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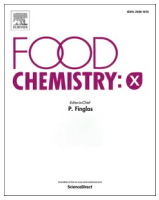

Influence of phloretin on acrolein-induced protein modification and physicochemical changes in a dairy protein model

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ABSTRACT

Acrolein (ACR) is an α,β-unsaturated aldehyde with high reactivity towards nucleophiles in proteins. In this study, a typical phenolic compound phloretin (Phl) was employed to counteract protein modification induced by ACR (1 mM) in whey protein isolate (WPI, 10 mg/mL). The addition of Phl (2 mM) significantly reduced ACR-induced surge of protein carbonyls (from 1.65 to 0.65 μmol/mg protein) and loss of protein total sulfhydryl content (from 0.28 to 0.24 μmol/mg protein) whilst contributing to further reductions in protein surface hydrophobicity and intrinsic fluorescence. The incorporation of ACR into WPI was effectively interrupted by Phl as visualized by Western blot. Only 2.87 % of ACR remained in the presence of 2 mM Phl with the generation of Phl-ACR adducts, suggesting Phl could partially alleviate protein modification by scavenging of ACR. These findings could have important implications for employment of natural phenolic nucleophiles against the adverse effects of ACR towards dietary proteins.

1. Introduction

Acrolein (ACR, CH_2 = CH–CHO, MW 56), a typical α,β-unsaturated aldehyde, is generated by incomplete combustion of organic compounds, the oxidation of unsaturated fatty acids and endogenous polyamine metabolism, etc. Dietary, smoking, and environmental sources are considered the most relevant pathway of human exposure to ACR (Zhu et al., 2011). A positive correlation was found between ACR and the development of various diseases such as Alzheimer's disease, atherosclerosis, diabetes, and cancer, etc. (Zhou et al., 2023) Since 2020, ACR has been classified as a "Group 2A carcinogen" instead of the previous "Group 3 carcinogen" by the IARC (Marques et al., 2021).

As an electrophilic agent, ACR has a conjugated C=C–C=O system containing a C=C double bond and a C=O group, which contribute to its high reactivity towards biological nucleophiles (Zhu et al., 2011). Nucleophilic sites such as thiol-, amino-, and imidazole in dietary proteins are the main targets of ACR (Wang et al., 2018). Michael addition and Schiff's base formation are two basic mechanisms involved in ACR's reaction with these residues. As for Michael addition, the nucleophilic groups in amino acids react with the β-carbon in C=C double bond of ACR to form 1, 4-addition products. Intermolecular and intramolecular protein-protein cross-links are also produced by the reaction between the aldehyde group of ACR and other nucleophilic groups in amino acids. As for Schiff's base formation, imines are generated via the reaction of the carbonyl group of ACR with primary amines. The facile reaction between ACR and amino acid residuals in biomolecules account for most of their potential damages, with adverse impacts on the nutritional quality and functional properties of the dietary proteins (Cai et al., 2009). Approximately 10–100 μg/kg ACR has been found in cheese, donuts, bread, potatoes and fish (Henning et al., 2017). Evidences indicate that ACR could induce protein carbonylation, protein aggregation and degradation of sulfhydryl groups, with decreased intrinsic fluorescence, surface hydrophobicity and solubility in some dietary proteins such as soy proteins, rabbit myofibrillar proteins and β-lactoglobulin (Lv et al., 2021; Wang et al., 2018; Wu et al., 2010).

To interrupt ACR-induced covalent modification on proteins, the application of nucleophiles as scavenging agent of ACR has been considered a promising strategy. Naturally-occurring polyphenolic

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nucleophiles derived from vegetables, fruits, and other edible plants were found to be excellent ACR scavengers (Zhu et al., 2009; Zhu et al., 2011). Ferulic acid, resveratrol, hesperetin, genistein, myricetin, cyanidin-3-O-glucoside and epigallocatechin-3-gallate (EGCG) were demonstrated to eliminate ACR in different bio-mimicked conditions (Jiang et al., 2024; Song et al., 2021; Tao et al., 2019; Wang et al., 2015; Zhang et al., 2020). In a structure-activity relationship study, phloretin, EGCG, phloridzin, theaflavin, and theaflavin-3,3′-digallate effectively scavenged ACR by acting as sacrificial nucleophiles, among which the most effective scavenger was found to be phloretin (Phl), a dihydrochalcone from apple. The phloroglucinol moiety (usually in A ring) is a structural requirement for scavenging of ACR (Zhu et al., 2009). Subsequent studies compared the reaction rates of Phl and certain amino acid (Nα-acetyl-L-lysine) with ACR. The formation of FDP-lysine (a typical ACR-lysine adduct) was substantially blocked by Phl, and Phl-ACR adducts were identified in the presence of amino acids, indicating a higher reaction rate of Phl than that of Nα-acetyl-L-lysine in covalent binding to ACR (Zhu et al., 2012). Primary study of the protective effects of Phl were conducted in protein models including the Phl-ACR-BSA (bovine serum albumin) and Phl-ACR-RNase A (bovine pancreas ribonuclease A) systems (Zhu et al., 2012). However, there have been no further studies about the impacts of Phl on physicochemical and functional properties changes of protein under exposure to ACR to date.

In this study, the effects of Phl were examined in an ACR-challenged food protein model, where whey protein isolate (WPI), a highly valued dairy protein, was adopted as a representative dietary protein. WPI is a health-promoting ingredient used in a wide range of food products such as beverages, creamers, sauces, baked products, and infant formula. High amino acid content makes it a target of free radicals and reactive carbonyl species during manufacture, storage, and utilization (Giblin et al., 2019). We infer that Phl's ACR-scavenging capability might contribute to the prevention of protein modification when WPI is exposed to ACR. The changes in protein's physiochemical, structural, and digestive properties during its co-incubation with ACR and Phl were investigated. The interactions between Phl and ACR were further characterized by tracing scavenged ACR using HPLC and identification of the Phl-ACR adducts by LC-MS. The results of this study are expected to facilitate understanding of the influences of phenolic compounds on dietary proteins when they are working as ACR-scavenging agents.

2. Materials and methods

2.1. Materials

Whey protein isolate (protein content: 90.46 %, dry basis) was a product of Milk Specialties Global, Inc. (USA). Phloretin, brilliant blue R250, 5,5′-dithiobis (2-nitrobenzoicacid), and 1-anilino-8-naphthalene-sulfonate were commercial products of Tokyo Chemical Industry Co., Ltd. (Japan). Porcine pepsin (≥250 units/mg), and pancreatin (8 × USP specifications) were obtained from Sigma-Aldrich Co., LLC (USA). Primary anti-acrolein antibody (ab37110) and the corresponding secondary antibody (ab205718) were purchased from Abcam (Cambridge, UK). Acrolein, trichloroacetic acid, 2,4-dinitrophenyl-hydrazine, β-mercaptoethanol, and HPLC-grade acetonitrile and other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (China).

2.2. Incubation of ACR in the absence and presence of Phl

The reaction model was adopted from previous studies (Wang, Yang, et al., 2019; Zhu et al., 2012). Briefly, WPI solution (final concentration: 10 mg/mL, dissolved in 0.02 mol/L PBS, pH 7.4) was incubated with ACR (final concentration: 1 mmol/L). Phl was added to the mixture at final concentrations of 0.1, 1 and 2 mmol/L, respectively. WPI control was prepared in the absence of ACR and Phl. Sodium azide (0.05 %) was used for inhibition of microbial growth. The reaction was allowed to proceed with constant shaking at 25 °C for 24 h.

2.3. Protein carbonyl content

A DNPH-derivatization assay was used to estimate the degree of ACR-induced protein carbonylation in WPI according to the method of Levine et al. (1990). Briefly, 0.2 mL protein sample (10 mg/mL) was incubated with equal volume of DNPH reagent (0.1 %, dissolved in 2 M HCl). Derivatization was conducted at room temperature in darkness for 1 h. The protein was then precipitated with 20 % trichloroacetic acid and centrifugated at 8000 g for 10 min. The resultant precipitate was extracted by 0.2 mL ethanol/ethyl acetate (1:1, *v*/v) solution at least 3 times and finally dissolved in 0.6 mL 8 M guanidine hydrochloride solution. UV absorbance of the derivatized protein samples were measured at 370 nm. Protein carbonyl content (nmol carbonyl/mg protein) was estimated using an absorption coefficient of 22,000 mol^{-1} cm^{-1} for protein hydrazones.

2.4. Total sulfhydryl content

Ellman's method was applied for measurement of total sulfhydryl content of WPI in different treatments (Ellman, 1959). 400 μL urea-SDS solution (8.0 M urea and 30 mg/mL SDS) was added to 100 μL sample in each treatment. 10 mmol/L 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) was mixed with sample solution and incubated at room temperature for 20 min in darkness. UV absorbance of the reaction mixture was recorded at 412 nm. Total sulfhydryl content of WPI (nmol/mg protein) was estimated by a molar extinction coefficient of 13,600 mol^{-1} cm^{-1}.

2.5. Surface hydrophobicity

Protein surface hydrophobicity in WPI was monitored according to the method of Li et al., with a fluorescence probe 1-anilino-8-naphthalene-sulfonate (ANS) (Li et al., 2015). PBS (0.01 mol/L, pH 7.0) was used to dilute the samples to a range of protein concentrations (0.005 to 0.5 mg/mL). Each diluted sample (0.2 mL) was mixed with 3 μL 8 mmol/L ANS, and the mixtures were allowed to react for 15 min in darkness. Fluorescence intensity was recorded (Ex/Em: 365/484 nm) by a Varioskan™ microplate reader (Thermo Scientific, Rockford, IL, USA). Index of protein hydrophobicity (H_0) was defined as the initial slope of fluorescence intensity against protein concentration (mg/mL) plot (calculated by linear regression analysis).

2.6. Protein solubility

The solubility of WPI was determined according to the method of Utrera and Estevez (2012). Briefly, 300 μL of each sample was centrifuged at 6000*g* for 15 min. Then, 100 μL Biuret reagent was mixed with 100 μL supernatant and the reaction proceeded for 30 min in darkness at room temperature. UV absorbance of the reaction mixture was monitored at 540 nm and protein solubility was expressed as the percentage of soluble protein in 100 g of protein.

2.7. Intrinsic fluorescence spectroscopy

The intrinsic tryptophan fluorescence of WPI was monitored according to the method of (Wu et al., 2009). Protein samples were diluted to 1 mg/mL by PBS before the fluorescent emission spectra were recorded in the range of 300 to 400 nm when excited at 283 nm by a Varioskan™ microplate reader (Thermo Scientific, Rockford, IL, USA).

2.8. Protein profile revealed by SDS-PAGE analysis

The protein profiles of WPI in different treatments according to the molecular weight distribution were monitored by SDS-PAGE. After incubation, reductive sample buffer (containing 0.05 % β-mercaptoethanol) was added to equal volume of protein samples, the protein content for SDS-PAGE separation was adjusted to 30 μL. Proteins were heated at

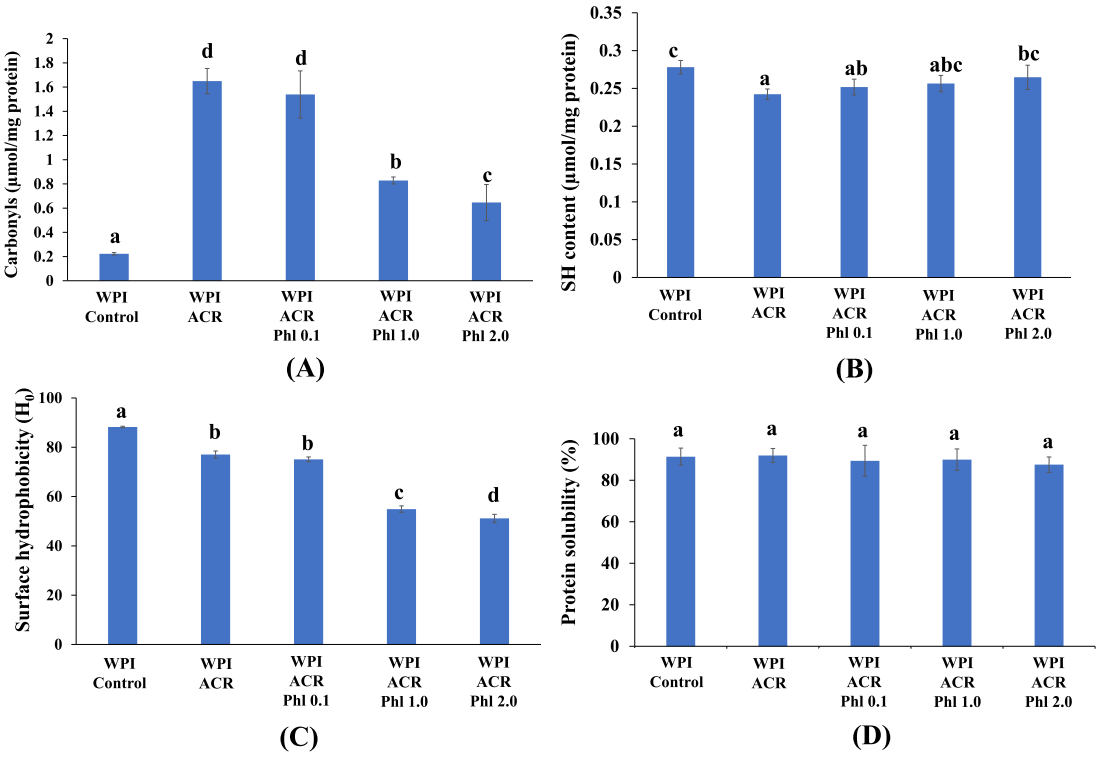

Fig. 1. Protein carbonyl content (A), total sulfhydryl content (B), protein surface hydrophobicity (C), and protein solubility (D) of WPI co-incubated with ACR (1 mmol/L) in the presence of Phl (0, 0.1, 1 and 2 mmol/L) at 25 °C for 24 h. Each value is expressed as average ($n = 3$) ± standard deviation. Means without common letters in the same parameter group differ significantly ($P < 0.05$).

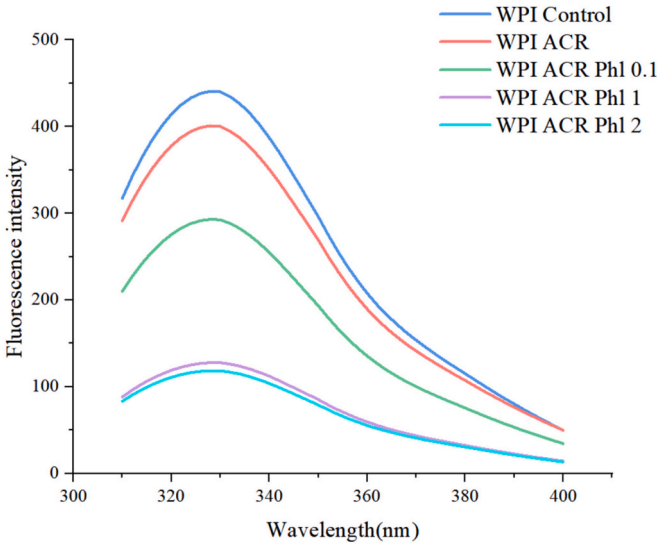

Fig. 2. Fluorescent emission spectra (in range from 300 to 400 nm) of WPI when co-incubated with ACR (1 mmol/L) and Phl (0, 0.1, 1 and 2 mmol/L) at 25 °C for 24 h. The fluorescence was excited at 283 nm.

95 °C for 5 min, loaded onto a 5 % polyacrylamide stacking gel and separated by a 15 % polyacrylamide resolving gel. Protein profile in different treatments was revealed by Coomassie brilliant blue R-250 staining after electrophoresis in a Mini-PROTEAN® Tetra Cell Systems (Bio-Rad, Hercules, CA, USA).

2.9. Western blot analysis of ACR-bound proteins

Proteins were transferred to a Millipore PVDF membrane after

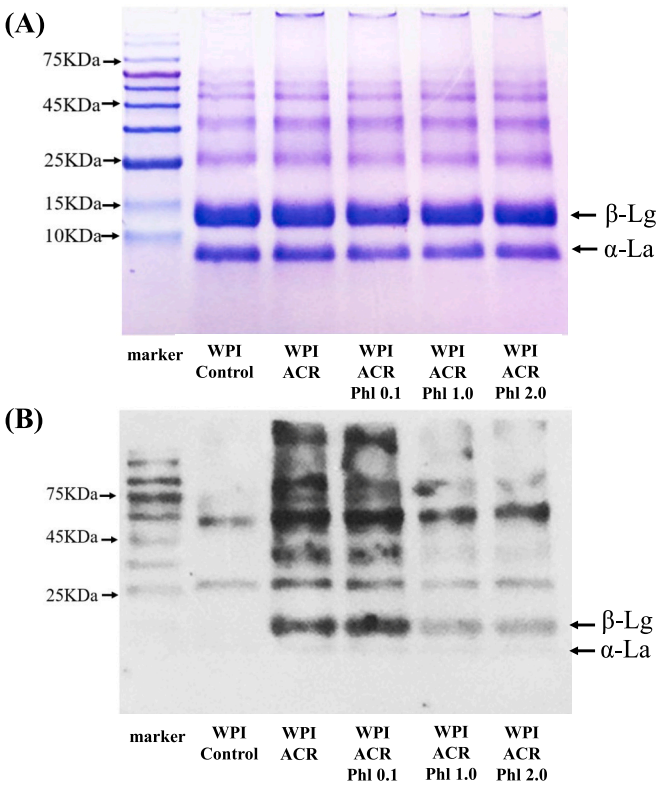

Fig. 3. Representative SDS-PAGE analysis of protein profile (A) and Western blot analysis of ACR-induced protein modification (B) in the presence of Phl (0, 0.1, 1 and 2 mmol/L) at 25 °C for 24 h.

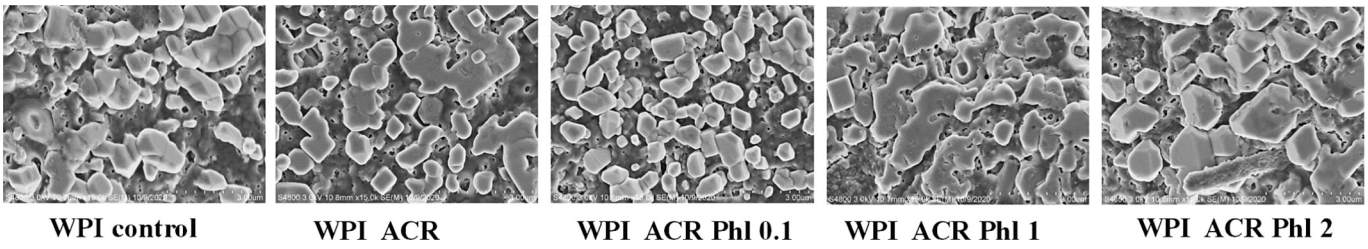

Fig. 4. SEM micrographs (magnification 1: 15000) of WPI co-incubated with ACR (1 mmol/L) in the presence of Phl (0, 0.1, 1 and 2 mmol/L) at 25 °C for 24 h. A: WPI control; B: WPI + ACR; C: WPI + ACR + Phl 0.1 mmol/L; D: WPI + ACR + Phl 1 mmol/L; E: WPI + ACR + Phl 2 mmol/L.

Table 1
In vitro digestibility of WPI co-incubated with ACR (1 mmol/L) in the presence of Phl(0, 0.1, 1, and 2 mmol/L).

samples	Intestinal tract		
	30 min	60 min	120 min
WPI Control	82.57 ± 1.19a	84.91 ± 2.43a	84.66 ± 0.56a
WPI ACR	83.48 ± 0.80a	83.48 ± 0.42a	83.51 ± 1.02a
WPI ACR Phl 0.1	82.56 ± 0.76a	83.20 ± 0.57a	84.56 ± 1.51a
WPI ACR Phl 1	83.77 ± 0.83a	84.08 ± 2.46a	84.34 ± 3.39a
WPI ACR Phl 2	82.66 ± 0.74a	83.32 ± 2.72a	82.41 ± 2.99a

All data were expressed as mean ± standard deviation (x ± SD) of at least three replicates for each treatment. Means without common letters in the same parameter group differ significantly ($P < 0.05$).

separation by SDS-PAGE. Nonspecific binding was blocked by 0.05 g/mL non-fat milk in PBST (0.05 % Tween 20 in PBS, pH 7.4) at 4 °C overnight with constant shaking. The PVDF membrane was washed with PBST three times and then incubated with 1:5000 primary anti-ACR antibody (ab37110) for 3 h and subsequently with corresponding secondary antibody (ab205718) for 1 h. All protein bands on the PVDF membrane were developed in A Kodak X-ray film after application of Pierce visualizer spray & glow ECL Western blotting detection kit.

2.10. Micrograph of protein surface

Micrograph of protein surface structures was obtained using an S-4800 high-resolution SEM (Hitachi, Tokyo, Japan) and the acceleration voltage was set at 10 kV. Sample powders were fixed on stubs by conductive plastic tapes before coating with gold-palladium. Images of surface microstructure were recorded under 15,000× magnification.

2.11. Protein digestibility

Protein digestibility was determined by characterizing the soluble protein fraction in trichloroacetic acid (TCA) according to the procedure of Lamothe et al. (2019). Briefly, WPI samples were diluted to 4 mg/mL (final protein concentration) with PBS. Each diluted protein sample was mixed with 2 M HCl until pH was adjusted to 1.5. Pepsin (1 mg/mL, dissolved in HCl) was added to simulate gastric phase digestion at 37 °C for 60 min (concentration of pepsin: 4 % *w*/w on protein basis). Pancreatin (4 %) was then used to start intestinal phase digestion after adjustment of pH to 7.4 by NaOH, and the digestion proceeded at 37 °C for 2 h. Finally, 30 % TCA solution was applied to terminate the simulated digestion. The resultant digests were obtained by centrifugation and redissolved in NaOH (1 M) before quantification of protein concentration by Biuret method. Ct and Cp represent the protein concentrations of total and TCA-precipitated protein and calculations were made by the following equation for protein digestibility:

$$\text{(Protein) digestibility (\%)} = \frac{C_t - C_p}{C_t} \times 100$$

2.12. HPLC quantification of residual ACR incubated with Phl

The reaction system of ACR and Phl was adopted from a previous study of Zhu et al. (2009). Briefly, ACR (1 mmol/L) and Phl (0, 0.1, 1, 2 mmol/L) were incubated in 0.01 mol/L PBS (pH 7.4), and the reaction was carried out at 25 °C for 24 h with constant shaking. After incubation, 0.5 mL of the reaction mixture was derived with 0.3 mL DNPH derivatization reagent for 1 h in darkness. The DNPH derivatization reagent was obtained by dissolving 125 mg of DNPH and 6 mL of 1 M HCl in 100 mL of acetonitrile. The resultant ACR-DNPH complex was monitored by an Agilent 1260 series HPLC system with a separation module (G7111B), an autosampler (G7129E) and a multiwavelength UV detector (G7115A). The reaction products were chromatographically separated by a YMC Pro C18 column (250 × 4.6 mm, 5 μm). The mobile phase was composed of solvent A (0.1 % formic acid in water) and solvent B (acetonitrile). Isocratic elution (flow rate: 1 mL/min) was applied with 40 % A and 60 % B, while the injection volume was 10 μL, and ACR-DNPH complex was detected at the wavelength of 372 nm. The amount of residual ACR was expressed as the percentage of peak area of ACR-DNPH in different treatments compared to that of the control group without Phl addition after incubation.

2.13. LC-MS analysis of ACR-Phl conjugates

The UPLC-MS analysis was performed on a Waters UPLC I-Class Xevo TQ-S micro system (Waters, Milford, MA, USA). The parameters for mass spectrometry analysis of ACR-Phl adducts were optimized as follows: negative ion mode (ESI-); capillary voltage, 4 kV; ion source temperature, 150 °C; scan range, 180–2000 Da. Chromatographic separation was performed using a Waters Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm). The UPLC analysis used the mobile phase of 0.1 % formic acid in water (solvent A) and acetonitrile (solvent B). Gradient elution was conducted at a flow rate of 0.2 mL/min with 10 % to 90 % solvent B from 0 to 10 min, and the injection volume was 2 μL. Masslynx 4.2 (Waters, Milford, MA, USA) was used for data acquisition and analysis.

2.14. Statistical analyses

All measurements were performed in triplicates with at least three independent tests. Data processing and analyses were carried out using a SPSS statistical package (SPSS Inc., Chicago, IL, USA). Shapiro-Wilk's test was used to ensure normal distribution of data inside each treatment group. Mean values of different treatments were compared by Duncan's tests. Significant difference was defined when $P < 0.05$.

3. Results and discussion

3.1. Protein carbonyl content

ACR is the most reactive α,β-unsaturated aldehyde, and exposure of proteins to ACR was reported to increase protein-linked carbonyl groups time-dependently (Uchida et al., 1998). In this study, the incubation of

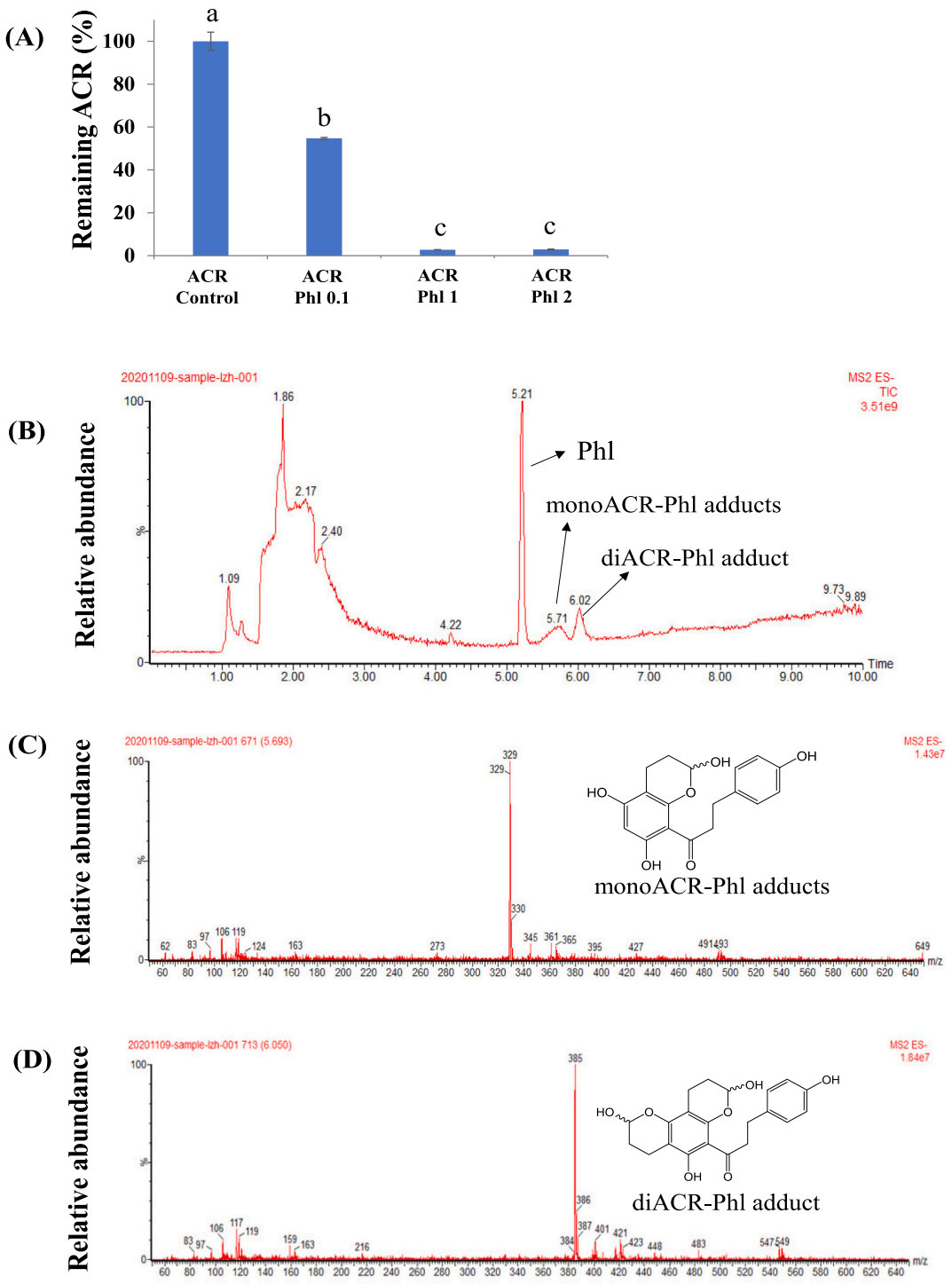

Fig. 5. HPLC quantifications of remaining ACR after incubation with Phl for 24 h at 37 °C (A) and the total ion chromatograms of the total ion chromatograms (TIC) of the reaction products in the co-incubation of WPI, ACR and Phl after removal of WPI (B). MS spectrum of monoACR-conjugated Phl ($[M-H]^-$ *m/z* 329) (C) and diACR- conjugated Phl ($[M-H]^-$ m/z 385.1) (D).

ACR (1 mmol/L) with WPI (10 mg/mL) at 25 °C for 24 h resulted in a drastic 8-fold increase in protein carbonyl level, from 0.22 to 1.65 μmol/mg protein (Fig. 1A). It has been proved amino acid residues could be selectively lost accompanying the incorporation of ACR into protein. The mechanism has been explained as ACR's reaction with amino acid residues with the formation of Michael-type adducts, preserving the functionality of aldehyde group in the modified protein, thereby introducing carbonyl groups into WPI (Aldini et al., 2011; Zhu et al., 2011). In the presence of Phl (0.1, 1, and 2 mmol/L), protein carbonyls significantly decreased to 1.54, 0.83, and 0.65 μmol/mg protein, respectively. The reduction on protein carbonyl content afforded by Phl was possibly mediated via its efficient scavenging of ACR, thus preventing WPI from carbonylation.

3.2. Total sulfhydryl content

Sulfhydryl groups in cysteine residues are considered the most reactive nucleophiles in proteins with facile reaction with ACR. It is

believed that preferential generation of Michael-type adducts on cysteine residues is the predominant way for ACR to exert its reactivity in biological systems (Aldini et al., 2011). In our study, ACR-treated WPI had a significant decline (12.8 %) of total sulfhydryl content, from 0.28 to 0.24 μmol/mg protein (Fig. 1B). Sulfhydryl groups are generally weak secondary bonds that might contribute to the stabilization of protein tertiary structures. Therefore, loss of sulfhydryl groups may lead to changed protein conformation of WPI. In the presence of Phl (0.1, 1, 2 mmol/L), total sulfhydryl content partially restored to 0.25, 0.26 and 0.27 μmol/mg protein, respectively, suggesting the protective effects of Phl on ACR's modification on the sulfhydryl groups of cysteine residue. It can be explained by a faster reaction rate of Phl than cysteine in reaction with ACR. Likewise, Phl was demonstrated to be "rapidly reacting with high capacity" and more active than cysteine in scavenging of the 1,2-dicarbonyl compound methylglyoxal (Comert & Gokmen, 2019). Similar to the results of this study, the protection of protein sulfhydryls was observed in ACR-incudced cytotoxicity tests in cultured MN9D cells and the enolate formation in ACR-Phl adducts was suggested as a prerequisite for thiol protection (LoPachin et al., 2016).

3.3. Surface hydrophobicity

ACR-induced alteration of protein physicochemical properties could be reflected by surface hydrophobicity, which was commonly used to assess the distribution of hydrophobic amino acid residues on protein surface. As illustrated in Fig. 1C, a significant decrease (12.7 %) in surface hydrophobicity was observed in ACR-treated WPI. Structural changes leading to decreased surface hydrophobicity have been observed in several reactive carbonyl compounds such as malondialdehyde, glyoxal, and methylglyoxal (Wu et al., 2009; Zhao et al., 2020). Protein surface hydrophobicity was not significantly changed when compared to ACR-treated WPI at the lowest concentration of Phl (0.1 mmol/L). Nevertheless, significant decline of surface hydrophobicity (37.7 % and 42.0 %) was observed at higher concentrations of Phl (1 mmol/L and 2 mmol/L), respectively. It has been reported that incubation of polyphenolic compounds (EGCG, quercetin, apigenin, and naringenin) with WPI can lead to the decrease of protein surface hydrophobicity (Liu et al., 2021). The introduction of polar hydroxyl groups from Phl into WPI might contribute to the further reduction of surface hydrophobicity in our study. Taken together, the reduced protein surface hydrophobicity is generally considered as a consequence of ACR's modification on WPI and phenol-protein interactions.

3.4. Protein solubility

As shown in Fig. 1D, the protein solubility of WPI control and ACR-treated WPI was 91.38 % and 91.95 %, respectively, indicating limited impact of low concentrations of ACR (1 mmol/L) on protein solubility. Co-incubation with Phl (0.1, 1, and 2 mmol/L) slightly but not significantly lowered the protein solubility to 89.38 %, 89.94 %, and 87.53 %, respectively. In general, protein solubility is affected by both intrinsic factors such as protein amino acid sequence/composition as well as extrinsic conditions (pH, temperature, and ionic strength). Depending on the nature and concentration of phenolic compounds, protein solubility may be improved or suppressed due to the modification in protein structure and extrinsic factors (Günal-Köroğlu et al., 2023). In this study, the addition of Phl hardly affected protein solubility and total sulfhydryl content although it can influence protein surface hydrophobicity.

3.5. Protein intrinsic fluorescence

Aromatic amino acids with benzene ring structure and conjugated double bonds including tryptophan, tyrosine, and phenylalanine contribute to the intrinsic fluorescence (Luna & Estevez, 2018). Major components of WPI such as α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) contain tryptophan and tyrosine residues which can be analyzed by intrinsic fluorescence profile (Baba et al., 2021). As shown in Fig. 2, WPI control exhibited maximum fluorescence emission around the wavelength of 330 nm, but the intrinsic fluorescence of tryptophan was significantly weakened by ACR treatment. Similar decay in intrinsic fluorescence has been observed in ACR (0.01 to 10 mmol/L) treated β-lactoglobulin in a dose-dependent manner, suggesting ACR's binding to protein altered the tertiary structure by partial masking of some nonpolar aromatic amino acid (Lv et al., 2021). Increase of steric hindrance induced by protein aggregation also contributes to the quenching of fluorescence intensity (Wu et al., 2010). In this study, with the presence of Phl (0.1, 1, and 2 mmol/L), the reduction in fluorescence intensity was noted in a dose-dependent way. Various evidence can confirm the quenching effects of polyphenols in protein intrinsic fluorescence (Geng et al., 2020). The lowering of fluorescence intensity was probably mediated by the attachment of phenolic moieties to protein (Feroz et al., 2012), leading to exposure of tryptophan residues to the more hydrophilic environments (Jiang et al., 2018).

3.6. SDS-page

ACR-induced protein modification was monitored by protein bands separated according to different molecular weight (Fig. 3A). Under reducing conditions, two major bands were identified as β-Lg and α-La by SDS-PAGE. Generally, there was no significant difference in protein profiles of all treatments in the separating gel. In ACR-treated samples, however, partly accumulation of protein aggregates at higher molecular weights was found and these protein aggregates even hindered to migrate across the stacking gel. Similar observations were reported in ACR treated soy proteins and rabbit myofibrillar proteins (Wang et al., 2018; Wu et al., 2010), when native proteins gradually disappeared with accumulation of protein-protein cross-linked derivatives at higher molecular weights, suggesting ACR-induced protein cross-linking, aggregation, polymerization, and even degradation.

3.7. Western blot analysis of ACR-bound proteins

Accurate targeting of ACR-bound proteins in WPI was achieved by Western blot analysis using anti-ACR antibody specifically binding to ACR-modified proteins. As shown in Fig. 3B, WPI control was hardly recognized by the antibody, and ACR-modified protein was observed in a wide range of molecular weight when ACR was incubated with WPI at 25 °C for 24 h. When compared to α-La, β-Lg is more vulnerable to modification by ACR. The appearance of higher molecular weight bands of ACR-bound proteins suggested formation of protein cross-links and aggregates. ACR-promoted protein cross-linking and aggregation might be mediated via Michael addition reaction between protein and the double bond of ACR molecule, together with reaction between other aldehyde groups of ACR and the ε-NH_2 groups of lysine residues in other protein through the formation of Schiff base. The addition of Phl (0.1, 1 and 2 mmol/L) significantly interrupted ACR's binding activity to WPI dose-dependently. The identification of ACR-bound proteins obtained in Western blot was consistent with the results of protein carbonyl detection in this study, suggesting that Phl is effective in scavenging of ACR and blocking its reactive sites towards proteins. In a previous study, Phl was found to be effective in inhibiting the formation of FDP-lysine (a typical ACR-lysine adduct) and blocking the remaining electrophilic sites in FDP-lysine (Zhu et al., 2012). For co-incubation of ACR, proteins and Phl, Phl was also capable of interrupting the introduction of carbonyl groups into BSA and oligomerization in RNase A (Zhu et al., 2012). These findings are in line with result of the present study, indicating protective effects of Phl on amino acids or proteins under ACR challenge via direct scavenging of ACR.

3.8. SEM

Scanning electron micrographs highlighted the impact of Phl on

surface morphology of ACR-treated WPI samples (Fig. 4). Relatively smooth and uniform surface structure was observed in native WPI, whereas smaller and scattered protein particles were found in ACR-treated WPI, suggesting possible disruption of the ordered structure of WPI by covalent interaction between ACR and WPI. In the presence of Phl, protein scattering was promoted at a low Phl concentration (0.1 mmol/L). Structural reorganization led to protein aggregation at higher concentrations of Phl (1 and 2 mmol/L), accompanied by the emergence of bulky proteins. Protein crosslinking and aggregation were frequently observed in *co*-incubation of protein and dietary polyphenols, and possible protein-polyphenol interactions might promote protein aggregation via covalent or non-covalent forces (hydrogen bonding, van der Waals forces, and hydrophobic bonding, etc.) (Liu et al., 2021). In our study, although the Phl could inhibit protein carbonylation and the formation of ACR-bound proteins, it could not restore the protein surface microstructure to its orderly and homogeneous state as shown in the untreated WPI control sample. Both ACR's modification on proteins and phenol-protein interactions contributed to the microstructure alternation in WPI.

3.9. Protein digestibility

The digestive property of WPI challenged by ACR in the absence or presence of Phl was evaluated and listed in Table 1. Protein digestibility is one of the most mportant indicators for its nutritional value which is closely related to amino acid composition and secondary structural features. After gastric digestion, at all-time points (30 min, 60 min and 120 min), the treatment of ACR in the absence and presence of Phl did not cause significant change in protein digestibility. Although limited information is available on the impacts of ACR in protein digestibility, there are many reports on other reactive carbonyl compounds such as malondialdehyde (MDA)-induced change of protein digestibility. Similar to ACR, MDA did not affect protein digestibility at a low concentration (0.5 mmol/L) (Niu et al., 2019), indicating enzyme-targeted amino acid residues were not severely damaged by these carbonyl compounds at low concentration. Meanwhile, protein digestibility was not affected by co-incubation with Phl, suggesting Phl did not inactivate digestive enzymes or produce nondigestible protein-polyphenol aggregates.

3.10. HPLC and LC-MS analyses

The ACR-scavenging capacity of Phl was probed with quantification of remaining ACR after incubation with Phl (Fig. 5A). After 24 h incubation with Phl (0.1, 1 and 2 mmol/L), remaining ACR dropped to 54.77 %, 2.74 %, and 2.87 %, respectively. In previous studies, Phl proved to be the most potent scavengers among several polyphenols with high reactivity towards ACR, and 99.6 % of ACR was scavenged at 90 min when the molar ratio of Phl to ACR was 2:1 (Zhu et al., 2009). Michael addition reaction was responsible for directly trapping of ACR, which mainly involves C-3 and/or C-5 in the A ring of Phl. Subsequently, nucleophilic attack at the terminal aldehyde carbon by an adjacent hydroxyl group at C-2 and/or C-4 in A ring lead to the formation of cyclic hemiacetal(s) as more stable final products. In this study, we examined whether Phl has better nucleophilic potential to ACR than WPI. The nucleophilic potential of Phl was accountable for the formation of conjugates between Phl and ACR. After 24 h co-incubation of Phl, ACR and WPI, formation of Phl-ACR adducts was identified by UPLC-MS. Total ion chromatograms (TIC) of the reaction products after removal of WPI was shown in Fig. 5B. Native Phl was eluted at the retention time of 5.21 min, while subsequent peaks at 5.71 min and 6.21 min represented Phl conjugates with ACR. As expected, the molecular weight of $[M\text{-}H]^-$ *m/z* 329 and m/z 385.1, were assignable to monoACR-conjugated Phl and diACR-conjugated Phl, respectively (Fig. 5C and D). The formation of ACR-Phl conjugates in co-incubation with WPI suggests Phl has stronger nucleophilic potential than WPI for Michael addition to ACR. Phl's protection on ACR-induced modification on WPI was probably mediated by Phl's role as a sacrificial nucleophile, which consequently rendered active site in ACR unavailable for covalent modification of WPI. In other studies, ACR-polyphenol conjugates have been identified in cakes (Sugimoto et al., 2021; Wang, Lu, et al., 2019), grilled chicken wings (Jiang et al., 2020), and roasted pork (Liu et al., 2023), suggesting polyphenols could eliminate ACR in complicated food matrix.

4. Conclusion

The α,β-unsaturated aldehyde acrolein (ACR) is endowed with high reactivity towards food ingredients, thereby lowering their nutrition values and even leading to potential risks in food safety. Elimination of ACR in the food system is proposed as an efficient strategy for prevention of its deleterious effects. Nowadays, progress has been made in the screening and development of novel ACR scavengers in chemical models. However, limited information is available on the effects of ACR scavengers in protein-based food models. Our study confirmed Phl's ACR-scavenging capability during the co-incubation of WPI, ACR and Phl, which therefore partially relieved carbonyl stress on protein. In ACR-challenged WPI, visualization of protein modification by Western blot further confirmed ACR's deleterious impact on WPI and the protective role of Phl in interrupting covalent binding of ACR to WPI. Protein physiochemical changes accompanying the formation of ACR-Phl conjugates were evaluated, including protein sulfhydryl content, surface hydrophobicity, protein solubility, tryptophan fluorescence, protein surface microstructure, and protein digestibility. This study may contribute to a better understanding of the role of natural polyphenolic RCS-scavengers in protein-based food system under carbonyl exposure.

CRediT authorship contribution statement

Yanming Zhang: Methodology, Investigation. **Xingya Hao:** Writing – original draft, Formal analysis. **Zhangjie Hu:** Validation, Data curation. **Wenhua Yao:** Investigation, Formal analysis. **Haihua Zhu:** Investigation, Data curation. **Zhongxu Du:** Investigation, Data curation. **Shuiping Ouyang:** Resources. **Shiqing Sun:** Resources. **Futing Huang:** Resources. **Qin Zhu:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Jun Xu:** Writing – review & editing, Resources, Project administration, Funding acquisition.

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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Data availability

Data will be made available on request.

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