

Effect of chlorogenic acid covalent conjugation on the allergenicity, digestibility and functional properties of whey protein

Haoxie Xu¹, Tingting Zhang¹, Yuqin Lu, Xiao Lin, Xiaopeng Hu, Lizhong Liu, Zhendan He, Xuli Wu*

School of Medicine, Shenzhen University, Shenzhen, Guangdong Province 518060, PR China

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ABSTRACT

We investigated the allergenicity, digestibility and functional properties of whey protein isolate (WPI) after covalent conjugation with chlorogenic acid (CHA). The covalent conjugation of CHA may cause an unfolded protein structure. The WPI-CHA conjugate showed lower IgE binding capacity but higher intestinal digestibility than unmodified WPI. Furthermore, after digestion, the IgE binding capacity of β -lactoglobulin and α -lactalbumin was lower in the digested WPI-CHA conjugate than digested WPI. Moreover, the solubility, emulsifying activity, foaming properties and antioxidant capacity of WPI were enhanced by covalent conjugation of CHA. Covalent conjugation with CHA might reduce the allergenicity *in vitro* of WPI by improving the functional properties of the protein.

1. Introduction

Whey protein isolate (WPI) is an important food ingredient, with versatile functional properties and high nutrition, and is widely used in the food industry (Smithers, 2015). However, the main proteins in WPI, β -lactoglobulin (β LG) and α -lactalbumin (α LA), are the main allergens in cow's milk (Restani, Ballabio, Cattaneo, Isoardi, Terracciano, & Fiocchi, 2004). Bovine serum albumin (BSA) in WPI is also a potential allergen (Restani et al., 2004). Cow's milk protein allergy is a common food allergy, the most prevalent allergy for young children and infants (Fiocchi et al., 2010). Because of its high allergenicity, WPI has greatly limited application in the food industry, especially for infant formula. Therefore, strategies for modifying or destroying the allergens in WPI have wide interest.

Some processing technologies such as heat treatment, high pressure, glycation, lactic acid fermentation and enzymatic hydrolysis can be used to reduce the allergenicity of milk proteins (Bu, Luo, Chen, Liu, & Zhu, 2013). However, these measures to control milk allergy have not been satisfactory (Villa, Costa, Oliveira, & Mafrá, 2018). Enzymatic hydrolysis is the technique most used for hypoallergenic formulas. However, while totally eliminating the protein allergenicity, the method also affects the sensory quality of formula, leading to a bitter taste (El Mecherfi et al., 2011). Therefore, to control milk allergy, novel methods to reduce the allergenicity of milk proteins must be found.

Dietary polyphenols can covalently or non-covalently interact with protein. Recently, it is found that some dietary polyphenols were able to interact with milk protein to form complexes, which may alter the protein functional properties (Ozdal, Capanoglu, & Altay, 2013; Budryn et al., 2015). The use of binding polyphenols to modify the structure of proteins might change the allergenic properties. Therefore, the interaction between dietary polyphenols and protein has been used to reduce the allergenicity of some food allergens. Pessato et al. reported that complexation with epigallocatechin gallate (EGCG) might reduce the IgE binding capacity of WPI (Pessato et al., 2018). However, these non-covalent protein-polyphenol interactions are reversible and might be easily affected by pH as well as salt, temperature and concentration, thus limiting the application for food processing. In contrast, for food processing, covalent protein-polyphenol interactions may generate stable covalent conjugates (Ozdal et al., 2013).

Most studies of covalent milk protein-polyphenol interactions are mainly related to the effect of the interaction on functional properties such as foaming and emulsifying properties of the proteins. Ali et al. found that some polyphenols formed covalent conjugates with WPI to improve its functional properties (Ali, Keppler, Coenye, & Schwarz, 2018; Jia et al., 2016). However, studies of the effect of whey-polyphenol covalent conjugates on the allergenicity of whey are scarce. In this study, we chose chlorogenic acid (CHA), the major active principle polyphenol in coffee bean and citrus fruits (Tajik, Tajik, Mack, & Enck,

* Corresponding author at: School of Medicine, Shenzhen University, Nanhai Ave 3688, Shenzhen, Guangdong 518060, PR China.

E-mail address: wxl@szu.edu.cn (X. Wu).

¹ Haoxie Xu and Tingting Zhang contributed equally to this work.

2017), to covalently interact with WPI. The IgE binding capacity, digestibility and functional properties of the WPI-CHA conjugate were investigated.

2. Materials and methods

2.1. Materials

CHA (purity > 99%), 1,10-diphenyl-2-picrylhydrazyl (DPPH) 2,2-azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), 3,3'-5,5'-tetramethylbenzidine (TMB) and Folin-Ciocalteu's phenol reagent were from Sigma Chemical Co. (St. Louis, MO, USA). ECL Reagent was from GE Healthcare Co. (GE Healthcare, Buckinghamshire, UK). NeutrAvidin-horseradish peroxidase (HRP)-conjugated antibody, HRP-conjugated goat anti-human IgE and mouse IgG-antihuman IgE antibody were from SouthernBiotech (Birmingham, AL, USA). WPI was kindly provided by Davisco Foods International Inc. (Le Sueur, MN, USA).

Serum from 10 children allergic to cow's milk (determined by UniCAP System assay for IgE) was obtained from Shenzhen Children's Hospital. Serum from nonallergic individuals (n = 3) was a negative control. All donors gave written consent. The availability of the serum was approved by the Medical Ethics Committee of Shenzhen University.

2.2. Sample preparation

The WPI-CHA covalent conjugate based on free radical grafting were prepared as described (Lu et al., 2018) with a minor change: 1 g WPI was dispersed fully in 200 ml distilled H₂O, then 0.25 g ascorbic acid and 1.0 ml of 5.0 M H₂O₂ were added. After stirred under atmospheric air at 25 °C for 2 h, 0.5 g CHA was added. The mixture was kept at 25 °C for 24 h. The unreacted CHA was removed by dialysis (3-kDa molecular weight cutoffs) with 8 changes of Milli-Q water for 48 h at 4 °C. Finally, the conjugate was obtained by lyophilization.

CHA in the conjugate was determined by the Foline-Ciocalteu method as we described (Lu et al., 2018).

2.3. Characterization of WPI-CHA conjugate

2.3.1. Sodium dodecyl sulfate polyacrylamide gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described (Wu et al., 2018) with minor modification. The sample solution (1 mg/mL) was mixed with loading buffer, then denaturized at 95 °C for 5 min. Then, aliquots (10 µg protein per well) were loaded on a gel (5% stacking gel and 12% separating gel). Electrophoresis was performed at 90 V for 2 h. Finally, proteins were stained for protein visualization with Coomassie Brilliant Blue R-250G, and destained in a H₂O: methanol: acetic solution (5:4:1 by volume).

2.3.2. Measurement of free amino, sulfhydryl thiol (SH) and tyrosine groups

The method based on using trinitro-benzene-sulfonic acid (TNBS) was applied to determine free amino groups in samples using the method previously reported (Adler-Nissen, 1972). The content of SH groups in samples was measured by using 5,5'-dithiobis (2-nitrobenzoic acid) as described (Lu et al., 2018). The procedure using nitric acid was used to determine tyrosine content using the method previously reported (Hassan, 1975).

2.3.3. Fluorescence

The fluorescence of samples was recorded at excitation wavelength 280 nm at 25 °C with a Hitachi-850 spectrofluorometer (Hitachi, Tokyo, Japan). The samples were dissolved in phosphate buffered saline (PBS, pH 7.2) and the protein concentration was 0.1 mg/mL. The emission was collected between 300 and 500 nm, with 5 nm emission and

excitation bandwidths.

2.3.4. Circular dichroism (CD) spectra

CD spectra were measured from 260 nm to 190 nm with a Jasco-810 spectropolarimeter (Jasco, Tokyo, Japan) at 25 °C. The samples were dissolved in PBS (pH 7.2) and the protein concentration was 0.015 mg/mL. Spectra were recorded with scan speed 100 nm/min and step resolution 0.2 nm.

2.3.5. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was performed at 25 °C with spectrophotometer (Shimadzu, Kyoto, Japan) in the range of 4000 cm⁻¹ to 400 cm⁻¹. Spectra were recorded with 32 scans and 4 cm⁻¹ resolution by using KBr pellets.

2.3.6. Differential scanning calorimetry (DSC) measurement

Thermal stability of the samples was measured by DSC with a DSC-60 thermal analyzer (Shimadzu, Tokyo, Japan). About a 5- to 6-mg sample was sealed in a hermetic aluminum pan. Thermal curves were established at a heating rate of 3 °C/min from 30 °C to 180 °C under nitrogen at flow rate 25 ml/min.

2.4. Effect on human IgE binding

2.4.1. Western blot analysis

To analyze human IgE binding, western blot analysis was performed as we described (Wu et al., 2018) with modification. In brief, after underwent SDS-PAGE, samples were transferred to polyvinylidene fluoride membranes and subsequently blocked in 1% fish gelatin. After 2 h, membranes were incubated with a mixture (v:v) of diluted sera (1:30) for 1 h, then washed with PBS containing 0.05% Tween 20 (PBST) 4 times. After incubation with a mouse IgG-antihuman IgE antibody (1:2000) for 1 h, the membranes washed in PBST 4 times and subsequently incubated with the diluted 1:2000 HRP-conjugated antibody for 1 h. After wash, the membranes were incubated with ECL Reagent, then exposed to X-ray film to detect protein bands.

2.4.2. Enzyme-linked immunosorbent assay (ELISA)

Competitive ELISA was performed as we previously described (Lu et al., 2018). In brief, samples were dissolved in PBS (pH 7.2) at 1 mg/mL. Single wells were coated with sample (100 µL) overnight at 4 °C. Plates were washed with PBST, which was used after each incubation step. The plates were blocked in 100 µL 2% fish gelatin. After 2 h, 50 µL sample solution (0.001–100 µg/ml) was added, then 50 µL mixture (v:v) diluted sera (1:30) was added. After incubation for 1 h, 100 µL of the diluted 1:10,000 HRP-conjugated goat anti-human IgE antibody was added. After incubation for 1 h, 50 µL solution of freshly prepared o-phenylenediamine containing H₂O₂ was added to plates and incubated for 15 min. Finally, 50 µL of 2 M H₂SO₄ was added to stop the reaction and optical densities were read at 450 nm.

The inhibition rate was calculated as follow:

$$\text{Inhibition(\%)} = \frac{1 - \text{absorbance value with inhibitor}}{\text{absorbance value without inhibitor}} \times 100$$

2.5. Simulation of digestion

In vitro simulated digestion was performed as described (Amigo-Benavent, Clemente, Ferranti, Caira, & Del Castillo, 2011) with modification. Briefly, for simulated gastric digestion, samples were dissolved in 120 mM NaCl at 0.8 mg/mL and adjusted to pH 2.0 with 5 M HCl. An 8-ml sample solution was mixed with 0.1 ml pepsin solution (2 mg/ml) and 20 ml of 120 mM NaCl. The mass ratio of pepsin: protein: pepsin was 1: 50 [w/w]. Pepsin digestion was carried out at 37 °C in a shaker. Aliquots of the digestion were periodically withdrawn at times of 5 min up to 120 min.

For intestinal digestion, an aliquot (10 ml) of the gastric digestion was withdrawn from simulated gastric digestion at 60 min as described above. The pH was adjusted to 6.0 with 1 M NaHCO₃. Then, 2.5 ml pancreatin-bile salts juice (2.5 mg pancreatin in 25 ml of 100 mM and NaHCO₃ 15 mg bile extract) was added to the aliquot. The mass ratio of pancreatin: protein was 1: 50 [w/w]. The volume of mixture was adjusted to 15 ml with 120 mM NaCl and the pH value was adjusted to 7.5 with 1 M NaOH. The reaction took place in a shaker at 37 °C. Aliquots of the digestion were periodically withdrawn at times of 5 min up to 120 min. The digestion was immediately quenched by heat treatment at 95 °C for 5 min. All aliquots of the digestion were collected for SDS-PAGE and western blot analysis as described above.

2.6. Effect on functional properties of WPI

2.6.1. Solubility

The solubility of WPI and its conjugate was determined as described (Jiang, Chen, & Xiong, 2009). Solubility was defined as the percentage of protein concentration in the supernatant (after centrifugation) to total protein in the sample (before centrifugation). In brief, the protein concentration in the original and in the supernatant samples was measured after centrifugation at 10,000 × g for 20 min.

2.6.2. Emulsifying properties

The emulsifying properties of WPI were measured as described (Huang et al., 2013) with modification. A 10 ml sample of soybean oil was added to 30 ml of 2 mg/ml sample protein sample in 50 mM PBS (pH 8.0). Then the mixture was homogenized for 2 min at 10,000 rpm at 25 °C. Subsequently, a 50-μl homogenized sample was removed at 0 and 10 min and diluted with 25 ml of 0.1% SDS solution. Finally, the absorbance of the diluted emulsion was measured at 500 nm.

The emulsifying stability index (ESI) was calculated as follows:

$$ESI(\min) = \frac{A_0 \times 10\min}{(A_0 - A_{10})}$$

where A₁₀ and A₀ are the absorbance at 500 nm at 10 and 0 min after homogenization, respectively.

2.6.3. Foaming properties

Foaming properties of WPI were measured as described (Huang et al., 2013). Briefly, 3 ml sample solution (2 mg/mL) was stirred at 3200 rpm for 10 min. The volumes at 0 min for foaming capacity (FC) and 30 min for foaming stability (FS) were measured. FC and FS were calculated as follows:

$$FC(\%) = \frac{\text{volume after stirring} - \text{volume before stirring}}{\text{volume before stirring}}$$

$$FS(\%) = \frac{\text{volume after stirring} - \text{volume before stirring}}{\text{volume before stirring}}$$

2.6.4. Antioxidant activities

ABTS⁺ radical scavenging was evaluated according to the method previously reported (Lu et al., 2018). Briefly, ABTS⁺ (7 mM) was prepared by mixing 2.45 mM potassium persulfate (2.45 mM) with 7 mM ABTS⁺ stock solution. After keeping in the dark for 16 h, the ABTS working solution was diluted with 5 mM PBS (pH 7.4) to absorbance 0.7 at 734 nm. An amount of 1 ml of 0.5 mg/mL sample solution was mixed with 3 ml ABTS working solution and then incubated at room temperature for 1 h. Finally, absorbance was measured at 734 nm. A standard curve was created by the method mentioned above by reacting a series of concentrations of Trolox.

DPPH radical scavenging was performed according to the method previously reported (Binsan et al., 2008). In brief, DPPH solution was freshly prepared in methanol at 1.75 × 10⁻⁴ M. An amount of 2 ml DPPH working solution was mixed with 2 ml of 0.5 mg/mL sample.

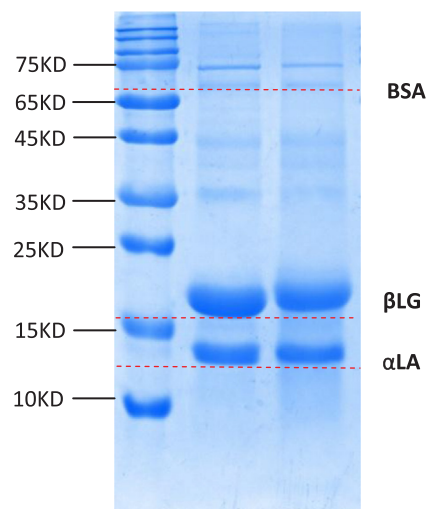


Fig. 1. SDS-PAGE analysis of molecular weights in WPI and WPI-CHA conjugate.

After keeping in the dark for 1 h, absorbance was measured at 517 nm. The calibration curve was prepared by using Trolox as described above.

2.7. Statistical analysis

All the measurements were carried out at least three times and data are expressed as mean ± SD. Differences between groups were assessed by one-way ANOVA. P < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Profile of WPI-CHA conjugate

We determined the change in protein profile of WPI after conjugation with CHA by SDS-PAGE. Three main bands were attributed to BSA (66.0 KDa), βLG (18.2 KDa) and αLA (14.2 KDa) (Fig. 1). As compared with native WPI, the bands corresponding to BSA, βLG and BSA slightly migrated up in after conjugation with CHA, which suggests increased molecular weight. Also, the conjugate was not separated by using mercaptoethanol or SDS, so the bonds formed between the CHA and WPI were covalent.

The mean CHA content of the conjugate was 53.85 ± 2.3 mg/g, which confirmed the conjugation of WPI with CHA. Our finding was similar to previous reports of the modified milk protein showing higher molecular mass than unmodified proteins after covalent conjugation with polyphenol (Wu et al., 2018).

As shows in Table 1, after conjugation with CHA, the content of the free amino and free thiol groups were decreased in WPI. To determine free amino groups in samples, we used TNBS, which can bind to the nucleophilic nitrogen in the ε-amino side chains of lysine. The reaction site for TNBS would be blocked due to the reaction of free amino groups with CHA. SDS was used to prevent non-covalent interactions. Therefore, a reduction in free amine groups suggested that CHA covalently

Table 1
Characterization of WPI and WPI-CHA conjugates.

Property/sample	WPI	WPI-CHA
Free amino groups (nmol/mg protein)	520.9 ± 14.4	469 ± 13.9*
Free thiol groups (nmol/mg protein)	33.4 ± 0.9	29.1 ± 0.5*
Tyrosine group (nmol/mg protein)	21.8 ± 0.4	12.4 ± 0.3*

Data are mean ± SD.

* p < 0.05 between WPI and WPI-CHA conjugate.

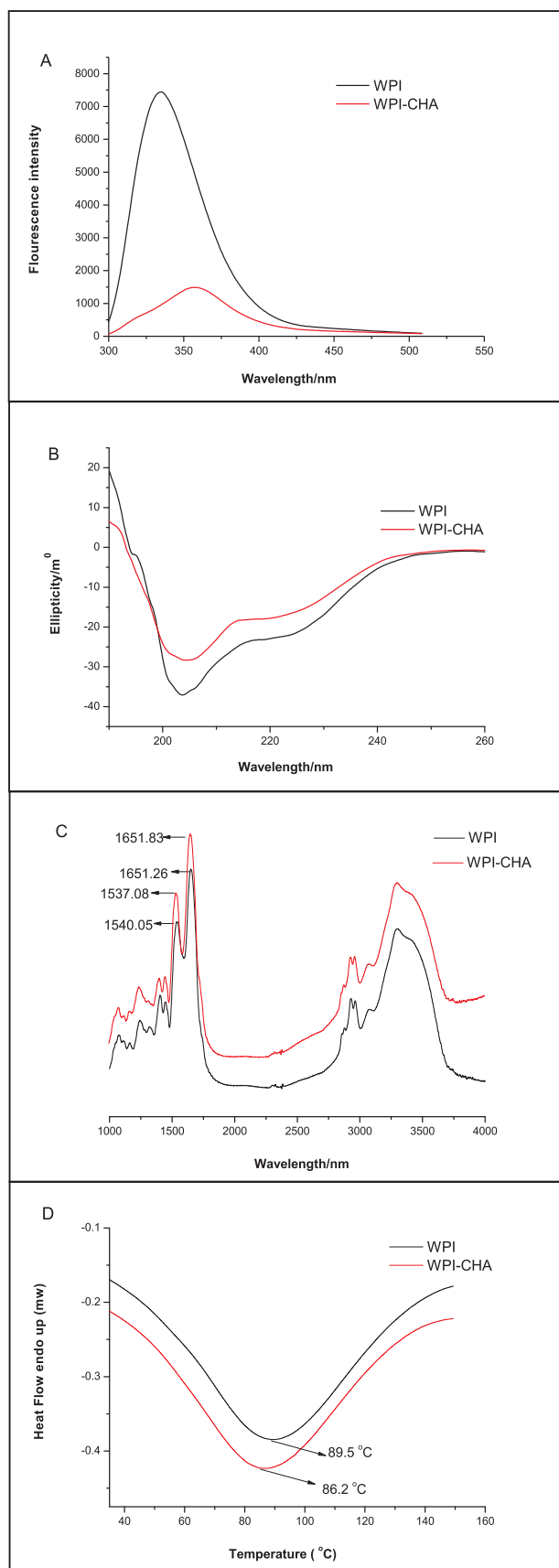


Fig. 2. Fluorescence (A), circular dichroism (CD) (B), Fourier transform infrared (FTIR) (C) and differential scanning calorimetry (DSC) spectra for WPI and WPI-CHA conjugate.

conjugated with some free amino groups in WPI. Moreover, because 8 M urea hindered the formation of disulphide bond from free thiol groups, a reduction in free thiol groups was caused by the covalent interaction between CHA and the free thiol groups in WPI. In addition, tyrosine group values were lower in the conjugate than the control WPI, which is similar to previous findings that oxidized phenolic compounds could react with tyrosine (Ali et al., 2018). Hence, CHA covalently interacted with the nucleophilic amino acid side chains of WPI.

3.2. Characterization of the change in structure of WPI-CHA conjugate

A fluorescence study was used to evaluate the conformational change of the protein. The fluorescence intensity was significantly decreased in the conjugate (Fig. 2A), which suggests that aromatic residues such as tryptophan and tyrosine were most likely involved in the interaction. A similar finding was observed when grafting polyphenols to egg protein (Lu et al., 2018). Furthermore, we observed an obvious red shift (from 334.6 to 357.6 nm) of maximum emission in the conjugate, which indicates that tyrosine and tryptophan residues transferred to a more hydrophilic environment.

CD and FTIR spectra were used to further analyze the changes in the secondary structure of WPI after conjugation with CHA. The CD spectra for WPI show a negative band at approximately 218 nm, which is mainly characteristic of β -sheet structures of proteins (Fig. 2B). The conjugation resulted in less absolute θ values from 200 nm to 260 nm, which suggests that the protein secondary structure was changed after conjugation with CHA. The second secondary structure contents were estimated by the CDSSTR method in DICHROWEB (<http://dichroweb.cryst.bbk.ac.uk>). Unmodified WPI contained 16.3% α -helices, 32.2% β -sheets, 24.0% turns and 27.5% unfolded structures and the conjugate contained 13.6% α -helices, 29.0% β -sheets, 24.2% turns and 33.2% unfolded structures. The α -helix and β -sheet contents were decreased in WPI after conjugation with CHA, with a parallel increase in unfolded structures, resulting from the covalent interaction between WPI and CHA.

In FTIR, the protein in amide I and II bands provide useful information about the secondary structure of proteins. The spectra for WPI exhibited two major bands at 1540.05 cm⁻¹ (amide II) and 1651.26 cm⁻¹ (amide I) (Fig. 2C). For the conjugate, these two major bands were changed in the peak positions, suggesting that the secondary structure in the conjugate was changed. The curve fitting method to the region of 1700–1600 cm⁻¹ and infrared self-deconvolution were used to analyze the protein secondary structures (Byler & Susi, 1986).

The unmodified WPI contained 24.7% α -helices, 22.9% β -sheets, 22.1% turns and 30.3% random coils and the conjugate contained 21.8% α -helices, 22.0% β -sheets, 21.7% turns and 34.5% random coils, which was consistent with CD spectroscopy results.

The thermal stability of the conjugate was characterized by DSC, which could also be used to analyze the protein structure. For the unmodified WPI and its conjugate, the thermal denaturation temperatures were 89.5 and 86.2 °C, respectively (Fig. 2D). As compared with the unmodified WPI, for the conjugate, the thermal denaturation temperatures were decreased, which indicates decreased tertiary conformation stability of WPI after covalent interaction with CHA.

The results of structure analyses for the WPI-CHA conjugate suggest that the covalent conjugation of CHA caused the unfolded protein structure.

3.3. Allergenic capacity

IgE binding of WPI and the WPI-CHA conjugate was tested by competitive ELISA. The WPI-CHA conjugate showed lower IgE-binding capacity than the unmodified WPI (Fig. 3): approximately 35% reduced IgE-binding capacity at 100 μ g/ml. In this work, the reducing IgE binding may be due to the conformational changes of WPI after

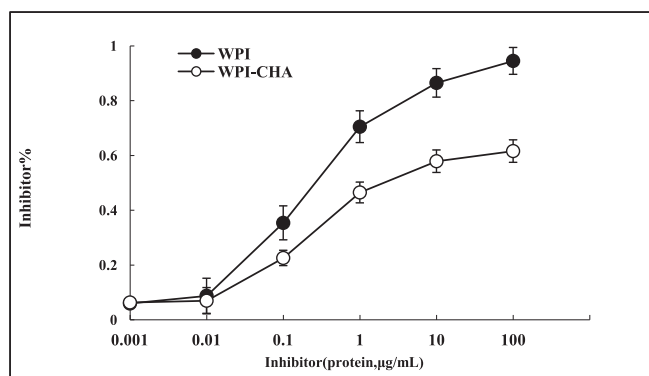


Fig. 3. Inhibition ELISA assay of IgE binding to WPI and WPI-CHA conjugate. Data are mean $\pm$ SD.

conjugation with CHA. Furthermore, the IgE-binding capacity of allergen is depend on conformational epitopes and also linear epitopes in the protein. CHA covalently interacted with the nucleophilic amino acid side chains of WPI. Therefore, these residues may form covalent bonds with CHA, which may affect the linear IgE epitopes to prevent IgE binding.

3.4. Simulated digestion with pepsin

Structural changes could affect the digestibility of the protein. We investigated the resistance to gastrointestinal digestion of WPI and its conjugate by simulated gastric digestion with pepsin. Fig. 4A shows the SDS-PAGE and western blot profiles of WPI with simulated gastric digestion. β LG (18.2 KDa) and α LA (14.2 KDa) were resistant to pepsin digestion, whereas BSA (66.0 KDa) was not detected in the corresponding digestion. Western blot bands corresponding to β LG and α LA were observed and showed an approximately 50% decrease at the maximum incubation time of 120 min mainly because the protein was not hydrolyzed by pepsin, but the conformation of the protein was changed, which resulted in reduced IgE binding capacity.

Fig. 4B shows the SDS-PAGE and western blot profiles of the WPI-CHA conjugate with simulated gastric digestion with pepsin. The bands according to β LG and α LA also showed resistance to pepsin digestion. However, as compared with the native WPI, for the conjugate, the bands in the western blot profiles for modified β LG showed a gradual decrease after 45-min incubation with pepsin, and with 120-min incubation, the band for modified β LG almost disappeared. The density of the western blot band for α LA weakened with time. The western blot of the conjugate did not exactly agree with SDS-PAGE findings. On comparing the western blot profiles of unmodified WPI (Fig. 4A), for the conjugate, β LG and α LA showed lower IgE-binding capacity than unmodified protein after pepsin digestion. This phenomenon may occur because the covalent conjugation of CHA caused the unfolded protein structure. Although the conjugate was resistant to pepsin digestion, some epitopes of modified β LG and α LA were destroyed more severely by pepsin than in the unmodified proteins.

After simulated gastric digestion for 60 min, the samples were added to simulated intestinal digestive juice for intestinal digestion. Fig. 4C and D show simulated intestinal digestion for the WPI and its conjugate, respectively. Although the profiles of the bands for unmodified β LG and α LA decreased gradually on SDS-PAGE (Fig. 4C), the bands still showed clearly at 120-min incubation. The bands in western blot analysis and SDS-PAGE showed the same trends. The western blot bands for unmodified β LG and α LA became weakened at 60 min; at 120 min, unmodified β LG and α LA were mostly not detected.

As compared with unmodified WPI, the conjugate showed a notable difference in intestinal digestion (Fig. 4D). Modified β LG and α LA started to be degraded at 5-min incubation, and the bands for these two proteins on SDS-PAGE were almost absent at 75-min incubation, which

suggests that the conjugate conferred higher intestinal digestibility than the unmodified WPI. On western blot analysis, the trace amounts of proteins were not mostly recognized by IgE at 75-min incubation.

The SDS-PAGE results showed no obvious difference in the peptic digestibility of the WPI after conjugation with CHA but improved pancreatic digestibility of the conjugate. The western blot results suggested that with prolonged digestion time, the IgE binding capacity was lower for β LG and α LA in the digested WPI-CHA conjugate than in digested WPI under the same gastrointestinal digestion condition.

Some allergens are resistant to gastrointestinal digestibility, easily passing through the gastrointestinal tract. Therefore, these allergens retain the conformational or linear epitopes to bind with IgE, thus triggering allergic responses (Bøgh & Madsen, 2016). β LG and α LA are resistant to gastrointestinal digestion (Villa et al., 2018). Fermentation (Fotschki, Szyk, & Wróblewska, 2015), glycation (Li, Luo, Feng, & Liao, 2013) and heat treatment (Peram, Loveday, Ye, & Singh, 2013), combined with simulated digestion, could increase the digestibility of milk protein, thus reducing the allergenicity of the protein. Similar to these findings, in this study, covalent conjugation with CHA could increase the digestibility of WPI, consequently reducing the IgE binding capacities. Covalent conjugation of CHA caused the unfolded protein structure of WPI, which may explain this phenomenon.

3.5. Functional properties

Fig. 5A shows that WPI solubility increased by 21% after conjugation with CHA, which might be by unfolding the protein structure after conjugation. Furthermore, the phenolic hydroxyl groups of CHA would enhance the hydrophilicity of the protein surface, which could increase the protein solubility.

As compared with the unmodified WPI, the conjugate showed higher emulsifying activity ($p < 0.05$) (Fig. 5B). Changing the conformational structure would affect the surface hydrophobicity of the protein, to affect the emulsifying properties of protein. Increasing exposed aromatic residues would increase the affinity of the proteins toward the oil/water interface, which could improve the emulsifying activity of WPI (Nor Afizah & Rizvi, 2014). In this study, the covalent conjugation of CHA caused the unfolded protein structure of WPI, therefore exposing more aromatic residues and improving the emulsifying activity of WPI.

Foaming capacity and foaming stability were used to estimate foaming properties of WPI and its conjugate (Fig. 5C). The foaming capacity and stability of the WPI-CHA conjugate were significantly greater than those of WPI alone ($P < 0.05$), which suggests that after conjugation with CHA, the foaming properties of WPI were improved. This finding agreed with Jia et al., who demonstrated improved foaming properties of WPI by covalent conjugation with EGCG (Jia et al., 2016).

The antioxidant activities were estimated by DPPH and ABTS radical scavenging activities. The antioxidant activities of the WPI-CHA conjugate were significantly higher than those of WPI ($p < 0.05$) (Fig. 5D). Phenolic compounds can conjugate to proteins to increase the antioxidant activity of the protein. The introduction of a higher number of hydroxyl groups is responsible for the improved antioxidant activities of the conjugate.

Our findings demonstrated enhanced solubility, emulsifying activity, foaming properties and antioxidant capacity of WPI by covalent conjugation of CHA, so covalent conjugation of CHA and WPI is a potential method for improving the functional properties of WPI.

WPI is present in many food products, and many efforts have been made to overcome cow's milk protein allergy. The application of food processing, such as high pressure, fermentation or heat treatment, has great potential for reducing milk allergenicity (Villa et al., 2018). However, these conventional food processing technologies might destroy some useful functions of milk protein and/or have adverse effects on taste in the product.

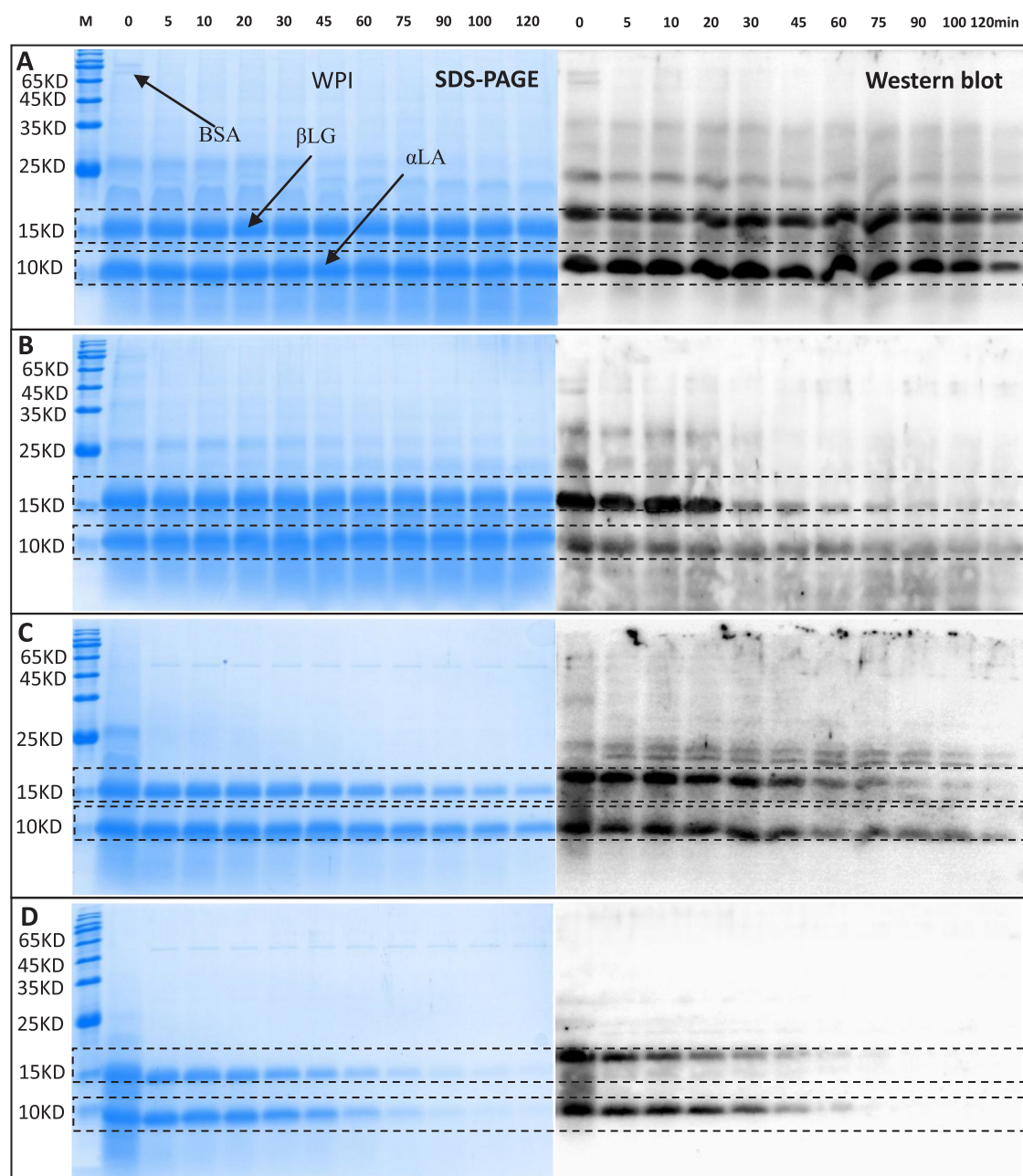


Fig. 4. SDS-PAGE and western blot profiles of unmodified WPI (A) and WPI-CHA conjugate (B) in simulated gastric digestion with pepsin and unmodified WPI (C) and WPI-CHA conjugate (D) in simulated intestinal digestion. M: protein marker; 0, 5, 10, 20, 30, 45, 60, 75, 90, 120: incubation time of simulated digestion (min).

The interaction between the allergen and polyphenols could reduce the allergenicity of allergens and might improve the functional properties of the protein (Pessato et al., 2018; Plundrich et al., 2015). However, these interactions were non-covalent and were reversible, which limited the application in food processing. In this study, covalent bonds formed between WPI and CHA to produce the conjugate, which was more stable than the complexes formed by non-covalent interaction. This stable conjugate showed low IgE binding capability but improved functional properties of the protein, which might be a promising approach to reduce the allergenicity of WPI.

4. Conclusion

In this study, CHA covalently interacted with nucleophilic amino acid side chains of WPI and caused an unfolded protein structure, which

may affect the conformational and linear IgE epitopes of the protein to prevent IgE binding. This covalent interaction reduced the IgE binding capacity and improved the digestibility, solubility, emulsifying activity, foaming properties and antioxidant capacity of WPI. This study indicated the potential applications of whey-CHA conjugates in producing food. Further research is needed to evaluate the allergenicity *in vivo* of modified WPI, for reducing food allergy in foods.

Declaration of Competing Interest

None.

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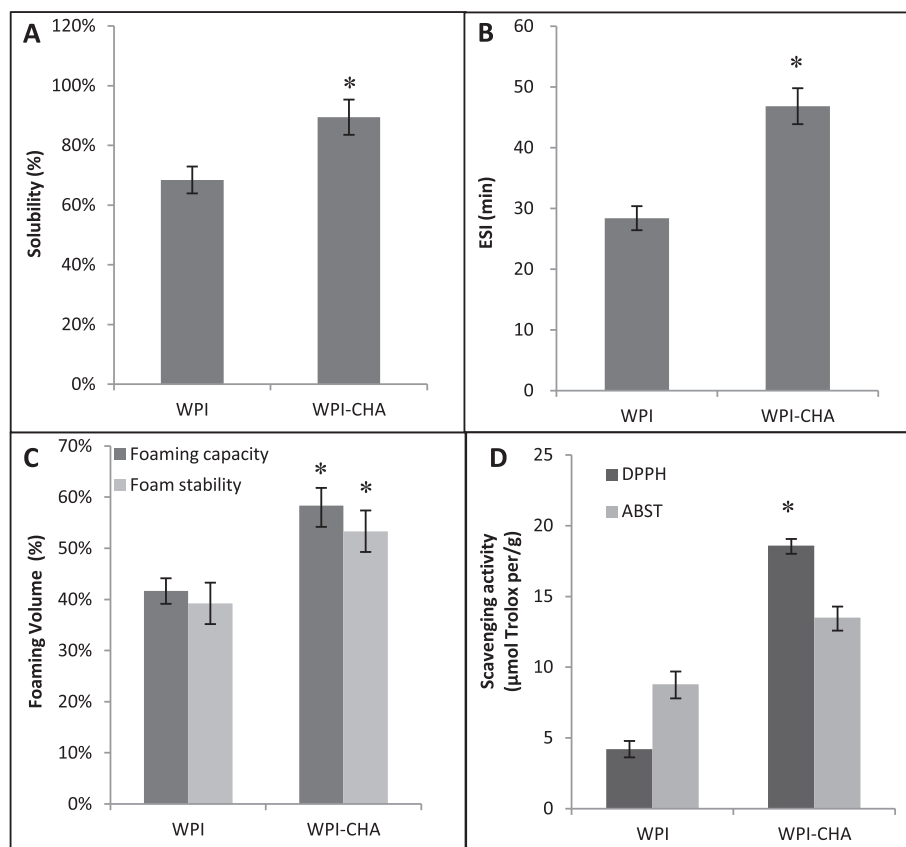


Fig. 5. Solubility (A), emulsifying properties (ESI) (B), foaming properties (C) and antioxidant activity (D) of WPI and WPI-CHA conjugates. Data are mean $\pm$ SD. *P < 0.05 between WPI-CHA and WPI.

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