Supplementary data for the article:

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SUPPORTING INFORMATION

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

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Supplementary material and methods

Materials

BLG was isolated from raw milk according to the published protocol (Stojadinovic et al., 2012). Green and black tea dried leaves, ground roasted coffee (mixture of Arabica and Robusta) and cocoa powder were obtained from the local grocery store. Pepsin A (from porcine stomach mucosa, 2300 U/mg solid), pancreatin (from porcine pancreas), ABTS (2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), gallic acid, Folin-Ciocalteu reagent, Trolox, all LC-MS grade solvents were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Isolation of phenolic extracts

Ten grams of ground teas or coffee were boiled for 10 min in 100 mL of de-ionized water (DIW). After cooling down to room temperature (RT), 40 mL (for tea PE) or 20 mL (for coffee PE) of CH_3OH were added and extraction proceeded for 1 h with constant shaking. Extracts were centrifuged and supernatants were lyophilized. Dried PEs were resuspended in DIW and storaged at -20 °C. CFPE was deffated with C_2Cl_4 before storage. Cocoa powder (5 g) was first extracted for 1 h with 20 mL of 16 mM HCl in 50 % CH_3OH (v/v). Supernatant was collected and pellet was further extracted with 20 mL of 70 % acetone (v/v) for 1 h. After centrifugation,

supernatant was pooled with the supernatant from the previous step and cocoa PE (CCPE) was freeze dried. CCPE pellet was resuspended in DIW and storaged at -20 °C. Concentration of phenolic compounds in PEs was determined using Folin-Ciocalteu reagent by the method reported by Chun et al (Chun, Kim & Lee, 2003) and expressed in µg/mL of gallic acid equivalents (GAE). Molar concentration was calculated using averaged molar mass (AMM) of polyphenols (BT- 420, GT-459.7, CF-387, CC- 487.63 g/mol) calculated from the data obtained from LC-MS analysis of the obtained polyphenol extracts (Tables S1-S4).

LC-MS analysis of phenolic extracts

Isolated PEs were characterized by LTQ Orbitrap XL hybrid FTMS (Thermo Fisher Scientific, USA). Samples were dissolved in 25 % methanol and introduced into mass spectrometer by Accela 600 UPLC system (Thermo Fisher Scientific, USA) via C18 Hypersil GOLD column, 50 x 2.1 mm, 1.9 µm particle size (Thermo Fisher Scientific, USA). For elution, water with 0.1 % formic acid (eluent A) and 98 % acetonitrile with 0.1 % formic acid (eluent B) were used at 400 µL/min flow in a linear gradient, with the eluent B rising from 5 to 95 % over 5 min. Ionization was performed by HESI (probe temperature 400 °C, voltage - 4 kV). Analysis was performed in negative mode in the 120 - 1000 m/z range. Data analysis was performed by Xcalibur software and ToxID Automated Screening Software (Thermo 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.

Fluorescence spectroscopy

Fluorescent spectra were recorded on FluoroMax-4 spectrofluorimeter (Horiba Scientific, Kyoto, Japan). Experiments were carried out at RT in a 3.5 ml quartz cuvette. 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 µg/mL (0.14 µM) protein solution (prepared in a suitable buffer just before the experiment) 10 aliquots of PEs (1 mg/mL) were added. Protein solution was titrated with 10 x 2 µL of PEs at pH 1.2, 2.5 and 10 x 1 µL of PEs at pH 7.2. After the addition of each aliquot fluorescent spectrum was immediately recorded under the conditions: ʎ excitation 280 nm, ʎ emission 290-410 nm. Between each measurement, the cell was washed three times with the buffer. 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. All experiments were performed in triplicate and the averaged data obtained from the binding studies were used for the calculations of the binding parameters.
At the excitation wavelength of 280 nm, both Trp and Tyr residues in BLG are excited, but tryptophans are mostly contributing to the measured fluorescence emission. Trp residues, major fluorophores in proteins, can solely be excited at 295 nm, though the intensity of emission is lower than when excited at 280 nm (Lakowicz, 1999).
Spectra were further analyzed in OriginPro 8 software package (Northampton, MA, USA).

Fluorescence quenching analysis

Fluorescent quenching is described by Stern-Volmer equation:

$$F_0/F = 1 + K_{SV} \times [Q] \quad \text{(Eq. 1) (Liang, Tajmir-Riahi \& Subirade, 2007)}$$

where Fo and F and the fluorescense intensitites before and after addition of a quencher; Ksv is the Stern-Volmer quenching constant and [Q] is the concentration of the quencher. Polyphenols are able to absorb energy at the proteins'excitation and to a lower extent emission wavelength (Shpigelman, Israeli & Livney, 2010). In order to overcome the inner filtering effect, the measured emission fluorescence intensity at 340 nm was corrected according to the equation 2:

$$F = Fu \times 10^{QL\times(\varepsilon_{\lambda ex}+\varepsilon_{\lambda em})} \text{ (Eq. 2) (Shpigelman et al., 2010)}$$

where Fu is the measured, uncorrected emission intensity, $\varepsilon_{\lambda ex}$ and $\varepsilon_{\lambda em}$ 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 obtained Stern-Volmer plots were linear at all the applied conditions. Thus, fluorescence quenching rate constant (k_q) can be calculated according to the Eq. 3 if τ_0 (fluorescence lifetime of fluorophore without a quencher) is known.

$$K_{SV} = k_q \times \tau_0 \text{ (Eq. 3) (Lakowicz, 1999; Soares, Mateus \& de Freitas, 2007)}$$

The maximum value possible of k_q for diffusion-limited quenching in water is ~10^{10} M^{-1} s^{-1}. Trp fluorescence lifetime depends on pH and buffer composition (Gudgin, Lopez-Delgado & Ware, 1981). Trp τ_0 in phosphate buffer pH 7.0 is 3.15 ns. At 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 et al., 1981). According to previous studies (Soares, Mateus & de Freitas, 2007), τ_0 of the Trp residues of BLG at neutral pH is 1.28 ns at λ_{ex} 280 nm. The lowest determined Ksv values in our study were in the range of 1 x 10^4 M^{-1} yielding k_q above 10^{12} M^{-1} s^{-1}, two orders of magnitude higher than the diffusion-limited quenching.

When the value of the bimolecular quenching rate constant is higher that 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.

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

$$\log[(F_0\text{-}F)/F] = \log Ka + n\log[Q] \quad \text{(Eq.4) (Lakowicz, 1999)}$$

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

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).

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 µg/mL. Samples were prepared in three different buffers of pH 1.2 (0.1 M HCl, 2 g/L NaCl), pH 2.5 (0.1 M glycine-HCl) and pH 7.2 (20 mM sodium-phosphate). Each spectrum was acquired two times, results were averaged and expressed as residue-average molar ellipticity (Stojadinovic et al., 2012). 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 Dunnetti test (compares all test samples to control one).

115 ***In vitro* pepsin digestibility assay**

116 Briefly, 40 µL of BLG (5 mg/mL) with or without 100 µl of PE solutions (2 mg/mL) were
117 diluted with 260 µL of DW and 200 µL of 4 x simulated gastric fluid, SGF, (0.4 M HCl with 8
118 g/L NaCl, pH 1.2 or 2.5), then solutions were warmed at 37°C and pH was checked and adjusted
119 if necessary to 1.2 or 2.5 by 0.5 M HCl. Prewarmed 200 µL of 4 g/L pepsin in 0.02 M HCl, 0.4
120 g/L NaCl pH 1.2 or 2.5 buffer were added to each reaction mixture. Final ratio of pepsin units to
121 µg of protein was 10, as recommended by Thomas et al (Thomas et al., 2004). Digestion
122 proceeded at 37 ^{o}C with continuous shaking and an aliquot (80 µL) of the digest was periodically
123 withdrawn at 0.25, 0.5, 1, 2, 3 and 6 h. The digestion was stopped with 11 µL of 2 M Na_2CO_3,
124 and samples were mixed with 22 µL of five times concentrated electrophoresis sample buffer
125 containing reducing agent 2-mercaptoethanol for SDS-PAGE analysis. Twenty µL of each
126 sample were applied per lane. A pepsin control was set up in the same manner but with no
127 addition of BLG. BLG control was prepared with the addition of DIW instead of the pepsin
128 solution. All digestions were done in duplicates.

129 ***In vitro* pancreatin digestibility assay**

130 Mixture containing 0.5 mg/mL of BLG and PE (0.5 mg/mL) in 20 mM sodium phosphate buffer
131 pH 7.2 was prewarmed at 37 ^{o}C. A 200 µL- aliqoute of mixture was taken and added to 200 µL
132 prewarmed pancreatin solution (0.4 mg/mL) in 80 mM sodium phosphate buffer, pH 7.2.
133 Digestion proceeded at 37 ^{o}C in closed tubes with vigorous shaking. Aliquots (40 µL) were taken
134 at 1, 5, 10, 15, 20, 30, 45 and 60 minutes and stopped by adding 10 µL of 5x reducing sample
135 buffer and boiling for 5 minutes at 95 ^{o}C. Twenty µL of each sample were analyzed by SDS-
136 PAGE. Pancreatin control was prepared with PE solution (0.5 mg/mL) in 20 mM sodium
137 phosphate buffer pH=7.2, instead of mixture of BLG and PE. BLG control was prepared by

adding mixture of BLG and PE to prewarmed 80 mM sodium phosphate buffer, pH 7.2. Control digestion was performed in the same manner but without PE. All digestions were done in duplicates.

Densitometry analysis

All gels were analyzed in Gel-Pro Analyzer 3.0 program (Media cybernetics, Bethesda, USA) , using single band analysis option. The bands of interest were outlined with the box AOI tool and the option lanes/bands were chosen. A monochrome image was created for the purpose of the analysis. The lanes and density centers of the bands were found automatically. The intensity of each band was presented relative to the band in the control lane designated as 100 %.

Precipitation of BLG and pepsin by polyphenols

PEs were diluted with buffers of pH 1.2 and 2.5 to final concentration of 0.33 mg/mL, if there was any need, pH was adjusted. To 150 µL of PEs, 50 µL of 1 mg/mL BLG or pepsin were added. After 2 h incubation at 37 °C with slight stirring, samples were centrifuged for 20 min at 12100 g and supernatant and pellet were carefully separated. The pellets were resuspended in 200 µL of 1x reducing sample buffer and to ~200 µL of each of the supernatants 50 µL of 5 x reducing sample buffer were added. Samples were analyzed on 14 % SDS-PAGE.

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

Antioxidant capacity (AC) was assayed by ABTS radical scavenging assay described by Re et al. (Re et al., 1999) modified for a microtiter plate. ABTS radical cation ($ABTS^{\bullet+}$) was produced by mixing 7 mM ABTS and 2.45 mM potassium persulfate, and used 12- 16 h after mixing. Trolox was used as a standard and Trolox equivalent antioxidant capacity (TEAC, µM Trolox/µM polyphenols) at pH 7.2 was determined for every PE. $ABTS^{\bullet+}$ solution was diluted with PBS pH 7.2 such that absorbance at 630 nm was 0.70 (± 0.02). PE and BLG final concentrations were

selected to produce between 20 %–80 % inhibition of the blank absorbance. For the masking experiments, to a mixture of 5 µL of 0.1 mg/mL PEs and 20 µL of BLG 5 mg/mL in PBS 125 µl of diluted $ABTS^{\bullet+}$ 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 triplicates.

Supplementary figures

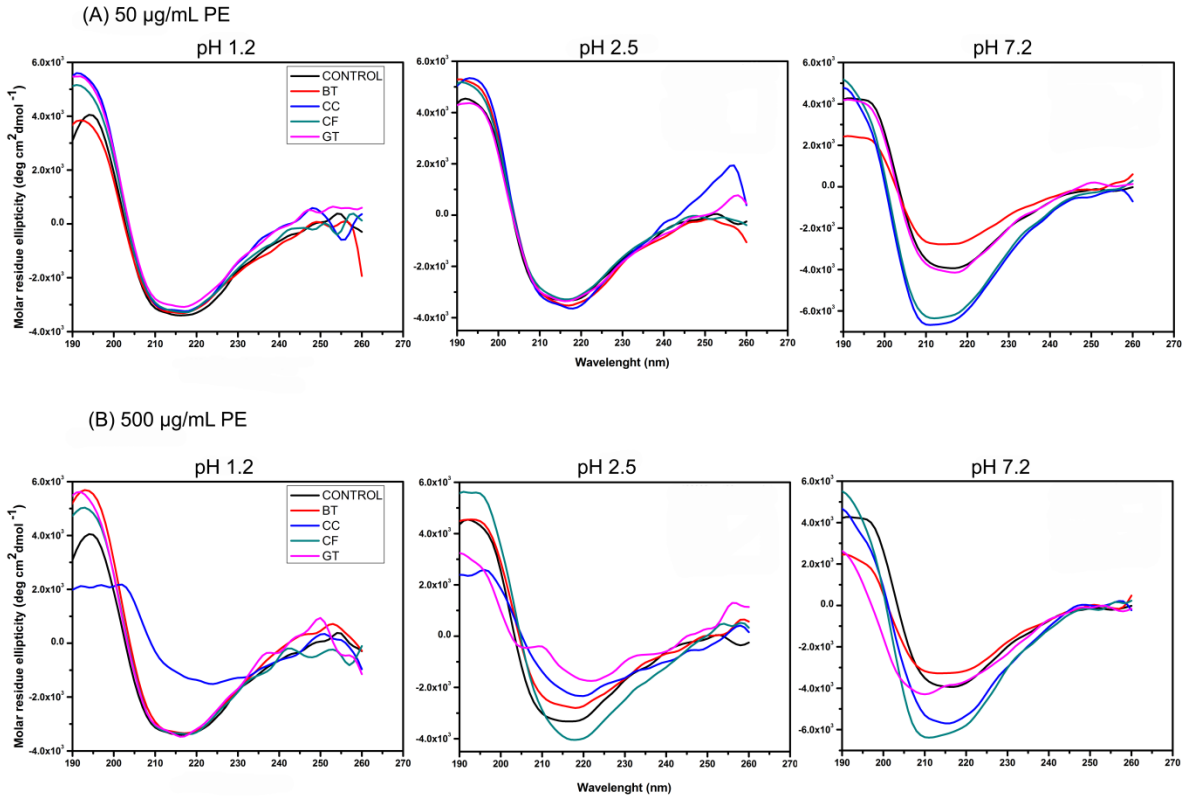

Figure S1. Influence of polyphenol binding on the protein secondary structure at pH 1.2, 2.5 and 7.2. BLG far UV CD spectra were recorded without (control) and with the addition of (A) 50 µg/mL or (B) 500 µg/mL of phenolic extracts. BT-black tea, CC-cacao, CF-coffee, GT-green tea.

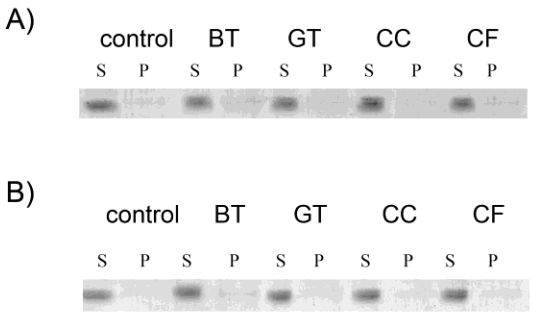

Figure S2. Precipitation of pepsin in the presence of polyphenol extracts of black tea (BT), green tea (GT), cocoa (CC) and coffee (CF) at pH 1.2 (A) and 2.5 (B). 5 μg of protein was analyzed by SDS PAGE. S-supernatant, P-pellet.

Tables

Table S1. Composition of black tea polyphenolic extract as determined by LC-MS. RT – retention time.

Compound Name	m/z	RT (min)	Abundance (%)
5-galloylquinic acid	343.067	0.57	5.18
Gallic acid	169.014	0.6	13.10
(-)-gallocatechin	305.067	1.34	2.62
3-caffeoyquinic acid	353.087	1.63	5.41
Quercetin-3-galactoside	463.182	1.72	0.40
(-)-epigallocatechin-3-gallate	457.077	1.79	5.86
5-O-p-coumaroylquinic acid	337.093	1.84	17.92
Theaflavin	563.14	1.9	3.58
Quercetin-rhamnose-hexose-rhamnose	755.204	2.04	1.60
Quercetin-rhamosylgalactoside Quercetin-3-rutinoside	609.146	2.06	12.29
Kaempferol-rhamnose-hexose-rhamnose	739.209	2.1	0.82
(-)-epicatechin-3-gallate	441.083	2.11	6.68
Kaempferol-3-rutinoside	593.151	2.19	10.67
(+)-catechin/(-)-epicatechin	289.072	2.21	2.18
Kaempferol-galactoside Kaempferol-3-glucoside	447.093	2.28	11.00
Quercetin-3-glucoside	463.255	2.52	0.14
5-caffeoyquinic acid	353.200	4.75	0.55

Table S2. Composition of green tea polyphenolic extract as determined by LC-MS. RT – retention time. b.d.l. - bellow detection limit.

Compound Name	**m/z**	**RT (min)**	**Abundance (%)**
Gallic acid	169.014	0.56	2.13
5-galloylquinic acid	343.067	0.57	1.99
5-O-p-coumaroylquinic acid	337.093	1.6	5.301
3-caffeoyquinic acid	353.087	1.69	3.75
Quercetin-3-galactoside	463.182	1.76	1.00
(+)-catechin/(-)-epicatechin	289.072	1.79	3.47
(-)-epigallocatechin-3-gallate	457.077	1.8	20.77
Theaflavin	563.14	1.92	8.21
Quercetin-rhamosylgalactoside	609.146	2.01	b.d.l.
Quercetin-3-rutinoside	609.146	2.01	b.d.l.
Quercetin-rhamnose-hexose-rhamnose	755.204	2.06	6.31
Quercetin-rhamosylgalactoside	609.146	2.07	8.38
Quercetin-3-rutinoside	609.146	2.07	n/a
(-)-epicatechin-3-gallate	441.083	2.12	18.12
Kaempferol-rhamnose-hexose-rhamnose	739.209	2.16	5.60
Kaempferol-3-rutinoside	593.151	2.19	8.30
Kaempferol-galactoside Kaempferol-3-glucoside	447.093	2.26	4.90
Quercetin-3-glucoside	463.255	2.65	0.82
5-caffeoyquinic acid	353.2	4.76	0.95

Table S3. Composition of coffee polyphenolic extract as determined by LC-MS. RT – retention time.

Compound Name	**m/z**	**RT (min)**	**Abundance (%)**
3-O-caffeoylquinic acid 4-O-caffeoylquinic acid	353.087	1.63	33.09
5-O-p-coumaroylquinic acid	337.092	1.84	5.86
3-O-feruloylquinic acid 4-O-feruloylquinic acid 5-O-feruloylquinic acid	367.103	1.94	28.89
Putative 3-O-caffeoylquinic lactone	335.077	2.01	12.34
Putative dicaffeoylquinic lactone	497.334	2.18	2.26
3,4-O-dicaffeoylquinic acid 3,5-O-dicaffeoylquinic acid 4,5-O-dicaffeoylquinic acid	515.119	2.23	13.19
3-O-feruloyl-4-O-caffeoylquinic acid 4-O-caffeoyl-5-O-feruloylquinic acid	529.135	2.46	4.43

Table S4. Composition of cocoa polyphenolic extract as determined by LC-MS. RT – retention time.

Compound Name	**m/z**	**RT (min)**	**Abundance (%)**
Luteolin-8-C-glucoside (orientin)	487.167	0.78	4.81
Apigenin-8-C-glucoside (vitexin)	431.133	0.89	6.57
Procyanidin dimmers	577.134	1.68	18.11
Catechin	289.072	1.78	19.77
Epicatechin	289.072	1.78	19.77
Procyanidin trimers	865.199	1.83	8.47
Quercetin-3-O-galactoside (hyperoside) Quercetin-3-O-glucoside (isoquercitrin)	463.088	2.12	4.72
Luteolin-7-O-glucoside	447.093	2.12	3.87
Luteolin-6-C-glucoside (isoorientin)	487.305	2.17	4.69
Quercetin-3-O-arabinoside	433.078	2.22	4.51

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