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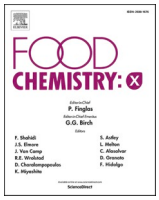

Altering functional properties of rice protein hydrolysates by covalent conjugation with chlorogenic acid

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ABSTRACT

Proteins and phenolic compounds are common components in foods that readily interact with each other to yield complexes, leading to changes in the functional properties. In this study, we investigated the effect of covalent conjugation of rice protein hydrolysates (RPH) with chlorogenic acid (CA) on the structural and functional properties of RPH. Three RPH-CA conjugates were prepared by the alkaline, enzyme, and free radical methods, respectively. Covalent conjugation decreased the content of free amino, thiol, and tyrosine groups, and increased in the amount of CA bounds from 15.23 to 21.11 nmol/mg. Moreover, the circular dichroism analysis revealed that covalent conjugation resulted in an increase of random coils. The emulsifying activity and antioxidant capacity of RPH were also improved by the covalent conjugation with CA. This work provides a better understanding of the formation of hydrolysates-chlorogenic acid conjugates, contributing to improving the functional properties of foods.

1. Introduction

Plant-based proteins, including soy, pea, and peanut, are increasingly attracting the interest of food manufacturers, because they have a lower production cost and environment-friendly impact compared with proteins from animal sources, such as meat, casein, whey, and egg (Day, 2013). In the food processing industry, plant proteins are widely utilized as emulsifiers and foaming agents, and they are considered important nutrition components in sports and fitness (Bessada, Barreira & Oliveira, 2019). Nevertheless, most plant proteins, which include wheat protein (Sievers et al., 2016), soy protein (L'Hocine & Boye, 2007), and peanut protein (Mondoulet et al., 2005), have been reported to contain many potential allergens. Unlike these plant proteins, rice proteins contain little known allergens listed in the database (https://www.Allergome.org/) and possess abundant essential amino acids; thus, they are recognized as a healthy protein source for human diets (Hirano et al., 2016). However, rice proteins have low solubility and thus poor functional properties, limiting their commercial application.

Protein modification technologies have attracted high interests from manufacturers, and a number of these technologies have been developed, such as freeze-milling, glycosylation, and enzyme treatments (Xu et al., 2016). Enzymatic hydrolysis is widely used to alter the molecular properties and improve the functional properties of proteins owing to its ability to steer the hydrolysis towards the desired hydrolysis products (Wouters et al., 2016). In our previous study, we reported that the limited enzymatic hydrolysis by trypsin improved the solubility and emulsifying activity of rice proteins (Pan et al., 2019a). Moreover, rice protein hydrolysates with strong antioxidant activity have been used to prevent lipid oxidation and improve the shelf life of meat products (Zhou, Canning & Sun, 2013).

Recently, the interaction between protein and phenolic compounds can be an effective method for improving the functional properties of proteins, especially for their covalent interactions. Chlorogenic acid, a polyphenol antioxidant that is found in coffee, fruits, and so forth, is well known to have a high affinity to binding protein (Gordon & Wishart, 2010). We prepared the covalent conjugates between rice protein hydrolysates and chlorogenic acid under alkaline conditions, and found they had high oxidative stability in the natural emulsions (Pan et al., 2019b). Oxidized chlorogenic acid, which acts as a protein cross-linker in protein-stabilized emulsions, has been reported to improve the storage stability and oxidative stability of porcine plasma protein hydrolysates emulsions (Chen et al., 2018).

Generally, phenolic compounds can covalently bind to proteins in the form of either phenolic quinone or radical (Prigent et al., 2007). In

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the phenolic quinone pathway, a phenolic compound is oxidized into quinone by alkali or enzyme (Fig. 1A), and the oxidized quinone derivatives then react with nucleophilic groups in the protein to form covalent bonds (Cao & Xiong, 2015). In the radical pathway, the reactive residues on the protein side chains are attacked by a radical (Fig. 1B), and the generated protein radical can react with a phenoxyl radical. It was reported that the antioxidant activity of covalent conjugate of ovotransferrin and catechin formed by the radical method is higher than that of the conjugate formed by the alkaline method (You et al., 2014). Moreover, Jing et al. (2020) have reported that egg white protein-tea polyphenol conjugates prepared using the alkaline method have higher surface hydrophobicity than those prepared by the radical method; however, the latter has a higher antioxidant activity. The conjugation methods have varying effects on the structures and properties of modified proteins due to different binding mechanisms. Therefore, an in-depth investigation is required to determine the suitable method for the preparation of protein–polyphenol conjugate.

Rice protein hydrolysates prepared with limited hydrolysis, have been shown to be a potential plant-based protein source in our previous study due to their increased solubility (Pan et al., 2019a). Covalent modifications with polyphenol are considered to be effective methods to further improve the functional properties of the protein. Considering a significant difference in binding mechanism, a comprehensive investigation of the effect of different covalent modifications with polyphenol on the functional properties of rice protein hydrolysates is required, to facilitate the development of new functional food ingredients in the food industry.

The objective of this study is to investigate the effects of covalent conjugation with chlorogenic acid by alkaline, enzyme, and free radical methods on the free amino, thiol groups and tyrosine contents and molecular weight of rice protein hydrolysates. Subsequently, the chemical structure, and functional properties, including thermal behavior, antioxidant activity, and emulsifying properties, of prepared covalent conjugates were also characterized.

2. Materials and methods

2.1. Materials

Rice protein powder was purchased from Pioneer Biotech Co., Ltd. (Xi'an, Shan'xi Province, China). The purity of the powder was calculated to be 90% by Kjeldahl analysis in our lab (N × 5.95). Trypsin (250 NFU/mg), laccase (200 U/g), and a protein marker (5–245 kDa) were purchased from Solarbio (Beijing, China). Chlorogenic acid with purity > 95% was purchased from Sigma (St. Louis, MO). Other used reagents were of analytical grade and purchased from Beijing Chemical Reagent Company (Beijing, China).

2.2. Preparation of rice protein hydrolysates

Tryptic hydrolysate of rice protein was produced as described in our previous study (Pan et al., 2019b). Rice protein powder (5 g) was added to 100 mL of distilled water. The mixture was continuously stirred for 2 h at room temperature and then heated to 50 °C; after that, its pH was adjusted to pH 8.0 using 0.5 mol/L NaOH. To start the hydrolysis, the mixture was mixed with trypsin (the mass ratio of enzyme to protein was 1: 250 (w/w)), during which the pH of the mixture was maintained at pH 8.0. To achieve the degree of hydrolysis of 4% (which produced strong surface hydrophobicity from our previous study) (Pan et al., 2019a), the hydrolysis reaction was held for 30 min. Then, the resultant hydrolysate was boiled for 10 min to inactive the trypsin and then cooled down to room temperature. Finally, the mixture was centrifuged at 10,000 × *g* for 15 min and the pH of the supernatant was adjusted to pH 7.0 before being freeze-dried.

2.3. Preparation of covalent conjugates between rice protein hydrolysates and chlorogenic acid

The preparation of covalent conjugate by the free radical method was carried out according to the modified method reported by Liu et al. (2015). Rice protein hydrolysates (1 g) and 0.25 g ascorbic acid were added to 99 mL of distilled water containing 1 mL of hydrogen peroxide, and the mixture was incubated at room temperature for 2 h.

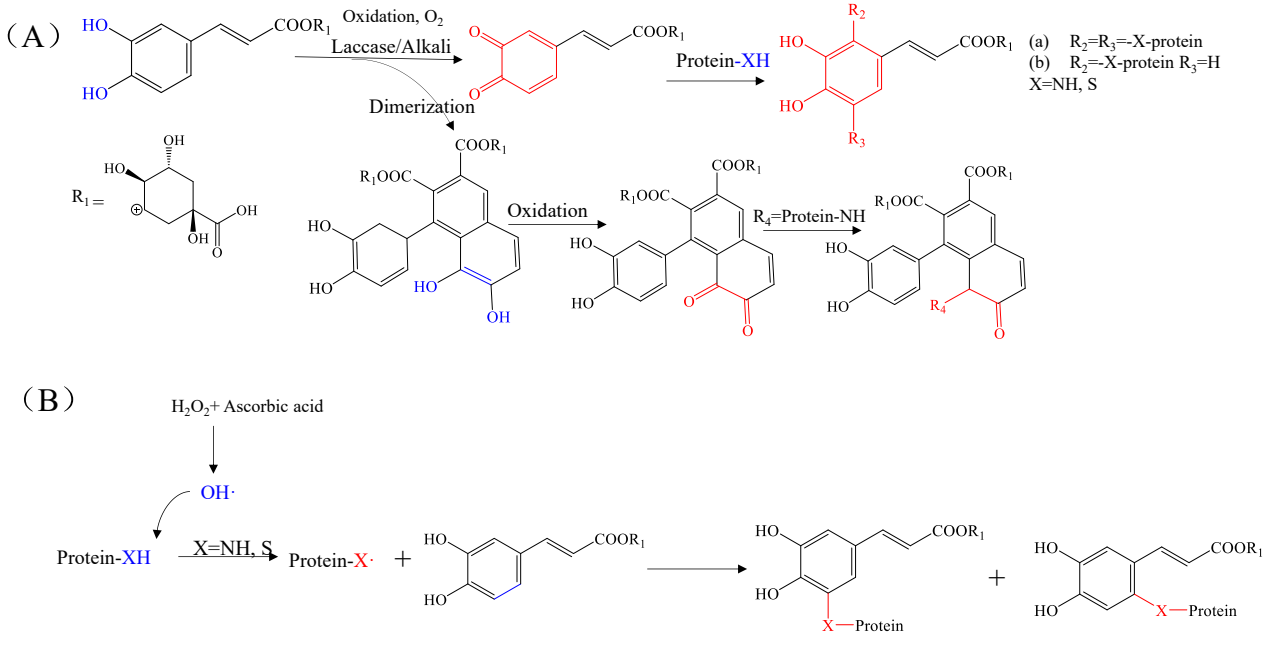

Fig. 1. Proposed formation mechanism for protein–chlorogenic acid conjugates prepared by radical method (A) and phenolic quinone method (B). Images were adapted from Rawel et al., (2002) and Prigent et al., (2007).

Subsequently, 10 mg of chlorogenic acid were added into the mixture and allowed to react for 24 h. The preparation of covalent conjugate by the alkaline method was conducted following the previously reported method (You et al., 2014). Rice protein hydrolysates (1 g) and 10 mg of chlorogenic acid were dissolved in 100 mL of phosphate buffer, pH 9. The mixture was stirred for 24 h at room temperature. Conjugate prepared by the enzyme method was prepared according to the method reported by Ma et al. (2011), with minor modifications. Chlorogenic acid (10 mg) and 0.4 mg of laccase were mixed with 50 mL of distilled water, and the mixture was stirred for 2 h. The mixture solution was mixed with 2% (w/v) hydrolysate solutions, and the two were allowed to react by stirring for 24 h. Untreated rice protein hydrolysate was used as a control. After the reaction, each complex was placed in a dialysis tube with a molecular weight cutoff of 1000 Da at 4 °C for 72 h to remove free chlorogenic acid (We change the dialysis bathwater four times without the dialysis bag being replaced). The control sample was also subjected to the same dialysis. All covalent conjugates were prepared in triplicate.

2.4. Physicochemical characterization of the modified covalent conjugates

2.4.1. Quantification of free amino, thiol groups, and tyrosine

To analyze the groups involved in the covalent reaction, the contents of free amino and thiol group content in the conjugates were determined according to the method reported by Wu et al. (2018) with a slight modification. For the measurement of amino group contents, *ortho*-phthaldialdehyde (20 mg) was dissolved in 0.5 mL of methanol. The resultant solution was then mixed with 1.25 mL of 20% (w/v) sodium dodecyl sulfate before being added into 12.5 mL of sodium borate buffer (1 mol/L, pH 9.85) containing 50 μL of β-mercaptoethanol. Next, 2 mL of the mixed solution was added to 200 μL of conjugate solution (5 mg/mL), and the reaction was allowed to occur at 35 °C in a water bath for 2 min. Subsequently, the absorbance at 340 nm of the sample was measured. Free amino groups were determined using the calibration curve of L-leucine as a standard. For the measurement of thiol group contents, each conjugate sample (15 mg) was mixed with 5 mL of tris (hydroxymethyl)-aminomethane hydrochloride buffer (50 mmol/L, pH 8) containing 8 mol/L urea to obtain a conjugate solution. Four milligrams of 5,5′-dithiobis (2-nitrobenzoic acid) and 1 mg of ethylene diamine tetraacetic acid (EDTA) were dissolved in 1 mL of tris (hydroxymethyl)-aminomethane hydrochloride buffer (50 mmol/L, pH 8). The mixture was allowed to react with 1 mL of the samples (5 mg/mL) for 1 h, and the absorbance at 412 nm (A_{421}) of the mixture was measured thereafter. The contents of the thiol group were calculated according to the following equation,

$$C_{thiol} = \frac{75.53 \times A_{421}}{C_{sample}}$$

The determination of tyrosine content in conjugates samples was conducted following the method of Wu et al. (2018) with minor modifications. Conjugates sample (0.9 mL, 1 mg/mL) was reacted with 16 mol/L nitric acids (1 mL) at 50 °C for 15 min; after that, the reaction mixture was cooled down to room temperature. Subsequently, 4 mL of ethyl alcohol and 4 mL of 5 mol/L NaOH were added to the mixture, and the absorbance at 360 nm (A_{360}) and 430 nm (A_{430}) of the resultant solution were recorded. The contents of tyrosine were calculated according to the following equation.

$$C_{tyrosine} = 0.5357 \times A_{430} - 0.35714 \times A_{360}$$

2.4.2. Quantification of bound chlorogenic acid content

The amounts of bound covalent phenolic in the conjugates were determined by the Folin-Ciocalteu method. Each diluted conjugate sample (0.5 mL, 1 mg/mL) was mixed with 2.5 mL of Folin–Ciocalteu reagent (10 N) for 2 min. Then, 2 mL of sodium carbonate solution (7.5% w/v) was added to the mixture, and the reaction was allowed to take place for 2 h in the dark. During the reaction in alkaline solutions, the electrons of phenolic compounds were transferred to phosphomolybdic/phosphotungstic acid complexes, which were determined spectroscopically at 765 nm using a spectrophotometer. The total content of phenolic groups was expressed as a chlorogenic acid equivalent by using the equations obtained from the calibration curves of chlorogenic acid, and the results were expressed as nmol chlorogenic acid/mg sample.

2.4.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

To confirm the formation of covalent bonding, the electrophoretic profiles of conjugate samples were examined by the method of Laemmli (1970) with minor modifications. Rice protein hydrolysates and their conjugates were dispersed in the loading buffer containing 0.5 M Tris-HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 5% (v/v) β-mercaptoethanol, 1% (w/v) bromophenol blue, and 10% (v/v) glycerol. The mixtures were incubated in boiling water for 5 min, and after being cooled down to room temperature, it was centrifuged at 10,000 × g for 10 min. The conjugate samples (containing 30 μg of protein), along with standard protein marker (5–245 kDa), were subjected to SDS-PAGE run at a constant voltage of 80 V. After electrophoresis, the gels have stained with a solution containing 0.5% (w/v) Coomassie Brilliant Blue R-250, 60% (v/v) methanol and 5% (v/v) acetic acid for 3 h and were then detained in the solution containing 60% (v/v) methanol and 5% (v/v) acetic acid for 4 h.

2.5. Structural characterization of covalent conjugates

2.5.1. Fluorescence spectroscopy

Fluorescence spectroscopy was performed on a fluorescence spectrophotometer (F-7000, Hitachi, Tokyo, Japan) following the method detailed in our previous study (Pan et al., 2019b) with minor modifications. Each conjugate sample was diluted to distilled water into a concentration of 1 mg/mL. All the measurements were carried out at the optimized excitation slit width of 5 nm and the emission slit width of 3 nm. The fluorescence intensity of conjugates was determined over an emission wavelength range from 310 to 400 nm and at an excitation wavelength of 280 nm.

2.5.2. Circular dichroism (CD)

The protein secondary structure of samples was determined by far-ultraviolet circular dichroism (Model MOS-450, France Biologic Company, Grenoble, French). Each conjugate sample (1 mg) was mixed with 10 mL of phosphate buffer (10 mM, pH 7.0) and then transferred to a 1 mm quartz cuvette. The circular dichroism spectra were recorded over a range of 190 nm to 250 nm under a nitrogen stream flowed at a flow rate of 100 mL/min. The analysis of protein secondary structure for the conjugates was carried out by the study by Lobley, Whitmore & Wallace, (2002), using the SELCON3 method on the Circular Dichroism website (http://dichroweb.cryst.bbk.ac.uk).

2.5.3. Fourier transform infrared (FTIR)

FT-IR measurements were conducted using a Fourier transform infrared spectrometer (Tensor 27, Bruker Optics Inc., Karlsruhe, Germany). The samples used for IR scans were prepared by mixing 1.5 mg of freeze-dried hydrolysates and conjugates with 198.5 mg of potassium bromide and then pressing the mixtures into tablets at 25 MPa. The spectra were collected at a range of 4000 cm^{-1} –400 cm^{-1} with an average of 64 scans, and potassium bromide was used as a reference.

2.5.4. Molecular docking

Considering the specificity of the trypsin and the complexity of the hydrolysate structure, the interactions between chlorogenic acid and rice protein by molecular docking were further evaluated. Rice glutelin as the most abundant protein in rice protein, showed the highest

sequence identity (46%) of pumpkin seed globulin (PDB: 2EVX) (Dai et al., 2019). In general, the sequence identity was higher than 40%, the homology model structure can be utilized in molecular docking studies. The crystal structures of the chlorogenic acid (CID: 6604240) were obtained from the PubChem database. Docking calculations were performed using the AutoDock 4.2 program package. A grid box was created that covered the entire protein and chlorogenic acid molecules, and the best docking results were obtained. Based on the noncovalent docking results, the covalent docking of rice protein and chlorogenic acid was conducted by AutoDock4 using the two-point attractor method. All docking results were visualized using PyMOL (v1.7.4, Schrödinger, LLC, Cambridge, MA, USA) and Discover Studio (v16.1, Accelrys, San Diego, CA, USA).

2.6. Analysis of functional properties of covalent conjugates

2.6.1. Thermal stability analysis

Thermal stability measurements were conducted using a DSC-60 thermal analysis system (Shimadzu, Tokyo, Japan) with a modified method by Xu et al. (2019). In brief, about 5.5 mg of samples were placed inside an aluminum pan and then sealed tightly with a perforated aluminum lid. Then, the conjugate samples were heated from 30 to 180 °C at a constant rate of 10 °C/min while being continuously purged with dry nitrogen at a rate of 30 mL/min. An empty aluminum pan was used as a reference. The peak denaturation temperature for each thermal curve was computed using the universal analysis software.

2.6.2. Assay of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity was determined based on the method described in Liu et al. (2015) with slight modifications. Briefly, 1 mL of samples (0.5 mg/mL) was mixed with 1 mL of DPPH solution (1.75×10^{-4} mol/L, freshly prepared in ethanol). The mixtures were allowed to react in the dark for 90 min, and their absorbance at 517 nm was recorded thereafter using a spectrophotometer to determine the residual DPPH concentration. As a blank control, the samples were replaced with distilled water; and additional control for each sample was also prepared by mixing each sample with 95% ethanol. The scavenging activity was determined based on the Trolox calibration curve and was expressed as μmol Trolox equivalent (TE)/mg sample.

2.6.3. Assay of (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) ABTS radical scavenging activity

The ABTS radical scavenging activity was measured following the method reported in Jing et al. (2020) with slight modification. To prepare the ABTS stock solution, ABTS (10 mg) was mixed with 2.6 mL of 2.45 mol/L potassium persulfate solution, and the mixture was incubated in darkness for 12 h. Then, 1 mL of the conjugate sample at a concentration of 0.5 mg/mL was mixed with 3 mL of ABTS solution (prepared by diluting the stock solution to a ratio of 1:3 (v/v)) and then allowed to react at room temperature for 1 h. After that, the absorbance at 734 nm of the mixtures was measured. The ABTS radical scavenging activity was determined based on the Trolox calibration curve and was expressed as μmol Trolox equivalent (TE)/mg sample.

2.6.4. Emulsifying activity

The emulsifying activity of conjugates was measured according to the method in our study previously reported (Pan et al., 2019b). A mixture containing 10%(w/w) oil phase (soybean oil) and 90% (w/w) aqueous phase of freeze-dried conjugates (2.5%, w/v) was homogenized using a high-shear mixer (T18, IKA, Staufen, Germany) at 10,000 × *g* for 3 min. The coarse emulsions were then transferred into a high-pressure homogenizer (DSJ-20L, ATS Engineering Inc, Shanghai, China) and further homogenized at a pressure of 60 MPa for 2 cycles. Next, the 50 μL emulsion was sampled from the bottom of the breaker at 0 and 30 min and then mixed with 5 mL of sodium dodecyl sulfate solution (0.1%, w/w). The emulsifying activity index (EAI) and the emulsifying stability index (ESI) were calculated using the absorbance at 500 nm at 0 (A0) and 30 (A30) min as follows:

$$EAI\ (m^2/g) = 2T \times \frac{A_0 \times N}{C \times \Phi \times 10000}$$

$$ESI\ (\%) = \frac{A_{30}}{A_0}$$

Where T = 2.303, N is the dilution factor, c is the weight of protein per unit volume (g/mL), and Φ is the oil phase volumetric fraction.

2.7. Statistical analysis

All experiments were conducted in triplicate. Analysis of variance (ANOVA) was carried out using the SAS software (Version 9.4, SAS Institute, Cary, NC). Means were compared by Duncan's multiple range test at a significant level of $p < 0.05$.

3. Results and discussion

3.1. Effect of covalent modification of chlorogenic acid on physicochemical properties of rice protein hydrolysates

3.1.1. Changes in the free amino, thiol groups, and tyrosine contents for rice protein hydrolysate conjugates

SDS is a denaturing agent that can destroy non-covalent interactions in proteins, and thus can be used to determine changes in the free amino group content for different conjugates that are prepared in this study. As presented in Fig. 2A, the covalent interaction caused a decrease in the free amino group content for rice protein hydrolysates ($p < 0.05$) from 712.53 nmol/mg sample to 675.87 nmol/mg sample or lower. This decrease implies the presence of covalent bonds in the conjugates. Ali et al. (2013) have reported that 5-caffeoylquinic acid covalently binds to β-lactoglobulin at the ε-amino in lysine groups. This leads to a decrease in the free amino group content for proteins, which is consistent with our results. Moreover, urea, which can prevent the formation of disulfide bonds between free thiol groups, was used to determine the structural changes in the thiol groups for protein hydrolysates (Xu et al., 2019). Fig. 2A also exhibited that the free thiol group contents in covalent conjugates were less than that in the control hydrolysates. Furthermore, the covalent interactions reduced the tyrosine contents in the hydrolysates. It has been reported the covalent conjugation between phenolic compounds and proteins occurs in the nucleophilic groups, such as tryptophan, tyrosine, and *N*-terminal proline, in proteins (Kroll, Rawel & Rohn, 2003).

As given in Fig. 2, the covalent conjugates prepared by the enzyme method had the lowest contents of free amino and thiol groups (420.39 ± 9.27 nmol/mg protein and 5.15 ± 0.15 nmol/mg protein) and tyrosine groups (8.21 ± 0.57 ng/mg protein) among all covalent conjugates. This suggests that the reactivity and affinity with free amino groups for each method are ranked in the following order: enzyme method > alkaline method > radical method. The covalent bond in the conjugates prepared by the radical method is mainly formed due to the attack of free radicals on the ortho- and *para*-positions of hydroxyl groups on the aromatic ring (Spizzirri et al., 2009). Nevertheless, the enzyme and alkaline methods induced the oxidization of phenolic compounds into quinone, thus leading to the cross-linking with proteins. Prigent et al. (2007) have found that phenolic dimers can more easily interact with the protein, compared with monomers due to their lower redox potential. They also reported that the intensity of modified protein seemed higher with alkaline modification than with polyphenol oxidase modification. But, You et al. (2014) found that alkaline conjugate causes a low degree of conjugation than radical modification. These findings were not consistent with our results, implying that the protein structure might be the key factor, followed by the modification methods.

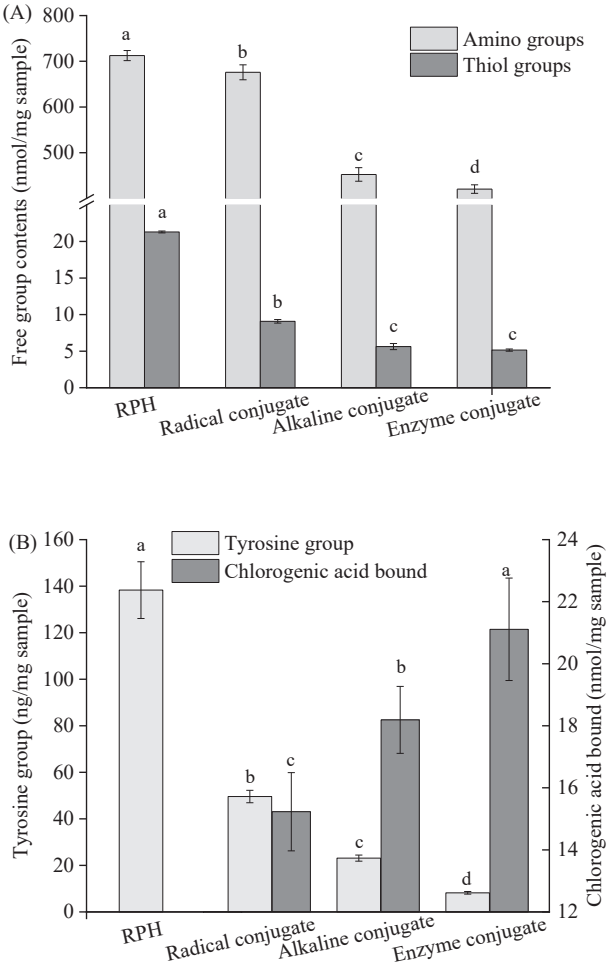

Fig. 2. Contents of free amino, thiol (A), and tyrosine groups and chlorogenic acid bound (B) of covalent conjugates prepared with rice protein hydrolysates (RPH) and chlorogenic acid (CA). Radical conjugate, RPH-CA conjugates formed by radical method. Alkaline conjugate, RPH-CA conjugates formed by alkaline method. Enzyme conjugate, RPH-CA conjugates formed by enzyme method. Different letters in the same property mean a significant difference by Duncan's multiple range test at $p < 0.05$.

3.1.2. Change in the molecular weight of rice protein hydrolysate conjugates

Fig. 3 presents the effect of covalent modification on the molecular weight of rice protein hydrolysates. The SDS-PAGE results showed that the main band in rice protein hydrolysates was about 16 kDa. After the hydrolysates covalently interacted with oxidized phenolic compounds, the molecular weight of rice protein hydrolysate conjugates prepared by the alkaline and enzyme methods slightly increased. Cao and Xiong (2015) have reported that the chlorogenic acid dimer has a function as the protein crosslinker. This crosslinker was involved in the conjugates prepared by the enzyme method since the structure of the protein in these conjugates had higher stability compared to other conjugates. Similar to our results, the molecular weight of whey protein conjugates slightly increased after interaction with polyphenol compounds (Wu et al., 2018). However, the change of molecular weight of conjugates prepared by the radical method was not obviously observed compared to the control sample, and the lower molecular weight was not detected in this conjugate. You et al. (2014) have reported that SDS breaks covalent bonds in the conjugates between protein and phenol with a low degree of conjugation. Hydrogen peroxide has been shown to lead to oxidative crosslinking in small protein molecules, causing the band of conjugates prepared by the radical method to move to a higher position in the gel.

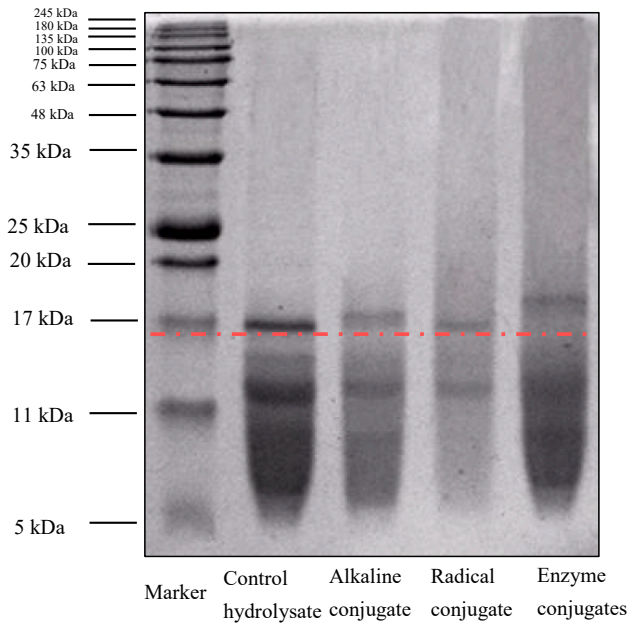

Fig. 3. SDS-PAGE of control rice protein hydrolysates (RPH) and RPH-chlorogenic acid (CA) conjugates prepared by different methods under reducing conditions (control, rice protein hydrolysates, marker: 5–245 kDa). The dotted red line means the low molecular weight of control rice protein hydrolysates.

Moreover, it should be noted that the molecular weight of covalent conjugates prepared by the enzyme method was about 18 kDa which was higher compared to other conjugates, implying that the enzyme method leads to the highest degree of crosslinking in proteins.

3.2. Effects of polyphenol covalent modification on the structure of rice protein hydrolysates

3.2.1. Fluorescence spectroscopic analysis

As shown in Fig. 4A, the covalent modification caused a significant decrease in the fluorescence intensity of rice protein hydrolysates, and the aromatic amino acids (*e.g.* tyrosine and tryptophan) were found to participate in the covalent interaction. Spizzirri et al. (2010) have reported that the covalent bonds between gelatin and gallic acid, or gelatin and catechin also result in a decrease in the fluorescence intensity of gelatin, which is consistent with our results. The conjugates prepared by the enzyme method exhibited the lowest fluorescence intensity. This is due to that they had the highest degree of integration among all the conjugates. Moreover, the maximum emission wavelength of rice protein hydrolysates, which was initially 342.8 nm, then was red-shifted to 346.0 nm, 362.6 nm, and 375.8 nm for the conjugates prepared by alkaline, radical, and enzyme method, respectively. Generally, the red shift indicated that tyrosine residues were more exposed to the solvent, or the redshift resulted from the transfer of tyrosine residues into a more hydrophilic environment (Pan, Gao & Ao, 2005). It suggested that the polarity around tyrosine and tryptophan residues of the conjugates in this study was increased and that these residues existed in a hydrophilic microenvironment, which agreed with previous studies that chlorogenic acid was grafted onto bovine serum albumin. Such covalent interactions can be confirmed by the decreased amount of free tyrosine in Fig. 2 (Rawel, Czajka, Rohn & Kroll, 2002), causing the different red shifts in conjugates. Hence, the quenching and redshift in the fluorescence emission are associated with the structural changes in rice protein hydrolysates after being conjugated with chlorogenic acid.

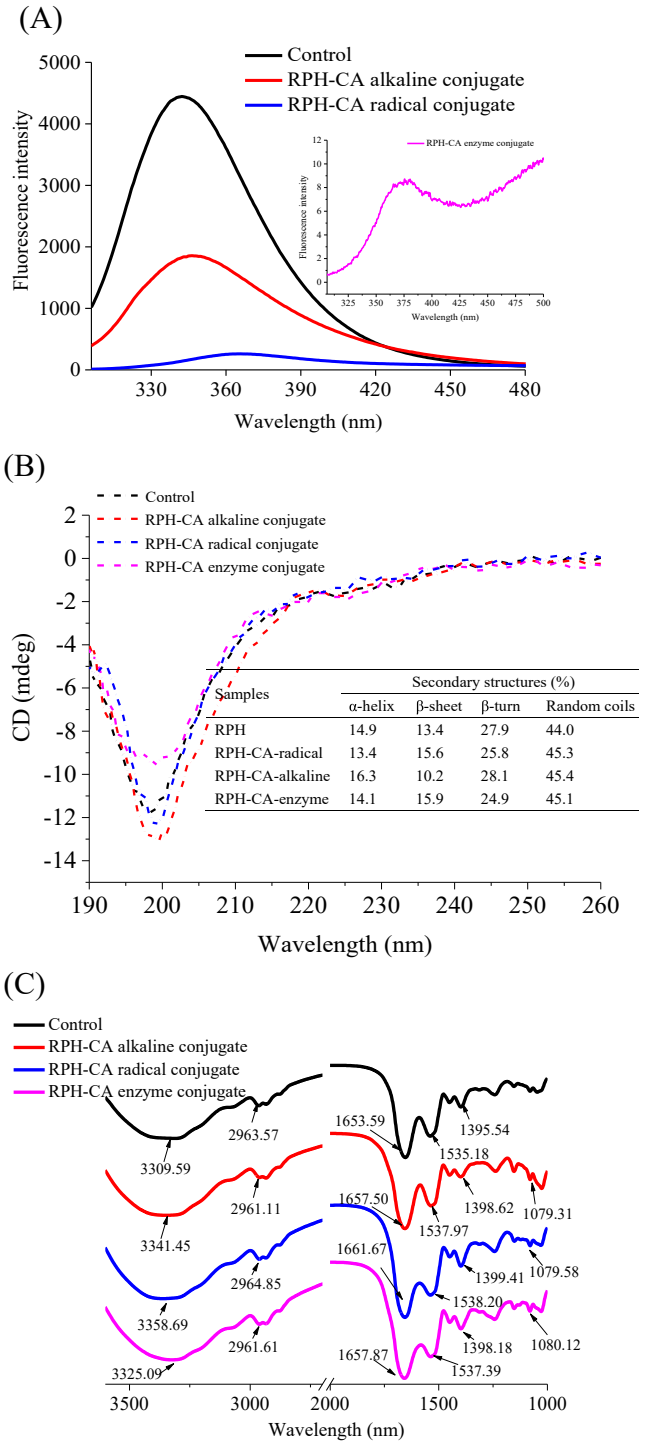

Fig. 4. Fluorescence (A), circular dichroism (B) and fourier transform infrared spectra (C) for control rice protein hydrolysates (RPH) and RPH-chlorogenic acid (CA) conjugates prepared by different methods (control, rice protein hydrolysates). Inset: Secondary structure content of conjugates.

3.2.2. CD analysis

The effect of covalent modification on the protein secondary structures of rice protein hydrolysates were determined by CD. As shown in Fig. 4B, the protein hydrolysates exhibited a negative band at 198 nm, which is the characteristic band of a random coil. Zheng, Li, Li, Sun, and Liu (2019) have also reported that enzyme-hydrolyzed black bean mainly has random coil conformation according to the CD observation. Moreover, the covalent interaction led to an obvious change in the peaks: the wavelength of negative minimum was shifted to a longer wavelength, suggesting that the protein secondary structure of samples was altered.

The CD spectra results were uploaded to the DICHROWEB to determine the absolute protein secondary structure contents. As shown in the inset of Fig. 4B, the control sample contained 14.9% α-helices, 13.4% β-sheets, 44.0% unfolded structures, and 27.9% β-turns. In general, β-sheet is considered to be relatively stable, whereas α-helix, β-turn, and random coils are relatively flexible and open (Xu, et al., 2019). In this study, the protein hydrolysates showed a decrease in the α-helix contents after the covalent conjugation by radical and enzyme method, respectively, and in turn, they had an increase in the unfolded structure contents. Nevertheless, our results showed the conjugates prepared by the alkaline method contained the highest α-helix content (16.3%) and the lowest β-sheet content (10.2%). This suggests that the alkaline method induced the conversion of β-sheets into α-helices. This finding is consistent with that observed in the grafting of tea polyphenol onto egg white proteins (Jing et al., 2020). Thus, we proposed that the unfolded structure of alkaline conjugates was caused by the increased contents of β-turn and random coils. Moreover, the α-helix content in the conjugates that were prepared by the radical method was lower than that is prepared by other methods. This is due to the difference in the reaction systems·H_2O_2 can generate a large amount of OH radicals; and these radicals can alter the protein's secondary structure (Liu et al., 2015). These results showed that the polyphenol conjugation caused the protein structure to be unfolded, which is consistent with the results from our fluorescence spectroscopic measurements.

3.2.3. FT-IR analysis

FTIR measurement was employed to further assess the structural changes in covalent conjugates. As presented in Fig. 4C, the control rice protein hydrolysate exhibited the main bands at 3309 cm^{-1} (amide A, due to the N—H stretching coupled with hydrogen bonding), 1653 cm^{-1} (amide I, due to the C—O stretching and hydrogen bonding coupled with COO–) and 1535 cm^{-1} (amide II, due to the C—N stretching coupled with NH bending modes). These amide bands are the important components for protein secondary structures due to their high sensitivity to hydrogen bonding, dipole–dipole interactions, and polypeptide backbone geometry (Carton, Bocker, Ofstad, Sørheim, Kohler, 2009). The covalent conjugation with chlorogenic acid caused the amide I band of conjugates that were prepared by alkaline, radical, and enzyme methods to move to 1657 cm^{-1}, 1661 cm^{-1} and 1657 cm^{-1}, respectively. A similar result, in which the amide II band of conjugates was moved to a longer wavelength, has also been found. Liu et al., (2015) have reported that a slight shift in peak positions in the lactoferrin was observed after covalent interaction with polyphenol. These findings suggest that the protein secondary structures for hydrolysates were interrupted by the covalent conjugation with chlorogenic acid.

3.3. Docking model analysis

As shown in Fig. 5A, the amino acid residues of rice protein interacted with the chlorogenic acid on the protein surface. From the observation of Fig. 5B, the complex formed by noncovalent interaction was mainly stabilized by hydrogen bonds at the THR 283, LYS 127, ILE 128, LEU 286, and ARG 285. Moreover, the van der Waals forces between chlorogenic acid and amino acid residues such as LEU 284, and PRO 130, were detected, while three electrostatic forces at the amino acid residues, LYS 127 and ARG 129, were also found. Chen et al. (2022) have reported that the hydrogen bonding and van der Waals forces as noncovalent interactive forces between protein and phenolic, and proposed that these noncovalent grafting reaction sites at Pro and Met might be the potential sites for covalent binding.

Generally, the covalent bonds were formed between the phenolic ring of chlorogenic acid and the nucleophilic side chain of proteins, to form a stable complex (Rohn, Rawel & Kroll, 2004). Hence, to better

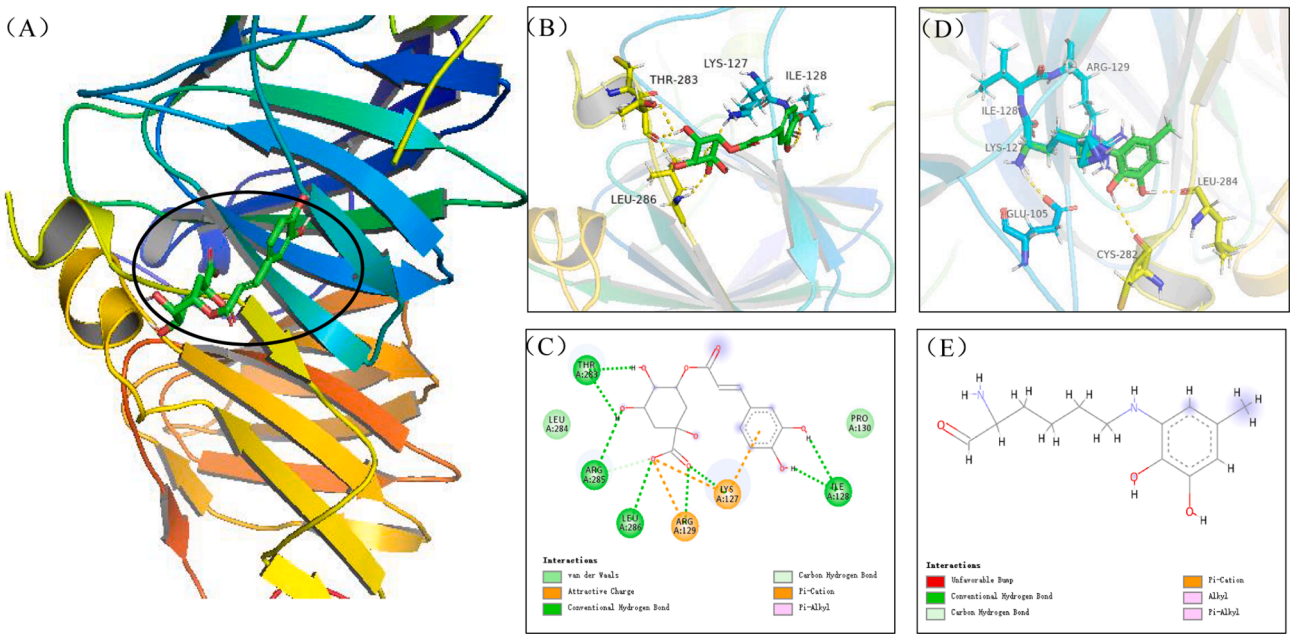

Fig. 5. Docking results of the complexes prepared with rice protein and chlorogenic acid. (A), Structure of protein showing the combined regions of chlorogenic acid. Noncovalent (B-C) and covalent interaction (D-E) between the hydrophobic surface of the protein and chlorogenic acid from 3D docking mode and 2D schematic diagram, respectively.

understand the covalent mechanism between the hydrolysates and phenolic acids, covalent docking would be carried out between the LYS 127 and the highly reactive phenolic ring in a chlorogenic acid through the nucleophilic substitution reaction. As shown in Fig. 5D and Fig. 5E, the above noncovalent interaction sites were significantly altered after covalent docking. New hydrogen bonds were established between the phenolic ring, the CYS 282 and the LEU 284. The exposure of the -SH group might lead to the following covalent interaction with the phenolic ring. Moreover, a new electrostatic force between ARG 129 and the phenolic ring still was formed, and it might be due to the changes in the position of phenolic structure during the covalent docking. Similarly, chlorogenic acid was also autoxidized to form a dimer by the alkaline and enzymatic method (Rohn et al., 2004), and its *para*-positions ortho- and *para*-positions in the phenolic ring also interacted with other nucleophilic side chains, resulting in the formation of protein aggregates. Generally, trypsin as a specific protease, can hydrolyze the carboxy terminus of ARG and LYS. The NH_2 of the binding site in protein-chlorogenic acid conjugates cannot be hydrolyzed by trypsin. Hence, we guessed that these results in docking might be still present in the interaction between chlorogenic acid and rice protein hydrolysate, which would be further verified in our followed study. These findings might be useful for explaining the difference in the structure of covalent modification by phenolic quinone or radical methods (Fig. 4).

3.4. *Effects of polyphenol covalent modification on the thermal stability of rice protein hydrolysates*

The thermal stability of protein hydrolysates is dependent on the protein structures; thus, the change in thermal stability can indirectly reflect the change in protein structure. As shown in Fig. 6A, the thermal denaturation temperature of control sample was 126.5 °C. After covalent conjugation with chlorogenic acid, the thermal denaturation temperature of protein hydrolysates was increased ($p < 0.05$) by about 5–14 °C, which showed that the covalent conjugation can improve the thermal stability of protein hydrolysates. It has been reported that the introduction of a carboxyl group and covalent and non-covalent interactions to hydroxyl groups results in an enhancement in the thermostability of soy protein (Guo, Bao, Sun, Chang, & Liu, 2020). Moreover, the enthalpy changes in all the conjugates were less than that in the control hydrolysates. This is in line with the observations that the conjugates had an unfolded structure (Fig. 4); therefore, low energy is required to open this structure. These results are similar to those reported in a previous study that covalent conjugates of protein and phenolic compounds formed by the enzyme or alkaline method were found to have a higher thermal denaturation temperature compared to the control sample (Prigent et al., 2007).

3.5. *Effects of polyphenol covalent modification on the in vitro antioxidant activity of rice protein hydrolysates*

As shown in Fig. 2B, the chlorogenic acid contents in the covalent conjugates were from 15.23 to 21.11 nmol/g. To evaluate the effect of covalent interaction on the antioxidant activity, *in vitro* antioxidant activities against DPPH and ABTS radicals of rice protein hydrolysates were determined. As shown in Fig. 6B, the antioxidant activity against DPPH of control sample was 87.37 μmol Trolox equivalent (TE)/g sample, whereas that of conjugate samples was in a range of 165.92 to 232.67 μmol Trolox equivalent (TE)/g sample. It has been reported that not all hydroxyl groups in phenolic compounds prepared in their oxidized form are converted to quinine (Balange & Benjakul, 2009). These findings showed that polyphenol conjugation improves the radical scavenging ability of DPPH since this produced a higher number of hydroxyl groups to prevent the radical chain reaction for DPPH. Hence, the radical scavenging activity of DPPH was affected by the content of bound polyphenols. However, covalent conjugates prepared by the alkaline method that was with a lower content of bound polyphenols, had the strongest radical scavenging ability of DPPH among all the conjugates. This was explained due to the structural difference between covalent conjugates. Conjugates of rice protein hydrolysates and chlorogenic acid prepared by the alkaline method had the most flexible structure (Fig. 6B) due to their highest contents of α-helix and random coils. Based on the docking results (Fig. 5), the antioxidant amino acid including the methionine and cysteine (Elias, Kellerby & Decker, 2008), might be exposed to the environment after covalent conjugation. The enzyme modification with the highest degree of crosslinking (Fig. 2) might lead to the covalent binding with these antioxidant amino acids,

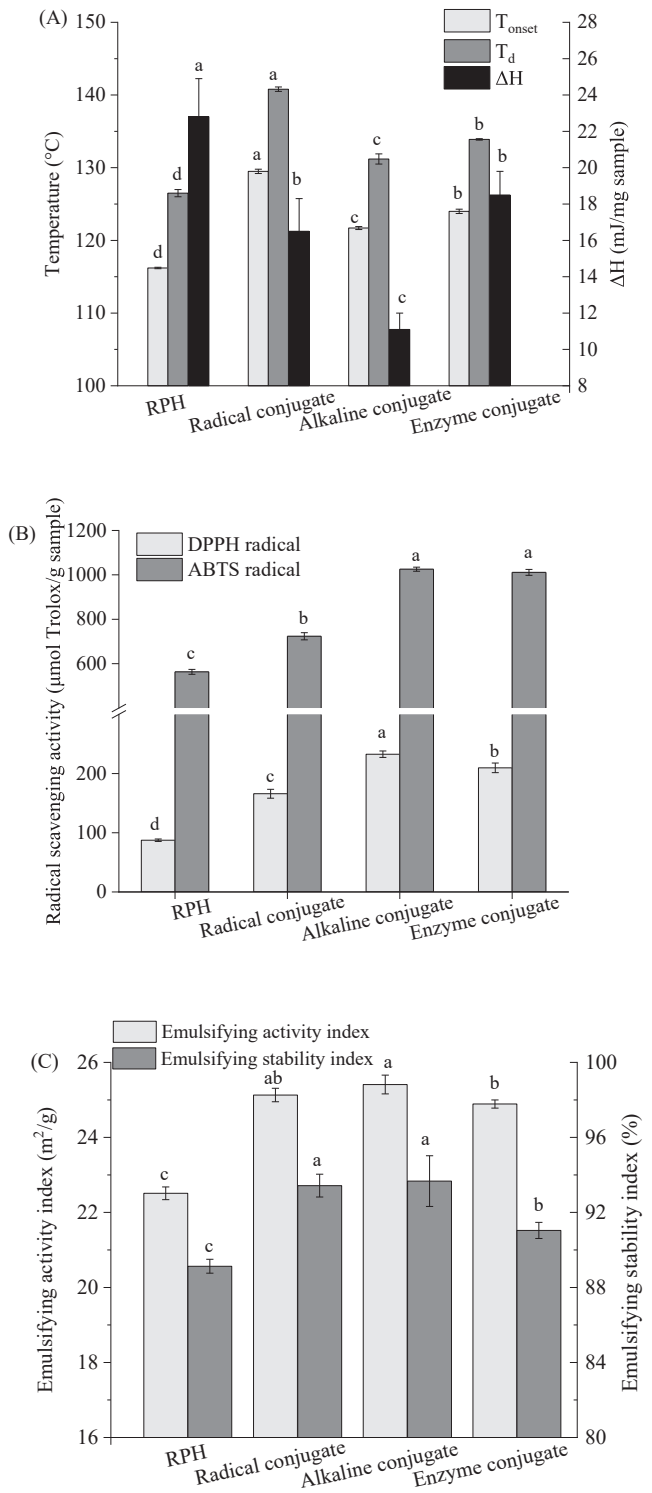

Fig. 6. Thermal transition characteristics (A), antioxidant activities (B) and emulsifying properties (C) of control rice protein hydrolysates (RPH) and covalent conjugates prepared with RPH and chlorogenic acid (CA) by different methods. Radical conjugate, RPH-CA conjugates formed by radical method. Alkaline conjugate, RPH-CA conjugates formed by alkaline method. Enzyme conjugate, RPH-CA conjugates formed by enzyme method. Different letters in the same property mean a significant difference by Duncan's multiple range test at $p < 0.05$.

resulting in lower DPPH scavenging activity than alkaline conjugate. Similarly, the radical scavenging activity of ABTS for conjugates was also increased ($p < 0.05$) compared to that for control samples (Fig. 6B). The difference in ABTS scavenging activity might be attributed to the varying chlorogenic acid contents in conjugates.

3.6. Effects of polyphenol covalent modification on the emulsifying activity of rice protein hydrolysates

As shown in Fig. 6C, the emulsifying activity index of rice protein hydrolysates was 22.51 m^2/g, but it was ($p < 0.05$) increased from 24.89 m^2/g to 25.41 m^2/g after covalent conjugation. Similarly, the emulsifying stability index of conjugates also increased. Emulsifying properties refer to the ability of a substance to form the adsorption films around oil globules for lowering the interfacial tension at oil–water interface (Bos & Van, 2001). In this study, the covalent interaction between rice protein hydrolysates and chlorogenic acid caused the unfolding of protein structure, resulting in the exposure of many aromatic residues. This also caused an increase in the affinity of proteins to the oil/water interface, which improved the emulsifying activity of proteins. The emulsifying activity of conjugate prepared by the alkaline method was higher than that of other conjugates (Fig. 3B). This is due to the highest content and flexibility of unfolded structure in the conjugate. This is consistent with the results from our study on the *in vitro* antioxidant activity of conjugates. Compared with other conjugates, the conjugate prepared by the enzyme method had the lowest fluorescence intensity and the highest content of β-sheet. The aromatic residues became more exposed due to the crosslinking that was developed by the enzyme treatment, as this conjugate appeared to be more stable compared to other conjugates. Proteins with more flexible structures have been reported to be more easily adsorbed onto the oil–water interface (Jiang et al., 2015). Thus, it is likely that the emulsifying properties of conjugates are dependent on the degree of unfolding and flexibility for of structure that is affected by the covalent conjugation.

4. Conclusion

In conclusion, rice protein hydrolysate-chlorogenic acid conjugates were prepared using the radical, enzyme, and alkaline methods. Chlorogenic acid covalently reacted with the nucleophilic amino acid side chains of rice protein hydrolysates, resulting in an increase of the contents of random coils and unfolded protein structures. Moreover, molecular docking revealed the mechanism of covalent interactions between LYS 127 site in the protein and that in the chlorogenic acid at a molecular level. Conjugate that formed by the alkaline method with 18.19 nmol/mg of polyphone bounds, possessed a stronger antioxidant capacity and better emulsifying properties compared to these formed by other methods. These findings demonstrate that the covalent conjugates formed by alkaline methods can be more appropriate in the use for improving the functional properties of protein hydrolysates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Ali, M., Homann, T., Khalil, M., Kruse, H. P., & Rawel, H. (2013). Milk whey protein modification by coffee-specific phenolics: Effect on structural and functional properties. *Journal of Agricultural and Food Chemistry, 61*(28), 6911–6920. https://doi.org/10.1021/jf402221m

Balange, A. K., & Benjakul, S. (2009). Effect of oxidised tannic acid on the gel properties of mackerel (*Rastrelliger kanagurta*) mince and surimi prepared by different washing processes. *Food Hydrocolloid, 23*(7), 1693–1701. https://doi.org/10.1016/j.foodhyd.2009.01.007

Bessada, S. M., Barreira, J. C., & Oliveira, M. B. (2019). Pulses and food security: Dietary protein, digestibility, bioactive and functional properties. *Trends in Food Science & Technology, 93*, 53–68. https://doi.org/10.1016/j.tifs.2019.08.022

Bos, M. A., & Van Vliet, T. (2001). Interfacial rheological properties of adsorbed protein layers and surfactants: A review. *Advances in Colloid and Interface Science, 91*(3), 437–471. https://doi.org/10.1016/S0001-8686(00)00077-4

Cao, Y., & Xiong, Y. L. (2015). Chlorogenic acid-mediated gel formation of oxidatively stressed myofibrillar protein. *Food Chemistry, 180*, 235–243. https://doi.org/10.1016/j.foodchem.2015.02.036

Carton, I., Bocker, U., Ofstad, R., Sørheim, O., & Kohler, A. (2009). Monitoring secondary structural changes in salted and smoked salmon muscle myofiber proteins by FT-IR microspectroscopy. *Journal of Agricultural and Food Chemistry, 57*(9), 3563–3570. https://doi.org/10.1021/jf803668e

Chen, L., Xie, X., Li, Y., Xiong, H., & Li, L. (2022). Activation mechanism of whey protein isolate mediated by free radicals generated in the ascorbic acid/hydrogen peroxide system. *Food Chemistry, 132533*. https://doi.org/10.1016/j.foodchem.2022.132533

Chen, Y., Wang, C., Liu, H., Liu, Q., & Kong, B. J. (2018). Enhanced physical and oxidative stability of porcine plasma protein hydrolysates based oil-in-water emulsions by adding oxidized chlorogenic acid. *Colloids and Surfaces A, 558*, 330–337. https://doi.org/10.1016/j.colsurfa.2018.08.067

Dai, T., Chen, J., McClements, D. J., Hu, P., Ye, X., Liu, C., & Li, T. (2019). Protein–polyphenol interactions enhance the antioxidant capacity of phenolics: Analysis of rice glutelin–procyanidin dimer interactions. *Food & Function, 10*(2), 765–774. https://doi.org/10.1039/C8FO02246A

Day, L. (2013). Proteins from land plants–potential resources for human nutrition and food security. *Trends in Food Science & Technology, 32*(1), 25–42. https://doi.org/10.1016/j.tifs.2013.05.005

Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition, 48*(5), 430–441. https://doi.org/10.1080/10408390701425615

Gordon, M. H., & Wishart, K. (2010). Effects of chlorogenic acid and bovine serum albumin on the oxidative stability of low density lipoproteins in vitro. *Journal of Agricultural and Food Chemistry, 58*(9), 5828–5833.

Guo, Y., Bao, Y., Sun, K., Chang, C., & Liu, W. (2020). Effects of covalent interactions and gel characteristics on soy protein-tannic acid conjugates prepared under alkaline conditions. *Food Hydrocolloid, 106293*. https://doi.org/10.1016/j.foodhyd.2020.106293

Hirano, K., Hino, S., Oshima, K., Nadano, D., Urisu, A., Takaiwa, F., & Matsuda, T. (2016). Evaluation of allergenic potential for rice seed protein components utilizing a rice proteome database and an allergen database in combination with IgE-binding of recombinant proteins. *Bioscience, Biotechnology, and Biochemistry, 80*(3), 564–573. https://doi.org/10.1080/09168451.2015.1116927

Jiang, Z. Q., Sontag-Strohm, T., Salovaara, H., Sibakov, J., Kanerva, P., & Loponen, J. (2015). Oat protein solubility and emulsion properties improved by enzymatic deamidation. *Journal of Cereal Science, 64*, 126–132. https://doi.org/10.1016/j.jcs.2015.04.010

Jing, H., Sun, J., Mu, Y., Obadi, M., McClements, D., & Xu, B. (2020). Sonochemical effects on the structure and antioxidant activity of egg white protein–tea polyphenol conjugates. *Food & Function, 11*(8), 7084–7094. https://doi.org/10.1039/D0FO01636E

Kroll, J., Rawel, H. M., & Rohn, S. (2003). Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Science and Technology Research, 9*(3), 205–218. https://doi.org/10.3136/fstr.9.205

L'Hocine, L., & Boye, J. I. (2007). Allergenicity of soybean: New developments in identification of allergenic proteins, cross-reactivities and hypoallergenization technologies. *Critical Reviews in Food Science and Nutrition, 47*(2), 127–143. https://doi.org/10.1080/10408390600626487

Liu, F., Sun, C., Yang, W., Yuan, F., & Gao, Y. (2015). Structural characterization and functional evaluation of lactoferrin–polyphenol conjugates formed by free-radical graft copolymerization. *RSC Advances, 5*(20), 15641–15651. https://doi.org/10.1039/C4RA10802G

Lobley, A., Whitmore, L., & Wallace, B. (2002). DICHROWEB: An interactive website for the analysis of protein secondary structure from circular dichroism spectra. *Bioinformatics, 18*(1), 211–212.

Ma, H., Forssell, P., Partanen, R., Buchert, J., & Boer, H. (2011). Improving laccase catalyzed cross-linking of whey protein isolate and their application as emulsifiers. *Journal of Agricultural and Food Chemistry, 59*(4), 1406–1414. https://doi.org/10.1021/jf103591p

Mondoulet, L., Paty, E., Drumare, M., Ah-Leung, S., Scheinmann, P., Willemot, R., … Bernard, H. (2005). Influence of thermal processing on the allergenicity of peanut proteins. *Journal of Agricultural and Food Chemistry, 53*(11), 4547–4553. https://doi.org/10.1021/jf050091p

Pan, B. F., Gao, F., & Ao, L. M. (2005). Investigation of interactions between dendrimer-coated magnetite nanoparticles and bovine serum albumin. *Journal of Magnetism and Magnetic Materials, 93*(1), 252–258. https://doi.org/10.1016/j.jmmm.2005.02.018

Pan, X., Fang, Y., Wang, L., Shi, Y., Xie, M., Xia, J., … Shen, X. (2019b). Covalent interaction between rice protein hydrolysates and chlorogenic acid: Improving the stability of oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 67*(14), 4023–4030.

Pan, X., Fang, Y., Wang, L., Xie, M., Hu, B., Zhu, Y., … Li, P. (2019a). Effect of enzyme types on the stability of oil- in- water emulsions formed with rice protein hydrolysates. *Journal of the Science of Food and Agriculture, 99*(15), 6731–6740. https://doi.org/10.1002/jsfa.9955

Prigent, S. V., Voragen, A. G., Visser, A. J., van Koningsveld, G. A., & Gruppen, H. (2007). Covalent interactions between proteins and oxidation products of caffeoylquinic acid (chlorogenic acid). *Journal of the Science of Food and Agriculture, 87*(13), 2502–2510. https://doi.org/10.1002/jsfa.3011

Rawel, H. M., Czajka, D., Rohn, S., & Kroll, J. (2002). Interactions of different phenolic acids and flavonoids with soy proteins. *International Journal of Biological Macromolecules, 30*(3–4), 137–150. https://doi.org/10.1016/S0141-8130(02)00016-8

Rawel, H. M., Rohn, S., Kruse, H. P., & Kroll, J. (2002). Structural changes induced in bovine serum albumin by covalent attachment of chlorogenic acid. *Food Chemistry, 78*(4), 443–455. https://doi.org/10.1016/S0308-8146(02)00155-3

Rohn, S., Rawel, H. M., & Kroll, J. (2004). Antioxidant activity of protein-bound quercetin. *Journal of Agricultural and Food Chemistry, 52*(15), 4725–4729. https://doi.org/10.1021/jf0496797

Sievers, S., Rawel, H. M., Ringel, K. P., Niggemann, B., & Beyer, K. (2016). Wheat protein recognition pattern in tolerant and allergic children. *Pediatric Allergy and Immunology, 27*(2), 147–155. https://doi.org/10.1111/pai.12502

Spizzirri, U. G., Iemma, F., Puoci, F., Cirillo, G., Curcio, M., Parisi, O. I., & Picci, N. J. B. (2009). Synthesis of antioxidant polymers by grafting of gallic acid and catechin on gelatin. *Biomacromolecules, 10*(7), 1923–1930. https://doi.org/10.1021/bm900325t

Spizzirri, U. G., Parisi, O. I., Iemma, F., Cirillo, G., Puoci, F., Curcio, M., & Picci, N. (2010). Antioxidant-polysaccharide conjugates for food application by eco-friendly grafting procedure. *Carbohydrate Polymers, 79*(2), 333–340. https://doi.org/10.1016/j.carbpol.2009.08.010

Wouters, A. G., Rombouts, I., Fierens, E., Brijs, K., & Delcour, J. A. (2016). Relevance of the functional properties of enzymatic plant protein hydrolysates in food systems. *Comprehensive Reviews in Food Science and Food Safety, 15*(4), 786–800. https://doi.org/10.1111/1541-4337.12209

Wu, X., Lu, Y., Xu, H., Lin, D., He, Z., Wu, H., … Wang, Z. (2018). Reducing the allergenic capacity of β-lactoglobulin by covalent conjugation with dietary polyphenols. *Food Chemistry, 256*, 427–434. https://doi.org/10.1016/j.foodchem.2018.02.158

Xu, H., Zhang, T., Lu, Y., Lin, X., Hu, X., Liu, L., … Wu, X. (2019). Effect of chlorogenic acid covalent conjugation on the allergenicity, digestibility and functional properties of whey protein. *Food Chemistry, 298*, Article 125024. https://doi.org/10.1016/j.foodchem.2019.125024

Xu, X., Liu, W., Liu, C., Luo, L., Chen, J., Luo, S., … Wu, L. (2016). Effect of limited enzymatic hydrolysis on structure and emulsifying properties of rice glutelin. *Food Hydrocolloid, 61*, 251–260. https://doi.org/10.1016/j.foodhyd.2016.05.023

You, J., Luo, Y., & Wu, J. (2014). Conjugation of ovotransferrin with catechin shows improved antioxidant activity. *Journal of Agricultural and Food Chemistry, 62*(12), 2581–2587. https://doi.org/10.1021/jf405635q

Zheng, Z., Li, J., Li, J., Sun, H., & Liu, Y. (2019). Physicochemical and antioxidative characteristics of black bean protein hydrolysates obtained from different enzymes. *Food Hydrocolloid, 97*, Article 105222. https://doi.org/10.1016/j.foodhyd.2019.105222

Zhou, K., Canning, C., & Sun, S. (2013). Effects of rice protein hydrolysates prepared by microbial proteases and ultrafiltration on free radicals and meat lipid oxidation. *LWT - Food Science and Technology, 50*(1), 331–335. https://doi.org/10.1016/j.lwt.2012.05.002