Colloids and Surfaces B: Biointerfaces 161 (2018) 387–393

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Full Length Article

Investigation of high pressure effect on the structure and adsorption of β-lactoglobulin

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ARTICLE INFO

Article history:
Received 28 June 2017
Received in revised form 3 October 2017
Accepted 30 October 2017
Available online 31 October 2017

Keywords:
Bovine β-lactoglobulin structure
High pressure β-lactoglobulin conformation
Adsorption
Electrophoretic mobility
QCM-D

ABSTRACT

β-Lactoglobulin, being one of the principal whey protein, is of huge importance to the food industry. Temperature/pressure effects on this small protein has been extensively studied by industry. To characterize biochemical properties of β-lactoglobulin after or during pressurization, a wide range of methods have been used thus far. In this study, for the first time, the pressure-induced conformation of β-lactoglobulin in the crystal state was determined, at pressure 430 MPa. Changes observed in the high pressure structure correlate with the physico-chemical properties of pressure-treated β-lactoglobulin obtained from dynamic light scattering, electrophoretic mobility and quartz crystal microbalance with dissipation monitoring measurements. A comparison between the β-lactoglobulin structures determined at both high and ambient pressure contrasts the stable nature of the protein core and adjacent loop fragments. At high pressure the β-lactoglobulin structure presents early signs of dimer dissociation, charge and conformational changes characteristic for initial unfolded intermediate as well as a significant modification of the binding pocket volume. Those observations are supported by changes in zeta potential values and results in increase affinity of the β-lactoglobulin adsorption onto gold surface. Observed pressure-induced structural modifications were previously suggested as an important factor contributing to β-lactoglobulin denaturation process. Presented studies provide detailed analysis of pressure-associated structural changes influencing β-lactoglobulin conformation and consequently its adsorption.

1. Introduction

Understanding the consequences of using new technologies for food products is an important scientific goal for the modern biological and medical research. Recent scientific advances in high pressure (HP) technologies for food processing and preservation have proven to have relatively minor effects on food composition and, hence, sensory and nutritional characteristics [1]. Thus, food and drink manufacturers have turned their attention to this novel preservation process, in order to satisfy the consumer's demand for additive–free products. β-Lactoglobulin (LGB), as a major component of the cow's milk whey, exemplifies the fact that noticeable structural and functional changes can be observed after pressurization. Pressure-modified LGB functional properties including: aggregation [2], enzymatic digestion [3], foaming [4] and denaturation [5] were reported to have both positively and negatively effects on the organoleptic and nutritional quality of milk products.

At neutral pH, β-lactoglobulin forms dimers that dissociate at acidic pH. The biological function of LGB is still unknown, the protein has the ability to bind an extended range of hydrophobic compounds such as retinol [6], fatty acids [7] and drugs [8]. The binding is regulated by a pH-dependent process known as a Tanford transition. At pH below 7.0, the EF loop blocks access to the binding pocket and opens it at higher pH values. LGB structures with ligands bound inside the binding pocket exhibit an open conformation of the EF loop. The most abundant bovine β-lactoglobulin isoforms, A and B, differ in amino acid sequence at positions 64 (Ala → Gly) and 118 (Val → Ala). It has a predominantly β-sheet

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https://doi.org/10.1016/j.colsurfb.2017.10.069
0927-7765/

structure with eight-stranded antiparallel β-barrel (A–H) and an additional β-strand (I) being a part of the dimer interface. One α-helix and two short 3_{10}-helices are also present in the molecule.

Since hydrostatic pressure can be successfully used as a tool to populate partially unfolded intermediate states, not detected when chemical denaturants or high temperature are applied, a number of studies of LGB under HP were performed to investigate its behavior when compressed. Compared with CD, SAXS, NMR and MD, high pressure protein crystallography (HPPX) is a superior and direct method capable to detect pressure-induced changes in protein hydration structure, as well as to identify unexplored conformations that provides new insight into protein function and dynamics.

In this study, we report on the application of HPPX and the physicochemical characterization for studying pressure induced β-lactoglobulin structural modifications. LGB crystals of suitable quality and high symmetry were obtained at ambient pressure (AP), both in the absence and presence of the chosen hydrophobic ligand. A single crystal of the liganded LGB was compressed to 430 MPa. To perform detailed analysis of the pressure influence on β-lactoglobulin structure, three crystal structures of LGB were determined: unliganded LGB at AP (AP-LGB), β-lactoglobulin complexed with dodecane at AP (AP-LGB-D12) and β-lactoglobulin complexed with dodecane at HP (HP-LGB-D12). In order to obtain a broad view of how HP influences the LGB molecule and its interaction, hydrodynamic radius, zeta potential and isoelectric point (i.e.p.) of LGB before and after pressurization were determined by dynamic light scattering (DLS) and electrophoretic mobility. Additionally, quartz crystal microbalance with dissipation monitoring (QCM-D) method was used to investigate the effect of pressure-induced LGB conformational changes on its adsorption onto the gold surface. Our results are discussed in terms of the β-lactoglobulin structural modifications caused by pressure that result in a pre-aggregated state, crucial for the amyloid fibril formation.

2. Material and methods

2.1. Chemicals

The isoform B of β-lactoglobulin was purchased from Sigma (Sigma-Aldrich Co, St. Louis, Mo) and used without purification. All the other chemical reagents used, were of analytical grade.

2.2. Crystallization and crystal mounting

Crystals were grown at room temperature using the hanging drop vapor diffusion method according to Wu et al.[9]. For high pressure data collection, a single crystal of the liganded LGB was mounted in the diamond anvil cell using the procedure previously described [10,11]. For more detailed information please see the supporting information (SI).

2.3. Data collection, processing and s***tructural analysis***

X-ray diffraction data was collected at 293 K on a Nonius diffractometer with the KappaCCD detector (Bruker-Nonius) using Mo radiation (55 kV, 30 mA) of wavelength λ = 0.71069 Å. For further details of data collection and processing, see SI.

2.4. DLS, electrophoretic mobility and QCM-D measurements

Native and pressure-treated β-LGB hydrodynamic radius at physiological pH were determined with a Zetasizer Nano-ZS from Malvern Instruments Ltd. (Worcestershire, UK) by dynamic light scattering and electrophoretic mobility [12,13]. Adsorption of the native and pressure-treated protein onto a gold surface was monitored using quartz crystal microbalance, using the dissipation monitoring Q-Sense E1 system (Q-Sense Biolin Scientific, Sweden) at pH 7.5 (at 25 °C) [14]. Details are provided in SI.

3. Results and discussion

3.1. X-ray diffraction limit and intensity

Crystals of β-lactoglobulin, both at ambient and high pressure, present the same symmetry of the space group $P3_221$. In both the HP and AP experiments, diffraction data was collected to the maximum resolution; which was 2.65 Å. Details of the data collection and refinement are summarized in Table S1. Resolution of the obtained data is similar to the resolution observed for other LGB structures determined at room temperature [15].

3.2. An overall and secondary structure

In all structures the asymmetric unit contains a single LGB dimer subunit, each subunit is at a distance of less than 4.0 Å away from three other molecules. The overall fold of the protein is retained in all three presented crystal structures as was confirmed by the Fourier maps. In all structures the EF loop adopts the so-called closed conformation. The superposition of Cα atoms excluding the GH loop gave RMS values that confirm the overall changes to the structures are moderate. For AP-LGB and AP-LGB-D12 structures, the RMS was 0.5 Å while for AP and HP structures the RMS value was 0.8 Å.

The *DSSP* program has been used to find all N—H···O hydrogen bonds that usually are involved in stabilization of secondary structure elements in the molecule. For comparison of AP and HP structures, we considered only those hydrogen bonds for which energy of the electrostatic interactions was lower than the limit. *DSSP* calculations revealed that number of the hydrogen bonds stronger than −1.0 kcal/mol is the same in both structures and totals 77. However, analyzing the number of bonds with an energy below −1.6 kcal/mol, we have noticed that it has decreased from 61 to 58 upon applying pressure. An even larger change of 49–43 was observed for bonds with an energy below −2.0 kcal/mol. It shows that HP has a destabilizing effect on the secondary structure and might be an indication of the proceeding unfolding of the protein.

Changes of the secondary structure were also reported for LGB molecules studied under pressure in solution [5]. Methods that utilize samples pressurized during entire measurement up to 300 MPa as HPPX, provide results that are in agreement with our observations. For instance NMR and SANS studies revealed that the pressure-induced denaturation of β-lactoglobulin at pH 7.0 is accompanied by an increase of disordered structure that is associated with decrease of β-sheets and α-helices fragments [16,17]. Our results also correspond well with FTIR studies revealing that the pressure-induced denaturation of β-lactoglobulin at pH 7.0 is accompanied by an increase in the fractional band intensity of disordered structures, while the intensity of the absorption bands associated with β-sheets and α-helices decreases [18].

3.3. Dimer interface

The dimer interface identified in the LGB crystal structure consists of β-strand I and its analog I', from the second subunit related by two-fold symmetry. Four hydrogen bonds between these β-strands and a number of weak interactions involving side chains of residues from β-strand I are observed in all dimers. The length of the hydrogen bonds between N(Ser150)-O(His146′) and N(Arg148)-O(Arg148′) is 2.93 Å and 3.04 Å in AP-LGB, 2.99 Å and 3.01 Å in