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Stability of pea protein isolate-γ polyglutamic acid stabilised O/W emulsion: Effect of oil fraction, salt, and temperature

[1]Zhang, B. B., [1]Zhu, H. S., [1]*Xie, X. H., [1]Liang, D., [2]Feng, J., [2]Shi, Q. S., [1]Liang, C., [1]Chen, K. and [3]Ji, H. F.

[1]*College of Food Science and Technology, Henan Agricultural University, Zhengzhou, China*
[2]*Institute of Microbiology, Guangdong Academy of Sciences, Guangzhou, China*
[3]*College of Tobacco, Henan Agricultural University, Zhengzhou, China*

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Abstract

Pea protein isolate (PPI) exhibits amphipathic properties and low allergenicity. Nonetheless, the limited solubility of PPI at its isoelectric point (pI = 4.6) may hinder its efficacy in emulsion applications. Therefore, addressing this limitation of PPI is imperative. The present work thus investigated the influence of γ-polyglutamic acid (γ-PGA) on the quality of PPI-based emulsion. The results showed that as the concentration of γ-PGA increased from 0.05 to 0.50%, turbidity measurements for a PPI system within a pH range of 4.0 - 5.0 decreased from 0.79 to a minimum of 0.24, indicating that γ-PGA enhanced the hydrophilicity of PPIs, which might have been due to γ-PGA's ability to promote conformational stretching of PPIs. When oil fraction volume was increased from 1 to 3% (v/v), the emulsion particle size at pH 4.5 decreased from 2233.33 to 710.11 nm; however, further increase in oil volume resulted in larger emulsion particles. Compared with other groups with different oil volumes, the emulsion containing 3% (v/v) oil volume exhibited the lowest separation index during 21-day storage. Additionally, particle size and ζ-potential exhibited an increase with increasing NaCl concentration, indicating that the presence of ionic strength decreased the stability of the PPI-γ-PGA emulsion. Similarly, a corresponding increase in particle size was noted with increasing temperature. Consequently, the interactions between γ-PGA and PPI indicated a substantial potential for utilising γ-PGA in the stabilisation of PPI-stabilised emulsions under acidic conditions. The present work also provided fundamental theoretical insights into the application of PPI-γ-PGA emulsions across diverse environmental contexts.

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Introduction

Proteins, including whey, soybean, and egg proteins, are currently employed to stabilise oil-in-water (O/W) emulsions owing to their excellent functional properties. However, these proteins have been identified as potential allergens (Foegeding *et al*., 2002; O'Sullivan *et al*., 2016). Consequently, interest in sourcing consumer and environmentally friendly proteins as alternatives to those derived from allergenic sources is growing amongst researchers and food industries. Peas represent a major pulse crop cultivated and consumed globally (O'Sullivan *et al*., 2016). Pea protein isolate (PPI) has garnered considerable attention due to its low allergenic potential and the sustainable sourcing from crops (Chaudhary *et al*., 2018; Ding *et al*., 2020; Gao *et al*., 2020) with good emulsifying properties (Aluko *et al*., 2009; Burger and Zhang, 2019).

During emulsion formation, proteins undergo reorientation at the oil-water interface, with hydrophobic residues oriented towards the oil phase, and hydrophilic residues directed towards the water phase. Therefore, proteins exhibiting strong emulsifying properties should rapidly migrate to the oil-water interface, and adsorb onto the periphery of oil droplets, thereby inhibiting coalescence and flocculation (Karaca *et al*., 2011; Shevkani *et al*., 2015; Stone *et al*., 2015). Optimal hydrophilicity and hydrophobicity are essential prerequisites for effective emulsification of proteins (Tang *et al*., 2020). At the protein isoelectric point (pI), the surface charge of the protein approaches neutrality, resulting in inadequate electrostatic repulsion between protein

*Corresponding author.
Email: xxinhua0515@163.com

molecules and consequently facilitating aggregation (Dickinson, 2008; FreitasMírian *et al*., 2017). However, the pI of PPI is 4.6, at which the hydrophobic groups of PPI are predominantly oriented towards the water phase, posing major challenges for its application in the food industry (Boukid *et al*., 2021).

Charged soluble polysaccharides interact with proteins, thereby imparting the pronounced hydrophilicity characteristic of polysaccharides to these proteins (Tran and Rousseau, 2013). Furthermore, the spatial site-blocking effect exerted by the polysaccharide facilitates the unfolding of protein structures, thereby exposing an increased number of hydrophilic groups and charged amino acid residues (Dickinson, 2008; You *et al*., 2018). Consequently, hypothesising that charged biopolymers possess the potential to modify the microenvironment surrounding proteins, and influence their spatial conformation and functional properties is reasonable. Poly-γ-glutamic acid (γ-PGA) is polymerised from D- and L-glutamic acids, possesses anionic characteristics (pKa~2.23), and contains a substantial number of hydrogen bonds (Luo *et al*., 2016). Furthermore, a previous study demonstrated that γ-PGA could improve the solubility and emulsification activity of soybean protein isolate at its pI (Xie *et al*., 2023). Nevertheless, the interaction between γ-PGA and PPI, and the potential influence of γ-PGA on the resistance of PPIs to processing environments, remain inadequately understood. Therefore, the present work investigated the effect of biopolymer ratio (PPI: γ-PGA) on emulsion stability, and the influence of oil fraction, temperature, and salt on PPI-γ-PGA stabilised O/W emulsions. The findings may enhance the understanding of the interaction between γ-PGA and PPI, and their application in the food industry.

Materials and methods

Materials

Pea beans were purchased from the market. γ-PGA was sourced from Xian Season Biotechnology Co., Ltd. Corn oil was obtained from COFCO Fortune Food Sales & Distribution Co., Ltd.

PPI preparation

PPI was isolated following the method of Stone *et al.* (2015), with some modifications. Whole pea beans were dehulled and milled using a mill machine. Then, the milled pea flour was sieved through a 1 mm screen, and collected. Next, 100 g of defatted pea flour was weighed and dispersed in 1,500 mL of water. A NaOH solution (0.5 mol L^{-1}) was employed to adjust the pH of the cloudy solution containing the aforementioned pea flour to 9.0. After the solution was stirred (500 rpm) for 2 h at 25°C, it was centrifuged at 5,000 *g* for 20 min at 4°C, and the supernatant was taken. The pH of the supernatant was adjusted to pH 4.5 using 0.5 mol L^{-1} HCl, and then centrifuged at 5,000 *g* for 20 min at 4°C. The precipitate was collected and dispersed in deionised water, with the pH adjusted to 7.0 using NaOH solution (0.1 mol L^{-1}), followed by freeze drying for 48 h, and it was considered as PPI (protein 83.60%).

Solution preparation

Various concentrations of γ-PGA (2.00, 1.00, 0.50, 0.10, 0.07, and 0.05%, w/v) and PPI (1.00%, w/v) were mixed and dissolved in water under gentle stirring at 25°C for 2 h at a speed of 500 rpm, following the method described by Kaushik *et al*. (2015). The pH of these mixtures was adjusted to a range between 2.0 and 7.0, using HCl (2 mol L^{-1}) or NaOH (2 mol L^{-1}). Finally, solutions were prepared and coded as P1γ2, P1γ1, P1γ0.5, P1γ0.1, P1γ0.07, and P1γ0.05, with PPI and γ-PGA serving as the controls. The effects of biopolymer ratio and pH (ranging from 2.0 to 7.0) on the turbidity of solutions were then investigated.

Emulsion preparation and treatment

The pH of the P1γ0.5 solution was adjusted to 4.5 using HCl (0.1 mol L^{-1}), after which varying volumes of corn oil were added to achieve final oil phase volume fractions of 1, 3, 5, 7, 9, and 11% (v/v) in the mixture. The mixture was homogenised for 2 min, using a high-speed disperser at a speed of 20,000 rpm to form a crude emulsion, followed by high-pressure homogenisation at 40 MPa for 4 min.

Next, varying concentrations of NaCl (0, 25, 50, 75, 100, or 150 mmol L^{-1}) were incorporated into the P1γ0.5 emulsion containing a 3% (v/v) oil fraction, and stirred at 500 rpm to ensure complete dispersion.

In addition, the freshly prepared P1γ0.5 emulsion with 3% (v/v) oil fraction was heated in a water bath at 50, 60, 70, 80, or 90°C for 30 min.

Turbidimetric analysis

The turbidity of the solutions was assessed using a UV/Vis spectrophotometer as a function of pH at the optical density of 600 nm. The turbidity of the homogeneous PPI or γ-PGA system was measured as control. All experiments were conducted at 25°C, and analysed in triplicate.

Fluorescence measurement

The intrinsic fluorescence emission spectra of the emulsions were recorded in the range of 320 - 340 nm using a Lumia fluorescence spectrophotometer (Thermo, USA). The excitation wavelength was optimised at 295 nm to reduce the emission from the tyrosine residues of PPI effectively. Simultaneously, 5 nm of excitation or emission slit was set to facilitate the passage of fluorescence. The pH values of the emulsions (2.5, 3.5, 4.5, 5.5, or 6.5) were adjusted using HCl or NaOH solutions. All measurements were conducted at 25°C in triplicate.

Particle size determination

The particle size of the emulsions was determined following the method of Cheng and Jones (2017). All measurements were conducted in triplicate using an LS-13 laser diffractometer (Beckman Coulter Commercial Enterprise Co. Ltd., China) at 25°C. To ensure homogeneity throughout the testing process, the emulsions were continuously stirred at a rate of 850 rpm.

Particle charge measurement

The ζ-potential of emulsions was measured using a modified method of Fainassi *et al.* (2021). Briefly, the emulsions were equilibrated at 25°C for 120 s. Subsequently, a specific volume of emulsion was transferred into the sample chamber of a microelectrophoresis device (Nano ZS instrument). The electrophoretic mobility of emulsions was determined, from which the ζ-potential (mV) was calculated.

CLSM of emulsion

The morphology of emulsions was examined using CLSM with a Nikon A1 with an argon laser, following the method of Huang *et al.* (2020). Briefly, the oil phase of the emulsion was stained with 10 μL of Nile red solution (0.02%, w/v), whilst the protein was labelled using 10 μL of Nile blue solution (0.1%, w/v). The excitation wavelength was set at 488 nm. Subsequently, 20 μL of the dyed sample was placed on a slide for CLSM imaging.

Emulsified phase volume fraction

The total height of each formulation (Ht) was measured. The nonemulsion phase (Hs) at specific storage intervals (1, 3, 7, 14, and 21 days) was recorded following a modified method (Xiao *et al*., 2016). The emulsified phase volume fraction was calculated using Eq. 1:

$$\text{Emulsified phase volume fraction} = \left(\frac{\text{Hs}}{\text{Ht}}\right) \times 100\% \quad \text{(Eq. 1)}$$

Statistical analysis

All experiments were conducted in triplicate, and data were expressed as mean ± SD. All data were assessed by One-way analysis of variance (ANOVA) with SPSS (SPSS, Chicago, IL), and Tukey's test for *post hoc* comparisons to determine the differences between means. Significant differences between means were considered at $p < 0.05$.

Results and discussion

Effect of biopolymer mixing ratio on turbidity of PPI-γ-PGA solutions

In the present work, the impact of biopolymer ratios (PPI: γ-PGA, 1:2~1:0.05) on solution turbidity as a function of pH were measured by turbidimetric analysis.

The turbidity of the homogeneous PPI solution exhibited a pronounced increase at pH 4.5, suggesting a considerable decrease in the solubility of PPI (Figure 1A). Additionally, Figures 1A and 1B indicate that the turbidity of homogenised γ-PGA is almost zero (≤ 0.02). Nevertheless, the interaction between γ-PGA and PPI resulted in a decrease in turbidity of PPI near its pI. When the ratio of PPI to γ-PGA ranged from 1:0.05 to 1:0.5, an increase in the concentration of γ-PGA correlated with a decrease in the turbidity of the PPI solution. For instance, when the addition of γ-PGA was increased from 0% (w/v, P1γ0.0) to 0.500% (w/v, P1γ0.5), the turbidity of the solution decreased from 0.79 to 0.23. Xie *et al*. (2023) reported that γ-PGA possesses a substantial number of hydrogen bonds and negative charges. Thus, γ-PGA enhances the solubility and intermolecular electrostatic repulsion amongst soybean protein isolates whilst concurrently diminishing the

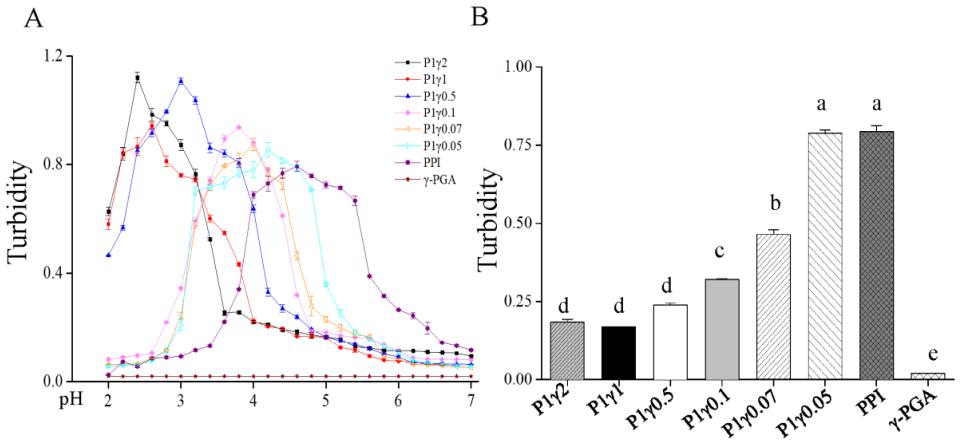

Figure 1. Effects of pH and γ-PGA concentration on turbidity of PPI solution **(A)**, and effect of γ-PGA concentration on turbidity of PPI solution at pH 4.5 **(B)**. P1γ2, P1γ1, P1γ0.5, P1γ0.1, P1γ0.07, P1γ0.05, PPI, and γ-PGA: solutions added with PPI (1.000 wt%) and 2.000, or 1.000, or 0.500, or 0.100, or 0.070, or 0.050%, w/v, γ-PGA, respectively. Different lowercase letters in Figure 1B indicate significant differences ($p < 0.05$) between solutions.

hydrophobic interactions amongst proteins. Furthermore, literature indicates that γ-PGA significantly reduces the charge content of PPI (Liang *et al.*, 2022). For the aforementioned reasons, the present work also found that increased concentrations of γ-PGA led to a decrease in turbidity levels in PPI solutions. Furthermore, the turbidity of P1γ0.5, P1γ1, and P1γ2 solutions exhibited no significant difference. These findings indicated that the solubility of PPI at its pI could not be further improved when the ratio of PPI to γ-PGA was less than 1:0.5.

Intrinsic fluorophore measurement and spectral analysis of PPI-γ-PGA solutions

Tryptophan fluorescence exhibits high sensitivity to the polarity of microenvironments along the tertiary structure transition (Viseu *et al.*, 2004). Charged polysaccharides may induce alterations in protein intermolecular hydrophobic, electrostatic, or protein-water interactions, thereby affecting the intensity and quantum yield of tryptophan fluorophores within proteins (Niu *et al.*, 2015). Therefore, analysing intrinsic tryptophan fluorophores serves as a useful method for evaluating the interactions between PPI and γ-PGA.

As illustrated in Figures 2A - 2E, the fluorescence intensity of the homogenous PPI system is higher than that of the P1γ0.5 solution at a pH range of 2.5 - 6.5. The wavelength corresponding to the maximum fluorescence intensity exhibited a slight red or blue shift (approximately 1 nm). These results suggested that the addition of γ-PGA induced conformational changes in PPI molecule. Meanwhile, when comparing the fluorescence intensity of PPI solution at its maximum wavelength at different pH levels, the fluorescence intensity of the P1γ0.5 solution at pH4.5 exhibited the most considerable decrease, indicating that the greatest conformational change in PPI molecule occurred at this pH in the presence of γ-PGA (Figure 2F). It has been proposed that electrostatic interactions between ovalbumin and gum Arabic, along with repulsive interactions amongst proteins, disrupt the environment of amino acid side chains. The addition of gum Arabic facilitates the relocation of partial tryptophan residues into a hydrophobic environment (within the proteins), and contributes to the unfolding of the protein's tertiary structure (Niu *et al.*, 2015). In the present work, the hydrophobic moieties of PPI were facing the water phase at pH 4.5, which impaired their ability to migrate and reorient at the oil-water interface (Shevkani *et al.*, 2015; Stone *et al.*, 2015); the addition of γ-PGA facilitated the encapsulation of tryptophan residues, resulting in the unfolding of the tertiary structure of PPI.

Effect of oil fraction on emulsion stability

In addition to the hydrophilicity of the protein, the volume fraction of the dispersed oil phase considerably influences the quality of the emulsion (Ho *et al.*, 2017). Therefore, the effects of oil fraction on the morphology, particle size, and ζ-potential of emulsions were investigated in the present work.

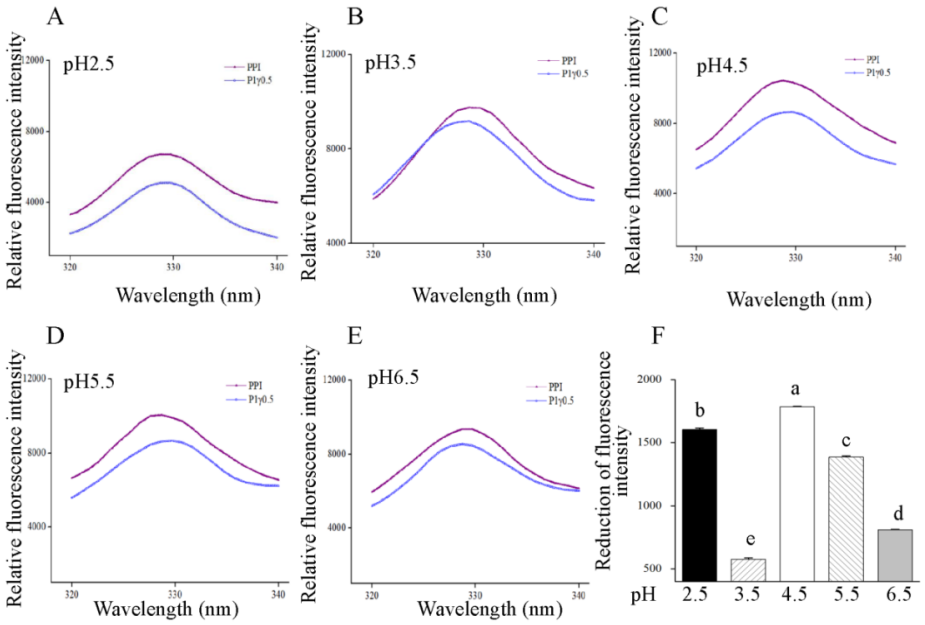

Figure 2. Effect of γ-PGA on tryptophan intrinsic fluorophores of PPI solution at pH 2.5 **(A)**, 3.5 **(B)**, 4.5 **(C)**, 5.5 **(D)**, 6.5 **(E)**, and on fluorescence intensity of PPI solution at different pHs **(F)**. P1γ0.5 and PPI: solutions added with PPI (1.000%, w/v) and 0.500 or 0.000% (w/v) of γ-PGA, respectively. Different lowercase letters in Figure 2F indicate significant differences ($p < 0.05$) between solutions.

As shown in Figure 3A, the oil phase is coloured red, PPI is in green, and the orange or light green hue indicates the mixed lipid and PPI phase. The results indicated that the P1γ0.5 emulsion containing varying volume fractions of the oil phase exhibited distinct morphological differences; for example, the emulsion with an oil phase volume fraction of 3% (v/v) appeared uniformly light green, suggesting that its lipid droplets were smaller than those in the other emulsions.

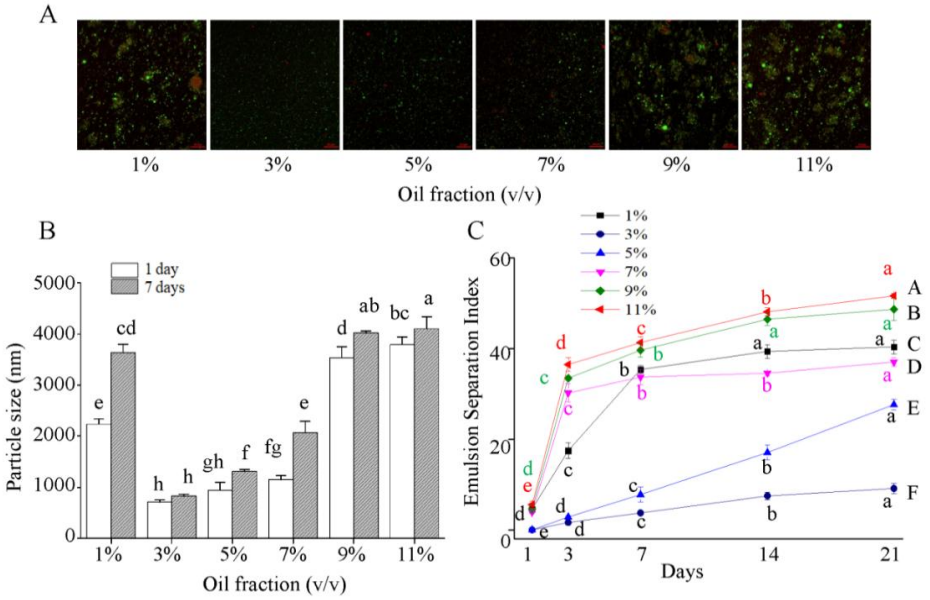

Figure 3. Effect of oil fractions (1, 3, 5, 7, 9, and 11%, v/v) on morphology **(A)**, particle size **(B)**, and emulsion separation index **(C)** of P1γ0.5 emulsion. P1γ0.5: emulsion added with PPI (1.000%, w/v) and γ-PGA (0.500%, w/v). Different lowercase letters in Figure 3B indicate significant differences ($p < 0.05$) between emulsions. Different lowercase letters in Figure 3C indicate significant differences ($p < 0.05$) in P1γ0.5 emulsion (added with fixing oil fraction) during 21-day storage. Different uppercase letters in Figure 3C indicate significant differences ($p < 0.05$) between P1γ0.5 emulsions (added with different oil fractions) after 21-day storage.

The droplet size of emulsion decreased from 2233 to 710 nm as the oil phase increased from 1 to 3% (v/v), and then increased instead as the oil fraction continued to increase (Figure 3B). The likely explanation for this phenomenon is the presence of excess non-adsorbed PPI present in emulsions with low oil content (1%, v/v) compared to those with high (3%, v/v) oil content, which leads to strong hydrophobic interactions and potential depletion flocculation (Dickinson, 1997). In addition, emulsions with 1% (v/v) oil content are not efficiently loaded with fat-soluble bioactives (Ho *et al*., 2017). However, when the oil fraction was greater than 3% (v/v), the PPI-γ-PGA complex molecules might have been insufficient to saturate the oil-water interface. Therefore, different oil droplets may share the proteinaceous material, which may lead to bridging flocculation (Dickinson, 2010; 2019). The result was different from the results of a stable soybean protein isolate (SPI, 1% w/v)-stabilised emulsion prepared from 10% plant-oil (Xie *et al*., 2023), possibly because of the different structures of PPI and SPI. Throughout the 7-day storage period, the particle size of the P1γ0.5 emulsion with a 3% (v/v) oil content consistently remained smaller than that of other emulsions. This decreased particle size may be indicative of an extended shelf life for the emulsion.

The measurements of the height of non-emulsified fraction during storage are summarised in Figure 3C. The height of non-emulsified fraction in emulsions containing 1, 7, 9, and 11% (v/v) oil fractions increased rapidly to 17.45, 30.18, 33.44, and 36.46%, respectively, following 3-day storage in comparison with emulsions containing 3% oil (v/v) fraction (1.62%). Oiling off was observed in all the emulsions containing varying oil fractions following 21-day storage. In comparison with the emulsion containing 1% (v/v) oil fraction, increasing the oil fraction to 3% (v/v) resulted in a decrease in the non-emulsified phase volume over 21-day storage, followed by an increase with further increments in the oil fraction up to 11% (Figure 3C). In conclusion, an appropriate amount of oil content (*e.g*., 3%, v/v) contributed to the stabilisation of the P1γ0.5 emulsion, and had the potential to encapsulate and stabilise fat-soluble components (Dickinson, 2019).

Effect of salt concentration on emulsion stability

The aqueous electrolyte conditions, specifically ionic strength, can alter the molecular interactions of the stabilising ingredients (Xiao *et al*., 2016). Therefore, the influence of salt concentration on the morphology, particle size, and ζ-potential of the P1γ0.5 emulsion was investigated in the present work.

As illustrated in Figure 4A, the emulsion exhibits a light green or orange hue at pH 4.5 that intensifies with increasing NaCl concentration (0 - 150 mmol L^{-1}), indicating a decrease in electrostatic interactions between PPI and γ-PGA. The particle size of the emulsion with different salt concentrations was consistent with the results obtained from CLSM analysis (Figure 4B). Concurrently, the net negative charges on the surface of PPI decreased with increasing NaCl concentrations (Figure 4C). As previously reported, increased NaCl concentrations may lead to increased Na^+ levels that decrease the electrical repulsions of sodium caseinate molecules and soy protein isolates (Dickinson, 2019), resulting in a decrease in the electrostatic repulsions between protein-stabilised emulsions. By contrast, the ionic strengths attenuated protein-protein interactions due to the presence of salt, which either induced increased resistance to flow or decreased protein mobility and flexibility, potentially leading to a less viscoelastic interfacial layer. The layer with low viscoelasticity could not resist the deformation of droplets during homogenisation or high-speed shear, then flocculation or aggregation could occur (Dickinson, 2019; Zhao *et al*., 2020). The present work contributed fundamental knowledge regarding the impact of ionic strength on P1γ0.5 emulsion stability.

Effect of temperature on emulsion stability

Heating is a critical technique employed in food processing and sterilisation, considerably influencing the interactions amongst polymer molecules. The effect of temperature (50, 60, 70, 80, and 90°C) on the stability of the P1γ0.5 emulsion was examined in the present work.

Figure 5A illustrates that the intensity of the orange or green colours increases with increasing temperature. In addition, the particle size of emulsion exhibited an increase from 696.26 to 751.33 nm as the temperature increased from 30 to 50°C (Figure 5B). The molecular motion is enhanced at elevated temperatures (Niu *et al*., 2014). The thermodynamic incompatibility of biomolecules results in the

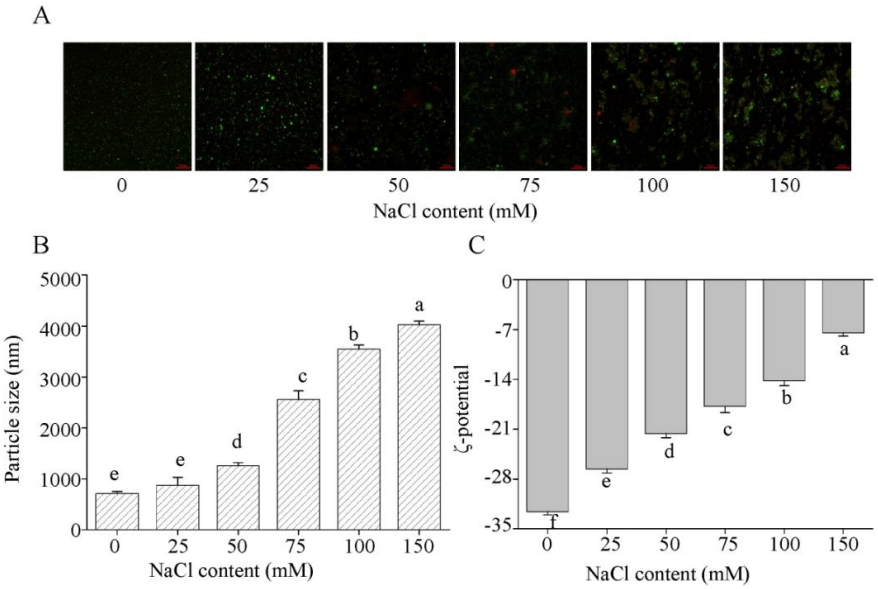

Figure 4. Effect of NaCl concentration on morphology **(A)**, particle size **(B)**, and ζ-potential **(C)** of P1γ0.5 emulsion. Different NaCl concentrations (0, 25, 50, 75, 100, or 150 mmol L^{-1}) were added into P1γ0.5 emulsion with 3% oil fraction. P1γ0.5: emulsion added with PPI (1.000%, w/v) and γ-PGA (0.500%, w/v). Different lowercase letters in Figures 4B and 4C indicate significant differences ($p < 0.05$) between emulsions (added with different NaCl concentrations).

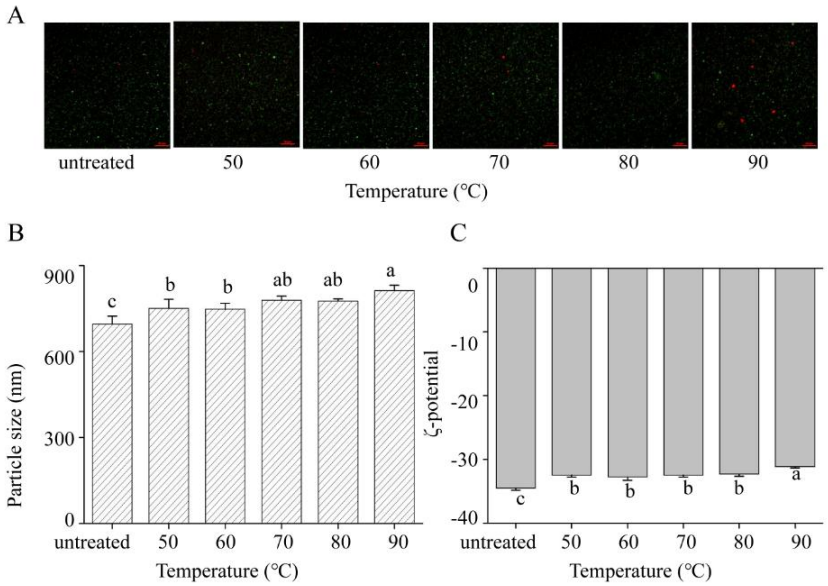

Figure 5. Effect of temperature on morphology **(A)**, particle size **(B)**, and ζ-potential **(C)** of P1γ0.5 emulsion. P1γ0.5 emulsion with 3% (v/v) oil fraction was heated in water bath at 50, 60, 70, 80, or 90°C for 30 min. P1γ0.5: emulsion added with PPI (1.000%, w/v) and γ-PGA (0.500%, w/v). Different lowercase letters in Figures 5B and 5C indicate significant differences ($p < 0.05$) between emulsions (heated in a water bath at different temperatures).

exposure of numerous hydrophobic amino acids within proteins to the aqueous phase at increased temperatures, thereby intensifying hydrophobic interactions (Tian *et al*., 2021). Consistent with our findings, the enhancement of hydrophobic interactions facilitated ovalbumin flocculation, leading to an increase in emulsion particle size with increasing temperature (Niu *et al*., 2014). The net negative charge on the PPI surface was contrary to the change trend of emulsion particle size (Figure 5C). This result indicated that the interactions amongst biological macromolecules, including hydrophobic and electrostatic interactions, were linked to particle dispersion (Dickinson, 2019).

Conclusion

When compared to whey, soybean, and egg protein, PPI was characterised by its hypoallergenic properties, and showed significant potential for application. The objective of the present work was to investigate the effect of γ-PGA on the hydrophilicity and tertiary structure of PPI, and the stability of PPI emulsions during processing and practical applications. The results suggested that both the biopolymer mixing ratio (PPI:γ-PGA) and pH considerably influenced the hydrophilicity of PPI, with turbidity reaching its maximum at a pH around 4.6. Furthermore, γ-PGA facilitated the folding of hydrophobic residues, such as tryptophan, into the interior of the protein, thereby enhancing PPI's hydrophilicity. Additionally, an optimal oil concentration (*e.g.*, 3%, v/v) proved crucial for emulsion stability; emulsions containing 1, 5, 7, 9, and 11% (v/v) oil fraction exhibited remarkably larger particle sizes and increased non-emulsified fraction over storage time. Analysis of particle size and ξ-potential revealed that emulsion stability decreased with increasing NaCl concentration. Moreover, emulsion stability decreased with a gradual increase in temperature. This understanding of interactions between PPI and γ-PGA can be leveraged to enhance specific functionalities of PPI in acidic environments, and facilitate their future applications.

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