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Ferulic acid alleviates the reduction of beef myofibrillar protein digestibility mediated by protein oxidation: insight from peptide release

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ABSTRACT

This study investigated the protective effect of ferulic acid (FA) on the digestibility of oxidized beef myofibrillar protein (MP). MP were treated with varying concentrations of FA (20, 40, and 80 μmol/g pro) and then exposed to oxidation. The results showed that FA inhibited carbonyl in a dose-dependent manner. Fluorescence spectroscopy and molecular docking studies revealed FA was bonded to MP *via* hydrophobic interactions and hydrogen bonds. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and transmission electron microscope analysis indicated that FA mitigated the aggregation of oxidized MP. Peptidomic analysis showed FA protected 34.24% peptides recover from oxidation loss, and theses recovered peptide were mainly distributed in hydrophobic regions and lysine sites. *In vitro* digestion showed FA remarkably improved digestibility of oxidized MP ($P < 0.05$), with 80 μmol/g pro FA mitigating 67.85% loss in digestibility. Overall, FA effectively preserved the release of peptides by inhibiting hydrophobic aggregation and oxidation of lysine residues, thereby alleviating the decrease in digestibility of oxidized MP.

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1. Introduction

Protein oxidation encompasses covalent modifications triggered by reactive free radicals, such as reactive oxygen and nitrogen species, and those indirectly mediated by lipid oxidation and myoglobin[1]. Animal-derived muscle serves as a crucial supplier of exceptional dietary protein for human consumption, and it is also rich in precursors or catalysts for the formation of reactive oxygen species (such as heme iron, transition metals, unsaturated fatty acids, and oxidases), which play a significant role in the process of protein oxidation[2]. Therefore, protein oxidation is a common phenomenon in the processing and storage of meat products.

Protein oxidation can cause irreversible damage to its structure, such as intramolecular and intermolecular crosslinking, amino acid side chain modifications, and peptide backbone fragmentation[1]. Digestibility is an important indicator for evaluating the nutritional value of dietary proteins, as proteins can only be absorbed by the small intestine after being digested into small peptides or amino acids. It is generally believed that moderate oxidation can enhance protein digestibility. However, excessive protein oxidation can reduce the digestibility of meat proteins by decreasing the accessibility of digestive enzymes to protein cleavage sites and shielding the digestion sites. In this regard, the reports on protein oxidation reducing the digestibility of dietary proteins have been widely documented in egg protein[3], beef protein[4], and fish protein[5]. The reduction of digestibility caused by protein oxidation is a waste of dietary protein resources. Moreover, undigested oxidized proteins reaching the colon are fermented by gut microbiota, potentially releasing

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toxic substances[6]. Therefore, it is necessary to take corresponding intervention measures to mitigate the adverse effects of protein oxidation on the digestibility of dietary proteins.

Ferulic acid (FA) is a phenolic acid derived from cinnamic acid isolated from natural plants. FA possesses diverse bioactive functions. Firstly, it exhibits potent antioxidant capabilities through scavenging free radicals, chelating metal ions, and regulating the activity of redox enzymes[7]. Additionally, FA demonstrates anti-inflammatory, antibacterial, and antitumor effects, providing adjunctive therapy for cardiovascular diseases, neurological disorders, and liver diseases[8]. FA is capable of interacting with proteins *via* both covalent and non-covalent, thereby enhancing the functional properties of proteins. Wang et al.[9] pointed out the appropriate concentration of FA could improve the emulsifying properties and antioxidant activities of rice bran protein hydrolysates. Yu et al.[10] reported FA effectively mitigated the accumulation of advanced glycation end-products during the thermal processing of food. The interactions between FA and protein, such as *β*-lactoglobulin, soy protein, have been widely reported[11-12]. However, there is few research about the effects of FA pertaining to the digestibility characteristics of oxidized meat proteins.

Therefore, the objective of study was to evaluate the protective effect of FA on the digestibility of oxidized myofibrillar proteins (MP). Molecular docking and peptidomics were employed to elucidate the potential mechanisms underlying the impact exerted by FA on the digestibility of MP mediated by protein oxidation. The findings of this study can broaden our knowledge on the antioxidant properties of polyphenols and meat proteins, meanwhile providing technical solutions to enhance the nutritional and safety levels of protein.

2. Materials and methods

2.1 Materials

Pepsin from porcine stomach mucosa, and trypsin from porcine pancreas, were purchased from Sigma (Shanghai, China). The *α*-chymotrypsin from bovine pancreas was from Beijing Solaibao Technology Co., Ltd. (Beijing, China). All other chemicals were of analytical grade chemicals, which were obtained from Nanjing Maibo Reagent Co., Ltd. (Nanjing, China).

Beef was procured from Henan Hengdu Food Co., Ltd., situated in Zhumadian, China. The slaughter process of cattle was performed in accordance with the Guide for cattle slaughtering (China, GB/T 19477−2018). After a post-mortem period of 48 h under 4 °C, semitendinosus muscle with a pH range of 5.5−6.0 was excised from the carcass. All samples were transported in a cooler containing ice to the laboratory for analyzing within 12 h.

2.2 Preparation of MP

The extraction of MP followed our previously established protocol[4]. The prepared MP was then dissolved in a solution of PBS (0.6 mol/L NaCl, 20 mmol/L K_2HPO_4/KH_2PO_4). This solution was saved at 4 °C and utilized within 48 h. To determine the protein concentration, a BCA kit sourced from Thermo Scientific was employed.

2.3 Incubation of MP with FA and oxidation treatment

FA was first dissolved in PBS (0.6 mol/L NaCl, 20 mmol/L K_2HPO_4/KH_2PO_4) to obtain storage solution (10 mmol/L). Then, took out appropriate amount of storage solution and mixed with MP solutions (10 mg/mL) to ensure the final concentration of FA is 20, 40, and 80 μmol/g pro. The selection of such concentration was based on our pre-experiments and previously studies[7,13]. Subsequently, the combined MP and FA solutions were oxidized according to the protocol outlined by Cao et al.[13]. Briefly, the oxidation was carried out for 24 h at 4 °C within a hydroxyl radical generating system, which comprised of 10 μmol/L $FeCl_3$, 0.1 mmol/L ascorbic acid, and 5 mmol/L H_2O_2. The oxidation reaction was terminated by adding Trolox (1 mmol/L, final concentration). The protein solution without FA and oxidant but containing Trolox was used as the non-oxidized control, and noted as control check (CK) group. The protein solution with oxidant but without FA was noted as oxidation (OX) group. The protein solution with oxidant and 20, 40 and 80 μmol/g pro FA was noted as OF_{20}, OF_{40} and OF_{80} group, respectively. The samples of the CK, OF_{20}, OF_{40}, and OF_{80} groups were equally divided into 2 parts. One half of the sample was freeze-dried using a vacuum freeze dryer (Alpha 2-4 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), and used for *in vitro* simulated digestion; while the other half was used for testing physicochemical indicators.

2.4 Carbonyl content

Carbonyl content was determined utilizing the methodology outlined by Xiao et al.[14]. Briefly, 5 mL of samples (concentrated at 5 mg/mL) were combined with an equal volume of 10 mmol/L 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2 mol/L HCl. This mixture was incubated under dark conditions at 25 °C for 1 h. After incubation, the mixture was added 5 mL of 24% trichloroacetic acid to precipitate protein and then centrifugated at 12 000 × *g* and 4 °C for 10 min. The precipitated samples underwent 5 washes using a wash solution composed of ethyl acetate and ethanol with a 1:1 volume ratio. After the final wash, the sediment was diffused in 2.5 mL of 6 mol/L guanidine hydrochloride. After that, centrifugation was performed at 8 000 × *g* for 10 min. The absorbance of the supernatant was recorded at 370 nm with a microplate reader (SpectraMax M2, Molecular Devices Ltd., Sunnyvale, CA, USA). Carbonyl levels were calculated with the molar absorption coefficient of 22 000 L/(mol·cm).

2.5 Sulfhydryl content

The sulfhydryl content was assessed following the protocol described by Feng et al.[15]. Briefly, 1 mL of sample (1 mg/mL) was combined with 10 mL of a buffer solution (pH 6.0) containing 20 mmol/L Tris-HCl, 10 mmol/L ethylene diamine tetraacetic acid, and 8 mmol/L urea. Then, 200 μL of 10 mmol/L DTNB solutions were added to the mixture. The resulting solution was incubated under dark at 25 °C for 30 min and subsequently centrifuged (10 000 × *g*, 10 min, 4 °C). The absorbance of the supernatant was quantified with a microplate reader at a wavelength of 412 nm. To calculate the sulfhydryl content, a molar absorption coefficient of 13 600 L/(mol·cm) was utilized.

2.6 Surface hydrophobicity (S_0)

The S_0 was measured following the protocol described by Chen et al.[16]. Shortly, 6 mL of samples (1 mg/mL) were combined with 30 μL of 15 mmol/L sodium 8-aniline-1-naphthalenesulfonate (ANS). The mixtures were then packed in tinfoil and incubated under dark (25 °C, 30 min). Fluorescence measurements were subsequently taken with a microplate reader, with excitation and emission wavelengths set at 380 nm and 410−570 nm, respectively.

2.7 SDS-PAGE

The 2 mL of sample (2 mg/mL) was combined with 2 mL of a loading buffer (pH 6.8) composed of 0.125 mol/L Tris, 20% glycerol and 4% sodium dodecyl sulfate. Subsequently, 5 μL of marker or 6 μL samples were loaded onto a 4%−10% gradient gel. Electrophoresis was performed initially at 70 V for 30 min, followed by 110 V for 80 min. After staining and decolorization procedures, the gel image was captured with a gel imaging system (Bio-Rad).

2.8 Transmission electron microscope (TEM)

The TEM observation was followed the method described by Zhu et al.[17]. Initially, the samples were diluted to a concentration of 10 μg/mL using 10 mmol/L PBS. Subsequently, 5 μL of the diluted samples were deposited onto a 200-mesh copper grid. These grids were then observed under a TEM (H-7650, Hitachi Corporation, Tokyo, Japan) operating at an accelerating voltage of 180 kV. The image scale bar was set to 100 nm, and the samples were visualized at a magnification of 25 000×.

2.9 Fluorescence spectroscopy

Varying quantities of FA were mixed with 5 mL of MP solution to get the concentration of FA to 0, 10, 20, 40, 60, 80, 100 μmol/g pro. The mixtures were maintained at room temperature (25 °C) for 10 min. Fluorescence spectroscopy measurements were conducted with an F7000 spectrofluorometer (Hitachi, Tokyo, Japan). The excitation wavelength was set at 280 nm, and the emission spectra was scanned across a range of 300−500 nm.

In order to ascertain the nature of quenching, the Stern-Volmer equation was employed[18]. This equation is expressed as follow:

$$F_0/F = 1 + K_q\,\tau_0[Q] = 1 + K_{sv}\,[Q] \quad (1)$$

Here, F_0 represents the fluorescence intensity of the protein solution without FA, while F corresponds to the intensity with FA. Additionally, [Q] denotes the concentration of FA, K_q signifies the rate constant for a bimolecular quenching process, and K_{sv} represents the quenching constant. The τ_0 represents the lifetime of the phosphor in the absence of a quencher, and the average life expectancy of macromolecules is approximately 10^{-8} s[18].

2.10 Molecular docking

The molecular docking was conducted using Discovery Studio 3.0 to foretell the potential interactions between FA and myosin. The three-dimensional (3D) structures of FA molecules were obtained from the Discovery Studio software repository. Additionally, the crystalline structures of myosin were retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/). Prior to docking, the myosin crystal structure was modified by removing all water molecules and appending hydrogen atoms. Both FA and myosin were subjected to energy minimization using the CHARMM force-field. During the docking process, a pose cluster radius of 0.5 was employed. The most favorable docking pose was determined based on the highest negative CDOCKER energy. Furthermore, the types of interactions between the FA and myosin were identified, and the docking outputs were visually rendered using appropriate software to generate comprehensible combination models.

2.11 Simulation of in vitro digestion

The *in vitro* digestion was followed the study of Minekus et al.[19]. The formula of gastrointestinal digestive fluid was as follows. Simulated gastric fluid (SGF, pH of 3.0) included 6.9 mmol/L KCl, 25 mmol/L $NaHCO_3$, 47.2 mmol/L NaCl, 0.9 mmol/L KH_2PO_4, 0.5 mmol/L $(NH_4)_2CO_3$, and 0.1 mmol/L $MgCl_2{\cdot}(H_2O)_6$. Simulated intestinal fluid (SIF, pH 7.0) included 38.4 mmol/L NaCl, 6.8 mmol/L KCl, 85 mmol/L $NaHCO_3$, 0.33 mmol/L $MgCl_2{\cdot}(H_2O)_6$, 0.8 mmol/L KH_2PO_4, and 8.4 mmol/L HCl. The 60 mg of freeze-dried samples were mixed with 12 mL of SGF. Subsequently, pepsin was added to the mixture at a concentration of 2 000 U/mL, and the mixture was agitated for 120 min (150 r/min, 37 °C). After that, the mixture was combined with 12 mL SIF. Trypsin and α-chymosin were then added to the mixture, adjusting their enzymatic activities to 100 and 25 U/mL, respectively. The mixture was further incubated at 37 °C, with agitation at 150 r/min for another 120 min. The 2 mL digestive products were withdrawn to evaluate gastrointestinal digestibility.

2.12 Protein digestibility

To assess protein digestibility, the degree of hydrolysis (DH) was measured following our prior research methodology[20]. Briefly, a volume of 100 μL of the digest was mixed with 100 μL 24% TCA to precipitate proteins. Subsequently, centrifugation was performed at 4 000 × g for 20 min at 4 °C. Then, 30 μL of either the supernatants or a standard *L*-leucine solutions ranging from 0.05 to 3 mmol/L were combined with 900 μL of 0.1 mol/L sodium tetraborate and 300 μL fluorescamine acetone solution (0.2 mg/mL). Fluorescence was quantified using a microplate reader, with excitation and emission wavelengths set at 390 and 480 nm, respectively. The DH was calculated using the following formula:

$$\mathrm{DH}\ (\%) = \frac{[-NH_2\ (h)] - [-NH_2\ (0)]}{[-NH_2\ (\infty)] - [-NH_2\ (0)]} \times 100 \quad (2)$$

Here, [$-NH_2$ (h)] indicates the concentration of primary amines post-digestion, [$-NH_2$ (0)] denotes the concentration prior to digestion, and [$-NH_2$ (∞)] signifies the concentration of primary amines subsequent to total hydrolysis achieved by exposure to 6 mol/L HCl at 100 °C for 48 h.

2.13 Peptidomics

The peptide profiles in the digested products were characterized through liquid chromatography tandem mass spectrometry (LC-MS/MS). Prior to the analytical procedure, a 100 μL aliquot of the sample was mixed with an equivalent volume of 0.2% formic acid solution. The mixture was subsequently shifted to a 0.5 mL ultrafilter tube with a 3 kDa cutoff membrane. Following centrifugation at 14 000 × *g* for 15 min at 4 °C, incompletely hydrolyzed macromolecules were effectively removed. For the separation of peptide samples, a liquid chromatography system equipped with an RP-C_{18} column (4.6 mm × 150 mm, 5 μm) was employed. The mobile phase A comprised 0.1% formic acid in water, while mobile phase B consisted of a mixture of 90% acetonitrile and 0.1% formic acid. A gradient elution program was designed as follows: initially, 2% of eluent B was used for 0−5 min; this was gradually increased to 40% over the next 80 min (5−85 min); subsequently, the eluent B concentration was raised to 80% for 20 min (85−105 min); finally, the concentration of eluent B was reduced back to 2% for the remaining 5 min (105−110 min). The identification of peptides was achieved using a Q Exactive mass spectrometer. The MS scans were acquired within the *m/z* range of 350−1 800, while the MS/MS scans were captured between *m/z* 150 and 1 800. The acquired mass spectrometry data were subsequently analyzed using Proteome Discover 1.4 software (Thermo Fisher Scientific). The search terms were established in the following manner: a mass tolerance of 20 × 10^{-6}, no specific protease selectivity, allowing for a maximum of 2 missed cleavages, and a false discovery rate threshold of 1%, and MS/MS tolerance was set at 0.1 Da.

2.14 Statistical analysis

Three separate replications were conducted, and the results are presented as mean values accompanied by their corresponding standard deviations (mean ± SD). For statistical analysis, the Duncan multiple range test was utilized within the SPSS 22.0 software package (SPSS, Chicago, IL, USA), and a *P*-value less than 0.05 was considered to indicate a statistically significant difference.

3. Results and discussion

3.1 Carbonyl value

Carbonyl group is an important indicator reflecting protein oxidation. As shown in Fig. 1A, consistent with previous reports, the carbonyl value of MP in the CK group was 0.94 nmol/mg pro[21]. Following oxidative treatment, the carbonyl value of MP increased to 3.83 nmol/mg pro, representing a 3.08-fold increase compared to the CK group, indicating the successful establishment of *in vitro* oxidation system. Under the hydroxyl radical oxidation system, the amino acid side chains containing NH or ε-NH_2 groups in proteins were tending to be attacked by free radicals, thus leading to the formation of carbonyl[22]. Additionally, the breakage of peptide chains during oxidation also contributed to the elevation of carbonyl values. Compared to the OX group, FA treatment markedly inhibited the generation of carbonyl in MP, and the carbonyl content progressively decreased as the concentration of FA increased. Specifically, the carbonyl content decreased to 1.63 nmol/mg pro in the OF_{80} group. The inhibitory effect of FA on MP oxidation might attribute to its strong abilities in scavenging free radicals and chelating metal ions. Our results align with a previous research recording that FA inhibited the protein oxidation of yak meat[23].

3.2 Sulfhydryl

Owing to the presence of sulfur containing amino acids, the sulfhydryl groups in proteins are susceptible to oxidation into sulfinic acid, sulfenic acid, and sulfonic acid, and disulfide bonds under hydroxyl radical oxidation system[21]. Therefore, changes in sulfhydryl groups in proteins are often employed to assess the level of protein oxidation. In this study, the sulfhydryl content in the CK group was 53.89 nmol/mg pro (Fig. 1B). Oxidative treatment led to a decrease in sulfhydryl groups to 27.63 nmol/mg pro, representing a 48.73% reduction compared to the CK group. Compared to the CK group, treatment with 20 μmol/g pro FA significantly inhibited the loss of sulfhydryl groups ($P < 0.05$). This phenomenon can be attributed to the antioxidant capacity of FA. However, the sulfhydryl content in the OF_{40} and OF_{80} groups were lower than that in the OF_{20} group ($P < 0.05$). This phenomenon might due to that FA formed C−S bonds with the sulfhydryl groups of cysteine, leading to a reduction in sulfhydryl content[9].

3.3 Surface hydrophobicity (S_0)

Hydrophobic amino acids are typically sequestered within the interior of proteins, and S_0 is commonly employed as a metric for characterizing pro conformations[16]. As shown in Fig. 1C, the fluorescence value of S_0 in the CK group was 9 200, and oxidation

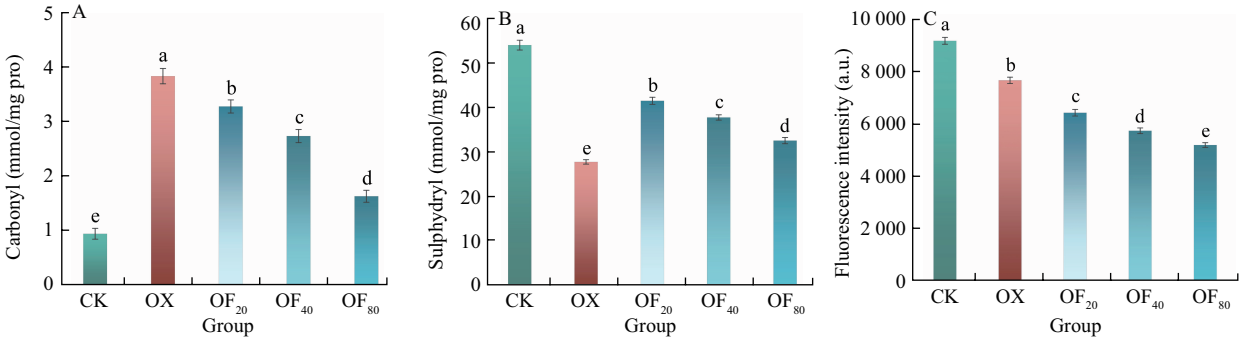

Fig. 1 Carbonyl (A), sulfhydryl (B), and surface hydrophobicity (C) of MP mediated by protein oxidation in the absence or presence of FA. Symbols (a, b, c, d, e) designate statistically significant differences at a level of $P < 0.05$.

treatment significantly reduced the S_0 of MP. The changes in S_0 are known well to be associated with the equilibrium between protein unfolding and aggregation[16]. The decreased S_0 in OX group suggested that the oxidation resulted in protein aggregation, which limited the accessibility of hydrophobic amino acids to ANS. It was suggested that the carbonyl groups formed in amino acid side chains was equivalent to introduction of hydrophilic groups, which might destroy the balance between hydrophilicity and hydrophobicity of protein, and thus leading to the protein aggregation[24]. In addition, the establishment of covalent bonds, including disulfide bonds and carbonyl-ammonia linkages during oxidation could also contribute to protein aggregation[25]. Compared with OX group, the fluorescence value of FA treatment group gradually decreased ($P < 0.05$), indicating that the addition of FA further reduced the surface hydrophobicity of MP. Our results are consistent with the report by Cao et al.[26], who reported that catechin led to the decrease in S_0 of oxidized pork MP. This phenomenon might be due to the binding of FA through both covalent and non-covalent interactions with the active functional groups present in MP, and therefore hindered the contact of ANS with hydrophobic amino acids.

3.4 SDS-PAGE and TEM

SDS-PAGE was performed to observe the changes in protein profiles. As shown in Fig. 2A, myosin heavy chain (MHC) and actin, the characteristic proteins of MP, can be clearly seen in the CK group. After oxidation treatment, the bands of MHC and actin were obviously lighter. Meanwhile, there was band appeared on the top of the separation gel in the OX group. This phenomenon could be explained by the fact that oxidation caused MP to form aggregates. In OF_{20} group, the lost MHC and actin were effectively recovered, and this phenomenon was further exacerbated in OF_{40} and OF_{80} group, indicating that FA could effectively alleviate the aggregation caused by protein oxidation. TEM was performed to further observe aggregation status in the different groups (Fig. 2B). As shown by the red arrows, the CK sample exhibited small-sized, dispersed spherical particles, and large pores were observed among particles. Upon oxidation treatment, the samples displayed aggregation, characterized by the presence of clumpy shadows, a mountain-like appearance. In the FA groups, the clumpy proteins were broken into branch-like appearance, and the size showed a decreasing trend with the increase of FA concentration. These observations align with the findings obtained from SDS-PAGE analysis (Fig. 2A), providing strong evidence that FA inhibited MP aggregation mediated by protein oxidation. The anti-aggregation effect of FA can be attributed to its antioxidant effect. As mentioned above, FA could eliminate free radicals and chelate metal ions, thereby inhibiting the formation of carbonyl-amine bonds and disulfide bonds during the oxidation process. Similarly, Li et al.[23] reported that FA inhibited the aggregation of yak meat MP caused by oxidation. However, Cao et al.[26] reported that the addition of catechin led to further aggregation of oxidized pork MP. The differences between these reports might be related to the degree of protein oxidation and the differences in phenolic structures. For example, catechin contains 6 hydroxyl groups. After providing electrons and hydrogen atoms, catechin is oxidized to the corresponding quinone substances, which can act as crosslinking agents to cause protein aggregation[13]. While, FA contains 1 hydroxyl group. As an antioxidant, FA was oxidized to the corresponding semiquinone substances, which usually did not cause cross-linking aggregation[27].

3.5 Fluorescence spectroscopy

Intrinsic fluorescence, a metric for assessing protein conformational state, exhibits sensitivity to the polarity of the tryptophan residue microenvironment. Therefore, the fluorometric analysis was performed to evaluate the FA-MP interactions. As shown in Fig. 3A, with the addition of FA, the fluorescence index of MP gradually decreased. Such phenomenon might be due to non-covalent binding between FA and tryptophan, leading to

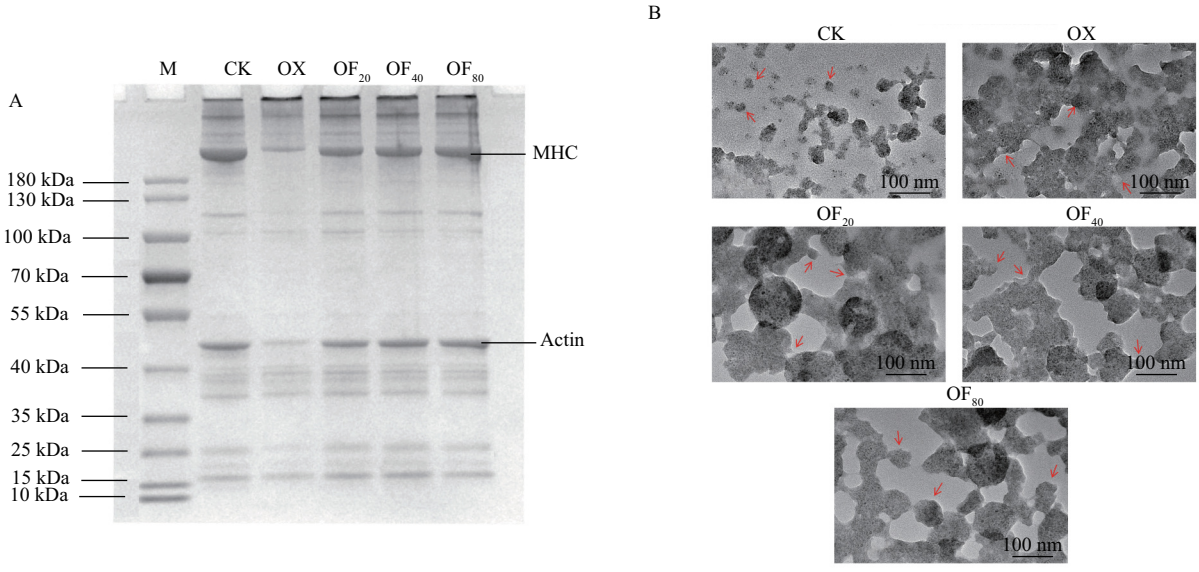

Fig. 2 SDS-PAGE (A), TEM (B) analysis of MP mediated by protein oxidation in the absence or presence of FA.

endogenous fluorescence quenching[11]. In addition, an observable red shift in the intense emission band was noted, indicating that the tryptophan residue came more exposed to the polar environment[27]. This observation concurs well with previous reports regarding the binding of FA to human serum albumin[8], and rice bran protein[9]. Fig. 3B presents the linear regression plot of F_0/F versus [Q]. As is known to us, the fluorescence quenching mechanism in proteins is classified into 2 primary categories: static quenching and dynamic quenching. Dynamic quenching entails collisional interactions between the fluorophore and quencher, whereas static quenching refers to the formation of a non-fluorescent complex between the quencher and the fluorophore. On the basis of results, the quenching constant K_q was 1.025×10^{12} L/(mol·s). Generally, the maximum diffusion collision quenching rate constant of biomacromolecules is approximately 2.0×10^{10} L/(mol·s) in the context of dynamic quenching[18]. In this study, the K_q value of FA and MP was much higher than the value of biomacromolecules, indicating that the fluorescence quenching observed in MP was attributed to a static quenching.

3.6 Molecular docking

Molecular docking serves as a powerful instrument for predicting the affinity and binding mode of proteins and ligands. Given that myosin is the main component of MP, molecular docking was used to further identify and visualize the preferred binding sites of FA on myosin. Fig. 4A displays the crystal structures of myosin and the structural formula of FA. Based on the lowest energy, the top predicted docking conformation for myosin-FA interactions was displayed in Fig. 4B. The binding energy between FA and myosin was −6.6 kcal/mol, indicating a strong binding interaction. As shown in Fig. 4C, FA was found to have hydrophobic interactions with Ile_{478}, Phe_{244}, Leu_{267}, Glu_{474}, and Ser_{242} in the head of myosin. As is known to us, Ile, Phe, Leu, Glu are non-polar amino acids, containing hydrophobic side chains. FA is a phenolic acid compound with the benzene ring being it's the dominant hydrophobic contributor. According to the hydrophobicity/hydrophilicity analysis by ExPASy ProtScale database, the hydrophobicity of the myosin head is much higher than that of the tail. Thus, it seems reasonable that FA bound to the head of myosin by hydrophobic interactions. According to Lu et al.[28], the head of myosin was prone to oxidation in the hydroxyl radical oxidation system. Therefore, the binding of FA with the head of myosin might contribute to exerting its antioxidant effects. In addition to hydrophobic interactions, FA could also bind to myosin by hydrogen bonds. The results showed that the carbonyl carboxyl group of FA can form a hydrogen bond with Ser_{180} and 2 hydrogen bonds with Arg_{243}; the hydroxyl group of FA can form one hydrogen bond with Gly_{245}. Our results agree with previous study, which reported that FA can be hydrogen-bonded to β-lactoglobulin[11]. Together, FA bound with the hydrophobic pocket of myosin through hydrogen bonding and hydrophobic interactions (Fig. 4D), which contributed to improving the stability of FA-myosin complexes. Such combination could help to explain the less aggregation in the FA treated groups than that observed in the OX group after suffer oxidation (Fig. 2).

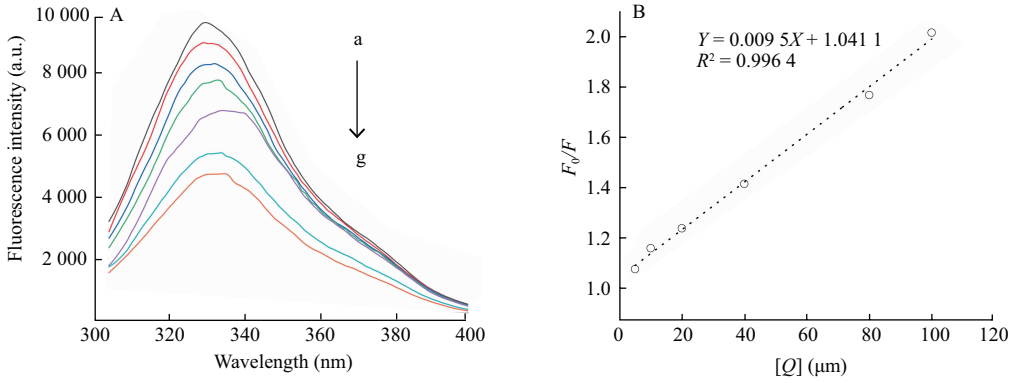

Fig. 3 Fluorescence spectra of MP treated with different concentrations of FA (A), a→g: 0, 10, 20, 40, 60, 80, 100 μmol/g pro. Stern–Volmer plots for the quenching of MP by FA (B).

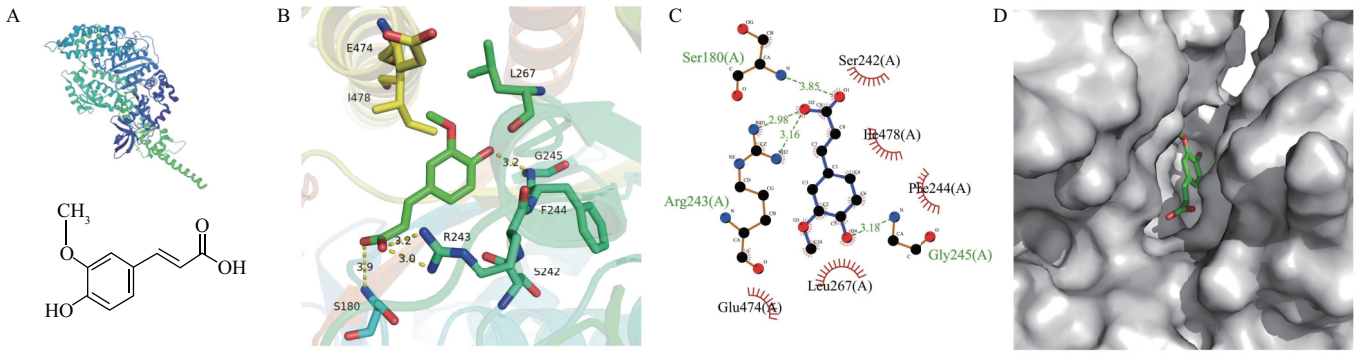

Fig. 4 Molecular docking analysis of myosin and FA. (A) Schematic diagram of myosin and FA, (B) interaction mode between myosin and FA in 3D display, (C) interaction mode between myosin and FA in 2D display, (D) the interaction mode between myosin and FA in surface form diagram.

3.7 Protein digestion

Protein digestibility serves as a pivotal metric for assessing the nutritional worth of dietary proteins, while DH represents a frequently employed tool for characterizing protein digestibility[20]. As shown in Fig. 5, the DH in CK group was 45.29%, protein oxidation reduced the DH to 29.63% ($P < 0.05$). It was suggested that protein oxidation may lead to crosslinking and aggregation of MP, which could hinder the accessibility of digestive enzymes to the hydrolysis sites of MP, resulting in decreased digestibility[29]. As expected, FA effectively inhibited the loss of digestibility caused by protein oxidation in a dose-dependent manner. Such protective effect of FA on the digestibility of MP might attribute to its anti-aggregation function. The results of SDS-PAGE and TEM can support this inference (Fig. 2). Exposure of sulfhydryl groups within a protein renders them susceptible to oxidation, resulting in the formation of disulfide bonds that causes an increase in protein aggregation[30]. It was suggested that FA could form C−S bonds with the sulfhydryl groups of cysteine, which contributed to reducing the formation of disulfide bonds and thereby inhibited protein aggregation[9]. Besides, oxidation caused the unfolding of hydrophobic amino acids, which increased hydrophobic interactions between amino acids and could also lead to MP aggregation. FA is a small phenolic molecule. The hydrophobic group (−OCH_3) of FA could interact with the hydrophobic amino acids of MP, which could contribute to alleviate the aggregation caused by the increase of hydrophobic interaction[17].

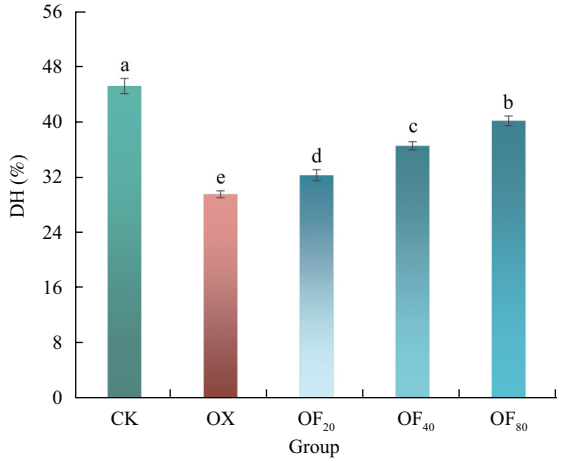

Fig. 5 DH of MP mediated by protein oxidation in the absence or presence of FA. Symbols (a, b, c, d, e) designate statistically significant differences at a level of $P < 0.05$.

3.8 Peptidomics

Peptidomics is a powerful tool for analyzing protein digestion products[31-32]. In order to further explore the influence that FA alleviated the reduction of MP digestibility caused by protein oxidation, peptidomics was employed for identifying peptides in digestion products. As shown in Table 1, a total of 1 495 peptides were identified in the CK group, while the number of peptides decreased to 1 127 in the OX group. This result was coincided with that ozone induced excessive oxidation reduced peptide release of myosin in silver carp[33]. This phenomenon might due to cross-linking caused by protein oxidation, which limited the accessibility of MP hydrolysis sites to digestive enzymes, resulting in the loss of peptide release in protein aggregation region[29]. In the OF_{80} group, the number of peptides recovered to 1 252, suggesting that FA effectively mitigated the reduction of peptide release caused by protein oxidation. Such phenomenon could be attributed to the antioxidant effect of FA, which reduced MP aggregation caused by protein oxidation. Additionally, there were remarkable differences in peptide composition among CK, OX, and OF_{80} group. Specifically, compared to the CK group, the OX group lost 595 peptides and gained 227 new peptides (Fig. 6A). Meanwhile, compared to the OX group, the OF_{80} group lost 244 peptides and gained 369 new peptides (Fig. 6B). According to secondary enzyme-substrate interaction, the modifications of residues that proximal to protease cleavage sites will greatly influence the hydrolysis kinetics of proteases[32]. Therefore, the oxidation of amino acids near potential cleavage sites of might change the action preference and hydrolysis kinetics of digestive enzymes, leading discrepancies in the digested peptides[33].

Table 1
Quantity of peptides in digestion products across various groups.

Group	Number of peptide		
	Total	Missing	New
CK	1 495	-	-
OX	1 127	595	227
OF_{80}	1 252	511	268

Note: Missing peptides denote the peptide that reduced in comparison to the CK group, while new peptides refer to the peptides that added in comparison with the CK group.

To further explore the mitigation of FA on the peptide release of oxidized MP, we matched the identified peptides with their mother proteins. Results showed that myosin released the highest quantity of peptides among the proteins constituting MP, with 668, 446, and 509 peptides identified in the CK, OX, and OF_{80} treatment group, respectively (Fig. 6C). This result was consistent with the findings of Zhao et al.[34], who reported that the peptides identified in chicken myofibrillar digests primarily originated from myosin. This is also in line with the fact that myosin is the predominant protein in MP. Myosin, a contractile protein situated within the thick filaments of the myofibrillar segment of muscle, comprises 6 subunits including 2 MHC and 2 pairs of myosin light chains (MLC). Notably, MHC exhibits several isoforms, each characterized by distinct properties that contribute to defining specific muscle attributes. In the present study, we identified three isoforms of MHC: MHC-1 (Q9BE40), MHC-2 (F1MRC2) and MHC-7 (F1MM07). Among these 3 isoforms, compared to the CK group, protein oxidation reduced the release of peptides by 25.59%, 43.33%, and 43.63% in MHC-1, MHC-2, and MHC-7, respectively (Fig. 6C). Such variations might be attributed to their differences in amino acid composition and oxidative sensitivity[28,35]. As is known to us, MHC is rich in oxidative sensitive amino acids such as tyrosine, cysteine, and lysine[29]. The cross-linking caused by these oxidation sensitive amino acids would reduce the peptide release of MHC during digestion[33]. Compared to the OX group, OF_{80} increased the release of peptides by 4.21%, 39.71%, and 25.81% in MHC-1, MHC-2, and MHC-7, respectively. This phenomenon might be due to the complex formed by MHC and FA helping to resist aggregation caused by protein oxidation, thereby protecting the release of peptides (Fig. 4)

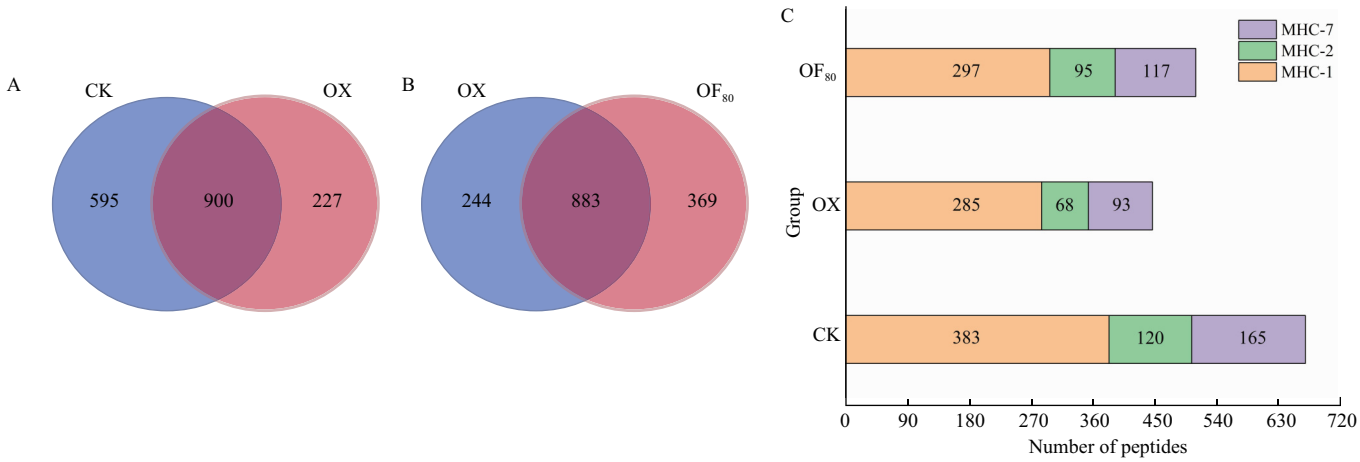

Fig. 6 Venn diagram analysis of peptide composition in MP digestive products. (A) CK group *vs*. OX group, (B) OX group *vs*. OF_{80} group; (C) number of peptide released from myosin.

Myosin constitutes the primary protein component in MP, and the peptide release from MHC-2 is largely influenced by both protein oxidation and FA. Therefore, the peptides released from MHC-2 were mapped to further analyses the mitigation of FA in peptides release of myosin mediated by protein oxidation. As depicted in Fig. 7, MHC-2 consists of 1 940 amino acids, composed with the head region (amino acids 1−842) and the tail region (amino acids 843−1 940). In total, compared with CK group, there were 53 peptides missing in OX group (marked with red lines, Fig. 7), and 29 miss peptides were recovered in OF_{80} group (marked with green lines, Fig. 7). Interestingly, as mapped in Fig. 7, FA restored 16 peptides from the head region of MHC-2, accounting for 55.17% of the total recovered peptides. Moreover, most of these recovered peptides were located in the hydrophobic amino acid region. For instance, FA restored peptides such as LPVYNPEVVTAY, LQSLNSADLL, LEKNKDPLNDTVVGL, and LFSGTPTGDSEASGGTK in the head region, which were initiated with the hydrophobic amino acid leucine. Peptides like FATIAVTGEKKKEEITSGK and VTKGQTVEQVTNAVGAL, initiated with hydrophobic amino acids phenylalanine and valine, were also recovered. Such phenomenon might due to the non-covalent binding of FA with the hydrophobic region of MHC, which inhibited the aggregation induced by oxidation (Fig. 2). The remaining 13 peptides were restored from the tail region of MHC-2. Considering that the head region only accounts for 43% of the total amino acids in MHC-2, therefore, it seems that the protective effect of FA on the head of MHC-2 was higher than the tail region. This result is consistent with molecular docking (Fig. 4), which might be attributed to the higher hydrophobicity of the head that favored the binding of FA[25]. As expected, FA effectively restored the peptides such as KEEFQKTKDELAK, KLFF, KNMEQTVK, KTLAF, and KVLNASAIPEGQY that loss in OX group. Interestingly, most these recovered amino acids were started with lysine. It was noted that trypsin prefers to cleave at lysine residues, and the oxidation of lysine residues reduced the hydrolysis efficiency of trypsin due to missing of cleavage sites[36]. In addition, the formation of carbonyl-ammonia bonds between lysine and adjacent amino acids due to oxidation increased steric hindrance of trypsin to hydrolysis sites, which could also lead to a decrease in protein digestibility. Therefore, the protective effect of FA on peptide release could be explained as FA inhibited the oxidation of lysine, thus allowing trypsin to successfully cleave peptides at lysine residues.

4. Conclusion

In this study, the effects of FA on the loss of beef MP digestibility mediated by protein oxidation and the inhibition mechanism were explored. The result showed that FA inhibited MP carbonyl formation in a dose-dependent manner. Besides, FA exhibited non-covalent interactions, including hydrophobic interactions and hydrogen bonds, with MP. Notably, SDS-PAGE and TEM analyses revealed that FA effectively countered MP aggregation triggered by protein oxidation. FA significantly increased digestibility of oxidized MP; specifically, 80 μmol/g FA alleviated 67.85% loss digestibility of oxidized MP. Furthermore, peptidomics analysis underscored FA's protective role in preserving peptide release within hydrophobic regions and lysine residue areas. In summary, FA preserves peptide release in lysine residues and hydrophobic regions through antioxidant and anti-aggregation, thereby mitigating the decline in protein digestibility caused by protein oxidation. Considering the higher concentrations of FA will affect the properties of MP, the protective effects of FA with higher concentrations on the digestibility of oxidized MP merit further investigation. In addition, future studies should assess the nutritional implications of combining dietary meat protein with FA *in vivo*.

Declaration of interests

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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Fig. 7 Location of differential peptide that released from MHC-2 in CK, OX, and OF_{80} group. The letters represent the amino acids of MHC-2, and numbers represent the arrangement order of amino acids. The red lines depict peptides absent in the OX group relative to the CK group, whereas the green lines represent peptides regained in the OF_{80} group compared to the OX group.

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