

Structural changes in emulsion-bound bovine beta-lactoglobulin affect its proteolysis and immunoreactivity



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ABSTRACT

Adsorption on the surface of sub-micrometric oil droplets resulted in significant changes in the tertiary structure of bovine beta-lactoglobulin (BLG), a whey protein broadly used as a food ingredient and a major food allergen. The adsorbed protein had increased sensitivity to trypsin, and increased immunoreactivity towards specific monoclonal antibodies. In spite of the extensive tryptic breakdown of emulsion-bound BLG, some sequence stretches in BLG became trypsin-insensitive upon absorption of the protein on the fat droplets. As a consequence – at contrast with free BLG – proteolysis of emulsion-bound BLG did not decrease the immunoreactivity of the protein, and some of the large peptides generated by trypsinolysis of emulsion-bound BLG were still recognizable by specific monoclonal antibodies. Structural changes occurring in emulsion-bound BLG and their consequences are discussed in comparison with those occurring when the tertiary structure of BLG is modified by lipophilic salts, by urea, or upon interaction with solid hydrophobic surfaces. Such a comparison highlights the relevance of situation-specific structural modifications, that in turn may affect physiologically relevant features of the protein.

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1. Introduction

Beta-lactoglobulin (BLG) is the major bovine whey protein, being present in the 2–3 g/l range in cow milk [1]. BLG is absent from human and mouse milk [2], and represents a major food allergen [3]. BLG belongs to the lipocalin protein family [2], and its physical and chemical properties are well characterized. However, its biological functions *in vivo* are still a matter of debate. It has been shown that BLG binds small ligands such as fatty acids and vitamins, as expected for lipocalins [4], as well as man-made hydrophobes [5], including various non-polar drugs [6]. Bound fatty acids are indeed present in as-isolated BLG, and contribute to the structural stability of BLG towards physical and chemical denaturation [7]. BLG also is an exceptionally acid-stable protein: a dimer at pH around neutrality, at pH 2 dissociates reversibly into monomers, but its structure remains native. This contributes to making BLG very resistant to pepsin degradation at low pH.

Resistance to peptic hydrolysis as well as to duodenal proteases allows some of the BLG in foods to remain intact in the digestive tract

[1]. This increases the probability that intact BLG will be absorbed and act as an antigen. In this frame, the presence of stabilizing agents – including physiologically relevant ligands – may contribute to the allergenicity of BLG, as structure-stabilizing agents may further protect BLG from proteolysis [7]. Treatments used in milk processing and in the preparation of dairy products may change the allergenic properties of BLG and its sensitivity to proteases. Many epitopic regions have been found in BLG, including both sequential and conformational epitopes [8]. Several of the conformational epitopes recognized by human IgE or by specific monoclonal antibodies are reportedly no longer present in BLG above a given threshold of treatment intensity [8–11], and proteolysis of partially unfolded BLG conformers has been shown to decrease BLG immunoreactivity [12,13]. However, others have reported that denaturation of BLG may expose epitopes that are not accessible in the native protein [9,11], and that specific IgE from cow's milk allergic patients may react with products of enzymatic breakdown of whey and milk proteins to a greater extent than the intact protein [14–17].

Many studies demonstrated that BLG undergoes extensive conformational changes after interacting at a water interface with either solid [18] or liquid [19] hydrophobic phases. Absorption of proteins on a liquid/liquid interface almost necessarily involves a concomitant mechanical denaturation of the protein. Whey proteins are often used as ingredients also in non-dairy foods, where they may interact with other food components either spontaneously or – more often – upon processing. Processing alters existing structures and forms new supra-

Abbreviations: BLG, beta-lactoglobulin; NP, nanoparticles; DTNB, 5, 5'-dithiobis-2-nitrobenzoic acid; ELISA, enzyme-linked immunoassay; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

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macromolecular architectures that contribute to the texture or appearance of a particular food. Most of these interactions occur after that processing conditions (temperature, shear forces, addition of other ingredients, changes in pH) have triggered conformational changes in the protein structure [20,21]. In many cases, process-dependent structural modifications produce protein conformers that are stabilized by interaction with other food components, such as lipid droplets in emulsions [22]. These interactions may limit (either kinetically or thermodynamically) the formation of completely unfolded forms of the involved proteins, and the ensuing aggregation events. Thus, partially unfolded proteins end up being trapped and stabilized at the interface, so that emulsion-bound proteins may have properties that are different from those of both their native forms and of those obtained by complete unfolding either in the laboratory or in industrial plants.

The structural features of BLG in emulsions have seldom been addressed from a molecular stand point [19,23], as most studies have been focused on practical issues related to emulsion stability [24,25]. Thus, there is very limited information as for: 1) the nature of epitopes exposed by BLG denatured at a liquid/liquid interface; 2) how the BLG conformers present in an emulsion-bound state may interact with proteases; and 3) the immunoreactivity of the proteolytic products derived from action of digestive-tract enzymes on BLG-stabilized emulsions.

Therefore, the aim of this work is to analyze the conformational changes in BLG resulting from the mechanical denaturation and the interaction with liquid hydrophobic interfaces when BLG/fat emulsions are formed. This work also addresses the consequences of structural modifications in BLG at the liquid/liquid interface in terms of altered susceptibility to proteases, and of altered immunoreactivity. Features of emulsion-bound BLG are discussed in comparison to those of other non-native forms of the protein, such as those generated in solution by treatment with urea and lipophilic ions [7,12,26], or upon adhesion of BLG to hydrophobic nanostructures [18], that have been characterized quite extensively in previous studies.

2. Materials and methods

2.1. Chemicals and proteins

All reagents used were in the highest degree of purity commercially available, and were purchased from Sigma-Aldrich (Milan, Italy), unless otherwise specified. To avoid the presence of the partially denatured BLG species found in commercial preparations, the protein was purified from unprocessed milk whey as reported in previous studies [7,18]. Hydrophobic ligands present in the as-purified BLG were removed on a Lipidex-1000 column (Sigma-Aldrich, Milan, Italy), as reported elsewhere [7,18]. The protein was then concentrated in an Amicon ultrafiltration device (Millipore, Billerica, MA, nominal cut-off 10 kDa), buffer-exchanged into distilled water, lyophilized, and stored at -24°C . BLG concentration in solutions was determined by using an extinction coefficient of $0.93\text{ g}^{-1}\text{ l cm}^{-1}$ at 280 nm.

2.2. Emulsion preparation and properties

Emulsions were prepared by pre-emulsifying 0.5 g of soy oil with 9.5 ml of a BLG solution (5 mg/ml in 0.05 M sodium phosphate buffer, pH 7.0) by using a Vortex dispersing unit for approximately 1 min. The pre-emulsion was immediately homogenized in ice by using a sonicator (MSE Soniprep 150, London, UK) for 5 distinct 1-min cycles (30 s interval) with a 14 μm amplitude intensity. Emulsion droplet size was measured using a Mastersizer instrument (Malvern Instrument Ltd., Malvern, UK) equipped with a Hydro SM manual small volume sample dispersion unit. The lipid-rich “cream” phase was separated from the aqueous “serum” phase by centrifugation at $13,200 \times g$ for 20 min. The bottom “serum” phase was carefully collected using a syringe. The “cream” phase was washed with water, and re-suspended in an appropriate volume of buffer (0.05 M sodium phosphate, pH 7.0)

to reach an oil content of 5% (i.e., the same of the original emulsion). The amount of protein present in each emulsion phase was determined by densitometric analysis of SDS-PAGE tracings, using known amounts of purified BLG for calibration. A 0.05 ml aliquot of either “cream” or “serum” samples (or of an appropriately diluted BLG standard) was treated with 0.05 ml of Laemmli denaturing buffer, boiled for 5 min, and aliquots of the resulting mixture were loaded on a 12% acrylamide gel. After the electrophoretic run (see below for detailed conditions) gels were stained with Coomassie Brilliant Blue prior to image analysis (Image Master 1D, GE Healthcare, Milano, IT).

2.3. Fluorescence spectroscopy

Tryptophan emission spectra (300–500 nm, scan speed 50 nm/min) were recorded in a Perkin-Elmer LS 50B spectrofluorometer (Perkin Elmer, Waltham, MA) equipped with a solid-state (front-face) cell holder for measuring the fluorescence of solid matrices. Excitation was at 280 nm, and emission and excitation slits were set at 2.5 nm. Fluorescence measurements were carried out on BLG solutions, on BLG emulsions, and on the “cream” and “serum” phases described in the previous subsection. All samples were adjusted to a protein concentration of 0.05 mg/ml by dilution in 0.05 M sodium phosphate buffer, pH 7.

2.4. Thiol accessibility studies

Determination of accessible –SH groups was performed by using 5,5'-dithiobis-2-nitrobenzoic acid as reported in previous studies [7, 18,26]. The time course of absorbance changes at 412 nm consequent to the addition of 0.5 mM DTNB (final concentration) to 3 ml of emulsions containing 1 mg of BLG in 0.05 M phosphate buffer, pH 7.0, was measured for 30 min at 25°C . Absorbance changes in the emulsion samples were followed by reflectance spectrophotometry using a properly equipped Lambda 650 UV-Vis spectrophotometer (Perkin Elmer, Waltham, MA).

2.5. Limited trypsinolysis

Limited proteolysis was performed by adding 0.01 ml of a trypsin solution (Sigma Chemical Co, St. Louis, MO, TCPK treated, 1 mg/ml in 0.025 M sodium acetate, pH 4.5) to 1 ml of BLG solutions, of “cream”, or of “serum”, as appropriate. All BLG-containing samples were diluted in 0.05 M sodium phosphate, pH 7.0, to achieve a final BLG concentration of 1 mg/ml. Thus, the protease/substrate ratio was kept constant at 1/100 w/w. Hydrolysis were carried out for 30 min at 37°C and stopped by addition of 0.02 ml of soybean Kunitz trypsin inhibitor solution (2 mg/ml in water). The resulting mixtures were used as such for immunoreactivity studies. Interfering lipids were removed from emulsion-derived trypsin-treated samples to be used for HPLC analysis and mass spectrometry studies by repeated extraction in hexane/acetone [18]. Lipid-free samples were lyophilized prior to further analysis.

2.6. Peptide characterization by RP-HPLC

Lipid-free lyophilized samples were dissolved in aqueous 0.1% trifluoroacetic acid (buffer A), clarified by centrifugation (15 min, $13,200 \times g$), and loaded on a Waters Symmetry 300™ C18 column (5 μm , $4.6 \times 250\text{ mm}$, Waters, Milan, IT). Separation was carried out in a HPLC system (mod. 515, equipped with a 717 autosampler and a 996 PDA detector, all from Waters, Milan, IT), operating at 0.8 ml/min, using a linear gradient from 100% A to 100% buffer B (0.1% trifluoroacetic acid in acetonitrile) in 125 min. Absorbance was monitored at 220 and 280 nm.

2.7. Peptide characterization by MALDI-TOF mass spectroscopy

Lipid-free lyophilized samples (see above) were dissolved in 0.1% aqueous TFA, cleaned on a C18 Zip Tip device (Millipore, Billerica, MA), mixed with the matrix (α -cyano-4-hydroxycinnamic acid) and loaded on MALDI plates. MALDI-TOF mass spectrometry was carried out on a PerSeptive BioSystems Voyager DE-Pro spectrometer equipped with a N₂ laser (337 nm, 3-ns pulse width, 20-Hz repetition rate), and operating at an accelerating voltage of 20 kV. Angiotensin I (m/z ¼ 1296.68), bovine α -s1-casein peptide 1-23 (m/z ¼ 2764.55) and bovine insulin (m/z ¼ 5730.61) were used for external mass calibration. Mass spectra were acquired in the reflector mode using Delay Extraction technology, and analyzed by using the software provided by the manufacturer.

2.8. ELISA (enzyme-linked immunosorbent assay)

Competitive capture ELISA tests were carried out by using two specific mouse anti-BLG monoclonal antibodies (mAb 5G6 and mAb 1E3, courtesy of Prof. Hanne Frøkiær, Department of Veterinary Disease Biology, Københavns Universitet, Copenhagen, DK) as done in previous studies [18,27,28]. BLG-containing samples were incubated for 3 h with the given monoclonal antibody prior to transfer into 96-well ELISA plates previously coated with BLG. Following various washing steps and incubation with an antimouse IgG-horseradish peroxidase conjugate (P260, Dako, Glostrup, DK), tetramethylbenzidine in hydrogen peroxide buffer was added, and plates were read after color development on an automated plate reader (Bio-Rad, Hercules, CA).

2.9. SDS-page and Western blotting

For SDS-PAGE and subsequent Western blotting, 0.05 ml of each sample was added to 0.05 ml of Laemmli denaturing buffer, boiled for 5 min, and then loaded to a 12% acrylamide gel. Electrophoresis was carried out for 1.5 h at 16 mA in a Mini Protean 3 apparatus (Bio-Rad, Hercules, CA). After the electrophoretic run, proteins were electroblotted (1 h at 100 V) on a nitrocellulose paper sheet (Protran®, 0.45 μ m, Millipore, Billerica, MA) using a Mini Trans Blot apparatus (Bio-Rad, Hercules, CA). The nitrocellulose sheets were blocked by overnight incubation with 5 mg/ml BSA in buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7, PBS). After extensive washing, sheets were incubated with the primary antibody solution for 2 h (mAb 5G6 or mAb 1E3, diluted 1:2000 in PBS), and washed extensively again. The secondary antibody was an anti-mouse IgG-horseradish peroxidase conjugate (P 260, Dako, Glostrup, DK, diluted 1:3000 in PBS), and incubation lasted 2 h. Membranes were then developed with a solution containing 0.6 mg/ml 4-chloro-1-naphthol, 10% (v/v) methanol, and 0.6% (v/v) H₂O₂ in PBS.

3. Results

3.1. Properties of BLG-stabilized emulsions

Emulsions formed by ultrasonic treatment of 5% oil dispersions in aqueous 5 mg/ml BLG had a relatively sharp size distribution, with an average droplet size of 249 nm. Particles of $\approx$ 200 nm were the most abundant species, although a shoulder at $\approx$ 1200 nm was also evident in distribution analyses, as shown in the Supplementary materials (Fig. S1, panel A). These dimensions are comparable with those of common food emulsions.

SDS-PAGE was used to quantify the amount of surface-bound BLG in the emulsions and of free BLG remaining in solution after separation of the fat-containing “cream” and the fat-free “serum” phases in the emulsions. Other procedures commonly used for protein quantification were found to be unreliable in this mixed-phase system. The approach used for SDS-PAGE quantification of the free and absorbed protein involved

digitalization and image analysis of the Coomassie Blue-stained gels, and the use of known amounts of pure BLG for constructing a calibration curve, as shown in the Supplementary materials (Fig. S1, panel B). In the emulsions used in this study, about 70% of total BLG was found absorbed on the oil droplet surface (i.e., in the “cream” phase), the remaining 30% being present as free BLG in the aqueous, lipid-free “serum” phase.

3.2. Front face fluorescence detects protein structural changes in emulsion-bound BLG

Structural features of BLG at the emulsion interface were studied by solid-state (front-face) fluorescence. This technique allows direct fluorescence measurements on solids or on multiphasic matrices [29–31], including liquid–liquid emulsions [23]. Tryptophan emission spectra of BLG in aqueous solution and in the different phases obtained by centrifugal separation are presented in Fig. 1. The red shifts observed in the tryptophan emission maximum when BLG is bound to the lipid droplets in the “cream” phase indicates that at least one of the two Trp residues in BLG had moved from a non-polar chemical environment to a polar one. This red shift is not observed for the protein remaining in the aqueous “serum” phase, indicating that the protein remaining in solution did not undergo severe conformational changes. According to recent reports [19], BLG at the surface of model emulsions undergoes a rearrangement of its secondary and tertiary structures, with a strongly decrease in the α -helix content and an increase in inter-molecular beta-sheets.

The findings reported here are in keeping with previous spectrofluorometric studies on the behavior of soybean proteins at a water/oil interface [22,31]. A red shift of the Trp emission maximum was reported also for BLG adsorbed on the surface of solid polystyrene nanoparticles [18], that is, under conditions where no portion of the protein may penetrate the (solid) hydrophobic phase. All these studies also reported an increase in Trp emission intensity, as observed here. In the case of BLG, the increased fluorescence yield was attributed to minor structural modifications involving either a movement of the surface-located Trp₆₁ away from the Cys₆₆–Cys₁₆₀ disulfide bridge, or a movement of the buried Trp₁₉ away from Arg₁₂₄. This latter residue can act as a fluorescence quencher (as typical also of disulfide bridges) and is located just 3.6 Å above the Trp₁₉ indole ring in the native protein. However, tryptophan fluorescence measurements on BLG adsorbed on n-hexadecane emulsions [23] have reported a blue-shifted emission

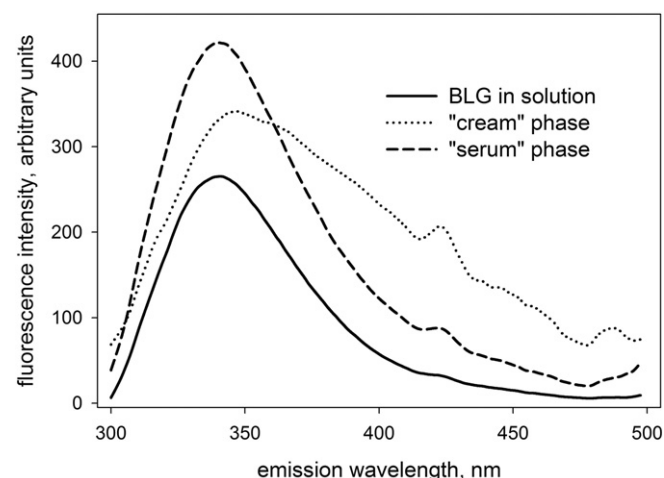


Fig. 1. Tryptophan emission fluorescence spectra of free and emulsion-bound BLG. Solid-state (front-face) tryptophan emission fluorescence spectra (excitation wavelength, 280 nm) of BLG in solution (full line), and of BLG present in the “cream” (dots) and “serum” (dashes) phases separated by centrifugation from BLG-stabilized oil emulsions. All samples were diluted in 50 mM phosphate buffer, pH 7.0, to a protein concentration of 0.05 mg/ml.

maximum, indicating incorporation of the protein Trp sidechain(s) in a non-polar environment, opposite to what reported here. This discrepancy may be consequent to the different hydrophobicity and residual surface charge of the non polar phases used in these studies, and of the different procedures used for emulsion preparation.

The data in Fig. 1 indicate that loss of the original structure accompanies stable incorporation of the protein into the fat droplet. Loss of structure may stem from the physical treatment (exposure to ultrasonic waves, in our case), that could generate transiently denatured BLG forms [12,32,33], that are characterized by exposed hydrophobic surfaces [5] and may be subsequently trapped at the lipid/water interface much more easily than the native protein, in which hydrophobic surfaces are typically most present in the protein interior. According to the incorporation data presented in the former subsection, 30% of BLG remains in solution as a conformer having spectroscopic features similar to those of native BLG. Whether this fraction of the protein did not respond to the physical treatment or reverted to a structure similar to the native one (at least from the standpoint of fluorescence spectroscopy) after transient deformation by the ultrasound treatment remains to be assessed.

One of the earliest steps in denaturation of BLG by either chemical agents or temperature has been reported to be a movement of the C-terminal helix away from the beta-barrel region of the protein. This results in exposure of the Cys₁₂₁ thiol (the only free thiol in BLG), that thus becomes accessible to suitable reagents [7,18,26]. Under appropriate conditions, the exposed free thiol may be involved in the subsequent formation of intermolecular disulfides through disulfide exchange reactions with other BLG molecules [34,35] or with other proteins.

Increased accessibility of the Cys₁₂₁ thiol has been observed for BLG adsorbed on nanoparticles [18]. Reaction rates with DTNB at room temperature for nanoparticle-adsorbed BLG (1.16 min^{-1}) were close to those measured for BLG heated in solution at 70°C (1.26 min^{-1}). Emulsion-bound BLG gave much lower rates (0.11 min^{-1}), very close to those measured for BLG heated in solution at 45°C [12,18,32,33]. This makes it evident that this peculiar region was differently affected by interaction with solid and liquid hydrophobic interfaces, respectively.

3.3. Trypsin proteolysis studies

Limited proteolysis studies offer a convenient way of addressing process-induced structural changes even in proteins lacking functional markers of their structural integrity, such as enzymatic or other biological activities. These studies are of particular relevance in the case of food proteins, as different digestibility ensuing from process-related structural modifications may affect their nutritional properties and/or other functions. In the case of BLG, marked changes in the pattern of proteolytic products have been reported for transient conformers generated during physical denaturation [12,13,27], but also as a consequence of the adsorption of the protein on solid hydrophobic surfaces [18].

As shown in Fig. 2, adsorption makes BLG quite susceptible to trypsinolysis, at difference with the native protein. This is evident from the substantial absence of intact BLG after treatment with trypsin in the emulsion state. Trypsinolysis of BLG in emulsion also gave origin to a pattern of peptides that only partially matched – in nature and relative abundance – those obtained from the protein in solution. Increased susceptibility of BLG to trypsin [25,36] and to pepsin [37] had already been reported after adsorption of BLG on fat and non-fat emulsion droplets.

MALDI-TOF mass spectroscopy was used to identify peptides released from trypsin action on free BLG and on BLG-stabilized emulsions. Table 1 lists the sequences identified in either mixture, along with those of the products originating from trypsin treatment of native BLG and of partially denatured forms of BLG obtained by interaction with lipophilic

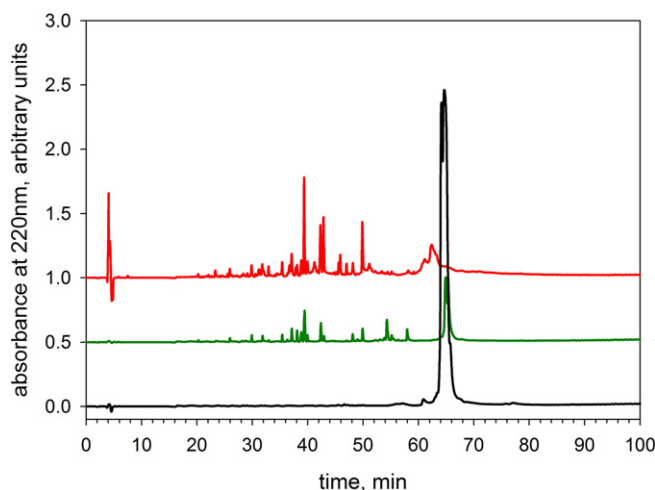


Fig. 2. Products of tryptic hydrolysis of free and emulsion-bound BLG. RP-HPLC traces (offset for clarity) of untreated BLG (black), and of reaction mixtures from tryptic hydrolysis of free BLG (green) and of a BLG-stabilized emulsion (red). Hydrolysis was carried out for 30 min at 37°C and stopped by addition of soybean Kunitz trypsin inhibitor. Lipids were removed from the tryptic digest obtained from emulsion-bound BLG by repeated extraction with hexane/acetone prior to RP-HPLC analysis.

salts or with chaotropes, or upon interaction with the hydrophobic surface of styrene nanoparticles.

Some of the peptides originating from emulsion-bound BLG do not match “classic” tryptic cleavage sites. Apparently, adhesion of BLG to the oil droplet makes it possible for trypsin to act on non-positively charged residues (Leu₃₁, Trp₆₁, Phe₁₀₅, Gln₁₂₀, Val₁₂₈). Whether this relates to altered accessibility of specific protein regions or to an interference of the oil phase with the enzyme itself remains to be established. As made evident in Table 1, non-canonical tryptic patterns have been reported previously for other denatured forms of BLG [12,18,26].

The data in Table 1 make it evident that the long stretch encompassing residues 61–105 may be found intact only in the trypsinolytic products from emulsion-bound BLG. This sequence contains eight lysine residues (at positions 69, 70, 75, 77, 83, 91, 100, 101) and no arginine, and corresponds to beta-strands located at the opposite side of the structure with respect to the dimerization interface for BLG in solution and to the major helix in native dimeric BLG, as depicted in the upper panel of Fig. 3.

Yet another possibility for explaining the lack of accessibility to trypsin of this region of BLG is that the positive charges on the lysine side chains may interact with residual free fatty acids in the oil droplet, thus limiting access to trypsin. However, it has to be noted that the titratable acidity of the refined soybean oil used in this study was extremely low (<0.05), thus contribution of electrostatic interactions in this system is expected to be minimal. However, this contribution may be much more relevant in food formulations using other types or grades of edible fats, where titratable acidity is much greater, or whenever charged amphiphilic molecules are used for emulsion stabilization.

Further analysis of the proteolysis products with reference to the native structure of the BLG monomer reveals that the lysine residues with limited trypsin accessibility found in emulsion-bound BLG are clustered on a specific side of the monomer (Fig. 3, lower panel), that is, opposite to the one where are located the “unusual” trypsin targets identified in this study (most of them hydrophobic, as mentioned above). The site with limited trypsin accessibility borders with the N-terminal side of the trypsin-resistant 61–105 stretch.

It is also worth noting that trypsin was found to be active on Lys₆₉ (but not on Lys₇₀) in two different partially unfolded forms of BLG, obtained in the presence of sub-denaturing concentrations of either urea or KSCN [26]. In those studies, the products of chaotrope-induced partial

Table 1

Masses and sequences of the tryptic peptides generated from various BLG conformers.

Measured molecular mass (Da) of BLG tryptic fragments in the given condition					Sequence	Non-canonical cuts and notes	Missedcuts
Native ^{a,b,c}	2 M KSCN ^a	8 M urea ^a	NP-bound ^b	Emulsion-bound ^c			
933.54	933.52	933.50	933.54		1–8	K ⁺ adduct	
		1587.93			1–14		Lys 8
	2102.40	2102.23			1–19	Trp 19	Lys 8, Lys 14
	2266.52	2266.32			1–20	Tyr 20	Lys 8, Lys 14
		4278.71			1–40		Lys 8, Lys 14
	4541.88	4540.99			1–42	Tyr 42	Lys 8, Lys 14, Arg 40
487.56					9–13	Gln13	
3362.97				3362.87	9–40		Lys 14
		1293.68			10–20	Gly9	Lys 14
		3926.71			10–45	Gly9	Lys14, Arg40
	3193.15				11–40	Leu10	
				1756.03	15–31	Leu31	
2707.37				2708.01	15–40		
	2379.53	2379.35			19–40	Thr18	
	1874.18	1874.98			21–39	Tyr20, Leu39	
2032.34			2032.93		21–40	Tyr20	
	3731.58	3731.21			21–55		Arg40
1759.48	1759.16	1759.01			24–40	Ala23	
	2022.30	2021.14			24–42	Ala23, Tyr42	Arg40
1627.23					25–40	Ala25	
2313.25			2310.38		41–60		
				3359.82	41–69		Lys60
3486.78					41–70		Lys60, 69
1192.54					61–70		Lys69
				5242.12	61–105	Phe 105	Lys69, 70, 75, 77, 83, 91, 100, 101
				5056.18	62–105	Trp61, Phe105	Lys69, 70, 75, 77, 83, 91, 100, 101
701.45				701.45	70–75		
				802.50	71–77		Lys75
	1458.80	1458.68			71–83		Lys75, Lys77
903.56				903.56	76–83		Lys 77
1801.13					76–91		
674.42				674.42	78–83		
916.47				916.47	84–91		
1065.58					92–100		Lys 101
2647.20					102–124		
				3074.58	102–128	Val 128	
	2999.94				113–124-S-S-149-162	Disulfide bridged	
				2722.15	118–141	Leu 117	
1245.58				1245.58	125–135		
1635.77				1635.77	125–138		
				2767.21	125–148		
			1153.60		139–148		
837.47				837.47	142–148		
		2478.40			142–162		
1658.78		1658.80	1661.73	1658.78	149–162		
			2769.95	2722.15	61–69-S-S-149-162	Disulfide bridged	
			8266.55		61–70-S-S-102-162	Disulfide bridged	

^a From [25].^b From [17].^c This work.

unfolding were found to differ remarkably in dependence of the chaotrope being used, and to generate polymeric fibrils having chaotrope-specific shapes, stability, and mechanism of formation and stabilization.

Somewhat in contrast with what were discussed above, the data in Table 1 reveal some similarities in the proteolysis pattern of emulsion-bound BLG (that underwent mechanical denaturation concomitant to exposure at the interface) and of BLG that unfolded “spontaneously” upon contacting the surface of polystyrene nanoparticles (and sticking to them in a partially denatured form) in a geometry-sensitive process reportedly much faster (although less complete and extensive) than denaturation by chaotropes [18].

As for the possible physiological significance of these findings, the trypsin-resistant 61–105 sequence present in emulsion-bound BLG contains epitopes reportedly recognized by the immune system of mice (sequences: 67–75, 71–79, 80–88) [38] and rat (sequences: 67–77, 82–92, 85–95) [39]. The 61–105 region also overlaps in part with the epitopic sequence 97–117, that has been reported as relevant to BLG

recognition by human T-cells [40,41], and with the epitopic region corresponding to residues 66–77 [11].

3.4. Immunoreactivity of emulsion-bound BLG and of its trypsinolysis products

Various reports demonstrated that denaturation processes can alter the immunological properties of proteins, including BLG. Extended or limited proteolysis [12,17], heat or pressure treatment in the absence/presence of concomitant proteolysis [12,13,42], and glycation [43] may modify the immunoreactivity of BLG.

To detect changes in immunoreactivity ensuing from adsorption of the protein at the oil–water interface, we developed a competitive ELISA assay using two specific monoclonal antibodies (mAb 5G6 and mAb 1E3, kindly provided by Prof. Hanne Frøkiær from the University of Copenhagen). Several types of antibodies have been developed against many epitopic regions of BLG [8], but the particular monoclonal antibodies used here are the same used in previous studies on the

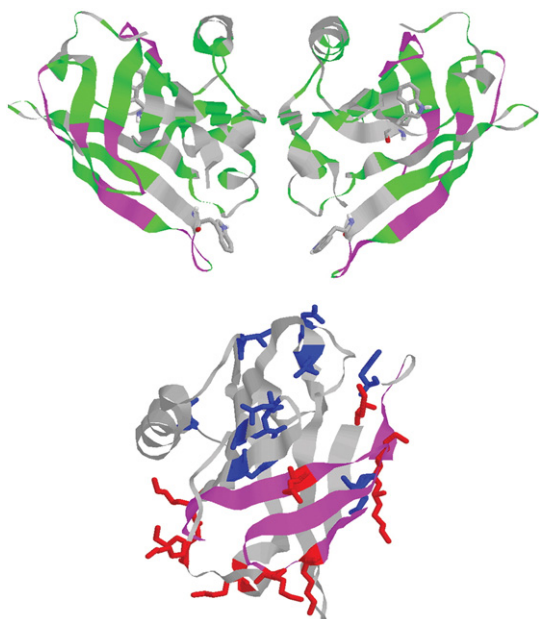


Fig. 3. Position of altered regions in the emulsion-bound state of BLG. Upper panel: a cartoon depicting the structure of the native BLG dimer. In each monomer, Trp 19 and 61 are represented as sticks in CPK colors, whereas the location of other hydrophobic amino acids is highlighted in green. Non-hydrophobic residues in the trypsin-resistant 61–105 sequence are in magenta. Lower panel: a ribbon representation of the structure of the native BLG monomer. Lysine residues that become trypsin-insensitive in emulsion-bound BLG are shown as red sticks. Residues undergoing unexpected hydrolysis by trypsin are represented as blue sticks. The trypsin-resistant 61–105 sequence found in BLG emulsions is in magenta. Cartoons were generated from accessions 1BEB (BLG dimer) and 3UEU (BLG monomer) in the Protein Data Bank database.

immunoreactivity of various process-modified forms of BLG [12,28]. Thus, the use of the same monoclonal antibodies all through these studies should simplify to a great extent any comparison of the changes in immunoreactivity associated with structural changes ensuing from absorption at a liquid/liquid interface with those associated with structural changes induced by other types of processing.

Fig. 4 compares competitive ELISA experiments carried out with BLG-stabilized emulsions and with free BLG. With either monoclonal antibody, emulsion-bound BLG seems more immunoreactive than free BLG, although the increase in immunoreactivity is much larger for mAb 1E3 than for mAb 5G6. This increase in immunoreactivity may be due to either an increase in the number of interfacial epitopes exposed after denaturation of the protein or to some sort of “structure locking” that stabilizes epitopes that are already present on the protein surface. This last hypothesis seems to offer a more creditable explanation,

since the exposure of new binding sites for the antibody would vary affinity constant of the system for the antibodies, thus resulting in a different slope of the inhibition curve. The “apparent” increase in the concentration of immunoreactive protein can be explained by stabilization of the BLG structure in emulsion as a single conformer, in contrast with the possible different immunoreactivity of BLG dimers and monomers, that are present in a concentration-dependent equilibrium in BLG solutions. Indeed, evidence has been provided for an increased immunoreactivity of BLG in solution upon stabilization of its dimeric form through denaturation-induced aggregation [12].

Fig. 4 also includes ELISA data (collected in separate experiments) for free and emulsion-bound BLG after proteolysis by trypsin. The whole hydrolysate was used in these experiments, with no attempt to separate intact BLG from proteolysis products. Consistent with previous reports, immunoreactivity of free BLG is sensibly decreased by proteolysis [12,13], in particular when the 5G6 monoclonal antibody was used. However, trypsinolysis does not seem to affect the immunoreactivity of emulsion-bound BLG towards either monoclonal antibody. This is likely consequent to the inaccessibility to proteolysis of specific epitopic sequences in emulsion-bound BLG, as discussed in the previous section, but it also may indicate that some of the proteolytic products remain stuck at the oil–water interface and interact among themselves in a fashion not dissimilar (at least from the standpoint of antibody recognition) from that observed for the non-nicked protein.

To address these issues, a Western blotting analysis was carried out on tryptic hydrolysates of free and emulsion-bound BLG using the same monoclonal antibodies used in the ELISA assays. The tracings in Fig. 5 make it evident that high-molecular-mass immunoreactive peptides are present only in the products obtained from proteolysis of emulsion-bound BLG, and that these peptides are identically recognized by either monoclonal antibody. Thus, impaired proteolysis of selected sequences in emulsion-bound BLG seems to be a major determinant of the retention of immunoreactivity after trypsin treatment. Although the possible physiological significance of the “protective” effect towards proteolysis ensuing from interaction with oil phases of various nature and composition and at different pH values in actual food systems remains to be verified, these findings confirm that even the simplest and mildest manipulation is capable of affecting some properties of proteins in a food system, with consequences that are of relevance from both a nutritional and an immunological standpoint.

4. Conclusions

This study was aimed at understanding some of the structural rearrangements of BLG when adsorbed onto a liquid/liquid interface, and evaluating whether these changes may be of some physiological relevance, given the role played by BLG-stabilized emulsions in many common foods and its role as an allergen. The reported intrinsic rigidity of

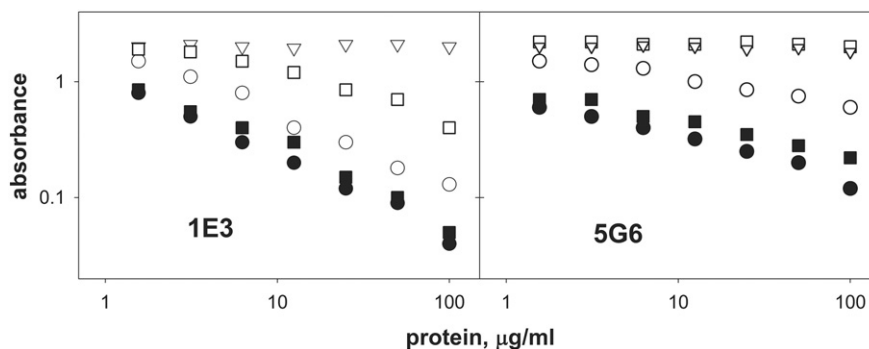


Fig. 4. Immunoreactivity of native and emulsion-bound BLG. ELISA inhibition curves for free BLG (○), for BLG-stabilized emulsions (●), and for samples derived from trypsin hydrolysis of free BLG (□) or of BLG-stabilized emulsions (■). Bovine serum albumin (▽) was used as a negative control. Proteins in each sample are present at the same concentration. Left panel, 1E3 monoclonal antibody; right panel, 5G6 monoclonal antibody.

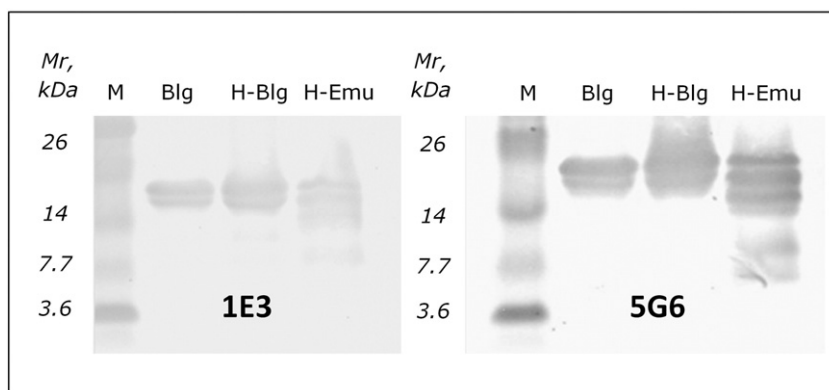


Fig. 5. Western blot of trypsin-hydrolyzed free and emulsion-bound BLG. Western blots of native BLG (Blg), of trypsin-hydrolyzed free BLG (H-Blg), and of trypsin-hydrolyzed BLG-stabilized emulsions (H-Emu) were developed using 5G6 (right panel) or 1E3 monoclonal antibodies (left panel).

the BLG structure [5–7,18] makes this protein a very suitable candidate for these studies, as structural changes ensuing from the treatments required for emulsion formation are apparently limited to the emulsion-bound protein (see Fig. 1) and do not affect the protein remaining in solution. This simplifies to a great extent further analysis of the system.

Adsorption of BLG on the surface of oil droplets in BLG-stabilized emulsions makes BLG more susceptible to proteolysis, as reported by other authors [24,25,36,37]. However, those studies did not address in detail the ensuing changes in the proteolytic pattern. The detailed analysis carried out in the study presented here suggests that adsorption prevents proteolytic action on sequences that include reported epitopic regions and regions involved in BLG recognition by components of the immune system [11,38–41]. The particular state of the emulsion-bound substrates also affects the specificity of the protease, resulting in “unusual” cuts.

We confirm here that BLG increases its immunoreactivity after interaction with an oil/water interface in microemulsions, that may be regarded as a model of food-relevant liquid/liquid interfaces. After hydrolysis with trypsin, the overall immunoreactivity of emulsion-bound BLG remains high, at least when compared with that of the protein treated with trypsin in solution. This appears to be a consequence of the presence of high molecular mass peptides in the hydrolytic products of emulsion-bound BLG, that are absent in the protein hydrolyzed in solution. These high-molecular-mass peptides remain recognizable by the monoclonal antibodies used in this study, as suggested also by Western blotting results.

The possible physiological relevance of these findings is linked to the common practice of using whey proteins as an emulsion stabilizer in

many food formulations, including many non-dairy products. In terms of practical relevance to human health, the interaction with fat droplets in BLG-stabilized emulsions significantly increases the chance that immunogenic portions of the protein reach the immune system in a non-degraded form.

Modeling the structure of BLG in BLG-stabilized emulsions appears a daunting task, also because of the likely occurrence of multiple BLG conformers at the emulsion interface. However, some information on the peculiar features of specific BLG conformers generated by various denaturation processes or agents may allow us to gather some information beyond the merely phenomenological description of events. Table 2 summarizes currently available information as for some structural features of BLG unfolded to different extents by interaction with specific chemical species or physical entities. Table 2 does not include temperature-dependent changes, as any analysis in this case is made complicated by concentration-dependence issues, and by the relatively fast occurrence of concentration-dependent aggregation events at pH values close to neutrality [13,34,35].

The data in Table 2 highlight a number of evident differences among the various unfolded forms of BLG. One of the most obvious regards the solvent exposure of “reporter” tryptophan residues, for which the denaturation-related emission red-shift is modest in the presence of an interface, but is very sensitive to the presence of sub-denaturing concentrations of urea (but not of KSCN). Thus, interaction with a hydrophobic interface does not result in “burying” in the hydrophobic phase of regions around Trp residues, but rather in making only slightly more hydrophilic the environment around Trp₁₉, which is reportedly responsible for most of the BLG fluorescence [7,18,42]. In this frame, it

Table 2
Relevant features of various unfolded forms of BLG.

Feature	Treatment				
	None ^{a,b,c}	2 M KSCN ^a	5 M urea ^a	Adhesion to polystyrene NPs ^b	Formation of oil-in-water emulsion ^c
Trp fluorescence emission λ_{max} , nm	338	338	355	344	340 (serum) 345 (cream)
Rate of Cys ₁₂₁ –SH reaction with DTNB	Very low	Low	Very high	High	Low
Significant trypsin-resistant sequences		1–45 113–124-(S-S)-149–162	1–45 142–162	139–148 61–70-(S-S)-102–162	61–105 61–69-(S-S)-149–162
<i>Estimated relative immunoreactivity</i>					
5G6 mAb	Intact	100	n.a. ^d	300	200
	Trypsin-treated	30	n.a.	300	180
1E3 mAb	Intact	100	n.a.	500	300
	Trypsin-treated	5	n.a.	n.a.	150

^a From [25].

^b From [17].

^c This work.

^d Not available.

is worth noting that increased solvent exposure of tryptophans in protein-stabilized emulsions has been reported also for “structurally stiff” proteins other than BLG, such as soybean conglutinin [22].

Also of relevance are the data on the accessibility of the Cys₁₂₁ thiol, that in native BLG is buried in a hydrophobic groove between the C-terminal alpha-helix and the beta-barrel main body, and thus is inaccessible to thiol reagents at acidic or neutral pH. Structural changes in this region have been shown to precede larger changes involving other regions of the protein in various types of unfolding conditions, including high temperature and high pH values [32,33,44,45].

Changes around Cys₁₂₁ are large upon treatment with urea and when the protein is stuck to a hydrophobic solid nanoparticle, but appear to be quite minor – at least in comparison – when the protein is unfolded by KSCN or when adsorbed onto an oil/water interface. Accessibility of the Cys₁₂₁ thiol is relevant for technological purposes, as this residue is central to the disulfide-exchange events involved in formation of covalently-bound BLG polymers and in the interaction of BLG with other proteins in dairy-based foods and in other food systems [33–35], in particular when thermal treatments are associated with mechanical ones. This combination of treatments is a common practice in food processing, where thermal treatment of emulsions is carried out both simultaneously to the emulsion production, in subsequent sanitization steps, or – in some cases – when a final cooking step is required.

Whether the molecular events analyzed here in detail are of actual significance to BLG-sensitive consumers remains to be ascertained. The data presented make it evident that any type of generalization when it comes to the consequences of protein unfolding events may be misleading, and that much more attention should be paid to situation-specific conformational changes that affect the outcome of the process and properties of the product that may be relevant to human health.

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Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

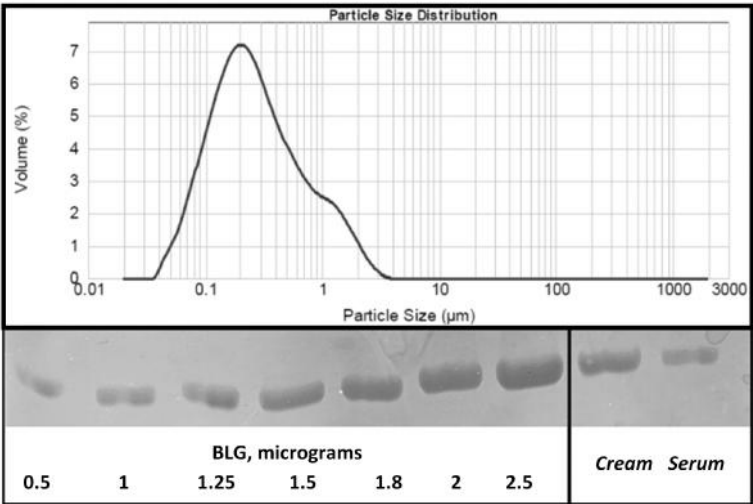
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Supplementary Materials



Supplementary Figure 1. Characterization of soybean oil emulsion: droplet size and BLG distribution. *Upper panel:* particle size distribution of soybean oil emulsions stabilized by BLG. *Lower panel:* SDS-PAGE of BLG standards (left), and of BLG in the “cream” and “serum” phases (right).