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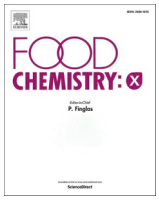

Preparation of bovine liver peptide-flavonoids binary complexes by free radical grafting: Rheological properties, functional effects and spectroscopic studies

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ABSTRACT

Free radical grafting is a green and fast method for modification of proteins and bioactive peptides. In this study, different flavonoids with flavonol, flavonoid glycoside and flavan-3-ol structures, such as quercetin (QC), rutin (RUT), and catechin (C), which are commonly used in food applications, were used as the research objects, and the binary systems of bioactive bovine liver peptides complexed with the flavonoids were prepared by free radical grafting method. The findings indicated that the affinity of catechin for bovine liver peptides markedly exceeded that of both quercetin and rutin. This observation was consistent with the extent of reduction in the content of free amino. The emulsion system formed using the binary complex was homogeneous and dense under optical microscopy, with reduced droplet diameters and significantly improved interfacial properties such as shear resistance. The combination of bovine liver peptides and flavonoids can be regarded as an effective means of modification.

1. Introduction

The principle of free radical grafting primarily involves the generation of free radicals using light, heat, or chemical initiators. These free radicals react with active sites (such as amino or hydroxyl groups) in food components (e.g., proteins), forming new free radicals. Subsequently, these newly formed free radicals undergo chain initiation and chain propagation reactions with functional monomers, resulting in the formation of graft copolymers. Finally, the grafting process is completed through chain termination reactions. Free radical grafting, involving the modification of molecules through grafting, coupling, or polymerization, represents a promising approach for the synthesis of novel materials or the modification of biomolecules with desired physical and chemical properties. This strategy is aimed at enhancing the properties of selected food ingredients or imparting new characteristics for specific applications (Spizzirri et al., 2009). The integration of natural food components with conjugated molecules can enhance the nutritional, organoleptic, physicochemical, and functional attributes of foods (Czubinski & Dwiecki, 2017). In numerous studies, free radical grafting has been utilized to modify proteins (Zheng et al., 2022). For instance, the antioxidant properties of faba protein are significantly enhanced through the free radical grafting method (Sharifimehr et al., 2025). Furthermore, the grafting of epigallocatechin gallate (EGCG) onto Ca^{2+}-promoted whey protein via the free radical grafting method expands its potential applications as a carrier for bioactive substance delivery (Wang, Guo, et al., 2024). The introduction of small molecule polyphenols at room temperature, using ascorbic acid and H_2O_2 as free radical initiators, enhances emulsification properties and antioxidant activity by attaching hydrophobic structural domains and hydroxyl groups to protein molecules (Fan et al., 2018), the process is considered a green, safe and convenient modification method.

Flavonoids are one of the most widely studied classes of polyphenols in terms of antioxidants and biological activities. According to the patterns of substitution and unsaturation, flavonols and related compounds such as flavanols may be formed, and they have potent antioxidant activity, scavenging superoxide, hydroxyl, and peroxyl radicals, etc., and

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chelating metal ions by oxidation-catalyzed complexation of metal ions and decomposition of peroxides (Maqsood et al., 2013). Among them, quercetin is a common flavonol with C6-C3-C6 basic skeleton, which has been proven to be an effective antioxidant and is widely distributed in tea and onion (Parolia et al., 2022). Rutin, also known as quercetin-3-O-rutinoside, is a glycoside formed by combining quercetin with rhamnose and glucose, specifically, it contains a rhamnose and a glucose molecule attached to position 3 of quercetin via a glycosidic bond, which is more water soluble than quercetin (Jiménez-Aliaga et al., 2011). Catechins have a 2-phenylbenzodihydropyran structure and are flavanols, and it was found that the introduction of other groups in the structure of catechin can effectively improve its stability and bioavailability, and specifically enhance the pharmacological effects of catechin, anti-tumour, antioxidant, anti-bacterial, as well as protection of cardio-cerebral organs (Meyer et al., 1998). Due to the different structures of the three flavonoids, their physicochemical and biological activities also showed some differentially, mainly determined by their polyhydroxy structures.

The liver has been demonstrated to possess excellent properties (Zou et al., 2021). However, liver still faces serious challenges in the field of processing applications in food, therefore, in order to effectively utilize the resources of bovine liver, to realize the functional value of bovine liver bioactive peptides, and to develop innovative and consumer-friendly functional ingredients, the present experiments cast the research attention on the preparation of hydrolysates and Flavonoid Complex by radical grafting method with hydrogen peroxide and ascorbic acid initiating the free radical reaction (He et al., 2023), to offer a novel perspective on the development of functional components from bovine liver peptides. Covalent couplings between peptides and polyphenols can be achieved via the free radical reaction of polyphenols with peptide (Quan et al., 2020). In recent years, numerous studies have demonstrated that proteins/peptides and polyphenols can interact (Zhao et al., 2021), polyphenols exhibit synergistic antioxidant activity with protein hydrolysates when couplings are formed (Jiang et al., 2018), and different structures of polyphenols can produce different couplings during the reaction process, in which the number of -OH groups plays a key role in the coupling of polyphenols and polypeptides (Hasni et al., 2011). Moreover, the functional properties of peptides/proteins, such as emulsification and emulsion stabilization activities, are influenced when they are coupled with phenolic compounds (Wang et al., 2019). Liu et al. examined the impact of porcine bone hydrolysate (PBPH) and PBPH-rutin coupling on the physical and oxidative stability of emulsions. Their findings demonstrated that the addition of the coupling agent facilitated additional interactions between adjacent droplets, thereby inhibiting lipid oxidation through electrostatic interactions (Liu et al., 2019). Chen et al. (2018b) investigated the effect of oxidized chlorogenic acid-modified porcine plasma protein hydrolysate on the stability of oil-in-water emulsions. Rapeseed oil emulsions stabilized with the addition of porcine plasma protein hydrolysate-chlorogenic acid coupling exhibited reduced aggregation and flocculation, along with smaller oil droplet sizes, compared to emulsions stabilized with the hydrolysate alone. Consequently, protein-polyphenol conjugates are frequently employed to enhance the stability of food emulsions, owing to their superior emulsifying properties.

The preparation of a complex of bovine liver peptide-flavonoid compounds by free radical grafting method, and the improvement of emulsifying antioxidant properties of bovine liver peptides by chemical modification, has significant implications in the field of food chemistry. It not only provides an innovative method for improving the functional properties bovine liver peptides but also opens new avenues for the development of efficient and stable natural food additives. This complex can be widely used in functional food, food and nutritional fortification, meeting the consumer demand for healthy, natural food, while promoting the high-value utilization of by-products from meat processing, with broad market prospects application potential. In this study, three flavonoids with different structures, quercetin [QC], rutin [RUT] and catechin [C], which are commonly found in food products, were selected to be complexed with bioactive peptides from bovine liver by free radical grafting method, the physicochemical and functional properties of the three binary complexes, as well as the interfacial behaviors of the emulsions, were evaluated. It provides a reference for the study of flavonoid-hydrolysate complexes as functional ingredients in different fields.

2. Materials and methods

2.1. Materials and chemicals

Bovine liver was sourced from Taohai Market (Lanzhou, China). Quercetin (purity ≥97 %), rutin (purity ≥95 %), catechin (purity ≥98 %) were purchased from Shanghai Yuanye Technology Co. Ltd. (Shanghai, China).

2.2. Preparation of bovine liver peptides

The purchased fresh bovine liver was rinsed with blood water, cut into pieces, and divided, and impurities were removed. A certain amount of bovine liver was weighed and homogenized (under the condition of ice bath). Based on the previous research content, the enzyme and ultrasound conditions were optimized respectively, and the fixed parameters for preparation were selected on this basis (Duan et al., 2024). The pH of the homogenized bovine liver was adjusted to about 8 and placed in a water bath at 50 °C to preheat the homogenized bovine liver. At the same time, 0.4 % of alkaline protease was added to carry out the enzymatic hydrolysis for 4 h, and at the same time, the centrifugation of supernatant obtained was placed in the ultrasound power of 500w to ultrasonicate for 20 min (HX-2000, Shanghai Huxi Industrial Co., Ltd). The obtained bovine liver peptide was lyophilized and stored at —18 to be analyzed. The obtained bovine liver peptides are represented as BLHs in the text.

2.3. Preparation of complexes

According to the previous research method slightly modified (Zheng et al., 2022), weighed three equal parts of 1 g of bovine liver hydrolysates (BLHs) dissolved in 49 mL of phosphate buffer solution (0.2 mol/L), while weighed 0.1 g of quercetin (QC), rutin (RUT), and catechins (C) dissolved in 50 ml of phosphate buffer, respectively, and the flavonoids to be dissolved were placed in a magnetic stirrer (B4-1 A, Shanghai Sile Instrument Co., Ltd.) at 3000 rpm with homogeneous mixing for 2 h, waiting for the sufficiently dissolved, the bovine liver peptide was mixed with quercetin (QC), rutin (RUT), and catechin (C), respectively, and added sodium propionate (0.2 %) preservative to the mixture with H_2O_2 (10 mol/L, 1 mL) and ascorbic acid (0.25 g). After fully reacting for 24 h, the reaction samples were dialyzed for 48 h. The dialysis bag is a cellulose dialysis bag with a molecular cutoff of 1000 Da (Yuanye, Shanghai). The dialysate was lyophilized to obtain the three substances BLHs-QC, BLHs-RUT, and BLHs-C, which were stored at -18 °C for further analysis.

2.4. Determination of polyphenol binding capacity

The polyphenol binding equivalents were determined using the Folin-Ciocalteu method (Fan et al., 2018). Briefly, 1 mL of the BLHs-polyphenol complex (1 mg/mL) was combined with 0.5 mL of Folin-Ciocalteu reagent (0.25 mol) and kept in the dark for 5 min. Subsequently, 1 mL of 15 % Na_2CO_3 was added, and the samples were heated in a water bath at 40 °C for 60 min before being cooled. The absorbance was then measured at 765 nm.

2.5. Determination of free amino groups

The extent of protein binding to polyphenols can be assessed by measuring their free amino groups, utilizing the o-phthalaldehyde (OPA) method (Liu et al., 2015). Specifically, 4 mL of OPA reagent was mixed with 200 μL of sample solution and reacted in a water bath at 35 °C for 2 min. The absorbance was then measured at 340 nm. As a control, 200 μL of water was added to the OPA reagent.

2.6. Fourier transform infrared spectroscopy

A certain amount of each of the four lyophilized samples, BLHs, BLHs-QC, BLHs-RUT, and BLHs-C, was mixed well with KBr and pressed into a transparent sheet. The infrared spectra were scanned using a Fourier transform infrared spectrometer (FTIR: Nicolet iS, Thermo Fisher Scientific) according to a spectral range of 4000 to 400 cm^{-1} with a resolution of 2 cm^{-1}.

2.7. Fluorescence spectroscopy

The four samples, BLHs, BLHs-QC, BLHs-RUT, and BLHs-C, were diluted to 0.1 mg/mL with phosphate buffer, and the changes of fluorescence intensity were recorded in the absorption wavelength range of 340–500 nm using a fluorescence spectrophotometer (F-7100, Hitachi High-Technologies Corporation). At this time, the excitation wavelength was 290 nm, and the slit widths for excitation and emission were 5 nm, respectively.

2.8. Surface hydrophobicity

The surface hydrophobicity of the samples was detected using an F-7100 fluorescence spectrophotometer (F-7100, Hitachi High-Technologies Corporation) with 1-aniline-8-naphthalenesulfonate (ANS) as a fluorescent probe with a 5 nm slit and excitation and emission wavelengths of 370 nm and 380–600 nm, respectively.

2.9. UV absorption spectroscopy

The samples were weighed and dissolved in phosphate buffer to a concentration of 1 mg/ml and detected by UV spectrophotometer (UV-1900i , Shimadzu) in the range of 180–500 nm and the spectral information was recorded.

2.10. Differential scanning calorimetry

Lyophilized samples (2–5 mg) were placed in an aluminum crucible in the reactor under flowing oxygen (100 ml/min). Flowing nitrogen (20 mL/min) was used as a protective gas. Subsequently, the thermal stability of the samples was determined by differential scanning calorimetry (DSC25 , Waters Corporation). DSC was performed from 25 °C to 180 °C with a heating rate of 10 °C /min.

2.11. Scanning electron microscope

The samples were processed and sprayed with gold, and the morphological features were observed at 1000×, 2000×, and 3000× magnification using an S-3400 N SEM scanner (Hitachi, Kyoto, Japan).

2.12. DPPH scavenging activity

Referring to previous studies and making some modifications (Jiang et al., 2016), 3.5 ml of DPPH solution (0.1 mmol /L) was mixed with 0.5 ml of the sample (1 mg/ml) and kept in the dark for 30 min. The absorbance at 517 nm was recorded as A_i; 3.5 ml of ethanol solution (95 %) was used instead of DPPH solution and the absorbance was recorded as A_j; 0.5 ml of ethanol solution (95 %) was added in place of the sample and was recorded as Ac.

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{A_i - A_j}{A_c}\right) \times 100\% \quad (1)$$

2.13. ABTS scavenging activity

The ABTS analysis was conducted as described by Jamróz et al. (2019). Prepare 7 mM of ABTS solution and 2.45 mM of potassium persulfate solution, mix ABTS solution with potassium persulfate solution 1:1 (*v*/v), and avoid light for 16 h to obtain $ABTS^{+}$ working solution. Dilute approximately 20 times with phosphate buffer before use until the absorbance value is 0.75 ± 0.02 at 734 nm. Take 2 ml of diluted ABTS solution and add it to an equal volume of 1 mg/ml of sample solution, leave it for 30 min and measure the absorbance at 734 nm, recorded as A1, using phosphate buffer instead of sample, recorded as A0.

$$\text{ABTS radical scavenging activity (\%)} = \frac{A0 - A1}{A0} \times 100\% \quad (2)$$

2.14. Reducing capability

The reducing power of BLHs was determined using the method described by Zhang et al. (2021).

2.15. Preparation of emulsions

The four samples were dissolved to form an aqueous solution at a concentration of 1 mg/ml and homogenized (HR-25, Shanghai Huxi Industrial Co., Ltd) with the soybean oil phase in a ratio of 9:1 for 3 min at 10,000 rpm using a high-speed homogenizer to form a crude emulsion, which was then sonicated (HX-1000, Shanghai Huxi Industrial Co., Ltd) for 10 min at 150 w ultrasonic power.

2.16. Emulsifying property

Measured with minor modifications based on previous research methods (Hrynets et al., 2010). Each 80 μL coarse emulsion was added to a 4.92 mL 0.1 % sodium dodecyl sulfate solution (SDS) solution, and the absorbance values A0 was measured at 500 nm. The emulsification activity (EAI) was calculated.

$$\text{EAI } (m2/g) = 2 \times 2.33 \times A0 \quad (3)$$

2.17. Rheological property

The rheometer (MCR302, Anton Paar, Graz, Austria) was used, the temperature of the sample stage was set to 25 °C, and the diameter of the parallel plate measurement system was 40 mm, the operation steps were as follows: the disposable dropper was used to suck up the appropriate amount of sample placed on the plate, and the excess sample was wiped away from the surrounding area, and the spacing between the parallel plates was set to 1 mm, the low-density silicone oil was coated around the plate samples with the dropper, to prevent the large error due to the evaporation of the sample water, the shear rate was increased from 0.1 s^{-1} to 100 s^{-1}, and the flow curve between shear rate and emulsion viscosity was recorded (Fei et al., 2021).

2.18. Dynamic viscoelasticity

Dynamic viscoelastic analyses were determined using a rheometer (MCR302, Anton Paar, Graz, Austria). Freshly prepared emulsions were added to the measuring platform, and the variation of energy storage modulus and loss modulus with angular frequency was determined in the linear viscoelastic region with a fixed oscillatory strain of 0.1 % and angular frequency of 0.1–100 rad/s (Sousa et al., 2015).

2.19. Optical microscopy

Use a 10 μL pipette gun to aspirate 5 μL of emulsion on a slide while a coverslip is slowly placed over it to prevent foaming. The distribution of BLHs, BLHs-QC, BLHs-RUT, and BLHs-C emulsion droplets was observed using an optical microscope (BM-2000 , Jiangnan Yongxin Optics Co., Ltd) under a 40× objective lens.

2.20. Statistical analysis

All the indicators and related data in this experiment were measured in three parallel groups. The data were analyzed, and graphs were plotted using SPSS and Origin software, respectively, and $p < 0.05$ was considered to be of difference.

3. Results and analyses

3.1. Bovine liver peptide-flavonoids binding capacity

The free radical grafting method was used, where hydrogen peroxide and ascorbic acid were added as initiators to generate hydroxyl radicals through redox reactions (Liu et al., 2018), hydroxyl radicals attack the hydrogen atoms of hydroxyl groups, amino groups, etc. on proteins to form intermediates (Curcio et al., 2009). Three complexes BLHs-QC, BLHs-RUT and BLHs-C were then prepared by reacting with three flavonoid substances. Polyphenol content reflect the polyphenol binding capacity (Dai et al., 2024). As shown in Fig. 2(A), the polyphenol binding capacities of the three complexes were in the order of BLHs-C > BLHs-QC > BLHs-RUT, with significant differences ($p < 0.05$). (See Fig. 1.)

The binding between the flavonoids and the bovine liver active peptide was further elucidated by determining the changes in free amino content. The results are shown in Fig. 2(B), after covalent modification of the three flavonoids, the free amino content of the complexes were all significantly lower than that of the bovine liver bioactive peptide ($p < 0.05$). This may be due to the involvement of SDS in the OPA reagent in the disruption of non-covalent bonds, where the phenolic hydroxyl group in the flavonoids covalently reacts with the free amino group in the side chain residues of the proteins to form a C—N bond to consolidate the structure of the complexes (Wang, Li, et al., 2024). Among them, the free amino content decreased in the order of BLHs-C > BLHs-QC > BLHs-RUT>BLHs, which was consistent with the order of polyphenol binding equivalents, suggesting that catechins reacted with bovine liver peptides with a stronger activity and binding strength than quercetin and rutin. In addition, quercetin is a flavonol, whereas rutin is a glycosidic form of quercetin, consisting of quercetin and sugar molecules. Therefore, the spatial site-blocking effect produced by the rhamnose structure of rutin is inevitable (Parolia et al., 2022). It can be inferred that the longer carbon chains of flavonoid polyphenols may relax their spatial structure upon binding to proteins, leading to the liberation of amino groups.

3.2. Fourier transform infrared spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is an analytical technique based on the transitions of molecular vibrational and

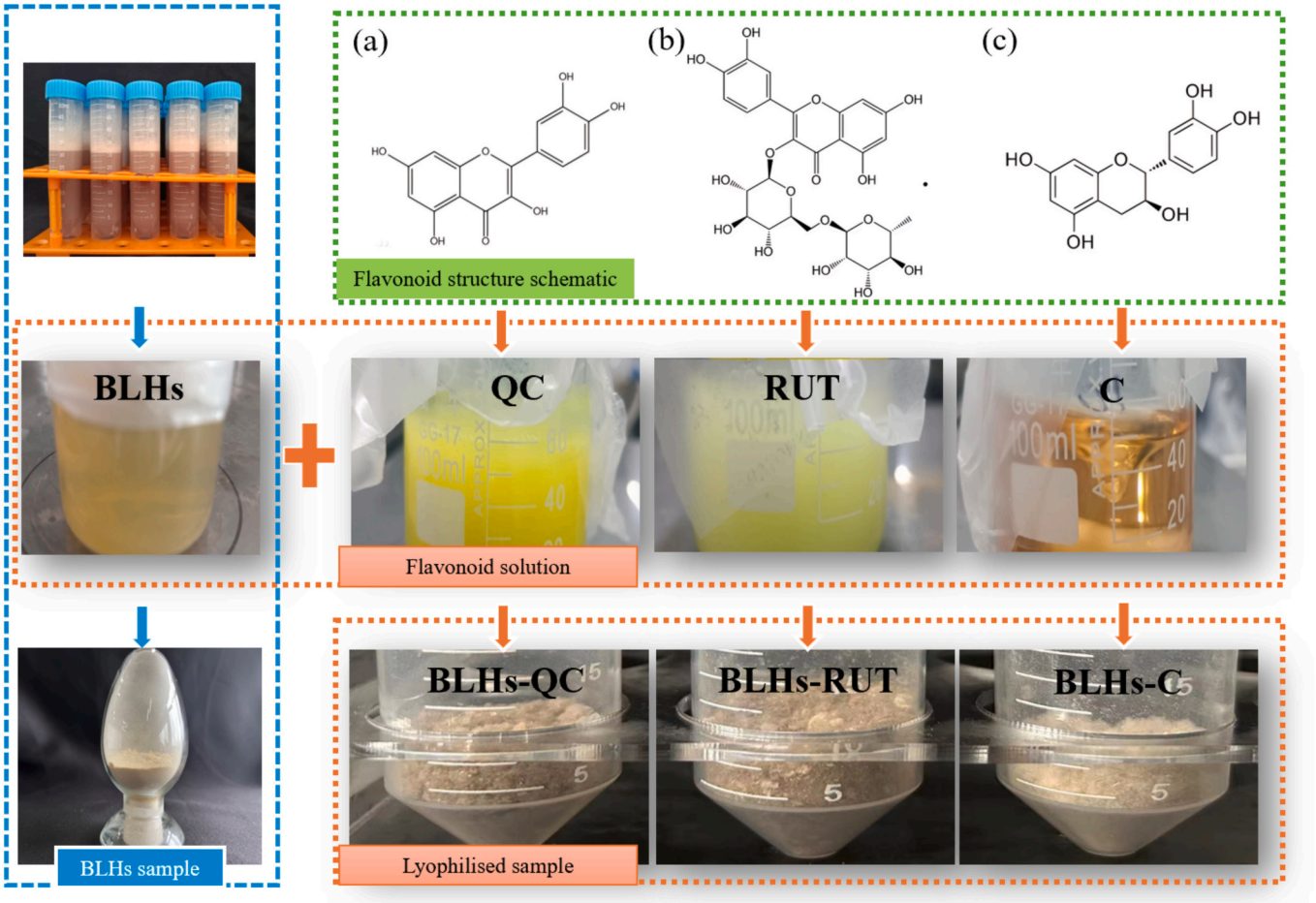

Fig. 1. Sample preparation process for BLHs, BLHs-QC\RUT\C, etc. (a). Representing the molecular structural formula of quercetin; (b). Representing the molecular structural formula of rutin; (c). Representing the molecular structural formula of catechin.

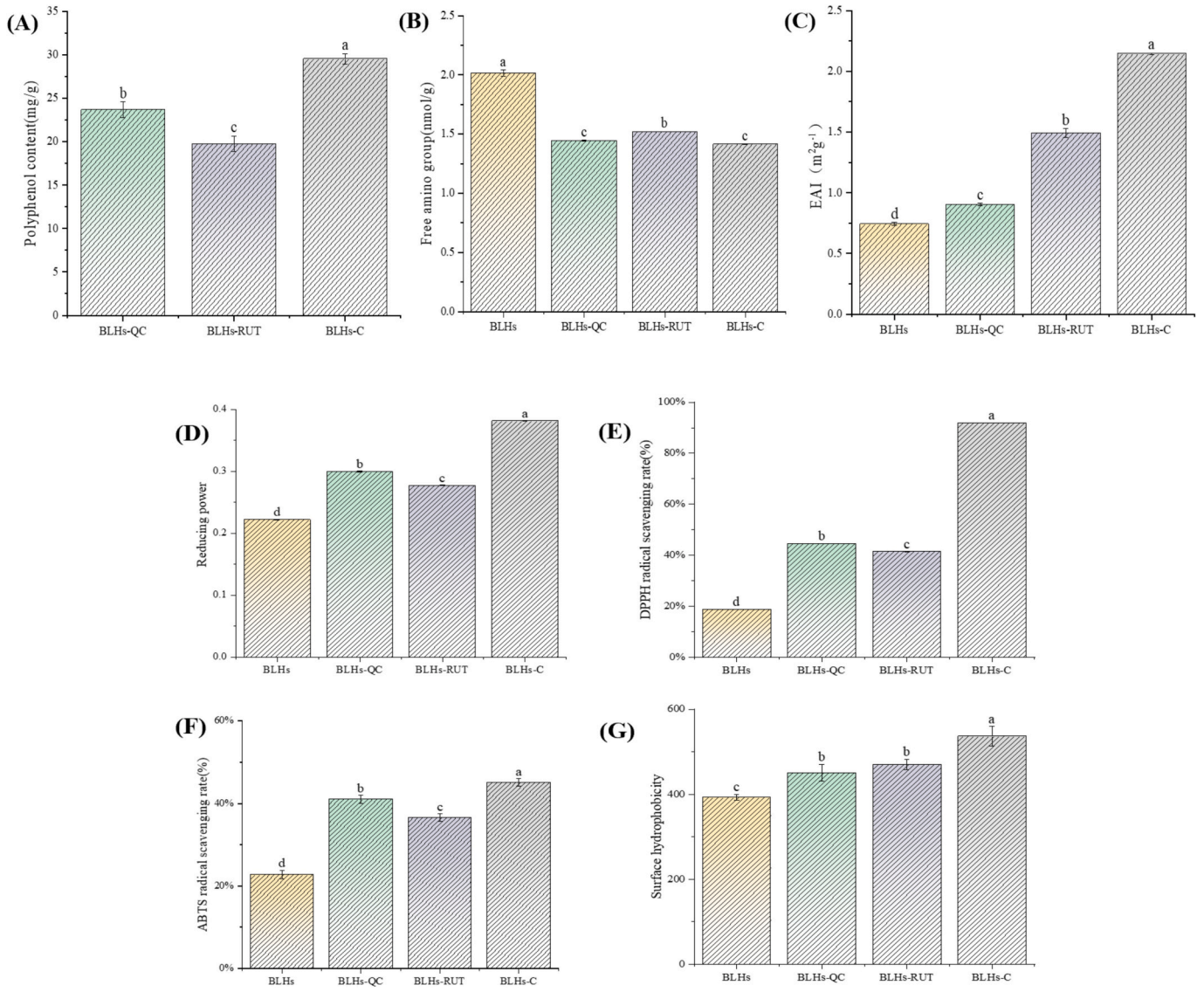

Fig. 2. (A) Polyphenol binding capacity; (B) Free amino acid content of bovine liver peptides and different complexes; (C) Emulsifying activity index; (D) Reducing ability; (E) Free radical scavenging ability of DPPH; (F) Free radical scavenging ability of ABTS; (G) Surface hydrophobicity.

rotational energy levels. It is mainly used to study the chemical structures of substances and intermolecular interactions. In this study, the FTIR spectroscopy was employed to analyze information about chemical bonds and functional groups. Fig. 3(B) demonstrates the FTIR spectra of BLHs and their complexes formed after radical grafting with three flavonoids such as QC, RUT, C. The FTIR spectra of BLHs showed major characteristic bands such as 3053.01 cm^{-1}, 1573.41 cm^{-1}.

The amide A band mainly originates from the N—H stretching vibration of the amide groups (-CONH-) in protein molecules. The vibration frequency of the N—H bond is influenced by hydrogen bonding. Compared to BLHs (3053.01 cm^{-1}), BLHs-QC (3270.88 cm^{-1}), BLHs-RUT (3270.88 cm^{-1}). BLHs-C (3259.49 cm^{-1}) complexes show a shift of the amide A band towards long waves. On the one hand, this may be due to OH stretching vibrations of the phenolic group (Liu et al., 2021). On the other hand, the protein secondary structure of bovine liver peptides is modified by covalent binding to flavonoids, specifically, a slight redshift was observed in the amide A band upon binding to QC, RUT, and C, suggesting that the BLHs-flavonoid covalent interaction may affect the N—H moiety of BLHs. The same phenomenon was found in the covalent coupling of hydrolyzed collagen from defatted sea bass skin with gallocatechin gallate (EGCG).Due to the coupling of EGCG to the N—H group of the peptide via hydrogen bonding, the characteristic peak at amide A vibrated to a higher wave number (Chotphruethipong et al., 2021).The stretching and bending vibrations of the peptide backbone in the amide I and amide II bands may represent different secondary structures, and in assessing the changes in the secondary structure, the characteristic peak of BLHs appeared at 1573.41 cm^{-1}, while BLHs-QC (1646.04 cm^{-1}), BLHs-RUT (1637.50 cm^{-1}), and BLHs-C (1639.37 cm^{-1}) showed new absorption peaks, and the amide I band of the binary complexes was more sensitive than the amide II band in terms of the change in characteristic absorption peaks. All the absorption peaks were red-shifted compared to BLHs, a phenomenon similar to the results of covalent binding of chlorogenic acid-wheat hydrolysis products (He et al., 2023). The shifts of the amide I and amide II bands indicate that the covalent reaction binds the flavonoids to the C═O, N—H, and C—N groups of the BLHs, reconfirming the conformational change of the BLHs.

3.3. *Intrinsic fluorescence spectroscopy*

Fluorescence spectroscopy is widely utilized to investigate interactions or conformational changes between proteins and small

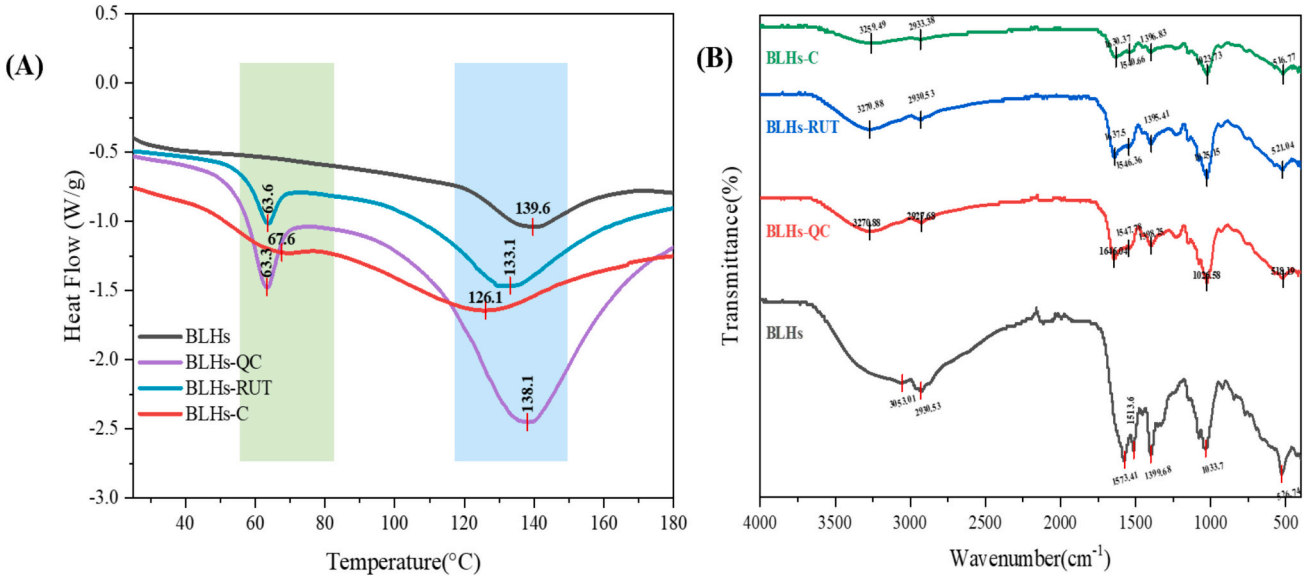

Fig. 3. (A) BLHs, BLHs-QC\RUT\C Thermal Stability Scans; (B) BLHs, BLHs-QC\RUT\C Infrared Spectroscopy Schematics.

molecules. The effect of flavonoid binding on the bioactive peptides of bovine liver was examined by measuring alterations in the fluorescence emission spectra of the samples. As shown in Fig. 4(A), the maximum fluorescence intensity wavelength of the fluorescence spectrum of the bovine liver peptide before compounding with the flavonoids was 358 nm.

After the addition of the three flavonoids, the fluorescence intensity of the bioactive peptide of the bovine liver was significantly affected, and the maximum wavelength shifted, which indicated that there was an interaction between the two classes of compounds (Lu et al., 2022). Compared with BLHs, the complexes BLHs-QC, BLHs-RUT, and BLHs-C had a slight redshift of about 2–5 nm, accompanied by a significant decrease in the maximum fluorescence intensity to 1/3, suggesting that the interactions of BLHs with all three flavonoids burst the intrinsic fluorescence of BLHs. These changes could be attributed to (1) the exposure of tryptophan-based fluorophores when the QC/RUT/C-induced proteins are partially or completely unfolded, leading to a decrease in the fluorescence intensity of the excited endogenous tryptophan (Huang et al., 2022). (2) Aggregation of the complex may increase the extent of light scattering, thereby reducing the magnitude of the emitted fluorescent signal (Yang et al., 2021).

3.4. *UV spectroscopy*

UV–visible absorption measurements are a simple technique for detecting structural changes and understanding the formation of complexes (Pan et al., 2011). The UV absorption spectra showed the effect of different flavonoids on bovine liver hydrolysate. The protein has an absorption peak at around 275 nm, which is due to the π-π jump of the carbonyl groups in the phenolic rings of tyrosine and tryptophan (Cai et al., 2015). The increase in the UV absorption intensity of proteins may be due to the hyperchromatic effect between flavonoids and bovine liver

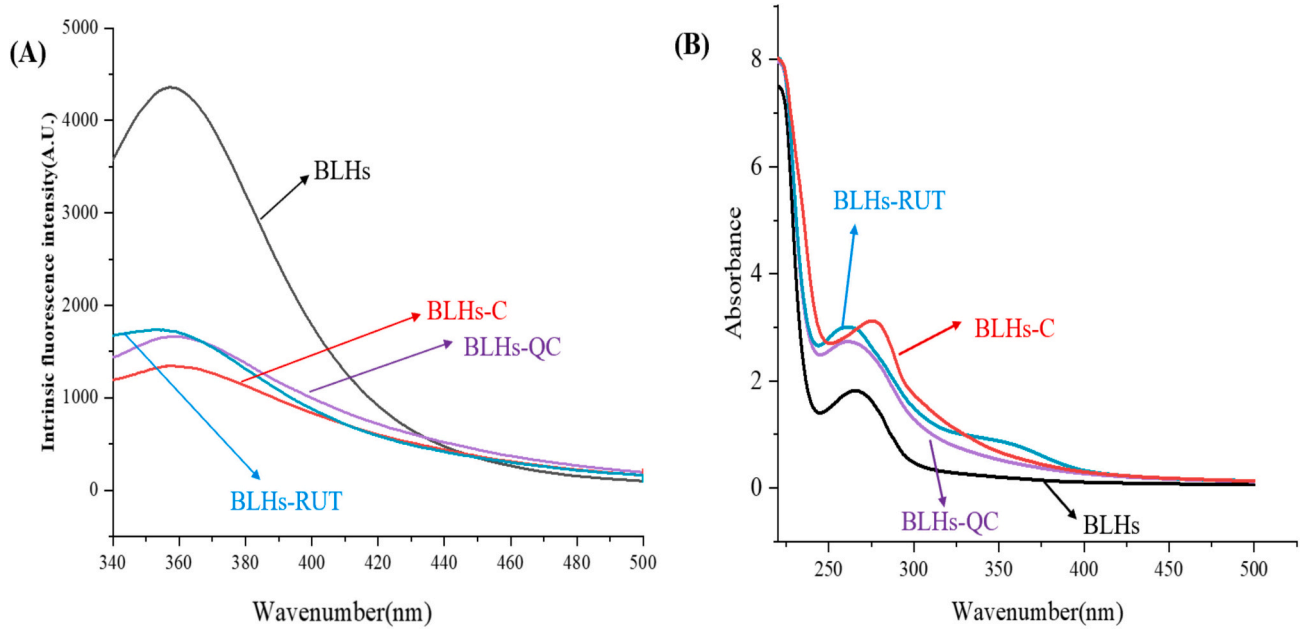

Fig. 4. Structural characterization of bovine liver peptide and its three flavonoid complexes. (A) Fluorescence spectroscopy; (B) Ultraviolet spectroscopy.

peptides because of hydrogen bonding that increases the intensity of the π-electron cloud on the aromatic ring of proteins and decreases the jump energy (Ye et al., 2013). Fig. 4 (B) shows that the absorption peak at 275 nm of bovine liver peptides was shifted by the addition of flavonoids. The spectral changes of the peaks can reflect the changes in protein structure and the changes in the microenvironment of protein aromatic acid residues. The blue shift of the maximum peak of the complex after the addition of RUT or QC indicates the movement of aromatic amino acid residues to a more hydrophilic environment (Zhao et al., 2023), whereas the red shift after the addition of C suggests an increase in the hydrophobicity of the microenvironment of the aromatic amino acid residues (Tang et al., 2016), and the change in the intensity of the absorption spectra and the shift of the peaks indicate the effective conjugation and unfolding of the backbone conformation of the bovine liver-flavonoid complexes (Vezie et al., 2016).

3.5. Surface hydrophobicity

The surface hydrophobicity of binary complexes formed by bovine liver peptide and bovine liver peptide-flavonoid was determined by ANS probe fluorescence method. In general, surface hydrophobicity reflects the number of hydrophobic groups exposed on the surface of a protein molecule. Therefore, measurements of surface hydrophobicity provide insights into protein amphiphilicity (Li et al., 2024). In addition, surface hydrophobicity affects the emulsification properties of peptides. The surface hydrophobicity of BLHs and BLHs-QC\RUT\C is shown in Fig. 2 (G). The coupling of flavonoids significantly increased the surface hydrophobicity of BLHs compared to that of BLHs ($p < 0.05$). This may be due to the fact that covalent binding prevents the attachment of hydrophilic groups such as amino groups (Chen et al., 2019); the production of quinone and the change in the conformation of some proteins leads to the exposure of hydrophobic sites (Liu et al., 2019); the presence of some macromolecules formed by the coupling of peptides with flavonoids, wherein the more hydrophilic amino acids tend to be embedded, which enhance the surface hydrophobicity of the complexes (He et al., 2023); and that covalent interactions can modulate the protein conformation and increase hydroxyl groups, thereby reducing interfacial tension (Wu et al., 2023).

3.6. Differential scanning calorimetry

Covalent binding of polyphenols to peptides or proteins can affect the thermal stability of the complexes. Usually, covalent interactions further improve the stability of the complexes, however, the thermal stability of bovine liver was not significantly enhanced after binding with polyphenols, as shown in Fig. 3(A), the peak temperature appeared around 139.6 °C when not bound with bovine liver peptide, and after the modification of quercetin, rutin, and catechins, new absorption peaks appeared in the binary complex's, meanwhile, the original peak temperatures decreased to 138.1 °C, 133.1 °C, and 126.1 °C. Therefore, the effect of binding of bovine liver peptides to flavonoids on thermal stabilization may be negative. Similar studies have shown that covalent couplings of perilla seed meal proteins modified with flavonoids are not only less thermally stable, but also require more energy to fold the protein structure (Wang, Guo, et al., 2024), providing support for similar results in this study. Based on its surface hydrophobicity, structural characterization and other indicators, it is speculated that the reduced thermal stability of the binary complexes may be related to (1) structural perturbation: the binding between the flavonoids and peptides may cause conformational changes in the peptide chain, and such changes may disrupt the original stable conformation, thus decreasing the peptide's thermal stability. (2) Changes in hydrophobic effects: the introduction of flavonoid molecules may interfere with these hydrophobic effects, occupying key hydrophobic sites originally used to maintain protein structure. However, the above speculations need to be confirmed by analyzing the sites of action of bovine liver peptides against their own structures.

3.7. Scanning electron microscope

By scanning electron microscopy (SEM) (magnification 1, 2, 3 k; Fig. 6), it was observed that BLHs were characterized by thick walls and rough surfaces, whereas BLHs bound to different flavonoids had weaker and thinner walls, more intensive fragmentation and larger surface areas. Meanwhile, spherical particles were produced along with the fragmentation, indicating that the ordered structure of the proteins had been disrupted during the formation of the complexes, while the unravelling of the protein chains resulted in a disordered laminar structure with a relatively smooth surface (Wu et al., 2023). Among them, BLHs-QC and BLHs-RUT differed significantly from BLHs-C in their microstructures, which is attributed to the fact that the degree of flavonoid binding to the protein/peptide depends on the flavonoid type (Parolia et al., 2022). By looking at the structural formulae of quercetin and rutin, it is easy to find that the structures possess duplicity. Therefore, the structural association of QC and RUT determines the existence of certain similarities in their complexes.

3.8. Antioxidant properties

Interaction with polyphenols affects the antioxidant potential of protein hydrolysates (Hernández-Jabalera et al., 2015), and in general, polyphenols with a second hydroxyl group in the neighboring or para-position have a high capacity to provide electrons and protons at these positions (Chotphruethipong et al., 2021). All complexes exhibited enhanced inhibition of free radical chain reactions compared to the bovine liver peptide control, which can be attributed to the binding of hydroxyl groups in the polyphenols to the system. Similar synergistic antioxidant effects have been previously shown for proteins/peptides bound to polyphenols (Ju et al., 2020).

In this study, the free radical scavenging (ABTS, DPPH) and reducing capacities of BLHs coupled with different flavonoids were determined. As shown in Fig. 2 (D—F), the binary complexes produced by coupling with different structural flavonoids significantly increased the antioxidant capacity of BLHs ($p < 0.05$). Among the three BLHs-flavonoid complexes, BLHs-C had the strongest antioxidant capacity of 45.09 % ($ABTS^{+}$ radical scavenging), and the presence of OH group at the B ring position was a key determinant of the radical scavenging activity, in addition to structural changes after covalent binding and consequent exposure to some antioxidant amino acids (He et al., 2023). To further verify their free radical scavenging ability, the DPPH free radical scavenging ability was determined for three complexes. DPPH is a stable free radical with a maximum absorbance of 517 nm in ethanol. When DPPH encounters the proton donor substance (H^{+}), the absorbance decreases, and the DPPH radical is scavenged and the color changes from violet to yellow. The highest scavenging capacity of DPPH can be achieved by BLHs-C up to 91.9 % at 1 mg/ml. The enhancement in the scavenging activity of the DPPH free radicals indicates that the hydroxyl groups of phenolic compounds play a key role in providing hydrogen and electrons, leading to the termination of free radical chain reaction (Liu et al., 2010). Meanwhile, the reducing power results also indicated that the presence of flavonoids such as catechin, rutin and quercetin increased the antioxidant activity of the complexes, where the reducing power of BLHs-RUT\QC\C was significantly higher than that of BLHs ($p < 0.05$), confirming the synergistic and potentiating effect of bovine liver hydrolysate with different flavonoid compounds. In a similar study, the antioxidant activity of egg white proteins was significantly increased by covalent coupling with tea polyphenols via an alkaline/free radical method (Jing et al., 2020), thus, the incorporation of flavonoids had a positive effect on the antioxidant effect of bovine liver peptides.

3.9. Rheological properties

Interfacial shear rheology enables the investigation of the adsorption kinetics and viscoelasticity of the interfacial film at the oil-water interface, and facilitates the analysis of the interactions among the adsorbed molecules (Wei et al., 2021). The apparent viscosity of the emulsion decreases with increasing shear rate and shows pseudoplastic flow behavior (Li et al., 2023). And then the apparent viscosity was basically maintained at a fixed value to keep constant, in which, as shown in Fig. 5 (A), the BLHs-C, BLHs-QC, and BLHs-C anti-shear weakened sequentially with the increase of the shear rate, but were still higher than the BLHs as a whole, indicating that the covalent binding of bovine liver peptide with quercetin, catechin, and rutin enhance the interfacial shear force of bovine liver peptide.

The modulus of elasticity (G') is the change in stored energy due to elastic deformation when a material undergoes deformation, thus reflecting the elastic strength of the material. The modulus of viscosity (G") is the energy lost due to viscous deformation of a material during deformation, which reflects the size of the material's viscosity. The ratio of the viscous modulus to the elastic modulus is known as the loss factor, which reflects the material viscosity to elasticity ratio (Zong et al., 2022).The results of the effect of different flavonoid modifications on G' and G" of crude emulsion of bovine liver peptide by increasing the angular frequency from 0.1 to 100 rad/ s at 25 °C and 0.1 % strain are shown in Fig. 5 (B, C). With the increase of scanning frequency, the elastic and viscous moduli of BLHs, BLHs-QC, BLHs-RUT and BLHs-C emulsions increased with the increase of application frequency (1–100 rad/s), i.e., both G' and G" increased, which indicated that the samples were in the flow state. And all G' values are higher than G" (Tan $\delta < 1$) .This behavior indicates that the emulsions such as BLHs-C, BLHs-QC and BLHs-C exhibit solid-like properties in the mixed system (Shekarforoush et al., 2016). As shown in Fig. 5(D), in the scanning frequency range from 0.1 to 1 rad/s, Tan δ decreases with increasing frequency, and at this stage, G' increases rapidly, where BLHs-QC > BLHs-C > BLHs-RUT>BLHs, and the elastic behavior is dominant, the treatment group is larger than the control group as a whole, in the 1 to 100 rad/s scanning frequency range, Tanδ increased sharply with the increase of frequency, and the viscous ratio increased, the increase of Tanδ indicated that the elastic ratio decreased more than the viscous ratio, and the degree of molecular cross-linking in the system became lower, and the value of Tanδ of BLHs-C was always at the minimum, which was obviously different from the other three groups of samples, indicating that the incorporation of catechins made the internal structure of bovine liver peptides more solid (Xu et al., 2017).

3.10. Emulsifying properties

Substances with high emulsifying activity can effectively reduce the

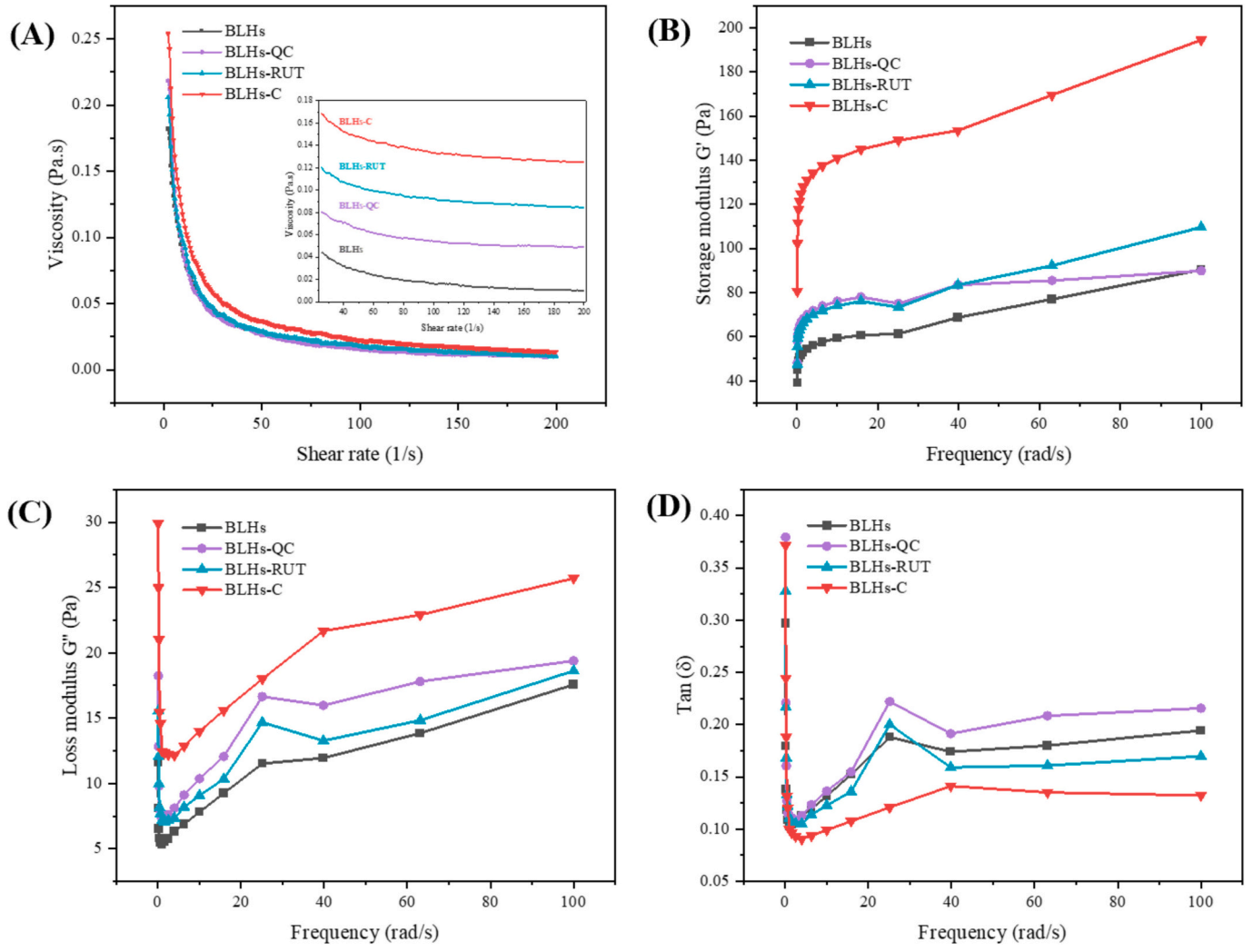

Fig. 5. Interfacial rheological characterization of (A) interfacial shear resistance; (B) characterization of elastic behavior; (C) characterization of viscous properties; (D) variation of loss tangent angle with frequency.

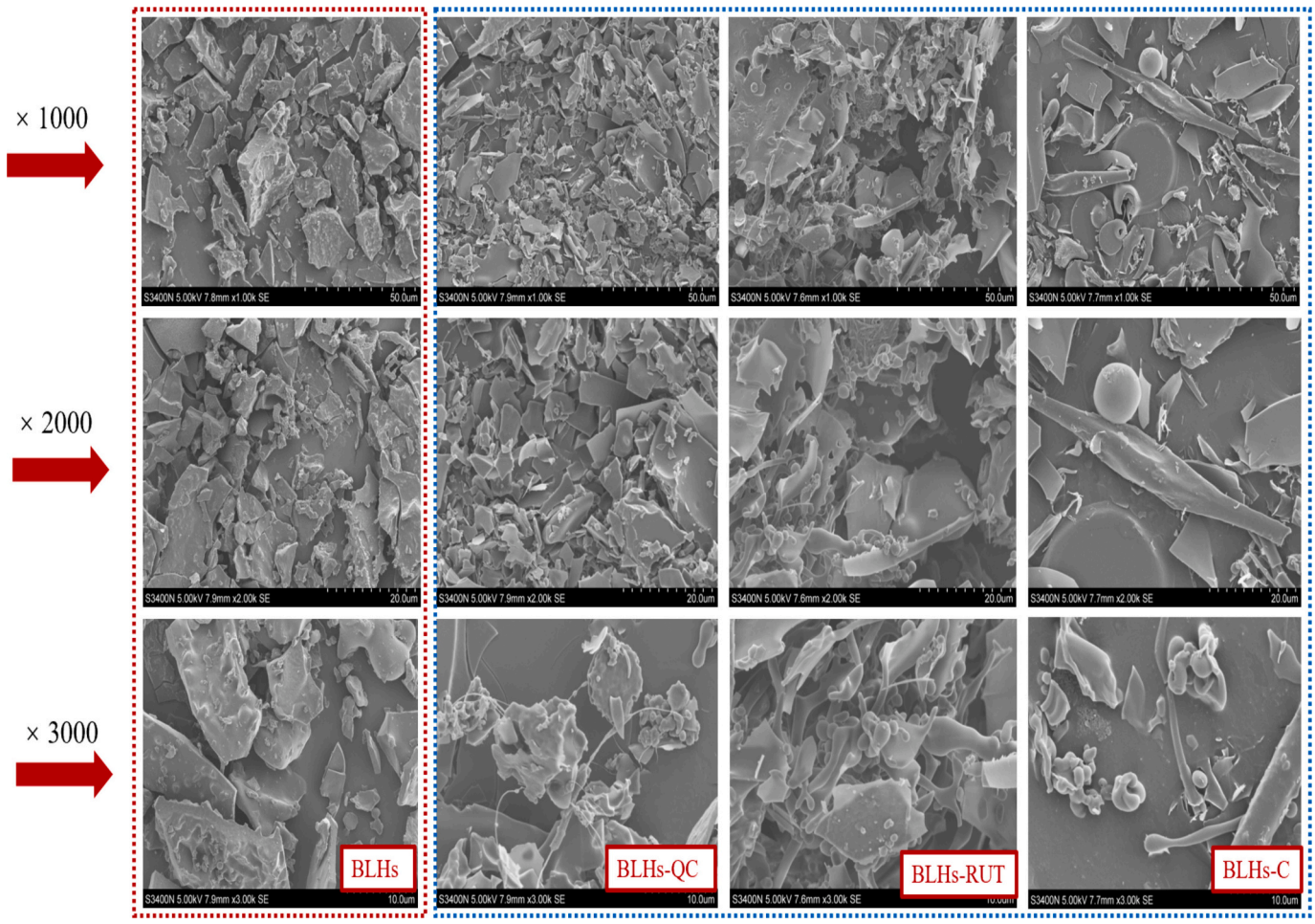

Fig. 6. Microstructural characterization of bovine liver peptide and three complexes.

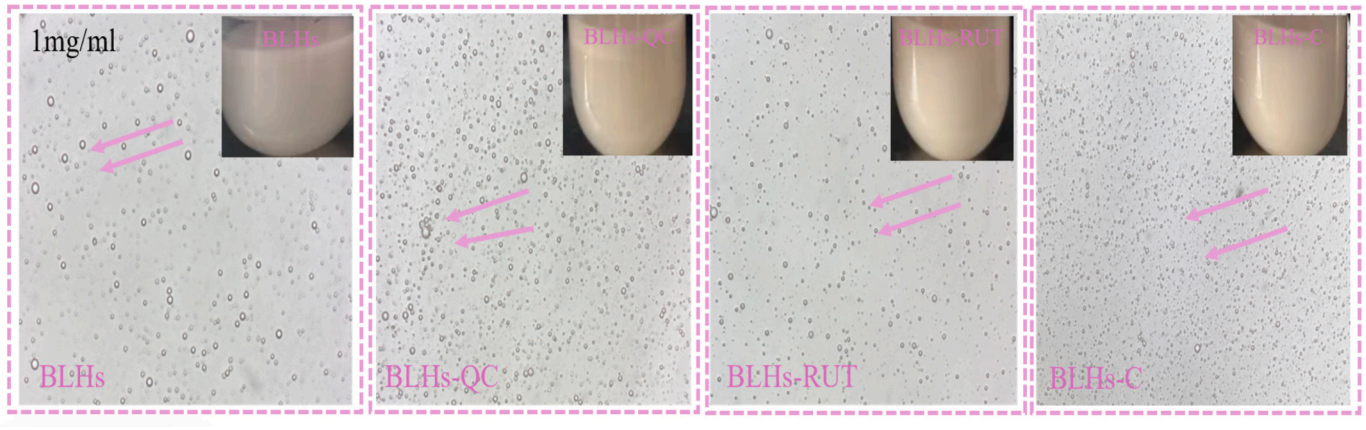

Fig. 7. Optical microstructures of bovine liver polypeptide emulsions and bovine liver-flavonoid complex emulsions.

interfacial tension at the oil - water interface and enhance the oil - water adsorption capacity. Consequently, smaller and more stable emulsion droplets are formed. The emulsification activity index (EAI) of different structural flavonoid-modified bovine liver peptides is shown in Fig. 2 (C). The EAI of unmodified bovine liver peptide was 0.74 m^2/g, and the overall EAI was significantly increased after coupling with catechin, quercetin, and rutin ($p < 0.05$). This is because the covalent attachment of the QC/RUT/C portion could change the bovine liver peptide conformation to form complexes with exposed hydrophobic structures, and these structural domains can migrate and localize at the water-oil interface. As a result, the complexes can adsorb rapidly at the oil interface, forming a film around the oil droplets and reducing the interfacial tension at the water-oil interface (Quan et al., 2020). These results coincide with the findings of Liu et al. (2015) who synthesized lactoferrin-polyphenol (EGCG, chlorogenic acid and gallic acid) by free radical grafting method, which showed better emulsification activity index than unmodified lactoferrin, thus BLHs - C exhibits promising potential in the preparation of emulsions.

3.11. Optical microscope

As shown in Fig. 7, from left to right, the imaging morphologies of the emulsions prepared from BLHs and three complexes (BLHs - QC, BLHs - RUT, and BLHs - C) under an optical microscope are presented in sequence. Through observation, it was found that the emulsion formed by BLHs - C exhibited the most ideal uniform dispersion state under the microscope. The uniform dispersion state of the BLHs - QC emulsion was the second best, while that of the BLHs - RUT emulsion was relatively poor. We hypothesize that the occurrence of this phenomenon can likely be ascribed to the polyhydroxy structure and specific functional - group distribution of catechin. On one hand, the numerous hydroxyl groups of catechin can form hydrogen bonds with proteins. On the other hand, its planar structure and the arrangement of functional groups enable simultaneous interactions with both the hydrophobic and polar regions of proteins. Consequently, catechin can form a more compact and stable complex with bovine liver peptide. In contrast, quercetin and rutin, although also able to bind to proteins, may not form as stable and effective complexes as catechins due to their structural limitations.

Compared to the crude emulsion prepared with bovine liver peptide, the crude emulsions formulated from the three complexes, namely BLHs - QC, BLHs - RUT, and BLHs - C, exhibit remarkable improvements in terms of emulsion aggregation and droplet size. The emulsion droplets formed by the three complexes are more uniform and denser can potentially be attributed to the unfolding of protein molecules, which enhances the attractive forces between bovine liver peptide and QC, RUT, and C respectively. A more robust interfacial layer is formed, enabling resistance to emulsion coalescence (Huang et al., 2022). Furthermore, previous applications of rice proteins and polyphenols in emulsions have demonstrated that the binding of proteins to polyphenols enhances the interfacial activity of proteins. This enhancement accelerates the interfacial adsorption process, ultimately resulting in a reduction in the particle size of the emulsion (Zong et al., 2022), this is consistent with the conclusions of this experiment.

4. Conclusion

BLHs-QC\RUT\C complexes were prepared by free radical grafting method, and the binding capacities were BLHs-C, BLHs-QC, BLHs-RUT in descending order. The results showed that the flavonoids were successfully covalently bound to bovine liver peptides by free radical grafting, which altered the secondary structure of bovine liver peptides, and, at the same time, there was a significant effect on the microenvironment of bovine liver peptide tryptophan residues. In addition, the observation of the complexes and their emulsion structures by optical microscopy and scanning electron microscopy, respectively, further confirmed the changes and generation of the new structures of the complexes. The antioxidant activities of the complexes such as BLHs-QC \RUT\C were quantified by DPPH, ABTS, and reducing power, with the antioxidant properties of BLHs-C being in the best, corresponding to the highest surface hydrophobicity and emulsifying activity. These results confirmed that the covalent modification of flavonoid compounds effectively enhanced the emulsifying activity and antioxidant properties of bovine liver peptides compared to bovine liver peptides alone and have good potential for application in novel antioxidants and emulsion application systems.

5. Research limitations and prospects

In the study of combining bovine liver peptides with flavonoids through free radical grafting to form a binary complex, this method has shown potential in improving the antioxidant and biological activities of the complex, there are still some limitations: the current methods for extracting and purifying bovine liver active peptides may have problems of low efficiency and high cost, which limits the feasibility of large-scale production. In the future, extraction efficiency and purity can be improved by introducing more efficient purification technologies (such as membrane separation, chromatography). In addition, although the free radical grafting method can effectively promote the combination of peptides and flavonoids, its reaction conditions (such as the concentration of free radical initiators, reaction time, temperature, etc.) have a significant impact on the structure and function of products, which may lead to side reactions or uneven products. It is necessary to further optimize the reaction conditions or explore milder and controllable grafting methods (such enzymatic grafting). Finally, the current structural characterization of the complex may not be comprehensive enough, especially the grafting sites, binding modes, and the higher order of the complex (such as spatial conformation) have not been fully elucidated. This requires in-depth research through more advanced analytical techniques (such as mass spectetry, nuclear magnetic resonance, molecular dynamics simulation). To further promote the development of this field, future research should carry out animal and clinical trials to evaluate the bioavailability of toxicological characteristics, and potential physiological functions (such as antioxidant, anti-inflammatory, immunomodulatory, etc.) of the complex. Combined with molecular biology technology and cell experiments, the molecular mechanism should be deeply studied. Through structural modification or process optimization, the functional properties of the complex (as stability, solubility, targetability) should be further improved. For example, attempts can be made to introduce nanocarrier technology or microencapsulation to enhance its application potential in food or medicine. In addition, by using the synergistic integration of food engineering and nutrition, the application of the complex in food should be optimized, and its stability, sensory characteristics, and health effects during the food processing should be studied to provide scientific basis for its development in functional food.

CRediT authorship contribution statement

Yufeng Duan: Writing – original draft, Conceptualization. **Xue Yang:** Conceptualization. **Ruheng Shen:** Conceptualization. **Li Zhang:** Project administration, Funding acquisition. **Xiaotong Ma:** Conceptualization. **Long He:** Conceptualization. **Yuling Qu:** Conceptualization. **Lin Tong:** Conceptualization. **Guangxing Han:** Conceptualization. **Xiangmin Yan:** Resources, Project administration.

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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