Effect of the non-covalent and covalent interactions between proteins and mono- or di-glucoside anthocyanins on β-lactoglobulin-digestibility

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1. Introduction

Anthocyanins belong to H2O-soluble flavonoids and structurally comprised of flavylum nucleus (2-phenyl-1-benzopyrylum) and organically concealed in numerous plant branches (Khalifa, Zhu, Li, 2018). Because of their appealing color and overall nontoxicity, anthocyanins are increasingly being employed instead of synthetic colors. Meanwhile, anthocyanins and make mulberry anthocyanins with high stability and acceptable qualities due to their heat sensitivity (Khalifa, Nie, Ge, Li, 2019). We recently noticed that mulberry anthocyanins mainly contain C3G and C3R (Khalifa et al., 2020). One of the most important proteins is β-lactoglobulin (β-Lg), which accounts for 80% of whey proteins. Because of its functional advantages, nutritional merits, and technical features, β-Lg is extensively used in the food processing sectors (Maticorena, Alarcón, Troncoso, & Zúñiga, 2018).

Proteins-anthocyanins complexation is being immensely investigated. Anthocyanins can non-covalently and covalently interact with proteins (Sui et al., 2018). The oxidation of phenol rings to quinone
derivatives, which are extremely reactive with proteins' nucleophilic groups, is permanently networked with the protein's structure (Cao & Xiong, 2017). Meanwhile, H-bonding, electrostatic assembly, van der Waals forces, and other hydrophobic forces might occur among anthocyanins OH-groups and the carbonyl groups of proteins (Khalifa, Nie, et al., 2018). Protein solubility, digestibility, and overall functioning were all, therefore, changed because of protein-anthocyanin interactions. For instance, the foaming, emulsifying, and antioxidant properties of soy proteins were enhanced after interacting with anthocyanins of black rice (Sui et al., 2018). The thermal steadiness of soybean proteins was improved by using C3G (Chen et al., 2019), while anthocyanins of mulberry fruits dropped the solidifying of whey proteins (Khalifa et al., 2019). Therefore, anthocyanins have numerous impacts on the proteins features that require additional studies.

Most notably, some reports have revealed that the conjugation of proteins with polyphenols may decline the in vitro digestion of proteins, through some studies, performed up to now on β-Lg-polyphenols mixtures. The non-covalent binding of tea, sour cherry, cocoa, and coffee polyphenols with β-Lg declined β-Lg-digestibility (Stojadinovic et al., 2013). It was determined that polyphenols could decrease the proteins' digestibility owing to their potential deactivation on the enzymes-related digestion and forming unsoluble polyphenol-protein accumulates (Cirkovic Velickovic & Stanic-Vunic, 2019). In contrast, some reports stated that polyphenols stimulate the digestibility of proteins. For example, rutin, quercetin, and chlorogenic acid increased the digestibility of whey proteins (Xu et al., 2019). Anthocyanins of black rice also increased the digestibility of soy proteins (Jakobek, 2015). In the meantime, the binding between anthocyanins and proteins seems to be responsible for the promoting/declining digestibility effects on proteins. Still, it is unclear whether this effect is due to the covalent or the non-covalent binding. Therefore, mostly importantly, the mode of action of the digestive influences of anthocyanins on β-Lg-proteins has not been yet explored.

Thus, we aimed to investigate the impacts of C3G and C3R on β-Lg-digestibility under covalent and non-covalent bondings. In vitro digestibility of β-Lg-C3G/R covalently and non-covalently conjugates and their antioxidant capacity were estimated via modeled gastrointestinal model systems. FTIR was also used to evaluate the secondary structural changes indigested and non-digested β-Lg-C3G/R conjugates. Meanwhile, fluorescence spectroscopy quenching, SDS-PAGE, nitroblue tetrazolium (SDS-PAGE/NBT), MALDI-TOF-MS, and molecular modeling approaches were employed to classify the interaction mode of action among β-Lg-C3G/R covalent and non-covalent conjugates.

2. Materials and methods

2.1. Materials

β-lactoglobulin (≥99% pure, Henan Tailijie Biotech Co., Ltd., China), while serine and O-phthalaldehyded (OPA) were bought from Career Henan chemical co. (Zhengzhou, China). Porcine pepsin (S10030, effectiveness 1:3000), pancreatic enzyme (S10031, effectiveness 1:300), and α-amylase (S10003, 50 Umg⁻¹) were purchased from Parchem Co. (New York, USA). HPLC and LC-MS ratings' diluents were attained from Sigma-Aldrich Inc., Missouri, USA. MilliQ-water (Millipore analytical grade). I. Khalifa et al. applied by a local company, based on the assay of Gras, Bogner, Carle, and Schweiggeert (2016). Anthocyanins were separated via a blend of HCl: MeOH (0.1:99.9) and subjected to sonication (RES50V2, Shanghai Yuanhui Industrial Co., Ltd., China) at 313.15 K/10 min. It was then rotary-vaporized (RE-52AA, Hecheng Co., Ltd., Hubei, China) at 311.15 K. To extract proteins and carbs, solid-phase separation based on AB-8 macroporous adsorption resins was utilized, while the other polyphenols were eliminated using liquid-liquid isolation with CH₂COO-CH₃-CH₂-OH. After that, anthocyanins were categorized by Waters alliance E2695-HPLC-PDA provided by a C18 column (250 mm × 4.6 mm, 5 μm, Agilent Technologies, Palo Alto, Inc. California, USA). Solvents A and B were the aqueous solution of HCOOH (5%, v/v) and the HPLC-grade of MeOH, respectively, with the gradient schedule of 0–10 min (5–20% B); 15–30 min (20–52% B); 35–40 min (25–33% B); 40–42 min (33–5% B). The stream rate was 1 ml per minute with an insertion amount of 10 μL and column temperature of 303.15 K. The recognition was performed at the wavelength of 200–600 with choosing 520 and 280 nm to verify the apparent appearance of other polyphenols. As charted in Fig. 1A, the raw anthocyanins comprised of 2 major peaks. Subsequently, we detected each peak via Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC-ESI-MS-MS) by the following circumstances: The m/z limit was 50–1000, positive ion type, the vaporizer force was 40 psi, gas stream ratio is 10 L per minute, the drier temperature was 623.15 K, and the vesel energy was 2500 V. As outlined in Fig. 1B, C3G, and C3R were recognized as the key anthocyanin’s portions of mulberries (Khalifa et al., 2020). Peak#1 exhibited an m/z rate of 449.1 [M + H]+ and a portion ion of 287, implying that it’s anthocyanin glucosides. This peak is C3G, according to the loss of 1 glucose fragment [M + H-162] + neutral portion at 449.1 m/z. Peak#2 has an m/z of 595.3 [M+H]+, and the molecular ion peak loses arhamnose molecule [M + H-146] + to get a portion ion of 449. To get C3R, drops of ahexose fragment [M + H-146 - 162] + produced a fragment ion with an m/z 287. In addition, we utilized preparative-HPLC-PDA (LC-100P, WINCOM Co., Ltd., China) to synthesize C3G and C3R under HPLC-PDA circumstances. After building appropriate amounts of C3G/R via an Agilent ZORBAX SB-C18 column (250 mm × 9.4 mm, 5 μm), HPLC-PDA showed that their integrity was over 97% (Fig. 1C). Both were further utilized to observe the digestion impacts of C3G/R on β-Lg-based models.

2.3. Fabricating β-Lg-C3G/R conjugates under covalent and non-covalent binding conditions

The β-Lg-C3G/R complexes were fabricated by non-covalent interactions (C3G-NON and C3R-NON) using our previous procedures (Khalifa, Nie, et al., 2018) with some adjustments. The β-Lg (2.5 g) was dissipated in sodium phosphate buffer (100 mL, 10 mM, pH 7.4) followed by incubation with 300 μM of either C3G or C3R at 298 K during 2 h in the O₂-free conditions to reach equilibrium. We selected this concentration (300 μM) of both C3G and C3R based on our pre-experiments, where we have been used several concentrations (data not showed), and most importantly to stimulate their concentrations in our previous energy ball models (Khalifa, Zhu, Li, & Li, 2021). Unconjugated β-Lg was also hatched using the same circumstances and considered a control. On the other hand, the β-Lg-C3G/R complexes fabricated by covalent interactions (C3G-CO and C3R-CO) were prepared using the assay of Sui et al. (2018). Concisely, 2.5 g of β-Lg was liquified in the same sodium phosphate buffer (100 mL, 10 mM, pH 7.4) at 298 K, and pH 9 was corrected by NaOH (0.5 M), then 300 μM of C3G or C3R added, and the whole blend was left at 298 K during 12 h. After fabricating β-Lg-C3G/R complexes, the C3G/R binding ratio was calculated as follows:

\[
\text{% C3G or C3R binding ratio} = \frac{\text{total } C3G/R - \text{C3G/R dialyzed}}{\text{total } C3G/R} \times 100
\]

The β-Lg-C3G/R conjugates were dialyzed via dialysis pipes 3 kDa molecular mass (Sigma-Aldrich Inc., Missouri, USA) versus MilliQ-H₂O overnight at 277 K. The dialyzed conjugates were then thinned to achieve absorbance evaluations inside the scale of the calibration curve.
2.4. Characterizing the binding of β-Lg-C3G/R

A F-4600 fluorescence spectrophotometer (Hitachi Ltd., Japan) was used for each mixture to evaluate the fluorescence strength at λex and λem of 280 and 290–420 nm, orderly, 277 K slit thickness of 5 nm, and an examining proportion of 1200 nm/min. Similarly, the fluorescence extinction of each blend was performed under the same conditions at 298, 308, and 318 K (Khalifa, Nie, et al., 2018). Anthocyanins are very
feebly fluorescent since the several vying fate of their excited asserts (Trouillas et al., 2016). Though to acquire high precise data, we amended the inner visual filter effect of C3G and C3R at λex and λem of 280 and 340 nm, orderly, followed by determining the quenching coefficients. The fluorescence quenching process was distinguished using Stern-equality. Based on twin-logarithmic calculations, Ks was calculated from y-axis deviation and plot slope of log (F0 - F)/F versus log [Q]:

$$\frac{F_0}{F} = 1 + K_s \tau_0 [Q] = 1 + K_{SV}$$

(2)

$$\log \left( \frac{F_0 - F}{F} \right) = \log K_n + n \log [Q]$$

(3)

Whereas Ks = binding constant, F0 and F = fluorescence intensity without and with quencher, [Q] = quencher (C3G/R) levels, KSV = Stern-Volmer’s constant, Ks = biomolecular quenching rate constant, and τ0 for a typical lifetime of Tyr for β-Lg-free quencher (τ0 = 3.37 ns) (Ren & Giusti, 2021).

Thermo Scientific FTIR Nicolet 470 was used to document the FTIR spectrum for the lyophilized β-Lg-C3G/R blends under covariant and non-covariant interaction conditions using a KBr disk method. The spectra were recognized in transmission mode with a determination of ±2 cm⁻¹ at 21 probes min⁻¹ from 4000 to 400 cm⁻¹. The Omnic programme automatically altered FTIR spectra lines and performed Fourier auto-deconvolution. On a J-1500 spectrometer, the far-UV CD spectra of each combination were also detected at 298 K using a 0.1 cm visual path quartz cell (195-240 nm) at a 2 nm bandwidth and a scan rate of 100 nm per minute (JASCO, Japan).

SDS-PAGE and SDS-PAGE/NBT of β-Lg-C3G/R blends were done using the assay of Shen, Xu, and Sheng (2017). After preparing all mixtures, ~10 mg (measured by Bradford protein method) of β-Lg was sedimented with trichloroacetic acid (10%, v/v), congregated through centrifugation at 14000g/20 min, splashed twofold via the icy acetone (5%, v/v), thinned 1:4 (v/v) with Laemmli sample buffer (Sigma-Aldrich Inc., USA), and heated at 373 K during 5 min. Sample (5 μL) of each blend or protein pointer (10-180 kDa) was inserted into an SDS-PAGE gel (12%), run on a perpendicular piece gel electrophoresis (Liuyi, Inc., USA), and heated at 373 K during 5 min. A 4 mL aliquot of each sample was obtained after each stage for 5 min. After each stage, the digestion was completed by heating at 368 K during 5 min. After each stage, the digestion was completed by heating at 368 K during 5 min. After each stage, the digestion was completed by heating at 368 K during 5 min. After each stage, the digestion was completed by heating at 368 K during 5 min. After each stage, the digestion was completed by heating at 368 K during 5 min. After each stage, the digestion was completed by heating at 368 K during 5 min.

2.5. In vitro gastrointestinal digestion of β-Lg-C3G/R complexes

Though in vitro digestion models provide different outcomes than in vivo digestion patterns, in vitro models are beneficial since they are less costly and may produce quicker findings. The in vitro digestion patterns contain reproduced buccal, gastric, and duodenal phases (Yan Zhang, Chen, Sui, & Jiang, 2018). After preparing each blend by conjugating 2.5 g of β-Lg with 300 μg of C3G or C3R as explained in detail in section 2.3, they were inserted to the in vitro imitation digestion process. Briefly, 10 mL of each blend was mixed with salivary juice (7.5 mL), α-amylase solution (0.5 mL, 100 U mL⁻¹), CaCl₂ (25 μL of, 0.3 M), and MilliQ-H₂O (0.975 mL). The mixture was incubated at 310 K in a stirring incubator (HHI208 Meena Medical Equipment, Inc, USA) at 120 rpm during 2 min. Afterward, the residual liquid was blended thoroughly with 6 mL of gastric liquid, pepsin solution (1.6 mL, 2500 U mL⁻¹), CaCl₂ (4 μL, 0.3 M), and MilliQ-H₂O were made up to 8 mL, followed by the adjustment of pH to 3.0 by HCl (1 M). Further, pH 7 was adjusted by using 1 M NaOH to stop digestion process. The mixture was likewise hatched for 60 min. The exceptional combination was mixed with duodenal juice (7.7 mL), pancreatin blend (3.5 mL, 800 U mL⁻¹), bile salt (1.75 mL, 160 mM), CaCl₂ (28 μL, 0.3 M), and MilliQ-H₂O (14 mL), followed by pH adjustment to 7.0 with NaOH (1 M), and the mixture was then hatched for 60 min. After each stage, the digestion was completed by heating at 368 K for 5 min. A 4 mL aliquot of each sample was obtained after each stage and stored at 253 K until needed (Fig. 2).

2.6. Characterizing the digested β-Lg-C3G/R complexes

1) The OPA method was followed to quantify the degree of hydrolysis (DH) of each assimilated mixture following the methodology of Rutherford (2010). The centrifugation of digested samples was done using a centrifuge (EN 61326, Dhruvidhi Lifecare Solutions India LLP, India) at 10,000 g during 10 min. After that, the thinned supernatant (400 μL) was blended with OPA-reactant (3 mL) for 2 min at 298 K until the absorbance at 340 nm. A serine concentration range (0–100 mg/L) was used as an ideal curve, and DH was calculated by this equation:

$$DH = \frac{h}{h_{100}}$$

(4)

Where h stands for the hydrolyzed bond numbers (Serine–NH₂–β)/α, α and β stands for the typical amount of dissociation of the α and β-NH₂. The Serine-NH₂ was calculated using the serine ideal curve. Then, htot equals 8.8 mmol g⁻¹ β-Lg, the sum of peptide bonds per protein counterpart (Gosh, Prasad, & Saha, 2017).

2) The solvable β-Lg in the digested supernatants, after centrifugation (4,000 g, 277 K, and 20 min), was measured following the study of Xing et al. (2017). The soluble β-Lg was spectrophotometrically computed at 595 nm via a microplate reader (Thermo Scientific, UK), using β-Lg as the ideal curve. β-Lg-solubility (%) was calculated and equated to β-Lg-free-C3G/R.

3) The SDS-PAGE assessment of each digested β-Lg-C3G/R was performed by combining the soluble supernatant β-Lg (1:4, v/v) with Laemmli buffer (S3401-10VL, Sigma) using the methodology of Shen.
et al. (2017). Once the electrophoresis steps were successfully finished with slab gel electrophoresis (as explained in section 2.4), the gels were scanned with Odyssey Fc-Imaging system. 

4) The particle-size distributions of the digested β-Lg-C3G/R mixtures were measured by light scattering spectrometer (Malvern, United Kingdom), using an angle of 173° + 633 nm and 298 K. The digested β-Lg-C3G/R was directly positioned into disposable cuvettes having an optical path of 1 cm. Each scan consisted of 11 runs, each lasting 10 s and is repeated three times. Afterward, the non-negative least squares method was used to calculate the Z-average diameter and polydispersity key (Carnovale, Britten, Couillard, & Bazinet, 2015).

Finally, using a fluorescent microscope (Nikon Instruments Inc., USA) and X 20- and 40-point lenses, the morphology, and microstructure of the digested β-Lg-C3G/R were examined. Before scanning, the assiliated β-Lg-C3G/R (2 mL) was combined with 40 μL of fluorescein isothiocyanate (FITC, 0.1%; w/v in DMSO, λex/λem = 494/518 nm) and left at 298 K during 1 min. A camera (Nikon DS-Fi 2.5, Tokyo, Japan) was used for taking pictures and then processed with NIS-Elements software. 

2.7. Measuring the conformational alterations of the digested β-Lg-C3G/R complexes 

To understand how C3G/R could alter β-Lg-structure after digestion, variations in surface hydrophobicity of the digested β-Lg-C3G/R were recorded by 1-anilinonaphthalene-8-sulfonic acid (ANS) (fluorescence probe) as reported by Dai et al. (2022). The dilution of digested β-Lg-C3G/R was done with PBS (100 mM, pH 7.0) to β-Lg-dose of 0.005–0.50 mg mL−1. A 4 mL of each diluent interacted with ANS blends (40 μL, 8 mmol/L), and their fluorescence strength was observed at λex/λem of 365 and 484 nm. Liner regression approach was adopted to fit the Fluorescence strength and the corresponding dose of β-Lg. The face hydrophobicity index of β-Lg was the slope of the curve. The free sulphydryl concentration of β-Lg-C3G/R was then investigated according to (Jiang, Zhang, Zhao, & Liu, 2018). Each sample (A 1 mL) was mixed with urea (2 mL, 8 M) and reacted with 5,5’-dithiobis-(2-nitrobenzoic acid) (0.5 mL, 10 mM). The absorbance was measured at 412 nm after 30 min. The free sulphydryl level was evaluated with molar extinction factor of 13,600 M−1 cm−1. Lastly, we used a J-1500 spectrometer at 298 K with a 0.1 cm optical route quartz cell from 195 to 240 nm at a bandwidth of 2 nm and a scan rate of 100 nm/min to assess the conformational fluctuations in the second assembly of β-Lg before and after 120 min of digestion using CD-spectroscopy. The secondary structure elements were then calculated and compared with the control matrix (Venkatachalam & Sathe, 2003).

2.8. Measuring the antioxidant capacity of the digested β-Lg-C3G/R complexes

The antioxidant capability of the digested peptides was assessed by adopting the protocol of Bao et al. (2016) based on the DPPH* assay. The absorbance of DPPH* solution reductions at 515 nm, and the calculation of DPPH radical-sifting ratio was done by Trolox ideal curve of Y = 0.001x + 0.657 (R2 = 0.999) and uttered as Trolox counterparts (mmol TE g−1).

2.9. Statistical analysis

All experiments were reiterated in triplicates, and all outcomes were reported as mean ± standard deviation, with P < 0.05 being regarded statistically significant. ANOVA was used to compare each parameter, tailed by a Tukey’s multiple analogy post-test using SPSS version 22. (IBM, USA). Origin Pro 9.2 (Origin Lab, Co. USA) was utilized to process the post-collection data.

3. Results and discussion

3.1. Mono- and di-glucoside anthocyanins covalently/non-covalently interacted with β-Lg-subunits

We first studied the covalent and non-covalent bonding potential of β-Lg with C3G and C3R using multi-dimensional approaches. Anthocyanins showed the ability to covalently and non-covalently bind together with β-Lg, where the competitive reaction between C3R and β-Lg have been peaked (Fig. 3A). The C3R was noted to interact with β-Lg more extremely than C3G under the non-covalent and covalent binding conditions with ratios of (59 and 81%) and (63 and 87%), respectively (Fig. 3A).

The inherent fluorescence of Tyr and Try remains of β-Lg-C3G/R complexes was utilized to classify the interaction of β-Lg-C3G/R complexes using fluorometric analysis (Ghalandari et al., 2014). As displayed in Fig. 3B, β-Lg regulated the fluorescence tend with the highest peak of 331.8 nm, where it was altered after conjugated with C3G or C3R with values of 332.6, 332.2, 332, and 333.2 nm for C3G-NON, C3G-CO, C3R-NON, and C3R-CO, orderly. Although, the fluorescence strength of the β-Lg-C3G/R matrix declined under the covalent conditions compared to the non-covalent ones. However, C3R-CO and C3G-CO declined the β-Lg fluorescence intensity, whereas C3R scavenge the fluorescence strength of β-Lg than C3G. For instance, 300 μM of C3G and C3R intensely dropped the fluorescence intensity by (80.44 and 70.95%) and (94.66 and 80.18%) under the covalent and non-covalent binding conditions, separately. As depicted in Fig. 3C, linear expression of the Stern–Volmer’s equation showed that the proportion of β-Lg-C3G/R conjugation was suitable (Mohd, Khan, Bano, & Siddiqi, 2010). It was also noticed that C3G and C3R might typically and extremely extinguish β-Lg under the covalent and non-covalent binding conditions because of their Kq-values which are upper than maximum diffusion collision quenching continual value (2.0 × 1010 L·M−1·s−1) (Wang et al., 2016). FTIR and CD spectrometry was employed to investigate the fluctuations in the secondary form of β-Lg (Tang, Li, Bi, & Gao, 2016). As shown in Fig. 3D, the most important three peaks of β-Lg were noticed at 1630, 1540, and 1320 cm−1, corresponding to amid-I (C=O), amid-II (N-H, C=H), and amid-III (N-H), separately. Contrary wise this, covalent and non-covalent interactions of β-Lg with 300 μM of C3G or C3R somewhat pushed the FTIR peak of each amid, representing a complication between C3G/R and β-Lg amides. The bands of C=O spreading of β-Lg-C3G/R developed to be wider and thicker than that of β-Lg-alone, implying that the active groupings of amino acids were conjugated with C3G/R and the secondary form of β-Lg was greatly influenced. Fig. 3E displays the CD spectrum of β-Lg and β-Lg-C3G/R blends. CD ranges of β-Lg presented dual (−) bands at 208 and 223 nm, describing its β-sheet form (Wang et al., 2016). Most importantly, C3R altered the secondary form element ratios of β-Lg better than C3G, suggesting the binding between β-Lg and C3G/R happened. However, this fluctuation had peaked under the covalent binding conditions. As found in Fig. 3D, integration of β-Lg with C3G and C3R slightly decrease α-helix, β-sheet, and random coil substances, while the ratio of β-turn was somewhat increased.

SDS-PAGE and SDS-PAGE/NBT assessments were carried out to catalogue and differentiate the binding of the β-Lg-C3G/R mixture under the covalent and non-covalent interaction conditions. At a basic pH, anthocyanins could catalyze redox cycling in the existence of glycinate and NBT produced superoxide to decrease NBT to an impenetrable for membrane, permitting the recognition of SDS steady, covalently, or firmly bound protein polyphenol conjugates (Yang, Zhe, Deng, Peng, & Li, 2017). As revealed in Fig. 4A, the intensity of SDS-PAGE regarding β-Lg-band marginally declined after non-covalently interacting with C3G and C3R. Conversely, the β-Lg-band considerably declined after covalently networked with anthocyanins, especially the di-glucose one like C3R, showing that the proteins belonging to these bands may be involved in the production of C3G.
and/or C3R cross-linked products. Meanwhile, 300 μM of C3G and C3R deeply generated NBT-stained conjugates with β-Lg under the covalent binding circumstances (Fig. 4 B, lane 3–4). In contrast, there was no obvious NBT staining band when the β-Lg-band was used alone. (Fig. 4 B, lane 1). These findings indicated that semi-solid SDS-hardy conjugate between C3G and/or C3R with β-Lg were developed. Phenols are easily converted to quinones or semi-quinones at physiological pH, with or without enzymic catalysis, which can react with proteins’ nucleophilic assembles (e.g., SH, NH). Comparable articles on the cross-connecting between a protein with anthocyanins were perceived (Jiang et al., 2019). We used MALDI-TOF/MS to validate the covalent adducts for formation between β-Lg with C3G and C3R structural units under the covalent conditions. In general, mass spectra exhibit peaks divided by multiple increases of the interacting molecules’ molecular weight (Ying Zhang et al., 2017). As indicated in Fig. 4 C, in the deficiency of anthocyanins structural units, β-Lg revealed the main peak [M + H]⁺ at m/z of 18356.74, in line with previous (Liu et al., 2018; Sheng et al., 2016). The reaction of β-Lg with C3G and C3R generated recent binary peaks at m/z rates of 18850.74 and 18651.14 that were possibly credited for adding aC3G molecule (449.38 Da) and C3R molecule (595.53 Da) to one β-Lg-backbone. The MALDI-TOF/MS analysis gave an additional aid for forming covalent adducts of β-Lg-C3G/R.

Theoretical chemistry can be a necessary complement to experimental work. It can aid in determining the interaction spots and driving forces of interaction between β-Lg and C3G or C3R. As 3D-diagrammed in Fig. 5 A&B, C3G and C3R conjugated with β-Lg by seven H-bonding, three van der Waals forces, and three π-π forces. Most notably, the interaction of β-Lg to both anthocyanins included roughly 23 amino acid residues, but C3G and C3R did not conjugate with Phe, Tyr, and Trp remains were favored as pepsin cleavage spots. This specified that C3G and C3R might not negatively impact the pepsinolysis of β-Lg. Additionally, C3G and C3R weekly interacted with the lysyl remains of β-Lg that make them available during the pancreatic hydrolyzing. These findings were validated using molecular modeling research to anticipate changes in the secondary structure of β-Lg. It was revealed that the β-LG secondary structure is a combination of α-helix, β-sheet, and random coil with about 162 amino acids (Fig. 5 C&D). Contrariwise, after β-Lg was conjugated with C3G or C3R, its secondary structure was intensely changed, again describing the complexation of β-Lg-C3G/R complexes.
Fig. 4. SDS-PAGE (A), SDS-PAGE/NBT (B), and MALDI-TOF-MS analysis (C) of β-Lg, β-Lg-C3G, and β-Lg-C3R mixtures.

Fig. 5. The schematic analysis of the interactions of C3G-β-Lg (A) and C3R-β-Lg (B) complexes based on the Libdock algorithm and their secondary structure predictable alterations (C) as well as entry’s sequences changes (D).
3.2. Both mono- and di-glucoside anthocyanins encouraged the digestion of β-Lg

In the current study, the effects of mono-glucoside (C3G) and di-glucoside (C3R) on the digestibility of β-Lg after complexing simultaneously under the covalent and non-covalent interaction conditions were studied. Fig. 6A displays the digestibility outlines of β-Lg non-covalently and covalently interacted with C3G and C3R that were conveyed as DH% through the three key stages of the digestion procedure. The gastric and intestinal digestions of all β-Lg-anthocyanin conjugates were steadily increased. However, the covalent interaction between anthocyanins, especially the di-glucoside one, and β-Lg displayed higher DH levels than the other matrix. For instance, 300 μM of C3R maximized the duodenal digestion of β-Lg by about 17.26% compared to the β-Lg-free-anthocyanins. Our findings are well-matched with the latest reports, where Jiang et al. (2019) hypothesized that the rate of protein hydrolysis in the soy-protein-anthocyanin conjugate was boosted by 19%. Small polyphenols, unlike tannins, are thought to have a negative impact on protein digestion (Duodu, Taylor, Belton, & Hamaker, 2003). Stojadinovic et al. (2013), in contrast, discovered that the digestibility of β-Lg was lowered in the existence of polyphenol extracts owing to anti-digestive enzyme effects. The kind of polyphenols and their actions during digestion might be one of the causes for the contradictory outcomes. Calculating the total soluble proteins may also be used to determine protein digestibility. Fig. 6B represented the protein solubility of β-Lg-C3G/R matrices, which was calculated and compared with the values of β-Lg-solution. The protein solubility was insignificantly boosted during the buccal stage without significance among β-Lg-conjugates. Anthocyanins, especially the di-glucoside one, gradually boosted the solubility of the protein throughout the gastric digestion phase. For instance, 300 μM of C3R considerably boosted the sums of solvable proteins by 75.2 and 71.5% after covalently and non-covalently interacting with β-Lg, separately. Likewise, the covalent interaction, especially with the di-glucoside anthocyanins, delivered the greatest amount of solvable β-Lg throughout the intestinal digestion. Pepsin, the acidic pH, and continuous mechanical vibrating were primarily accountable for releasing solvable proteins.

SDS-PAGE is widely used to determine protein breakdown patterns during gastrointestinal digestion. The intensity of β-Lg-bands progressively decreased throughout digestion from the buccal to the intestinal phases, as seen in Fig. 6C. Despite this, the β-Lg-band is still visible following pancreatic hydrolysis, probably because of β-Lg’s digesting difficulties. A similar occurrence was noticed by Tantoush et al. (2011), who noted that β-Lg was very resilient to pepsin and pancreatin hydrolysis. Complexing between β-Lg and C3G/R largely decomposed its bands throughout the digestion, equated to the β-Lg-free-anthocyanins. For example, 300 μM of C3R covalently conjugated with β-Lg reduced its band intensity through gastric and intestinal digestion by 28.23 and 34.54%, equated to the β-Lg-free-C3R which was declined by 22.55 and 31.07%, separately. Furthermore, more protein subunits digested into lesser and lighter peptides were noted in the existence of C3R-Co. These findings proved that C3R could stimulate the digestion of β-Lg more than C3G, especially under the covalent binding conditions, according to the DH and solubility findings (Fig. 6A and B).

Because of their association with protein breakdown during digestion, particle size and fluorescence microscopy pictures are two more direct confirmations that might be utilized to evaluate the in vitro digestion process. Fig. 7 shows the particle size circulations and the fluorescent microscopy snaps of β-Lg-C3G/R under the covalent and non-covalent interaction conditions through the three key steps of the digestion process. During the buccal digestion stage, there was a substantial difference in particle size between the control and β-Lg-C3G/R (P < 0.05). C3G/R boosted the particle size of β-Lg with appreciable coalescence, mostly due to its binding with C3G/R which was previously shown to increase the particle size of protein particles through hydrophobic interactions, H-bonding, and van der Waals forces (Xing et al., 2017). The particle size circulation of β-Lg-C3G/R through the buccal digestion was monodispersed that was well-fitted with their fluorescent snaps (Fig. 8). The particle size of β-Lg-C3G/R also dropped significantly from the buccal to the duodenum due to the enzymatic digestion of β-Lg.
digested, the bulky β-Lg agglomerations became little and medium particles because of the pancreatolysis protein breakdown (Fig. 8). The particle size of β-Lg-C3R-Co was smaller than that of the control after 120 min of the duodenal phase, with D_{4,3} rates of 2.2 and 2.4 μm, separately. In general, particle size and fluorescence snaps indicated that the β-Lg-C3G/R was more digested than the control, with aggregates disappearing mainly during duodenal digestion. When all the findings were considered, it was discovered that C3G/R might improve protein digestibility. On the other hand, the mechanism of anthocyanins’ digestive effects on β-Lg, on the other hand, is yet unknown. As a result, we looked at the impacts of C3G/R on the secondary assemblies of β-Lg, as well as their impact on protein folding and unfolding.

3.3. C3G and C3R fluctuated the conformational structure of β-Lg after gastrointestinal digestion

For the first time, we discovered that in the existence of C3G/R, the digestibility of β-Lg was heightened, particularly following the covalent interaction with C3R. The alterations in the secondary structure of β-Lg caused by C3G/R-complexation most likely caused the improvements in protein digestibility. However, no study has looked at the structural changes in β-Lg-digest caused by anthocyanins, which might improve β-Lg-proteolysis under covalent and non-covalent binding circumstances. Thus, we furtherly investigated the alterations in the secondary structure of digested and non-digested β-Lg in the existence and deficiency of C3G/R under the covalent and non-covalent interaction conditions. Surface hydrophobicity has a thoughtful impact on the interfacial behavior of β-Lg. Up to 72.25 and 70.41% decreases in surface hydrophobicity were noticed of β-Lg solutions after covalently interacting with C3R and C3G, respectively (Fig. 9A). The addition of phenolic chemicals decreased the protein surface hydrophobicity comparable to soy protein (Jiang et al., 2018). This could be accounted for by considering that upon exposure of the hydrophobic groups in the protein structure, the surface hydrophobicity decreased, and the solubility increased. The insertion of polar groups (-COOH and –OH groups) from C3G/R is thought to have reduced surface hydrophobicity. Collectively, the surface hydrophobicity data suggest that β-Lg digestion with C3G/R is different, and the structural motifs available for pepsin attack in each case may differ significantly and warrant further investigations.

Furthermore, the fluorescent probe ANS would have fewer binding sites since anthocyanins connect to hydrophobic amino acid side chain groups on the protein surface (especially the aromatic rings). After interacting with C3G/R, free sulphydryl groups gradually increased, and up to 70% of sulphydryls were lost after joining with C3R under the covalent interaction (Fig. 9B). Partial unfolding of protein structure caused by C3G/R probably helped in the conversion of -SH to S= S, as evidenced by the alterations in fluorescence spectra. Furthermore, C3G/R might be oxidised to the equivalent quinone, catalyzing the shift from sulphydryl to disulfide, resulting in further loss of sulphydryl. The UV-Far-CD spectroscopy was utilized to estimate the secondary structure elements and then to predict the folding/unfolding β-Lg. As portrayed in Fig. 9C, β-Lg significantly (p < 0.05) dropped their secondary structure elements after digestion. The α-helix, β-sheet, and β-turn were greatly reduced, but the random coil was somewhat increased. After 120 min of digestion, C3G/R helped to break down the secondary structural parts of β-Lg. After digestion, α-helix and β-turn peaks were still visible in β-Lg, although they were marginally reduced in the β-Lg-C3R-Co model. For example, Venkatasahalam and Sathe (2003) reported that by treating phaseolin with HCl reduced α-helix and β-sheet by 14.2 and 3%, respectively, while increasing random coils by 10.4%. This could be recognized as the unfolding effects of C3G/R on β-Lg-structure.

Consequently, we validated the alterations in the secondary structure of β-Lg stimulated by C3G/R via the molecular models. We employed the β-Lg homology model and the C3G/R structure to do this. It was proven that modifying the β-sheet, β-turn, and random coil parts

![Fig. 7. The particle size of the digested β-Lg and β-Lg-C3G/R mixtures after buccal (2 min) (A), gastric (60 min) (B), and duodenal (120 min) (C) digestion stages.](image-url)
of C3G and C3R might affect the secondary structure of β-Lg (Fig. 5D). C3G/R was discovered to be able to modify the α-helix component of β-Lg across the entry’s sequences, which matched the experimental data well. Previous findings revealed that partly unwinding the β-Lg structure via C3G/R might aid digestion of β-Lg, particularly under covalent binding circumstances (Khalifa, Zhu, Li, & Li, 2021).

3.4. C3G/R synergistically intermixed with β-Lg-digest and increased its antioxidant capacity

The protein-polyphenol bindings may boost the free radical-scavenging efficiency of proteins. We, therefore, measured the antioxidative effects of peptides released by in vitro gastrointestinal digestion. The buccal digestion negligibly increased the DPPH-values of the digested β-Lg-peptides (Fig. 10). Additionally, the antioxidant activity of β-Lg-digest was increased during gastric and intestinal digestion. Notably, C3G/R significantly increased the radical-scavenging capacity of β-Lg-digest, especially under the covalent binding conditions (P < 0.05). In addition, C3G/R exhibited a synergistic antioxidant effect with β-Lg-hydrolysates in a structure-dependent manner. For instance, 300 μM of C3G and C3R significantly increased the antioxidant effects of β-Lg-hydrolysates by 46.05 and 67.39%, after 120 min of digestion under the covalent binding conditions, respectively. It was also noticed that soy protein-anthocyanin conjugates displayed higher antioxidant capacity than the protein-free-anthocyanin mixtures after gastric and intestinal digestion. A similar synergistic antioxidant phenomenon of β-Lg with tea polyphenols, chlorogenic acid, and sour cherry polyphenols has been described (Cao et al., 2017; Jiang et al., 2018, 2019; Tantoush et al., 2011). It has been suggested that free –OH groups of polyphenols could boost the radical-scavenging capacity of polyphenol-protein mixtures and form stable reaction products (Almajarano, Delgado, & Gordon, 2007).

Fig. 8. The fluorescence microscopy clicks of the digested β-Lg in the presence and absence of C3G and C3R after buccal (2 min) and duodenal (120 min) digestion stages. The snaps were captured using an inverted microscopy (Nikon Eclipse Ti-S, Japan) at 10 x/0.25 magnification.
4. Conclusion

This is the first study of the in vitro digestibility of protein-anthocyanins under the covalent and non-covalent interaction conditions. The study reports molecular mechanisms of the digestive impacts of C3G/R on β-Lg-protein and their utilization as functional ingredients to boost protein digestibility. The study found significant evidence that C3G/R interacted with β-Lg via non-covalent and covalent binding to generate complexes with a partly unfolded structure and higher surface hydrophobicity. The β-Lg/C3G/R binding and structural significantly enhanced solubility, degree of hydrolysis, and protein dissemination during in vitro gastrointestinal digestion. The simulated gastrointestinal digestion experiment further explained that C3G/R helped the digestion of β-Lg into small peptides that were able to synergistically and effectively scavenge the free radicals, especially the di-glucoside anthocyanins under the covalent interaction conditions. Hence, C3G/R have potential to be utilized in protein-rich diets to expand the solubility and digestibility features of proteins and to improve the products oxidative stability.

Declaration of competing interest

No conflict of interest among authors.

Data availability

Data will be made available on request.

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