



Binding affinity between dietary polyphenols and β -lactoglobulin negatively correlates with the protein susceptibility to digestion and total antioxidant activity of complexes formed

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ARTICLE INFO

Article history:

Received 6 July 2012

Received in revised form 5 September 2012

Accepted 11 September 2012

Available online 18 September 2012

Keywords:

Antioxidant
 β -Lactoglobulin
Digestion
Interaction
Quenching
Polyphenol

ABSTRACT

Non-covalent interactions between β -lactoglobulin (BLG) and polyphenol extracts of teas, coffee and cocoa were studied by fluorescence and CD spectroscopy at pH values of the gastrointestinal tract (GIT). The biological implications of non-covalent binding of polyphenols to BLG were investigated by *in vitro* pepsin and pancreatin digestibility assay and ABTS radical scavenging activity of complexes formed. The polyphenol–BLG systems were stable at pH values of the GIT. The most profound effect of pH on binding affinity was observed for polyphenol extracts rich in phenolic acids. Stronger non-covalent interactions delayed pepsin and pancreatin digestion of BLG and induced β -sheet to α -helix transition at neutral pH. All polyphenols tested protected protein secondary structure at an extremely acidic pH of 1.2. A positive correlation was found between the strength of protein–polyphenol interactions and (a) half time of protein decay in gastric conditions ($R^2 = 0.85$), (b) masking of total antioxidant capacity of protein–polyphenol complexes ($R^2 = 0.95$).

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1. Introduction

Polyphenols, including phenolic acids, flavonoids, proanthocyanidins and resveratrol, are a large and heterogeneous group of phytochemicals abundantly present in foods and beverages such as tea, coffee, wine, cocoa, cereal grains, soy, fruits and berries.

Non-covalent interactions between polyphenols and globular proteins may result in complexation (Chaudhuri, Chakraborty, & Sengupta, 2011; Li et al., 2010), stabilization of protein structure (Kanakis et al., 2011), protein unfolding and precipitation (Ma, Yin, Liu, & Xie, 2011; Papadopoulou & Frazier, 2004; Siebert, Troukhanova, & Lynn, 1996). The strength of interactions depends on the size of polyphenols, polyphenol structure and amino-acid sequence of proteins (Frazier et al., 2010).

Many *in vitro*, *in vivo* and epidemiological studies have suggested that dietary polyphenols induce beneficial effects on human health, treatment and prevention of cardiovascular disease and cancer (Lambert & Elias, 2010; Yang, Wang, Lu, & Picinich, 2009). These effects are mainly due to their antioxidant and anti-

inflammatory properties (Tachibana, 2009; Wu, Guo, Ren, Guo, & Meydani, 2009). The inhibition of digestive enzymes involved in carbohydrate, lipid and protein metabolism by dietary polyphenols may be another important mechanism for the health benefits attributed to a diet rich in fruit and vegetables (Hanhineva et al., 2010; McDougall, Kulkarni, & Stewart, 2008).

The structural characterization of the interactions between globular food proteins and polyphenols is a major step in elucidating the effect of polyphenols on globular protein structure. Tryptophan residues are intrinsic fluorophores in proteins and spectroscopic techniques measuring quenching of Trp fluorescence in proteins have been applied to studies of polyphenol binding to various proteins, such as milk caseins and β -lactoglobulin (BLG), lysozyme, hemoglobin, serum albumins and gamma globulins (Chaudhuri et al., 2011; Rahimi Yazdi & Corredig, 2012; Wang et al., 2011; Xiao et al., 2011; Yuksel, Avci, & Erdem, 2010; Zorilla, Liang, Remondetto, & Subirade, 2011). The quantum yield and emission maximum wavelength associated with intrinsic Trp fluorescence are very sensitive to the polarity of the environment and the structural changes in biomacromolecules (Chaudhuri et al., 2011; Ma et al., 2011).

Complexation of polyphenols and proteins can affect the antioxidant activity of polyphenols by affecting their electron donating capacity and reducing the number of hydroxyl groups available

Abbreviations: BLG, beta-lactoglobulin; GIT, gastrointestinal tract; PE, polyphenol extract; BTPE, black tea PE; GTPE, green tea PE; CFPE, coffee PE; CCPE, cocoa PE.
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in solution (Arts et al., 2002; Niseteo, Komes, Belščak-Cvitanović, Horžić, & Budeč, 2012). However, due to the prolonged life of polyphenols in complexes, the effect of complexation may be beneficial for the overall antioxidant activity of the polyphenols.

Some recent studies have demonstrated new important biological effects of protein–polyphenol non-covalent interactions. Polyphenols exhibited a potent dose-dependent inhibitory activity on alpha Synuclein aggregation and were capable of disaggregating the pre-formed alpha Synuclein oligomers (Caruana et al., 2011). A positive correlation was also found between the degree of polyphenol oligomerization and inhibition of elastase due to an increased number of protein interacting groups (Brás et al., 2010). In addition, protein–polyphenol complexation may reduce IgE binding of allergens due to irreversible precipitation of allergens (Chung & Champagne, 2009; Chung & Reed, 2012).

Most of these reports focused on the interactions of pure polyphenol compounds and a model protein. In the GIT, dietary protein encounters mixtures of polyphenolic compounds present in various foods. Therefore, our approach was to examine the interactions between a model globular whey protein (β -lactoglobulin) and complex polyphenol mixtures as isolated from various polyphenol rich sources (green and black tea, coffee, cocoa). All the mixtures were analyzed for their exact compositions by high resolution mass spectrometry.

We demonstrated that changes in pH that occur in the human GIT (1.2–2.5 in the stomach and 7.2 in the intestine and saliva) affect the binding affinity of polyphenolic compounds to the protein. The most profound effect of pH on the binding affinity was observed for polyphenol extracts composed mainly of phenolic acids. The binding constants obtained were analyzed relative to the known biological effects of polyphenols – i.e., protein digestion inhibition and masking of the antioxidative power of complexes formed. All polyphenol extracts (PE) tested, regardless of chemical composition, protected protein secondary structure at an extremely acidic pH of 1.2. A positive correlation was observed between the binding strength of protein–polyphenol interactions, half time of protein decay during gastric digestion and masking of the total antioxidant capacity of protein–polyphenol complexes.

2. Materials and methods

The details of the experiments are given in the [Supplementary material](#) available online ([Supplementary material: Materials and methods](#)).

2.1. Isolation of phenolic extracts

Dietary polyphenols were isolated by methanol extraction and quantified using Folin-Ciocalteu reagent. Molar concentration was calculated using averaged molar mass (AMM) of polyphenols (BTPE – 420, GTPE – 459.7, CFPE – 387, CCPE – 487.63 g/mol) calculated from the data obtained from LC–MS analysis of the obtained PE ([Supplementary material: Tables S1–S4](#)).

2.2. LC–MS analysis of phenolic extracts

Isolated PEs were characterized by LTQ Orbitrap XL hybrid FTMS (Thermo Fisher Scientific, USA). Compounds were identified by combining exact masses, molecular formulae derived from Mass Frontier Spectral Interpretation Software (Thermo Fisher Scientific, USA) and spectra from LTQ Orbitrap XL hybrid FTMS with findings from the literature and internal database of spectra of standard polyphenol compounds.

2.3. Fluorescence spectroscopy

Fluorescent spectra were recorded on a FluoroMax-4 spectrofluorimeter (Horiba Scientific, Kyoto, Japan). The following buffers were used: 0.1 M HCl with 2 g/L NaCl pH 1.2, 0.1 M glycine-HCl pH 2.5 and phosphate buffered saline (PBS) pH 7.2. To 2.5 mL of 2.5 μ g/mL (0.14 μ M) protein solution (prepared in a suitable buffer immediately before the experiment) 10 aliquots of PEs (1 mg/mL) were added. Protein solution was titrated with $10 \times 2 \mu$ L of PEs at pH 1.2, 2.5 and $10 \times 1 \mu$ L of PEs at pH 7.2. After the addition of each aliquot fluorescent spectrum was immediately recorded under the conditions: λ excitation 280 nm, λ emission 290–410 nm. Because some polyphenols possess intrinsic fluorescence, a blank was made for each polyphenol concentration, in which protein solution was replaced with appropriate solvent, as described previously (Soares, Mateus, & de Freitas, 2007). The blank spectrum was automatically subtracted from the emission spectrum of the corresponding solution. Spectra were further analyzed using an OriginPro 8 software package (Northampton, MA, USA).

2.4. Fluorescence quenching analysis

Fluorescent quenching is described by the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV} \times [Q] \quad (1)$$

(Liang, Tajmir-Riahi, & Subirade, 2007) where F_0 and F are the fluorescence intensities before and after addition of a quencher; K_{SV} is the Stern-Volmer quenching constant and $[Q]$ is the concentration of the quencher. Polyphenols are able to absorb energy both at the proteins' excitation and to a lower extent emission wavelength. In order to overcome the inner filtering effect, the measured emission fluorescence intensity at 340 nm was corrected according to Eq. (2):

$$F = F_u \times 10^{QL \times (\epsilon_{\lambda_{ex}} + \epsilon_{\lambda_{em}})} \quad (2)$$

(Shpigelman, Israeli, & Livney, 2010) where F_u is the measured, uncorrected, emission intensity, $\epsilon_{\lambda_{ex}}$ and $\epsilon_{\lambda_{em}}$ are the molar extinction coefficients of polyphenols at the excitation and emission wavelengths, $[Q]$ is the quencher concentration and L is the path length of the cell.

A linear Stern-Volmer plot indicates that one mechanism of quenching occurs – static (complex formation) or dynamic. The Stern-Volmer plots obtained were linear at all the applied conditions (Fig. 1). Thus, the fluorescence quenching rate constant (k_q) can be calculated according to Eq. (3) if τ_0 (fluorescence lifetime of fluorophore without a quencher) is known.

$$K_{SV} = k_q \times \tau_0 \quad (3)$$

(Lakowicz, 1999; Soares et al., 2007)

The maximum value possible for diffusion-limited quenching in water is $\sim 10^{10} \text{ M}^{-1} \text{ s}^{-1}$.

Trp fluorescence lifetime depends on pH and buffer composition. Trp τ_0 in phosphate buffer pH 7.0 is 3.15 ns. At the acidic pH of 1.2 in hydrochloric acid Trp τ_0 is 0.75 ns and at pH 2.5 Trp τ_0 is 2.33 ns (citric acid-phosphate buffer) (Gudgin, Lopez-Delgado, & Ware, 1981). According to previous studies (Soares et al., 2007), τ_0 of the Trp residues of BLG at neutral pH is 1.28 ns at λ_{ex} 280 nm. The lowest K_{SV} values determined in our study were in the range of $1 \times 10^4 \text{ M}^{-1}$ yielding k_q above $10^{12} \text{ M}^{-1} \text{ s}^{-1}$, two orders of magnitude higher than the diffusion-limited quenching.

When the value of the bimolecular quenching rate constant is higher than diffusion-limited quenching, it could mean that there is a complex formation between a protein and a quencher, corresponding to a static mechanism of the fluorophore quenching.

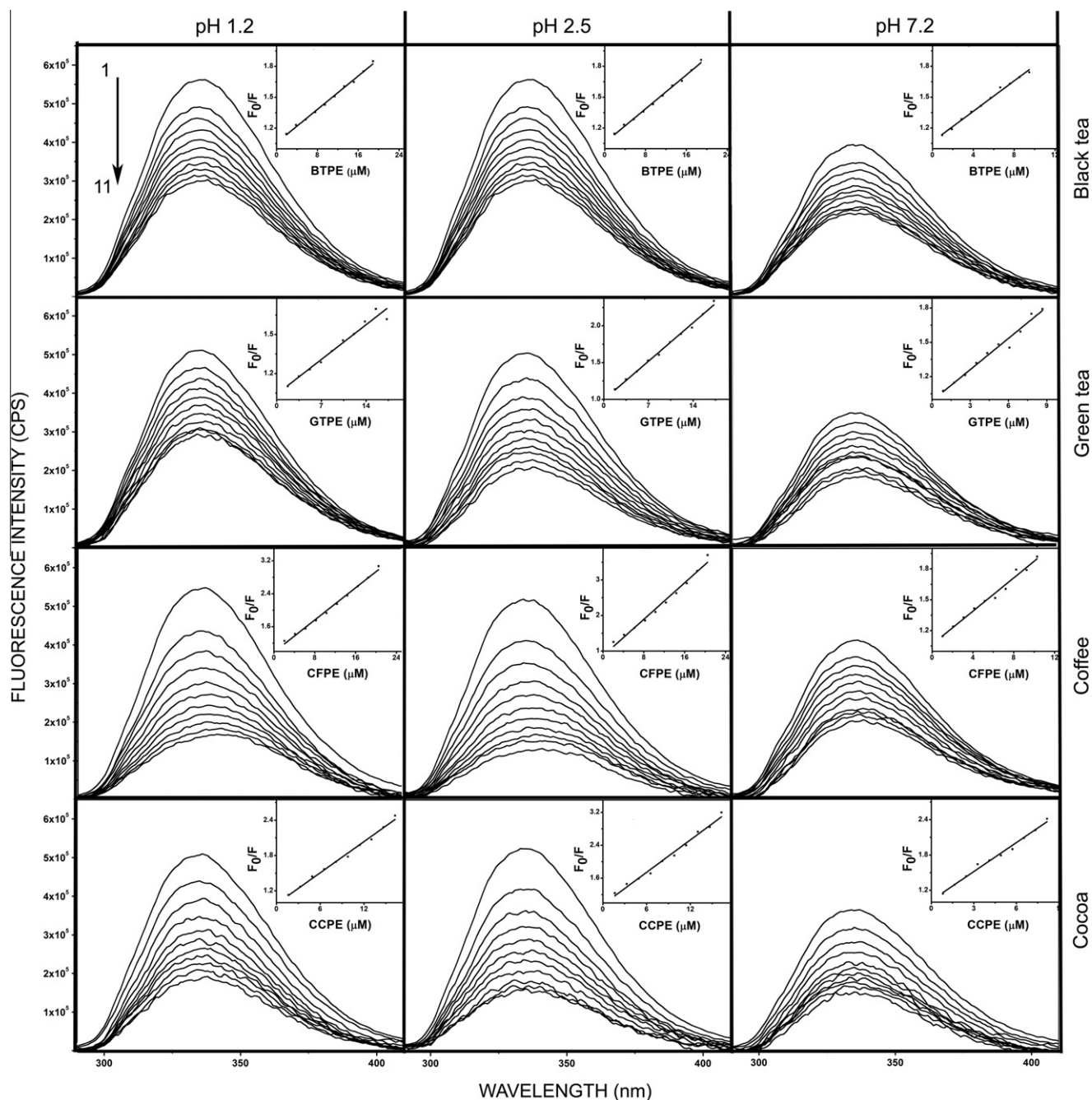


Fig. 1. The quenching effects of black tea (BTPE), green tea (GTPE), coffee (CFPE) and cocoa (CCPE) polyphenolic extracts on β -lactoglobulin (BLG) intrinsic fluorescence. Fluorescence spectra of BLG without (1) and with the addition of polyphenolic extracts (2–11) at pH 1.2, 2.5 and 7.2. 1: 0 $\mu\text{g/mL}$ PE; 2: 0.8 $\mu\text{g/mL}$ PE; 3: 1.6 $\mu\text{g/mL}$ PE; 4: 2.4 $\mu\text{g/mL}$ PE; 5: 3.2 $\mu\text{g/mL}$ PE; 6: 4.0 $\mu\text{g/mL}$ PE; 7: 4.8 $\mu\text{g/mL}$ PE; 8: 5.6 $\mu\text{g/mL}$ PE; 9: 6.4 $\mu\text{g/mL}$ PE; 10: 7.2 $\mu\text{g/mL}$ PE; 11: 8 $\mu\text{g/mL}$ PE; at pH 1.2 and 2.5. At pH 7.2 the final concentration of PE was two times lower for each aliquot. Inserts: the Stern-Volmer plots of F_0/F at 340 nm and room temperature as function of concentration of quencher as per Eq. (1).

For the static quenching, the binding constant K and a number of binding places can be calculated according to a double-logarithmic equation:

$$\log[(F_0 - F)/F] = \log K_a + n \log [Q] \quad (4)$$

(Lakowicz, 1999).

The slope of the double logarithmic Stern-Volmer plot yields the number of binding sites and the intercept provides the binding constant (K_a).

All plots were created and analyzed in OriginPro 8. Every experiment was repeated three times and statistical analysis was

performed using one way ANOVA followed by Bonferroni test (compares selected pairs of samples).

2.5. Circular dichroism

CD spectra of BLG/ BLG-PE samples were recorded on a JASCO J-815 spectropolarimeter (JASCO, Japan) at 25 °C in 1 nm steps at a rate of 50 nm/min over the wavelength range 185–260 nm using a quartz cell with a path length of 0.01 cm in nitrogen atmosphere. Protein concentration was 1 mg/mL whilst PE concentration was 50 and 500 $\mu\text{g/mL}$. To determine the percentage of secondary

structure motifs CONTIN software was used and SP29 protein set provided in CDPro software package. Statistical analysis was performed using one way ANOVA followed by the Dunnett test (compares all test samples to the control sample).

2.6. *In vitro* pepsin digestibility assay

Forty microliters of BLG (5 mg/mL) with or without 100 μ L of PE solutions (2 mg/mL) were diluted with 260 μ L of DIW and 200 μ L of 4 $\times$ simulated gastric fluid, SGF, (0.4 M HCl with 8 g/L NaCl, pH 1.2 or 2.5). Then, solutions were warmed at 37 °C and the pH was checked and adjusted if necessary to 1.2 or 2.5 by 0.5 M HCl. The final ratio of pepsin units to μ g of protein was 10. Digestion proceeded at 37 °C with continuous shaking and an aliquot (80 μ L) of the digest was periodically withdrawn at 0.25, 0.5, 1, 2, 3 and 6 h. Digests were analyzed by SDS–PAGE and the bands obtained were analyzed by densitometry.

2.7. *In vitro* pancreatin digestibility assay

A mixture containing 0.5 mg/mL of BLG and PE (0.5 mg/mL) in 20 mM sodium phosphate buffer pH 7.2 was pre-warmed at 37 °C. A 200 μ L-aliquot of this mixture was taken and added to 200 μ L pre-warmed pancreatin solution (0.4 mg/mL) in 80 mM sodium phosphate buffer, pH 7.2. Digestion proceeded at 37 °C in closed tubes with vigorous shaking. Aliquots (40 μ L) were taken at 1, 5, 10, 15, 20, 30, 45 and 60 min and analyzed by SDS–PAGE and densitometry.

2.8. Precipitation of BLG and pepsin by polyphenols

PEs were diluted with buffers of pH 1.2 and 2.5 to final concentrations of 0.33 mg/mL. To 150 μ L of PEs, 50 μ L of 1 mg/mL BLG or pepsin were added. After 2 h incubation at 37 °C samples were centrifuged for 20 min at 12,100g, supernatant and pellet were then separated and analyzed by SDS–PAGE.

2.9. Masking of total antioxidant capacity by the interactions of BLG with phenolics

Antioxidant capacity (AC) was assayed by the ABTS radical scavenging assay using Trolox as a standard. Trolox equivalent antioxidant capacity (TEAC, μ M Trolox/ μ M polyphenols) at pH 7.2 was determined for every PE. For the masking experiments, to a mixture of 5 μ L of 0.1 mg/mL PEs and 20 μ L of BLG 5 mg/mL in PBS 125 μ L of diluted ABTS⁺ solution were added and absorbance was measured after 6 min. AC of PEs or BLG only was assayed in the same way, except that PBS was added instead of the other component. Masking of total AC was calculated as percent difference between (AC_{PE} + AC_{BLG}) and AC_(BLG+PE). All experiments were conducted in triplicate.

3. Results and discussion

3.1. Composition of phenolic extracts of common beverages

The polyphenol composition of green tea (GTPE), black tea (BTPE), cocoa (CCPE) and coffee extracts (CFPE) was determined by LC–MS and the results are presented in [Supplementary information, Tables S1–S4](#).

The major phenolic components of GTPE are catechins with EGCG accounting for 50–60% of the total catechin mass (Cabrerá, Artacho, & Giménez, 2006). Different approaches in the manufacture of green tea (unfermented) and black tea (fully fermented) result in reduced content of catechins and appearance of their

oxidized high molecular weight polymers in BTPE (Obanda, Owuor, & Taylor, 1996).

CFPE is rich in phenolic acids; chlorogenic acid is the major component that during roasting generates caffeic, quinic acid and lactones as main byproducts (Clifford, 1999; Farah, de Paulis, Trugo, & Martin, 2005). The main polyphenols present in CCPE are flavanols, which include monomers (catechins) and more abundant polymers (proanthocyanidins) (Porter, Ma, & Chan, 1991).

3.2. Interaction between BLG and polyphenols

BLG has two Trp and four Tyr residues that give raise to protein intrinsic fluorescence. Upon excitation at 280 nm both residues are activated, but most of the emission yield is ascribed to Trp residues (Liang & Subirade, 2012). Trp19, emission region 340 nm, is buried inside BLG at the bottom of the calyx and contributes to 80% of the total fluorescence. Trp61 (emission region 350 nm) is partly exposed to solvents, near the disulfide bridge Cys66–Cys160, and has a minor contribution to Trp fluorescence (Liang et al., 2007). Quenching of emission at 340 nm was used to estimate the strength of interaction between BLG and PE by applying the Stern–Volmer equation and analysis. Intensity of quenching, presented as a linear ratio of F_0/F versus concentration of the quencher, gives the Stern–Volmer quenching constant (Fig. 1).

The linear Stern–Volmer plot is generally indicative of a single class of fluorophore in a protein, or one mechanism of quenching, while deviation from linearity indicates the presence of more than one class of fluorophore, which are not equally accessible to the quencher, or two mechanisms of quenching (Faridbod et al., 2011). In many instances, the fluorophore can be quenched both by collision and by complex formation with the same quencher. As indicated by a linear Stern–Volmer plot, polyphenols quench BLG fluorescence by one mechanism of quenching which allowed calculation of quenching rate constants and analysis of the binding affinities (Fig. 1). Calculated bimolecular quenching rate constants were two orders of magnitude higher than the maximum diffusion controlled limit, thus allowing application of the double logarithmic Stern–Volmer equation for the static mechanism of quenching and calculation of binding constants (K_a) of the complexes formed.

K_a values of all tested PEs were in the range of 10^3 – 10^5 M^{−1} (Table 1). The association constants obtained were in agreement with previously published data determined for BLG and various dietary polyphenols by applying the same methodology e.g. resveratrol (10^4 – 10^6 M^{−1}) (Liang et al., 2007), epigallocatechin-3-gallate (10^4 – 10^5 M^{−1}) (Kanakakis et al., 2011; Shpigelman et al., 2010), catechin and epicatechin (1.1×10^3 M^{−1}) (Kanakakis et al., 2011) at neutral pH. Various polyphenolic compounds tested in previous studies (myricetin, daidzein) were not released from BLG at pH 2, suggesting that binding occurs at the surface of the protein, and not to the central calyx (Riihimäki et al., 2008).

BLG has a protein conformation highly dependent on the pH and ionic strength of the solution. Below pH 2, BLG is a monomer retaining most of its secondary structure, but losing its native tertiary structure. Near pH 3, the protein dimerizes with little alteration in structure and, followed by Q–N transition at pH 4.5–6, where Q is BLG in expanded acidic form at pH 4.5 and N is BLG in more compact native form at pH 6.5 without any significant changes in secondary structure. Between pH 6–8 Tanford transition occurs with its peak at 7.5 (Taulier & Chalikian, 2001). Major structural change during Tanford transition involves rearrangement of the EF loop (amino acid residues 85–90) that closes the protein interior below pH 7 and opens it at higher pH causing the barrel aperture to become negatively charged due to the deprotonation of Glu89 (Tanford, Bunville, & Nozaki, 1959). The decrease in 340 nm peak intensity observed in free protein emission spectra at pH 7.2 (Fig. 1) is the result of dimerization induced self-quenching of

Table 1Calculated binding constants (K_a) for the polyphenol- β -lactoglobulin complexes and the number of polyphenols bound per a molecule of protein (n).

		pH 1.2	pH 2.5	pH 7.2
BTPE	K_a (M^{-1}) ^a	$(3.08 \pm 1.54) \times 10^3$	$(4.13 \pm 0.60) \times 10^3$	$(5.85 \pm 4.40) \times 10^3$
	n	0.77 ± 0.06	0.78 ± 0.02	0.75 ± 0.10
GTPE	K_a (M^{-1})	$(3.82 \pm 2.77) \times 10^3$	$(3.53 \pm 2.87) \times 10^4$	$(6.54 \pm 5.82) \times 10^4$
	n	0.76 ± 0.08	1.03 ± 0.07	0.88 ± 0.21
CFPE	K_a (M^{-1})	$(4.35 \pm 0.90) \times 10^4$	$(1.44 \pm 0.41) \times 10^5$	$(3.11 \pm 4.13) \times 10^4$
	n	0.93 ± 0.02	1.04 ± 0.04	0.84 ± 0.11
CCPE	K_a (M^{-1})	$(8.15 \pm 4.50) \times 10^4$	$(9.56 \pm 1.81) \times 10^4$	$(1.46 \pm 0.81) \times 10^5$
	n	0.98 ± 0.07	0.96 ± 0.01	0.99 ± 0.05

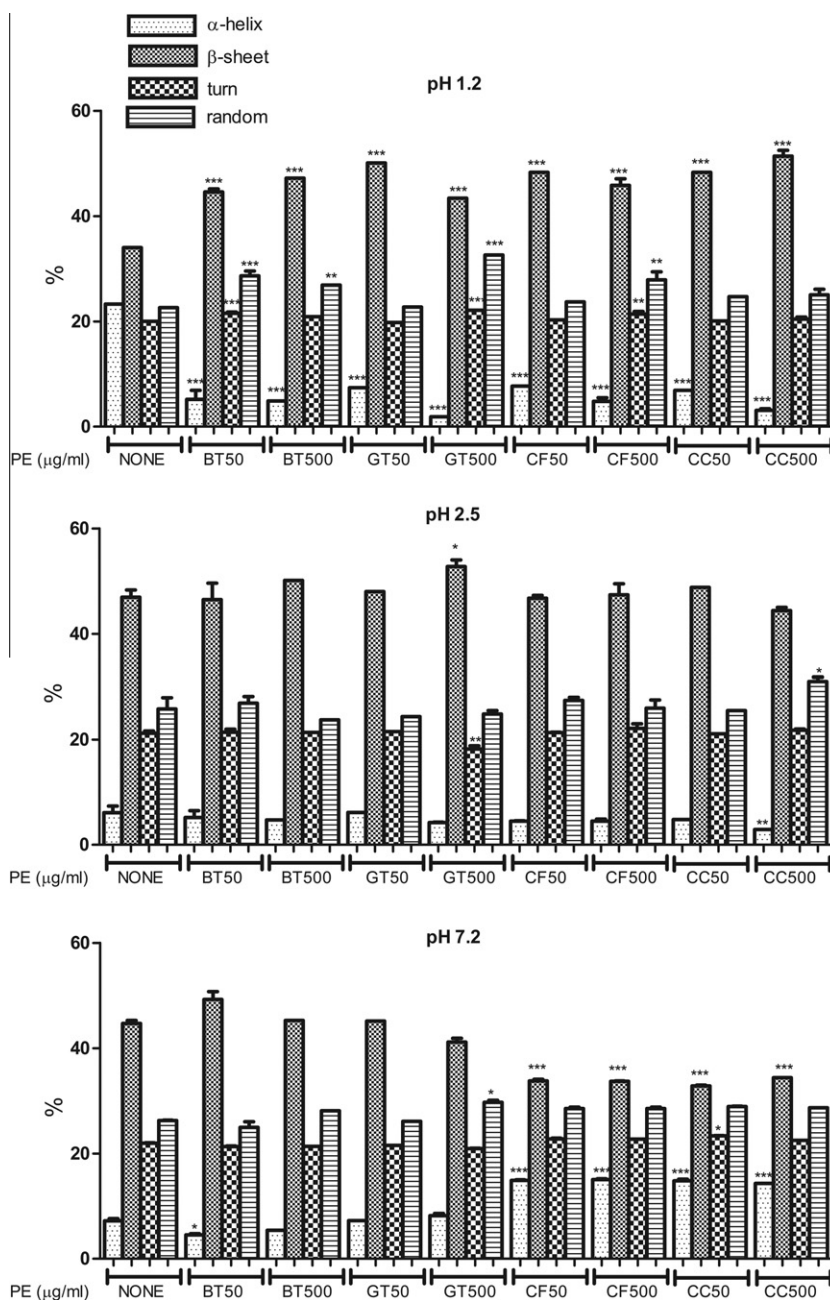
^a Values represent mean values $\pm$ std. deviation.**Fig. 2.** Influence of polyphenol binding on the protein secondary structure at pH 1.2, 2.5 and 7.2. Percentage of secondary structure motifs were calculated from CD spectra. Polyphenolic extracts (PE) at two concentrations 50 and 500 μg/mL. BT-black tea, CC-cacao, CF-coffee, GT-green tea. Results are expressed as mean $\pm$ standard deviation, and are statistically compared to the control, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

Table 2Pepsin and pancreatin digestion half-times ($T_{50\%}$) of β -lactoglobulin in the presence of polyphenolic extracts of green tea, black tea, cocoa and coffee.

PE:	$T_{50\%}$ (min) ^b			% BLG at the end of the digestion period ^a	
	Pepsin pH 1.2	Pepsin pH 2.5	Pancreatin pH 7.2	Pepsin pH 1.2	Pepsin pH 2.5
0	72 ± 2	82 ± 5	6.5 ± 0.5	3.0 ± 0.1	4.4 ± 0.9
GTPE	100 ± 3	95 ± 6	2.1 ± 0.5	12 ± 4	15 ± 10
BTPE	117 ± 17	129 ± 45	6.6 ± 0.8	8 ± 1	7 ± 1
CFPE	164 ± 83	264 ± 35	10.8 ± 0.8	21 ± 3	39 ± 5
CCPE	164 ± 13	185 ± 10	11.1 ± 0.2	22 ± 3	26 ± 2

^a Remaining amount of BLG at the end time of pepsin digestion is shown as % of the initial amount of protein. Results are expressed as mean ± standard deviation.^b Results are expressed as mean ± standard deviation.

Trp19 (Renard, Lefebvre, Griffin, & Griffin, 1998). All these pH derived structural changes might influence polyphenol–BLG binding affinity.

Changes in binding parameters are not dramatic, but some of them should not be neglected and are statistically significant. An increase in pH from 1.2 to 2.5 and 7.2 caused a change of the binding parameters near the pH of BLG conformation transition sites. Change in pH did not greatly influence the binding of tea and cocoa polyphenols with the highest K_a values observed at pH 7.2. BLG structure at pH 7.2 seems to be more accommodating for tea and cocoa polyphenols than for coffee polyphenols. The largest effect of pH on binding strength was observed for CFPE. At physiological pH of 7.2, negatively charged phenolic acids from CFPE were repulsed by the negatively charged protein. Chlorogenic acid pK_a value lies between 3.4 and 3.6 (Clifford, 1999), and the pK_a of caffeic and quinic acid are 4.6 and 3.4 (Farah et al., 2005), respectively. Since at both acidic pH values, CFPE acids are protonated, the higher binding affinity at pH 2.5 might be due to the structural requirements for polyphenol binding that are disrupted at very acidic pH (i.e., absence of tertiary structure of BLG).

3.3. Influence of polyphenol binding on the protein secondary structure

Far CD spectra provided an insight into changes in BLG secondary structure upon polyphenol binding at different pH (Supplementary Fig. S1). BLG retains its secondary structure at pH 7.2 and 2.5, but at pH 1.2 the protein is destabilized and the α -helix content increases to around 15%, mainly due to the β -sheet (Fig. 2). BLG is an interesting protein because of its high preference for α -helical structures that occur as intermediates in the unfolding pathway (Ragona et al., 1999). All PEs, regardless of their polyphenol content and binding strength, exhibited the same protein stabilizing behavior at pH 1.2 and prevented β – α non-native transition in a protein. At pH 2.5, binding of polyphenols did not additionally stabilize protein structure, but at pH 7.2, CFPE and CCPE significantly destabilized BLG structure increasing α -helix and random coil while decreasing β -sheet content. Around pH values of 6.8–7.5 BLG conformation is changing, calyx is being more open and some hidden amino acid residues are being deprotonated. Negatively charged phenolic acids and hydrogen bond donors and acceptors formed electrostatic bonds and hydrogen bonds with the protein influencing protein α -helical intermediates formation.

3.4. Impact on the pepsin digestion of BLG

Human gastric juices can be of various acidity, ranging physiologically from pH 1.2–2.5 up to pH 4.0 in the case of pathophysiological conditions (Ulleberg et al., 2011). Thereby, gastric digestion was tested at the physiological range of pH 1.2 and 2.5. In simulated gastric conditions BLG digestion by pepsin followed a first order decay which was quantified by densitometry in order to

estimate a half-life of BLG ($T_{50\%}$) and a residual amount of intact BLG after a 6 h digestion.

In the presence of all tested PEs, the BLG decay slowed down under both test conditions (Table 2). CCPE and CFPE slowed the pepsinolysis of BLG by up to 2 times at pH 1.2 and the effect was most pronounced at pH 2.5 for the CFPE. The highest amount of protein at the end of the incubation period of 6 h was 39% for the CFPE at pH 2.5. Good correlation ($R^2 = 0.85$) was obtained between the binding constants of polyphenols and the half-life of BLG decay for all tested conditions (Fig. 3A). Polyphenols bound to BLG provided protection of enzyme target sites in a protein and resulted in prolonged survival of intact protein in gastric fluids. Due to the enhanced intrinsic resistance of BLG to proteolytic degradation, this protein can be a good carrier of various polyphenols in the GIT.

Formation of non-covalent protein–polyphenol interactions can lead to the formation of aggregates that precipitate from the reaction mixture. It can be one of the reasons for a reduction in protein digestibility in the digestive tract, and it has been frequently described for polymerized polyphenols (Martínez & Moyano, 2003) which form a coat around a protein by a surface adsorption mechanism (Dobrev et al., 2011). Following interactions of BLG and PE of higher molecular weight (i.e., tannins), or polymerized by phenol oxidase, precipitation may occur, thus rendering the protein insoluble and so protected from pepsin digestion (Tantoush et al., 2012). We analyzed by centrifugation if a fraction of BLG became insoluble after incubation with various PE at the concentration used in the pepsin digestibility assay by SDS–PAGE analysis following precipitation. No significant precipitation of BLG occurred in the presence of any PE tested in the study at pH 1.2. Precipitation of BLG occurred only in the presence of BTPE at pH 2.5 (Fig. 3B). A deviation from the linear trend that occurred for BTPE in the correlation between K_a and $T_{50\%}$ could be assigned to the ability of BTPE to precipitate BLG. Upon exclusion of BTPE, the correlation coefficient obtained was 0.92.

Addition of PEs did not cause pepsin precipitation at all tested conditions (Supplementary material: Fig. S2).

3.5. Impact of non-covalent protein–polyphenol interactions on the pancreatin digestion of BLG

The *in vitro* intestinal digestion of proteins is often monitored by digestion with pancreatin at pH 7.2. The delayed intestinal digestion of BLG was noticed only in the presence of CCPE and CFPE but the overall impact was not as pronounced as for the pepsin digestion. Despite the relatively strong binding affinity of CCPE and CFPE to BLG at pH 7.2, which might shield the protein from the proteolytic enzymes, their destabilizing effect on BLG secondary structure (Table 2) negatively contributed to the digestion stability of protein resulting in an overall minor delay in intestinal digestion of the protein.

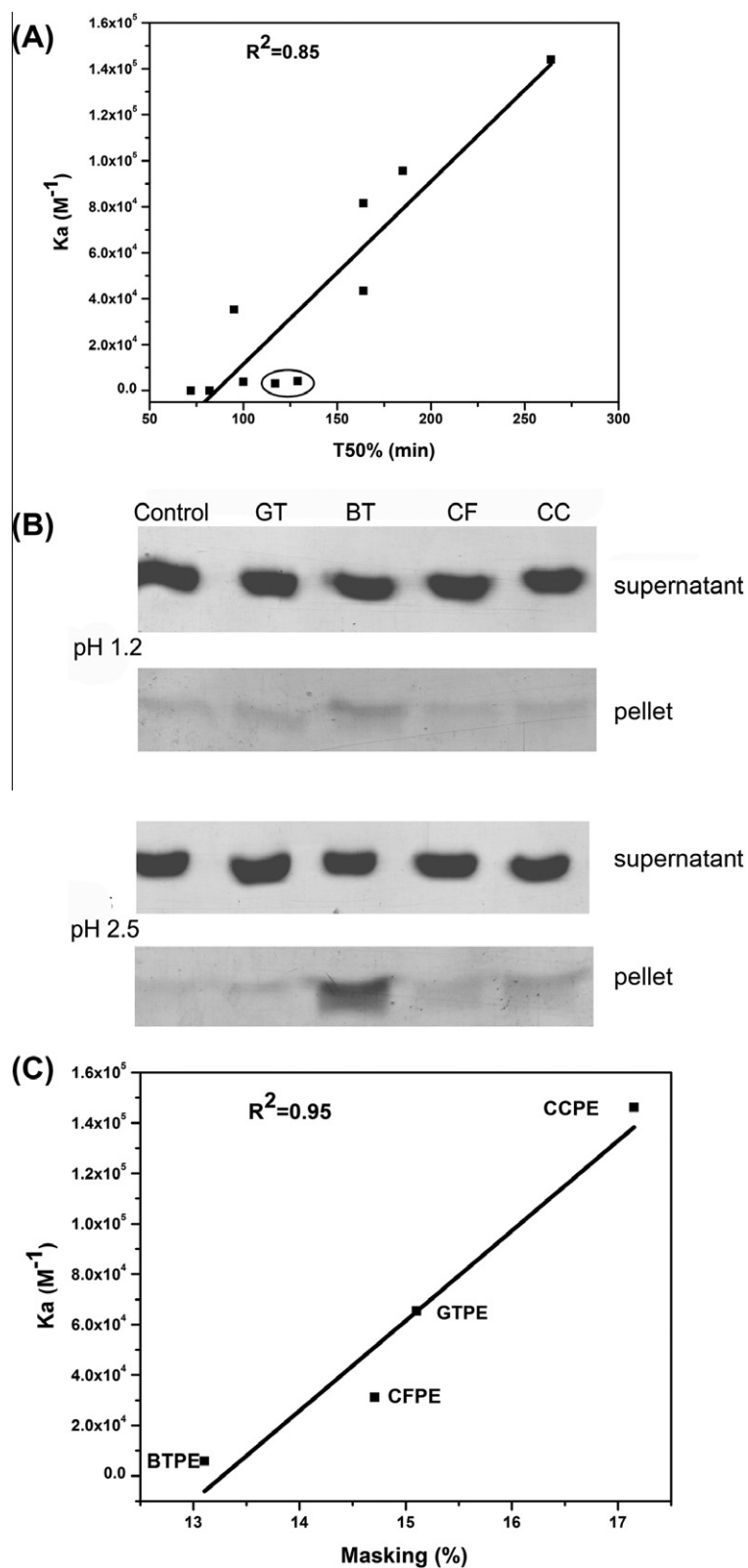


Fig. 3. (A) Correlation between the binding constants (K_a) and the half time of protein decay ($T_{50\%}$) at pH 1.2 and 2.5. When black tea is excluded (circled dots) correlation coefficient is 0.92. (B) Precipitation of β -lactoglobulin in the presence of phenolic extracts (PE) at pH 1.2 and 2.5. Control- β -lactoglobulin, GT-green tea PE, BT-black tea PE, CF-coffee PE, CC-cocoa PE. (C) Correlation between the binding constants (K_a) and masking of the total antioxidant capacity of protein–polyphenol complexes at pH 7.2. Black tea (BTPE), green tea (GTPE), coffee (CFPE) and cocoa (CCPE) polyphenolic extracts.

3.6. Masking of the total antioxidant capacity of protein–polyphenol complexes positively correlates with the binding affinity of polyphenols to the protein

Antioxidant activity of phenolic compounds can be modulated through intra or intermolecular hydrogen bonding of active or neighboring –OH groups (Amorati & Valgimigli, 2012). The decrease in antioxidant activity observed when polyphenols are non-covalently bound to biomacromolecules such as proteins, has been reported previously (Khan, Rakotomanomana, Dufour, & Dangles, 2011). It has also been shown that antioxidative capacity of polyphenols and blood plasma is not additive due to masking effects of interactions with proteins (Arts, Haenen, Voss, & Bast, 2001). Decrease in the total antioxidant capacity (AC) can be calculated from the ABTS^{••} radical scavenging assay by subtracting AC of the mixture of components from the sum of ACs of each component determined separately. Under physiological conditions, due to PE–BLG interactions, the masking effect on the antioxidant activity was in the range 13–15% and correlated well with the data obtained for similar polyphenol–protein systems (Arts et al., 2002). The degree of AC masking was strongly dependent on the binding affinity of PE to BLG, with the correlation coefficient of 0.95 (Fig. 3C). Calculated Trolox equivalent antioxidant capacity (μM Trolox/ μM polyphenol) for green tea was 1.70 (± 0.55), black tea 0.79 (± 0.22), coffee 1.16 (± 0.32) and cocoa 2.10 (± 0.52). GTPE and CCPE had the highest AC, and were most influenced by the binding to BLG's amino acid side chain residues.

4. Conclusion

Polyphenol–BLG complexes form at both acidic and neutral pH values of the GIT. All polyphenol extracts examined provided protective shielding of a protein from pepsin digestion and, additionally, at an acidic pH of 1.2 prevented β – α non-native transition in a protein, thus enhancing its stability to digestion at the extreme pH of the GIT. Antioxidant activities of all components, protein and polyphenols, were influenced by non-covalent bonding resulting in reduced radical scavenging activity of the complexes formed, when compared to the individual components. The strength of non-covalent interactions between dietary polyphenols and β -lactoglobulin governs the biological effects examined: protein susceptibility to pepsin digestion and radical scavenging capacity of complexes formed.

Acknowledgements

The authors acknowledge support of the GA No. 172024 of the Ministry of Education and Science of the Republic of Serbia and FP7 RegPot project FCUB ERA GA No. 256716. The EC does not share responsibility for the content of the article. The authors are participants in the COST Action FA1005 Infogest on food digestion.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2012.09.040>.

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