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Effect of Processing on Physicochemical Characteristics and Bioefficacy of β-Lactoglobulin–Epigallocatechin-3-gallate Complexes

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ABSTRACT: Varying amounts of epigallocatechin-3-gallate (EGCG) were encapsulated in β-lactoglobulin (β-Lg) nanoparticles, either native or processed, denoted as heated or desolvated protein. The stability, physical properties, and bioactivity of the β-Lg–EGCG complexes were tested. Native β-Lg–EGCG complexes showed comparable stability and binding efficacy (EGCG/β-Lg molar ratio of 1:1) to heated β-Lg nanoparticles (1% and 5% protein w/w). The sizes of heated and desolvated β-Lg nanoparticles were comparable, but the latter showed the highest binding affinity for EGCG. The presence of EGCG complexed with β-Lg did not affect the interfacial tension of the protein when tested at the soy oil–water interface but caused a decrease in dilational elasticity. All β-Lg complexes (native, heated, or desolvated) showed a decrease in cellular proliferation similar to that of free ECGC. In summary, protein–EGCG complexes did not alter the bioefficacy of EGCG and contributed to increased stability with storage, demonstrating the potential benefits of nanoencapsulation.

KEYWORDS: *β-lactoglobulin, EGCG, nanoencapsulation, fluorescence spectroscopy, interfacial tensiometry, bioefficacy, Caco-2 cells*

1. INTRODUCTION

Tea (*Camellia sinensis*) has been consumed for thousands of years and is the most common beverage after water.[1] Green tea extracts have shown antibacterial and antiviral activity[2] and also have been associated with a decreased risk of cancer or cardiovascular disease.[3] These activities are mainly attributed to the presence of polyphenolic compounds in tea leaves (i.e., flavonoids). Flavonoids contribute to color and taste of foods[3] and are widely found at high concentrations in plant-derived materials.[4]

In green tea, flavonoids are a large and heterogeneous group of molecules. Catechins account for 80% of total green tea flavonoids and include the majority of bioactive molecules in tea.[4] Epigallocatechin-3-gallate (EGCG) is the most prevalent (approximately 50%) and bioactive tea catechin[5] and is a highly water-soluble compound. As an anticarcinogen, EGCG inhibits the growth of cancer cells, such as human cervical cancer cells or colon cancer cells, and although its mechanisms of action are not fully understood, it has been shown to induce apoptosis and regulate gene expression in such cells.[6] However, recent in vivo studies on prostate cancer have led to the conclusion that green tea may not be considered yet as a chemopreventive agent.[7]

As an antioxidant, EGCG was demonstrated to act up to 10 times as effectively as L-ascorbate or β-carotene,[8] as assessed by the survival of *Escherichia coli* incubated with alkyl peroxyl radical (pH 7.3, 37 °C, 35 min). EGCG can be easily oxidized in aqueous environments, rendering it highly unstable in aqueous solutions, especially at neutral and basic pH.[9,10] The aqueous stability of EGCG can be improved by the addition of metal ion scavengers (such as ethylenediaminetetraacetic acid, EDTA) or other antioxidants (such as vitamin C) in the medium.[11] It is important to note that the bioavailability of EGCG depends not only on its stability but also on its complexed state in the food matrix, the extent of its intestinal absorption, and the extent of its bioconversion.[3]

In addition to their nutritional value, proteins are utilized for a variety of functional properties in foods, such as gelation, emulsification, or foam formation. They have the ability to bind with small hydrophilic and hydrophobic molecules, making them potential carriers of bioactive molecules in the human body[12] and in the creation of functional foods.[13−15] β-Lactoglobulin (β-Lg) is the major whey protein with an approximate concentration of 1 $g \cdot L^{-1}$ in milk and a molecular mass of 18 400 Da.[16] Its ability to act as a nanocarrier system has been shown with model compounds such as bixin,[17] vanillin,[18] resveratrol,[19] and tea polyphenol extracts.[20]

The structure and functionality of β-Lg is highly dependent on environmental conditions such as pH and temperature. Previous studies showed that the EGCG-binding characteristics of β-Lg can be enhanced up to 3.5 times after thermal denaturation of the whey proteins.[9] The binding of polyphenols to proteins occurs mainly through hydrophobic interactions,[16] hydrogen bonding,[21] and, in some instances, electrostatic forces.[22] These interactions depend on the size and structure of the polyphenol molecules. EGCG has the highest binding affinity to whey proteins among tea catechins,[16] because of its esterification with gallic acid, which creates a galloyl functional group with multiple peptide binding sites, able to form hydrophobic interactions and hydrogen bonds with the proteins.[23]

The utilization of β-Lg as a delivery system for EGCG could potentially improve the stability of the polyphenol in a wider pH range, limit its gastric degradation, and enhance its

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intestinal uptake. A well-designed β-Lg−EGCG system should not negatively affect the digestibility of the protein and the health-promoting properties of EGCG. The effect of tea polyphenols on digestive enzymes remains controversial, as shown by Shpigelman et al.[24] According to their results, complexation of β-Lg with EGCG did not significantly affect in vitro gastric digestion of the protein. Similar conclusions were drawn for casein−EGCG complexes: the presence of EGCG did not affect casein hydrolysis, in two different in vitro models.[25] In the literature, the role of β-Lg−EGCG interactions in the prevention of EGCG degradation in aqueous medium remains controversial regarding the extent to which EGCG is protected. It was demonstrated that the binding of EGCG to heat-denatured (85 °C, 20 min) β-Lg can effectively reduce the extent of oxidative degradation (up to 33 times) in 30 mM phosphate-buffered saline (PBS, pH 6.9), especially over the first 8 days.[24] However, it was also reported that degradation of EGCG over time is not significantly limited by binding with native or heated β-Lg.[4]

In vitro assays have been conducted to measure EGCG antioxidant activity as well as that of β-Lg−EGCG complexes.[26] To the best of our knowledge, the antioxidant activity of β-Lg−EGCG complexes has not been studied in cell culture experiments.

In this study, the interactions between β-Lg and EGCG as influenced by processing conditions were investigated. Thermal processing and desolvation were utilized in order to manufacture protein−EGCG complexes along with the unprocessed protein. The physicochemical characteristics of such complexes including encapsulation efficiency, particle size, and charge were studied by a variety of analytical techniques. It has been repeatedly shown in the literature that utilization of cell culture models in the testing of bioefficacy of medicinal drugs or bioactive compounds is a valuable approach prior to animal or human trials as an efficient and cost-effective strategy.[27,28] The bioefficacy of these complexes was studied in Caco-2 cells. This cell line was chosen as representative of epithelial tissues; it is one of the most commonly utilized cell culture models in current pharmacological studies. The effect of free EGCG, or EGCG associated with native protein, as well as protein nanoparticles, was tested by measuring their ability to inhibit cellular proliferation.

2. MATERIALS AND METHODS

2.1. Sample Preparation. Highly pure β-Lg (>99%) used in the experiments was purified from whey protein isolate (New Zealand Dairy Products, NZMP, Palmerston North, New Zealand) by preparative ion chromatography on Q-Sepharose (GE Healthcare) as previously reported.[29] β-Lg stock solution (2−5% w/w) was prepared in a buffer medium (see below) and kept refrigerated overnight (4 °C) to ensure complete hydration. The stock solution was then filtered through a 0.45 μm filter (Millipore, Millex HV, Billerica, MA). EGCG (min. 94%) was a gift of DSM Nutritional Products (Teavigo, Ayr, Ontario, Canada) and its stock solutions (0.1−5 mg·mL^{-1}) were freshly prepared and filtered as described for β-Lg solutions. Unless otherwise stated, all solutions were prepared in sodium phosphate buffer (20 mM, pH 7.0) that contained sodium azide as bacteriostat (0.02%). β-Lg stock solution was equilibrated at room temperature (30 min) before it was mixed with varying volumes of EGCG solutions to obtain different EGCG/β-Lg molar ratios (0−10). Each sample was immediately vortexed for 5 s.

2.2. Fluorescence Spectroscopy. β-Lg−EGCG complexes were prepared by mixing 10.9 μM β-Lg solution with equal volumes of diluted EGCG stock solutions ranging from 0 to 135.9 μM. Eleven different samples with EGCG/β-Lg molar concentration ratios of 0−10 were prepared, and 200 μL aliquots of each sample were transferred to a 96 well fluorescence plate (Fisher Scientific). The protein intrinsic fluorescence spectra (300−450 nm) were directly recorded with a Synergy H4Microplate Reader (Biotek, Winooski, VT) utilizing an excitation wavelength of 280 nm and slit width of 5 nm. The Stern−Volmer equation was used to determine the quenching mechanism that takes place due to EGCG binding to β-Lg molecules:

$$\frac{F_0}{F} = 1 + K_{SV}[\mathrm{EGCG}] \quad (1)$$

where F_0 is the initial fluorescence intensity of 0.02% (w/w) β-Lg solution, F is the fluorescence intensity of the sample at the peak (325 nm emission), and K_{SV} is the Stern−Volmer constant.[30] The binding constant and stoichiometry were calculated as follows:

$$\log\left(\frac{F_0 - F}{F}\right) = \log(K_s) + n \log[\mathrm{EGCG}] \quad (2)$$

where K_s is the static binding constant and n is the binding number.[20] All measurements were carried out at least in duplicate.

2.3. Preparation of EGCG-Bearing β-Lactoglobulin Nanoparticles. *2.3.1. Thermal Processing.* Thermal processing of β-Lg solutions was carried out at 85 °C (20 min, 400 rpm) with a thermomixer (Eppendorf 5436, Brinkmann Instruments, Mississauga, ON, Canada). The samples were immediately transferred to an ice bath and were mixed with EGCG solutions at room temperature at a molar ratio ranging from 0:1 to 3:1 (EGCG/β-Lg). The final β-lactoglobulin concentrations in the mixture were 1% or 5% (w/w) in all cases.

2.3.2. Desolvation Procedure. Whey protein nanoparticles were prepared by an established procedure.[14] To maximize electrostatic repulsion between protein molecules, pH of the native β-Lg solutions was adjusted to pH 9 with concentrated sodium hydroxide solution (2 M). Pure ethanol was then added to 2 mL of β-Lg solution at a rate of 1 mL·min^{-1}. The final volume of ethanol added was 5 times the volume of β-lactoglobulin solution (i.e., 10 mL). Immediately after desolvation, the mixture was diluted 20 times in 20 mM sodium citrate buffer (pH 3) to stabilize the desolvated particles. In this case, as heating was not applied, EGCG was dissolved in the dilution buffer prior to mixing (0−190.7 μM). Due to the high extent of dilution in the desolvation protocol (i.e., 120 times), all desolvation experiments were conducted at an initial protein concentration of 5% (w/w).

2.4. Interfacial Tensiometry. To further probe the formation of complexes and possible changes in the protein functionality, interfacial tensiometry was applied to determine changes in protein adsorption at the interface. This technique is based on analysis of the size and shape of a dispersed phase droplet (i.e., soy oil) suspended in a continuous phase (i.e., aqueous suspension of β-Lg−EGCG complexes). Interfacial tension was measured for at least 2 h to ensure that equilibrium was established. Immediately afterward, the oil droplet was sinusoidally oscillated around its initial volume (±10%) to measure the elastic modulus. A dilational amplitude range of 0−40% was within the linear viscoelastic range (data not shown).

The interfacial activity of EGCG-bearing β-Lg nanoparticles was determined by drop shape tensiometry at the aqueous dispersion−soybean oil interface (25 °C) (Tracker, IT Concept, Longessaigne, France). The oil phase was purified prior to the experiments by mixing it with Florisil (Sigma−Aldrich, catalog no. 46385, particle size 149−250 μm)[31] at a ratio of 10:1, shaking for 2 h (60 rpm) on a shaking plate, and then removal of Florisil by filtering (Whatman cellulose filter #52, 7 μm). The purification process was repeated three times prior to all measurements. A soy oil droplet (approximately 6 μL) was automatically formed at the tip of a syringe, which was immersed in a cuvette containing the aqueous dispersion of protein−EGCG complexes or protein only. The assembly of the cuvette and syringe was monitored by a charge-coupled device (CCD) camera for acquisition of high-quality images, and the shape of the oil droplet was automatically analyzed in order to calculate the time dependence of the interfacial tension up to a drop age of 10 000 s. Since the calculation of dynamic interfacial tension was based on the Young−

Laplace equation, the shape of the drop was periodically monitored to ensure a Laplacian shape and the absence of partial detachment of the drop throughout the run. All the measurements were carried out in duplicate. The focus, verticality of the syringe tip, and measured image volume were reset or recalibrated daily.[32]

Dilational elasticity gives an indication of the ability of the adsorbed moieties to stabilize the interface during droplet volume changes. The method utilized here is based on the automatically controlled, sinusoidal compression−expansion of the aqueous droplet at a defined oscillatory frequency and amplitude of dilation.[33] The interfacial modulus of dilational elasticity was calculated from the change in interfacial tension (dγ) relative to the change in droplet surface area (dA):

$$\varepsilon = \frac{d\gamma}{d \ln A} \tag{3}$$

Heated and desolvated β-Lg particles were prepared and EGCG was added to prepare complexes of these particles and along with native protein, as detailed above. The interfacial activity of the complexes was measured. A molar ratio range of 0:1 to 3:1 (EGCG/β-Lg) was utilized throughout the experiment. All measurements were carried out at least in duplicate.

2.5. Determination of Apparent Particle Diameter and ζ-Potential by Dynamic Light Scattering. The particle size distribution and ζ-potential of EGCG-bearing β-Lg nanoparticles was determined by dynamic light scattering (DLS) on a Zetasizer Nano (Malvern Instruments, Worcestershire, U.K.). No further dilution was carried out prior to DLS measurements due to the low extent of scattering of the protein dispersions. All particle size distributions reported here were determined on the basis of volume frequency, and the apparent mean diameter was measured.

2.6. Separation of Uncomplexed Polyphenols. To determine the amount of encapsulated EGCG, free EGCG was separated from bound EGCG on a desalting column (HiTrap desalting column, product no. 17-408-01, GE Healthcare, Uppsala, Sweden). The extent of EGCG encapsulation was then quantified on the basis of absorbance measurements (at a wavelength of 280 nm). The complexes were eluted with 20 mM sodium phosphate buffer (pH 7) that contained 50 mM NaCl. Free EGCG was removed from the column with 20% ethanol, and the absorbance measurements were carried out in ethanolic solution on a UV−visible spectrophotometer (RF-5301PC, Shimadzu, Japan). A range of EGCG controls (0−0.2 mg/mL) was also studied along with the samples.

2.7. Cellular Proliferation Assays. Caco-2 cells were provided form CRIFS culture collection (Canadian Research Institute for Food Safety) and were maintained in Dulbecco's modified Eagle medium (DMEM; Sigma−Aldrich Corp., Oakville, ON, Canada) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Invitrogen Canada Inc., Burlington, ON, Canada), 2 mM L-glutamine, 1% (v/v) penicillin−streptomycin, and 1% (v/v) essential amino acids as well as 0.25% (v/v) *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) buffer. Cells were passaged weekly (80−90% confluence) with 0.25% trypsin−EDTA (Invitrogen) and medium was changed every other day. Cells were kept daily at 37 °C in humidified atmosphere in 5% CO_2. Cells at passage 29−38 were used for the proliferation tests.

Freshly prepared EGCG−β-Lg and respective aged samples (24 h at 4 °C) were administered to the Caco-2 cell culture. Sulforhodamine B (SRB) assay was used to evaluate the influence of the samples on the proliferation rate of Caco-2 cancer cells.[34] Briefly, Caco-2 cells at a concentration of 6×10^3 cells/well were seeded on clear bottom 96 well plates (Fisher Scientific, Mississauga, ON, Canada) and allowed to adhere for 24 h. Afterward, fresh samples were added to the cells at a dilution rate of 1:5.6 in the medium (sample/medium v/v) and incubated for 24 h. Loading on the cells was calculated on the basis of the amount of EGCG originally present in the sample. The amount of protein β-Lg in final dilution in the well was 1.8 μg/mL for native and heated samples, while for desolvated samples it was 0.08 μg/mL. EGCG concentrations were 0.05 and 0.13 μg/mL for EGCG/β-Lg molar ratios of 1 and 3, respectively, when complexed with native or heated β-Lg. On the other hand, the EGCG concentration was 0.006 μg/mL when complexed with desolvated β-Lg to maintain a EGCG/β-Lg molar ratio of 3. Control samples (cells with medium only) along with blank samples (medium only) were also tested. The optical density of plates was measured on an automated 96-well plate reader (Synergy H4Microplate Reader, Biotek, Winooski, VT) at 570 nm. Results were expressed as percentage proliferation with respect to control wells grown with no sample treatments.

2.8. Statistical Analyses. Unless otherwise stated, all the experiments were carried out in triplicates and the results are expressed as means ± standard deviation (SD). Statistical differences were evaluated by analysis of variance (ANOVA) (XL Stats [7.5.2], Addinsoft, Paris, France). The differences were considered to be significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Fluorescence Spectroscopy. Figure 1 shows the changes in intrinsic fluorescence in native β-Lg solutions (0.02% w/w) as a function of EGCG concentration (0−0.05 mg·mL^{-1}, corresponding to EGCG/β-Lg molar ratios 0−10). For EGCG/β-Lg ratios that are lower than 4:1 (0.02 mg/mL EGCG), the absorbance values recorded for the mixture were lower than 0.1 (data not shown), thus rendering the measurements valid in this concentration range. In all cases, the intensity of the intrinsic fluorescence intensity of the protein decreased with EGCG addition. The linearity of the Stern−Volmer plot (Figure 1, inset) suggested that all fluorophores in the protein were equally accessible to the EGCG molecules.[30] The quenching was attributed to binding of EGCG to β-Lg.

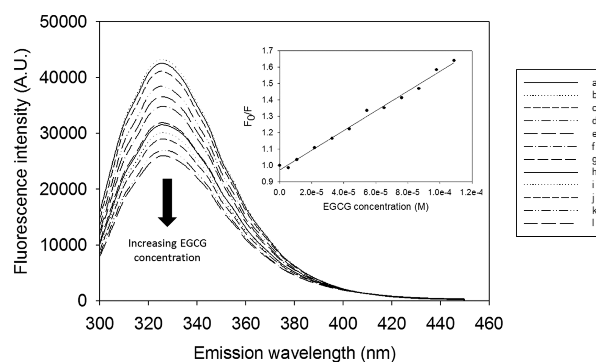

Figure 1. Fluorescence spectra of 0.02% (w/w) β-lactoglobulin and the influence of EGCG concentration (millimolar) on intrinsic fluorescence of the protein. The molar ratio range tested was 0−10. Curves a−l respectively correspond to EGCG/β-Lg molar ratios 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10. Excitation was carried out at 280 nm. Representative runs are shown. (Inset) Stern−Volmer plot (eq 1) derived from fluorescence intensity data.

Figure 2 illustrates the linearity of the double logarithmic plot (eq 2), employed to determine the binding constant (K_s) and number of EGCG binding sites per β-Lg molecule (n). These values were 4.3×10^4 M^{-1} and 1.2, respectively. These figures were found to be comparable with those reported in previous studies where a similar fluorescence method was used: 1.25 × 10^4 M^{-1} and 0.94[4] and 1.3×10^4 M^{-1} and 1.3,[16] respectively, for binding constant and number of binding sites. On the basis of these findings, each native β-Lg molecule can be expected to bind approximately one EGCG molecule (i.e., 1:1 on a molar concentration basis).

Previous authors reported an increase in the EGCG binding capabilities of β-Lg with heating.[9] In the present work, the

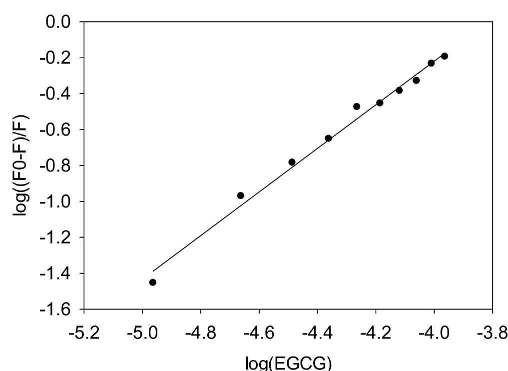

Figure 2. Double logarithmic plot of changes in fluorescence intensity as a function of EGCG concentration (10.9−108.8 μM). The binding constant and number of binding sites of β-Lg for EGCG binding can be inferred from the intercept and slope, respectively (eq 2).

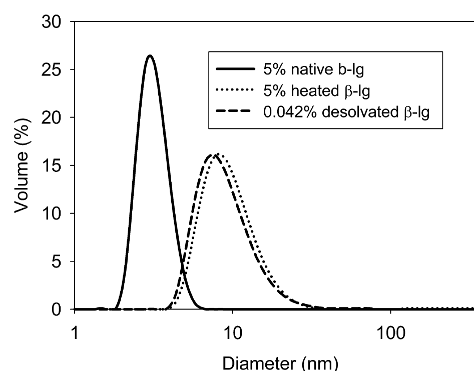

Figure 3. Particle size distribution of native (—), heated (85 °C, 20 min) (···), or desolvated (5× ethanol) (− − −) β-lactoglobulin solutions mixed with EGCG solutions at molar concentration ratio (EGCG/ β-Lg) of 3 as determined by dynamic light scattering (DLS). The protein concentration was 5% for native and heated protein, whereas it corresponded to 0.042% due to extensive dilution requirement for desolvated samples. Representative runs are shown.

association of EGCG with β-Lg was studied after preparation of protein nanoparticles by either thermal processing or desolvation techniques. We hypothesized that β-Lg−EGCG complexes could be obtained and that, in the complexed form, EGCG could maintain its bioefficacy in a cell culture model for intestinal cells (i.e., Caco-2 culture).

3.2. Physicochemical Characteristics of EGCG-Bearing β-Lactoglobulin Particles. The average apparent diameter for nanoparticles is summarized in Table 1, and Figure 3 shows the particle size distribution of EGCG-bearing β-Lg nanoparticles (heated or desolvated, 1:3 molar ratio of protein/ EGCG) or native β-Lg, as measured by dynamic light scattering. Both heating and desolvation resulted in significantly larger protein complexes but <100 nm in average apparent diameter. The experiments were conducted at 10 and 50 μg·mL^{-1} protein in the case of solutions heated at 85 °C for 20 min and at 50 μg·mL^{-1} for desolvated nanoparticles (due to the high dilution in pH 3 buffer; see Materials and Methods). Native β-Lg solutions showed an average apparent diameter between 11 and 14 nm, and heating significantly increased the size to about 30 nm for 10 μg·mL^{-1} and 40 nm for 50 μg·mL^{-1} protein. The presence of EGCG did not affect the particle size significantly at the protein concentration levels studied (Table 1). This finding might be attributed to the molecular mass of EGCG (458 Da), which is considerably smaller compared to that of β-Lg (18.4 kDa) and the low extent of EGCG binding on a molar basis as shown by fluorescence measurements (i.e., n = 1.2). Furthermore, EGCG interactions with the protein did not cause aggregation of the particles.

Desolvated β-Lg particles (with a starting solution of 50 μg·mL^{-1}) showed a significantly higher apparent diameter than both heated and native particles, with a larger standard deviation between experiments (p < 0.05). This was due to a small fraction (1.2% of the volume) of larger particle (>1000 nm) in some of the replicate runs (data not shown). These results are in agreement with the observation of desolvated β-Lg samples by cryo-transmission electron microscopy (cryo-

Table 1. Particle Size, ζ Potential, and Encapsulation Efficiency of EGCG-Bearing Native, Heated, or Desolvated Nanoparticles[a]

β-Lg concn (%)	EGCG/β-Lg molar ratio	diameter (nm)	ζ potential (mV)	encapsulation efficiency (% EGCG)	loading EGCG/β-Lg (μg·mg^{-1})
			Native[b]		
1	0	14 ± 1 D	−17 ± 4 C		
1	1	11 ± 1 D	−21 ± 3 CD	91 ± 13 A	23 ± 3
1	3	12 ± 4 E	−27 ± 2 E	69 ± 9 AB	52 ± 7
5	0	12 E	−10 ± 2 B		
5	1	10 ± 0 E	−16 ± 3 C	89 ± 12 A	22 ± 3
5	3	11 ± 0 E	−21 ± 2 C	22 ± 9 C	16 ± 7
			Heated[b]		
1	0	30 ± 0.5 CD	−17 ± 5 C		
1	1	30 ± 0.5 D	−21 ± 4 C	94 ± 8 A	21 ± 5
1	3	38 ± 8 BCD	−27 ± 3 E	54 ± 13 BC	44 ± 8
5	0	41 ± 1 BC	−27 ± 2 E		
5	1	42 ± 1 B	−26 ± 2 DE	79 ± 2 AB	20 ± 1
5	3	42 ± 3 B	−30 ± 3 E	27 ± 3 C	20 ± 2
			Desolvated[c]		
0.042	0	71 ± 24 A	28 ± 1 A		
0.042	3	101 ± 38 A	26 ± 2 A	91 ± 18 A	68 ± 13

[a]Different letters in the same column indicate a statistically significant difference. [b]Native β-Lg or heated β-Lg nanoparticles at final protein concentrations of 1% and 5% (pH 7). [c]β-Lg nanoparticles prepared with desolvation at a final protein concentration of 0.042% (pH 3).

TEM) showing small spherical particles and some larger particles.[14]

The thermal denaturation point of β-Lg is ∼76 °C.[35] Previous studies[35,36] showed that the first steps of denaturation lead to formation of surface-exposed hydrophobic clusters that increase protein−protein interactions. At temperatures higher than 60 °C, β-Lg monomers are partially unfolded, exposing the thiol group.[37] The free sulfhydryl group of β-Lg becomes reactive and causes thiol/disulfide interchanges with the other β-Lg monomers.[37,38] Heating caused aggregation of β-Lg, and the size of the aggregates significantly increased with protein concentration from 10 to 50 $\mu g \cdot mL^{-1}$ ($p < 0.05$). This observation was previously attributed to an acceleration of the polymerization reaction with increasing protein concentration.[38] Although the tertiary structure of β-Lg is affected by heating, as shown by Fourier transform infrared (FTIR) measurements, a reversible change in secondary structure will take place once β-Lg is cooled down quickly after heating.[26] Furthermore, it was reported that binding of EGCG did not affect the secondary structure of the protein, which is composed primarily of β-strands (55%).[26] This was in contrast with the findings of Shpigelman et al.[24] who suggested, using attenuated total reflectance (ATR)-FTIR, that binding of EGCG on heated β-Lg changed the protein secondary structure toward a higher extent of random coils. In the case of desolvated nanoparticles, however, the addition of ethanol to a β-Lg solution triggers a change in solvent polarity that affects the protein secondary structure and its ability to bind with other molecules. It has been shown that, in the presence of ethanol, β-Lg increases its α-helix content, and the structural changes lead to aggregation of the monomers.[39]

The ζ-potential of native β-Lg and heated β-Lg nanoparticles was found to be negative at pH 7, for example, −17 mV for 1% native and heated protein alone. When measured at a higher protein concentration, the values for native β-Lg particles were significantly lower; this may indicate the presence of residual ions in the purified protein fractions, which increases the overall ionic strength in the medium with protein concentration. In all cases, the presence of EGCG significantly increased the magnitude of ζ-potential of native β-Lg, as well as for the heated β-Lg nanoparticles (Table 1). This is consistent with results reported by Thongkaew et al.[40] when adding catechins to whey protein isolate. At 50 $\mu g \cdot mL^{-1}$, the heated nanoparticles were significantly more negatively charged than the native proteins, and the ζ-potential was also found to significantly increase in magnitude with EGCG concentration (Table 1). These results were consistent with a previous study[24] showing that the stability of EGCG-bearing heated β-Lg complexes was greater than that of heated β-Lg alone, which demonstrated that EGCG tends to stabilize β-Lg particles. It can be explained by the fact that, at pH 7, EGCG phenolic groups are deprotonated, generating an oxygen center of negative charges that will prevent further aggregation of β-Lg−EGCG complexes.[37] A recent work also pointed out a change in the structure of the protein upon polyphenol addition, namely, an increase in α-helix and β-sheet secondary structures and a decrease in random coil.[16] This could also stabilize the β-Lg−EGCG complexes. However, at high polyphenol concentration (1% w/v), precipitation of whey protein isolate has been reported and attributed to the aggregation of soluble complexes forming insoluble flocs.[37] In the case of desolvated particles (Table 1), since pH 3 is below the isoelectric point of β-Lg, their ζ-potential was positive, as previously demonstrated.[14] The value did not significantly vary with the presence of EGGC. In all cases, the particles showed a charge of about +26 mV, which indicates stability of the particles.

3.3. Encapsulation Efficiency of EGCG-Bearing β-Lg Nanoparticles. The amount of EGCG associated with the protein was determined after separation of the protein fraction from the unbound EGCG by a gel-filtration method. The values of EGCG recovery and encapsulation efficiency are summarized in Table 1 for 10 and 50 $\mu g \cdot mL^{-1}$ protein. The amount of EGCG recovered with β-Lg in 10 $\mu g \cdot mL^{-1}$ solutions was not significantly different between native and heated β-Lg (Table 1). At a molar ratio of 1:1 (EGCG/β-Lg), most of the EGCG was found associated with the protein, which was consistent with the fluorescence data reported in Figures 1 and 2. Since the fluorescence measurements indicated a molar binding ratio of approximately 1.2, the two sets of measurements are coherent. However, at the same β-Lg concentration, upon further addition of EGCG to the system (EGCG/β-Lg molar ratio increasing from 1:1 to 3:1), the encapsulation efficiency (percent) significantly decreased to about 60%, which implied the saturation of complexes. On a weight basis, the extent of binding increased from about 20 to 50 $\mu g \cdot (\text{mg of protein})^{-1}$ when the EGCG/β-Lg molar ratio increased from 1:1 to 3:1.

Table 1 also summarizes the association behavior of EGCG at 50 $\mu g \cdot mL^{-1}$ β-Lg. At this concentration, again, as for the 10 $\mu g \cdot mL^{-1}$ samples, there was no significant difference between heated and unheated β-Lg particles, and in all cases, the percent encapsulated was about 80% for EGCG/β-Lg molar ratio 1:1. These results are in contrast with earlier reports showing that EGCG binding of β-Lg increases after thermal denaturation of the protein.[9] At the higher molar ratio, the amount of encapsulated EGCG significantly decreased to about 25%, and the amount of loading was consistent at approximately 20 μg of EGCG/mg of protein. When 1% native dispersion and heated β-Lg particles complexed with EGCG were compared, at a molar concentration ratio of 1, almost complete encapsulation of EGCG took place, whereas at higher EGCG/β-Lg ratios, part of the EGCG remained free in solution. These findings are in full agreement with the fluorescence data shown in Figures 1 and 2 (i.e., $n = 1.2$). The findings can be attributed to increased extent of protein−protein interactions upon thermal denaturation, which could limit protein−polyphenol interactions.

Desolvated β-Lg particles showed very different encapsulation behavior. Due to their high extent of dilution (to a final protein concentration of 0.42 $\mu g \cdot mL^{-1}$), the percentage encapsulated was tested only for a molar ratio of 3:1 (EGCG/β-Lg). The encapsulation efficiency for these samples was approximately 90%, which was significantly higher than the other methods. It has been previously demonstrated that desolvated whey protein particles show high encapsulation efficiency.[15] The data shown in Table 1 clearly demonstrate that most of the EGCG added was found associated with the desolvated protein particles. The amount of EGCG bound was 68 μg of EGCG/mg of β-Lg, which was significantly higher than the values obtained with heated β-Lg particles. Compared to native β-Lg, which can bind approximately 1 EGCG molecule/β-Lg protein molecule (as shown in Figures 1 and 2), desolvated particles can bind approximately 2.7 EGCG molecules/β-Lg protein molecule. The different pH value (pH 3) could clearly affect the structural attributes of the protein, including surface charge and secondary structure.[41] However, the high dilution ratio utilized here may limit the applicability in

food systems. Although the ethanolic desolvation procedure enhanced the molar encapsulation ratio and the encapsulation efficiency on a weight basis (mg/mg), the stabilization of desolvated proteins and the reduction in ethanol content require extensive dilution, which in turn limits the total amount of EGCG that can be encapsulated. However, this approach could extend the EGCG encapsulation characteristics of β-Lg to acidic pH values and relevant food products.[10]

3.4. Interfacial Characteristics of EGCG-Bearing β-Lactoglobulin Nanoparticles. β-Lactoglobulin is known for its interfacial properties, and it is commonly found as an ingredient in emulsion systems, due to its ability to adsorb at interfaces and stabilize oil droplets or air bubbles. The interfacial characteristics of β-Lg were studied to determine whether the interfacial characteristics of the protein could be modified by formation of a complex with EGCG. The interfacial characteristics of the samples (at EGCG/β-Lg molar ratios of 0:1 and 3:1) were studied at the soy oil−dispersion interface by drop shape tensiometry. It has been previously reported that EGCG in isolation does not demonstrate interfacial activity.[42]

In all experiments, β-Lg reduced the interfacial tension of the oil−water interface, initially measured at 30.7 $mN \cdot m^{-1}$,[32] regardless of presence or absence of EGCG (Table 2). The interfacial tension data show that there were no significant differences in equilibrium interfacial tension values between native, heated, or desolvated protein particles. In the absence of EGCG, interfacial tension values for the oil−water interface were approximately 16 $mN \cdot m^{-1}$. This was consistent with previous findings on desolvated whey protein particles.[14] The presence of EGCG did not significantly affect the equilibrium interfacial tension for any of the samples, which implied that EGCG binding does not limit the extent of protein adsorption at the oil−dispersion interface.

Table 2. Interfacial Tension and Dilational Elasticity Characteristics of EGCG-Bearing β-Lg Dispersions at the Soy Oil−Dispersion Interface as a Function of Composition and Processing Conditions[a]

EGCG/β-Lg molar ratio	interfacial tension ($mN \cdot m^{-1}$)	dilational elasticity ($mN \cdot m^{-1}$)
	Native	
0	17.5 ± 2.1	32.8 ± 12.9
3	16.3 ± 2.4	21.2 ± 4.3
	Heated	
0	15.8 ± 1.2	33.3 ± 6.3
3	16.0 ± 1.6	23.7 ± 7.6
	Desolvated	
0	14.7 ± 0.5	34.8 ± 11.7
3	17.4 ± 0.0	29.3 ± 1.4

[a]Native and heated samples contained 1% protein, whereas due to extensive dilution, desolvated samples had a β-Lg concentration of 0.042%. Dilational elasticity was measured at a dilational frequency of 10 mHz, and the extent of dilation was 10% in all cases.

Dilational elasticity was also tested for native β-Lg dispersions, β-Lg nanoparticles, and their corresponding EGCG complexes, as shown in Table 2. The method of sample manufacture did not significantly alter the dilational elasticity characteristics of the interface. The presence of EGCG significantly decreased the dilational modulus in all cases. In the absence of EGCG, the elasticity modulus was approximately 33 $mN \cdot m^{-1}$, while at a molar ratio of 3:1 (EGCG/β-Lg), dilational elasticity decreased to 21, 23, and 29 $mN \cdot m^{-1}$ (Table 2), for native dispersion and heat-induced and desolvated particle systems, respectively. It was concluded that the association of EGCG with β-Lg may decrease the ability of the protein to interact with other protein molecules at the interface and therefore hinder the creation of a rigid interface. These results are in contrast with the study of Rossetti et al.,[42] who have shown that EGCG increases elasticity when complexed with other proteins (saliva proteins from whole human saliva). This may be due to the differences in proteins and environments. We can infer from the current findings that the presence of EGCG did not significantly affect interfacial tension but decreased the extent of protein−protein interactions at the interface. These results demonstrated that EGCG binding only slightly affected the functional, and possibly nutritional, properties of the protein.

3.5. Bioefficacy of β-Lactoglobulin−EGCG Complexes. Proliferation tests were conducted on confluent Caco-2 cells after 24 h of growth. The viability (percent) of Caco-2 cells that were administered freshly prepared or aged samples (24 h, 4 °C) after incubation for 24 h is shown in Table 3. Since EGCG is known for its anticancer properties on the vast majority of cancer cell lines,[6,43,44] in this study, its antiproliferative capacity was investigated in isolation or complexed with native β-Lg or desolvated or heated β-Lg nanoparticles. In the case of

Table 3. Caco-2 Cell Proliferation Measured for EGCG Complexed with Native β-Lg or Heated or Desolvated β-Lg Nanoparticles after 24 h of Incubation[a]

amt loaded ($\mu g \cdot mL^{-1}$)		cell proliferation (%)	
protein	EGCG	fresh (t = 0)	aged (t = 24 h)
		Native	
1.8	0	106.3 ± 3.5	105.9 ± 1.6
1.8	0.05	18.4 ± 0.3	34.9 ± 2.1
1.8	0.13	17.1 ± 0.8	23.4 ± 1.9
		Heated	
1.8	0	92.1 ± 11	106.2 ± 12
1.8	0.05	27.3 ± 0.6	42.8 ± 5.8
1.8	0.13	19.9 ± 0.8	34.7 ± 1.7
		Desolvated	
0.08	0	104.7 ± 15	94.6 ± 5.5
0.08	0.006	34.8 ± 0.9	34.1 ± 15
		EGCG (pH 7)	
0	0.05	20.5 ± 0.7	87.5 ± 16
0	0.13	34.1 ± 1.9	94.1 ± 19
		EGCG (pH 3)	
0	0.05	31.3 ± 2.2	81.2 ± 7.5
0	0.13	29.4 ± 5.0	94.3 ± 18
		Phosphate Buffer	
0	0	102.1 ± 4.5	NA
		Citrate Buffer	
0	0	105.2 ± 4.9	NA
		Citrate Buffer + EtOH	
0	0	100.1 ± 7.4	NA

[a]Final β-Lg and EGCG concentrations in the medium were calculated respectively on the basis of the amount loaded initially for 1 and 3 protein/EGCG molar ratios after final dilution in the medium (1:5.6 v/v). A high dilution ratio (120×) was applied on desolvated protein−EGCG complex. Controls of respective buffers and EGCG solution pH 3 and 7 were also tested.

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unheated β-Lg and heated β-Lg nanoparticles, 1.8 $\mu g \cdot mL^{-1}$ solutions were tested at EGCG/β-Lg ratios of 1 and 3. On the other hand, for the desolvated nanoparticles, due to dilution effects, final dilutions brought the β-Lg concentration to 0.08 $\mu g \cdot mL^{-1}$, and a molar ratio of 3 was employed. In all cases, about 90% of the EGCG was encapsulated (see Table 1). Control samples containing buffer at pH 7 or citrate buffer at pH 3 (with or without traces of ethanol) were tested, and they did not affect Caco-2 viability. Free EGCG showed a significant decrease in cell proliferation in fresh samples ($p < 0.05$); however, in all cases, both at pH 7 and 3, the decrease in efficacy after storage of the EGCG solution at 4 °C was noticed. When EGCG was associated with native β-Lg, there was a clear decrease in cell proliferation. The values were comparable to those obtained with free EGCG. Similarly, heated β-Lg−EGCG complexes showed a statistically significant decrease in cell proliferation ($p < 0.05$). All the samples of EGCG complexed with proteins showed bioefficacy also after 24 h at the incubation temperature. In the desolvated β-Lg samples, due to the extensive dilution requirements in buffer at pH 3, a smaller amount of EGCG was added to the cell culture wells (see Table 3). The bioefficacy of EGGC in the desolvated β-Lg nanoparticles was fully maintained, with results not significantly different in fresh and aged samples. In both cases, the cell viability decreased up to 35%. Encapsulation of EGCG in nanostructures such as liposomes or casein micelles also has been shown to maintain its antiproliferative capacity on colon carcinoma cells.[45,46]

In this work we demonstrated that β-Lg, both native and after heat or desolvation treatment,can be an effective, protective carrier for EGCG. The bioactivity of β-Lg−EGCG complexes tested on Caco-2 cell monolayers did not alter its antiproliferative activity, and in all cases this activity was maintained. Furthermore, the results of this study confirm previous suggestions by Shpigelman et al.[9] that β-Lg−EGCG complexes provide considerable protection against oxidative degradation of EGCG. As shown in Table 3, such complexes were able to preserve the bioactivity of EGCG after 24 h storage of the solutions, compared to free EGCG solutions. Further studies should be conducted to investigate whether such processing conditions are able to protect EGCG activity after prolonged storage, if such complexes were to be considered in beverage formulations.

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■ ABBREVIATIONS

EGCG, epigallocatechin-3-gallate; β-Lg, β-lactoglobulin

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