Co-assembled whey protein and proanthocyanidins as promising biocarrier for hydrophobic pterostilbene: Fabrication, characterization and cellular antioxidant potential

Weigang Zhong, Qi Wang, Min Li, Xuming Deng,* and Xue Shen* 

aDepartment of Food Science, College of Food Science and Engineering, Jilin University, Changchun, Jilin, 130062, China 
bKey Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun, 130062, China

ABSTRACT

The usage of food-derived polyphenols with different polarities has been limited by their instability and incompatibility. Therefore, a biocarrier was developed by co-assembly of whey protein isolate (WPI) and hydrophilic proanthocyanidin (PC) for loading hydrophobic pterostilbene (PTE). Such biocarrier has superior affinity for PTE than WPI alone as determined by encapsulation efficiency (EE) and loading capacity (LC) assay, fluorescence quenching analysis and molecular docking, while assembly process was characterized by particle size and zeta-potential, 3D fluorescence and SEM. CD and FTIR spectra confirmed the α-helix to β-sheet and random coil transition of proteins during the nanocomplexes formation. WPI acted as a mediator through altering the binding mode of PC and PTE, allowing them to perform significant synergistic effects in enhancing ABTS and DPPH radical scavenging and reducing H$_2$O$_2$-induced cell damage. This research may serve to develop new protein/polyphenol co-loading systems and offer a reliable nutritional fortification.

Key words: Whey protein, Co-assembly, Proanthocyanidins, Pterostilbene, Nanocomplexes

INTRODUCTION

Polyphenols, naturally found largely in fruits, cereals, tea, coffee, and beverages, are gaining much attention due to their health-promoting effects and potentials to prevent several human diseases (Silva et al., 2020). In particular, proanthocyanidins and pterostilbene, both derived from berries like grapes and lingonberries, are typical polyphenols with different polarities that attracted great interest as nutraceutical agents in the food, supplement, and pharmaceutical industries owing to their favorable bioactivity (Koh et al., 2021; Hou and Wang, 2022). Proanthocyanidins (PC), also known as procyanidins and condensed tannins, possess a variety of biological activities such as antioxidant, antithrombotic, anti-inflammatory and potential as natural food pigment (de Mejia et al., 2020). Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene, PTE) is a hydrophobic natural stilbene compound and dimethyl ether analog of resveratrol (Huang et al., 2021) that has been extensively studied for its antioxidant, anti-aging effects, cardioprotective and neuroprotective properties (Penalva et al., 2015). Unlike resveratrol, the hydroxyl and dimethyl ether structure of pterostilbene ensures higher bioavailability and more optimal metabolic stability (Wang and Sang, 2018), which renders pterostilbene a new alternative to resveratrol as a nutraceutical, functional food or treatment for diseases.

Although both PC and PTE may exhibit potential health benefits when consumed at sufficiently high levels, they are difficult to use directly in food because of their susceptibility to some environmental conditions (e.g., Light, heating, oxygen and extreme pH values), coexistence in a single system, palatability (astringent taste for PC) and low bioavailability (Liu et al., 2017; Nagarajan et al., 2022). Protein/polyphenol complexes are favored due to its easy to prepare, enhanced polyphenolic stability and antioxidant activity, simultaneous delivering hydrophilic and hydrophobic polyphenols, and improved protein functionality and food flavor (Lila et al., 2022). This strategy may make it possible to produce additive-free food ingredient with healthy dietary proteins and concentrated polyphenols, often known as “clean label” or “green product,” to satisfy public demand for natural and healthy food. Whey proteins isolate (WPI), consisting mainly of 50–70% β-lactoglobulin (18.4 kDa), 20% α-lactalbumin (14.2 kDa) and 5% bovine serum albumin (66 kDa) (Meng and Li, 2021), appear to be excellent amphiphilic biological carriers for the protection and delivery of several bioactive substances. Its advantages not only in terms of biodegradability and biocompatibility as carriers of...
active substances (Farooq et al., 2019), but also in its high nutritional values and unique properties as food ingredients (Zhao et al., 2022). The incorporation of polyphenols with various polarity into whey proteins to create multi-bioactives and protein complexes can overcome these applications' limitations to enhance their stability and efficacy in food products and boost their bioavailability (Kong et al., 2022). On the one hand, the complexes produced by self-assembly of proteins with PC can reduce PC astringency, improve its environmental stability and lead to a sustained release of PC (Liu et al., 2017, 2019; Olatujoye et al., 2020). On the other hand, their binding has been shown to improve functional properties of proteins such as emulsification, foaming and digestive properties (Ozdal et al., 2013; Li et al., 2021). Furthermore, PC has been reported to lead to the transition of the secondary structure of proteins from ordered to disordered (Zhang et al., 2022), thereby leading to the exposure of the internal hydrophobic structural domains, which created favorable conditions for the loading of other active substances. However, the use of PC-induced protein complexes as biocarrier for loading other active substances has not been carried out.

In this study, PC plays a dual role including susceptible hydrophilic bioactive as well as unique cross-linking agent to co-assemble with the whey proteins. We hypothesized that WPI co-assembled with PC can alter the structure and encapsulate lipophilic nutraceuticals, such as PTE, thereby endowing whey protein particles loaded with polyphenols with different polarities. This study systematically investigated the mechanism of the formation of nanocomplexes between WPI and PC and serving as a biocarrier for the loading of PTE. Further, the biocompatibility and protection of these complexes against H2O2-induced oxidative damage in Caco-2 cells were also assessed. The WPI/PC-PTE system developed in this study could provide unique insights into the establishment of protein/multipolar polyphenol co-loading systems and contribute to food, beverage and biomedical applications.

MATERIALS AND METHODS

Materials

Whey protein isolate (purity of 93.14%) was obtained from Fonterra Co-operative Group (Auckland, New Zealand). Proanthocyanidins (PC, from grape seeds, purity of 95%, 3.5 mean polymerization degree), pterostilbene (PTE, purity of 97%) and Cellulosic dialysis membranes (cut off 3500 Da) were purchased from YuanYe Bio-Technology Co., Ltd. (Shanghai, China). Phosphate buffer at pH 7 (PB, 10 mM) were obtained from Coolaber Science & Technology (Beijing, China). Potassium bromide (KBr) was purchased from Sigma-Aldrich (St. Louis, MO, USA). High-glucose and L-glutamine Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Co. (Waltham, MA, U.S.A.). All other chemicals used were of reagent grade and purchased through Sigma (St. Louis, MO, U.S.A.).

Preparation of WPI/PC biocarrier and WPI/PC-PTE nanocomplexes

The WPI solution (1 mg/mL, wt/vol) was prepared from dissolving WPI powder in phosphate buffer solution (PB, 10 mM, pH 7.4) at room temperature under magnetic stirring (700 rpm) for 2 h and stored overnight at 4 °C to complete the hydration. The apparent molecular weight of WPI was assumed to be 20 kDa, which was estimated from the weighted average of the constituent proteins of WPI. Therefore the molar concentration of the WPI solution in this assay was about 50 µM (Meng and Li, 2021). Subsequently, varying amounts of PC were added to the WPI solution and fully stirred in the dark for 2h to reach concentrations of 0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5 and 100 µM, referring to the method of Li et al. (Li et al., 2021) with some modifications.

For the WPI/PC-PTE complexes preparation, the WPI/PC solution was quantitative fabricated by adding 62.5 µM PC to the WPI solution based on the results of Section 3.1.1 and the PTE solution (10 mM) was developed with anhydrous ethanol. Similarly, WPI/PC solution were titrate with different concentrations of PTE ethanol solution in dark to obtain ternary complexes with a PTE concentration of 0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5 and 100 µM, then ethanol was then removed using a vacuum rotary evaporator at a vacuum at 35 °C for 10 min. The ethanol concentration of the solution was less than 1% to prevent denaturation of the protein. Additionally, solutions of WPI alone with the same concentration range of PTE were also prepared to compare the contribution of PC to PTE loading.

According to the results of Section 3.1.1, WPI/PC-PTE and WPI/PTE complexes prepared from 75 mM PTE were selected for subsequent measurements. In addition, equal amounts of PC, PTE as well as PC/PTE were used as comparisons for the antioxidant sections.
Encapsulation efficiency (EE) and loading capacity (LC)

The determination of EE and LC of WPI to PC was based on the method of Li et al. (Li et al., 2020) with slight modifications. Briefly, binary complexes at different PC concentrations were dialyzed through dialysis bags (cut off 3500 Da) for 48 h and sodium azide (0.02%, wt/wt) was added to prevent sample spoilage. The double-distilled water for dialysis was changed every 6 h. Afterward, the PC content bound to the proteins was measured by a microplate reader (Synergy HT, BioTek, USA) and developed a standard curve of PC.

The EE and LC of WPI/PC complexes loaded with different concentrations PTE was determined by the method of Zhan et al. (Li et al., 2020) with some modifications. In short, all dispersions were centrifuged at 14,000 g to remove excess PTE and added with anhydrous ethanol to reach the ethanol concentration of 80% (vol/vol) and fully vortexed to extract PTE in complexes. Then they were centrifuged at 4000 g for 10 min to remove any precipitates. The supernatant was diluted with anhydrous ethanol to the appropriate concentration and the absorbance at the wavelength of 317 nm was recorded using a microplate reader (Synergy HT, BioTek, USA). The amount of PC and PTE was quantified with a standard curve of both. EE and LC were calculated according to the following equations:

\[
EE (\%) = \frac{\text{Encapsulated Polyphenol (mg)}}{\text{Added Polyphenol (mg)}} \times 100\% ,
\]

\[
LC (\%) = \frac{\text{Encapsulated Polyphenol (mg)}}{\text{Protein (mg)}} \times 100\% .
\]

Fluorescence spectroscopy

The changes of intrinsic fluorescence intensity of samples were performed with a fluorescence spectrophotometer (RF-5301 PC, Shimadzu, Japan). Excitation wavelength was set at 280 nm and emission was recorded in range of 300–450 nm with a fixed slit width of 5 nm. All samples were measured at 298 K, 304 K and 310 K. The internal filter effect should be considered since the compounds have absorption near the excitation and emission wavelengths. Thus, all fluorescence values are corrected by the following equation:

\[
F_{\text{cor}} = F_{\text{obs}} \times 10^{(A_{\text{ex}} + A_{\text{em}})/2},
\]

where \(F_{\text{cor}}\) and \(F_{\text{obs}}\) represent the corrected and measured fluorescence intensities respectively; while \(A_{\text{ex}}\) and \(A_{\text{em}}\) denote the absorbance of the binary and ternary complexes at the excitation and emission wavelengths respectively.

Fluorescence quenching mechanism and binding constant

The Stern-Volmer equation were employed to determined the origin of the quenching mechanism:

\[
\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{SV} [Q],
\]

where \(F_0\) and \(F\) was the fluorescence intensity of protein or protein/ligand (in the case of single or double ligand complexes) before and after quenching by different concentrations of additives. \([Q]\) denotes to the quencher (PC or PTE) concentration. \(K_{SV}\) and \(K_q\) respectively represents the Stern-Volmer quenching constant and the biomolecular quenching rate constant and \(\tau_0\) (10⁻⁸ s) is the average lifetime of protein.

To further understand the interaction between protein or protein/ligand (in the case of single or double ligand complexes) and additives (PC or PTE) after the determination of the static quenching mechanism, the number of binding sites (n) and the binding constant (\(K_a\)) were obtained by the double logarithmic equation:

\[
\log \left( \frac{F_0 - F}{F} \right) = n \log [Q] + \log K_a.
\]

Thermodynamic parameters

The driving force for protein/ligand binding was identified by the thermodynamic parameters calculated from the van’t Hoff equation and the Gibbs function:

\[
\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R},
\]

\[
\Delta G = \Delta H - T\Delta S,
\]

where \(\Delta H\) and \(\Delta S\) are the binding enthalpy and entropy; \(\Delta G\) is the Gibbs free energy; \(T\) is the absolute temperature (K); \(K_a\) is the calculated binding constant at different temperatures; and \(R\) is the gas constant (8.314 J·mol⁻¹·K⁻¹).
Three-dimensional (3D) fluorescence spectroscopy

The samples measured were further diluted to 0.05 mg/ml to reduce the intensity of the Rayleigh scattering peak. The excitation wavelength was set as 220–350 nm, the emission wavelength was 220–450 nm. The other parameters are the same as the parameters described at the beginning of Section 2.6.

Particle size and zeta potential measurements

Particle size, polydispersity index (PDI) and zeta potential of samples were recorded at a backscatter detection angle of 173° using a Malvern Nano Zetasizer (Zetasizer Nano ZS90, Malvern Instruments Ltd., Worcestershire, UK). Each results was obtained from the average of at least triplicate measurements.

Far-UV Circular Dichroism (CD) and fourier transform infrared (FT-IR) spectroscopy

All samples was loading to a quartz cell with a 0.1 cm path length and scanned in a wavelength range of 190 to 260 nm in Far-UV region using a Spectropolarimeter (MOS-500, Bio-logic, Seyssinet-Pariset, France). Spectral data analysis via online Dichroweb to obtain further details of the secondary structure of the samples. FT-IR measurements were performed on an IRPRESTIGE-2 FTIR spectrometer (Shimadzu, Tokyo, Japan). First, 2.0 mg of sample was mixed with 198.0 mg of pure KBr powder and pressed into tablets. FTIR spectra were recorded from 400 cm⁻¹ to 4000 cm⁻¹ at a resolution of 4 cm⁻¹. The amide I band (1620–1700 cm⁻¹) was extracted to calculated the secondary structures by the Gaussian function using OriginPro 2022 software (Meng and Li, 2021).

Scanned Electron Microscope (SEM)

Lyophilized samples were attached to a conductive carbon tape and sprayed with a layer of gold metal under high vacuum, The micromorphology of the samples was analyzed using SEM (S-4800, Hitachi, Japan) at 3 kV.

Molecular docking studies

Crystal structure of bovine α-LA (PDB:1F6S) and β-LG (PDB:3NPO) was obtained from Research Collaboratory for Structural Bioinformatics (http://www.rcsb.org). The structures of procyanidine (CID:107876) and pterostilbene (CID:5281727) used for docking were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov) and optimized. Simana was employed for molecular docking in this study, and the docking results were analyzed with Schrödinger and LigPlus software. The proteins was first docked with proanthocyanidin, then the docked Binary complexes and the pure protein (served as a control) was docked with pterostilbene as a receptor. The xyz axis coordinates of α-LA and β-LG center were (37.17, 53.11, 41.02) and (24.38, 37.08, 33.58). The exhaustiveness was set to 80, and 20 conformations were generated at a time.

Free radical scavenging ability

DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) methods are both scavenging activity-based antioxidant assays and involve a mixed mode of hydrogen atom transfer (HAT)/single electron transfer (SET) in the reaction process. However, DPPH focuses more on the SET mechanism, whereas ABTS is inclined to HAT. The differences in scavenging mechanisms lead to the fact that the 2 usually need to be assayed jointly (Masters et al., 2020). Briefly, ABTS (Liu et al., 2022) and DPPH (Parolia et al., 2022) work solutions were prepared exactly following the previous study. For ABTS investigation, samples (50 µL) were mixed with the working solution (150 µL) and incubated for 7 min at ambient temperature in a dark environment, PB was used as a control. Afterward, the absorbance of the sample at 734 nm was measured by a microplate reader (Synergy HT, BioTek, USA). DPPH solution were fully mixed with samples in equal volume, then samples were rested for 30 min at room temperature in dark and anhydrous ethanol was used as control and the absorbance of the sample was recorded at 517 nm. The free radical scavenging capacity was derived from the following formula:

.Scavenging capacity (%) = \left(1 - \frac{A_1}{A_0}\right) \times 100% 

Where A₁ and A₀ are the absorbance of the samples and control at a specific wavelength.

Cellular antioxidant activity

Cytotoxicity assay. Cytotoxicity of samples toward Caco-2 cells were performed using the well-established MTT assay. The Caco-2 cells were incubated in DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin at 37°C and 5% CO₂. The cells were seeded in 96-well plates with a density of 2.0 × 10⁴ cells/well and cultured for 48 h. Then, the cells were treated with the samples (20 µL) diluted in DMEM
for 12 h. DMEM was used as a control. Afterward, 200 µL of MTT was added to each well for 4 h. The UV absorbance at 570 nm was recorded by a microplate reader (Synergy HT, BioTek, U.S.A.) to calculate the cell viability.

Cytoprotective effects. The cellular antioxidant activity was determined according to a method described by Li et al. with some modifications (Li et al., 2022). First, the oxidative damage of Caco-2 cells induced by H₂O₂ (0.1–1 mM) were performed. The cytoprotective effects of protein-polyphenol samples on the H₂O₂ treated Caco-2 cells were then evaluated. The cells were seeded in 96-well plates with a density of 2.0 × 10⁴ cells/well and cultured for 48 h. After pre-incubation with samples and damage by H₂O₂ as mentioned above, the cells were gently washed by HBSS. After incubation with 25 µM DCFH-DA, the fluorescence intensity was measured at an emission wavelength of 525 nm and an excitation wavelength of 488 nm. The ROS intensity was measured at an emission wavelength of 525 nm and an excitation wavelength of 488 nm. The ROS levels were calculated by comparing the fluorescence intensity of damage group with or without sample pre-incubation to that of blank control group.

Determination of intracellular ROS. The cells were seeded in 96-well black plates with a density of 2.0 × 10⁴ cells/well and cultured for 48 h. After pre-incubation with samples and damage by H₂O₂ as mentioned above, the cells were gently washed by HBSS. After incubation with 25 µM DCFH-DA, the fluorescence intensity was measured at an emission wavelength of 525 nm and an excitation wavelength of 488 nm. The ROS levels were calculated by comparing the fluorescence intensity of damage group with or without sample pre-incubation to that of blank control group.

Determination of enzyme activity. The enzyme activities of SOD, CAT, and GSH-Px were determined according to a method described by Li et al. with some modifications (Li et al., 2022). Briefly, Caco-2 cells were seeded in 6-well plates at a density of 5 × 10⁵ cells/well and cultured for 48 h. The cells were treated with samples and subjected to H₂O₂ (400 µM) for another 1 h to induce oxidative stress. The cells were lysed and centrifuged at 10,000 g, 4 °C for 5 min. The supernatant was collected to determine superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) enzymatic activities using colorimetric kits (Beyotime Biotech, Shanghai, China) according to the manufacturer’s procedure.

Cellular absorption. Caco-2 cells were first inoculated at a density of 2 × 10⁵ cells per well in 6-well culture plates (Corning, NY) and incubated overnight to adhere to the culture plates. Fluorescent labeling of the WPI/PC-PTE nanocomplexes was performed by adding 20 µL Rhodamine B (0.5 mg/mL) to 1 mL nanocomplexes and incubating for 1 h, and the mixture was centrifuged at 12,000 g and refreshed the sample supernatant to remove excess rhodamine B. Then, 20 µL stained WPI/PC-PTE nanocomplexes were added into each well. After 2 h of incubation, cultures were aspirated and then cells were gently rinsed 3 times with PBS to remove unabsorbed nanocomplexes. Finally, treated Cells were imaged under both bright field and fluorescence at 20 × by a fluorescence microscope (IX-83P2ZF, Olympus).

Statistical analysis.
Experiments were carried out 3 times and data were expressed as mean ± standard deviation. Statistical significance (P < 0.05) was determined using SPSS 21. Least squared difference (LSD) or Dunnett’s C was employed for difference test between means according to the homogeneity of variance.

RESULTS AND DISCUSSION

Fabrication of WPI/PC biocarrier and WPI/PC-PTE nanocomplexes
EE and LC. Encapsulation efficiency (EE) and loading capacity (LC) are of practical importance for the application of complexes in nutrient delivery systems. WPI spiked with different PC concentrations were dialyzed to remove unreacted polyphenols, and then the total phenolic content of the samples was measured to determine the variation of EE and LC as a function of PC addition (Figure 1A). The EE of WPI were all higher than 50% when PC concentration increased from 12.5 to 75 µM, while the LC increased as PC accumulating on the protein and stabilized close to a PC of 62.5 µM with a LC value of 20%, indicating that the LC loading capacity of the WPI approached saturation. Therefore, 62.5 µM of PC were selected for forming WPI/PC biocarriers to study their binding behavior to PTE (molar ratio of PC to WPI was approximately 1.25), while WPI alone was used as a control to determine the contribution of PC to PTE loading. As shown in Figure 1B, there was no significant difference between the EE of WPI/PC biocarriers and WPI alone at low PTE concentrations (<62.5 µM) while the LC increased rapidly, which may be due to the fact that both were able to fully bind to PTE. The WPI/PC biocarriers then showed significantly higher EE and LC than WPI, evidencing the higher affinity for PTE, and notably, the EE and LC of both stabilized after PTE concentrations above 75 µM, which indicated that the PTE loading had reached saturation.
**Fluorescence spectroscopy analysis.**

Conformational analysis and quenching mechanism The fluorescence assay was used to investigate conformational changes in protein upon addition of phenolic compounds. The fluorescence spectra of WPI with various concentrations of PC (0–100 µM) are shown in **Figure 2A**. WPI alone showed a strong fluorescence emission peak located at about 333 nm due to the presence of aromatic amino acid residues such as Trp, Tyr, and Phe (Razzak and Cho, 2022). Upon increasing the PC concentration from 0 to 100 µM, a dose-dependent decrease in WPI fluorescence intensity was observed, while the maximum peak of WPI was shifted to 330 nm. This result indicated that PC could quench the intrinsic fluorescence of WPI resulting from molecular interactions between PC and protein leading to the fluorophore residues in a more hydrophobic environment (Lu et al., 2022). The fluorescence spectra of WPI/PC biocarriers and WPI alone with addition of different concentrations of PTE (0–100 µM) were plotted in the **Figure 2D and 2E**. The natural PTE appeared a sudden characteristic peak at 380 nm after excitation at 280 nm, while the fluorescence intensity was negligible until 350 nm (Liu et al., 2020), which provided an opportunity to employ fluorescence spectroscopy to analyze the binding of PTE to WPI/PC biocarriers. Similarly, the fluorescence intensity of both decreased in a dose-dependent manner with increasing PTE concentration (0–100 µM), in contrast to the addition of PC, the PTE addition shifted the maximum peak values of WPI/PC biocarriers and WPI alone from 330 nm to 332 nm and 333 nm to 335 nm, respectively, which suggested that PTE was instead bound to the hydrophobic region of both, thereby increasing the surrounding hydrophilicity.

**Binding parameters and thermodynamic analysis** Typically, fluorescence quenching can be distinguished between dynamic quenching from intermolecular collisions and static quenching from intermolecular binding once the effects of internal filtering have been excluded (Liu et al., 2020). To clarify the potential quenching mechanisms of WPI/PC, WPI-PTE and WPI/PC-PTE interactions, the Stern-Volmer equation were employed to analyze the fluorescence data at 298, 304, and 310 K (**Figure 2B and 2F**). All the plots showed good linear curves (R² > 0.9) at tested temperatures. Besides, the minimum quenching rate constants Kq for WPI/PC, WPI/PTE, and WPI/PC-PTE interactions were much higher than the maximal dynamic quenching constant (2.0 × 10^10 L·M⁻¹·S⁻¹) at tested temperatures (**Table 1**), which suggested that static quenching mechanism involved in the binding process between whey proteins and PC and/or PTE interactions, and stable complexes were formed during this reaction.

The double logarithmic equation was applied to calculate the apparent binding constants (Kₐ) and the number of available binding sites (n) at the 3 temperatures through data treatment (**Figure 2C and G**). The number of binding sites (n) of WPI was approximately 1.3 (298 K) for PC, indicating that WPI/PC complexes were formed with a molar ratio of 1:1.3, which agreed with the results of EE and LC. The relevant binding parameters of WPI/PC complexes were then further compared with those of WPI alone. Interestingly, the WPI/PC complexes (1.68 and 6.58 × 10⁷ L/mol) had a higher binding constant for PTE as well as a higher
number of binding sites than WPI alone (1.45 and 9.65 × 10^6 L/mol) at 298 K, thus it can be assumed that the presence of PC facilitated the binding of PTE in some way. One possibility, combined with the conformational analysis, is that the surface microenvironment of WPI became more hydrophobic upon binding of PC, and another is that PC itself may contribute to the binding of PTE. In addition, the high Ka (10^6-10^7) values of the complexes were believed to allow the complexes to remain stable during storage and transport (Razzak et al., 2019).

The thermodynamic parameters (ΔH, ΔS, and ΔG) were obtained based on the results of fluorescence quenching at 298 K, 304 K, and 310 K (Table 1). In general, 4 types of non-covalent interactions including hydrogen bonding, hydrophobic interactions, electrostatic forces and van der Waals interactions are responsible for the complexation between small molecules and proteins. Insights into the origin of the forces involved can be obtained by determining the signs of the ΔH and ΔS. As calculated by the Van't Hoff equation, both ΔH and ΔS exhibited negative values in binding of PC and/or PTE to WPI, which confirmed that hydrogen bonding or van der Waals forces played a major role in the binding process (Qie et al., 2020). Moreover, all the protein-polyphenol interactions had negative ΔG values that decreased with increasing temperature, evidencing that the non-covalent binding was spontaneous and exothermic since lower temperatures favored their binding (Ren et al., 2022). Similar binding approaches have been found in many protein-polyphenol combinations such as glabridin/human serum albumin (Razzak et al., 2019).

**Table 1** Fluorescence quenching parameters (Kₐ, Kₐ and n) and related thermodynamic parameters (ΔH, ΔS and ΔG) of WPI/PC, WPI/PTE and WPI/PC-PTE nanocomplexes

<table>
<thead>
<tr>
<th>T (K)</th>
<th>Kₐ (10¹² L/mol)</th>
<th>Kₐ (10¹⁴ L/mol)</th>
<th>n</th>
<th>ΔH (KJ/mol)</th>
<th>ΔS (KJ·mol⁻¹·K⁻¹)</th>
<th>ΔG (KJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPI/PC</td>
<td>298</td>
<td>0.83 ± 0.03</td>
<td>14.32 ± 2.80</td>
<td>1.32 ± 0.02</td>
<td>-187.59 ± 15.89</td>
<td>-0.53 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>304</td>
<td>1.22 ± 0.01</td>
<td>1.04 ± 0.22</td>
<td>0.99 ± 0.02</td>
<td>-25.26 ± 0.17</td>
<td>-22.06 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>310</td>
<td>1.29 ± 0.02</td>
<td>0.78 ± 0.18</td>
<td>0.95 ± 0.02</td>
<td>-22.06 ± 0.44</td>
<td></td>
</tr>
<tr>
<td>WPI-PTE</td>
<td>298</td>
<td>1.79 ± 0.17</td>
<td>9.65 ± 3.73</td>
<td>1.45 ± 0.04</td>
<td>-47.87 ± 6.56</td>
<td>-0.03 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>304</td>
<td>1.64 ± 0.06</td>
<td>6.92 ± 0.73</td>
<td>1.42 ± 0.03</td>
<td>-39.89 ± 0.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>310</td>
<td>1.45 ± 0.02</td>
<td>5.13 ± 0.84</td>
<td>1.41 ± 0.02</td>
<td>-39.89 ± 0.61</td>
<td></td>
</tr>
<tr>
<td>WPI/PC-PTE</td>
<td>298</td>
<td>1.40 ± 0.07</td>
<td>6.53 ± 0.51</td>
<td>1.68 ± 0.01</td>
<td>-118.94 ± 0.26</td>
<td>-0.26 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>304</td>
<td>1.01 ± 0.01</td>
<td>0.86 ± 0.14</td>
<td>1.51 ± 0.01</td>
<td>-37.27 ± 0.28</td>
<td></td>
</tr>
</tbody>
</table>
et al., 2019). Notably, the binding of WPI to PTE was hardly affected by temperature, whereas the binding of WPI to PC and the binding of the WPI/PC biocarrier to PTE was more pronounced upon changes in temperature. Higher temperatures weakened the binding of PC to WPI and thus affected the loading of PTE, which laterally supported the hypothesis that PTE may bind in the vicinity of PC.

**Molecular docking analysis**

α-LA and β-LG as the main components of WPI were used for molecular docking assays (Figure 3). Earlier studies have determined that the main binding site of α-LA is located in the cavity at the top of its surface, which mainly includes: α-helix H2 (His32 and Thr33), chains S1 and S2 (Val42, Asn44 and Glu49), aromatic cluster II (Phe53, Gln54 and Trp104) and flexible loops (Ala106 and Leu110) (Chrysina et al., 2000) (Figure 3A). The docking results demonstrated that PC has high affinity for α-LA (−9.0 kcal/mol), which formed hydrogen bonds with His32, Glu49, Leu105, and Tyr103, respectively, and was affected by hydrophobic forces of Thr33, Val42, Phe53, Gln54, Trp104, Ala106, and Ala109. Among them, Leu105 was thought to form amide-π stacked bonds with PC thereby reducing the fluorescence intensity (Figure 3B). The binding posture of α-LA/PC versus α-LA alone to PTE was then further compared, PTE bound to the PC-loaded α-LA via hydrogen bonding (Asn44 and Glu49) as well as hydrophobic interaction (Val42) (Figure 3C), suggesting that PTE was located near the S2 chain and adjacent to the PC (Unl1). In contrast, for α-LA alone, PTE bound near α-helix h2 (Figure 3D). This confirmed that the presence of PC altered the binding site of PTE and increased the PTE affinity score from -5.6 kcal/mol to -6.0 kcal/mol. Previous studies have reported the common binding sites for β-LG include: the interior of β-barrel (calyx), the hydrophobic region in the groove between α-helix and β-barrel, and the external surface at Trp19-Arg124 (Roufik et al., 2006). As shown in the Figure 3F, PC bound to the outer surface of β-LG (Trp19-Arg124) by hydrophobic interaction and forming hydrogen bonds with Thr18, Thr19, Val43, Glu44, and Glu157, achieving an higher affinity than α-LA (−10.3 kcal/mol) (Figure 3G). Subsequent addition of PTE similarly bound to β-LG via hydrophobic interaction (Thr18). Interestingly, this process involved the formation of a hydrogen bond between PC and PTE (Unk0) (Figure 3H). Similarly, the β-LG/PTE docking was also performed to compare the PTE binding posture (Figure 3I). PTE alone bound above the β-barrel (calyx) and forming hydrogen bonds with Lys69, Asn109, Ser116 (Figure 3J). The distinction in docking posture similarly allowed the PC-loaded β-LG to possess a higher affinity for PTE (−6.7 kcal/mol) than β-LG alone (−6.6 kcal/mol).

Overall, hydrophobic interaction forces and hydrogen bonding play an important role in PC binding, which was consistent with thermodynamic analysis. The presence of PC enhanced the affinity of the protein for PTE by both altering the binding posture of PTE to protein and by providing hydrogen bonds to PTE, which provided a strong evidence for the excellent loading capacity of WPI/PC biocarriers to PTE. Thus, the formation process of WPI/PC-PTE nanocomplexes will be further characterized.

**Characterisation of WPI/PC-PTE nanocomplexes**

**Particle size and zeta potential** The effect of PC and PTE addition on the particle size and polydispersity index (PDI) of WPI were investigated by DLS. As shown in Figure 4A, WPI/PC complexes showed a significantly (P < 0.05) larger mean particle size and a higher PDI value than WPI alone, suggesting the formation of protein agglomerates induced by PC. Similar process was also found in the binding of PC to bovine serum albumin (Carballo et al., 2017) and soy proteins (Gong et al., 2023). In contrast, the combination with PTE did not result in any significant changes in mean particle size and PDI values (P > 0.05). Furthermore, zeta potential did not change after adding PC and PTE, which remained around -30 mV (Figure 4B). This was expected because PC and PTE molecules have no net charges.

**Three-dimensional fluorescence spectroscopy**

The 3-dimensional fluorescence spectra during the formation of WPI/PC-PTE nanocomplexes are plotted in Figure 4C-4F. The “pencil-type” peak a is the Rayleigh scattering peak (λex = λ em), while the “fingerprint-type” peak b mainly related to the spectral characteristic of Trp and Tyr residues (λex = 280 nm). The peak c (λex = 230 nm) is considered to be associated with the fluorescence spectral behavior of the peptide backbone structure (C = O) (Pan et al., 2011). The successive addition of PC and PTE resulted in a substantial quenching of the fluorescence intensity of peak b accompanied by a slight fluctuation in λem, while peak c almost completely disappeared, which evidenced that PC and PTE effectively bound to WPI and modified the structure of the protein. It is worth mentioning that with the addition of PTE, the 2 characteristic peaks appearing at λem = 380 nm in the 3D spectrum coincided with the results of the fluorescence spectra.
Figure 3. Molecular docking result and 2D schematic interaction diagram for the ternary complexes formed by α-LA (A-C) or β-LG (F-H) with PC and PTE as well as the binary complexes formed by α-LA (D and G) or β-LG with PTE (H and I).
**SEM**

The morphological features of the WPI/PC-PTE nanocomplexes formation were characterized by SEM. The natural WPI was in the form of stacked granules (Figure 4F), whereas WPI/PC biocarriers existed as spherical aggregates, suggesting that PC induced the self-assembly of WPI particles (Figure 4G). The subsequent formation of the WPI/PC-PTE nanocomplexes exhibited a larger and irregular shape, which was consistent with a trend in particle size, demonstrating successful binding to PTE (Figure 4H), moreover the presence of PTE may result from the conformational change of the protein. It is worth noting that the freeze-drying of samples may lead to secondary aggregation of particles, which can result in differences in mean particle size and size in SEM images (Razzak et al., 2019).

**Secondary structure variation of WPI during the formation of WPI/PC-PTE nanocomplexes**

**Far-UV Circular Dichroism (CD) spectroscopy.** CD spectroscopy has been employed to track

---

**Figure 4.** Particle size and polydispersity index (A), zeta potential (B), 3-dimensional fluorescence spectra (C, D and E) and SEM micrographs (F, G and H) during the formation of WPI/PC-PTE nanocomplexes.
secondary structure alterations in WPI induced by PC and PTE. The positive peak at 190 nm and the negative peaks at 208 nm and 220 nm are used to characterize the α-helical structure, while the peak at 215 nm is associated with β-folding (Araújo et al., 2020). The values of the negative ellipticity decreased with the addition of PC and PTE, which revealed some changes in the secondary structure of the protein after binding reaction (Figure 5A). The results were subsequently quantified and the changes in the secondary structure of the WPI in the different samples were obtained (Figure 5B). In the absence of the polyphenol, free WPI contained around 11% α-helix, 33% β-sheet, 23% β-turn, and 33% random coil. With the successive addition of PC and PTE, the content of α-helix significantly decreased, while corresponding β-turn and random coil of WPI increased significantly. These results indicated that the protein-polyphenol interaction induced a conformational change in the polypeptide chain that involved breaking up the hydrogen-bonding networks that stabilized the helical structures (Araújo et al., 2020).

Fourier transform infrared spectroscopy (FTIR). FTIR is also used to footprint the subtle variations in secondary structure of WPI before and after binding to PC and PTE (Figure 5C). The broad peak of WPI at 3300 cm⁻¹ is reported to be associated with the stretching vibration of -OH groups, while the peak at 2925 cm⁻¹ is ascribed to C-H stretching vibrations. The significant spectral features of the protein are the strong amide I (1600–1700 cm⁻¹, C = O stretch) and amide II bands (1500–1600 cm⁻¹, C–N stretch and N–H bending), which are sensitive to changes in secondary structure. In particular, bands at 1610–1640 cm⁻¹, 1640–1650 cm⁻¹, 1650–1660 cm⁻¹ and 1660–1695 cm⁻¹ are correspond to β-sheet, random coil, α-helix and β-turn, respectively (Meng and Li, 2021). PC showed multiflavonoid-related absorption peaks at 3200–3600 cm⁻¹, 1602 cm⁻¹ and 1438 cm⁻¹, while PTE showed a series of characteristic absorption bands at 1585 -, 1514−, and 1458 cm⁻¹. With the incorporation of PC and PTE, the characteristic peaks of WPI located in the -OH region and the characteristic peak of the amide I band both fluctuated, while the peak located at the

Figure 5. CD spectra (A) and secondary structure percentages (B), FTIR spectra (including pure PC and PTE) (C) and deconvoluted amide I band spectra (D) during the formation of WPI/PC-PTE nanocomplexes.
strong amide II remained unchanged. These changes suggested that hydrogen bonding and hydrophobic forces might be the main driving force for binding (Liu et al., 2022). Further peak fits were performed for the amide I region (1620–1700 cm\(^{-1}\)) (Figure 5D), and all results were iterated to convergence. The incorporation of PC as well as PTE resulted in a further increase in the percentage of random coil, while the content of β-sheet fluctuated, probably due to a slight defolding of the protein caused by the polyphenols addition (Meng and Li, 2021). The same results were found for the soybean isolate/catechin (Dai et al., 2023) and epigallocatechin gallate/β-lactoglobulin (Qie et al., 2020) systems. Generally, the α-helix of the protein binding site are associated with the stability of the protein conformation, while the random coils relate to the flexibility of the proteins (Xu et al., 2018). In this regard, PC led to a more flexible WPI structure, which may contribute to further loading of PTE.

**Free radical scavenging ability.** The antioxidant activity of nanocomplexes in different stages of formation (WPI, WPI/PC and WPI/PC-PTE) were evaluated using DPPH and ABTS radical scavenging methods, and equal amounts of free PC, PTE, and PC/PTE mixtures were used as controls to determine the contribution of the WPI carrier to the antioxidant capacity of the 2 actives individually as well as under coexisting conditions (Figure 6). PC exhibited the strong free radical scavenging capacity, which can be attributed to the presence of a large amount of aromatic ring hydroxyl groups in the PC structures (Parolia et al., 2022). The DPPH and ABTS radical scavenging capacity of WPI was 6.12% and 4.22%, respectively, which was significantly improved by addition of PC. It is likely that the non-covalent attachment of polyphenols to moderate π-electron donors on WPI (such as aromatic amino acids) may result in a more stable phenoxy radical formed after H-atom abstraction (Razzak and Cho, 2022). Similarly, Dai et al. have found that the presence of rice gluten enhanced the DPPH radical scavenging activity of procyanidin (Dai et al., 2019). Interestingly, rather than exerting the additional radical scavenging ability of PC and PTE, the PC/PTE mixture even showed an antagonistic effect, probably due to their different polarities and poor coexistence in one system. In contrast, WPI/PC-PTE nanocomplexes showed a higher scavenging ability of DPPH and ABTS radicals than any of the samples. It has been reported that binary antioxidant systems usually exhibit antagonistic effects due to the bonding of reactive groups (e.g., phenolic hydroxyls) (Liang et al., 2021). However, proteins as a mediator may contribute to the redox potential as well as to the group orientation (Qi et al., 2023), which explains that binding with WPI may enhance the electron supplying capacity and the antioxidant activity of the PC/PTE system.

**Cellular antioxidant activity.** Caco-2 cells were selected to simulate the efficacy of nanocomplexes in the intestine. Similarly, the cytotoxicity of nanocomplexes in different stages of formation (WPI, WPI/PC and WPI/PC-PTE) and equivalent amounts of its constituents (PC, PTE and PC-PTE mixtures) on Caco-2 cells was first detected by a MTT assay (Figure 7A). The cell viability of Caco-2 cells was greater than 95% after incubation with all tested samples, indicating that WPI and its complexes with PC and/or PTE were nontoxic and biocompatible at tested concentrations. The protective effects of these samples on the oxidative damage of Caco-2 cells induced by H\(_2\)O\(_2\) were further evaluated. Compared with the H\(_2\)O\(_2\)-damaged group (52.84%), the group incubated with samples showed a improved cell viability (Figure 7A), where the final WPI/PC-PTE nanocomplexes formed reached the highest viability of 79.39% after treatment, indicating their protective effect against H\(_2\)O\(_2\)-induced cell apoptosis and necrosis (Li et al., 2022). Exogenous H\(_2\)O\(_2\) stress may disrupt the redox balance of the endogenous antioxidant defense system, resulting in elevated intracellular ROS (Liang et al., 2022). Thus, the effect of these samples on ROS levels in Caco-2 cells induced by H\(_2\)O\(_2\) were quantified using a cell model owing to the strong inhibitory effect of PC and PTE on intracellular ROS production induced by H\(_2\)O\(_2\) (He et al., 2018, 2021). During the formation of the nanocomplexes, the ROS levels of the complexes were continuously reduced, implying their potent cytoprotective effect. Interestingly, similar to the results for free radical scavenging capacity, the ROS scavenging capacity of WPI/PC-PTE nanocomplexes was significantly higher than that of PC-PTE mixture despite the fact that WPI alone has almost no ROS scavenging capacity, which again proved that the nanocomplexes exerted a higher antioxidant capacity by optimising the binding of PC to PTE. To further investigate role of nanocomplexes in the balanced free radical metabolism, the intracellular antioxidant enzymes including SOD, CAT, and GSH-Px in Caco-2 cells were evaluated under oxidative stress. As depicted in Figure 7B, Caco-2 cells treated by H\(_2\)O\(_2\) showed a significantly higher intracellular ROS level than that of untreated ones. Unsurprisingly, the ROS level of group incubated with samples was much lower than H\(_2\)O\(_2\)-treated group, indicating their inhibitory efficiency on H\(_2\)O\(_2\)-induced ROS production, which has been confirmed in various cell models owing to the strong inhibitory effect of PC and PTE on intracellular ROS production induced by H\(_2\)O\(_2\) (He et al., 2018, 2021).
Figure 6. ABTS and DPPH scavenging capacity of samples (A). Cytotoxicity and cytoprotective effects of samples on H$_2$O$_2$-treated Caco-2 cells (IC$_{50}$ = 400 µM) (B), quantitative analysis of the effect of samples on ROS levels in Caco-2 cells (C), protective effect of samples on the activity of antioxidant enzymes SOD, CAT and GSH-Px (D, E and F). Bright field (G), fluorescence (H) and merge (I) images at 20 × of Caco-2 cells treated with WPI/PC-PTE nanocomplexes. The mechanism of cellular antioxidant capacity enhancing by the WPI/PC-PTE nanocomplexes (J).
activity under oxidative stress. Besides, the protective effect of nanocomplexes on SOD, CAT and GSH-Px activities was similarly more pronounced than that of free PC-PTE, indicating a synergistic effect of hydrophilic PC and hydrophobic PTE complexed with WPI. These results suggest that WPI/PC-PTE nanocomplexes can exert the protective effect against H$_2$O$_2$-induced oxidative stress by enhancing the activities of antioxidant enzymes (Figure 9F).

**Cellular absorption.** Typically, the bioavailability of hydrophilic and hydrophobic active ingredients (e.g., PC and PTE) is dramatically limited by their gastrointestinal absorption properties. Herein, the cellular absorption of WPI/PC-PTE nanocomplexes stained with rhodamine B was observed by fluorescence microscopy (Figure 9G-9I). The bright-field and fluorescence images were merged to show that the nanocomplexes carrying the 2 polyphenols can be effectively absorbed by cells and thus exert antioxidant effects. In relation to the cellular antioxidant activity, the results implied the potential of WPI/PC-PTE nanocomplexes for practical application as a nutritional supplement.

**CONCLUSIONS**

In summary, we facilitated the co-loading of PTE effect of PC on the structure of WPI. PC induced WPI complexes exhibited a superior affinity for PTE than WPI alone. Meanwhile, PC and PTE promoted the irregularization of the structure of WPI. More importantly, WPI, as a mediator, was able to eliminate the antagonistic effects of PC and PTE by altering their binding modes, thus enabling them to exert significant synergistic effects in enhancing free radical scavenging capacity, cellular uptake, and cytoprotective effects. This study may provide unique insights into the establishment of protein/multipolar polyphenol co-loading systems and contribute to food, beverage and biomedical applications.

**ACKNOWLEDGMENTS**

The research was financially supported by the National Natural Science Foundation of China (31902321), and the National and Scientific and Technological Project of Jilin Province of China (No.20230101143JC).

Declaration of competing interest The authors declare no competing financial interest.

**REFERENCES**


Zhang et al.: Co-assembled whey protein...

[1021/acs.jfc.1c05580].
https://doi.org/10.1021/jacs.j.1c05580.
https://doi.org/10.3390/molecules27196316.
Ozdal, T., E. Capanoglu, and F. Altay. 2013. A review on protein–phe-
https://doi.org/10.1016/j.foodres.2013.02.009.
Pan, X., P. Qin, R. Liu, and J. Wang. 2011. Characterizing the In-
teraction between Tartrazine and Two Serum Albumins by a Hy-
https://doi.org/10.1021/jf200907x.
Penalva, R., I. Espana, E. Larrañeta, C. J. González-Navarro, C. Gamazo, and J. M. Irache. 2015. Zein-based nanoparticles improve the oral bioavailability of resveratrol and its anti-inflammatory ef-
https://doi.org/10.1021/acs.jafc.1c05694.
Qi, X., H. Liu, Y. Ren, Y. Zhu, Q. Wang, Y. Zhang, Y. Wu, L. Yuan, H. Yan, and M. Liu. 2023. Effects of combined binding of chloro-
ogenic acid/coffee acid and gallic acid to trypsin on their synergis-
tions: the antioxidant capacity and effects of polyphenols under different heating conditions in polyphenolic–protein in-
teractions. Food Funct. 11:3867–3878. 
https://doi.org/10.1039/D0FO00627K.
lease behaviour of curcumin-loaded lactoferrin nanohydrogels into food simulants. Food Funct. 11:303–317. 
https://doi.org/10.1039/C9FO01963D.
https://doi.org/10.1016/j.foodhyd.2019.01.031.
Roufik, S., S. F. Gauthier, X. Leng, and S. L. Turgeon. 2006. Thermo-
https://doi.org/10.1021/bm050229c.
Silva, A. S., P. Reboredo-Rodríguez, I. Simnár, A. Sureda, T. Bel-
https://doi.org/10.1111/1541-4337.12629.
Wang, P., and S. Sang. 2018. Metabolism and pharmacokinetics of res-
https://doi.org/10.1002/biof.1410.
ae synergistically improves gel properties of oxidized myofibril-
https://doi.org/10.1016/j.foodchem.2022.133262.

ORCIDs

Xue Shen ☑ https://orcid.org/0000-0002-7145-7601

Journal of Dairy Science Vol. TBC No. TBC, TBC