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6 **Establishment of a stable complex formed from whey protein isolate and**
7 **chitosan and its stability under environmental stresses**
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Abstract

This study aimed to investigate the stability of a complex formed with whey protein isolate (WPI) and chitosan under environmental stress. The optical density, particle size, zeta potential, chemical characteristics, electrostatic interactions, and surface morphology were evaluated for the stable complexes; the optimum conditions for the generation of the stable complex were 0.2% (wt/wt) whey protein with 0.05% (wt/wt) chitosan at pH 5.7. Under these conditions, the complex particle size was 217.8 ± 11.3 nm and the zeta potential was 16.7 ± 0.92 mV. The complex was formed through electrostatic interactions between the amine groups of chitosan ($-\text{NH}_3^+$) and carboxyl groups of whey protein ($-\text{COO}^-$), and contained a porous network interspaced by heterogeneously sized vacuoles. The complex displayed stable physiochemical characteristics under environmental stresses including NaCl (0–75 mM) or sugar (0–5%) at ambient temperature and upon heating for 15 min at 25–65 °C, up to 65 °C for 30 min. Moreover, the complex could be stably stored for 30 d at 4 °C and for 20 d at 25 °C. The present results provide theoretical insights into the industrial production of chitosan-protein complexes and for microencapsulation of sensitive food or medicinal ingredients to increase their intestinal absorption.

Key words: Whey protein isolate; Chitosan; Complex stability; Environmental stress

1 Introduction

Chitosan (CN) is a unique cationic polysaccharide with mucoadhesive and biological activity [1-3] and is extensively used in theoretical medicine and food processing [4]. When dissolved in aqueous solutions at $\leq$ pH 6.5, CN acquires a positive charge owing to protonation of its amino residues, which forms the basis of immobilization of core substances such as protein, drugs, and other nutrients [5]. In recent years, numerous complexes based on CN have emerged through coacervation, including CN-gum Arabic [6], CN-hsian-tsao [7], gum CN-casein [8], CN-carboxymethyl konjac glucomannan [9], and CN-canola protein isolate [10].

Whey proteins are frequently used in food systems. They aggregate among themselves and with other food particles (e.g., casein micelles or emulsion) upon thermal denaturation [1, 2]. In particular, they can interact with polysaccharides to form soluble or insoluble complexes, depending on the colloidal properties of the protein-polysaccharide systems. These properties are associated with not only the individual functionality of proteins and polysaccharides, but also the nature and strength of their interactions. Whey protein isolates (WPI) are filtered whey proteins containing protein-rich in α -lactalbumin and β -lactoglobulin, with less than 1% lactose and 1% milk fat. WPI harbors charged hydrophilic and hydrophobic regions and is used to stabilize food emulsions, which potentially increase the bioavailability and stability of hydrophobic nutrients [11, 12]. However, WPIs are sensitive to thermal processing [13], pH, and ionic strength [14, 15], thus limiting their application. In particular, WPIs and polysaccharides can interact to form soluble or insoluble complexes, depending on the colloidal properties of the protein-polysaccharide system.

Numerous studies have focused on encapsulation techniques with proteins and polysaccharides and their complexes [11, 16-18]. In particular, the phase behavior of CN-protein complexes and the effects of pH, ionic strength, and macromolecular structure on the formation of CN-protein complexes including ovalbumin-CN, pea protein isolate-CN, glycinin-CN [19-21], and caseinate-CN [5] have received increasing attention. Recently, de Souza et al. explored the interaction between WPI and CN at different deacetylation degrees and reported that the WPI-CN interactions were the strongest at pH 6 [22]. Furthermore, Laplante et al. reported that 0.1% CN with WPI/CN >5 formed a soluble complex at pH 5.5 [23].

In this study, we comprehensively analyzed (**Scheme. 1**) the optimum concentration,

WPI-CN interactions, and their characteristics and stability under different environmental stresses, to formulate a stable complex resilient during thermal processing, with a prolonged shelf life and extensive industrial applications.

2 Material and methods

2.1 Materials and reagents

WPI containing 97.5% protein was kindly provided by Jinan SAN Chemical Co., Ltd (Jinan, China). CN (75–85% deacetylation degree) was obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). All other chemicals were of analytical grade. Ultrapure water was obtained from a Milli-Q water filtration system. A WPI (1 wt%) stock solution was prepared by dissolving 10 g of WPI in 980 g of distilled water; thereafter, 10 g of 0.02 wt% NaN_3 was added into the solution, stirred with a magnetic stirrer (200 rpm) for 3 h at ambient temperature, and stored at 4 °C. Similar to the 1 wt% WPI stock solution, a 0.25 wt% CN stock solution was prepared by dissolving 2.5 g of CN and 10 g of NaN_3 in 987.5 g of 1% acetic acid. Individual WPI (0.05 wt%, 0.1 wt%, 0.2 wt%, 0.3 wt%, 0.4 wt%, 0.5 wt%, and 1 wt%) and CN (0.01 wt%, 0.02 wt%, 0.03 wt%, 0.04 wt%, 0.05 wt%, 0.1 wt%, 0.15 wt%, and 0.2 wt%) solutions were prepared (Data in Brief, Section 1-2) [45]. Thereafter, WPI and CN complexes of different concentrations were prepared at different pH 3–7, and the WPI solution was gradually added to the CN solution (Data in Brief, Section 3) [45] and stirred using a magnetic stirrer for 2 h (150 rpm) and finally stored overnight at 4 °C for subsequent experiments.

2.2 Measurement of absorbance, particle size, and zeta potential of the WPI-CN complex

The absorbance, particle size, and zeta potential of individual WPI, CN, and their complex were determined at different pHs (3, 4, 5, 5.7, 6.5, and 7), NaCl concentrations (0, 25, 50, 75, 100, 150, 200, 250, and 300 mM), and sugar concentrations (0~5%) for 15 min at different temperatures (4–95 °C), heating at 65 °C up to 1 h, and storage at 4 °C or 25 °C for up to 30 d (Data in Brief, Sections 4-6) [45]. The absorbance was measured using an AGILENT 8453 UV-visible spectrophotometer at 500 nm. Droplet size was determined through dynamic light scattering with a Zetasizer Nano-ZS90

(Malvern Panalytical, Malvern, UK) at a detection angle of 90° to reduce multiple scattering effects. The zeta potential was determined using a Zetasizer Nano-ZS90 and analyzed with its software (Malvern Panalytical, Malvern, UK) based on the electrophoretic mobility of complexes. To minimize multiple scattering effects, samples were diluted 100-fold with deionized water before each measurement. Each measurement was obtained in triplicate. Mean $\pm$ standard deviation values were determined for the particle size and distribution. The polydispersity index (PDI) is the distribution of molecular mass in a given polymer number sample. It is calculated by the weight of average molecular weight (M_w) to the number of average molecular weight (M_n). It is used to indicate the broadness of molecular weight distribution. The larger the PDI, the broader the molecular weight. The software of the machine could automatically calculate both M_w and M_n .

2.3 Fourier transform infrared spectroscopy (FTIR), X-ray diffraction, rheological analysis, and assessment of surface morphology

Individual freeze-dried samples of CN, WPI, and WPI-CN complexes were used for these measurements. Samples were mixed with a pure KBr pellet and compressed on a transparent sheet. The infra-red (IR) spectra were measured via FTIR in the region of $400\text{--}4000\text{ cm}^{-1}$ (Perkin-Elmer, Beaconsfield, UK). X-ray diffraction was performed using an advanced X-ray diffractometer. An appropriate amount (about 10–15 mg) of lyophilized samples of WPI, CN, and WPI-CN complexes was placed on a sample plate and flattened using a glass slide. The polymethyl methacrylate holder was filled with the sample and scanned at diffraction angle of $2^\theta=5^\circ\text{--}60^\circ$ at $5^\circ/\text{min}$. Rheological analysis was performed using a controlled stress rheometer AR2000 (TA Instruments Inc., New Castle, DE, USA) at a shear rate of $10^{-3}\text{--}2000\text{ Hz/s}$ at 25°C for sample equilibration (15–17 g), and the mixtures were retained at ambient temperature for 15 min before each measurement. Surface morphology was assessed using a Hitachi S-3400N scanning electron microscope at an accelerated voltage of 10 kV. The sample was lightly sprinkling on an aluminum stub, covered with double-sided tape, and the stub was coated with a gold sputter coater. The sample was then visualized.

2.4 Assessment of the stability of the WPI-CN complex at different pH, NaCl and sugar concentrations, heating temperatures, thermal processing conditions, and different storage temperatures

The stability of the WPI-CN complex (20 g) was assessed under the following environmental conditions. 1) The pH of the mixtures was adjusted to pH 4, 5, 5.7, 6.5, and 7 with NaOH or 1% citric acid in a 50-ml centrifuge tube and stored at 4 °C for 24 h before analysis. 2) NaCl was added into the complex solution to a final concentration of 0, 25, 50, 75, 100, 150, 200, 250, and 300 mM; sugar, 0, 1%, 2%, 3%, 4%, and 5%. Thereafter, the complex solution was stored in 50-ml tubes at 4 °C before analysis. 3) The complex solutions were incubated for 15 min at 4 °C, 25 °C, 35 °C, 45 °C, 55 °C, 65 °C, 75 °C, 85 °C, and 95 °C and then immediately cooled to 4 °C before further analysis, as previously reported [24]. 4) The complex solutions were heated in a water bath at 65 °C for 0, 15, 30, 45, and 60 min and immediately cooled to 4 °C before further analysis. 5) The mixtures were stored at 4 °C and 25 °C for 0, 5, 10, 15, 20 and 30 d. Thereafter, the absorbance, particle size, and zeta potential of the WPI-CN complex samples were determined to assess their stability.

3 Results and discussion

3.1 The optimum WPI and CN concentrations and appropriate pH for forming a stable WPI-CN complex

To form a stable WPI-CN complex, the effects of pH and polymer concentration on the absorbance, zeta potential, particle size, and PDI of WPI-CN complexes were investigated, and the respective results of the mixtures of 0.2 wt% WPI with CN at different concentrations (0.01 wt%; 0.02 wt%; 0.04 wt%; 0.05 wt%; 0.1 wt%; and 0.2 wt%) are shown in **Fig. 1 (A-D)**.

The pH of the emulsion potentially affects the coacervation strength by influencing the charge intensity of biopolymers [5]. Turbidity or the sediment was observed in complex of 0.2 wt% WPI with 0.01 wt% CN at $\text{pH} \geq 5.0$, with 0.02 wt% CN at $\text{pH} \geq 5.7$, with 0.04 wt% CN and 0.05 wt% CN at $\text{pH} > 5.7$, and with 0.1 wt% CN at $\text{pH} > 5.7$. However, complex of 0.2 wt% WPI and 0.2 wt% CN were always clear at all pH

values (See **Data in Brief, Section 8**) [45]. Anal [5] reported that stable complexes were formed without phase separation or sediments. These results indicate that the emulsion of the complexes of 0.2 wt% WPI interacted with 0.04–0.2 wt% CN at pH 4–5.7 without phase separation or sediments and remained stable.

The pH of the emulsion strongly affects the charge density of biopolymers and the strength of their interactions. Because WPI harbors negative charges only beyond its isoelectric point, i.e., pH 4.9 [25], CN can only be dissolved in acidic solutions. In a narrow range of pH (5.34–6.5), negatively charged WPI and positively charged CN would potentially interact with one another through electrostatic interactions [26, 27] below the dissociation constant of CN ($pK_a = 6.5$) and beyond the isoelectric point of WPI. Previous studies have reported that at pH 5.3–6.5, CN and β -Lg prefer to interact with each other through electrostatic attractions, since they are oppositely charged [28]. At pH 4.0, the emulsion was almost clear, since WPI and CN harbored similar charges, indicating towards the dissociation of the WPI-CN complexes.

The zeta potential of the complexes of 0.2 wt% WPI with 0.1 wt% CN and 0.2 wt% CN were stable at pH 4–7 and that of complexes of WPI with 0.04 wt% or 0.05 wt% CN (**Fig. 1B**) was stable at pH 4–5.7, while that of complexes of 0.2 wt% WPI with 0.01 wt% and 0.02 wt% CN decreased with an increase in pH from pH 4 to pH 7. Furthermore, at the same pH, the zeta potential increased with an increase in the CN concentration, indicating that owing to complex interactions between WPI and CN, treatment of WPI with CN significantly increased the charge density on the surface of the complexes. This could partly explain the aforementioned findings (also indicated in the **Data in Brief, Section 8**). The sediment was formed in the complex of 0.2 wt% WPI with 0.01 wt% CN at $pH \geq 5.7$, with CN (0.01 wt% to 0.02 wt%) at $pH \geq 6.5$, with CN (0.01–0.05 wt%) at pH 7, but not in an emulsion of 0.2 wt% WPI and 0.1 wt% CN at pH 4–5.7; however, the emulsion of 0.2 wt% WPI and 0.2 wt% CN was always clear at tested pH values, indicating that WPI and CN concentrations are important to maintain a stable zeta potential, which is important for the stability of the complex.

The average diameter and PDI of the particles of the complex comprising 0.2 wt%

WPI with CN (0.01~0.2 wt%) at different pHs is depicted in **Fig. 1C-D**. PDI is used to assess the range of the molecular weight distribution determined as the polymer mass/molecular weight. The smaller PDI, the better the particle dispersion. The particle size of the complexes gradually decreased with an increase in pH from pH 4.0 to pH 5.7, then increased with an increase in pH from pH 5.7 to pH 7. Mixtures of 0.2 wt% WPI and CN (0.04~0.2 wt%) displayed a smaller average particle size ranging 200–330 nm, and their diameter distribution was relatively uniform at pH 5.7. Therefore, to maximally strength interactions between WPI and CN, pH 5.7 was selected for the analysis.

The biopolymer concentration markedly influenced the aggregation and stability of the complex. Their effects on the absorbance, zeta potential, and particle size of their complexes at pH 5.7 are shown in **Fig. 2(A-D)**. Mixtures of WPI (0.4~1 wt%) and CN (0.01~0.2 wt%), 0.1 wt% WPI and 0.01 wt% CN, 0.2 wt% WPI and CN (0.01~0.03 wt%), and 0.3 wt% WPI and CN (0.01~0.05 wt%) were unstable, as the turbidity and sedimentation were observed (data not shown). Complexes containing WPI and CN at other concentrations were stable. This abrupt increase and reduction in absorbance (**Fig. 2A**) is primarily attributed to the phase separation (aggregation of particles); sedimentation, owing to electrostatic bridging effects and charge neutralization.

The zeta potential of mixtures of WPI (0.05–1 wt%) with CN (0.01–0.2 wt%) at pH 5.7 are depicted in **Fig. 2B**. The zeta potential increased with an increase in the CN concentration, based on the WPI:CN ratio, indicating that complexation occurred through electrostatic interactions [22]. The average diameter of the particles of WPI (0.05–0.5 wt%) and CN (0.01–0.2 wt%) at pH 5.7 is depicted in **Fig. 2C**. The size of particles comprising 0.2 wt% WPI and CN (0.03 wt%, 0.04 wt%, and 0.05 wt% [see the framed image (**Fig. 2C**)] was significantly smaller than those comprising WPI and CN at other concentrations; in particular, the size of particles comprising 0.2 wt% WPI and 0.05 wt% CN was the smallest (217.8 ± 11.3 nm), and these particles had the most uniform size distribution and the lowest PDI value, and their zeta potential was 16.7 ± 0.92 mV. Masarudin et al. [29] reported that greater cross-linking between

the anionic groups of the WPI and the free primary amino groups of CN led to more homogeneous particles. These results indicate that except for pH, the biopolymer ratio markedly influenced the aggregation and the stability of the complexes. Based on these results, complexes were prepared using 0.05 wt% CN and 0.2 wt% WPI at pH 5.7 and their characteristics and stability were further investigated.

3.2 Characterization of the complex formed by WPI-CN interactions

The IR spectra, X-ray diffraction, and micrographs of particles comprising 0.05 wt% CN and 0.2 wt% WPI and their complex were further assessed. The IR spectra for WPI (**Fig. 3Aa**) displayed absorption bands associated with C=O stretching at 1646 cm^{-1} and N-H bending (amide II) at 1533 cm^{-1} . The absorption band at 1398 cm^{-1} was attributable to N-H bending and C-N stretching (amide III) vibrations [30]. The IR spectra for CN (**Fig. 3Ab**) displayed robust amino characteristic peaks at approximately 3437 cm^{-1} , stretching vibration of the C-H bond at 2936 cm^{-1} and a strong band associated with the amide III ($-\text{NH}_3^+$ groups) at 1578 cm^{-1} . The band contributes to vibration of the -OH and -CH groups at 1411 cm^{-1} , the symmetrical stretching of C-O-C at 1152 cm^{-1} and C-O stretching vibration at 1043 cm^{-1} [31]. The IR spectra of the WPI-CN complex (**Fig. 3Ac**) displayed a band attributable to the stretching vibrations of $-\text{NH}_3^+$ and $-\text{COO}^-$ groups at 1579 cm^{-1} . The spectra of the complexes in the carbonyl-amide region were altered owing to the interaction between WPI and CN (**Fig. 3Ac**). The obliteration of asymmetric and $-\text{COO}^-$ stretching vibration at 1646 and 1533 cm^{-1} of WPI and the peak of $-\text{NH}_3^+$ groups at 1578 cm^{-1} of CN slightly changed in the complexes, respectively, indicating the electrostatic interaction between the amine groups of CN ($-\text{NH}_3^+$) and the carboxyl groups of WPI ($-\text{COO}^-$). Furthermore, the spectra of the WPI-CN complex compared to that of WPI and CN displayed a broad band at approximately $3000\text{--}3600\text{ cm}^{-1}$, which enhanced hydrogen bonding. These results indicate that hydrogen bonding was involved during the interaction between WPI and CN.

De Queiroz et al. [32] reported that X-ray diffraction helps analyze the chemical interactions between WPI and CN through its variation in the structure of the material,

which affect the physical properties of the material. **Fig. 3B** a, b, and c show the X-ray diffraction pattern for WPI, CN, and their complexes, respectively. At pH 5.7, WPI displayed diffraction signals at 8° and 20° with in the predominantly amorphous state, the CN had a crystalline structure characterized by sharp peaks at 9°, 20°, 26°, and 30°. The complexes of WPI and CN presented a crystalline structure, indicating the presence of crystalline planes in the biopolymer material matrices. Compared with WPI and CN, the board peak of WPI was obliterated, the intensity of the sharp peak increased at 9° and decreased at 20°, 26°, and 30°. Therefore, intermolecular interactions between pure polymers were replaced by interactions between WPI and CN molecules, predominantly with electrostatic interactions [33, 34].

Scanning electron microscope analysis revealed that WPI particles were flat, large, and uneven fragments (**Fig. 4A**), while CN particles resembled broken pieces of thin cloth (**Fig. 4B**). The WPI-CN coacervates displayed a porous network structure interspaced by heterogeneously sized vacuoles (**Fig. 4C**). It is the freezing that perturbs the structure because of the formation of ice crystals. Therefore, the structure seen with scanning electron microscope is not of the complexes as they are in water. However, it is good to see their structure after they were freeze-dried. Huang et al. [35] reported the interactions between soybean protein isolate and CN, resulting in a porous network interspaced by heterogeneously sized vacuoles. The interaction between two oppositely charged macromolecules yields a complex condensed network entrapping water molecules in the coacervate phase, thus increasing the complexity of the structure.

3.3 Stability of the complexes under various environmental stresses

The stability of WPI-CN complexes was further investigated when its emulsion contained NaCl (0~300 mM, **Fig. 5**) or sugar (0~5%, **Fig. 6**), incubated 15 min at different temperatures (4~95 °C) (**Fig. 7**), heated at 65 °C for up to 60 min (**Fig. 8**), at different pHs (pH 3~7) (**Fig. 9**) and stored for different durations at 4 °C or 25 °C (**Fig. 10**). As shown in **Fig. 5**, on increasing the NaCl concentration from 0 mM to 75 mM, the absorbance, particle diameter, and zeta potential of the WPI-CN complex at pH 5.7 increased, but decreased at NaCl concentrations of >75 mM. As expected, complexation was affected by the ionic strength. With <75 mM NaCl, Na⁺ and Cl⁻ ions promoted the

complexation between WPI and CN. However, at NaCl concentrations >75 mM, the interaction was gradually inhibited. Weinbreck et al. [36] reported the same trend for the WPI–carrageenan coacervation system. McClements [37] reported that attractive interactions among the complexes, van der Waals interactions, and hydrophobic interactions were insufficient to overcome the repulsive force (space, statics and fluctuation) in an emulsion system. Attractive interatomic forces led to droplet aggregation; however, repulsive interactions among similar charges opposed droplet aggregation. The WPI-CN complex exhibited high stability with 0–5% sugar (**Fig. 6**). The absorbance, the zeta potential, and diameter of the particles increased from 0 to 1% with an increase in the sugar concentration and remained consistent at sugar concentrations greater than 1%. As expected, the sugar concentration did not significantly affect the stability of the WPI-CN complex.

The absorbance slightly increased with an increase in temperature from 4 °C to 65 °C, while the zeta potential slightly decreased, and the particle diameter remained constant. At temperatures greater than 65 °C, the absorbance, the zeta potential, and the particle diameter increased linearly with an increase in temperature (**Fig. 7**). Liu et al. [38] reported that hydrogen bonding, hydrophobic interactions, and electrostatic attractions are involved in protein–polysaccharide complexation. Generally, the extent of hydrogen bonding in protein–polysaccharide coacervates increases at lower rather than higher temperatures, at which hydrophobic interactions become more prevalent [39]. For WPI and CN interactions, the high temperature weakened the strength of coacervation. This temperature-dependent interaction was also observed in β -lactoglobulin and low/high-methylated pectin pairs, wherein elevated temperatures decreased the coacervation strength of β -lactoglobulin and highly methylated pectin complexes [40].

The absorbance, the zeta potential, and particle diameter significantly increased with an increase in the treatment duration at 65 °C, although no stratification or sedimentation were observed, and turbidity increased with an increase in the treatment duration (**Fig. 8**). These results indicate that thermal treatments reduced the stability of WPI-CN mixtures owing to the denaturation of WPI in the complex or free WPI in solution heating at 65 °C [41].

The effects of pH on the stability of the complex prepared from 0.05 wt% CN and

0.2 wt% WPI are depicted in **Fig. 9**. On changing the pH from pH 4.0 to pH 5.7, the absorbance of the mixture did not change significantly; however, the zeta potential drastically decreased from 25.23 ± 0.50 mV to 15.15 ± 0.79 mV ($p < 0.01$), and the particle diameter significantly decreased from 589.1 ± 31.33 nm to 264.65 ± 34.66 nm ($p < 0.01$). However, the absorbance, zeta potential, and particle diameter markedly increased at $> \text{pH } 5.7$. Phase separation and sedimentation were observed at pH 7. These results indicate that the WPI-CN complexes exhibited high stability under acidic conditions at pH 3.0–5.7, primarily because WPI and CN harbor positive charges and display limited interactions under these conditions. However, on increasing the pH to pH 6.5 and pH 7, approaching the pKa of CN, the mixtures were destabilized and sedimentation occurred, and at $\leq \text{pH } 6$, the amino group in CN started losing its positive charge, thus losing its protection and resulting in the loss of the electrostatic repulsion in CN, thereby decreasing the solubility of CN [8]. In **Figure 1B** and **Figure 9B**, the zeta potential of complexes of 0.2%WPI with either 0.1% or 0.2% CN were positive at pH 7. The appropriate condition to form the complexes was 0.2%WPI-0.05%CN at pH 5.7, in which there is no precipitation, but a few precipitation was appeared at pH 6.5, and more at pH 7. In addition, these also appeared similar when 0.2%WPI-0.1%CN or 0.2%WPI-0.2%CN at pH 5.7, 6.5 and 7. This may be caused by the excess CN particles existed in the complexes emulsion due to their inappropriate ratio to form the complexes at the pH 6.5 and 7. Furthermore, the zeta potentials of individual 0.05%, 0.1%CN, and 0.2%CN solution were positive with obviously higher at pH 7 than at pH 6.5 ((See **Data in Brief, Section 26**) [45]. This also explains why the zeta potential of complexes of both the 0.2%WPI-0.1%CN and 0.2%WPI-0.2%CN was positive when the pH was greater than 5.7.

The stability of WPI-CN mixtures stored at 4 °C and 25 °C is depicted in **Fig. 10**. The absorbance of the complex remained constant for 15 d at 4 °C and then significantly increased on day 20; the zeta potential slightly but not significantly decreased during 30 d; the particle diameter remained stable during the first 20 d the then significantly increased from 200.6 ± 17.26 nm on day 0 to 274.85 ± 8.84 nm ($p < 0.01$) on day 30, indicating that the mixture was stable for up to ~30 d at 4 °C (**Fig. 10A, C**). At 25 °C,

the absorbance significantly increased with an increase in the storage duration after day 10 ($p < 0.05$), and the zeta potential remained unchanged between days 0 and 20, followed by a reduction from 16.35 ± 0.92 mV at day 0 to 11.8 ± 0.57 mV on day 30 ($p < 0.01$), while the particle diameter remained consistent for 20 d and then significantly increased from 209.55 ± 17.26 at day 0 to 298.35 ± 20.58 nm upon 30-d storage ($p < 0.01$) (**Fig. 10B, D**), indicating that the mixture was stable for at least 20 d at 25 °C.

In general, at a higher relative zeta potential, the dispersion is more stable. At pH 5.7, WPI-CN complex had adequate zeta potentials, indicating their potential to be stable during storage. With an increase in the storage duration, the zeta potential for WPI-CN complexation at a storage temperature of 4 °C and 25 °C decreased. These results indicate that the stability of the complex is decreased with an increase in the storage duration. However, the stability of the complex was higher upon storage at 4 °C than at 25 °C, indicating that temperature plays an important role in the stability of complex during storage. Kenar et al. [42] reported that at a high temperature, the frequency of the particle collisions is increased, thus promoting particle aggregation.

Figs. 10G, H show the flow curves of the WPI-CN complex upon increasing the shear rate during storage at different temperatures. Viscosity decreased with an increase in the shear rate, and when the shear rate approached a certain value, the viscosity tended to stabilize. These results indicate that the composite exhibits shear olefination, indicating that the complex has characteristics typical of non-Newtonian fluids, probably owing to changes in the structure of the coacervates owing to the induction of the shear rate. Electrostatic interactions between WPI and CN could have led to an attraction of the protein and polysaccharide chains, thus potentially disrupting the deformation of the polysaccharide [42]. However, with an increase in the storage duration at 4 °C, the viscosity was markedly increased, and markedly increased at 25 °C. Moreover, the zeta potential of the complex decreased with an increase in the storage duration at both 4 °C and 25 °C (**Fig. 10C**, between days 0 and 30, $p > 0.05$; **Fig. 10D**, between days 0 and 30 to day 30, $p < 0.01$), probably because of the changes in the complex, with the zeta potential decreasing, the internal structure of the composite was more dense, thus increasing the viscosity [43]. Niu et al. [44] reported that the viscosity primarily increased owing to stronger interactions between ovalbumin and gum Arabic

and since the particles aggregated more easily when their surface charges were close to neutral. Furthermore, Weinbreck et al. [43] reported that the viscosity of the composite is associated with the degree of electrostatic interactions between the materials. The stronger the electrostatic interaction, the greater the viscosity of the composite.

4 Conclusions

This study systematically investigated the characteristics and stability of the nanoparticle complex developed from WPI and CN. The stability of the complexes was maximum when they were prepared using 0.2 wt% WPI and 0.05 wt% CN at pH 5.7. The particle size of the complex was 217.8 ± 11.3 nm, and its zeta potential was 16.7 ± 0.92 mV. The complex was formed through electrostatic interactions between the carboxyl groups of WPI ($-\text{COO}^-$) and the amine groups of CN ($-\text{NH}_3^+$). The freeze-dried complex was a porous microstructure interspaced by heterogeneously sized vacuoles. The physiochemical characteristics of the complex were stabilized 1) with NaCl (0–75 mM), 2) sugar (0–5%), 3) heating for 15 min at 25–65 °C, 4) at thermal processing at 65 °C for 30 min, 5) exhibiting high stability under acidic conditions at pH 3.0–5.7, and 6) stable upon storage at 4 °C for up to 30 d and 25 °C for up to 20 d.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Scheme 1. Schematic representation of the study outline.

Fig. 1 Effects of pH and polymer concentration on the absorbance (A), zeta potential (B), particle diameter (C), and polydispersity index (PDI) (D) of whey protein isolate-chitosan (WPI-CN) mixtures prepared with 0.2 wt% WPI with different concentrations of CN (●, 0.01 wt%; ■, 0.02 wt%; *, 0.04 wt%; ◆, 0.05 wt%; ▲, 0.1 wt%; ×, 0.2 wt%) at different pH.

Fig. 2 Effects of polymer concentration on the absorbance (A), Zeta potential (B), particle diameter (C) and polydispersity index (PDI) (D) of whey protein isolate-chitosan (WPI-CN) mixtures, described as the absorbance of mixtures of various concentrations of WPI (◆, 0.05 wt%; ■, 0.1 wt%; ▲, 0.2 wt%; ×, 0.3 wt%; +, 0.4 wt%; —, 0.5 wt%; ●, 1 wt%) and with various concentrations of CN (0.01 wt% ~ 0.2 wt%) at pH 5.7.

Fig. 3 Fourier transform infrared spectra (A) and the X-ray diffractograms (B) for 0.2 wt% whey protein isolate (WPI) (a), 0.05 wt% chitosan (CN) (b) and 0.2 wt% WPI-0.05 wt% CN complexes (c) at pH 5.7.

Fig. 4 Scanning electron micrographs of freeze dried 0.2 wt% whey protein isolate (WPI) (A), 0.05 wt% chitosan (CN) (B), and 0.2 wt% WPI-0.05 wt% CN complexes (C). (Magnification, 200×).

Fig. 5 Effect of NaCl concentrations on the absorbance (A), the particle diameter, and zeta potential (B); the morphology (C); the particle size distributions (D) of 0.2 wt% whey protein isolate (WPI)-0.05 wt% chitosan (CN) complexes at pH 5.7.

Fig. 6 Effects of sugar concentration on the absorbance (A), the particle diameter, and zeta potential (B); the morphology (C) ; the particle size distributions (D) of 0.2 wt% whey protein isolate (WPI)-0.05 wt% chitosan (CN) complexes at pH 5.7.

Fig. 7 Effects of temperature on the absorbance (A), the particle diameter, and zeta potential (B); the morphology (C); the particle size distributions (D) of 0.2 wt% whey protein isolate (WPI)-0.05 wt% chitosan (CN) complexes at pH 5.7.

Fig. 8 Effects of heat treatment duration on the absorbance (A), the particle diameter (B), and zeta potential; the morphology (C); the particle size distributions (D) of 0.2 wt% whey protein isolate (WPI)-0.05 wt% chitosan (CN) complexes at pH 5.7.

Fig. 9 Effect of pH on the absorbance (A), the particle diameter, and zeta potential (B); the morphology (C); the particle size distributions (D) of 0.2 wt% WPI-0.05 wt% CN complexes.

Fig. 10 Effect of storage at 4 °C and 25 °C on the absorbance (A, B), the particle diameter and zeta potential (C, D); the polydispersity index (E, F) and flow curves of viscosity (G, H) of 0.2 wt% whey protein isolate (WPI)-0.05 wt% chitosan (CN) complexes.

Figures

Establishment of a stable complex formed from whey protein isolate and chitosan and its stability under environmental stresses

Weili Xu, Yinchao Tang, Yang Yang, Guijie Wang, Shaobo Zhou

Scheme 1

**Establishment of a stable complex formed from whey protein isolate and
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Figure 9

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Figure 10

Fig. 10.