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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.

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 $g^{-1}\,l\,cm^{-1}$ 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 ×*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 ×*g*), and loaded on a Waters Symmetry 300™ C18 column (5 μm, 4.6 × 250 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.