



Covalent conjugation with (–)-epigallo-catechin 3-gallate and chlorogenic acid changes allergenicity and functional properties of Ara h1 from peanut

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ABSTRACT

Ara h1 is a major allergen from peanut. We investigated the effect of covalent conjugation of Ara h1 and dietary polyphenols on allergenicity and functional properties of Ara h1. Enzyme-linked immunosorbent assay revealed that the covalent conjugation of dietary polyphenols significantly reduced the IgE binding capacity of Ara h1. Covalent binding of dietary polyphenols with Ara h1 reduced histamine release by 40% in basophils. The decreased IgE binding capacity of Ara h1 could be ascribed to changes in protein conformation. The IgE epitope of Ara h1 might be blocked by polyphenols at the binding site. Analysis of pepsin digestion of Ara h1–polyphenol conjugates indicated that the covalent binding increased pepsin digestibility and reduced IgE binding capacity. Furthermore, covalent conjugation of Ara h1 with polyphenols decreased denaturation temperature and increased antioxidant activity. Ara h1 conjugated with polyphenols may be a promising approach for reducing the allergenicity of Ara h1.

1. Introduction

Over the past decade, the prevalence of peanut allergy has increased; peanut allergy affects 1% to 3% of the population in western countries, usually in early childhood (Fisher, Keet, Lack, & du Toit, 2019). Peanut-induced allergic diseases seriously reduce the quality of life of affected individuals (Shemesh et al., 2013). Therefore, investigating a reduction in peanut allergenicity has great significance.

Ara h1 is the most abundant allergen in the peanut kernel and plays an important role in the sensitization process (Tian, Rao, Zhang, Tao, & Xue, 2018). Ara h1 accounts for 12% to 16% of total protein in peanut kernels; it is a glycoprotein of molecular mass 63.5 kDa with an isoelectric point of 4.55 (Montserrat, Mayayo, Sanchez, Calvo, & Perez, 2013) and can be used as a common model for studying peanut allergy (Chruszcz et al., 2011).

In the food industry, processing methods such as heating, high pressure and enzymatic hydrolysis are often used to reduce the allergenicity of peanut (Koppelman, Hefle, Taylor, & de Jong, 2010;

Montserrat et al., 2013). However, those methods have certain deficiencies: some heating treatments cannot reduce Ara h1 sensitization but can increase the IgE binding capacity of Ara h1 protein (Koppelman et al., 2010; Maleki et al., 2000), and high pressure and enzyme hydrolysis can significantly reduce the IgE binding capacity of allergen but destroy the functional properties and produce a bad taste (Wu et al., 2018). Numerous studies have shown that methods of covalent modification can reduce food allergenicity, which might be a promising method for desensitization during food production (Huang et al., 2012). Covalent modification including glycation (Wu et al., 2013), PEGylation (Zhong et al., 2016), reduction and alkylation (Apostolovic et al., 2013) and polyphenol conjugation (Wu et al., 2018) can reduce allergenicity.

Polyphenols can conjugate with proteins covalently (Ozdal, Capanoglu, & Altay, 2013). Recently, our studies showed that polyphenols covalently conjugated with β -lactoglobulin and ovalbumin reduced IgE binding capacity, while improving functional properties in the proteins (Wu et al., 2018; He et al., 2019). Hence, covalently

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conjugating polyphenols with peanut allergen to reduce its allergenicity and increase nutritional properties of peanut is a promising approach.

(-)-Epigallocatechin gallate (EGCG) and chlorogenic acid (CHA) are common dietary polyphenols in many plant foods. Both EGCG and CHA have multiple biological activities that are beneficial for human health, such as anti-oxidizability, antitumor and antimicrobial activity (Naveed et al., 2018). To our knowledge, the covalent binding of Ara h1 with polyphenols has not been reported.

In this study, we investigated the covalent interaction of Ara h1 with EGCG and CHA. The aim of this work was to assess the effect of conjugation with polyphenols on the structure, IgE-binding and functional properties of Ara h1.

2. Materials and methods

2.1. Materials and reagents

Fresh peanuts (Shenzhen, Guangdong, China) were purchased from a local supermarket. Ara h1 was purified as previously described (Montserrat et al., 2013). EGCG (purity $\geq 98\%$), CHA (purity $\geq 99\%$), diammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) and bovine serum albumin (BSA) were from Sigma-Aldrich Co (St. Louis, MO, USA). 1,10-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu phenol reagent and 3,3',5,5'-tetramethylbenzidine (TMB) were from Solarbio Co. (Beijing, China). Enhanced chemiluminescence (ECL) substrate was from Bio-Rad Co (Hercules, CA, USA). The BCA protein quantitative test kit was from Thermo Fisher Scientific (Waltham, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-human IgE was from SouthernBiotech (Birmingham, AL, USA). All other chemicals were of analytical grade.

2.2. Human serum

Serum samples from peanut-allergic patients for enzyme-linked immunosorbent assay (ELISA), western blot analysis and histamine release experiments were kindly provided by Shenzhen Children's Hospital. Pooled serum was collected from 15 patients who were allergic to peanut. All serum-sample IgE levels to peanut were > 5 KU/L and were stored at -80°C . The research was approved by the Medical Ethics Committee of Shenzhen Children's Hospital and all patients knew about the use of their serum.

2.3. Preparation of Ara h1-polyphenol conjugates

Ara h1-CHA and Ara h1-EGCG conjugates were synthesized by the alkali method as described (Rawel, Rohn, Kruse, & Kroll, 2002) with modification. In brief, 0.64 g Ara h1 was dissolved in 100 ml distilled H_2O , and the pH value of protein samples was adjusted to 9 by using 0.5 M NaOH. Then 242.7 mg EGCG or 172.7 mg CHA was added and continuously stirred at room temperature (25°C) with free exposure to air for 24 h. The sample was dialyzed with deionized water at 4°C for 24 h to remove the free CHA and EGCG. The vaporous solid was obtained by lyophilization. Control Ara h1 was treated by the same method but without EGCG or CHA.

2.4. Chemical characterization of Ara h1-polyphenol conjugates

2.4.1. Determining the content of polyphenol of the Ara h1-polyphenol conjugate

The Foline-Ciocalteu method was used to determine total polyphenol content in Ara h1-polyphenol conjugates as described (Xu et al., 2019) with minor modification. Briefly, 5 ml Foline-Ciocalteu phenol reagent (200 mM) was mixed thoroughly with 1 ml Ara h1 or Ara h1-polyphenol conjugates (2 mg/ml) at 25°C for 5 min. Then, 4 ml of 7.5% sodium carbonate solution was added and the mixture was placed in the dark for 2 h with continuous shaking at 25°C . Finally, 760 nm

absorbance of the sample was detected and the amount of EGCG and CHA was detected according to the standard calibration curve of EGCG and CHA. The grafting efficiency % was determined as polyphenol sample/polyphenol content in the reactants before dialysis $\times 100\%$.

2.4.2. Profile analysis of Ara h1-polyphenol conjugate by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

A 100- μl amount of sample (1 mg/ml) solution of native Ara h1, control Ara h1 and Ara h1-polyphenol conjugate was mixed with 25 μl of $5 \times$ loading buffer, then denatured by water bath heating at 100°C for 5 min. A 15- μl sample solution was loaded into each well of SDS-PAGE gel (12% separating gel and 5% stacking gel) for 120 constant voltage electrophoresis for 60 min. After gels were stained with Coomassie Brilliant Blue R-250G for 3 h at room temperature, they were destained with acetic acid decolored solution for protein visualization.

2.4.3. Molecular weight identification of Ara h1-polyphenol conjugate by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS was used to detect the molecular weight of Ara h1-polyphenol conjugates as described (Lu et al., 2018). In brief, 1-mg samples were dissolved in 1 ml of 0.1% acetonitrile/trifluoroacetic acid with 2 mg dithiothreitol. Then, 0.5 μl solution was placed on the target and covered with 0.5 μl 2,5-dihydroxybenzoic acid as the matrix compound. Then samples were air dried before measurement. MALDI MS involved using a TM MALDI-TOF/TOF system (Bruker Daltonics, Billerica, MA, USA).

2.4.4. Measurement of free amino groups

The trinitro-benzene-sulfonic acid (TNBS) method was used to determine amino groups as described (Rawel et al., 2002) with modification. Briefly, a 50- μl sample (1 mg/ml) was mixed with 500 μl phosphate buffered saline (PBS; pH 8.2) in the test tube, then 400 μl of 0.1% TNBS solution was added and the test tube was placed in a 50°C water bath for 60 min with shaking. Afterward, 0.8 ml of 100 mM HCl was added to end the reaction, then absorbance was detected at 340 nm. The lysine solution of 0.2 mg/ml was prepared with distilled water and diluted to 0.1, 0.05, 0.025, 0.0125 mg/ml. The 340-nm absorbance value of lysine was detected by the same method and the standard curve was established. Then the content of free amino groups was calculated according to the standard curve of lysine.

2.4.5. Determination of sulfhydryl thiol (SH) groups

5, 5-dithiobis nitro-benzoic acid (DTNB) was used to measure the content of SH groups as described (Liu, Sun, Yang, Yuan, & Gao, 2015) with modification. Briefly, 50 μl DTNB reagent (5 mg/ml DTNB dissolved in 50 mM Tris-HCl buffer containing 1 mM EDTA) was added to 1 ml samples (0.5 mg/ml) in a test tube and mixed rapidly. After 1 h of incubation, the 412 nm absorbance of the samples was measured by using a Hitachi-850 spectrofluorometer (Hitachi Co., Tokyo). The sulfhydryl thiol group content ($\mu\text{mol/g}$) was calculated as $75.53 \times A_{412}/C$, where C is the concentration of protein, mg/ml.

2.4.6. Determination of tyrosine groups

The content of tyrosine groups in samples was measured as we previously described (Wu et al., 2018). In brief, 0.5 ml nitric acid (16 M) was added to a 0.5-ml sample (1 mg/ml) in a tube, then the mixture was heated at 50°C for 15 min. Thereafter, 2 ml ethyl alcohol and 2 ml NaOH (5 M) were added to end the reaction. Finally, 360 and 430 nm absorbance was detected by using a Hitachi-850 spectrofluorometer (Hitachi Co., Tokyo). Then the tyrosine group content was calculated as $0.5357 \times A_{430} - 0.35714 \times A_{360}$.

2.5. Structural analysis of the Ara h1–polyphenol conjugates

2.5.1. Fluorescence analysis

Fluorescence of samples was determined as described (He et al., 2019). The fluorescence of native and control Ara h1 and Ara h1–polyphenol conjugates was measured by using a RF-5301PC fluorescence spectrometer (Shimadzu, Kyoto, Japan). Samples (0.5 mg/ml) were dissolved in PBS (0.01 mol/l, pH 7.0). A 1-ml amount of sample solution was incubated in a 1-cm quartz cell at 25 °C. PBS was the blank solution for all samples. Then fluorescence spectra were collected at emission wavelength 300 to 500 nm and excitation wavelength 295 nm. All samples were analyzed in triplicate.

2.5.2. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy of samples was performed as described (Lu et al., 2018). The FTIR spectra for native and control Ara h1 and Ara h1–polyphenol conjugates were determined by using a FTIR-8300PCS spectrometer (SHIMADZU Co., Kyoto, Japan). The samples were mixed with KBr pellets equally at 25 °C. Then infrared spectra were collected from the spectral range 400–4000 cm^{-1} . KBr was a reference.

2.5.3. Circular dichroism (CD)

CD spectroscopy detection was performed as described (Xu et al., 2019). The CD spectra for native and control Ara h1 and Ara h1–polyphenol conjugates were obtained by using a Jasco J-815 CD spectrometer (Jasco Co., Japan) at room temperature. Samples (0.5 mg/ml) were dissolved in PBS (pH 7.2) with a 1-ml amount of sample solution in a 1-cm quartz cell, under a nitrogen atmosphere. Then CD spectra were collected from 190 to 250 nm with a 100-nm/min scan rate. The secondary structure of the protein was analyzed by using DichroWeb as described (Wu et al., 2014).

2.5.4. Scanning electron microscopy (SEM)

SEM observations were performed as described with some modification (Liu, Ma, McClements, & Gao, 2017). Microstructures of native and control Ara h1 and Ara h1–polyphenol conjugates were obtained by SEM (SU8000, Hitachi, Japan). The samples (0.2 mg/ml) were dissolved in PBS (pH 7.2). A 5- μl sample solution was dispersed in a 5 × 5-mm monocrystalline silicon piece and vacuum-dried at low temperature. Digital images of samples were collected and representative images are presented.

2.5.5. Determination of thermal stability by differential scanning calorimetry (DSC)

DSC was used to analyze the thermal stability of native and control Ara h1 and Ara h1–polyphenol conjugates by using a DSC-60 thermal analysis system (Shimadzu, Tokyo) as described (He et al., 2019). A 5-mg protein sample was tightly packed onto the aluminum plate, and nitrogen at the rate of 30 ml/min was used as the transfer gas. Thermal stability analysis was performed from 40 to 150 °C at a rate of 2 °C/min.

2.6. Analysis of sites in Ara h1 – polyphenol conjugates by liquid chromatography/tandem mass spectrometry (LC-MS/MS)

LC-MS/MS was used to analyze sites of Ara h1–polyphenol conjugates as we previously described (He et al., 2019). The protein samples of native Ara h1, Ara h1–CHA and Ara h1–EGCG were cut from the SDS-PAGE gel and transferred to a 1.5-ml centrifuge tube and rinsed with Mill-Q water twice, then gel was de-stained with 100 μl of 50 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) and incubated with occasional vortexing for 30 min, depending on the staining intensity. Then the de-stained solution was discarded, washed twice with 200 μl Mill-Q water, and 400 μl of 100% acetonitrile was added for 15 min to dry the gel spots in a SpeedVac for 10 min. Finally, gels were rehydrated in a minimal volume of sequencing-grade porcine trypsin or chymotrypsin (Promega, USA) solution (20 $\mu\text{g/ml}$ in 25 mM

NH_4HCO_3) and incubated at 37 °C overnight. The supernatant was transferred into a 200- μl microcentrifuge tube and gels were extracted once with extraction buffer (67% acetonitrile containing 1% trifluoroacetic acid). The peptide extract and supernatant of the gel were combined, then completely dried in a SpeedVac. The lyophilized tryptic peptides were resuspended in 2% acetonitrile containing 0.1% formic acid. A 4- μl amount of this solution was injected onto a ChromXP C18 (120 Å, 3 μm) trap column. Then operational steps were performed according to (He et al., 2019). Finally, The MS/MS raw data were submitted to ProteoWizard Software to generate Mascot generic format (mgf) files for further identification of conjugation sites of polyphenols in Ara h1.

2.7. Allergenic capacity of Ara h1–polyphenol conjugates

2.7.1. ELISA

The IgE-binding capacity of native and control Ara h1 and Ara h1–polyphenol conjugates was analyzed by ELISA as we previously described (Lu et al., 2018) with modification. In brief, 100 μl Ara h1 solution (1 mg/ml) was added to 96-well plates, which were placed in a 4 °C refrigerator for 16 h. The Ara h1 solution was removed and wells were washed with PBS tween-20 (PBST) three times. A 200- μl amount of 3.0% BSA in PBST was added to wells and blocked with blocking buffer at 37 °C for 2 h. The blocking buffer was removed and samples were washed with PBST three times, each for 5 min. Then 50 μl sample (0.02–20 $\mu\text{g/ml}$) was mixed with 50 μl serum from peanut-allergic patients (diluted 1:100). The mixture was added to each well and incubated at room temperature for 1 h. Then 100 μl HRP-conjugated goat anti-human IgE antibody (1:2000) was added to 96-well plates and incubated at 37 °C for 60 min. Antibody solution was removed and samples were washed with PBST three times, each 5 min. Then 50 μl TMB color liquid was added to each well and incubated at 37 °C for 10 min. Finally, 50 μl stopping solution (H_2SO_4 , 2 M) was added to end the enzyme reaction. The 450 nm absorbance was measured.

2.7.2. Histamine release experiments

The content of histamine release was measured as described (Meng et al., 2016) with some modification. Briefly, human basophilic KU812 cells were cultured in Iscove's modified Dulbecco's medium (Gibco, Shanghai) with 10% fetal bovine serum (Gibco, Australia) at 37 °C under a humidified atmosphere with 5% CO_2 . KU812 cells (1.0×10^6 cells/ml) were seeded in 48-well culture plates for 24 h. Serum from patients with peanut allergy (v/v = 20:1) was added to each well and incubated for 24 h, then wells were stimulated with native Ara h1 and Ara h1–polyphenol conjugates (0.01–100 $\mu\text{g/ml}$) for 4 h. A 10- $\mu\text{g/ml}$ amount of BSA was a negative control. An ELISA kit (Immuno-tech, Marseille, France) was used to detect the histamine release of samples according to the manufacturer's instructions.

2.7.3. Simulated gastric fluid (SGF) digestion and western blot analysis

Ara h1 was digested by using pepsin as described (Koppelman et al., 2010) with minor modifications. In brief, the SGF for gastric digestion consisting of 76 $\mu\text{g/ml}$ pepsin dissolved in 35 mM NaCl with pH 1.2 was prepared. For the digestion stability assay, 1.7 ml simulated gastric fluid was added to a 2-ml centrifuge tube, preheated at 37 °C for 10 min. Then 90 μl of 5 mg/mL test protein was added and the tube was centrifuged at time $t = 0$. Thereafter, 200 μl sample was collected at 0.5, 1, 2, 4, 8, 15, 30 and 60 min. The digestion was carried out at 37 °C with continuous shaking and was stopped at the appropriate time by mixing with 70 μl of 200 mM NaHCO_3 (pH 9.0). Then 70 μl of the five times of loading buffer was added to the digestion samples and boiled for 7 min. All digestion samples underwent SDS-PAGE analysis as described above.

Western blot analysis was performed to analyze the specific IgE binding ability of digested samples. The protein in 12% SDS-PAGE gels was electrophoretically transferred onto polyvinylidene fluoride

membranes. BSA TBST at 5% was used to block the non-specific binding sites in membranes for 2 h at room temperature. Then membranes were incubated with serum from peanut-allergic patients (diluted 1:100) for 1 h, washed in PBST for 10 min and repeated three times. Then HRP-conjugated goat anti-human IgE antibody (1:2000) was added to membranes and incubated for 1 h at room temperature. Finally, the ECL analysis system (GE Healthcare, Buckinghamshire, UK) was used to detect protein bands, representing the specific IgE-binding capacity of samples.

2.8. Antioxidant activity analysis of Ara h1-polyphenol conjugates

2.8.1. DPPH scavenging activity analysis

The antioxidant activity of the native and control Ara h1 and Ara h1-polyphenol conjugates was measured by DPPH scavenging activity as we previously reported (Xu et al., 2019) with minor modifications. Briefly, 3 ml sample (0.5 mg/ml) was mixed with 3 ml DPPH (1.75×10^{-4} M, methanol), then placed in the dark at room temperature for 1 h. Thereafter, absorbance at 517 nm was detected. The DPPH free radical scavenging rate % was calculated as $[A_0 - (A_t - B)] / A_0 \times 100\%$, where A_0 is the absorbance value of control DPPH, A_t is the absorbance value of the sample after 1-h reaction, B is the absorbance value of the protein sample without DPPH. The radical scavenging activity was expressed as Trolox equivalents. The Trolox calibration curve was calculated by the same method to calculate DPPH radical scavenging activity.

2.8.2. ABTS⁺ scavenging activity

ABTS⁺ scavenging activity of Ara h1 and Ara h1-polyphenol conjugates was detected as described (He et al., 2019) with some modification. In short, 1 ml sample (0.5 mg/ml) was mixed with 3 ml ABTS reagent. The mixture was incubated at 25 °C for 1 h. Finally, the absorbance at 734 nm was detected. The formula and calculation of DPPH was fitted for the expression of the ABTS⁺ radical-scavenging capacity.

2.9. Statistical analysis

All measurements were performed at least in triplicate. All data are expressed as mean $\pm$ SD. Data were analyzed by one-way ANOVA followed by multiple comparisons with post-hoc Tukey's test by using SPSS 20.0. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Molecular weight change of Ara h1

SDS-PAGE and MALDI-TOF MS were used to analyze the molecular weight change of the Ara h1-polyphenol conjugates. The main band for native and control Ara h1 was about 63.5 kDa, whereas the bands for Ara h1-EGCG and Ara h1-CHA conjugates migrated upward slightly (Fig. 1A), which suggests that the molecular weight of Ara h1 by covalent conjugation with EGCG and CHA. The finding was similar to a previous report (Wu et al., 2018), in which CHA increased the molecular weight of OVA by covalent conjugation.

MALDI-TOF MS was used to further analyze polyphenol conjugates with Ara h1 (Fig. 1B). The mass spectrum of unmodified protein showed a peak at about 61.331 kDa. Ara h1-EGCG and Ara h1-CHA conjugates showed the presence of peaks around 61.972 and 61.69 kDa, respectively, corresponding to additions of about 600 and 400 Da. These mass additions may due to the addition of one EGCG (458.4 Da) and one CHA (354.3 Da) molecule covalently conjugated with Ara h1, which were consistent with SDS-PAGE findings. MALDI-TOF MS is a non-quantitative method highly dependent on the ionisation ability of the modified protein type (Prigent, Voragen, Visser, van Koningsveld, & Gruppen, 2007). So we estimated the polyphenol contents of Ara h1-polyphenol conjugates and content of free amino, thiol groups and

tyrosine residue change of Ara h1 to analyze the degree of protein modification. The Folin-phenol reagent method was used to further analyze the polyphenol contents of Ara h1-polyphenol conjugates. The polyphenol contents of the Ara h1-EGCG and Ara h1-CHA conjugates were 19.17 ± 0.67 mg EGCG/g and 7.37 ± 0.27 mg CHA/g, respectively, and the mean polyphenol binding efficiency was $4.93 \pm 0.19\%$ and $2.71 \pm 0.10\%$ (Table 1). These results further confirmed that the EGCG and CHA conjugation with Ara h1 was consistent with the results of SDS-PAGE and MALDI-TOF MS.

3.2. Content of free amino, thiol groups and tyrosine residue change of Ara h1

We detected the content changes of thiol groups, tyrosine residue and free amino (Table 1) to further study the interactions of EGCG and CHA with Ara h1. The polyphenol conjugates with Ara h1 showed decreased content of free amino, which suggests that EGCG and CHA can react with side-chain amino acids of Ara h1 with alkaline treatment. The interaction between Ara h1 and polyphenols is a nucleophilic addition reaction and leads to polyphenol covalent modified proteins. These results were consistent with our previous report of EGCG conjugated with β -lactoglobulin decreasing the free amino groups of the protein (Wu et al., 2018). In this study, to avoid the free thiol groups converting to a disulphide bond, 8 M urea was added to prevent the production of disulphide bonds. The content of free thiol groups was lower in Ara h1-polyphenol conjugates than in native and control Ara h1 (Table 1), which suggested that CHA and EGCG interacted with free thiol groups in Ara h1 by covalent binding. Furthermore, the content of free tyrosine groups was lower in Ara h1-EGCG and Ara h1-CHA conjugates than control Ara h1. In aerobic conditions with alkaline treatment, polyphenol could react with amino acid residues of protein, such as histidine, tryptophan, tyrosine and N-terminal proline (Kroll, Rawel, & Rohn, 2003; Liu et al., 2017). Thus, the interaction between Ara h1 and polyphenols is via covalent binding, with reduced content of free amino, thiol groups and tyrosine residues.

3.3. Conjugate site analysis of Ara h1-polyphenol conjugates by LC-MS/MS

LC-MS/MS was used to analyze the binding site of Ara h1-polyphenol conjugates after trypsin digestion. For Ara h1-CHA, a new peak was identified at m/z 864.9533 (Fig. 2A), which could be the conjugation position of CHA in Ara h1. From MS/MS spectra for the ions, the charged ion at m/z 864.9533 was identified as LKEGDVFIMPAA-HPVAINASSWLHLLGFGINAENNH-chlorogenic acid RIFLAGDK (residues 499–542). For Ara h1-EGCG, a new peak was identified at m/z 867.4180, and the charged ion at m/z 867.4180 was identified as LKEGDVFIMPAAHPVAINASSWLHLLGFGINAENNH-RIFLAGDK(–)–Epigallocatechin-3-gallate (residues 499–542). Therefore, CHA and EGCG possibly conjugated with His494 and Lys542, respectively, in Ara h1.

Covalent binding of proteins to polyphenols can be prepared by radical or alkaline treatment (He et al., 2019). In this study, conjugates of Ara h1 and polyphenols were prepared by alkaline treatment because the radical method will cause Ara h1 precipitation under acidic conditions. Polyphenols in the presence of alkaline condition can be oxidized to their respective quinones, which could react with the nucleophilic amino acid residue of protein chains such as tyrosine, histidine, tryptophan, and cysteine (Kim & Cavaco-Paulo, 2012; Rohn, Rawel, & Kroll, 2004). Thus, in this work, CHA and EGCG were altered to their respective quinones under the alkaline condition and reacted with His 494 and Lys 542, respectively, finally generating the Ara h1-CHA and Ara h1-EGCG conjugates.

3.4. Structural analysis of the Ara h1-polyphenol conjugates

Fluorescence spectroscopy was used to measure the interaction

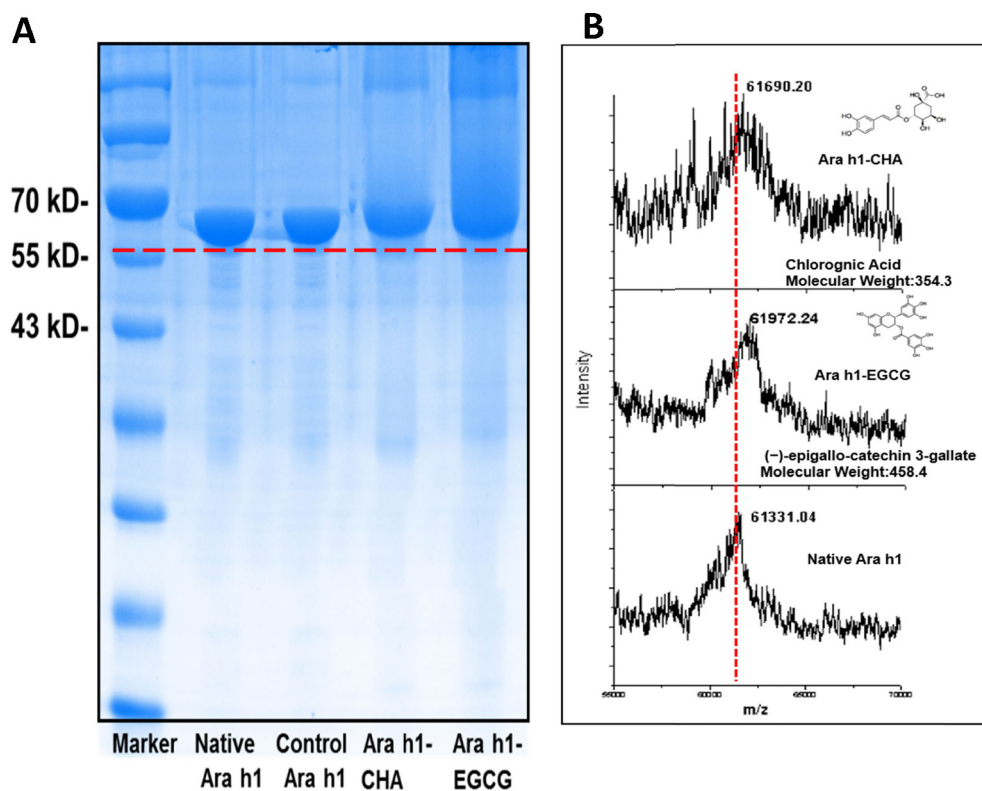


Fig. 1. SDS-PAGE (A) and MALDI-TOF-MS spectra (B) for native Ara h1, control Ara h1, Ara h1-CHA and Ara h1-EGCG. MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; EGCG, (-)-epigallocatechin gallate; CHA, chlorogenic acid.

Table 1
Characterization of Ara h1 and Ara h1-polyphenol conjugates.

Property/sample	Native Ara h1	Control Ara h1	Ara h1-CHA	Ara h1-EGCG	P
Free amino groups (nmol/mg protein)	472.9 ± 1.52	478.56 ± 1.15	407.84 ± 2.65 ^a	436.13 ± 1.53 ^{b,c}	< 0.01
Free thiol groups (nmol/mg protein)	24.37 ± 0.46	24.87 ± 0.57	17.47 ± 0.48 ^a	12.94 ± 0.62 ^{b,c}	< 0.01
Tyrosine group (nmol/mg protein)	14.9 ± 0.87	15.17 ± 0.72	11.17 ± 0.14 ^a	10.81 ± 1.21 ^{b,c}	< 0.01
Polyphenols bound (mg/g protein)	–	–	7.37 ± 0.27	19.17 ± 0.67 ^{b,c}	< 0.01
Grafting efficiency (%)	–	–	2.71 ± 0.10	4.93 ± 0.19 ^{b,c}	< 0.01
α-Helix (%) by CD	33.5 ± 0.2	32.4 ± 0.1	23.3 ± 0.3 ^b	22.5 ± 0.4 ^b	< 0.01
β-Sheet (%) by CD	16.8 ± 0.3	15.6 ± 0.6	17.4 ± 0.1 ^a	17.3 ± 0.1 ^b	< 0.01
β-Turn (%) by CD	12.4 ± 0.2	12.8 ± 0.2	14.5 ± 0.2 ^a	13.7 ± 0.2 ^{b,c}	0.02
Random coil (%) by CD	37.3 ± 0.1	39.2 ± 0.5	44.8 ± 0.2 ^a	46.5 ± 0.5 ^{b,c}	0.03
DPPH scavenging activity (μmol Trolox per g sample)	84.45 ± 3.01	86.25 ± 2.82	166.79 ± 5.86 ^a	207.58 ± 6.39 ^{b,c}	< 0.01
ABTS scavenging activity (μmol Trolox per g sample)	32.31 ± 1.64	33.01 ± 1.62	82.29 ± 3.47 ^a	122.35 ± 3.29 ^{b,c}	< 0.01

Data are mean ± SD.

CHA: chlorogenic acid.

EGCG: (-)-epigallocatechin gallate.

CD: circular dichroism.

DPPH: 1,10-diphenyl-2-picrylhydrazyl.

ABTS: diammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate).

^a Statistical difference (p < 0.05) between Ara h1-EGCG and control Ara h1.

^b Statistical difference (p < 0.05) between Ara h1-CHA and control Ara h1.

^c Statistical difference (p < 0.05) between Ara h1-CHA and Ara h1-EGCG.

between Ara h1 and polyphenols. Ara h1 has 5 Trp residues and 9 Tyr residues with intrinsic fluorescence. Therefore, detection of intrinsic fluorescence intensity in Ara h1 can be used to analyze the structure changes of Ara h1. The maximum emission wavelengths for native Ara h1, control Ara h1, Ara h1-CHA and Ara h1-EGCG were 360.6, 360, 365.4 and 369.9 nm, respectively (Fig. 3A). As compared with native and control Ara h1, the emission maxima for the Ara h1-CHA and Ara h1-EGCG conjugates showed an obvious red shift, with lower fluorescence intensity, which could be due to the Ara h1-polyphenol conjugate increasing the exposure of Trp residues of Ara h1. This finding agreed with a previous report of covalent binding of polyphenols resulting in

the emission maxima of OVA with a red shift and lower fluorescence intensity (Lu et al., 2018). Hence, the Ara h1 conjugate with CHA and EGCG resulted in a conformation change of Ara h1 by unfolding protein structures.

CD spectroscopy was used to study the secondary structure changes of native Ara h1, control Ara h1, and Ara h1-CHA and Ara h1-EGCG conjugates. The CD spectra of samples showed a negative band at 208 nm (Fig. 3B), which is a characteristic of α-helical structures of protein in the ultraviolet region (Xu et al., 2019). As compared with native and control Ara h1, both Ara h1-CHA and Ara h1-EGCG conjugates showed a significant reduction in absolute θ values of the 208-

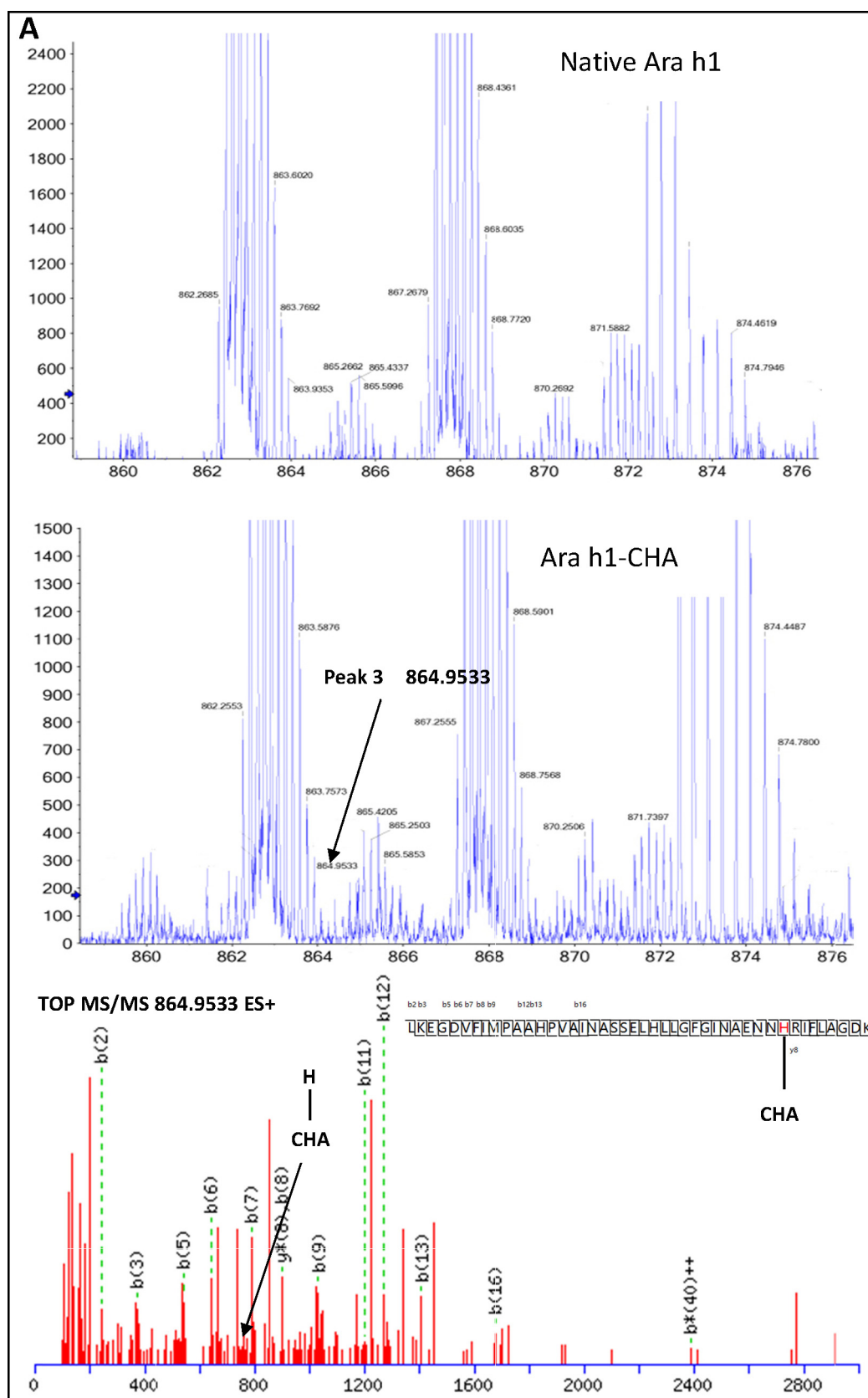


Fig. 2. LC-MS/MS spectra of Ara h1 and Ara h1-polyphenol conjugate; interpretation of LC-MS/MS spectra of Ara h1-CHA (A) and Ara h1-EGCG (B). LC-MS/MS, liquid chromatography/tandem mass spectrometry.

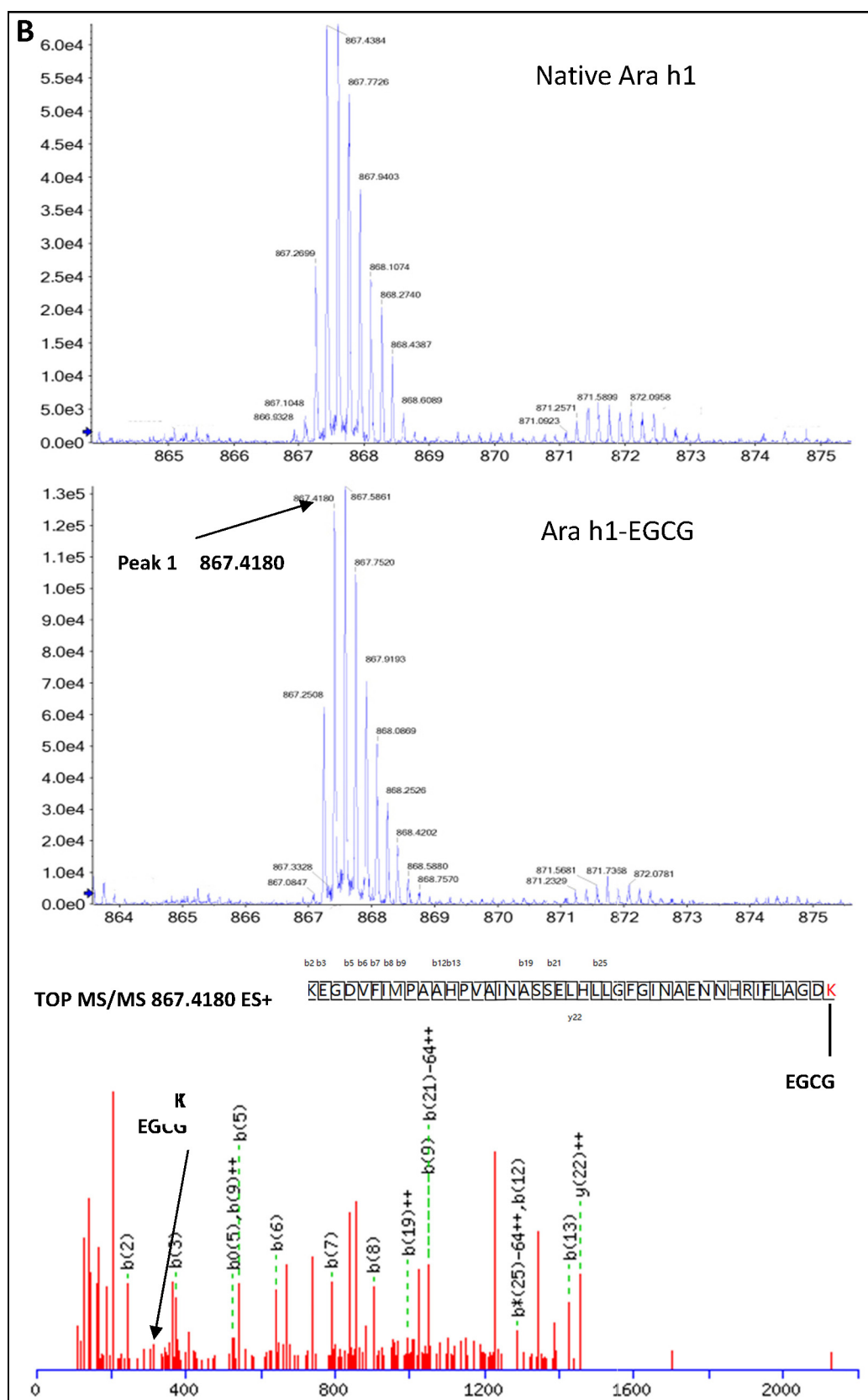


Fig. 2. (continued)

nm negative band, which suggests that some α -helical structures in the Ara h1-polyphenol conjugates were lost. The secondary structure of native Ara h1, control Ara h1, Ara h1-CHA and Ara h1-EGCG

conjugates were calculated by the SELCON3 method in DICHROWEB. The native Ara h1 contained 33.5% α -helix, 16.8% β -sheet, 12.4% β -turn, and 37.3% random coil content (Table 1). For control Ara h1,

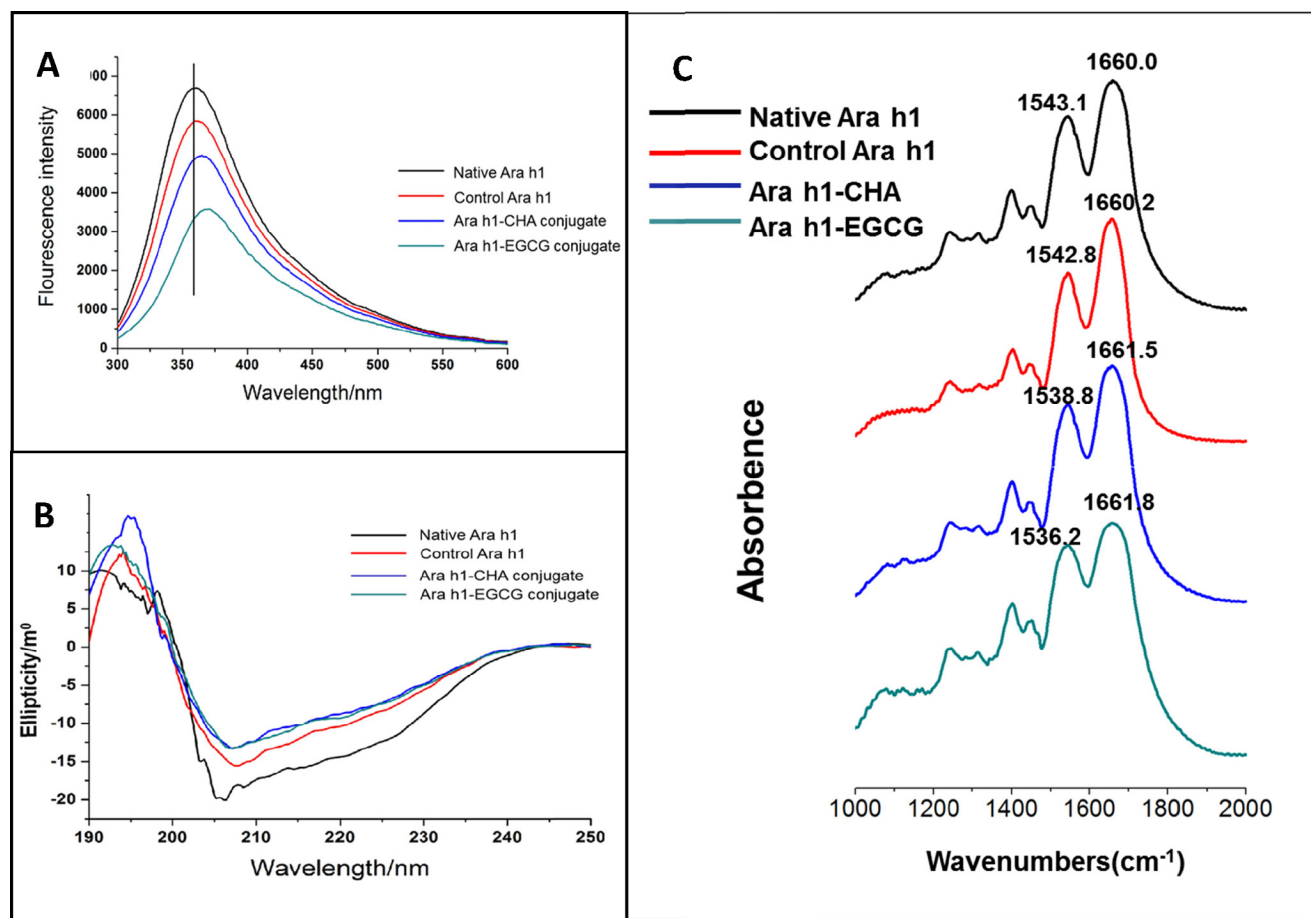


Fig. 3. Fluorescence (A), CD (B), FTIR (C) for native Ara h1, control Ara h1, Ara h1-CHA and Ara h1-EGCG. CD, circular dichroism; FTIR, Fourier transform infrared.

alkaline treatment slightly reduced the content of α -helices (33.5% to 32.4%) and β -sheets (16.8% to 15.6%), whereas the content of random coils was increased slightly (37.3% to 39.2%) and that of β -turns hardly changed, which may due to the alkaline microenvironment changing the structure of protein. After conjugation with EGCG and CHA, the α -helix content decreased, that of β -sheets and β -turns increased slightly, and that of random coils increased greatly. These data suggest that with polyphenol conjugation with Ara h1, the conformation was more unfolded, which is consistent with the results of fluorescence spectroscopy.

FTIR analysis was used to further study the structural changes of Ara h1 covalently conjugated with polyphenol. The native Ara h1 exhibited amide I and amide II peak positions at 1660.0 and 1543.1 cm^{-1} , respectively (Fig. 3C). The amide I and II bands of the control Ara h1 moved to 1660.2 and 1542.8 cm^{-1} , respectively, with no significant difference between native and control Ara h1. As compared with control Ara h1, for the Ara h1-CHA conjugation, the amide I changed to 1661.5 cm^{-1} and amide II changed to 1538.8 cm^{-1} . For Ara h1-EGCG, the amide I and amide II bands moved to 1661.8 and 1536.2 cm^{-1} , respectively. The results suggest that conjugation of Ara h1 with polyphenols changed the secondary structure of Ara h1.

Scanning electron microscopy (SEM) was used to observe the microstructures of native Ara h1, control Ara h1, Ara h1-CHA and Ara h1-EGCG. The morphology of Ara h1-based colloidal particles is shown in Fig. 4A. The native and control Ara h1 formed irregularly ellipsoid in sheets with different size, whereas the Ara h1-polyphenols formed a number of additional small spherical particles, which suggested that covalent conjugation with polyphenols altered the self-assembly of the Ara h1 molecules. This result was similar to a previous report showing that covalent modification by polyphenols changed the morphology of

particles of zein molecules (Liu et al., 2017). The morphology change of molecular particles of Ara h1-polyphenol conjugates may be attributed to the altered conformation of Ara h1.

Thermal stability of protein is closely related to structure. To analyze the change in thermal stability by covalent conjugation of polyphenols in Ara h1, differential scanning calorimetry (DSC) was used to evaluate the thermal stability of native Ara h1, control Ara h1, Ara h1-CHA and Ara h1-EGCG (Fig. 4B). The thermal denaturation temperature of native and control Ara h1 was at 74.47 °C and 69.12 °C, which suggests that alkaline treatment decreased the thermal stability of Ara h1 and is consistent with the fluorescence and CD spectra results. For Ara h1-CHA and Ara h1-EGCG, the thermal denaturation temperature was reduced to 65.8 °C and 64.47 °C, respectively, which suggests that polyphenol conjugation decreased the thermal denaturation temperature of Ara h1. This result agrees with our previous report that the thermal stability of ovalbumin decreased by covalent conjugation with chlorogenic acid (Lu et al., 2018).

3.5. Allergic capacity of Ara h1-polyphenol conjugates

The IgE binding capacity of Ara h1, control Ara h1, Ara h1-CHA and Ara h1-EGCG was analyzed by ELISA (Fig. 5A). IgE binding capacity was lower for both Ara h1-EGCG and Ara h1-CHA conjugates than control Ara h1, which suggests that conjugation with polyphenol reduced the allergenicity of Ara h1. IgE binding capacity was lower for Ara h1-EGCG than Ara h1-CHA perhaps because EGCG may have more phenolic hydroxyl groups than CHA, which results in greater degree of antigenic epitope structural modification.

Histamine release is a characteristic and critical function of basophils and can be used to assess allergenicity of allergens. To further

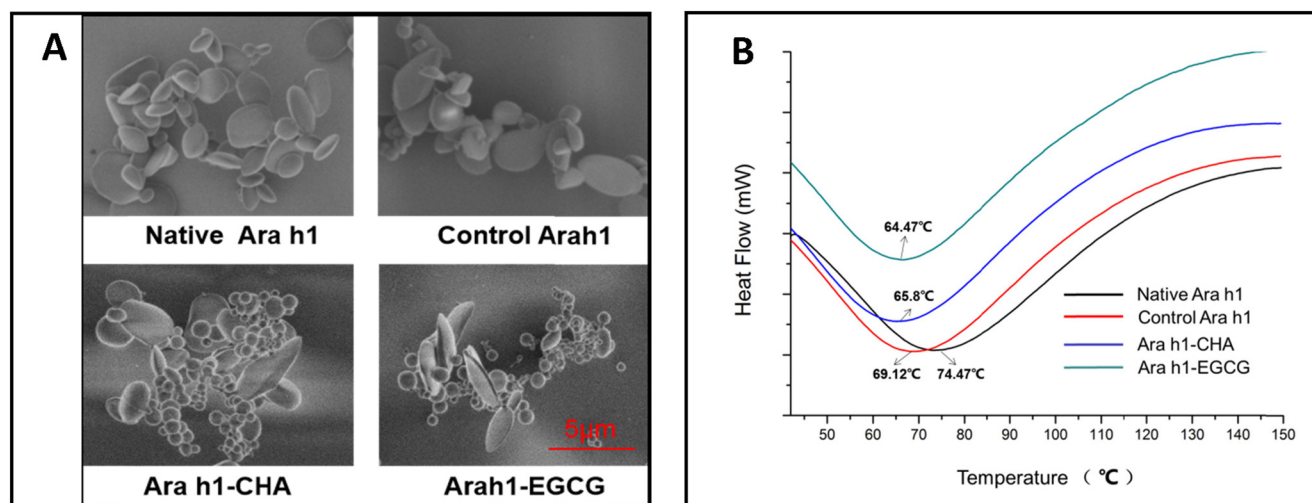


Fig. 4. DSC (A) and SEM (B) for native Ara h1, control Ara h1, Ara h1-CHA and Ara h1-EGCG. DSC, differential scanning calorimetry; SEM, scanning electron microscopy.

evaluate the change in allergenic capacity, we used histamine release assay of human basophil KU812 cells (Fig. 5B). The Ara h1-polyphenol conjugates significantly reduced histamine release. A 10-μg/ml amount of native and control Ara h1 stimulated KU812; the average OD450 of histamine release was about 1, whereas for Ara h1-polyphenol conjugates, the OD450 was reduced to about 0.6, about 40% decreased as compared with control Ara h1. Thus, conjugation with polyphenols may decrease the cross-linking of IgE on FcεR in basophils and result in reduced histamine release. These results were consistent with ELISA findings (Fig. 5A).

The digestibility of a protein is related to structural characteristics. To further study the digestibility and IgE binding capacity of native Ara

h1, control Ara h1 and Ara h1-polyphenols, samples were treated with simulated gastric digestion, then underwent SDS-PAGE and western blot analysis. All protein samples were time-dependently hydrolyzed to smaller molecules (Fig. 5C, left). However, the enzymatic hydrolysis into peptides was more rapid for Ara h1-CHA and Ara h1-EGCG than control Ara h1. The interaction bands of native and control Ara h1 at about 63.5 kDa disappeared at 30 min, whereas for Ara h1-CHA and Ara h1-EGCG, the bands disappeared at 8 min, so Ara h1 conjugated with polyphenols increased the digestibility of Ara h1. This result agrees with our data showing that partial unfolding of Ara h1 protein facilitated digestion and with a previous report finding that polyphenol treatment increased the digestibility of peanut allergens (Plundrich,

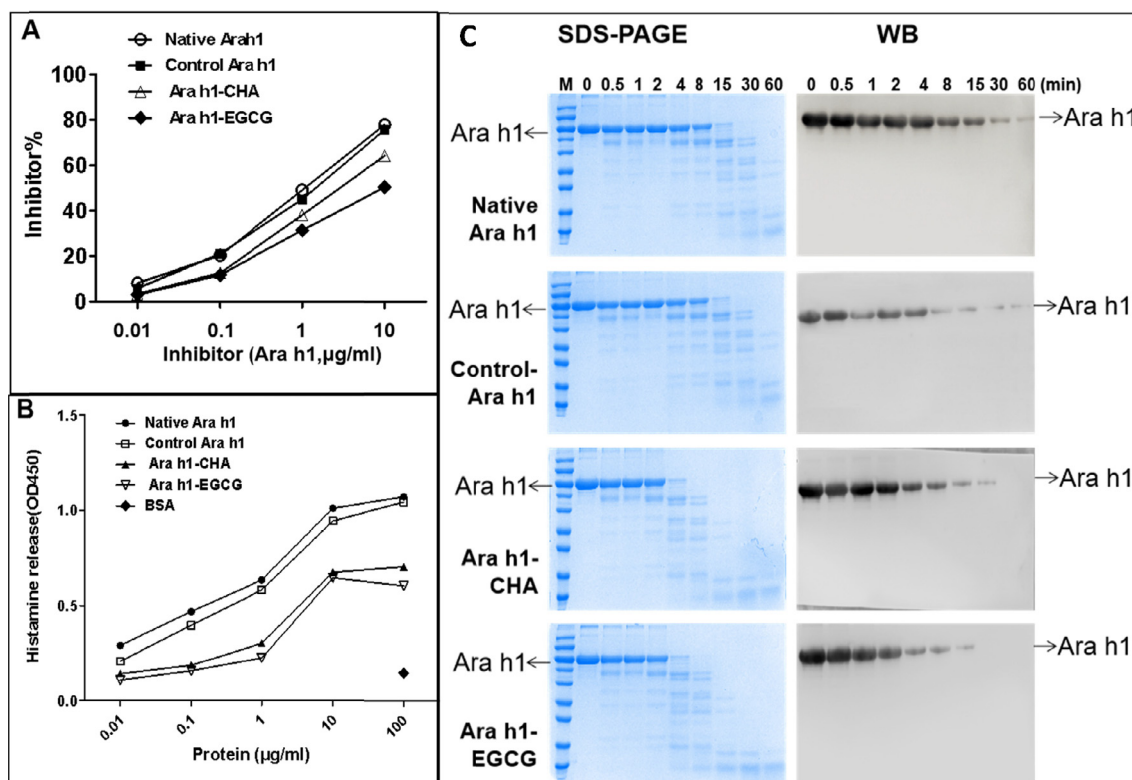


Fig. 5. ELISA (A), histamine release (B) and pepsin digestion characteristics by SDS-PAGE and western blot analysis (C) for native Ara h1, control Ara h1, Ara h1-CHA and Ara h1-EGCG.

White, Dean, Davis, & Lila, 2015).

Western blot analysis was used to evaluate the IgE binding capacity of Ara h1 and Ara h1–polyphenols treated with simulated gastric digestion. The intensity of the band of Ara h1 at 63.5 kDa decreased gradually with increasing SGF digestion time (Fig. 5C, right), which differed from the SDS-PAGE result of no new band at the low-molecular-weight position. These results suggest that the new peptide fragments produced by enzymatic hydrolysis under the condition did not react with serum IgE from patients with peanut allergy. For native and control Ara h1, the 63.5-kDa position still showed slight IgE binding bands at 60 min, which differed from corresponding SDS-PAGE results perhaps because western blot analysis is a more sensitive method of detection. For Ara h1-CHA and Ara h1-EGCG, the bands of IgE immunoblotting completely disappeared at 60 and 30 min, respectively. As expected, covalent conjugation with CHA and EGCG increased the digestibility of Ara h1 with reduced IgE binding capacity.

3.6. Antioxidant activity of Ara h1–polyphenol conjugates

We examined ABTS and DPPH radical scavenging activities to assess the antioxidant activities of native Ara h1, control Ara h1, Ara h1–CHA and Ara h1–EGCG (Table 1). These assays are associated with the hydrogen-donating ability of the tested samples. The mean DPPH and ABTS⁺ values for control Ara h1 were 86.25 ± 2.82 and 33.01 ± 1.62 $\mu\text{mol Trolox equivalent/g sample}$, respectively, with no significant difference between native and control Ara h1. For Ara h1-CHA and Ara h1-EGCG, the DPPH and ABTS⁺ values increased 2 to 4 times as compared with control Ara h1. These results suggest that CHA and EGCG conjugated with Ara h1 increased the antioxidant activity of Ara h1, which agreed with some previous reports that covalent conjugation with EGCG and CHA increased the antioxidant activity of OVA (Lu et al., 2018; He et al., 2019). EGCG decreasing more radicals than CHA in conjugation with Ara h1 may be due to EGCG having more phenolic hydroxyl groups than CHA (Pervin, Unno, Takagaki, Isemura, & Nakamura, 2019; Naveed et al., 2018).

Interaction of polyphenols can affect the allergenicity of food allergens (Singh, Holvoet, & Mercenier, 2011). Previous studies demonstrated that polyphenols can interact with the allergens in peanuts and reduce the allergenicity of peanut (Chung & Champagne, 2009; Plundrich et al., 2015; Bansode, Randolph, Plundrich, Lila, & Williams, 2019). Recently, Plundrich et al. found that certain *Vaccinium* fruit polyphenols can inhibit IgE recognition of Ara h2 by obstructing linear IgE epitopes (Plundrich, Cook, Maleki, Fourches, & Lila, 2019). These studies found that the interactions of peanut proteins with polyphenols might lead to the formation of protein–polyphenol complexes and reduce the allergenicity of peanut. A study also found that some polyphenol interactions with peanut proteins are covalent in nature (Plundrich, Bansode, Foegeding, Williams, & Lila, 2017). Polyphenols can interact non-covalently or covalently with proteins, and covalent interaction irreversibly affects the properties of proteins, which suggests that covalent interaction of polyphenols with protein may generate more stable conjugates than non-covalent interaction (Rohn, 2014). Therefore, covalent interaction with polyphenols seems good to produce hypoallergenic food in food processing. However, study of the main allergen present in peanut and detailed polyphenol covalent interactions are lacking. In this study, we investigated the covalent interaction between Ara h1 and EGCG and CHA in detail and evaluated the allergenicity of the conjugates. To understand the reduced IgE binding abilities of Ara h1 with the covalent interaction of polyphenols, we investigated the conjugate site and changes of protein structure by using LC-MS/MS, fluorescence CD, FTIR, SEM and DSC. EGCG and CHA covalently conjugated with amino acid residues on Ara h1 resulted in an unfolded protein structure, which might affect the conformational and linear IgE epitopes on Ara h1. Furthermore, the results of basophil activation tests indicated that EGCG and CHA conjugation reduced the ability of Ara h1 to trigger cell degranulation, which agreed with IgE

binding capacity testing by ELISA. Our previous study and this study indicated that the reduced IgE binding abilities may due to the shielding of the linear IgE epitope and changes in the protein structure by covalent interaction with polyphenols (Lu et al., 2018). A similar phenomenon was found by the glycation method, which can alter IgE binding abilities by disruption of the conformational and linear epitopes (Teodorowicz, van Neerven, & Savelkoul, 2017). However, advanced glycation endproducts have been proposed as potential toxic compounds formed during the Maillard reaction (Cepas, Collino, Mayo, & Sainz, 2020), but covalent interaction with polyphenol might be a safe method.

4. Conclusion

Ara h1 is the most abundant allergen in peanut and has been used as an important model for studying food allergens. In this study, we investigated the interaction between Ara h1 and dietary polyphenols with alkaline treatment. Both EGCG and CHA could covalently conjugate with Ara h1, which increased the unfolded protein structure and changed the secondary structure by reducing α -helix content and increasing random coil content. The IgE binding capacity of Ara h1 was decreased by covalent conjugation with polyphenols. Moreover, the histamine release of basophil (KU812) cells was reduced significantly on conjugation with polyphenols. EGCG and CHA covalent conjugation with Ara h1 increased antioxidant activity and the digestive capacity of pepsin, with lower thermal stability. This research provides a promising method for reducing the allergenicity of Ara h1 while enhancing the functional properties of the protein.

CRediT authorship contribution statement

Weiyi He: Investigation, Writing - original draft. **Tingting Zhang:** Investigation, Data curation, Visualization. **Tanja Cirkovic Velickovic:** Methodology, Writing - review & editing. **Shuiming Li:** Investigation. **Yansi Lyu:** Investigation. **Linlin Wang:** Validation. **Jiang Yi:** Formal analysis, Data curation. **Zhigang Liu:** Resources. **Zhendan He:** Resources. **Xuli Wu:** Methodology, Project administration, Supervision, Funding acquisition, Writing - review & editing.

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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