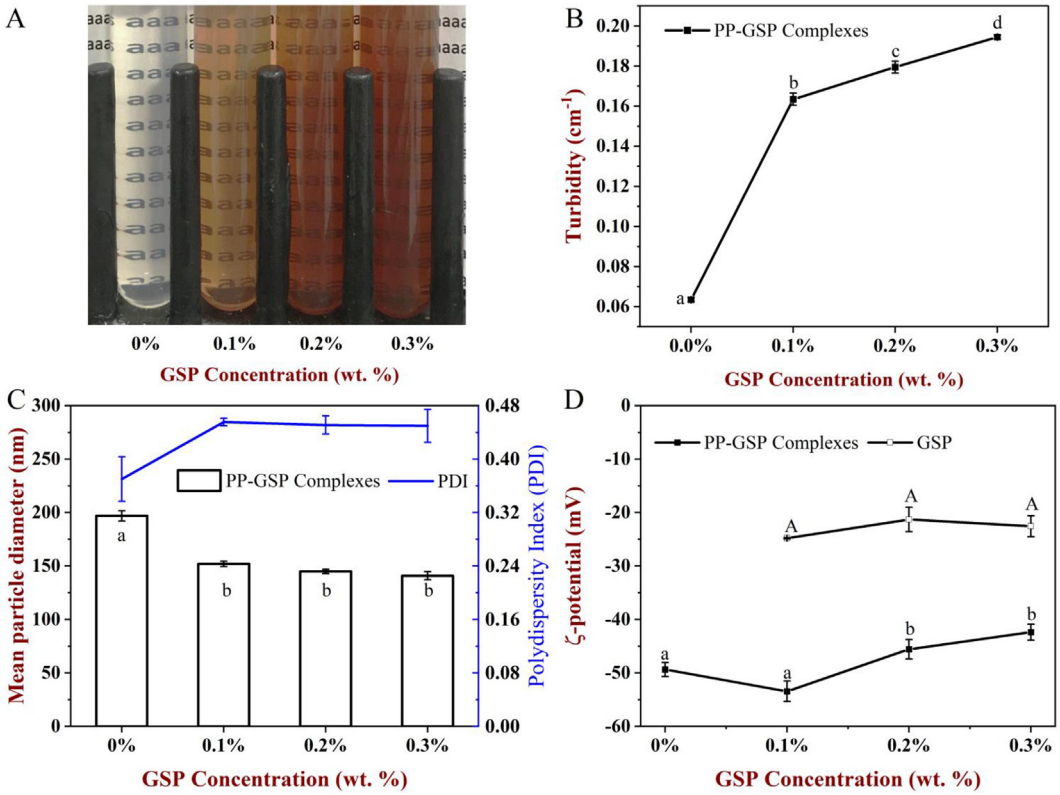

Fig. 1. The effect of GSP concentration on appearance (A), turbidity (B), mean particle diameter (C) and ζ-potential (D) of PP-GSP complex. Different letters in the same figure represent significant ($p < 0.05$).

solution (800 μM) was placed into a 500 μL syringe and PP solution (10 μM) was placed in a 1.45 mL reaction cell. The titration experiment was then carried out by injecting 29 small volumes (10 μL) of GSP solution into PP solution, with 240 s waiting time between each injection. The samples contained within the titration cell were subjected to constant stirring (315 rpm) during the injections. The ITC instrument recorded the change in heat flow over time profile and then converted it into an enthalpy change *versus* GSP-to-PP molar ratio curve by integration (Microcal ORIGIN 7.0, Northampton, MA). A one-site thermodynamic model was used to determine the binding constant (K_a), enthalpy change (ΔH), entropy change (ΔS), Gibbs free energy change (ΔG), and number of binding sites (n). In this case, the number of binding sites corresponds to the moles of GSP bound per mole of PP.

2.6. Molecular docking

Analysis of the polyphenol-protein interactions was carried using a commercial molecular docking program (CDOCKER, Discovery Studio version 3.0, BIOVIA, United States). The protein structure used in the simulations was based on the reported crystal structure of pea protein 11 s (PDB:3KSC), since it is one of the main constituents of pea protein . The proanthocyanidin structure (CAS: 4852–22-6) was obtained from the Pubchem NCBI database maintained by the National Institutes of Health (US) (https://pubchem.ncbi.nlm.nih.gov/). The structures of the pea protein and proanthocyanidin were then imported to the software program. The energy was optimized to a minimum and the pH was adjusted to 7.0 using the CHARMm force field. The pea protein and proanthocyanidin were then allowed to interact with each other using the CDOCKER Docking protocol described earlier (Dai, Li et al., 2019). The interactions between the pea protein and proanthocyanidin were visualized using a higher version of the software (Discovery Studio 2019 Client).

2.7. Emulsion preparation and characterization

A two-stage process was utilized to prepare the flaxseed oil-in-water emulsions: (i) an emulsion containing relatively large oil droplets was created by blending 3% (w/w) oil phase (flaxseed oil) and 97% (w/w) aqueous phase (either 0.3% PP or 0.3–0.3% PP-GSP mixtures in double distilled water) for 2 min using a high-speed mixing device (M122/1281-0, Biospec Products, Inc., ESGC, Switzerland); (ii) the droplet size was then reduced by passing these emulsions through the interaction chamber of a microfluidizer (M110P, Microfluidics, Newton, MA) three times at 12 000 psi (83 MPa). After preparation, the emulsions were kept under ambient temperature conditions in the dark for 21 days and any changes in their properties were measured. The following properties of the emulsions were measured using the experimental protocols described in detail in our previous studies (Li, Peng, et al., 2019; Liu, Tan, Zhou, Mundo, & McClements, 2019; Xu, Sun, & McClements, 2019): (i) mean particle diameter ($D_{4,3}$) and particle size distribution (PSD) by laser diffraction; (ii) ζ-potential by particle electrophoresis; (iii) L*a*b* color coordinates by instrumental colorimetry; (iv) microstructure by confocal laser scanning microscopy.

2.8. Environmental stress stability

The stability of 3 wt% flaxseed oil-in-water emulsions prepared using either 0.3 wt% PP or 0.3–0.3% PP-GSP as an emulsifier was determined when they were exposed to a range of solution and environmental conditions as described previously (Li, Peng, et al., 2019; Xu et al., 2019). Briefly, the pH-stability (pH 2–9, 0 mM NaCl, 25 °C), salt-stability (0 to 0.3 M NaCl, pH 7.0, 25 °C), and thermal-stability (30 to 90 °C, 30 min, pH 7.0, 0 mM NaCl) of the PP- and PP-GSP-emulsions were determined.

2.9. Statistical analysis

All experiments were carried out using two or more samples, and then the mean and standard deviation of each sample were calculated. Statistical analysis software was used for analyzing the data (SPASS 25.0, SPSS Inc., Chicago, IL). Significant differences between sample means ($p < 0.05$) were established using a one-way ANOVA test (Tukey test).

3. Results and discussion

3.1. Formation of PP/GSP complexes

It should be noted that PP/GSP complexes were prepared by adding GSP to PP, because previous studies have shown that this order-of-addition sequence should be followed to avoid the formation of unstable colloid suspensions (Su et al., 2015). The turbidities of colloidal dispersions containing PP/GSP complexes prepared with varying proanthocyanidin concentrations were measured. Visually, the turbidity of the PP/GSP suspensions increased with increasing GSP concentration (0 to 0.3%), at a fixed PP concentration (0.3%) (Fig. 1AB). No obvious precipitation or sedimentation was observed in any of the mixtures after 24 h storage at ambient temperature. This observation suggests that the PP/GSP complexes formed colloidal particles with good resistance to gravitational forces. The size and charge characteristics of the PP/GSP complexes were also measured (Fig. 1CD). The mean diameter of the PP particles decreased from around 200 to 150 nm (PDI = 0.4) after binding to proanthocyanidin (Fig. 1C). This result suggests that the bound proanthocyanidin molecules altered the ability of the pea proteins to assemble into colloidal particles. Similarly, previous researchers have reported that the presence of proanthocyanidin or tannic acid reduced the size of the colloidal particles formed by gelatin (Huang et al., 2017).

The electrophoresis measurements indicated that the PP/GSP complexes all had relatively high negative charges (-42 to −54 mV) (Fig. 1D). This phenomenon occurs because the pea proteins are highly negative charge under neutral conditions, since this pH is well above their isoelectric point (pH 4.5). Moreover, the surface potential measurements of the pure polyphenol solutions (no protein) showed that the proanthocyanidins were also highly negative charge (about −25 mV). The surface potential of the PP/GSP complexes decreased somewhat as the proanthocyanidin level was increased. This phenomenon may have occurred because a number of the polyphenols bound to the pea proteins and reduced the overall net negative charge at the outer surfaces of the colloidal particles. In other words, the PP/GSP complexes had a lower net negative charge than the PP alone because GSP has a lower charge magnitude than PP. Previous researchers have reported that the net charge on gelatin/GSP complexes was less than that on gelatin alone, which was also attributed to the lower negative charge on the polyphenol than the protein (Huang et al., 2017).

3.2. Stability of complexes to environmental stresses

For these assays, colloidal complexes were prepared at a mass ratio of 1:1 PP-to-GSP and then their stability to pH, salt, and heating were determined.

3.2.1. pH-stability

The protonation status of proanthocyanidin impacts its ability to interact with proteins because this influences the molecular interactions involved (Zhou et al., 2017). The extinction coefficient (turbidity + absorbance) of solutions containing either PP or PP/GSP complexes was therefore measured over a range of pH values (Fig. 2A). The presence of the polyphenols clearly altered the aggregation behavior of the proteins. For the PP/GSP systems, the extinction coefficient remained relatively low when the system was decreased from pH 9 to 6, increased steeply from pH 6 to 3, but then decreased at pH 2. Conversely, for the PP solutions, the extinction coefficient (in this case turbidity, since there was no absorbance) stayed fairly low from pH 9 to 6, increased sharply from pH 6 to 4, but then decreased sharply from pH 4 to 2. These results suggest that the proanthocyanidins reduced the pea protein's isoelectric point, which agrees with previous studies on other protein–polyphenol mixtures (Huang et al., 2017). From pH 2 to 5, a thin white layer was seen at the test tube bottoms, indicating that large dense complexes had formed and then sedimented. From pH 6 to 9, the samples appeared clear, which indicated that the complexes formed under these conditions were small enough to not scatter light strongly or to sediment due to gravitational forces.

3.2.2. Thermal-stability

The extinction coefficient of PP or PP/GSP solutions were monitored across a range of temperatures (30 to 90 °C). The extinction coefficient (in this case turbidity) of the PP solution stayed constant across the whole range of temperatures used, suggesting that heating did not promote pea protein aggregation under these conditions (Fig. 2B). This phenomenon may be due to the high thermal denaturation temperature of pea proteins (Mession, Sok, Assifaoui, & Saurel, 2013). The initial extinction coefficient of the PP/GSP was much higher than that of the PP solutions, which is because they contained GSP chromophores that absorbed light. A slight increase in the extinction coefficient of the PP/GSP solutions occurred when they were heated above 60 °C but no sediments could be seen. These results suggest the PP/GSP complexes were fairly resistant to heating. The slight increase in extinction coefficient of the PP/GSP solutions observed upon heating may have been due to an increase in the binding capacity of the proteins at higher temperatures (Ozdal, Capanoglu, & Altay, 2013).

3.2.3. Salt-stability

The impact of salt addition on the extinction coefficient of PP/GSP solutions was monitored at pH 7 and 25 °C (Fig. 2C). PP solutions stayed fairly transparent from 0 to 0.3 M NaCl, suggesting salt addition did not induce pea protein aggregation under these conditions. On the other hand, the extinction coefficient of the PP/GSP solutions became appreciably higher as the NaCl level was increased, which is consistent with the formation of larger colloidal particles at higher salt levels. Other researchers have also reported an increase in extinction coefficient of polyphenol-biopolymer solutions when the ionic strength was increased (Li, Zeng, et al., 2019). Salt addition presumably modulated the physical forces between the PP and GSP molecules, thereby altering their tendency to associate with each other (McClements, 2015). In particular, it may have increased hydrogen bonding and hydrophobic attractions by altering water structure, thereby promoting the generation of larger PP-GSP complexes. Conversely, the addition of salt could have reduced the electrostatic repulsive forces acting amongst the PP-GSP complexes, thereby promoting their aggregation.

3.3. Characterization of pea protein/proanthocyanidin interactions

Isothermal titration calorimetry (ITC) and molecular docking were employed in combination to obtain some insights into the character of the interactions between the pea protein and proanthocyanidin molecules. ITC was used to provide information about the thermodynamic characteristics of the interactions (such as stoichiometry, Gibbs free energy, enthalpy, entropy, and equilibrium binding constants), whereas molecular docking was used to provide insights into the functional groups and molecular forces involved in the interactions (Ferreira, dos Santos, Oliva, & Andricopulo, 2015).

3.3.1. ITC analysis

ITC was used to measure the heat flow *versus* time profiles when small aliquots of GSP solution were titrated into a PP solution (Fig. 3A). The interaction enthalpy *versus* PP/GSP molar ratio was then calculated

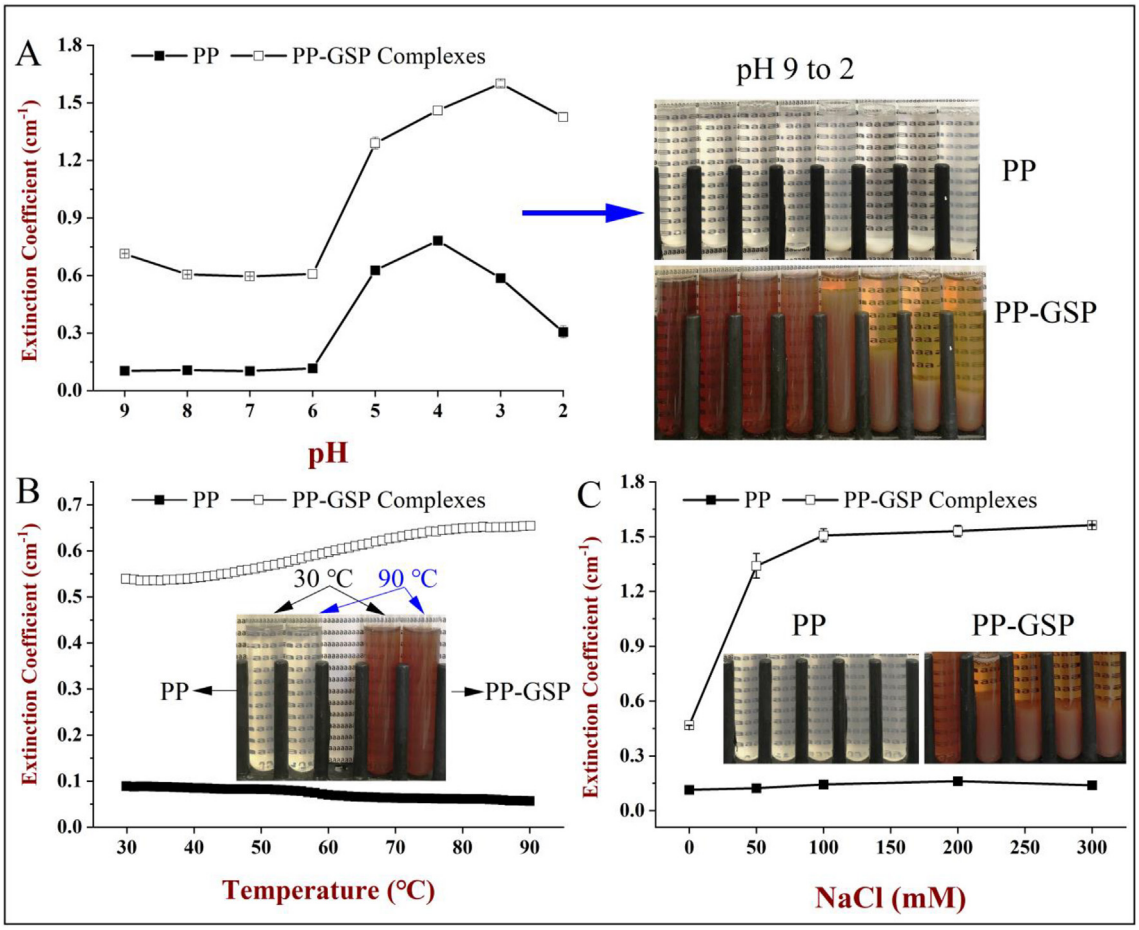

Fig. 2. The pH (A), Temperature (B), NaCl (C) effect on extinction coefficient of PP-GSP complex.

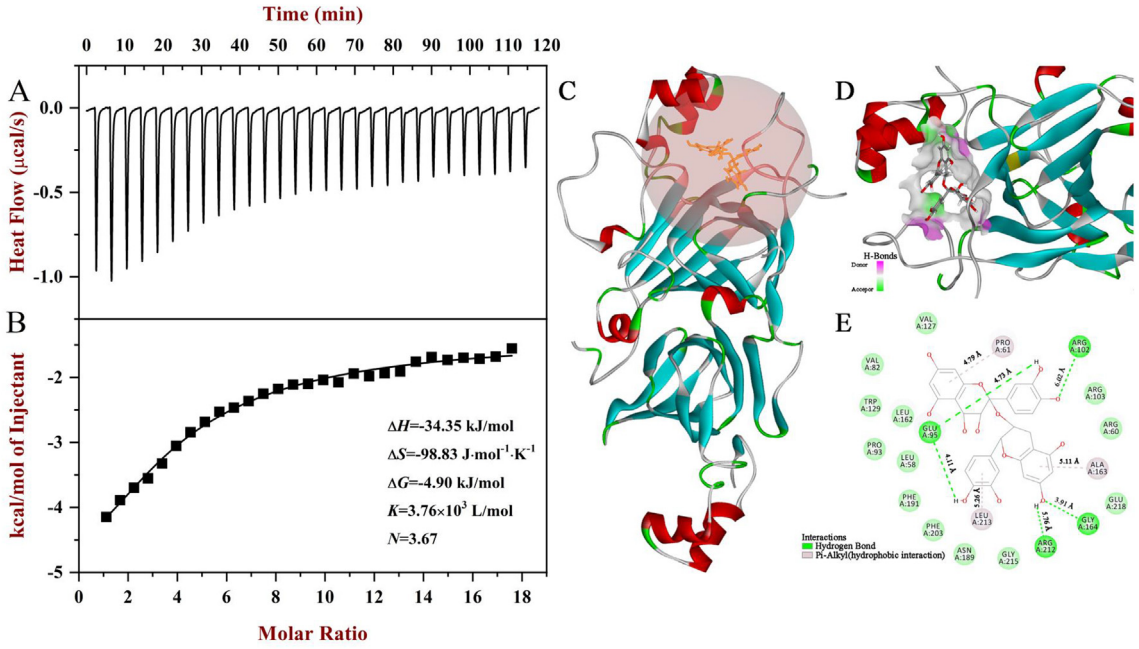

Fig. 3. Raw data plot of heat flow against time (A) and the binding isotherms (B) corresponding to the titration of 0.8 mM GSP into 0.01 mM PP at 25 °C. The 3D binding mode and site between PP and GSP (C), partial 3D bind mole with hydrogen bond surface affinity (D), 2D schematic interaction diagram between PP and GSP, the color of amino acid residue is drawn by interaction (E).

by integration of the heat-time profiles, and then the thermodynamic parameters were determined by model fitting (Fig. 3B). The binding interaction was found to be highly exothermic (ΔH = -34.4 kJ/mol), which is consistent with hydrogen bond formation between proanthocyanidin and protein molecules (Huang et al., 2017). The negative Gibbs free energy change ($\Delta G = -4.90$ kJ/mol) indicates that the GSP and PP molecules spontaneously interacted with each other. The negative entropy change ($\Delta S = -98.8$ J·mol^{-1}·K^{-1}) suggests that the interaction was mainly enthalpy-driven (rather than entropy-driven) because the molecular order actually increased after formation of the complexes. The estimated stoichiometry (N = 3.67) of the interaction suggested that PP had about four binding sites for GSP. The binding constant for PP-GSP (3.76×10^3 L/mol) was relatively high, being greater than that reported for BSA-GSP (1.5×10^3 L/mol) but smaller than that for gelatin-GSP (3.3×10^5 L/mol) (Frazier et al., 2010; Huang et al., 2017).

3.3.2. Molecular docking

A molecular docking simulation provided additional insights into the character of the interactions between the proanthocyanidin and pea protein molecules. CDOCKER is a high precision docking algorithm based on the CHARMm forcefield, which has been widely used to model protein-small molecule interactions (Zhou et al., 2018). According to CDOCKER analysis, the molecular arrangement with the lowest energy score is taken to be the most likely one adopted by the system (Xiong, Liu, Zhou, Zou, & Chen, 2016). In our case, CDOCKER energy = −24.1 kcal/mol and CDOCKER interaction energy = −50.56 kcal/mol. The polyphenol-protein interaction energy was then taken to be this latter value. The docking location (transparent zone) within the overall complex is shown in Fig. 3C, while a more detailed visualization of the interaction zone is shown in Fig. 3D. These images clearly shown that proanthocyanidin is bound to the pea protein surface. A 2D schematic of the interaction indicates that the polyphenol molecule interacts with the following amino acid groups on the protein (Fig. 3E): Leu 58, Pro 93, Glu 95, Leu 162, Trp 129, Val 82, Val 127, Pro 61, Arg 102, Arg 103, Arg 60, Ala 163, Glu 218, Gly 164, Arg 212, Gly 215, Asn 189, Leu 213, Phe 203, Phe 191. All of these amino acids are therefore potentially involved in the interactions between the two molecules. Among these, five hydrogen bonds were identified between the proanthocyanidin and the Glu 95, Arg 102, Arg 212, and Gly 164 groups with bond lengths of 4.11, 4.73, 6.02, 3.91, and 5.76 Å, respectively. Moreover, we identified three hydrophobic interactions (Pi-Alkyl) between the proanthocyanidin and the Pro 61 (4.79 Å), Ala 163 (5.11 Å), and Leu 213 (5.26 Å) groups on the protein surface. The main interaction force between the proteins and polyphenols therefore appeared to be hydrogen bonds, which agreed with the ITC results, but hydrophobic forces may also have played some role.

3.4. Utilization of PP-GSP complexes to form and stabilize emulsions

3.4.1. Particle characteristics

The influence of proanthocyanidin concentration within the PP-GSP complexes on emulsion formation and stability was investigated. The particle characteristics of the emulsions were measured at neutral pH and ambient temperature (Fig. 4). Interestingly, the mean particle diameter ($D_{4,3}$) slightly decreased with increasing proanthocyanidin concentration in the initial emulsions (Fig. 4A), which suggested that the presence of the polyphenol may have promoted droplet disruption or inhibited droplet aggregation during homogenization (Li, Zhao, et al., 2020; Wang et al., 2019). Emulsions formed using either PP or PP/GSP complexes contained relatively small droplets ($D_{4,3} < 0.3$ μm) with monomodal particle size distributions, and high surface potentials (absolute value > 50 mV), throughout storage. Thus, both the PP- and PP/GSP-coated droplets were relatively resistant to aggregation, consistent with their visual appearances and microstructures (Fig. 4E). Even so, the $D_{4,3}$ values of the PP-emulsions determined by light scattering increased appreciably after 21 days storage, which suggested some aggregation of the droplets occurred (Fig. 4). Conversely, the $D_{4,3}$ values of all the PP/GSP-emulsions did not change during storage, suggesting that oil droplets coated by protein–polyphenol complexes were more resistant to aggregation. Thus, the addition of the polyphenols appears to have improved the resistance of the protein-coated droplets to aggregation during storage.

3.4.2. Optical properties

The optical properties of the emulsions changed appreciably after addition of the grape seed proanthocyanidin used to assemble the polyphenol-protein complexes (Fig. 5). In general, the lightness (L*) of the emulsions decreased with proanthocyanidin concentration, which is due to the presence of a greater number of chromophores to absorb the light waves (McClements, 2002). Consequently, less light was reflected from the surfaces of the emulsions making them appear darker. The change in lightness of the emulsions during storage depended on the proanthocyanidin concentration. There was a slight increase in lightness after storage in the presence of 0 and 0.1 wt% proanthocyanidin, but a slight decrease in the presence of 0.2% and 0.3% (Fig. 5A). The origins of this phenomenon are unknown at present but they could be due to changes in light absorption and/or scattering caused by the presence of the polyphenols.

The chromatic properties (a* and b*) of the emulsions also depended on proanthocyanidin concentration (Fig. 5B). In general, both the a* and b* values of the emulsions became increasingly positive as the level of proanthocyanidin added was increased. Again, this effect can be attributed to an increase in selective absorption of light waves by the chromophores as the level of pigment present was raised. The fact that the a*-value increased with increasing proanthocyanidin indicates that the color of the emulsions changed from white to red. Similarly, the fact that the b*-value increased indicates that the emulsion color changed from white to yellow. There was some change in the color of the emulsions containing the complexes during storage. In particular, both the redness and yellowness increased slightly after 21 days storage, which suggests that there may have been some change in emulsion microstructure or proanthocyanidin chemical stability (Cai, Sun, Schliemann, & Corke, 2001). Overall, our results indicate that the grape seed proanthocyanidins may be used as natural pigments in food emulsions, provided that a red-yellow color is desirable. In other applications, the strong color of the proanthocyanidins would be a disadvantage to their utilization.

3.4.3. Emulsion microstructure

Confocal fluorescence microscopy was used to give additional insights about the impact of emulsifier type on emulsion microstructure (Fig. 4E). The microscopy images of the PP- and PP/GSP-emulsions indicated that they all contained relatively small oil droplets that were not associated with each other. These results suggest that proanthocyanidin addition did not promote aggregation of the protein-coated oil droplets, which is consistent with the particle size analysis and visual appearance of the emulsions

3.5. Influence of environmental stresses on emulsion stability

In this series of experiments, we compared the environmental stability of emulsions coated by 0.3% PP or 0.3%PP-0.3%GSP. The emulsions were exposed to changes in their pH (2–9), temperature (30–90 °C) and ionic strength (0–0.3 M), and then their physicochemical properties were measured.

3.5.1. pH-stability

The dependence of the particle size (Fig. 6A) and charge (Fig. 6D) of the PP- and PP/GSP-emulsions on pH were measured. All the emulsions were highly unstable to aggregation below pH 6, with the largest particle sizes being observed around pH 4 to 5. Conversely, all the

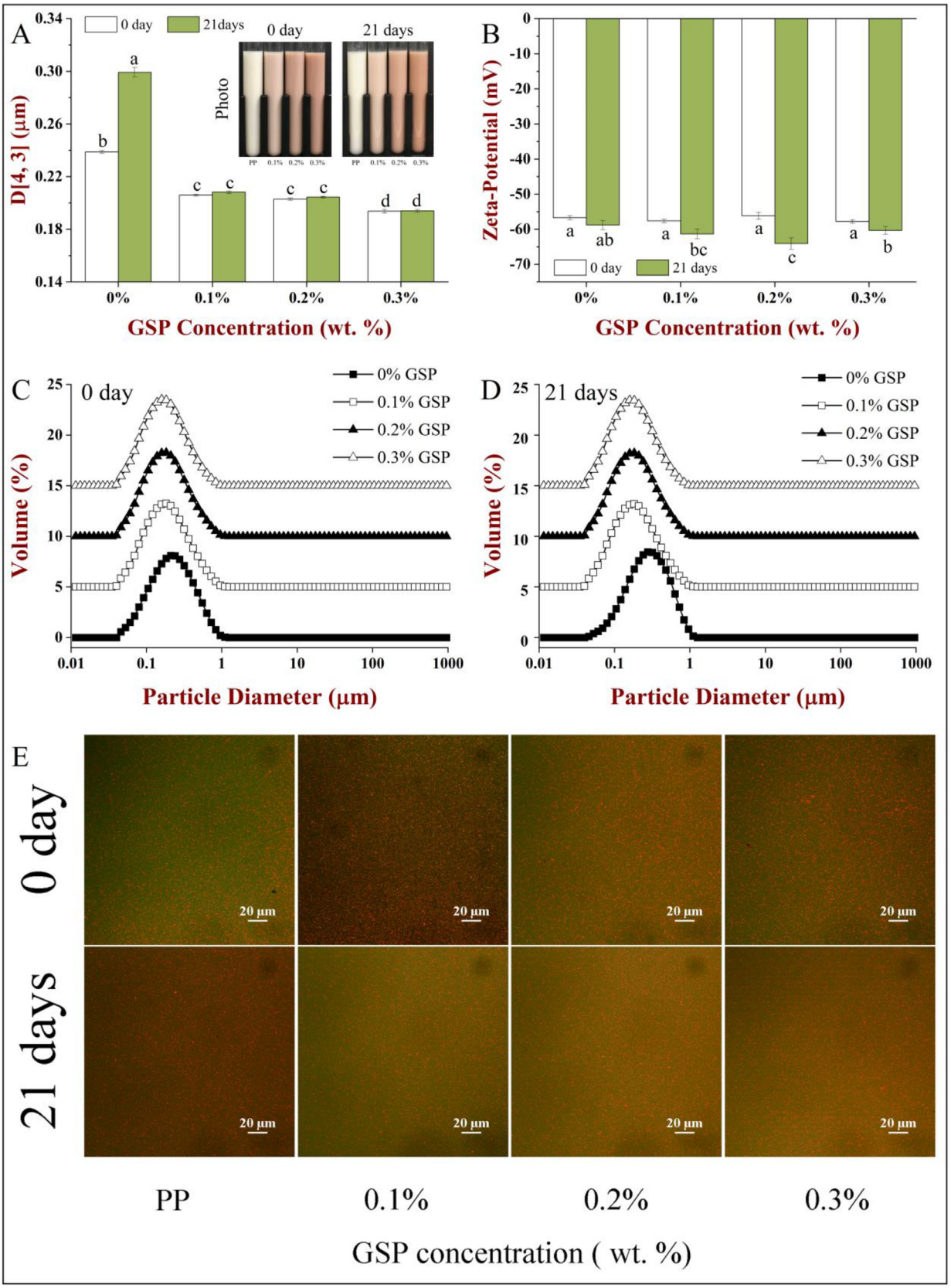

Fig. 4. D[4,3] (A), ζ-potential (B), and particle size distribution of PP emulsions or PP-GSP emulsions during storage time 0 days (C) and 21 days (D) at 25 °C. The confocal microscopy images (E) of PP or PP–GSP emulsions during storage time (21 days at 25 °C).

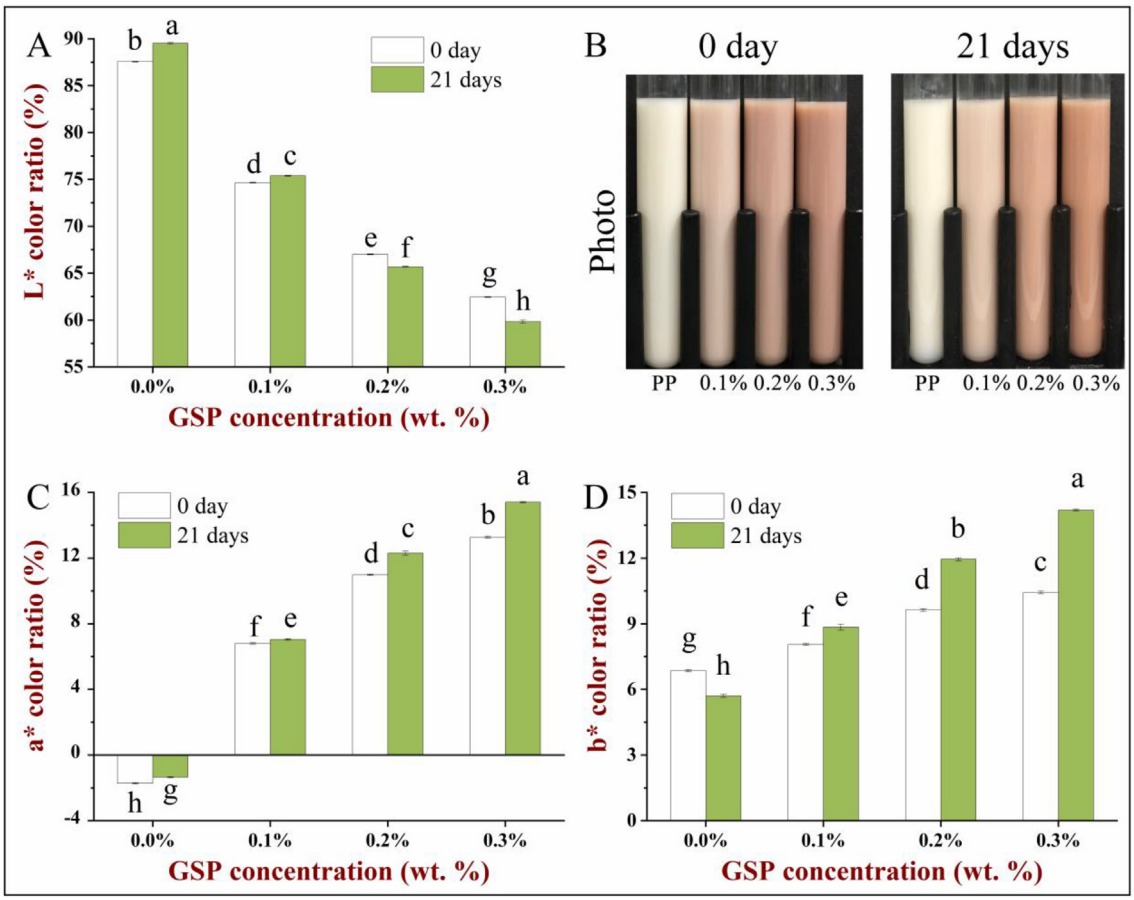

Fig. 5. The effect of GSP concentration on color including L* (A), a*(C), b*(D) of PP-GSP emulsions. The photographs (B) of PP or PP-GSP emulsions during storage time (21 days at 25 °C).

emulsions were relatively stable to aggregation from pH 6 to 9, with the mean particle diameter being around 200 nm. The ζ-potential measurements indicated that the emulsions had little net charge around pH 4, which is close to the isoelectric point of the pea proteins. As a result, there would only have been a weak electrostatic repulsion between them, which would account for their poor aggregation stability in this pH range. Interestingly, the presence of the polyphenols caused a slight decrease in the pH where a net zero charge was observed, as well as in the magnitude of the positive charge observed under acidic conditions. This effect may have been due to the binding of anionic polyphenols to cationic groups on the proteins.

3.5.2. Thermal-stability

The impact of heating (30 to 90 °C) on the particle size and charge of the PP- and PP/GSP-emulsions was measured (Fig. 6B and E). These thermal treatments had no significant influence ($p > 0.05$) on the mean particle diameter or surface potential of any of the PP-emulsions, suggesting that there the electrostatic repulsion was still strong enough to overcome any other attractive colloidal interactions. Conversely, the mean particle diameter of the PP/GSP-emulsions remained relatively small when they were heated to temperatures below 80 °C, but increased appreciably when they were heated to higher temperatures (especially 90 °C), which suggested extensive droplet aggregation had occurred. Interestingly, there was no visible evidence of creaming at this higher temperature and the ζ-potential did not depend strongly on temperature. It is possible that the presence of the polyphenols lowered the thermal denaturation temperature of the proteins, thereby causing there to be an increase in surface hydrophobicity when they unfolded (Sun, Gidley, & Warren, 2017). As a result, there was some aggregation at the highest holding temperature due to a strong hydrophobic attraction between the droplets. The fact that no creaming was observed may have been due to the creation of a 3-network of cross-linked oil droplets.

3.5.3. Salt-stability

Finally, the salt-stability of the PP- and PP/GSP-emulsions was measured (Fig. 6C). In the absence of salt, both types of emulsion were relatively stable to aggregation and phase separation. In the presence of salt, even relatively low levels (100 mM NaCl), extensive droplet aggregation ($D_{3,2} \sim 10$ μm) and creaming were observed. This result suggests that the presence of salt ions reduced the electrostatic repulsion between the emulsion droplets, thereby causing them to aggregate (Li, Peng, et al., 2019). In summary, emulsions stabilized by pea proteins alone or by pea protein–polyphenol complexes were highly unstable to salt addition.

4. Conclusion

The purpose of this study was to prepare and characterize plant-based protein–polyphenol (PP-GSP) complexes, and then to examine their ability to form and stabilize oil-in-water emulsions. The PP and GSP spontaneously assembled into colloidal particles in aqueous solutions, which was attributed to a combination of attractive hydrogen bonds and hydrophobic interactions. The colloidal particles formed were relatively small, had a high negative charge, and were resistant to sedimentation. Environmental stability tests on the colloidal particles suggested that proanthocyanidin changed the isoelectric point, decreased the thermostability, and reduced the salt-stability of the pea proteins. In emulsions, the presence of the proanthocyanidins had little impact on the pH and salt-stability, but reduced their thermal stability.

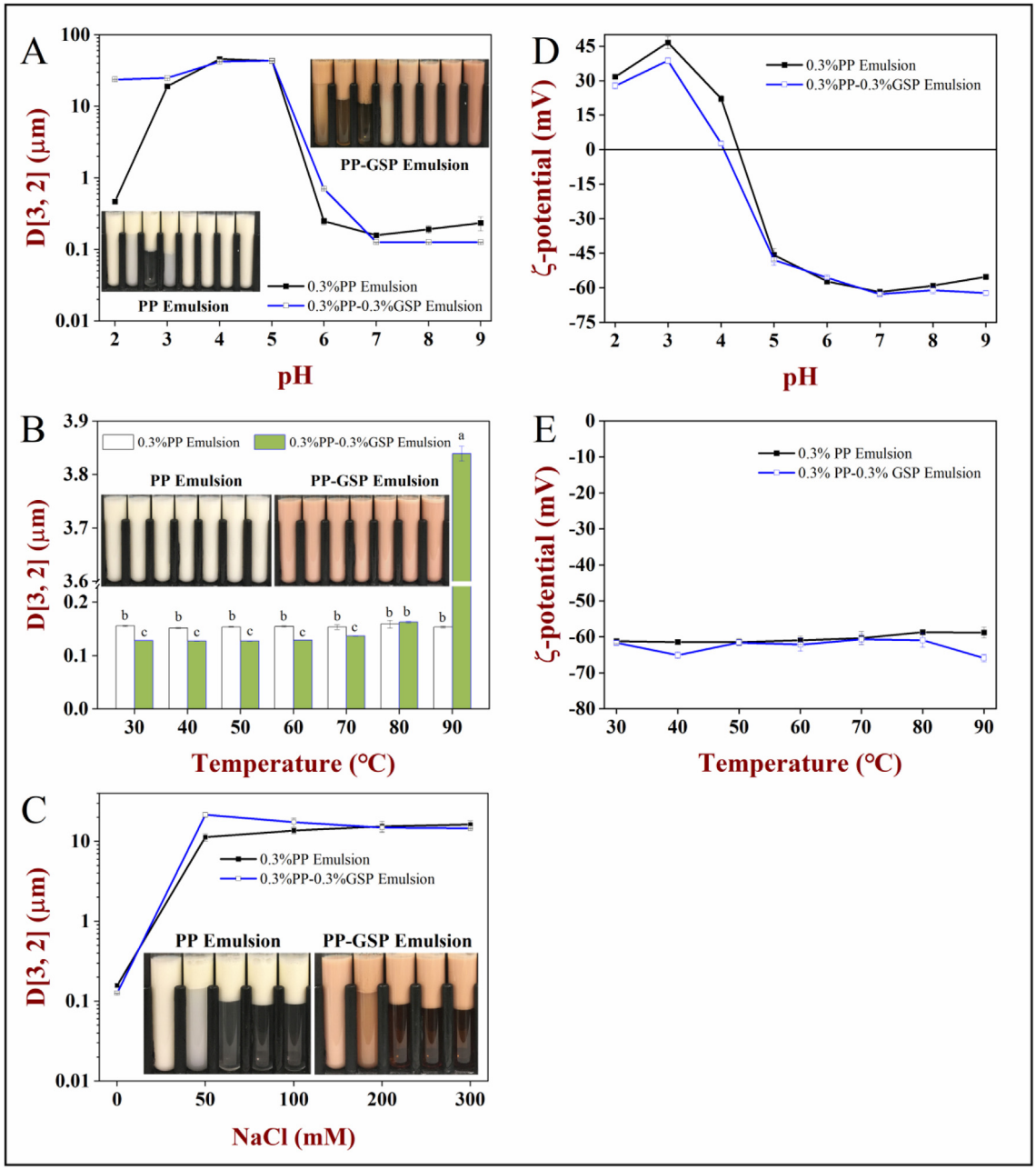

Fig. 6. The pH (A), Temperature (B), NaCl (C) effect on particle diameter of PP or PP-GSP emulsions. The pH (D) and Temperature (E) effect on zeta-potential of PP or PP-GSP emulsions.

Conversely, the proanthocyanidins improved the long-term storage stability of the emulsions. It is important to note that the incorporation of the proanthocyanidins into the emulsions caused a pronounced alteration in their appearance. In particular, they had a salmon color (red-yellow), which may be advantageous for some applications but a limitation for others. Overall, our results have important implications for the design and development of effective delivery systems for proanthocyanidins, as well as the development of plant-based food emulsions with improved physicochemical properties and stability.

CRediT authorship contribution statement

Taotao Dai: Conceptualization, Methodology, Writing - original draft. **Ti Li:** Software. **Ruyi Li:** Data curation. **Hualu Zhou:** Formal analysis. **Chengmei Liu:** Visualization, Investigation. **Jun Chen:** Supervision, Validation. **David Julian McClements:** Visualization, Writing - review & editing.

Declaration of Competing Interest

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.

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