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Mitigation of malondialdehyde-induced protein lipoxidation by epicatechin in whey protein isolate

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ABSTRACT

Malondialdehyde (MDA) can induce lipoxidation in whey protein isolate (WPI). The physicochemical changes in this reaction with or without the presence of a phenolic compound epicatechin (EC) were characterized in this study. Results suggested the content of MDA was significantly reduced during co-incubation of MDA and EC. The addition of EC dose-dependently alleviated MDA-induced protein carbonylation, Schiff base formation and loss of tryptophan fluorescence. The interruption of MDA-binding to WPI was directly visualized by immunoblotting analysis. Observation of the surface microstructure of WPI showed that MDA-induced protein aggregation was partially restored by EC. Meanwhile, EC was found to promote loss of both protein sulfhydryls and surface hydrophobicity due to possible phenol-protein interactions. These observations suggested the potential of EC in the relief of MDA-mediated protein lipoxidation.

1. Introduction

Malondialdehyde (MDA, OCH-CH_2-CHO, MW 72; pKa 4.45), is the most intensively studied secondary products produced in both foodstuff and living organisms as a consequence of lipid peroxidation. Being one of the most abundant aldehydes produced in oxidative degradation of unsaturated fatty acids (both ω-3 and ω-6) with three and more double bonds, MDA is widely accepted as a typical marker of oxidative stress (Esterbauer, 1993; Zhou et al., 2020). MDA can be found in almost all lipid-containing foods, especially in high-fat foods such as vegetable oils, animal oils, meat products, and fried foods, with a content ranging from 0.1 to 10 mg/kg (Zhou et al., 2020). However, only free MDA is estimated in this well-accepted lipid oxidation marker, without considering the existence of bound forms of MDA (Vandemoortele, Babat, Yakubu, & De Meulenaer, 2020). The formation of bound MDA is ascribed to its high electrophilic reactivity towards biological nucleophiles (proteins, nucleic acids, and phospholipids, especially amino groups), with adverse impacts on the structure and function of biomolecules (Esterbauer, 1993). Nucleophilic protein degradation mediated by MDA was implicated in decreasing the bioavailability and nutritional value of dietary proteins (Zamora, Navarro, Aguilar, & Hidalgo, 2015). Furthermore, MDA-modified proteins were also suggested to be involved in the pathogenesis of some human diseases such as atherosclerosis, diabetes, Alzheimer's disease, and cancer, as well as aging (Kanner, 2007; Lankin, Tikhaze, & Melkumyants, 2022). Although there are no officially published detailed MDA limits, the European Food Safety Authority (EFSA) Scientific Committee recommended a 30 μg/kg bw/day threshold of toxicological concern (TTC) for MDA (Zhou et al., 2020).

Upon formation, MDA can diffuse and react with adjacent proteins. Such post-translational non-enzymatic modification of proteins by reactive or electrophilic lipid species is termed as protein lipoxidation. The process involves the formation of Schiff's bases or Micheal adducts by reactions between lipid oxidation-derived carbonyls (aldehydes or ketones) and primary amines (Zhou et al., 2020). MDA covalently binds to protein ε-amino and thiol groups with modification on protein side chains, leading to protein cross-linking, misfolding, aggregation, and loss of enzymatic activity (Huang, Sarkhel, Roy, & Mohan, 2023).

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Various intervention strategies were developed to delay and interrupt the lipoxidation process. Antioxidant phenolic additives have been demonstrated to behave as a triple barrier (Zamora & Hidalgo, 2016), whereas the roles of chelator, free radical scavenger and lipid-derived carbonyl scavenger, are all responsible for minimizing the consequence of lipoxidation. The reaction mechanisms of phenolics towards lipid peroxidation-derived carbonyls including α, β-unsaturated aldehyde (4-hydroxy-*trans*-2-nonenal and acrolein), di-aldehyde (glyoxal) have been elucidated with identification of aldehyde-phenol adducts in chemical and food models. However, the interaction mechanism between phenolic compounds and MDA has not yet been described clearly due to the complexity of reaction pathways. Until recently, the main adducts formed in the reaction between the phenolic compounds (2,5-dimethylresorcinol, orcinol, olivetol, and alkylresorcinol) and MDA were produced via the reaction of one molecule of the phenol, one molecule of MDA, and additional one molecule of acetaldehyde (which is absent in other carbonyl-phenol reaction) (Zamora, Alcon, & Hidalgo, 2023).

The recognition of phenols as MDA-trapping agents justifies their application in retarding MDA-induced lipoxidation. Theoretically, fast scavenging of MDA by phenol contributes to protection on proteins by inactivation or removal of MDA. Nevertheless, in a complicated food system with co-existence of protein, MDA, and phenol, multiple reactions happen among these compounds. The A ring of phenol, with OH groups at *meta*-positions, is involved in trapping of MDA through nucleophilic addition. The B ring of phenols, with OH groups at the *ortho*-positions, is often engaged in antioxidant activities donating H-atoms to free radicals, leading to the formation of corresponding quinones with further reactivity towards nucleophilic groups on proteins (Farooq, Abdullah, Zhang, & Weiss, 2021). Hence, the variations in protein physicochemical properties introduced by phenolic MDA-trapping agents need to be evaluated to avoid potential adverse effects on protein quality.

Epicatechin (EC), as a major ingredient in popular tea beverages, was selected as the potential MDA-trapping agent in this study. The position OH groups in A ring of EC which meets the structural requirement (*meta*-diphenols) in trapping of MDA. However, the *ortho*-positions OH groups in B ring may implicate possible phenol-protein interaction which could cause protein cross-linking and aggregation. Therefore, the effects of EC in a typical dietary protein (whey protein isolate, WPI) under exposure to MDA were investigated. The efficiency of EC in eliminating MDA was measured by HPLC and the resultant mitigation on MDA-induced lipoxidation was visualized by Western blot analysis. The physiochemical changes were revealed by monitoring protein carbonylation, sulfhydryl content, surface hydrophobicity as well as fluorescence profiles of intrinsic tryptophan and Schiff base adducts. The influence of EC on protein surface microstructure and protein hydrolysis were also evaluated. The findings of the present study may provide a theoretical basis for the development of phenolic MDA-trapping agents in controlling lipid oxidation product-mediated protein damage in food system.

2. Materials and methods

2.1. Material

WPI (> 92% protein in dry basis) was supplied by Hilmar Ingredients (Hilmar, CA, USA). 1,1,3,3-tetramethoxypropane, the enzymes used for simulated gastrointestinal digestion, including pepsin (≥250 units/mg, solid), trypsin (8 × USP specifications), were the products of Sigma-Aldrich Co., LLC (United States). The rabbit polyclonal specifically binds to MDA-modified proteins (Ab27642) and the goat anti-rabbit IgG H&L (HRP) secondary antibody were purchased from Abcam (Cambridge, UK). 1,1,3,3-tetramethoxypropane,1-anilino-8-naphthalenesulfonate, trichloroacetic acid, acetonitrile, 5,5′-dithiobis (2-nitrobenzoicacid), 2,4-dinitrophenylhydrazine (DNPH), guanidine hydrochloride, Brilliant blue R250, and other reagents were all purchased from Sinopharm Chemical Reagent Co., Ltd. (China) or Tokyo Chemical Industry Co., Ltd. (Japan). All chemicals used in the study were of analytical grade and acetonitrile was of HPLC grade.

2.2. Preparation of MDA

For each experiment, MDA was freshly prepared by acidic hydrolysis of 1,1,3,3-tetramethoxypropane (TMP) according to the procedure described in a previous study (Kikugawa, Tsukuda, & Kurechi, 1980). Briefly, acid hydrolysis was carried out by the incubation of 845 μL TMP and 1 mL HCl (1 M) in 3.16 mL ultrapure water for 2.5 min at 40 °C. 6 M NaOH was used to adjust the pH to 7.4. The obtained MDA solution was diluted 200 times with 10 mM phosphate buffer (pH 7.4) and the MDA concentration was measured at the UV absorbance of 267 nm and calculated with a molar extinction coefficient value of 31,500 mol^{-1} cm^{-1}.

2.3. Retained MDA content after the incubation with EC

To understand the interactions between MDA and EC, MDA (1 mM) was incubated with EC (0.1, 1, and 2 mM) in 10 mM PBS (pH 7.4) at 25 °C for 24 h. The residual MDA was monitored by the Agilent 1260 Infinity II HPLC system with a separation module (G7111B), an autosampler (G7129E), and a multiwavelength UV detector (G7115A). Reaction mixtures were chromatographically separated by a YMC Pro C18 column (250 × 4.6 mm, 5 μm). The flow rate was 1 mL/min, and the column temperature was kept at 25 °C. The mobile phase was composed of deionized (DI) water with 0.1% formic acid (solvent A) and acetonitrile (solvent B). Gradient elution (flow rate: 1 mL/min) was carried out for 98% solvent A in 0 to 8 min, 80% solvent A in 8 to 20 min, and 98% solvent A in 20 to 30 min. MDA was monitored the at the wavelength of 245 nm according to a previous study of Cai et al. (2011).

2.4. MDA-induced protein lipoxidation

MDA-induced protein lipoxidation reaction system was established according to a previous report by Zheng et al. (2022). In 10 mM phosphate buffer (pH 7.4), MDA (1 mM) was co-incubated with WPI (6 mg/mL) and EC (0, 0.1, 1, and 2 mM). The reaction was carried out at 25 °C for 24 h in the darkness with the addition of 0.02% sodium azide as germicide.

2.5. Protein carbonylation

Protein carbonyl content in WPI was measured according to the procedure of Levine (Levine, Wehr, Williams, Stadtman, & Shacter, 2000). Briefly, 0.2 mL DNPH derivatization reagent (0.1% *w/v* dissolved in 2 M HCl) was added to 0.1 mL of the reaction mixture. The derivatization process was last for 1 h at room temperature. Carbonylated proteins were then precipitated by 20% TCA solution (*w/v*) and washed with ethanol/ethyl acetate (1:1, *v*/v) at least 3 times. Finally, the obtained protein precipitate was dissolved in guanidine hydrochloride (8 M). The UV absorption was monitored at 370 nm and the protein carbonyl content (nmol carbonyl/protein) was calculated as follows:

$$\text{Protein carbonyl content (nmol carbonyl/protein)} = \frac{A_{370}}{\varepsilon} \times \frac{10^6}{C.}$$

where A_{370} is the UV absorbance at 370 nm, C is the MP concentration, and ε is the absorption coefficient of 22,000 mol^{-1} cm^{-1}.

2.6. Protein sulfhydryls

The total sulfhydryl content of WPI was measured using Ellman's method (Ellman, 1959). Briefly, urea-SDS solution was prepared by dissolving 8.0 M urea and 0.03 g/mL SDS in 0.1 M phosphate buffer (pH

7.4). 100 μL sample was mixed with 200 μL urea-SDS solution. 200 μL DTNB (10 mM) was added and the reaction lasted for 15 min before centrifugation (6000 *g*, 5 min). The absorbance of the supernatant was monitored at 412 nm. A molar extinction coefficient of 13,600 M^{-1} cm^{-1} was applied to calculate the sulfhydryl content (mmol/g protein).

2.7. Protein surface hydrophobicity

Protein surface hydrophobicity was determined by the methods of Li et al. (2015). Each sample was diluted to 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL and reacted with 1-anilino-8-naphthalenesulfonate (ANS) for 3 min before the fluorescence intensity was recorded at an emission wavelength of 484 nm when excited at 365 nm. The slope of the linear regression of fluorescence intensity vs. protein concentration (mg/mL) was calculated as the index for protein hydrophobicity index (H_0).

2.8. Protein fluorescence spectra of tryptophan and Schiff base

The spectra of intrinsic tryptophan fluorescence in different treatments were recorded according to the method (Wu, Zhang, & Hua, 2009) using a Hitachi-F4600 model fluorescence spectrometer by the excitation wavelength fixed at 295 nm and emission wavelength recorded from 300 nm to 400 nm. The emission spectrum of Schiff base adducts ranged from 400 nm to 600 nm at the excitation wavelength of 375 nm (Chen, Zhao, Sun, & Zhao, 2013).

2.9. SDS-PAGE analysis of protein profile

The protein profile of MDA-modified WPI was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). 10 μL sample was mixed with the same volume of sample buffer (containing 10% β-ME), and the mixture was heated at 95 °C for 5 min. Separation of proteins was carried out in a Mini-PROTEAN 3 Cell apparatus (Bio-Rad) using 15% separation gel and 5% concentrated gel. After electrophoresis, all the protein bands were characterized by Coomassie brilliant blue R-250 stain solution.

2.10. Determination of MDA-bound proteins by Western blot

After electrophoresis, the protein was transferred to a PVDF membrane. The PVDF membrane was put into freshly prepared 5% skim milk and blocked overnight at 4 °C, and the primary antibody of Rabbit polyclonal to MDA modified protein (ab27642, 1: 5000) was added at 37 °C for 3 h, followed by incubation with secondary antibody (1:2000, HRP-conjugated goat anti-rabbit IgG) for 1 h. All the immunoreactive bands were visualized by Pierce ECL Western blot Substrate in a Bio-Rad ChemiDoc XRS+ system.

2.11. Observation of protein microstructure

The microstructure on the surface of WPI was observed by a Scanning Electron Microscope instrument (SEM, model S-4800, Hitachi Co., Tokyo, Japan). The sample was evenly coated on the conductive tape and dried before it was glued to the sample table with conductive adhesive. The gold about 15 nm thickness was sprayed in the vacuum spray plating instrument. Protein surface microstructure was observed and photography under 10,000 × magnification at an acceleration voltage of 10 kV.

2.12. Measurement of protein hydrolysis (DH)

O-phthaldialdehyde (OPA) was employed for the quantification of the degree of protein hydrolysis (DH) to the procedure of Church, Swaisgood, Porter, and Catignani (1983). Briefly, HCl (6 mM) was added to each sample for adjusting the pH to 1.5. Porcine pepsin solution (4% *w*/w protein basis) was added to start gastric digestion for 60 min at 37 °C. Intestinal digestion was subsequently performed by adjusting the pH to 7.4 to inactivate pepsin and adding 4% pancreatin (*w*/w protein basis) for the following 180 min. TCA (30%) was used to terminate the gastrointestinal digestion and digests were re-dissolved in NaOH after centrifugation. OPA reagent was prepared with 0.08% OPA (*w/v*), 0.088% dithiothreitol (w/v) and 0.1% SDS (w/v) in 2% ethanol (*v*/v). 0.6 mL OPA reagent was added to 30 μL each sample for DH measurement for 2 min at 37 °C. UV absorbance was taken at 340 nm and serine was used for the construction of a standard curve. The DH% was calculated as follows:

$$W_{serine-NH_2} = C_{serine-NH_2} \times \frac{V \bullet N}{X \bullet P\%}$$

$$h = \frac{W_{serine-NH_2} - \beta}{\alpha}$$

$$DH = \frac{h}{h_{tot}}$$

Here, $W_{serine-NH_2}$ stands for the amount of serine-NH_2 (g-1 protein) per gram of protein; V (L) stands for sample volume; N stands for sample dilution factor; X (g) stands for sample mass; P% stands for the mass fraction of protein in the sample; h (mmol/g) stands for the number of peptide bonds broken per gram of protein during digestion; h_{tot} (mmol/g) stands for the total number of peptide bonds per gram of protein. The h_{tot} of WPI is 8.8; α and β are represented by constants 1 and 0.4, respectively.

2.13. Statistical analysis

All the tests were performed in triplicates and the values were expressed as mean ± standard error (SD). Statistical analysis was performed with SPSS Statistics 20 software (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was applied to compare significant differences at the level of 0.05 using one-way analysis of variance (ANOVA).

3. Results and discussion

3.1. HPLC quantification of MDA with co-incubation of EC

Fig. 1A displayed the residual MDA (%) after the incubation of EC (0, 0.1, 1, and 2 mM) at 25 °C for 24 h. When MDA was incubated with 0.1 mM EC, 84.03% MDA remained with a significant difference compared with control. Higher disappearance rate of MDA was observed at the concentration of 1 mM and 2 mM EC, and the remaining MDA were 64.78 and 65.41%, respectively. The result was consistent with previous studies, suggesting the potential of tea polyphenols in lowering MDA content (Cai et al., 2011; Zhang, Zhao, Ohland, Jobin, & Sang, 2019). In a recent study of Zamora, phenolic compounds were demonstrated to react with MDA by producing stable adducts (Zamora et al., 2023). The chemistry of the reaction between phenolics and MDA was far more complex than the chemistry previously unraveled in the reaction between phenolics with other lipid oxidation derived carbonyls (HNE, acrolein, and glyoxal). Different from the direct nucleophilic additions, MDA was firstly degraded to acetaldehyde and oligomerized into dimers and trimers before all these intermediates took part in the formation of aldehyde-phenolic adducts. The atoms in the contiguous aromatic carbon of the phenolic were participated in the MDA/phenolic reaction at both high temperature (100 °C) and room temperature (Zamora et al., 2023; Zamora, Alcon, & Hidalgo, 2024). In the present study, EC, with the hydroxyl groups at the *meta*-position of A-ring, could react with MDA through similar pathways and contribute to the reduction of MDA content during the co-incubation of EC and MDA.

3.2. Protein carbonylation

MDA might attack nucleophilic groups in protein (mainly cysteine,

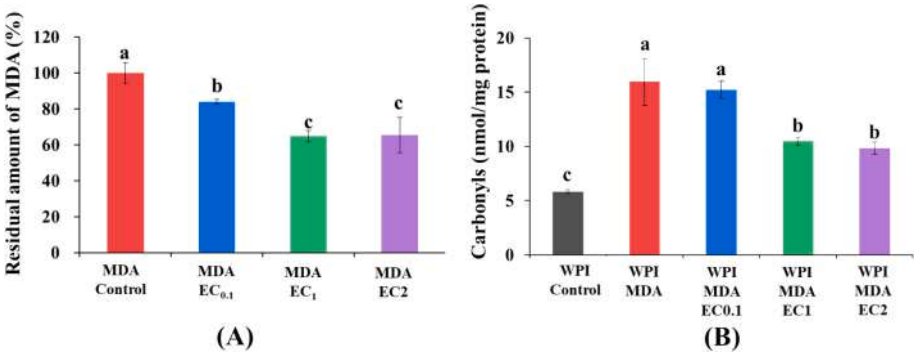

Fig. 1. HPLC quantification of the remaining MDA (%) in the reaction system containing MDA (1 mM) and EC (0, 0.1, 1, and 2 mM) at 25 °C for 24 h (A); and protein carbonyl content of WPI (6 mg/mL) co-incubated with MDA (1 mM) in the presence of EC (0, 0.1, 1, and 2 mM) at 25 °C for 24 h (B). Values are expressed as average ($n = 3$) ± standard deviation (SD). In the same parameter group, means without common letters differ significantly ($P < 0.05$).

lysine, and histidine residues), with the promotion of Schiff base formation, resulting in protein carbonylation (Gurbuz & Heinonen, 2015). The changes in MDA-induced protein carbonylation were presented in Fig. 1B. As expected, compared to native WPI (5.81 nmol/mg protein), the concentration of protein carbonyls was sharply increased to 15.94 nmol/mg protein with incubation of MDA (1 mM). Protein carbonyl content was slightly decreased to 15.22 nmol/mg protein with the addition of 0.1 mM EC and further decreased to 10.46 and 9.85 nmol/mg protein when the concentration of EC was 1 and 2 mM, respectively. The inhibition of protein carbonylation was also observed in addition of purslane polyphenols (mainly comprised of chlorogenic acid, caffeic acid, ferulic acid, rutin, kaempferol, and quercetin) in rabbit meat myofibrillar protein under MDA stress (Chen, He, Wang, & Li, 2023a). These results suggested the protective effects of phenolic compounds in avoiding further introduction of carbonyls into other food ingredients.

3.3. Protein sulfhydryls

As shown in Fig. 2A, no significant difference was found in MDA-treated WPI (42.84 nmol/mg protein) compared with native WPI (42.13 nmol/mg protein). The result is consistent with a previous study, indicating low concentrations of MDA (0.5 to 5 mM) had negligible impacts on protein sulfhydryl (SH) groups, while higher concentrations (> 10 mM) of MDA led to a progressive depletion of protein SH groups (Niu et al., 2019). In this study, with the co-incubation of MDA (1 mM) and EC (0.1, 1, and 2 mM), the SH content was further declined to 41.18, 40.70 and 38.04 nmol/mg protein, respectively. The plausible explanation is the SH groups in WPI was susceptible to Michael addition reactions by equivalent quinones derived from the oxidation of phenols (Lund, 2021). Another study reported elevated SH content in polyphenols-incorporated rabbit myofibrillar protein under MDA-induced oxidation. The concentration of these polyphenols (chlorogenic acid, caffeic acid, ferulic acid, rutin, kaempferol, and quercetin) was 20 μM. Hence, the impact of phenols on protein SH content is variable according to the types and dosage of phenols (Chen et al., 2023a). The dosage of polyphenols should be optimized in order to refrain from depletion of protein SH groups.

3.4. Protein surface hydrophobicity

Surface hydrophobicity of MDA-treated WPI in the presence and absence of EC was shown in Fig. 2B. MDA-induced protein oxidation led to an increase in protein surface hydrophobicity from 8.20 to 9.34 ($P < 0.05$), suggesting the exposure of hydrophobic amino acids to the polar surface of protein. Elevated surface hydrophobicity was also observed in MDA-modified soy protein isolates (Wang, Zhao, Qiu, & Sun, 2018), rabbit and sturgeon myofibrillar proteins (Chen, He, Wang, & Li, 2023b; Shen et al., 2022). MDA-induced protein oxidation might disrupt electrostatic interactions or hydrogen bonds between proteins, leading to the unfolding of proteins and exposing non-polar amino acids to the protein surface with a resultant increase in surface hydrophobicity (Wang, He, Zhang, Chen, & Li, 2021). In the presence of EC (0.1 mM), protein surface hydrophobicity was reduced to 8.53, comparable to native WPI, suggesting the protection of EC on MDA-modified WPI. A significant decrease was observed in higher concentrations of EC (1 mM and 2 mM), with protein surface hydrophobicity significantly declined to 7.16 and 6.32, respectively. It is concluded that the influence of polyphenols on protein surface hydrophobicity is closely related to conformational changes (protein unfolding), covalent blocking of hydrophobic residues and the binding sites available for ANS (the fluorescent probe of surface hydrophobicity) (Günal-Köroğlu, Manuel Lorenzo, & Capanoglu, 2023). Therefore, the covalent or non-covalent

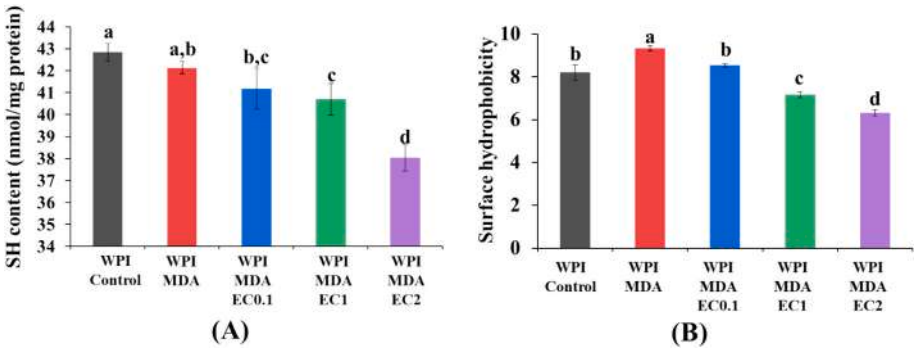

Fig. 2. Protein total sulfhydryl contents (A) and surface hydrophobicity contents (B) of WPI (6 mg/mL) in the absence and presence of EC (0.1, 1, and 2 mM) during the co-incubation with MDA (1 mM) at 25 °C for 24 h. Values are expressed as average (n = 3) ± standard deviation (SD). In the same parameter group, means without common letters differ significantly ($P < 0.05$).

phenol-protein interactions contribute to the reduction of protein surface hydrophobicity in the present study.

3.5. Protein fluorescence spectra of tryptophan and Schiff base adducts

When native WPI was excited at the wavelength of 280 nm, the maximum fluorescence emission wavelength was observed around 330 nm (Fig. 3A). An obvious descent in protein endogenous fluorescence was observed in MDA-treated WPI, indicating the fluorescent tryptophan residues were sensitive to MDA. The indole nitrogen of tryptophan is the modification site of MDA (Foettinger, Melmer, Leitner, & Lindner, 2007), and MDA-induced protein conformational changes also contribute to the loss of tryptophan fluorescence (Huang et al., 2023). A dose-dependent recovery of tryptophan fluorescence was found when MDA-treated WPI was co-incubated with EC (0.1, 1, and 2 mM). The contribution of EC in regaining tryptophan fluorescence was also found in WPI under oxidative stress (Fenton system) (Lian et al., 2023), suggesting the protective effects of EC in lipoxidation-induced oxidative modification on tryptophan residues.

A variety of covalent dihydropyridine (DHP)-type products including Schiff base adducts and cross-links have been identified through the reaction of electrophilic groups in MDA with protein nucleophilic groups (N-termini of peptides and ε-amino groups on amino acid side-chains) (Gurbuz & Heinonen, 2015). When excited at 350 nm, the fluorochrome in Schiff base's conjugated structure (–N=CH-CH=CH–) in MDA-treated WPI had a maximum emission wavelength around 460 nm, which was absent in native WPI (Fig. 3B). MDA triggered Schiff base formation was dose-dependently alleviated by EC, which is in accordance with the EC's contribution to the restoration of protein tryptophan fluorescence. In a previous study, the inhibition of lipofuscin-like fluorescence intensity was reported in MDA-modified human serum albumin by tea catechins (Cai et al., 2011). The antioxidant effects, direct MDA-scavenging capacity and neutralizing the amino-carbonyl cross-linking reaction all contribute to the reduction of Schiff base adducts formation.

3.6. SDS-PAGE and Western blot analysis

The representative SDS-PAGE profile was presented in panel A of Fig. 4. WPI was composed of two major proteins, β-lactoglobulin (β-Lg, 18 KDa) and α-lactalbumin (α-La, 14 KDa). The separation of proteins by SDS-PAGE revealed that the co-incubation of WPI with MDA (1 mM) did not induce significant changes in the appearance and intensity of the protein bands. Other studies reported the fade or disappearance of native proteins occurred at high concentration of MDA (> 3 mM) (Chen et al., 2013; Niu et al., 2019).

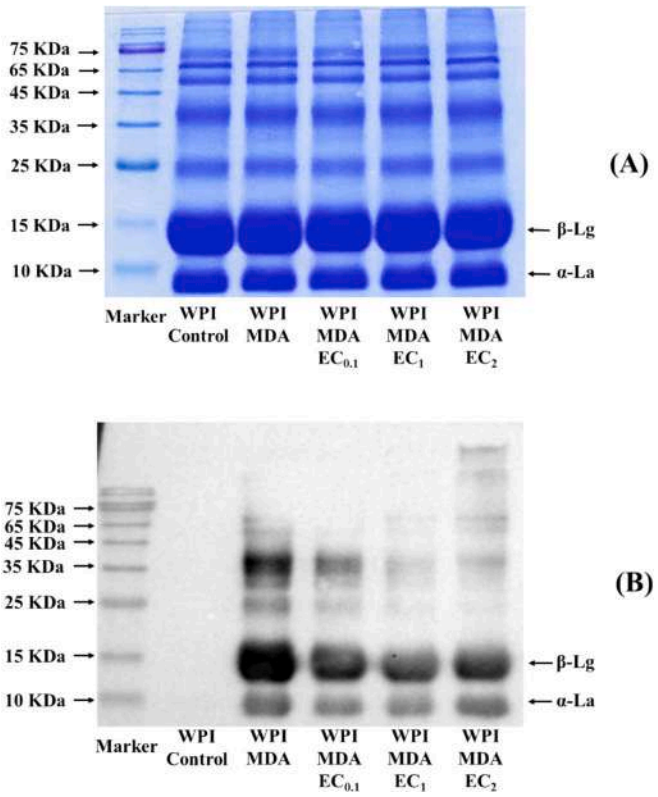

Fig. 4. Representative SDS-PAGE analysis of protein profile (A) and Western blot analysis of MDA-bound proteins (B) when WPI (6 mg/mL) was co-incubated with MDA (1 mM) and EC (0, 0.1, 1, and 2 mM) at 25 °C for 24 h.

The existence of MDA-bound protein was detected by an immunological technique specifically targeted at MDA-bound proteins. As shown in Fig. 4B, no MDA-bound protein was detected in native WPI. The treatment of MDA (1 mM) led to the existence of MDA-bound proteins which occurred mainly in β-Lg. MDA-induced protein covalent modification also appeared at higher molecular weights, indicating intermolecular protein cross-linking and protein aggregation. In the presence of EC (0.1, 1, and 2 mM), the immunoreactivity of the MDA-bound protein was significantly weakened. This is in agreement with the results of HPLC quantification on remaining MDA and protein

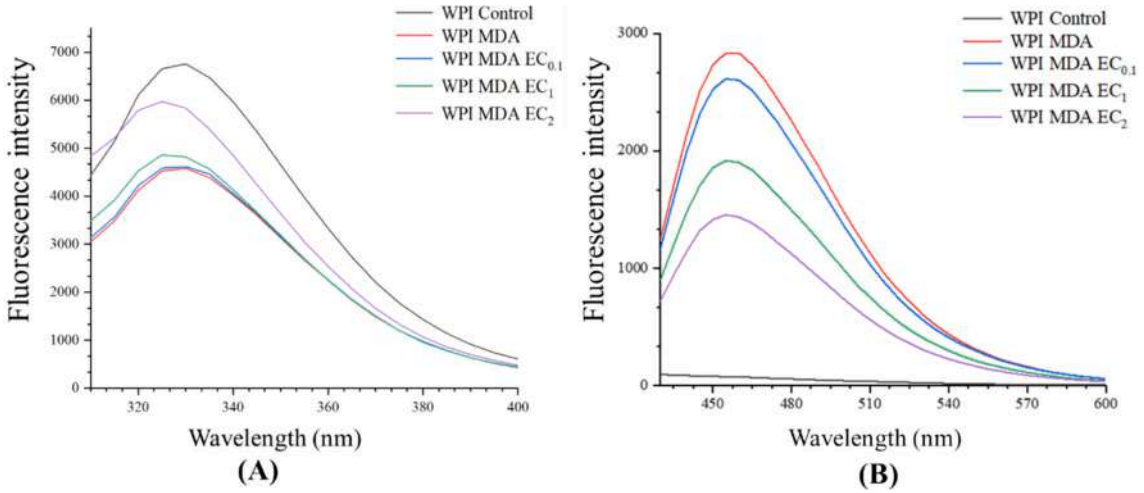

Fig. 3. Representative fluorescence emission spectra of intrinsic tryptophan (A) and Schiff base adducts (B) of WPI (6 mg/mL) during the co-incubation with MDA (1 mM) and EC (0, 0.1, 1, and 2 mM) at 25 °C for 24 h. The fluorescence of was excited at 295 nm and 375 nm respectively.

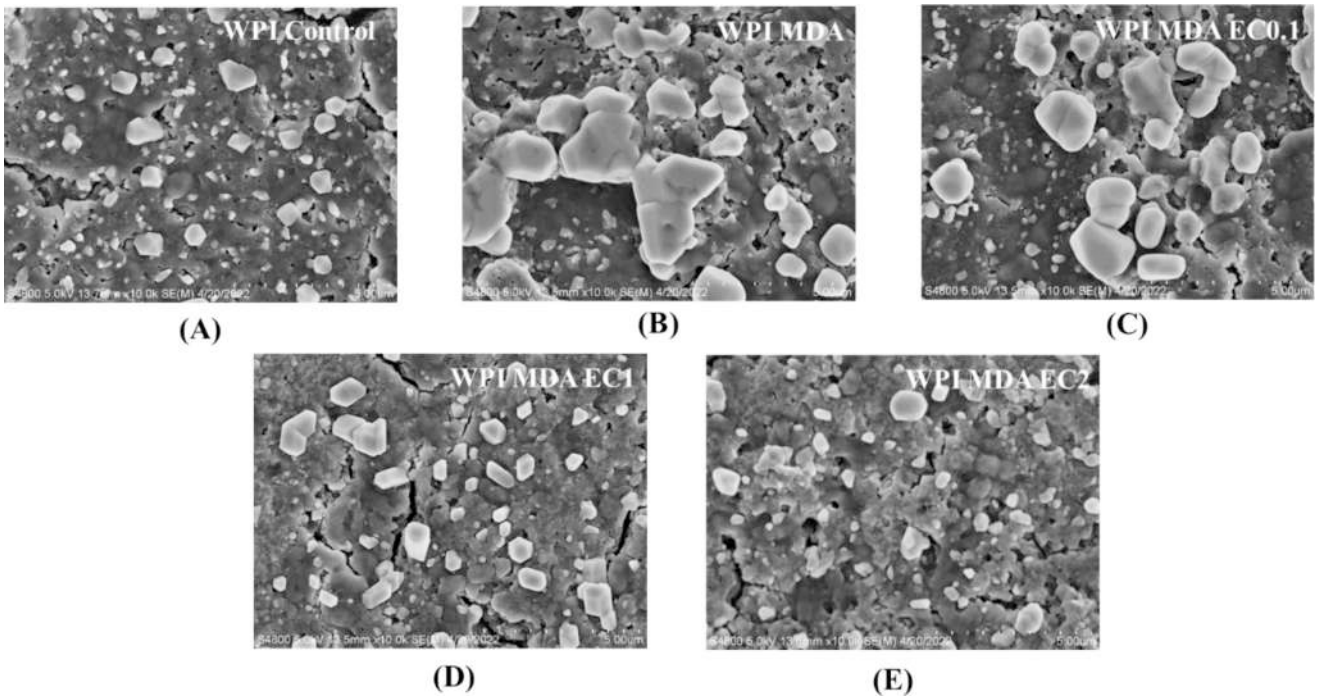

Fig. 5. SEM viewing (magnification 1: 10000) on protein microstructure when WPI (6 mg/mL) was co-incubated with MDA (1 mM) in the presence of EC (0, 0.1, 1, and 2 mM) at 25 °C for 24 h. A: WPI control; B: WPI + MDA; C: WPI + MDA + EC 0.1 mM; D: WPI + MDA + EC 1 mM; E: WPI + MDA + EC 2 mM.

carbonylation measurement, suggesting the potential of EC in interrupting MDA's covalent binding to proteins. In a recent study conducted in a cracker model, MDA and other short chain aldehydes were trapped by a *m*-diphenol olivetol, with decreased content of toxic aldehydes. Once generated, MDA is easily incorporated into other food ingredients such as fats, proteins, emulsifiers, and phenolics. The reaction rates of MDA towards different ingredients are influencing factors for the level and distribution of MDA in food matrixes (Vandemoortele, Babat, Yakubu, & De Meulenaer, 2017; Vandemoortele & De Meulenaer, 2015; Zamora et al., 2024). In this study, working as a sacrificial nucleophilic agent, the reaction rate of EC with electrophilic MDA may be faster than nucleophilic amino acid residuals in WPI, resulting in the mitigation of MDA-induced protein modification.

3.7. Protein microstructure

The changes in MDA-induced protein surface structural properties were clearly depicted by scanning electron micrographs. Native WPI exhibited small and uniform particles with clear and distinct boundaries (Fig. 5A). MDA-modification led to the disappearance of the boundary between WPI particles, with obvious cross-linked protein aggregates and large blocky particles without regular morphology (Fig. 5B). A low concentration of EC (0.1 mM) partially disrupted the protein cross-linking (Fig. 5C), and higher concentrations of EC (1 mM and 2 mM) minimized the tendency of protein aggregation and restored the protein morphology back to its original pattern (Fig. 5D, E). The morphology changes observed in SEM are consistent with the results of protein carbonylation and MDA-bound proteins, suggesting the role of EC in interrupting the binding of MDA to protein with subsequent protein cross-linking and aggregation. The result was also in agreement with our previous study, confirming the potential of EC in maintaining protein microstructure in an oxidizing environment (Lian et al., 2023).

3.8. Protein hydrolysis (DH)

Protein hydrolysis was expressed as the percentage of released α-amino groups during simulated gastric and intestinal digestion by the OPA method. As shown in Table 1, DH was slightly reduced ($P > 0.05$) during gastric digestion in the time points of 30 min and 60 min, indicating that MDA-induced protein lipoxidation had little effect on the susceptibility of WPI to proteolytic enzymes. A significant increase of DH was only observed in WPI co-incubation with 1 mM MDA and 2 mM EC in gastric digestion. During subsequent intestinal digestion, there are almost no significant differences in all the treatments, and a slight increase of DH was found with the addition of EC. The above results partially agreed with previous studies (Jiang, Zhang, Zhao, & Liu, 2018; Xu et al., 2020), suggesting the introduction of phenolic compounds into protein led to greater accessibility of the enzyme-binding sites with

Table 1
Protein hydrolysis (%) of WPI incubated with 1 mM MDA in the absence and presence of EC (0, 0.1, 1, and 2 mM). Different letters in the same column showed significant differences ($P < 0.05$), $n = 3$.

Samples	Time(min)				
	Gastric tract		Intestinal tract		
	30	60	90	120	180
Control WPI	6.38 ± 0.34 b	6.58 ± 0.26 b	10.81 ± 0.39 b	13.22 ± 1.03 a	15.43 ± 1.51 b
WPI + MDA	5.76 ± 0.16 b	6.24 ± 0.07 b	9.58 ± 1.11 a	11.43 ± 0.32 b	13.97 ± 1.02 a
WPI + MDA + $EC_{0.1}$	5.84 ± 0.34 b	6.32 ± 0.45 b	9.72 ± 1.09 a	11.44 ± 1.36 b	14.01 ± 0.67 a
WPI + MDA + EC_1	6.21 ± 0.21 b	6.74 ± 0.42 b	10.35 ± 1.02 a	12.47 ± 0.41 a, b	15.96 ± 1.11 a
WPI + MDA + EC_2	7.38 ± 0.66 a	7.92 ± 0.08 a	11.52 ± 1.13 a	14.08 ± 0.79 a	16.13 ± 0.96 a

improved DH in dairy matrices. Although it is generally accepted that the presence of polyphenols is likely to reduce protein DH because of the non-covalent and covalent phenol-protein interactions (Lund, 2021). On the other hand, phenol-protein interactions might also cause protein unfolding, with increased exposure of cleavage sites of the digestive enzymes, resulting in an improved DH (Cirkovic Velickovic & Stanic-Vucinic, 2018). The types and concentrations of oxidizing agents, polyphenols and protein substrates are influential factors to protein DH.

4. Conclusion

Many investigations on natural-occurring polyphenols have been concentrated on the B ring structure which is responsible for the antioxidant properties. On the other hand, in the past decade, the *meta*-position structure of A ring with high nucleophilic centers has been associated with direct scavenging of lipid oxidation-derived reactive carbonyl species (RCS). Not all phenolic compounds have RCS-trapping abilities due to the structural variation in A ring. In this study, EC-mediated MDA-phenol reactions might be faster than MDA-amine reactions, therefore contributing to the lessening of MDA's toxicity towards proteins. These findings provide new insights into the intervention strategy in food quality deterioration and health risks associated with unwanted lipid oxidation-derived RCS (especially MDA and HNE). In fact, the formation of RCS was accelerated by pepsin at low pH during gastrointestinal digestion (Bolea, Ginies, Vallier, & Dufour, 2019; Larsson, Harrysson, Havenaar, Alminger, & Undeland, 2016; Wang, Wu, Tu, & Xu, 2023). Hence, our future work will focus on the carbonyl-phenols interaction and the fate of reaction products during the digestive process.

CRediT authorship contribution statement

Wenhua Yao: Writing – original draft, Methodology, Investigation, Formal analysis. **Xingya Hao:** Writing – original draft, Methodology, Investigation, Formal analysis. **Zhangjie Hu:** Methodology, Investigation, Formal analysis. **Zhenghao Lian:** Validation, Methodology, Investigation. **Yue Cao:** Software, Investigation. **Rong Liu:** Software, Methodology, Investigation. **Xiaoying Niu:** Methodology, Formal analysis. **Jun Xu:** Writing – review & editing, Resources, Project administration. **Qin Zhu:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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