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Phycocyanobilin-modified β-lactoglobulin exhibits increased antioxidant properties and stability to digestion and heating

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ABSTRACT

β-lactoglobulin (BLG) is a major whey protein with numerous techno-functional properties desirable for the food industry. Phycocyanobilin (PCB), a bioactive pigment of *Arthrospira platensis* with health-promoting effects, covalently binds to BLG at physiological pH. This study investigated the effects of this covalent modification on BLG functional properties. The BLG–PCB adduct possesses enhanced antioxidant properties, and bound PCB protects BLG against free radical-induced oxidation. Despite the similar thermal stabilities of BLG and BLG–PCB, BLG–PCB is less susceptible to covalent and noncovalent aggregation under moderate heat treatment (63 °C, 30 min). Blocked thiol group and reduced hydrophobicity due to hindering of hydrophobic residues by bound PCB, as well as the heat-induced transition of β-sheet to α-helix, contributed to the low susceptibility of BLG–PCB to aggregation. BLG–PCB has a higher resistance to pepsin and pancreatin digestion than BLG and unaltered IgE-binding properties. The improved functional properties of BLG–PCB make it a useful ingredient in the food industry.

1. Introduction

In the recent years, several studies have focused on the development of food ingredients with improved functional properties through physical, chemical, or enzymatic treatments. One of the strategies for improving the functional properties of food is to use food proteins as vehicles for the incorporation and efficient delivery of bioactive food components (Cirkovic Velickovic et al., 2019; Liu, Ma, Gao, & McClements, 2017). Bovine β-lactoglobulin (BLG) is a protein widely employed as a bioactive food component carrier and often used in food fortification. BLG is a globular protein comprising 162 amino acid residues, with a molecular weight of 18.4 kDa in its monomeric state (Kontopidis, Holt, & Sawyer, 2004). It accounts for about 50–60% of total whey protein and is frequently used as an additive in the food industry due to its high nutritional value and many desirable techno-functional properties. It is soluble over a wide pH range, forms gels that provide structure to foods, and possesses excellent emulsification properties as well as water-binding and foaming activities (Singh, 2011). Moreover, BLG proteolysis releases peptides with numerous biological activities, such as antihypertensive, antioxidant, and antimicrobial activities (Hernández-Ledesma, Recio, & Amigo, 2008). High resistance to pepsin digestion is another important feature of BLG, and with its ability to bind numerous ligands, BLG is a potent and stable vehicle for integrating bioactive food components and their controlled release in the gastrointestinal tract (GIT) (Kontopidis et al., 2004). However, BLG is regarded as one of the main whey allergens, and many methods have been explored to reduce its allergenic potential (Shao, Zhang, Zhu, Liu, & Tu, 2020; Wu et al., 2018; Xu et al., 2019). BLG is absent from human milk, and due to its high resistance to pepsin digestion can reach intestinal mucosa almost intact, inducing allergenic reactions in 36% of patients with IgE-mediated cow milk allergy (Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001; Shek, Bardina, Castro, Sampson, & Beyer, 2005).

BLG application as a transporter for noncovalently bound ligands has

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been investigated in many studies (Shpigelman, Cohen, & Livney, 2012; Stojadinovic et al., 2013; Teng, Xu, & Wang, 2015), while covalent BLG modification is a recent approach for tailoring its performance as a food additive. In comparison to noncovalent modifications, covalent modifications, being irreversible in most cases, provide more stable conjugates that are less sensitive to changes in pH and temperature occurring during food processing, making covalent conjugates more suitable as food ingredients. Covalent BLG modifications can occur naturally as well as develop during food processing through glycation in the Maillard reaction (Jiang & Brodkorb, 2012; Karbasi et al., 2020) and in reaction with electrophilic compounds such as aldehydes (Meynier, Rampon, Dalgalarrondo, & Genot, 2004) and quinones (Ali, Homann, Khalil, Kruse, & Rawel, 2013; Sladić et al., 2004). To the best of our knowledge, only a few studies have investigated covalent BLG modifications through binding of bioactive ligands via lysine residues, such as avaron (Sladić et al., 2004) and polyphenols, such as caffeic acid (Abd El-Maksoud et al., 2018), (−)-epigallocatechin-3-gallate (EGCG) (Tao et al., 2019; Wu et al., 2018), and gentisic acid (Li, Pan, Yang, Rao, & Chen, 2019); cysteine, such as shikonin (Albreht, Vovk, & Simonovska, 2012), dihydrolipoic acid (Wijayanti, Oh, Sharma, & Deeth, 2013), allicin, and diallyl disulfide (Wilde, Keppler, Palani, & Schwarz, 2016a); or both, such as allyl isothiocyanate (AITC) (Keppler et al., 2014; Rade-Kukic, Schmitt, & Rawel, 2011), flavor compounds (Anantharamkrishnan, Hoye, & Reineccius, 2020), and chlorogenic acid (Xu et al., 2019).

Using BLG as a bioactive agent carrier improves not only ligand stability and sensory properties, such as undesirable taste and smell (Rade-Kukic et al., 2011; Wilde, Keppler, Palani, & Schwarz, 2016b), but also enhances the functional properties of proteins. Moreover, BLG conjugates with phenolic compounds possess improved antioxidant properties than free protein (Abd El-Maksoud et al., 2018; Ali et al., 2013; Tao et al., 2019) and produce stable foams, while simultaneously improving the solubility and emulsifying properties of proteins (Xu et al., 2019).

Phycocyanin (PC) is a chromoprotein derived from the blue-green alga *Arthrospira platensis*, which contains an open-chain tetrapyrrole chromophore phycocyanobilin (PCB) covalently attached to the apoprotein. Several studies have shown that PC has antioxidant, anti-inflammatory, neuroprotective, and hepatoprotective properties (Romay, González, Ledón, Remirez, & Rimbau, 2003), mainly ascribed to its chromophore PCB. PC is also widely used as a natural protein dye in the food and cosmetic industries to replace often harmful synthetic dyes (Sekar & Chandramohan, 2008).

We have recently shown that PCB covalently binds to the free Cys residue of BLG under physiologically relevant conditions. Considering that the BLG tertiary structure slightly changes upon binding to PCB (Minic et al., 2018), this study sought to further investigate the effects of covalent BLG modification by PCB on some functional properties, such as antioxidant properties, heat-induced changes, *in vitro* pepsin and pancreatin digestibility, and IgE-binding properties, that could affect the usage of PCB-modified protein in the food industry.

2. Materials and methods

2.1. Materials and equipment

PCB was isolated from the protein extract of commercial Spirulina powder (from Hawaiian *Spirulina pacifica*, Nutrex-Hawaii; Kailua-Kona, HI, USA) according to a method described previously (Fu, Friedman, & Siegelman, 1979). PCB concentration was determined using a molar absorption coefficient of 37,900 M^{-1} cm^{-1} at 680 nm (Cole, Chapman, & Siegelman, 1967). BLG was isolated and purified from raw cow milk as previously described (Stojadinovic et al., 2012), and the protein concentration was determined spectrophotometrically using an extinction coefficient of 17,600 M^{-1} cm^{-1} at 280 nm. All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. Ultrapure water (Barnstead Smart2Pure Water Purification System, Thermo Fischer Scientific; Waltham, MA, USA) was used in all experiments. All fluorescence, circular dichroism (CD), and spectrophotometric measurements were performed using a FluoroMax-4 spectrofluorometer (HORIBA Scientific; Kyoto, Japan), a Jasco J-815 spectropolarimeter (JASCO; Tokyo, Japan), and a NanoDrop 2000c spectrophotometer (Thermo Fischer Scientific; Waltham, MA, USA), respectively.

2.2. Covalent BLG modification with PCB

A covalent BLG–PCB adduct was prepared and characterized exactly as described in Minic et al. (Minic et al., 2018), by incubating a mixture of 200 μM BLG and 300 μM PCB in 20 mM sodium phosphate buffer (pH 7.2). The reaction yield of the obtained conjugate was around 70%. The details of BLG–PCB preparation are provided in the Supplementary material.

2.3. Antioxidant properties of BLG–PCB

2.3.1. Antioxidant assays

The fluorescence-based oxygen radical absorbance capacity (ORAC) assay was performed based on a previously proposed method (Ou, Hampsch-Woodill, & Prior, 2001). The radical scavenging activity of 30 μM BLG and BLG–PCB was assayed using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) as described previously (Re et al., 1999). The reducing power of 150 μM BLG and BLG–PCB samples was determined by slightly modifying a previously reported method (Chawla, Chander, & Sharma, 2009). The experimental details of the antioxidant assays are provided in the Supplementary material.

2.3.2. BLG and BLG–PCB oxidation by free radicals

BLG and BLG–PCB radical-mediated oxidation were monitored by measuring the decay of protein intrinsic fluorescence over time. The experimental details are provided in the Supplementary material. The results were fitted to first-order kinetics for calculating the fluorescence decay rate constants, while areas under respective curves (AUC) were used for calculating the protective effect (PE) of PCB against BLG oxidation according to the following equation:

$$PE(a.u) = \frac{AUC_{\mathrm{BLG-PCB}} - \mathrm{AUC_{BLG}}}{\mathrm{AUC_{BLG-PCB}}} \times 100\%$$

2.4. Heat-induced changes of BLG and BLG–PCB

2.4.1. Heat treatment of BLG and BLG–PCB

BLG and BLG–PCB samples (both 70 μM) in 20 mM sodium phosphate buffer (pH 7.2) were heat-treated under conditions resembling those during batch pasteurization (63 °C for 30 min).

2.4.2. Sodium dodecyl sulfate (SDS)– and native–polyacrylamide gel electrophoresis (PAGE) of heated BLG and BLG–PCB

The degree and profile of heat-induced protein aggregation of BLG and BLG–PCB was assessed by denaturing and native PAGE, and protein components were resolved on 14% polyacrylamide gels. Reducing SDS–PAGE was performed with the addition of β-mercaptoethanol to the sample buffer. Native PAGE was performed without SDS and β-mercaptoethanol in polyacrylamide gels and sample buffer.

2.4.3. Surface hydrophobicity

The surface hydrophobicity of heated BLG and BLG–PCB was investigated using 1-anilino-8-naphthalensulfonate (ANS) as a hydrophobic probe. The fluorescence spectra of 10.9 μM BLG and BLG–PCB saturated with 80 μM ANS in 20 mM sodium phosphate buffer (pH 7.2) were recorded in the 360–620 nm range, using excitation at 350 nm, under thermostatic conditions (25 °C). The bandwidths of the slits were set to 5 nm. Unheated samples were also included as a control. The

contribution of protein emission was subtracted from the fluorescence spectra of the corresponding protein samples containing ANS. The results are expressed as the ratio of fluorescence intensities at 494 nm, corresponding to the wavelength of emission maximum of ANS bound to protein, and 524 nm, corresponding to the emission maximum of free ANS (F_{494nm}/F_{524nm}).

2.4.4. CD spectroscopy

Far-UV CD spectra of heated 70 μM BLG and BLG–PCB samples at pH 7.2 were recorded in the 200–260 nm range under thermostatic conditions (25 °C). The CD spectra of control, unheated, BLG, and BLG–PCB samples were also recorded. The spectra were recorded in 0.1 nm steps with 50 nm/min scan speed, using a quartz cell with 0.01 cm path length. The obtained spectra are the averages of two accumulated scans. The spectra of the buffer and PCB were subtracted from the spectra of BLG and BLG–PCB, respectively. The secondary structure content was calculated using the CONTIN algorithm available in the CDPro software package. SP37 database was used.

2.4.5. Detection of amyloid-like fibrillar protein structures

To examine the possible formation of fibrillar structures in solution upon thermal treatment, thioflavin T (ThT) binding was monitored after heating BLG and BLG–PCB for 30 min at 63 °C or 2 h at 85 °C. BLG and BLG–PCB (3.5 μM) were added to 20 μM thioflavin T in 20 mM sodium phosphate buffer (pH 7.2). The fluorescence emission intensity at 485 nm was monitored using an excitation wavelength of 435 nm (bandwidths of 5 nm). The fluorescence spectra of the protein samples in the absence of ThT and the spectra of ThT alone were subtracted.

2.4.6. Thermal stability of BLG and BLG–PCB

Thermal denaturation of 2 μM BLG and BLG–PCB at pH 7.2 (20 mM sodium phosphate buffer) and 2.5 (100 mM Gly-HCl) was analyzed at 35–91 °C (with 2 °C increments). The fluorescence spectra were measured between 310 and 370 nm with excitation at 280 nm. The equilibration time at each temperature was set to 1 min. The contribution of PCB fluorescence at each temperature was subtracted from the corresponding BLG–PCB spectra. The data were expressed as temperature dependencies of the fluorescence ratios F_{350nm}/F_{336nm}, normalized to 0–100%. The fluorescence intensity at 350 nm (F_{350nm}) corresponds to the emission maximum of free tryptophan in a water solution (Albani, 2014), and the fluorescence intensity at 336 nm (F_{336nm}) corresponds to the emission maximum of BLG. The obtained curves were fitted with a sigmoidal function, with the inflection point representing the melting temperature (T_m) of BLG.

2.5. Pepsin and pancreatin in vitro digestibility assays

The pepsin and pancreatin digestibility assays were performed using simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), respectively. BLG and BLG–PCB digestibility was estimated using SDS–PAGE under reducing conditions. The experimental details are provided in the Supplementary material.

2.6. IgE-binding properties of BLG and BLG–PCB

2.6.1. Western blot

Following SDS–PAGE, BLG and BLG–PCB were transferred on a polyvinylidene difluoride (PVDF) membrane using an electroblotting system (VWR; Darmstadt, Germany). Sera from 10 individuals previously shown to react to milk using immunoCAP were pooled and used further. Pooled serum was prepared by mixing equal amounts of 10 persons' sera with total IgE levels to milk determined to be: 10.5 kU/L, 47.3 kU/L, 18.4 kU/L, >100 kU/L, 20 kU/L, 71 kU/L, 12.9 kU/L, 5.3 kU/L, >100 kU/L, 9.67 kU/L. The membrane was probed with the serum pool diluted four times in 0.1% BSA in Tris-buffered saline containing Tween-20 (TBST; 20 mM Tris buffer, 0.9% NaCl, 0.2% Tween-20; pH 7.4) to examine the binding of IgE from the allergenic serum pool. The bound IgE was detected using rabbit anti-human IgE antibodies (MIAB; Uppsala, Sweden; 2000 times diluted in TBST containing 0.1% BSA), followed by incubation with alkaline phosphatase-labeled goat anti-rabbit IgG antibodies (AbD Serotec; Kidlington, United Kingdom; 10,000 times diluted in TBST containing 0.1% BSA). Immunoblot was visualized with a substrate solution containing 1.5 mg 5-bromo-4-chloro-3-indolyl phosphate and 3 mg nitroblue tetrazolium in 10 mL 100 mM carbonate-bicarbonate buffer containing 5 mM $MgCl_2$, pH 9.5.

2.6.2. ELISA inhibition

The IgE-binding properties of BLG and BLG–PCB were quantitatively assessed using an inhibition ELISA. A 96-well microplate (NUNC Maxisorp, Thermo Fischer Scientific; Roskilde, Denmark) was coated with 100 μL/well 25 μg/mL BLG diluted in coating buffer (50 mM carbonate-bicarbonate buffer; pH 9.6) and incubated overnight at 4 °C. The remaining binding sites were blocked at room temperature with 300 μL 1% BSA in TBST for 1 h. Serial three-fold dilution of BLG and BLG–PCB (initial concentration: 1.8 mg/mL) was pre-incubated 1:1 (V:V) with the serum pool (final dilution of serum pool: 8-fold in blocking buffer) for 1 h at 37 °C before adding to the plate for overnight incubation at 4 °C. The wells were washed three times with TBST between each step. The bound IgE was detected by incubation with 100 μL mouse anti-human IgE monoclonal antibody (Abcam; Cambridge, UK; 2000 times diluted in TBST containing 0.1% BSA) conjugated to horseradish peroxidase for 1 h at room temperature. ELISA was visualized with 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate (0.1% TMB, 0.006% H_2O_2 in 100 mM citrate-phosphate buffer; pH 5). The reaction was stopped 20 min after substrate addition with 30 μL 2 M H_2SO_4, and the absorbance at 450 nm was measured. IgE-binding inhibition was calculated as $[(OD_{no\ inhibitor} - OD_{inhibitor})/OD_{\ no\ inhibitor}] \times 100\%$. The results were fitted to a five-parameter logistic curve. The concentration needed to inhibit 50% of the signal was calculated (IC_{50}) and used to compare the IgE-binding potencies of the samples.

2.7. Statistical analysis

The data are presented as the mean ± S.D. Unless otherwise stated, all experiments were conducted three times. Data were analyzed using Student's t-test or one-way ANOVA with Tukey's multiple comparison test at a significance level of 0.05.

3. Results

3.1. Antioxidant properties of BLG–PCB

3.1.1. Antioxidant potential of BLG–PCB

The antioxidant potential of BLG–PCB and BLG was evaluated *in vitro* using ORAC, ABTS radical scavenging activity, and reducing power assays.

For the ORAC test, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was used as a peroxyl radical source that directly reacts with fluorescein, producing an oxidatively modified non-fluorescent product. The fluorescence intensity at 511 nm as a function of time (Fig. 1A) demonstrated that fluorescence decay was delayed in the presence of antioxidants (BLG, BLG–PCB, and Trolox), with BLG–PCB being more potent than free BLG in delaying fluorescence decay. Although BLG quenched peroxyl radicals (scavenging activity 5.3 ± 0.1 Trolox equivalents [TE]), BLG–PCB showed significantly higher ($p < 0.05$) radical scavenging activity (7.6 ± 0.3 TE).

The ABTS radical scavenging capacities of BLG and BLG–PCB are shown in Fig. 1B. The ABTS radical scavenging activity of BLG–PCB was 41.1 ± 0.6%, which was significantly higher ($p < 0.05$) than that of the control BLG sample (21.3 ± 0.5%).

The reducing power of BLG and BLG–PCB (Fig. 1C) are in accordance

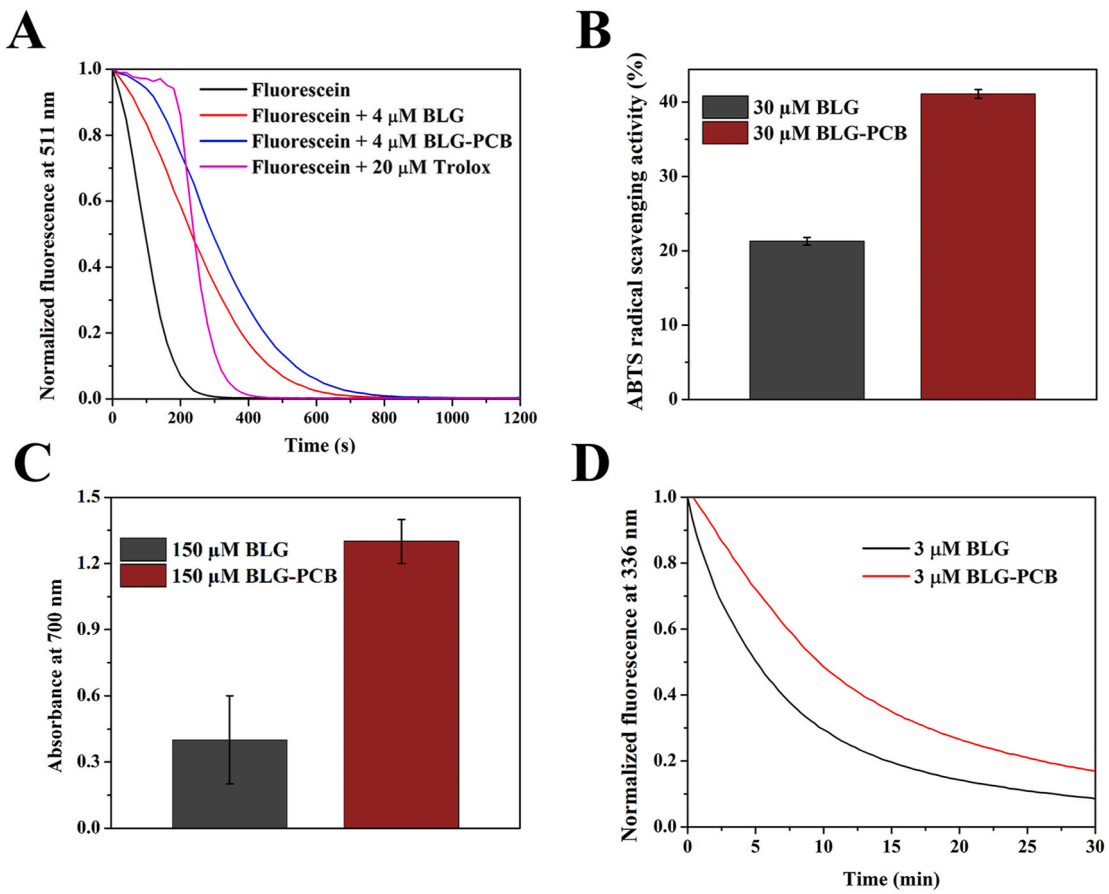

Fig. 1. (**A**) 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced fluorescein oxidation in the absence and presence of 4 μM β-lactoglobulin (BLG), 4 μM BLG–phycocyanobilin (PCB) and 20 μM Trolox. (**B**) 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity of BLG and BLG–PCB (both 30 μM). (**C**) Reducing power of BLG and BLG–PCB (both 150 μM). (**D**) AAPH-induced fluorescence decay of BLG and BLG–PCB (both 3 μM).

with the results of the ORAC and ABTS radical scavenging activity assays. BLG–PCB had a high reducing power, reflected through the significantly higher ($p < 0.05$) absorbance at 700 nm in the presence of BLG–PCB (1.3 ± 0.1) than in the presence of equivalent BLG amount (0.4 ± 0.2), suggesting the exceptional antioxidant capacity of BLG–PCB.

3.1.2. *Protective effect of PCB against free radical-induced BLG oxidation*

Protein oxidation is assessed by monitoring changes in tryptophan fluorescence upon free radical-induced oxidation (Fuentes-Lemus et al., 2016). Free radical-induced BLG and BLG–PCB oxidation was monitored by measuring the changes in protein intrinsic fluorescence upon adding AAPH, a free radical generator. The curves of the AAPH-induced decrease in fluorescence at 336 nm (BLG emission maximum upon excitation at 280 nm) in both BLG and BLG–PCB (Fig. 1D) follow

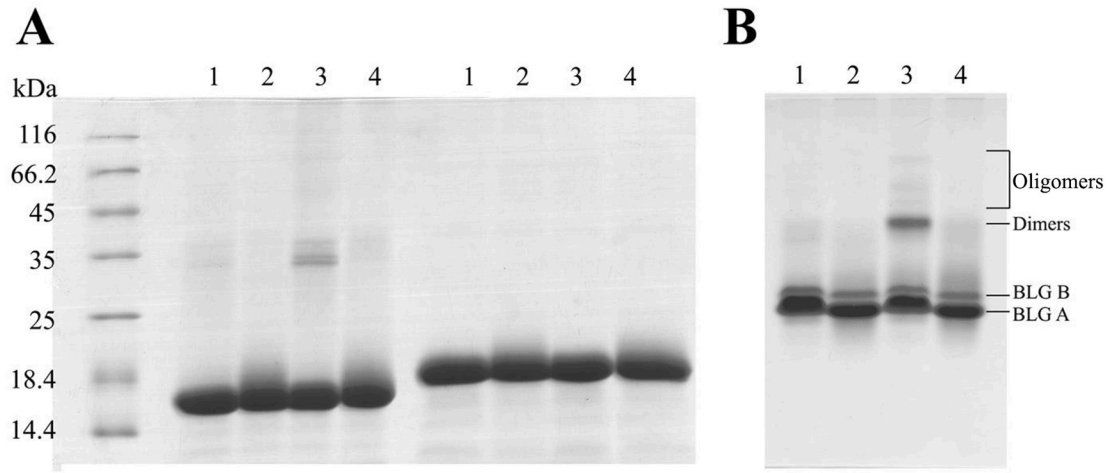

Fig. 2. Electrophoresis of (1) unheated β-lactoglobulin (BLG) and (2) BLG–phycocyanobilin (PCB) and (3) BLG and (4) BLG–PCB heated at 63 °C for 30 min under (**A**) denaturing (**left**) non-reducing and (**right**) reducing and (**B**) native conditions.

first-order kinetics. The calculated fluorescence decay rate constant for BLG–PCB was 0.068 ± 0.016 min^{-1}, which was significantly ($p < 0.05$) lower than that of BLG (0.117 ± 0.014 min^{-1}), indicating that covalently bound PCB protects BLG against oxidation. The PE of PCB was calculated to be 33.6 ± 4.9%.

3.2. Characteristics of BLG–PCB upon heat treatment

3.2.1. BLG–PCB aggregation upon moderate heating

To gain insight into the susceptibility of BLG–PCB to aggregation upon moderate heat treatment of importance for the food industry, unmodified BLG and BLG–PCB were subjected to batch pasteurization conditions (heated at 63 °C for 30 min). Electrophoretic protein profiles under denaturing conditions (Fig. 2A) show that covalent BLG modification slightly smears the band corresponding to the BLG monomer toward higher molecular mass due to attachment of one or more 586.7 Da-large PCB molecules, as obtained in our previous work (Minic et al., 2018). SDS–PAGE of heated BLG under non-reducing conditions (**A left**) showed a 36 kDa band corresponding to BLG dimer mass. On the contrary, only traces of dimers in heated BLG–PCB clearly show that covalently modified BLG cannot form dimers. SDS–PAGE of the same samples under reducing conditions (**A right**) unambiguously demonstrate that dimers present under non-reducing conditions are bonded by disulfide linkages. The almost complete absence of dimers in heated BLG–PCB is expected, as the free thiol group of BLG forms a covalent bond with PCB and cannot form a disulfide bond with another BLG molecule.

However, native PAGE (Fig. 2B) revealed the presence of non-covalent high molecular weight aggregates in heated BLG that were not present in BLG–PCB, implying that covalent modification not only affects the ability of BLG to form disulfide dimers, but also reduces soluble noncovalent aggregate formation. Native PAGE also demonstrated slightly higher mobility of BLG–PCB, despite its higher mass due to PCB binding. Moreover, the isoelectric point of BLG shifted toward an acidic pH, as confirmed by isoelectric focusing in an immobilized pH gradient (IPG) strip (Fig. S1; experimental details are provided in the Supplementary material). Therefore, PCB binding shifts the isoelectric point of BLG as a consequence of the presence of two carboxyl groups in PCB.

3.2.2. Surface hydrophobicity of BLG and BLG–PCB upon moderate heating

The binding of the hydrophobic probe ANS, whose fluorescence intensity increases upon binding to the hydrophobic sites on protein surfaces, is a sensitive tool for monitoring protein surface hydrophobicity. Differences in fluorescence intensities upon ANS binding to heated or unheated BLG and BLG–PCB (Fig. 3A) show that covalent BLG modification with PCB significantly ($p < 0.05$) decreases ANS fluorescence, indicating the lower surface hydrophobicity of BLG–PCB than that of the unmodified protein. This can be explained by the hindrance of hydrophobic residues by bound PCB as the PCB binding site lies in a highly hydrophobic cavity of BLG (calyx) (Minic et al., 2018). Upon heating BLG, ANS fluorescence slightly decreased ($p < 0.05$), while it did not change in BLG–PCB. This can be explained by the oligomerization/aggregation of BLG upon heating, which reduces the availability of hydrophobic patches for ANS binding. In contrast, an almost complete absence of oligomerization/aggregation in heated BLG–PCB enables the level of ANS binding to be similar to that of unheated BLG–PCB.

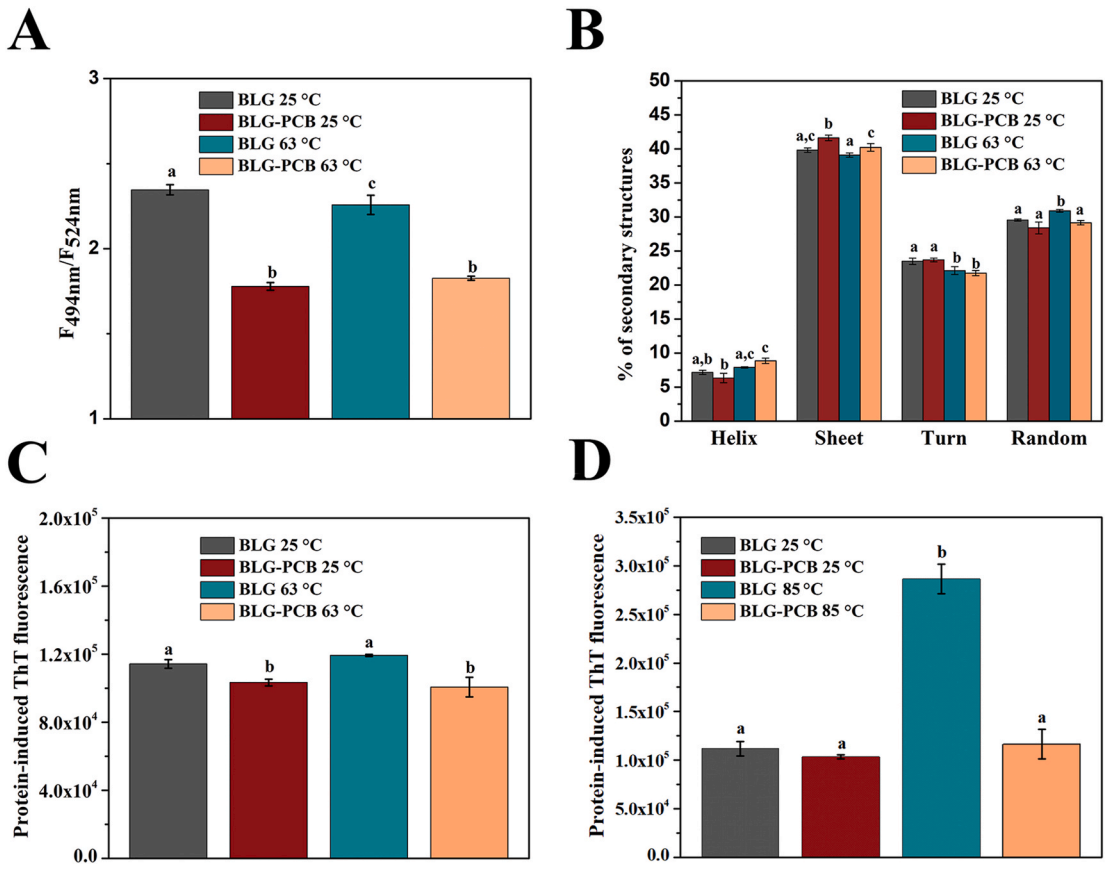

Fig. 3. The behavior of β-lactoglobulin (BLG) and BLG–phycocyanobilin (PCB) before and after heating at 63 °C for 30 min or 85 °C for 120 min. The fluorescence intensities due to (**A**) 1-anilino-8-naphthalenesulfonate (ANS) binding, (**B**) secondary structure content, and thioflavin T (ThT) fluorescence enhancement upon heating at (**C**) 63 °C and (**D**) 85 °C. Data were analyzed using one-way ANOVA with Tukey's multiple comparison test at a significance level of 0.05. Means with different letters (a to c) differ significantly ($p < 0.05$).

3.2.3. The changes in secondary BLG–PCB structure upon moderate heating

Far-UV CD spectroscopy was employed to investigate the structural changes upon heating BLG and BLG–PCB at 63 °C for 30 min. Secondary structure content estimation (Fig. 3B) shows that moderate BLG heating increases random coils at the expense of β-turns, without affecting the helical and β-sheet content. Covalent BLG modification with PCB alters the heat-induced behavior of the protein, since heating BLG–PCB induces β-strand–α-helix transition with a slight decrease in β-turns, but without changing the random coil content. This implies that while moderate heating decreases the ordered structures in unmodified BLG, it only rearranges the secondary structures in BLG–PCB, suggesting that bound PCB maintains the ordered structure content.

3.2.4. BLG–PCB amyloid-like structure formation upon moderate heating

BLG fibrils are extensively formed under extreme conditions (low pH, prolonged heating at temperatures above its denaturation temperature, low ionic strength, and the presence of denaturants). However, as BLG is prone to fibrillation, BLG fibrils have also been reported to form at a lower extent under more moderate conditions (neutral pH and shorter incubation times) (Sardar, Pal, Maity, Chakraborty, & Halder, 2014; Zúñiga, Tolkach, Kulozik, & Aguilera, 2010). ThT is a commonly used fluorescent probe that specifically binds to amyloid fibrils and similar β-sheet-rich oligomeric structures because its fluorescence intensity at 485 nm is enhanced upon binding to such structures (Biancalana & Koide, 2010). Cross β-structure formation upon heating BLG and BLG–PCB at 63 °C for 30 min was monitored by ThT binding. Unheated BLG–PCB demonstrated significantly ($p < 0.05$) lower ThT emissions than unheated BLG (Fig. 3C). This result is in accordance with the low surface hydrophobicity of BLG–PCB. In fact, in addition to binding to the cross β-structure of amyloid fibrils, ThT also binds to the hydrophobic structures (Biancalana & Koide, 2010). However, no statistically significant change was observed for either BLG or BLG–PCB (Fig. 3C) upon moderate heat treatment. Zenker et al. observed only a slight increase in ThT emission after heating BLG at 60 °C for 24 h (Zenker et al., 2020). This suggests that although PCB binding slightly changes the BLG structure, it does not increase its susceptibility to fibril formation induced by mild heat treatment over a short time. On the contrary, heat treatment at 85 °C for 120 min (Fig. 3D) significantly increased ThT emission ($p < 0.05$) of BLG, while it did not affect ThT emission of BLG–PCB upon the same heat treatment ($p > 0.05$), implying that BLG–PCB is less susceptible not only to aggregation but also to amyloid fibrils formation, indicating that covalent BLG modification by PCB reduces BLG susceptibility to fibril formation induced by high-temperature treatment for a long period.

3.2.5. Thermal stability of BLG–PCB

To investigate the effects of PCB conjugation on the thermal stability of BLG, the decrease in intrinsic BLG fluorescence (emission at 336 nm, excitation at 280 nm) was monitored during thermal denaturation at pH 7.2 and pH 2.5. The melting temperature of BLG–PCB was similar to that of BLG both at pH 7.2 (76.3 ± 1.1 °C and 75.9 ± 0.7 °C, respectively; $p > 0.05$; Fig. 4) and pH 2.5 (84.3 °C ± 3.3 and 85.3 °C ± 2.0, respectively; $p > 0.05$; Fig. S2), indicating that thermal stability is not affected by ligand binding.

3.3. Impact of covalent modification by PCB on BLG digestibility

BLG and BLG–PCB digestibility was assessed by simulated gastric and intestinal *in vitro* digestion, and the relation between the BLG band (about 18 kDa) intensity and digestion time was monitored by SDS–PAGE under reducing conditions.

3.3.1. Pepsin digestibility of BLG–PCB

The electrophoretic profiles of BLG and BLG–PCB during 6 h of pepsin digestion are shown in Fig. 5A. BLG–PCB is more resistant to pepsinolysis than BLG, as evidenced by the persistence of 18.4 kDa band

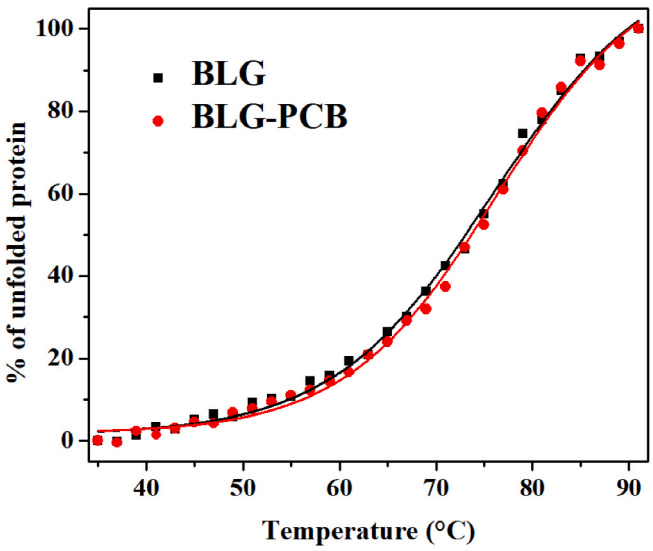

Fig. 4. Thermal stability of 2 μM β-lactoglobulin (BLG) and 2 μM BLG–phycocyanobilin (PCB) (excitation at 280 nm) at pH 7.2.

throughout digestion. Changes in 18.4 kDa band intensity, obtained from the densitometric analysis of the gels, were plotted against digestion time (Fig. 5B). The rate of decrease was fitted to a first-order equation: The half-life ($T_{50\%}$) of unmodified BLG was estimated to be 18.1 ± 3.9 min, while covalent modification by PCB significantly ($p < 0.05$) prolonged the half-life to 40.6 ± 0.7 min.

3.3.2. Pancreatin digestibility of BLG–PCB

The electrophoretic profiles of BLG and BLG–PCB during 4 h of pancreatin digestion are shown in Fig. 5C. Covalent modification by PCB attenuated BLG digestion by pancreatin, but the effect was not as pronounced as the effect on gastric digestion. The densitometrically obtained band intensity at each time point was plotted against the digestion time (Fig. 5D). Under the given experimental conditions, the half-life of unmodified BLG was estimated to be 2.7 ± 0.9 min, while BLG–PCB had a half-life of 5.1 ± 0.6 min.

3.4. Impact of covalent modification by PCB on the IgE-binding properties of BLG

BLG and BLG–PCB were assayed by Western blot and inhibition ELISA for the binding to milk-allergic patients' IgE. Western blot probed with the serum pool from milk-allergic human subjects revealed that both BLG and BLG–PCB bind to the IgE from the milk-allergic serum pool (Fig. 6A). Additionally, the IgE-binding properties of proteins were assessed quantitatively using inhibition ELISA. As shown in Fig. 6B, both BLG and BLG–PCB show the same pattern of dose-dependent inhibition of milk-allergic patients' IgE binding to unmodified BLG, with their inhibition plots virtually overlapping, indicating that IgE-binding potency does not change significantly upon BLG modification by PCB. Indeed, the IC_{50} value of BLG was $6.4 \times 10^{-5} \pm 6.9 \times 10^{-7}$ mg/mL, while that of BLG–PCB was $6.2 \times 10^{-5} \pm 2.1 \times 10^{-7}$ mg/mL ($p > 0.05$, $n = 2$).

4. Discussion

In this study, we investigated the functional properties of conjugates formed spontaneously at physiological pH and 37 °C between the free cysteine (Cys 121) of food protein BLG and PCB, the bioactive chromophore of *A. platensis*. Having in mind that the BLG structure is slightly changed upon binding to PCB, positioned in BLG calyx (Minic et al., 2018), some functional properties that could affect the usage of PCB–modified protein in the food industry were investigated.

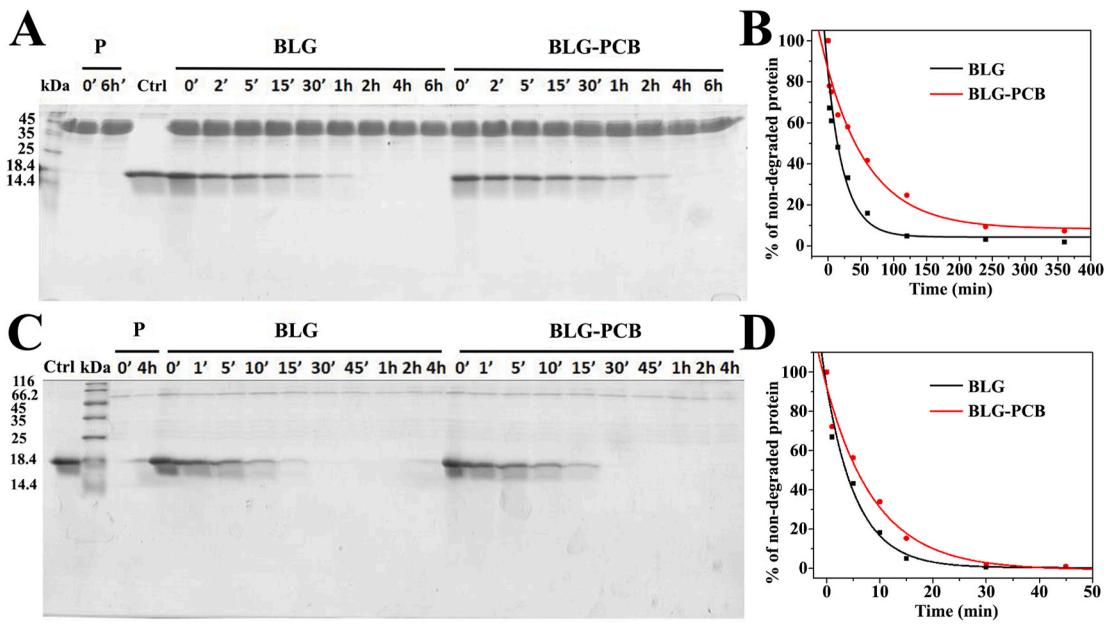

Fig. 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis (16% gel) of β-lactoglobulin (BLG) and BLG–phycocyanobilin (PCB) digested with (**A**) pepsin and (**C**) pancreatin. P – pepsin/pancreatin control; Ctrl – protein stability control. Time-dependence of undigested BLG and BLG–PCB during (**B**) 6 h of pepsin and (**D**) 4 h of pancreatin digestion. BLG/BLG–PCB band intensity at 0 h was considered 100%.

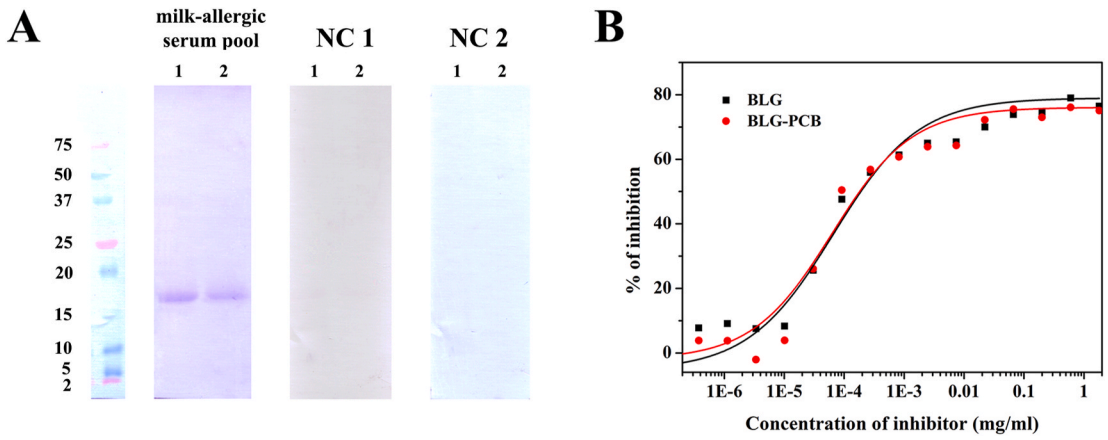

Fig. 6. (**A**) Western blot analysis of (1) β-lactoglobulin (BLG) and (2) BLG–phycocyanobilin (PCB) probed with milk-allergic patients' serum pool and rabbit anti-human IgE, followed by incubation with goat anti-rabbit IgG-AP conjugate. NC 1 – negative control 1, serum of non-allergic person; NC 2 – negative control 2, no patient serum. (**B**) ELISA inhibition: IgE-binding of milk-allergic patients' serum pool to unmodified BLG was inhibited by both BLG and BLG–PCB.

BLG exhibits high radical scavenging activity due to the presence of four Met, two Trp, two His, four Tyr, and primarily one free sulfhydryl of Cys-121, all of which have electron/hydrogen atom donating ability and are therefore responsible for the radical scavenging activity of BLG in the ORAC assay (Zheng, Zhao, Dong, Su, & Zhao, 2016). Although the free Cys residue in BLG–PCB is blocked by forming thioether bond with PCB, the antioxidant activity of this BLG adduct is strikingly higher than that of unmodified protein, due to the presence of PCB, which possesses exceptional antioxidant capacity like other tetrapyrroles, such as bilirubin (Benedetti, Benvenuti, Scoglio, & Canestrari, 2010; Jansen et al., 2010). Thus, despite BLG having more than 10 amino acid residues that can contribute to total ORAC activity, binding of only one PCB molecule per BLG molecule increases its antioxidant activity by almost 50%, contributing to the ORAC value of the modified protein by more than 30%. Moreover, the ABTS assay further confirmed the excellent antioxidant activity of BLG–PCB, which was almost two-fold higher than that of unmodified BLG. As Cys residue is the only residue showing notable reducing power in the reducing power assay (Zheng et al., 2016), blocking the free –SH group of the protein through the formation of a covalent bond with PCB might decrease the total reducing power of the protein. However, the reducing power of BLG–PCB was almost three times that of free protein, suggesting that most BLG–PCB antioxidant potential originates from bound PCB *per se*. Indeed, it was shown that the reducing power of the total pepsin digest of C-phycocyanin almost entirely originates from PCB (Minic et al., 2016). Moreover, covalent BLG modification by PCB efficiently inhibited free radical-induced oxidation of the protein itself, where bound PCB has shown a PE of 31.5 ± 4.4%.

As BLG is used as an emulsifier and stabilizer of oil-in-water emulsions, several studies have attempted to increase its antioxidant capacity by covalent modification, such as glycation in the Maillard reaction (Chobert, Gaudin, Dalgalarrondo, & Haertlé, 2006) and conjugation with polyphenols (Abd El-Maksoud et al., 2018). In addition, BLG covalently modified with catechin can be used as an emulsifier in oil-in-water nanoemulsion delivery systems, stabilizing β-carotene more efficiently than unmodified protein (Yi, Zhang, Liang, Zhong, & Ma,

2015). Therefore, BLG–PCB could be widely used as an effective surface-active agent with extraordinary antioxidant capacity. Moreover, it also has excellent potential for developing BLG-based edible films for food packaging, providing efficient protection against the oxidation of sensitive food components.

BLG aggregation is initiated by unfolding of its globular structure, which exposes hydrophobic surfaces and a reactive free thiol group during heating. This thiol group is involved in sulfhydryl–disulfide exchange reactions to form polymers with other BLG molecules or other proteins, while the exposed hydrophobic surfaces are engaged in noncovalent aggregate formation. Several studies have attempted to protect BLG during thermal processing and prevent its denaturation and aggregation, such as adding sulfhydryl reagents because of their ability to form covalent derivatives with BLG free thiol group that cannot participate in aggregation reactions. Food-compatible sulfhydryls like dihydrolipoic acid and glutathione were investigated to reduce BLG and whey protein isolate aggregation during heating (Wijayanti, Bansal, Sharma, & Deeth, 2014; Wijayanti et al., 2013). This study demonstrates that upon moderate heating, under conditions resembling those during batch pasteurization (63 °C for 30 min), the formation of both covalent and noncovalent heat-induced protein polymers and aggregates proceeds at a much lower extent in BLG–PCB than that in unmodified BLG. A low level of covalent disulfide polymers in BLG–PCB is a consequence of the blocked free thiol group by bound PCB. The reduced extent of high molecular weight noncovalent aggregate formation in BLG–PCB is due to its low surface hydrophobicity, resulting from the hindering of hydrophobic residues by bound PCB. Moderate heating decreased the ordered structure, but not β-sheet, content in BLG. In contrast, the content of ordered structures in heated BLG–PCB was maintained, but with the transition of β-sheet to α-helix, which could contribute to the reduced susceptibility of BLG–PCB to aggregation. The α-helix-rich non-native BLG conformation is resistant to aggregation, and BLG becomes prone to aggregation when its non-native α-helical structure is converted to a β-sheet structure (Pourjabbar, Hassani, & Sajedi, 2015). Interestingly, the thermal stability of the protein did not change upon PCB binding. Although mild heat treatment for a short time (63 °C for 30 min) did not result in fibril-like structure formation, treatment at high temperature for a long time (85 °C for 120 min) resulted in a lower increase for heated BLG–PCB than for BLG. The steric constraints of the large tetrapyrrole moiety could also contribute to the lower heat-induced amyloid-like fibril formation in BLG–PCB. BLG glycation was shown to inhibit is fibrillation, and lactosylation had a more substantial effect than glucosylation, irrespective of the glycation degree (Dave, Loveday, Anema, Jameson, & Singh, 2014). Therefore, this study suggests that, in addition to dihydrolipoic acid and glutathione, PCB is a promising food-grade sulfhydryl modifying agent that can reduce BLG polymerization, aggregation, and fibril formation during heating.

BLG has a remarkably stable tertiary structure at acidic pH because of the presence of many charged and polar amino acid residues, high rigid β-structure content, and two disulfide bridges that stabilize its structure, thus making BLG highly resistant to pepsin digestion (Kontopidis et al., 2004). On the contrary, it undergoes several conformational changes as pH increases (Qin et al., 1998), making it susceptible to pancreatin digestion (Schmidt, Meijer, Slangen, & Van Beresteijn, 1995). Covalent modification by PCB significantly affected both pepsin and pancreatin digestibility of BLG, with the PCB-modified protein being slowly digested in both cases, particularly with pepsin. PCB binding to BLG might have caused steric hindrance of proteolytic cleavage sites, thus limiting the access to enzymes, which reduces digestion. Similarly, covalent BLG modification by glycation with galactooligosaccharides reduced BLG digestion (Sanz, Corzo-Martínez, Rastall, Olano, & Moreno, 2007). The high resistance of covalently modified BLG to pepsin and pancreatin could thus be used to efficiently deliver bioactive PCB to distal parts of the GIT, contributing to GIT health. Jiang & Jiu suggested that the BLG-conjugated linoleic acid (CLA) complex could be used to deliver CLA to target colon cancer (Jiang & Liu, 2010). Due to its resistance to proteolytic degradation, BLG has been suggested as a potent vehicle for the delivery of various bioactive ligands (Teng et al., 2015). In its covalently modified form, it seems to be a promising vehicle, as EGCG release from BLG–chlorogenic acid (CA) conjugate-based nanoparticles was slower and less than that from BLG nanoparticles alone in simulated gastrointestinal digestion fluid due to the high resistance of BLG–CA to proteolysis (Fan, Zhang, Yokoyama, & Yi, 2017). Therefore, the low propensity of the modified BLG to enzymatic digestion could enable prolonged delivery of not only modifying bioactive molecules, such as PCB, but also encapsulated bioactive molecules, as well as bioactive peptides originating from BLG itself. Moreover, we have previously shown the cytotoxic activities of chromopeptides, obtained after pepsin digestion of phycocyanin, toward human epithelial colonic carcinoma cells (Minic et al., 2016). Similar bioactive chromopeptides can arise by the pepsin digestion of PCB-modified BLG.

BLG is one of the main allergens in cow milk, and 36% of patients with IgE-mediated cow milk allergy react to BLG (Shek et al., 2005). Since covalent BLG modifications, such as modification by glycation (Zhong et al., 2013; Zhong, Tu, Liu, Luo, & Liu, 2015) or polyphenols (Wu et al., 2018), could influence its allergenic potential, the assessment of IgE-binding properties of this important food allergen upon PCB modification is of great interest. Modification of the allergenic proteins by the Maillard reaction could even enhance the allergenicity of allergenic food proteins (Costa et al., 2021). Therefore, from the point of possible application in the food industry, it was important to demonstrate that the IgE binding was not negatively influenced by the PCB modification. The IgE binding was not altered, and the IgE binding ability correlated well with the proven structural similarity of the modified protein to the unmodified one. Tryptic fragment (102–124) containing free Cys residue of BLG, the target of modification by PCB, has been reported as one of the major BLG epitopes, both in terms of intensity and frequency of IgE responses (Sélo et al., 1999). The lack of difference in IgE-binding properties of BLG and BLG–PCB implies a minimal steric hindrance of this IgE-binding epitope by a single chromophore and/or lower contribution of this epitope in patients whose sera were used for testing.

5. Conclusions

In this study, we investigated the functional properties of BLG covalently modified with PCB. Covalent BLG modification by PCB develops a modified chromoprotein with enhanced antioxidant activity and oxidative stability, reduced tendency toward heat-induced polymerization, aggregation, and fibril formation, with thermal stability similar to that of the native protein, exhibiting higher proteolytic stability and unaltered IgE-binding properties. Thus, covalent BLG modification with PCB could be an innovative approach for preparing multifunctional food protein, potentially making it a valuable ingredient in the food industry. This study opens up new avenues for food fortification with proteins with bioactive tetrapyrrole chromophores. Further studies should evaluate the techno-functional properties of BLG–PCB to explore its full potential as a food ingredient.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2021.107169.

Author contributions

Mirjana Radomirovic: Conceptualization, Formal analysis, Investigation, Writing – original draft. Simeon Minic: Conceptualization, Formal Analysis, Investigation, Writing – review & editing. Dragana Stanic-Vucinic: Supervision, Writing – review & editing. Milan Nikolic: Writing – review & editing. Sam Van Haute: Resources, Writing – review & editing. Andreja Rajkovic: Writing – review & editing, Tanja Cirkovic Velickovic: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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