Formation and characterization of noncovalent ternary complexes based on whey protein concentrate, high methoxyl pectin, and phenolic acid

Yuanyuan Zhang,^{1,2} Siyao Li,¹ Yang Yang,¹ Cuina Wang,^{1*} and Tiehua Zhang^{1*}

¹Department of Food Science, College of Food Science and Engineering, Jilin University, Changchun, Jilin, 130062, China

²School of Grain Science and Technology, Jilin Business and Technology College, Changchun, Jilin, 130507, China

ABSTRACT

Protein-polysaccharide-polyphenol noncovalent ternary complexes possess unique physicochemical, structural, and functional properties. In the present study, ternary complexes based on whey protein concentrate (WPC; 2%, wt/vol) and high methoxyl pectin (HMP; 0.5%, wt/vol) complexes and 0.2 to 0.6% (wt/vol) chlorogenic acid (CA) or rosmarinic acid (RA) were formed and characterized at 3 pH values (4, 4.5, and 5). The pH conditions were decided according to phase diagram of WPC and HMP during acidification. Fluorescence quenching experiments indicated that WPC-HMP complexes bound RA stronger than CA and the binding constant increased with increasing pH for both phenolic acids. Particle size of ternary complexes decreased and absolute ζ -potential increased with pH values changing from 4 to 5, and RA influenced the particle size of WPC-HMP complexes greater than CA. The CA and RA in ternary complexes showed good stability against UV light with pH order of pH 5 > pH 4.5 > pH 4. Fourier-transform infrared spectroscopy spectra indicated the involvement of hydrogen bonding between WPC-HMP and CA or RA. Antibacterial tests showed that ternary complexes had good antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* at concentrations of 6.2 mg/mL and the ability increased with decreasing pH values. All ternary complexes possessed strong scavenging radical capacities with median inhibitory concentration (IC₅₀) values ranging from 2.71 ± 0.05 to 6.20 ± 0.41 µg/mL. Antioxidative ability of ternary complexes increased as pH went up and WPC-HMP-RA showed significantly higher antioxidative property compared with WPC-HMP-CA. Data may provide useful information for rational design of ternary complexes and applications of the formed complexes in food matrices such as beverages and emulsions.

Key words: whey protein concentrate, high methoxyl pectin, chlorogenic acid, rosmarinic acid, ternary complex, pH effect

INTRODUCTION

Proteins and polysaccharides have great influences on the structure, texture, and stability of foods. In aqueous solution, proteins and polysaccharides can interact through electrostatic forces, hydrogen bonds, and hydrophobic effects (Le et al., 2017), forming either soluble complexes or coacervates depending on several intrinsic or extrinsic factors such as biopolymer charges and medium conditions. In a system where proteins, polysaccharides and polyphenols exist simultaneously, possible interactions between the 3 compounds may occur mainly via noncovalent binding such as hydrophobic forces and hydrogen bonds. Accordingly, studies on combining the 3 compounds are gaining attention. Protein-polysaccharide complexes have a good protective effect on polyphenols against degradation under harsh conditions (Wei and Huang, 2019). Ternary complexes of proteins, polysaccharides, and polyphenols can be served as functional materials to stabilize emulsion, foam, and colloidal systems (Yang et al., 2020). Polysaccharides exhibited the role of inhibiting protein-polyphenol complexes precipitation (Chen et al., 2019). Polyphenols would strengthen noncovalent binding between proteins and polysaccharides (Aberkane et al., 2012). Due to the electrostatic interaction nature of proteins and polysaccharides, the formation and properties of the formed ternary complexes were influenced by the solution pH values. Wang et al. (2016) found that quercetin (a characteristic flavonol with a structure similar to quercetin) showed different binding affinities and affected the physicochemical and structural properties of soy protein isolate-κ-carrageenan complexes differently at pH 2.3 and pH 6.5. In addition, the ternary complexes were also greatly dependent on the polyphenol type used. A previous study showed that polyphenolic substances including catechin, tannic acid, grape seed or hibiscus extract affected the particle size, surface charge and stability of whey protein

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*Corresponding authors: wanguina@jlu.edu.cn and zhangth@jlu.edu.cn

concentrate (**WPC**)-high methoxyl pectin (**HMP**) complexes differently due to their different molecular structures (Thongkaew et al., 2014).

Whey protein and pectin are commonly used models of electrostatic complexation due to their characteristic and definite structure. Whey protein is an important fraction of proteins in milk and is a byproduct during cheese making. It is mainly composed of β -LG, α -LA, BSA, and immunoglobulin, as well as a small amount of lactoferrin and lactoperoxidase (Madureira et al., 2007). It has high nutritional value, and its essential AA structure is more reasonable and perfect than that of plant-derived proteins (Zhao et al., 2021a). In addition, whey protein is helpful for improving human immunity (Legrand, 2016), protecting the heart (van der Ven et al., 2002), preventing cancer (Baena Ruiz and Salinas Hernández, 2014), providing antiaging effects (Camargo et al., 2020), and promoting health and controlling weight (Pal et al., 2010). Pectin is a polysaccharide composed of a linear chain of galacturonic acid units joined by α -1–4 links, interspersed by rhamnose units linked by α -1–2, in which the carboxylic groups of galacturonic acid may be esterified by methyl groups (Thakur et al., 1997). Pectin with a degree of esterification (**DE**) higher than 50% is considered as HMP. Below the isoelectric point of the protein, whey protein, and pectin form complexes principally via electrostatic effects. However, above the isoelectric point, negatively charged pectin interacts with the local positive patches in whey protein to form interpolymer complexes. These formed complexes showed useful applications in food industries such as bilayer emulsion, delivery agents, and fat replacers, and for isolating acid whey protein from cheese making (Raei et al., 2018; Wefers et al., 2018).

Phenolic acids are secondary metabolites that are widely present in plants and play an important role in plant survival and defense (Zhao et al., 2021b). In the human diet, phenolic acids, which account for approximately 30% of the total phenols (Yang et al., 2001), are strong antioxidants that people are interested in (Zhao et al., 2021b). Depending on the position and number of hydroxyl groups on the aromatic ring, phenolic acids are classified as hydroxybenzoic acids and hydroxycinnamic acids (Rashmi and Negi, 2020). Chlorogenic acid (**CA**) and rosmarinic acid (**RA**) are representative nonflavonoid compounds of hydroxycinnamates. Chlorogenic acid is formed by caffeic acid and 1-hydroxy hexahydrogallic acid and is mainly derived from coffee, tea, fruits, and vegetables (Meinhart et al., 2019). Rosmarinic acid consists of caffeic acid and 3,4-dihydroxyphenyllactic acid (Ngo et al., 2018) and is widely distributed in plant species of the Lamiaceae and Boraginaceae families (Marchev et al., 2021). Both

CA and RA have various beneficial effects on health, such as anti-inflammatory (Bao et al., 2018; Zhao et al., 2018), antioxidative (Wongsa et al., 2012; Žugić et al., 2014), antidiabetic (Ong et al., 2013; Ngo et al., 2018), and anticarcinogenic (Feng et al., 2005; Shi et al., 2016) properties.

In this study, we aimed to identify conditions where stable WPC-HMP and phenolic acid ternary complexes could be formed and their possible applications to meet the increasing demand for food formulations with active ingredients. Noncovalent ternary complexes were fabricated by mixing WPC (2%, wt/vol) and HMP (0.5%, wt/vol) complexes with CA or RA at different concentrations (0.2–0.6%) and pH values (4, 4.5, and 5). The formed ternary systems were analyzed for physicochemical properties, structure, phenolic acid stability, antimicrobial activity and antioxidative properties.

MATERIALS AND METHODS

Materials

The WPC was purchased from Hilmar, and the protein purity of 80% was provided by the manufacturer. The HMP (DE of 62%) was provided by Davisco Foods International Inc. The CA (purity of 95%) and RA (purity of 98%) were purchased from Coolaber Science & Technology. Deionized water (conductivity is below 18.2 milliohms) was obtained using a Milli-Q deionization reversed osmosis system (Millipore Corp.).

Sample Preparation

The WPC stock solution (4%, wt/vol) was made by slowly dissolving WPC powder into ultrapure water, then stirred at room temperature ($25 \pm 1^\circ\text{C}$) for 2 h at a speed of 700 rpm with the help of a magnetic stirrer (IKA Ared). The HMP stock solution (1%, wt/vol) was prepared by dropping pectin powder into ultrapure water under agitation (700 rpm) and heated at 65°C for 2 h (Kord Heydari et al., 2021). Stock solutions were stored at 4°C overnight to ensure complete rehydration.

Ternary systems were prepared according to a previous study (Wang et al., 2016) with some modifications. Briefly, WPC-HMP complexes (mass ratio of 4:1) at total concentration of 2.5% were prepared at pH 4, 4.5, and 5, respectively. The CA or RA powder was added into the binary complex solutions, readjusted to pH 4, 4.5, and 5 and then mixed via a vortex shaker at 3,000 rpm for 1 h. The final concentrations of phenolic acid in the ternary systems were 0.2 to 0.6% (wt/vol). When weighing the CA or RA powder, the purity of CA or RA was taken into consideration.

Turbidity Measurement

The WPC, HMP, and WPC-HMP mixtures (mass ratio of 4:1, 0.1%) were adjusted from pH 7 to pH 2 at 0.2 intervals with 0.2 M HCl. Turbidity of all the samples was monitored by measuring absorbance at 600 nm (1 cm path length optical cuvette) using a UV-visible spectrophotometer (UV2550, Shimadzu). Distilled water was set as control and results were the mean of triplicate measurements.

Fluorescence Spectroscopy

Fluorescence spectra of WPC-HMP complexes in presence of various concentrations of CA or RA in emission wavelength range of 290 to 450 nm were recorded at excitation wavelength of 280 nm using a fluorescence spectrophotometer (Hitachi). The slit width for excitation and emission was 5 nm and the path length was 1 cm. Ultrapure water and CA or RA solutions with corresponding concentrations (0, 10, 20, 30, 40, 50, 60 $\mu\text{mol/L}$) were also scanned for subtraction of raw complex for spectra analysis. Stern-Volmer equation and double logarithmic curve equation were used to explore the fluorescence quenching mechanism and compute binding constant and binding number according to our previous study (Zhang et al., 2021b).

Particle Size Measurement

Particle size of ternary complexes was measured using a Laser Particle Analyzer (Mastersizer 3000) based on static light scattering technique. Samples were dropped into a Hydro 2000SM sampling device until the shading rate achieved 8%. The mean diameter of the complexes was expressed as volume mean diameter (d_{43}):

$$d_{43} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3, \quad [1]$$

where n_i is the number of particles, and d_i is the diameter of particles.

Surface Charge Measurement

Average surface electrical charge (ζ -potential) of samples was determined using a Malvern Nano Zeta-sizer (Malvern Instruments Ltd.) with a U-shaped cuvette. For phase diagram establishment, ζ -potential of WPC-HMP mixtures (0.1%, wt/vol) from pH 7 to pH 2 was determined. Ternary systems were measured for ζ -potential after diluting 25-fold with deionized water at corresponding pH values of 4, 4.5, and 5.

Fourier-Transform Infrared Spectral Measurement

Fourier-transform infrared (FTIR) spectra of samples were recorded using an IRPrestige-2 FTIR spectrometer (Shimadzu) at a resolution of 4 cm^{-1} and an average of 16 scans from 400 to 4,000 cm^{-1} . Freeze-dried WPC-HMP complexes and ternary complexes with CA or RA (0.6%, wt/vol) were ground into powder with KBr powdered at a mass ratio of 1:100. After each sample was mixed with anhydrous KBr, the mixtures were compressed into thin slices. Collected data were plotted as a function of transmittance and the peak positions were picked using Origin 21 software (Origin-Lab Corp.).

Secondary Structure Analysis

A Far-UV circular dichroism spectropolarimeter (MOS-500, Bio-Logic) was used to analyze the secondary structure of whey protein in the range of 190 to 260 nm using a 1-mm cell. The WPC-HMP complexes and complexes with CA or RA (0.6%, wt/vol) were diluted 25-fold with deionized water at pH 4, 4.5, and 5. The HMP and CA or RA solutions at pH 4, 4.5, and 5 were considered as blank and subtracted from raw files. Secondary structure portion of whey protein based on circular dichroism (CD) spectra was estimated using online DichroWeb (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>).

Colloidal Stability of Ternary Complexes and Photostability of Phenolic Acid

Colloidal stability of ternary complexes was assessed via observing appearance by taking pictures at different days after production. Light stability of CA and RA was tested based on previous studies (Wang et al., 2016; Yi et al., 2018) with some modifications. All ternary complexes with 0.6% CA or RA were exposed in controlled UV light cabinet (365 nm) at intensity of 6 W at room temperature and determined for CA or RA contents every 8 h for a total of 64 h. Then the CA or RA was separated from ternary complexes suspension according to a previous study (Tan et al., 2014). The CA or RA was dissociated from the ternary complex by ultrasound for 20 min, and then the protein and pectin were precipitated by centrifugation at $14,000 \times g$ for 20 min at room temperature. The CA or RA was in the supernatant. The supernatant was diluted 100-fold and determined for absorbance at 324 nm (CA) and 328 nm (RA). The supernatant was calculated for CA or RA content based on established standard curves ($R^2 >$

0.99). Free CA and RA experienced the same UV light irradiation was set as control.

Antimicrobial Activity

Samples were assessed for antimicrobial activities against *Staphylococcus aureus* and *Escherichia coli* according to a previous study with some modifications (Su et al., 2020). Nutrient broth solution inoculated with bacteria (1×10^8 cfu/mL) in presence of WPC-HMP complexes or ternary complexes (0.6% CA or RA) were incubated at 37°C for 12 h in 24-well plates. The final concentration of ternary complexes in the system was 6.2 mg/mL. Supernatants in the bacteria suspension were taken out and measured for absorbance at 600 nm using a microplate reader (Synergy HT, BioTek) every 1 h for a total of 12 h. Kanamycin sulfate in broth (2 µg/mL) was used as positive control (Li et al., 2021).

Antioxidative Properties

Antioxidative properties of samples were tested according to protocols described in our previous study (Gao et al., 2018) with some modifications. Samples were diluted to concentrations of 1 to 15 µg/mL followed by adjustment to corresponding pH values. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution (0.2 mM) was prepared by dispersing the powder into methanol. Samples and DPPH solution at volume ratio of 1:1 was mixed and stored in dark for 30 min. Absorbance of all solutions was measured by a microplate reader (Synergy HT, BioTek) at 517 nm.

2,2'-Azinobis(2-ethylbenzothiazoline-6-sulfonate) (ABTS) radical action solution was generated by mixing ABTS (7 mM) and potassium persulfate (2.45 mM) and stored in dark at 4°C for 12 to 16 h. Samples (50 µL) were blended with 150 µL of activated ABTS radical and incubated for 30 min. The absorbance of all samples was recorded at 734 nm. Both DPPH and ABTS scavenging activity (SA) was calculated according to the following equation and median inhibitory concentration (IC₅₀, µg/mL), the value required to scavenge 50% of radicals, was calculated:

$$SA(\%) = 1 - (A_S - A_B)/A_R \times 100, \quad [2]$$

where A_s , A_B , and A_R represent the absorbance of samples with DPPH or ABTS, blank, and DPPH or ABTS solutions, respectively.

Statistical Analysis

Data were the mean of data for at least 2 batches. Each batch was consisted of 3 biological replicates and

each biological replicate included 3 technical replicates. All data from analysis were expressed as mean $\pm$ standard deviation and were analyzed for significant difference at $P = 0.05$ using software of SPSS 20 (IBM Corp.). Data were first checked for homogeneity of variance using Levene model. Least squared difference and Dunnett's C were used for post hoc multiple comparisons for data with homogeneity or inhomogeneity of variance, respectively. Origin 2021 (OriginLab Corp.) was used to draw figures.

RESULTS AND DISCUSSION

Phase Diagram Analysis of Whey Protein and Pectin Mixture

Electrostatic interpolymeric complexing between WPC and HMP during acidification was monitored via measurements of optical features and charge profiles. The turbidity magnitude of a protein-polysaccharide mixture in diluted suspension, which mainly originates from the size and concentration of the particles (Chylek and Zhan, 1990), can be taken as a sign of complex formation or dissociation, and was therefore used to monitor structural transitions (Zhong et al., 2021). Figure 1A displays the turbidity profiles of the WPC, HMP, and WPC-HMP mixture during acidification from pH 7 to pH 2 with intervals of 0.25. Maximum turbidity for WPC was observed at pH 4.5, which was attributed to the aggregation of whey protein due to the weak repulsion between neighboring protein molecules close to the isoelectric point (IEP) of proteins (Zhao et al., 2021c). However, the optical values of HMP remained negligible during the whole process due to the weak light scattering ability. In comparison to the individual polymers, turbidity of the WPC-HMP mixture first increased and then decreased and peaked at approximately pH 3.75, where precipitation was visibly observed. During attractive interactions, proteins and polysaccharides were held together mainly by electrostatic forces and can appear as coacervates or complexes. Correspondingly, the critical phase transition points of pH_c and pH_c, which signify the onset of soluble intramolecular complexes and complex coacervates (Souza and Garcia-Rojas, 2017), were graphically identified to be 5.5 (pH_c) and 4.5 (pH_c), respectively. The mixture turbidity remained almost constant at pH values above pH_c and then increased from pH_c to pH_c. Subsequently, a sharp increase in turbidity was observed at pH values lower than the second critical pH (pH_c) until pH 3.75. Thereafter, the resultant assembled biopolymer complexes formed at pH 4, 4.5, and 5, which correspond to coacervates, coacervates and soluble complexes were selected for subsequent experiments. In addition, complexes formed

Figure 1. Absorbance at 600 nm (A) and ζ -potential (B) of whey protein concentrate (WPC), high methoxyl pectin (HMP), and WPC-HMP mixture during acidification from pH 2 to pH 7. Concentrations of WPC, HMP, and WPC-HMP mixture were 0.1% (wt/vol). pH_{ϕ} indicates the onset of complex coacervates; pH_c indicates the onset of soluble intramolecular complexes. a.u. = arbitrary units.

at the 3 selected pH values may find broad applications in the food industry because most foods are weakly acidic in conditions of pH 4 to 5 (Ueno et al., 2020).

Changes in the pH of an aqueous system comprising charged proteins and polysaccharides can modulate the charge profile on surface of molecules, supplying a pH scale for complexation between these biopolymers (Zhang et al., 2021a). Surface charge expressed as ζ -potential was obtained from the perturbations of Brownian diffusivity under a pulsating electrical field and has been used for biopolymers to evaluate electrostatic interaction and reflects the stability of mixed dispersion. As depicted in Figure 1B, WPC carried negative surface charge and then positive charge

with IEP where ζ -potential becomes zero located at about pH 4.5, in line with results for turbidity. Individual HMP displayed an initial ζ -potential of -45.24 ± 1.29 mV (pH 7.0) and the surface remained negatively charged throughout the whole titration process attributed to distribution of carboxyl ($-\text{COO}^-$) group on backbone. The middle charge curve of WPC-HMP mixture indicated the electrostatic interactions between the 2 polymers and introduction of HMP added more negative charges to the system. The large difference at pH 3.75 revealed the strong attraction between polymers, corresponding to the maximum turbidity. In pH range of 4.5 to 5.5, above the IEP of protein, complexes were still formed via so-called charge patch model due to heterogeneous charge distribution of protein (Zhong et al., 2021).

Binding Ability of WPC-HMP to CA or RA

A fluorescence quenching experiment was applied to explore the binding parameters between WPC-HMP and CA or RA because all major components of WPC contain tryptophan and tyrosine, which possess intrinsic fluorescence. As shown in Figure 2, upon excitation at 280 nm, WPC-HMP showed a maximum emission wavelength at 330 to 334 nm for all pH values, and a slight redshift peak position was observed after phenolic acid addition, conveying a change in the tertiary structure of the protein and transfer of the fluorophore from hydrophobic to a more hydrophilic environment (Wang et al., 2021a). Concurrently, both phenolic acids regularly quenched the fluorescence intensity of WPC at an excitation wavelength of 280 nm (Wang et al., 2016). Fluorescence quenching may result from various processes, such as molecular collision, ground-state complex formation or energy transfer (Zhang et al., 2014) and can be categorized into dynamic and static quenching types. K_q , which can be used to judge quenching type, can be calculated by the Stern-Volmer equation in accordance with our previous study (Zhang et al., 2021b). The computed K_q values of all samples (Table 1) were much higher than the maximal dynamic quenching constant (2.0×10^{10} mmol/L per second), suggesting that the main mechanism of static quenching and ternary complexes was formed (Li et al., 2013).

Fluorescence data were further analyzed to estimate the binding constant (K_a) and the number of binding sites (n) using double logarithmic curve equation (Zhang et al., 2021b) with coefficient of determination ($R^2 > 0.99$) as shown in the inset of Figure 2. Table 1 showed that affinity of WPC-HMP for phenolic acid was considerably dependent on phenolic acid type and solution pH. Generally, WPC-HMP bound RA stronger than it bound CA, which may be due to the different

Figure 2. Fluorescence emission spectra of whey protein concentrate (WPC) in WPC-high methoxyl pectin (HMP) complexes (0.1%, wt/vol) with various concentrations of chlorogenic acid (CA; panels A, B, C) and rosmarinic acid (RA; panels D, E, F) at pH 4, 4.5, and 5. [CA] and [RA] = 0, 10, 20, 30, 40, 50, 60 $\mu\text{mol/L}$ (curves a $\rightarrow$ g). The inserted linear plot of $\lg [(F_0 - F)/F]$ versus $\lg [CA/RA]$ was drawn using the change in maximum fluorescence intensity of WPC. F_0 = fluorescence intensity of WPC-HMP complexes in the presence of CA; F = fluorescence intensity of WPC-HMP complexes in the presence of RA; a.u. = arbitrary units.

Table 1. Quenching constants (K_{sv} , K_q , and K_a), coefficients of determination (R^2), and number of binding sites (n) for the interaction between CA or RA and WPC-HMP complexes at different pH values¹

Compound	pH	K_{sv} (10^4 L/mol)	K_q (10^{12} L/mol per s)	R^2	K_a (L/mol)	n	R^2
WPC-HMP-CA	4	2.64	2.64	0.9973	4.31×10^4	1.06	0.9914
	4.5	3.88	3.88	0.9941	3.89×10^5	1.24	0.9958
	5	4.47	4.47	0.9912	4.37×10^6	1.48	0.9902
WPC-HMP-RA	4	4.02	4.02	0.9900	7.34×10^4	1.07	0.9944
	4.5	4.32	4.32	0.9958	4.12×10^5	1.26	0.9994
	5	5.43	5.43	0.9905	4.32×10^7	1.69	0.9902

¹WPC = whey protein concentrate; HMP = high methoxyl pectin; CA = chlorogenic acid; RA = rosmarinic acid.

number and position of phenolic hydroxyl groups (Li et al., 2020). Binding constant increased with increasing pH as a result of alternation of electrostatic interactions between polymer chains, indicating that strong electrostatic interaction was unfavorable for binding with phenolic acid. This result can be explained that the binding site with CA or RA on the protein polymer may be shielded by interacting with HMP. Similarly, binding site number increased with increasing pH in despite of the fact that all the values were approximately equal to 1. Similar result was reported in previous study (Wang et al., 2016).

Particle Size and ζ -Potential of Ternary Complexes

Ternary complexes of WPC-HMP with phenolic acids at 0 to 0.6% were characterized for particle size and ζ -potential. As indicated in Figure 3A, the mean diameter (d_{43}) of WPC-HMP complexes varied as a function of pH and phenolic acid type. In general, a lower pH resulted in a higher particle size due to the stronger attraction between WPC and HMP, in line with the turbidity results (Figure 1A). At pH 5, the different types and concentrations of phenolic acids at levels of 0.2% and 0.4% did not influence the particle size and RA addition up to 0.6% resulted in aggregates with larger particle sizes. Compared with the control, a sharp increase in the particle size was observed after CA or RA addition at pH 4 and 4.5. Continuous growth in the particle size of ternary complexes with rising CA or RA concentration was observed at pH 4.5, whereas a peak bottom value was observed for samples with 0.4% phenolic acid at pH 4 due to the shrinkage of particles caused by expulsion of water (Wang et al., 2016). Additionally, it is worth mentioning that RA showed a higher particle size enhancing effect than CA, which may be attributed to the increased binding amount of RA molecules as the pH decreased (Ferraro et al., 2015).

Figure 3B shows the effects of 2 types of phenolic acids on the ζ -potential of WPC-HMP electrostatic complexes at 3 pH values. Overall, the absolute ζ -potential

of the complexes increased with increasing pH, in line with the results for the ζ -potential of the WPC-HMP mixture (Figure 1B). Phenolic acid affected the surface charge of WPC-HMP differently. The 3 selected pH values of 4, 4.5, and 5 were below, equal to, and above the IEP of WPC, respectively. At pH 4, below the IEP of the protein, whey protein molecules carry a positive surface charge and the addition of negatively charged phenolic acids neutralizes a part of the positive charge, causing a more negative surface charge of ternary complexes. At pH 4.5, the slight changes may be due to the electric neutrality of the protein. At pH 5, the less negative surface charge may be due to protonation of carboxyl groups in both WPC and HMP (Zhong et al., 2021).

Colloidal Stability of Ternary Complexes and Photostability of CA and RA in WPC-HMP Complexes

Figure 4 shows photos of ternary samples just after production, stored for 7, 14, and 28 d. All freshly prepared samples were homogeneous and appeared as milky white without any precipitation. After production for 14 d, slight separation occurred in samples of WPC-HMP-RA at pH 5 due to different affinities between polymers and solvent (Hadian et al., 2016). The rest of the samples remained totally homogeneous, lacking any sign of phase separation throughout the whole storage period. These results undoubtedly indicated the colloidal stability of the ternary complexes of WPC-HMP with CA or RA during storage.

In recent years, the utilization of soluble or insoluble complexes arising from protein-polysaccharide interactions for the purpose of encapsulating hydrophilic and lipophilic molecules has been studied (Ron et al., 2010). Both CA and RA are vulnerable to harsh environments including UV light due to the existence of unsaturated bonds in the molecules (Halliwell, 2008). The stabilization efficiencies of complexes for CA and RA under UV light at various pH values were studied and the results are shown in Figure 5. All phenolic acids experienced

Figure 3. (A) Particle size (d_{43} = volume mean diameter), and (B) ζ -potential of whey protein concentrate (WPC)-high methoxyl pectin (HMP) complexes and WPC-HMP complexes with chlorogenic acid (CA) or rosmarinic acid (RA; 0–0.6%, wt/vol) at pH 4, 4.5, and 5.

physicochemical deterioration to various extents during irradiation for 64 h. Free RA showed the most rapid degradation rate and approximately 50% (pH 4), 47% (pH 4.5), and 44% (pH 5) were decomposed at the end of the test, which may be due to more unsaturated bonds in the molecules. Predictably, the photostability of RA was enhanced by incorporation into WPC-HMP complexes, and the protecting ability of the systems revealed a positive correlation with pH for both phenolic acids because of the strengthened binding constant at high pH values (Table 1). Similar changing pattern was observed for CA. Furthermore, contrastive analysis of Figure 5A and Figure 5B uncovered that binary WPC-HMP complexes protected CA better than RA,

which may be due to their different molecular structure characteristics.

FTIR and Far-UV CD Spectral Analysis

Samples were measured for FTIR spectra to probe the possible interactions between WPC-HMP and CA or RA at 3 pH values. Figure 6 shows that WPC-HMP complexes at 3 pH values had a peak at $1,659\text{ cm}^{-1}$, which was attributed to the stretching vibration of C=O double bond (Fang and Dalglish, 1997). As CA was added, peaks were all shifted to $1,641\text{ cm}^{-1}$, which was independent of the pH values. Similarly, RA addition shifted the peak to $1,649\text{ cm}^{-1}$, $1,641\text{ cm}^{-1}$, and $1,639\text{ cm}^{-1}$ for samples at pH 4, 4.5, and 5, respectively. Meanwhile, the peak at $1,234\text{ cm}^{-1}$ for the WPC-HMP complex assigned to the stretching vibration of C–N bond (Albano and Nicoletti, 2018) moved to $1,271\text{ cm}^{-1}$ and $1,261\text{ cm}^{-1}$ after the addition of CA or RA, respectively. These changes indicated that phenolic acids associated with WPC-HMP complexes and hydrogen bonds may be involved (Flammini et al., 2020).

Far-UV CD spectroscopy is commonly used to acquire information on alterations of proteins occurring at a secondary level. The CD spectra of all samples in Supplemental Figure S1 (<https://data.mendeley.com/datasets/79zsmrvx8y/1>; Zhang, 2021) indicated that whey protein showed broad positive and negative peaks at approximately 191 and 220 nm which represent the main α -helix and β -sheet contents, respectively. The fluctuation of CD curves revealed the alteration of protein secondary structure caused by pH and phenolic acid type variation. To further confirm changes in secondary structure contents, fractions of α -helices, β -sheets, β -turns, and random coils were estimated by the Dichroweb website program (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>). The quantitative analysis (Figure 6) showed that the α -helix of the protein in WPC-HMP was significantly decreased when the pH changed from 4 to 4.5 and 5 ($P < 0.05$) with concomitant increase in β -sheets, indicating the transition from α -helices to β -sheets, and more compact coacervates were formed (Ren et al., 2019). After the addition of phenolic acid, CA and RA affected the secondary content of protein in different ways. In terms of α -helices, significant difference was only observed for WPC-HMP with CA at pH 4.5 and 5, where CA incorporation significantly increased α -helices ($P < 0.05$). The β -sheet of whey protein in both ternary complexes significantly increased compared with that of WPC-HMP at the 3 tested pH values ($P < 0.05$). These results suggested that whey protein in ternary complexes was more stable than in corresponding binary complexes. Comparative analysis of WPC-HMP-CA and WPC-HMP-RA at

each pH value revealed that both ternary complexes were affected by pH to a lesser degree than WPC-HMP, which may be due to the stabilizing effect of phenolic acids (Gharehbeglou et al., 2019).

Antimicrobial Activity Analysis of Ternary Complexes

Staphylococcus aureus and *E. coli*, representatives of gram-positive and gram-negative bacteria, respectively, are common foodborne pathogens. Ternary complexes were assessed for antimicrobial activity against these 2 bacteria by monitoring changes in optical density, which is a common technique to quantify cell growth (Myers et al., 2013). As shown in Figure 7, both microorganisms in the control group began to enter the growth

period after 2 to 3 h and then the population increased gradually in the following 9 h. The optical density of the bacterial suspension with kanamycin sulfate maintained a constant closing baseline during incubation due to the high degree of transparency, indicating that *Staph. aureus* and *E. coli* were killed by kanamycin sulfate at 2 µg/mL, within the range of 0.25 to 32 µg/mL reported in a previous study (Voicu et al., 2013). The midway curves between the control and kanamycin sulfate data indicated that microorganism growth was significantly inhibited by all ternary complexes (Figure 7). Generally, all samples showed stronger antimicrobial activity against *Staph. aureus* than against *E. coli*. This may be due to the fact that *Staph. aureus* lacks an LPS layer in the extracellular membrane, resulting in higher sensitivity to environmental pressure (Rao et al., 2019).

Figure 4. Appearance of whey protein concentrate (WPC)-high methoxyl pectin (HMP) complexes with chlorogenic acid (CA) or rosmarinic acid (RA; 0.6%, wt/vol) stored for 28 d at 4°C.

Figure 5. Retention rate (%) of (A) chlorogenic acid (CA), and (B) rosmarinic acid (RA) in ternary complexes after exposure to UV light over 64 h. WPC = whey protein concentrate; HMP = high methoxyl pectin.

Ternary complexes had antimicrobial activity to various extents depending on phenolic acid type and pH (Figure 7). Upon comparing the antimicrobial activity of WPC-HMP-CA and WPC-HMP-RA, the difference in potency was found to be highly significant: CA was more effective than RA at each tested pH value for both bacteria. The results also showed that samples exerted more potent antimicrobial activity at low pH values. Phenolic compounds can form hydrogen bonds with enzymes, modifying the permeability of microorganism cell membranes and altering various intracellular functions. Additionally, polyphenols also change cell wall rigidity, and consequently integrity losses due to a variety of interactions with the cell membrane (Cushnie

and Lamb, 2011; Khochapong et al., 2021). A previous study showed that acids entered cells in hydrophobic unionized form and then dissociated protons in the cells, lowering the intracellular pH (Ueno et al., 2020). Therefore, antimicrobial activities of the samples were enhanced as pH of samples was lowered.

Antioxidative Properties

Antioxidants play a crucial role in postponing, controlling, or hindering oxidation processes perceived to cause deterioration of food quality (Shahidi and Zhong, 2015). Whey protein is abundant in amino acids such as histidine, phenylalanine, and tryptophan, which can exert their antioxidant activity by hydrogen atom transfer or electron transfer mechanisms (Corrochano et al., 2019). Pectin has antioxidant properties which may be related to the presence of hydroxyl group (Yeung et al., 2021). Both CA and RA showed strong DPPH and ABTS scavenging abilities (Hyatt et al., 2021; Wang et al., 2021b) might be due to the generation of stable semiquinone compounds through reaction of phenolic hydroxyl groups and autologous groups, thus terminating the free radical chain reaction (Çelik et al., 2011). Thus, the combination of the 3 compounds has huge potential for application as an antioxidant in food industry.

The DPPH and ABTS radical scavenging assays with different mechanisms were applied to evaluate the in vitro antioxidant activity of different samples. The radical scavenging ability of the samples was studied at various concentrations, dose-dependent DPPH and ABTS radical scavenging behaviors at concentrations of 1 to 15 $\mu\text{g/mL}$ were observed, and the established standard curves all showed good correlation ($R^2 > 0.99$). Accordingly, the median inhibitory concentration (IC_{50}) was calculated as per the standard curves, and the results are shown in Figure 8. Overall trend analysis showed that all samples possessed strong antioxidant properties with IC_{50} values ranging from 2.71 ± 0.05 to $6.20 \pm 0.41 \mu\text{g/mL}$, which were significantly lower than those of the corresponding ascorbic acid control ($P < 0.05$) except WPC-HMP-CA in terms of DPPH scavenging ability.

For DPPH, the color changed from purple to yellow by acceptance of hydrogen radicals from ternary systems, and DPPH became a steady diamagnetic molecule (Shang et al., 2020). The scavenging ability of samples was significantly strengthened with increasing pH values ($P < 0.05$). The changed DPPH cleaning percentage may be due to the accessibility of free hydroxyl groups and antioxidative amino acids in the ternary system. At low pH values, especially at pH 4, WPC-HMP coacervates with packed structures were

formed and exposure of active hydroxyl groups was hindered by the formation of intramolecular hydrogen bonds between pair of compounds, resulting in a suppression of antioxidant ability (Yeung et al., 2021). In addition, antioxidative amino acids in proteins may be masked in compact structures at low pH values. Most importantly, the phenolic hydroxyl group in CA and RA may form hydrogen bonds with WPC-HMP complexes at low pH values.

At each pH value, WPC-HMP-RA showed stronger antioxidative properties than WPC-HMP-CA. This result may be due to that RA is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, which has a better antioxidant efficiency than the composed units of CA, which are caffeic and quinic acids (Chen and Ho, 1997). Similar changing tendencies as pH variation for DPPH analysis were observed for ABTS experiments.

CONCLUSIONS

Ternary complexes based on WPC-HMP complexes and CA or RA were prepared and characterized at pH values of 4, 4.5, and 5. The complexes suspension showed good stability and superior attributes such that they could be introduced as new products to the market. Both CA and RA were protected against UV light irradiation for 64 h by binding with WPC-HMP complexes. Ternary complexes inhibited the growth of *Staph. aureus* and *E. coli* at a relatively low concentration, and antibacterial activity increased with decreasing pH values. Ternary complexes showed strong DPPH and ABTS radical scavenging ability, which increased with increasing pH. These data may provide useful information for the practical applications of ternary systems in acidic foods and beverages.

Figure 6. Fourier-transform infrared spectroscopy (FTIR) spectra (A) and secondary structure content (B) of whey protein concentrate (WPC)-high methoxyl pectin (HMP) complexes and WPC-HMP complexes with chlorogenic acid (CA) or rosmarinic acid (RA; 0.6%, wt/vol) at pH 4, 4.5, and 5. Lowercase letters indicate significant differences ($P < 0.05$) between samples at the same pH; uppercase letters indicate significant differences ($P < 0.05$) between samples at different pH values. a.u. = arbitrary units.

Figure 7. Growth curve (measured as optical density at 600 nm, OD_{600nm}) of *Staphylococcus aureus* (A, B, C) and *Escherichia coli* (D, E, F) in the presence of whey protein concentrate (WPC)-high methoxyl pectin (HMP) complexes and WPC-HMP complexes with chlorogenic acid (CA) or rosmarinic acid (RA) at pH 4, 4.5, and 5.

Figure 8. Changes in scavenging ability (median inhibitory concentration, IC_{50}) against (A) 2,2-diphenyl-1-picrylhydrazyl (DPPH) and (B) 2,2'-azinobis(2-ethylbenzothiazoline-6-sulfonate) (ABTS) of ternary complexes at different pH values. Lowercase letters indicate significant difference ($P < 0.05$) between samples at different pH values. uppercase letters indicate significant difference ($P < 0.05$) between samples at same pH values. WPC = whey protein concentrate; HMP = high methoxyl pectin; CA = chlorogenic acid; RA = rosmarinic acid.

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