Antioxidative capacity and binding affinity of the complex of green tea catechin and beta-lactoglobulin glycated by the Maillard reaction

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Abstract

Major green tea catechin, epigallocatechin-3-gallate (EGCG), binds non-covalently to numerous dietary proteins, including beta-lactoglobulin of cow's milk. The effects of glycation of proteins via Maillard reaction on the binding capacity for polyphenols and the antiradical properties of the formed complexes have not been studied previously.

Binding constant of BLG glycated by milk sugar lactose to EGCG was measured by the method of fluorophore quenching. Binding of EGCG was confirmed by CD and FTIR. The antioxidative properties of the complexes were examined by measuring ABTS radical scavenging capacity, superoxide anion scavenging capacity and total reducing power assay.

Glycation of BLG does not significantly influence the binding constant of EGCG for the protein. Conformational changes were observed for both native and glycated BLG upon complexation with EGCG. Masking effect of polyphenol complexation on the antioxidative potential of the protein was of the similar degree for both glycated BLG and native BLG.

Key words: beta-lactoglobulin, epigallocatechin-3-gallate, glycation, Maillard reaction, protein polyphenol interactions, tea

Chemical compounds studied in this article: epigallocatechin-3-gallate (PubChem CID: 65064)

1. Introduction

Naturally occurring polyphenols can form complexes with globular proteins and such interaction may result in complexation, protein unfolding and precipitation (Ma, Yin, Liu, & Xie, 2011). Biological consequences of protein-polyphenol interactions are diverse and range from the direct effect of proteins on antioxidant power of polyphenols to inhibition of enzymatic activity (Sakulnarmrat, Srzednicki, & Konczak, 2014; Stojadinovic, et al., 2013).

Tea polyphenols have profound effects on human health (Butt & Sultan, 2009). (-)-Epigallocatechin-3-gallate (EGCG) is major active principle of green tea (Butt & Sultan, 2009; Yang, Wang, Lu, & Picinich, 2009) and shows strong affinity for dietary proteins, such as ovalbumin, peanut conglutins, alpha-lactalbumin, beta-lactoglobulin (BLG) and caseins (Al-Hanish, et al., 2016; Hasni, Bourassa, Hamdani, Samson, Carpentier, & Tajmir-Riahi, 2011; Kanakis, Hasni, Bourassa, Tarantilis, Polissiou, & Tajmir-Riahi, 2011; Keppler, Sonnichsen, Lorenzen, & Schwarz, 2014; Ognjenovic, et al., 2014; Vesic, Stambolic, Apostolovic, Milcic, Stanic-Vucinic, & Cirkovic Velickovic, 2015). Binding process between green tea polyphenols and proteins, such as the binding constant, binding site, forces involved in binding, as well as effects of complexation on antioxidant activity of polyphenols and its stability have been studied thoroughly (Al-Hanish, et al., 2016; Keppler, Martin, Garamus, & Schwarz, 2015; Ognjenovic, et al., 2014; Wu, Dey, Wu, Liu, He, & Zeng, 2013; Zorilla, Liang, Remondetto, & Subirade, 2011).

In particular, numerous reports characterized binding of EGCG to BLG, the major protein of milk whey (Keppler, Stuhldreier, Temps, & Schwarz, 2014; Keppler, Martin, Garamus, & Schwarz, 2015; Keppler, et al., 2014; Lestringant, Guri, Gulseren, Relkin, & Corredig, 2014; Li, Du, Jin, & Du, 2012; Shpigelman, Israeli, & Livney, 2010). BLG is an 18.4 kDa protein consisting of 162

43 amino acids which accounts for about 10% of the total milk proteins, i.e. approximately 50–55%
44 of the total whey proteins.

45 Delivery of sensitive water-soluble compounds in foods is an important challenge. Green tea
46 catechins are stable under acidic conditions but with the increase of pH, oxygen concentration or
47 temperature they degrade faster (Shpigelman, Cohen, & Livney, 2012). Stabilization of EGCG by
48 binding to native or heated BLG has been demonstrated in previous studies (Lestringant, et al.
49 2014). It has also been shown that dietary proteins can be good vehicles for transport and
50 preservation of antioxidant capacity of bioactive compounds, such as EGCG. For that purpose,
51 thermally-treated beta-lactoglobulin has been described as a promising nanovehicle, able to bind
52 EGCG with a higher affinity than native protein and protect the polyphenol from degradation
53 (Shpigelman, et al., 2010).

54 For dietary proteins, heating in the presence of reducing sugars is a reason for non-enzymatic
55 browning of foods and glycation of proteins due to Maillard reaction (MR). The non-enzymatic
56 browning is largely encountered during industrial processing and particularly during heat treatment
57 of foods rich in reducing sugars.

58 Glycation of BLG is described to readily occur in the presence of different mono-, di- and oligo-
59 saccharides (Chobert, Gaudin, Dalgalarrondo, & Haertle, 2006), also during food processing and
60 in neutral aqueous environment (Stanic-Vucinic, Prodic, Apostolovic, Nikolic, & Cirkovic
61 Velickovic, 2013). Glycated BLG shows antiradical properties, which intensity depends on the
62 sugar employed for modification of the protein (Chobert, et al., 2006).

63 The aim of this study was to examine the effects of MR of BLG in the presence of milk sugar
64 (lactose) on binding capacity of the protein for EGCG and the antiradical properties of the formed

complex. Glycated BLG was prepared in the presence of milk sugar lactose and characterized. The binding constant was measured by the method of fluorophore quenching. Binding to the glycated protein, as well as the effects of EGCG binding on the stability of BLG and its glycated form, was analyzed by far UV-CD and FTIR and the antioxidative properties were examined by ABTS radical scavenging capacity, superoxide anion scavenging capacity and total reducing power assay. We demonstrate in our study that processed forms of whey protein we frequently encounter in our diet do not loose potency to bind the most abundant and active principle of green tea.

2. Materials & methods

2.1. Materials

Native BLG (natural mixture of genetic variants A and B) was purified according to a previously published method, with minor modifications (Stojadinovic, et al., 2012). Protein purity was assessed by SDS PAGE. (-)-Epigallocatechin-3-gallate (EGCG) and sodium dihydrogen phosphate were purchased from Sigma Aldrich (St. Louis, MO, USA). Deionized water (DW) used in the experiments was purified by a Milli-Q system (Millipore, Molsheim, France).

2.2. Glycation of beta-lactoglobulin and characterization of glycated product

Beta-lactoglobulin was glycated with lactose in solution (20 mg/ml BLG, 1 M lactose in 50 mM phosphate buffer pH 8.0) by heat treatment at 60 ˚C during 10 days. To remove unreacted lactose and small reaction products dialysis was performed against 20 mM phosphate buffer pH 7.2. Glycated BLG was characterized by SDS PAGE and spectrofluorimetry measurements as described previously (Perusko, Al-Hanish, Cirkovic Velickovic, & Stanic-Vucinic, 2015). The degree of glycation was estimated by determination of remaining free amino group content (Perusko, et al., 2015).

2.3. Fluorescence spectroscopy

Fluorescent spectra were recorded on FluoroMax-4 spectrofluorimeter (Horiba Scientific, Kyoto, Japan). Experiments were carried out at room temperature in a 3.5 ml quartz cuvette. Samples were prepared in 20 mM phosphate buffer, pH 7.2 – BLG as 25 µg/ml, and LacBLG as 75 µg/ml. Protein concentrations were chosen as to obtain similar fluorescence intensity of in both cases (~ 2 x 10^6 CPS). To 2.5 ml of each protein solution 8 x 2 µl aliquots of EGCG (2.5 mg/ml) were added. After the addition of each aliquot fluorescent spectrum was immediately recorded under the conditions: ʎ excitation 280 nm, ʎ emission 290 - 500 nm. Because some polyphenols possess intrinsic fluorescence, a blank was made for each polyphenol concentration, in which protein solution was replaced with phosphate buffer, as described previously (Soares, Mateus, & Freitas, 2007). Blank spectrum was subtracted from the emission spectrum of the corresponding protein:EGCG solution. All experiments were performed in triplicate and averaged data obtained from the binding studies were used for the calculations of the binding parameters.

2.4. Fluorescence quenching analysis

2.4.1. Stern-Volmer equation

Fluorescence quenching is described by Stern-Volmer equation (Eq. 1) (Liang, Tajmir-Riahi, & Subirade, 2008):

$$\frac{F_0}{F} = 1 + K_{sv} \times [Q]$$

where F_0 and F and 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.

A linear Stern-Volmer plot can be obtained in cases of collisional or static quenching (Lakowicz, Gryczynski, Gryczynski, & Dattelbaum, 1999). Collisional quenching is a diffusion-controlled process that occurs when a quencher molecule is within a certain minimal distance from the fluorophore at the time of excitation. In contrast, static quenching results from the formation of a complex between fluorophore and quencher (Johansson, 1997).

The Stern-Volmer constant $\boldsymbol{K_{sv}}$ can be interpreted as the binding constant of the complex formation, assuming the observed changes in fluorescence come from the interaction between EGCG and protein (Hasni, et al., 2011).

The Stern-Volmer plots obtained here were linear (Fig. 2C). Thus, the fluorescence quenching rate constant ($\boldsymbol{k_q}$) can be calculated if $\boldsymbol{\tau_0}$ (fluorescence lifetime of fluorophore without a quencher) is known according to Eq. 2 (Lakowicz, et al., 1999):

$$K_{sv} = kq \times \tau_0$$

When the value of the bimolecular quenching rate constant ($\boldsymbol{k_q}$) 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.

Diffusion-limited quenching in water has a maximum value of 10^{10} M^{-1} s^{-1} (Soares, et al., 2007). While $\boldsymbol{\tau_0}$ of the BLG Trp residues at neutral pH is 1.28 ns at λ_{ex} 280 nm (Soares, et al., 2007). The lowest $\boldsymbol{K_{sv}}$ values determined in our study were in the range of 3 x 10^4 M^{-1} yielding $\boldsymbol{k_q}$ above 10^{13} M^{-1} s^{-1}, three orders of magnitude higher than the diffusion-limited quenching. EGCG quenches both native and glycated BLG fluorescence by a static mechanism, most likely through complex formation.

2.4.2. Double-logarithmic equation

For the static quenching, the binding constant $\boldsymbol{K_a}$ and number of binding sites ($\boldsymbol{n}$) can be calculated according to a double-logarithmic equation (Eq.3) (Lakowicz, et al., 1999):

$$log\frac{F_0 - F}{F} = \log K_a + n \times log[Q]$$

The slope of the double logarithmic plot yields the number of binding sites and the intercept provides the binding constant ($\boldsymbol{K_a}$).

2.4.3. Statistics

All fluorescence spectroscopy experiments were performed in triplicate and the averaged data obtained from the binding studies were used for all the calculations. LacBLG:EGCG binding parameters were compared to BLG:EGCG binding parameters. Statistical analysis was performed using descriptive statistics and two sample *t*-test, where $p < 0.05$ was considered as significant. All statistical analysis was performed by OriginLab 8.5.1 (http://www.originlab.com). All data are shown as mean ± standard error of mean (SEM).

2.5. Circular dichroism measurements (CD)

Recording of CD spectra was performed on the JASCO J-710 spectropolarimeter (JASCO, Tokyo, Japan) which was calibrated with a 0.6 g/l solution of ammonium D-10-camphorsulfonate, which has a known ellipticity of + 190.4 mdeg at 290.5 nm. Far-UV spectra were collected in 0.1 nm steps at a rate of 20 nm/min over the wavelength range 185–260 nm. The samples were analyzed at 25 °C in a 0.1 mm path length quartz cell. CD spectra were obtained for both 1.00 mg/ml BLG and Lac:BLG in 20 mM PBS buffer (pH 7.2) in the absence and presence of EGCG in 1:1 and 1:10 molar ratio. Calculation of percent's of secondary structures was performed with CONTIN

algorithm, the reference protein set SP29 (29 soluble proteins) was selected (Stojadinovic, et al., 2012).

2.6. FTIR spectroscopy measurements

FTIR data were obtained on a Nicolet 6700 FTIR spectrometer (Thermo, USA) equipped with a Germanium attenuated total reflection (ATR) accessory, a thermoelectrically cooled deuterated triglycine sulfate (DTGS TEC) detector and a XT-KBr beam splitter. Spectra were collected *via* the ATR method with a resolution of 2 cm^{-1} and 64 scans. The infrared spectra of LacBLG (72 μM) and the LacBLG:EGCG complex (the molar ratios of LacBLG to EGCG were 1:1 and 1:10) were obtained in the featured region of 4000–400 cm^{-1}. Same conditions were applied for recording spectra of BLG in presence and absence of EGCG with the same molar ratios. Corresponding absorbance contribution of buffer and free ligand solution were recorded and subtracted to get the FTIR spectra of protein and of protein:EGCG complexes, respectively. The subtraction was performed in order to obtain baseline in the region between 2000 and 1750 cm^{-1} (Dong, Huang, & Caughey, 1990).

The protein secondary structure composition was determined from the shape of the amide I band, located between 1600 and 1700 cm^{-1}. Fourier self-deconvolution and secondary derivative were applied to the range of 1700–1600 cm^{-1} to estimate the number, position, and areas of component bands. The peaks corresponding to β-sheet (1613–1637 cm^{-1}), random coil (1637–1644.5 cm^{-1}), α-helix (1644.5–1662 cm^{-1}), β-turn (1662.5–1682 cm^{-1}), and β-antiparallel sheet (1682–1689 cm^{-1}) (Goormaghtigh, Cabiaux, & Ruysschaert, 1990) were adjusted and the area was measured with the Gaussian function. The area of individual band assigned to a given secondary structure was then divided by the total area. The curve-fitting process was carried out by PeakFit 4.12 software

(SeaSolve software Inc., USA) to get the optimal Gaussian-shaped curves that fit the original protein spectrum.

2.7. Antioxidant capacity

Antioxidant capacity (AC) of BLG or LacBLG in the presence or absence of EGCG in molar ratio 5:1 was determined by the ABTS radical scavenging capacity, superoxide anion scavenging capacity and total reducing power.

ABTS radical scavenging assay was performed according to Stojadinovic et al. (Stojadinovic, et al., 2013), with minor modifications. 25 µl of BLG sample (1.00 mg/ml) was mixed with 125 µl of ABTS•+ solution with absorbance at 670 nm around 0.9, and allowed to react for 6 min. Absorbance was measured at ELISA reader with 670 nm filter. Masking effect (ME) of EGCG on antioxidant capacity of BLG was determined according to formula $ME(EGCG) = AC_{(BLG)} + AC_{(EGCG)} - AC_{(BLG + EGCG)}$

The capability of BLG or LacBLG to neutralize superoxide anion formed by the reduction of nitroblue tetrazolium (NBT) with NADH mediated by phenazine methosulfate (PMS) under aerobic conditions was conducted according to the method of Nishikimi *et al*. with slight modifications. The mixture of 120 µl of BLG samples (2.00 mg/ml), 30 µl NADPH (2 mM), 30 µl of NBT (0.5 mM), and 4 µl freshly prepared PMS (1.5 mM) was incubated for 10 min. Control probe was prepared by substituting BLG or LacBLG with 20 mM phosphate buffer pH 7.2. Absorbance was measured at 540 nm.

The reducing power of BLG or LacBLG (1.00 mg/ml) was determined according to Stanic-Vucinic *et al*., 2013.

3. Results and discussion

3.1. Maillard reaction induced high degree of beta-lactoglobulin glycation

BLG was thermally glycated with lactose under wet conditions. Formation of MR products were monitored by measuring fluorescence emission at 435 nm, when the mixture was excited at 350 nm. A significant increase in fluorescence at 435 nm in treated LacBLG suggests formation of early Maillard reaction products (MRPs) (Fig. 1A). The progress of the MR was also monitored by the loss of available $-NH_2$ groups after heat treatment in the presence of lactose (Fig. 1B). Heat treatment induced a significant ($p < 0.05$) loss of BLG amino groups, as only 30% of amino groups of the protein remained free.

SDS PAGE under non-reducing (Fig. 1C) and reducing (Fig. 1D) conditions shows that band corresponding to BLG smeared to higher molecular masses, due to conjugation to lactose. Non-reducing gel shows monomer and dimer BLG forms in BLG sample, while reducing gel shows only monomeric BLG, indicating that dimer BLG form is disulfide linked. LacBLG is present in a form of a monomer, dimer and higher molecular masses, under both non-reducing and reducing conditions, indicating formation of covalent bonds other than disulfide bonds. Smear toward higher molecular masses and formation of covalent bonds other than disulfide, suggest that Maillard reaction occurred in LacBLG sample to a high extend, and it is in accordance with increased fluorescence and dramatic decrease of free amino group content.

3.2. Binding affinity to LacBLG was determined by fluorescence quenching analysis

The structural characterisation of the interaction between globular food proteins and polyphenols is often monitored by the method of fluorescence quenching. This technique measures binding affinities, can determine number of binding places and thermodynamical parameters of binding

(Lakowicz, et al., 1999). Tryptophans are intrinsic fluorophores in proteins and spectroscopic techniques measuring quenching of tryptophan fluorescence in proteins have been applied to studies of polyphenols binding to various proteins, such as milk caseins and BLG, ovalbumin, alpha-lactalbumin, peanut conglutins, lysozyme, hemoglobin, serum albumins and gamma globulins (Chaudhuri, Chakraborty, & Sengupta, 2011; Ognjenovic, et al., 2014; Vesic, et al., 2015; Xiao, Kai, Yang, Liu, Xu, & Yamamoto, 2011; Zorilla, et al., 2011).

BLG intrinsic fluorescence comes from its two Trp and four Tyr residues (Liang & Subirade, 2012). Although both residues are activated at 280 nm, most of the emission yield comes from Trp residues (Liang et al., 2012). Trp19 (emission region 340 nm) is buried inside BLG and contributes to 80% of the total fluorescence with the characteristic emission maximum at 340 nm. Trp61 is partly exposed to solvents, has a minor contribution to Trp fluorescence (Liang et al., 2012) and show an emission maximum at 350 nm. Emission maximum of BLG is at 335 nm, while LacBLG shows a slight red shift with emission maximum at 347 nm (Fig. 2A). This is indicative of Trp residues becoming more exposed to the solvent as a consequence of glycation reaction with lactose.

Quenching of emission at the aforementioned emission maxima were used to estimate the strength of interaction for EGCG with BLG (Fig. S1, SOM) and LacBLG (Fig. 2B) by applying Stern-Volmer (Eq. 1) and double logarithmic (Eq. 3) equations and analysis. Intensity of quenching, presented as a linear ratio of Fo/F versus concentration of the quencher, gives the Stern-Volmer quenching constant (Eq. 1 and Fig. 2C). As indicated by a linear Stern-Volmer plot (Fig. 2C), EGCG quenches LacBLG fluorescence by one mechanism of quenching (Faridbod, et al., 2011) which allowed calculation of quenching rate constants and analysis of the binding affinities of EGCG for BLG and LacBLG (Table 1). Calculated bimolecular quenching rate constant was three orders of magnitude higher than the maximum diffusion controlled limit, which allowed

application of the double logarithmic equation (Eq. 3) and calculation of binding constant (K_a) and number of EGCG binding sites (n) (Table 1).

The intensity of quenching, presented as a linear ratio of F_0/F versus concentration of the quencher, gives the Stern-Volmer quenching constant (K_{SV}) of 2.65 x 10^4 M^{-1} (Table 1 and Fig. 2C). BLG:EGCG also gave a linear Stern-Volmer plot (data not shown) with K_{SV} value of 3.1 x 10^4 M^{-1}. Our data are in agreement with data published by other authors for BLG:EGCG binding constant (Keppler, et al., 2014; Wu, et al., 2013) (Table 1). There is a statistically significant difference between the quenching constants for BLG:EGCG and LacBLG:EGCG complexation, as determined by t-test (Fig. S2A, SOM).

Application of double logarithm plot (Eq. 3 and Fig. 2D) yielded the number of ligand binding of 1.05 and association constant of 5 x 10^4 M^{-1} for LacBLG:EGCG (Table 1). Both experimentally determined (Table 1) and literature data (Keppler, et al., 2014; Wu, et al., 2013; Zorilla, et al., 2011) for EGCG binding sites on BLG are very similar to those we calculated for LacBLG:EGCG complex. There was no statistically significant difference for either parameter obtained by use of double logarithm equation for EGCG binding to LacBLG versus BLG (Fig. S2 B and C, SOM).

Thus, we conclude that a stable LacBLG:EGCG complex is formed in solution and that EGCG quenches protein's fluorescence by static mechanism. Determined association constants were in 10^4 M^{-1} range, which is in accordance with previously published data determined for BLG by the same methodology. Our results suggest that EGCG binds to LacBLG and forms a stable complex in solution with a similar binding affinity as described for BLG.

BLG contains in its primary sequence 19 potentially reactive sites (N-terminal, 15 lysine and three arginine residues). Previous mass spectrometry analysis of highly lactosylated BLG showed a total

of 17 different modified amino acid residues (Leu1, all 15 Lys residues and Arg124) with usually attached 6-15 lactose units per BLG molecule (Fenaille, Morgan, Parisod, Tabet, & Guy, 2004). Lactosylated amino acids residues lie on the protein surface, exposed to solvent, without interfering with amino acid residues of hydrophobic cavity that are responsible for EGCG binding (Wu, et al., 2013). These data support our observation that lactosylation of BLG does not significantly affect its binding to EGCG.

3.3. Secondary structure changes of LacBLG in a complex with EGCG

Complexation of protein and polyphenols may influence proteins' secondary structure (Ognjenovic, et al., 2014; Vesic, et al., 2015). This phenomenon was monitored by CD spectroscopy and FTIR.

BLG, upon binding of EGCG to BLG, a predominantly beta-sheet protein, induces an increase in α-helix content on the account of random coil (Table 2 and Fig. S3, SOM), which is in agreement with previously published data (Kanakis, et al., 2011). A non-native transition of α-helix to β-sheet, is an event that might proceed precipitation and aggregation of the tested protein, a phenomenon known to occur in higher polyphenol:protein ratio. We wanted to examine the effect of EGCG binding to high affinity binding sites and analyzed effects of only up to 1:10 molar ratio of protein:polyphenol.

LacBLG shows similar secondary structure content as native protein (Table 2 and Fig. S3, SOM). Due to the heat-induced dimerization and/or denaturation, small changes in the protein fold can be observed. Calculation of secondary structures showed that glycation of BLG induces beta-sheet to alpha-helix transition (non-native), accompanied with an increase in random coil (Table 2 and Fig. S3, SOM). With the results obtained by monitoring Trp fluorescence in LacBLG, it is evident that

glycation induces changes in protein fold and causes non-native transitions of protein secondary structures. These effects could mainly be attributed to the heat-induced denaturation of BLG, as the changes in the protein fold due to increase of temperature occur already at 60 ^{o}C (Seo, Hedoux, Guinet, Paccou, Affouard, Lerbret, et al., 2010). On the other hand, interactions of EGCG with LacBLG further induced a decrease of β-sheet and increase of α-helix and random coil, thus further contributing to glycated protein unfolding. A different pattern of secondary structure changes as a consequence of complexation of BLG with EGCG could also be observed (Table 2 and Fig. S3, SOM).

Additional evidence regarding the effect of EGCG binding on BLG and LacBLG structures came from FTIR results. Infrared spectra of proteins exhibit a number of the amide bands, which represent different vibrations of the peptide moiety. The amide I peak with position in the region 1600–1700 cm^{-1} (mainly C=O stretch), is commonly used for determination of secondary structures in proteins. BLG has peak position at 1634 cm^{-1} (Fig. 3A), confirming that beta sheets prevail in its structure. However, after glycation, amide I peak position shifts to 1642 cm^{-1} (Fig. 3D), indicating beta sheet to random coil transition in modified protein.

EGCG induced change of the amide I band shape in the spectrum of BLG, indicating that the secondary structure of BLG is altered upon EGCG binding (Fig. 3C and D). A quantitative analysis of the BLG secondary structure before and after interaction with EGCG is given in Fig. 3 and Table 2**.** The curve-fitted results (Table 2) show that α helix is slightly increased for 1%, upon EGCG binding. Beta sheet content increased from 27.9% (BLG) to 31.7% (BLG:EGCG 1:10 molar ratio), while beta-antiparallel sheet percentage decreased from 12.5% (BLG) to 7.8 % (BLG:EGCG 1:10 molar ratio). It can be observed that content of beta-antiparallel sheets is substantially changed upon EGCG binding. These results are in agreement with previous study (Kanakis, et al., 2011).

The curve fitted results (Table 2) show that LacBLG has a higher percentage of random coil (30.5%) in comparison to BLG (22.9%), in agreement with CD data. However, similarly to BLG, alpha helical content was only slightly affected upon EGCG binding (Table 2). Moreover, in the presence of EGCG beta sheet content increased in comparison to LacBLG, while beta-antiparallel sheet percentage decreased (Table 2). Therefore, FTIR results confirmed that glycation produces substantial changes in secondary structures of BLG. Binding of polyphenol to BLG, as well as, LacBLG, induces subtle changes in secondary structures of protein, mostly characterized by a decrease in antiparallel-beta sheet content and a small increase in α-helix content.

3.4. Antioxidative capacity of the LacBLG:EGCG complex

BLG is a mild temperature-dependent antioxidant and the free thiol group is likely to be involved in this antioxidant activity (Liu, Chen, & Mao, 2007). Glycated BLG shows antiradical properties which intensity depends on the sugar used for modification of the protein (Chobert, et al., 2006).

Even though the exact mechanism of how proteins influence antioxidative effect of polyphenols is still not yet known, the decrease in total antioxidant capacity when the polyphenols are bound to proteins is very well studied (Ozdal, Capanoglu, & Altay, 2013). Therefore, we wanted to examine the effect of glycation on the total antioxidative capacity of glycated protein-polyphenol complex and the magnitude of the masking effect of LacBLG:EGCG complex.

Antioxidant capacity of BLG and LacBLG in the absence or presence of EGCG in molar ratio protein:EGCG 5:1, was assessed in ABTS+ radical scavenging assay (Fig. 4A). LacBLG showed higher capacity to scavenge ABTS radical than BLG, and complex formation with EGCG increases this capacity in both cases. However, ABTS scavenging activity of the complexes is lower than the sum of protein activity and EGCG activity. Masking effect due to BLG:EGCG interactions was

12.67%, and due to LacBLG:EGCG interactions was 13.15%, which is consistent with the data obtained for the interactions of different polyphenols with BLG (Stojadinovic, et al., 2013).

The superoxide anion radical scavenging activity, followed a similar pattern (Fig. 4B), with masking effects of 8.87% and 7.53% for BLG:EGCG and LacBLG:EGCG, respectively. Reducing power (Fig. 4C) of BLG was negligible and so was the masking effect of BLG:EGCG interactions. Glycation of BLG notably increased its reducing power. Interactions of LacBLG:EGCG complex gave rise to masking effect of 10.05%.

Melanoidins and heterocycles formed in the advanced stages of the MR possess a hydrogen-donating ability, explaining the reducing power of LacBLG and its increased ability to react with radical compounds (Chevalier, Chobert, Genot, & Haertle, 2001). Complexation of EGCG to BLG or LacBLG led to a similar degree of antioxidant capacity masking effect, correlating well with determined association constants in 10^4 M^{-1} range for both BLG forms. Strong correlation between antioxidant masking effect and binding affinity of polyphenols to BLG has been reported previously (Stojadinovic, et al., 2013), therefore similar binding affinity determined in our study also correlates well with the similar extents of the masking effect of EGCG binding to the proteins.

4. Conclusions

The interaction of EGCG with various proteins, including BLG, is now widely accepted and proposed to contribute to diverse biological activities of this molecule. Whey proteins are frequently used as food additives and Maillard reaction occurs due to food processing or heating and results in protein glycation. Glycation can influence the structural and functional properties of the proteins. Our findings indicate that glycation of the major whey protein does not affect its complexation with the EGCG. The effects of the complexation on the protein fold differ between

the native and glycated protein. Masking effect of polyphenol binding on the antioxidative properties and reducing power of the protein are in the same range for both the glycated protein, and the native one. Glycated BLG possesses improved techno-functional properties with retained ability to bind and transport EGCG, making glycated BLG an exceptional food additive and carrier for nutraceuticals.

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Appendix

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Figure captions

Figure 1. Characterization of LacBLG. Fluorescence emission spectra of BLG and LacBLG (A). Free amino group content of LacBLG compared to BLG (B). SDSPAGE under non-reducing (C) or reducing conditions (D).

Figure 2. Fluorescence quenching analysis of LacBLG:EGCG complex. Fluorescence of BLG and LacBLG upon excitation at 280 nm (A); Quenching of LacBLG (75 µg/ml) by EGCG (0 – 34.7 µM) (B); LacBLG:EGCG complex formation described by fluorescence quenching analysis: Stern-Volmer plot (C) and Double logarithm plot (D).

Figure 3. The curve-fit amide I (1700–1600 cm^{-1}) region with secondary structure determination of the BLG (A), BLG:EGCG complexes, with molar ratios: 1:1 (B) and 1:10 (C), LacBLG (D), LacBLG:EGCG complexes, with molar ratios: 1:1 (E) and 1:10 (F).

Figure 4. Antioxidant activity of BLG, BLG:EGCG, LacBLG, LacBLG:EGCG and EGCG measured as A) ABTS radical scavenging activity, B) Superoxide anion scavenging activity and C) Reducing power. Results are expressed as mean ± standard deviation, and are statistically

compared by student's t-test. *, **, ***, and ns, represent significance at $p \leq 0.05$, 0.01, 0.001 and not significant, respectively.

Table 1. Binding parameters obtained for LacBLG:EGCG complex determined by the fluorescence quenching and application of two different mathematical models.

Table 2. Contents of different secondary structures of BLG and LacBLG in the presence and absence of EGCG, obtained using FT-IR and CD spectroscopy.

Table 1

Binding parameters obtained for LacBLG:EGCG complex determined by the fluorescence quenching and comparison to reference values.

Equation	Stern-Volmer		Double logarithm			
	Ksv (M^{-1})	**SEM**	**Ka (M^{-1})**	**SEM**	**n**	**SEM**
Experimental values						
LacBLG/EGCG	2.65×10^4	7×10^2	5×10^4	2×10^4	1.05	0.04
BLG/EGCG	3.1×10^4	1×10^3	3.1×10^4	7×10^3	1.00	0.02
Reference values for BLG/EGCG complex						
Wu et al., 2013	5.64×10^5	R = 0.9885	1.09×10^5	n/a	1.08	n/a
Keppler et al., 2014	1.70×10^4	6×10^2	1.7×10^4	2×10^3	0.82	0.05
Zorilla *et al.*, 2011	n/a	n/a	1.25×10^4	1×10^2	0.94	0.01
Jia, Gao, Hao, & Tang, 2017	4.37×10^4	n/a	5.8×10^4	n/a	1.04	n/a

Abbreviations: K_{SV} - Stern-Volmer quenching constant, K_a – binding constant, n – number of EGCG binding sites on a protein, SEM – standard error of mean, n/a - not available

Supplementary material

Antioxidative capacity and binding affinity of the complex of green tea catechin and beta-lactoglobulin glycated by the Maillard reaction

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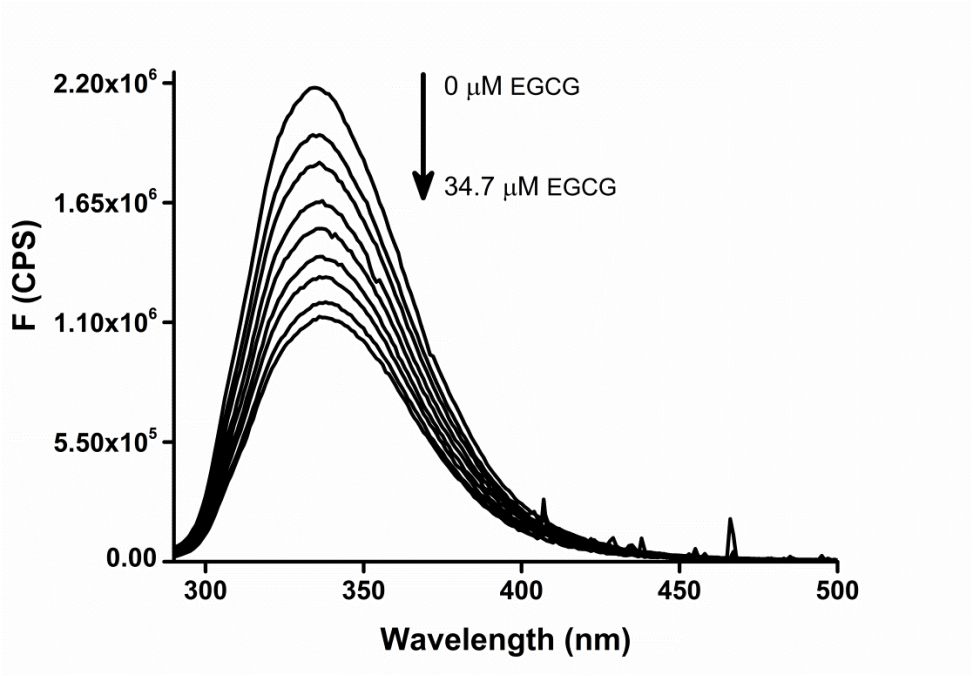

Figure S1. Fluorescence quenching of native β-lacoglobulin (25 μg/mL) by EGCG (0 – 34.7 μM)

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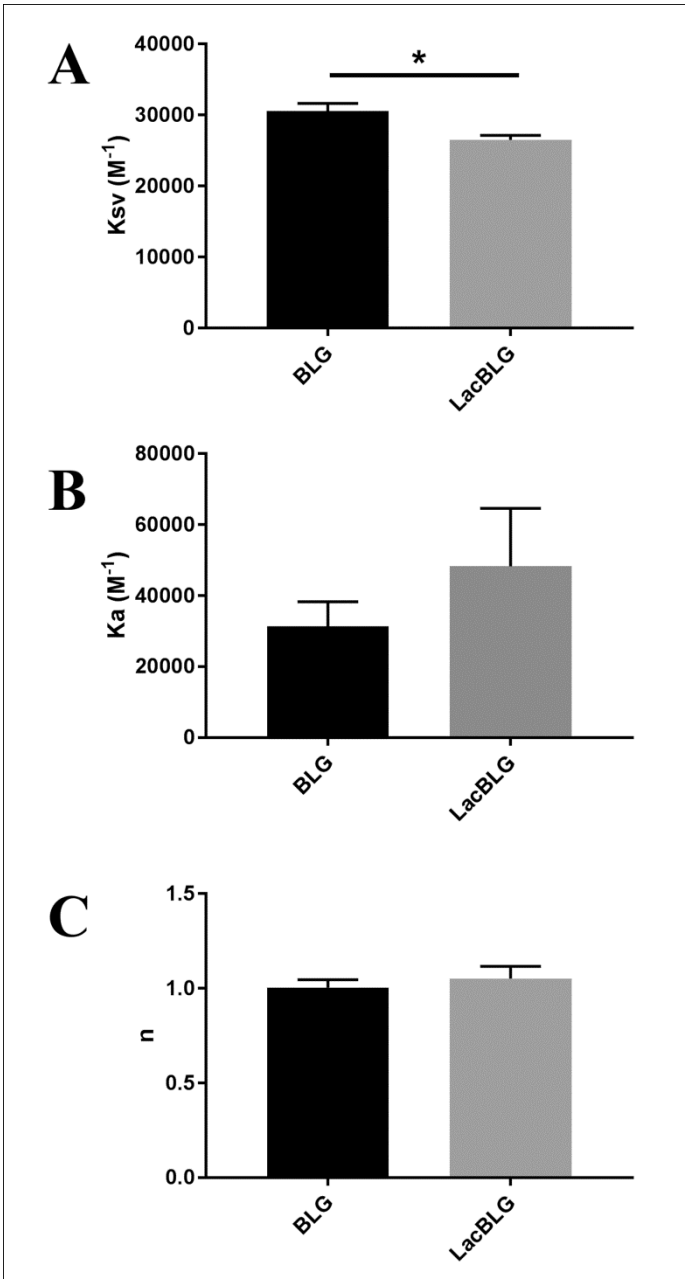

Figure S2. Statistical analysis of EGCG binding parameters to native (BLG) and glycated (LacBLG) β-lactoglobulin. Two sample *t*-test only showed significant difference between Ksv of the two BLG forms (A), while there was no significant difference in the Ka (B) or the number of binding sites (C) at $p < 0.05$.

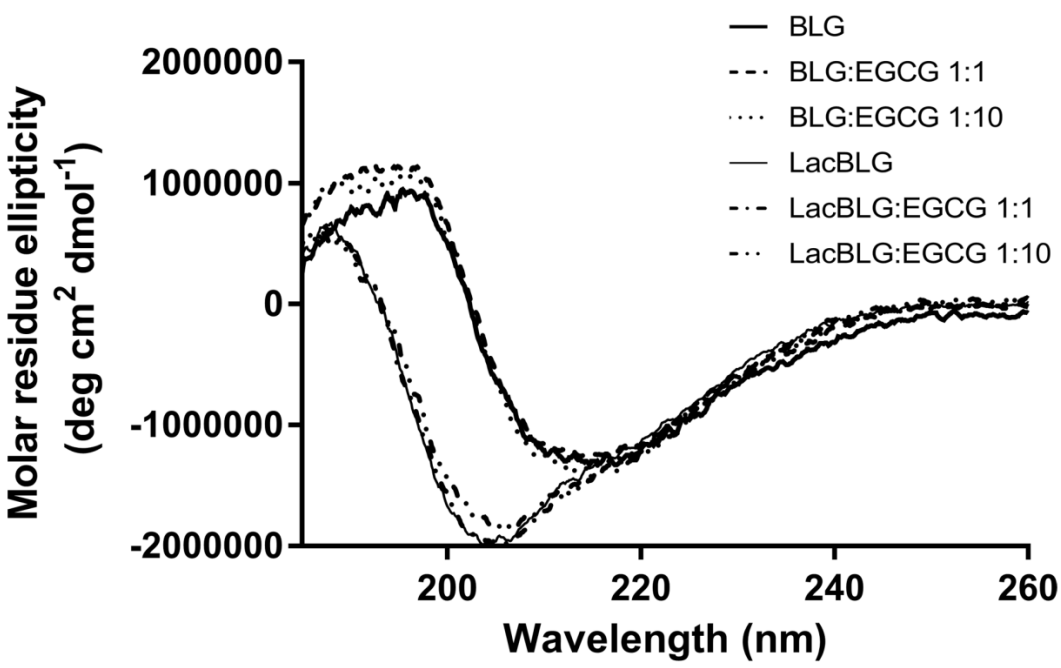

Figure S3. EGCG induces conformational changes in both forms of BLG as shown by CD spectra. The protein CD spectra were recorded in 20 mM PBS buffer (pH 7.2) with and without EGCG. The following molar ratios were used: BLG (——), BLG:EGCG 1:1 (- - -), BLG:EGCG 1:10 (· · ·), LacBLG (——), LacBLG:EGCG 1:1 (–·–), LacBLG:EGCG 1:10 (– · ·)