

Towards understanding the effect of high pressure on food protein allergenicity: β -lactoglobulin structural studies

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ABSTRACT

A number of studies were devoted to understanding an immunological effect of pressure-treated β -lactoglobulin. In our previous work we have proved that high pressure significantly modifies β -lactoglobulin conformation and consequently its physicochemical properties. Here, structure of β -lactoglobulin complex with myristic acid determined at the highest accepted by the crystal pressure value of 550 MPa is reported. Our results structurally prove that pressure noticeably modifies positions of the major β -lactoglobulin epitopes. Considering the biological impact of observed changes in epitope regions, high pressure β -lactoglobulin structure presents a step forward in understanding the pressure modification of food protein allergenicity. The conformational changes of pressurized β -lactoglobulin did not support the hypothesis that proteolytic digestion facilitated by pressure is caused by an exposure of the digestive sites. Our findings demonstrate that high pressure protein crystallography can potentially identify the most pressure-sensitive fragments in allergens, and can therefore support development of hypoallergenic food products.

1. Introduction

Approximately 2–3% of children under 2 years of age and even 1% of the adults experience allergic reactions to cow's milk (Björnsson, Janson, Plaschke, Norrman, & Sjöberg, 1996). The major allergen involved in allergy to cow's milk proteins is bovine β -lactoglobulin (LGB). β -Lactoglobulin, together with several allergens of animal origin, belongs to the lipocalin family, which share a similar tertiary structure (β -barrel) and high affinity for hydrophobic ligands (Mäntyjärvi, Rautiainen, & Virtanen, 2000). The ruminant β -lactoglobulin is a globular protein composed of 162 amino acids with a molecular weight of 18 kDa. It contains 5 cysteine residues, 4 of which are forming disulfide bridges. β -Lactoglobulin is predominantly a β -sheet protein with nine β -strands and one major α -helix (Brownlow et al., 1997). As many allergens, LGB is a transient dimer (Niemi et al., 2015). In physiological conditions β -lactoglobulin is in the form of non-covalent dimers, whereas at low pH it forms monomers that preserve its native state (Taulier & Chalikian, 2001). Additionally, β -lactoglobulin undergoes a conformational change, the so-called Tanford transition, that is also

associated with pH and occurs around neutral pH (Sakurai & Goto, 2006). Dimeric β -lactoglobulin molecules exist in the open conformation at basic pH, while the closed conformation is observed at acidic pH. This open-close conformational change involves displacement of the EF-loop (residues 85–90) and regulates access to the binding pocket located in the β -barrel.

The epitopes of LGB have been characterized in many studies during the last two decades. The IgE strong epitope peptides identified by Järvinen and co-workers (Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001) are located on the DE loop (Lys75-Asp85) and in the α -helix H3 (Glu127-Pro144). Three weaker epitope peptides were also proposed: Leu31-Pro48, Lys47-Lys60, and Leu57-Ile78. In another study, Clement et al. have characterized the IgG epitope map of LGB by using a competitive immunoassay (Clement et al., 2002). According to those studies, the most antigenic regions are located in the α helix and on the external loops, including Glu127, Asp130 and Glu134 residues, as well as in the β -strand B where a minor antigenic area Glu44-Glu45 was identified (Fig. 1). Recently, Niemi et al. reported the crystal structure of an IgE Fab fragment in complex with β -lactoglobulin

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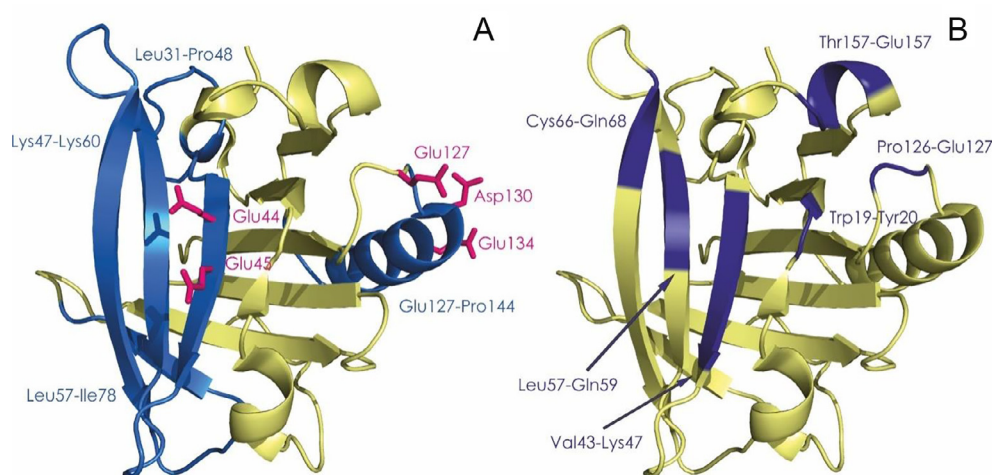


Fig. 1. LGB epitopes: (A) functional epitopes on LGB regarding interaction with IgE (blue) and IgG (pink) antibodies, according to Järvinen et al. (2001) and Clement et al. (2002), respectively; (B) structural epitopes defined by the set of LGB atoms considered to be in contact with atoms of the antibody Fab-IgE identified by Niemi et al. (2007). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Niemi et al., 2007), which revealed that 15 amino acid residues of LGB molecule form contacts with the antibody. Those interactions between LGB and an IgE fragment include 2 salt bridges and 13 hydrogen bonds. Large fragments of β -strands were reported as epitopes: Trp19-Tyr20 (β -strand A), Val43-Lys47 (β -strand B), Leu57-Gln59 (β -strand C) and Cys66-Gln68 (β -strand D). In addition, the loop between β -strand H and the following helix H3 (Pro126-Glu127), and the short C-terminal α -helix H4 (Thr154-Glu157) were reported to be a part of the epitope.

Like most food allergens, LGB is very stable at low pH and resistant to degradation (Bu, Luo, Chen, Liu, & Zhu, 2013). Therefore, different strategies for destroying or modifying β -lactoglobulin allergenicity, as well as digestibility including heat treatment (Bu, Luo, Zheng, & Zheng, 2009), proteolysis (Chicón, Belloque, Recio, & López-Fandiño, 2006) and high-pressure treatment (López-Expósito, Chicón, Belloque, López-Fandiño, & Berin, 2012) were investigated. High pressure is known to affect the structure of proteins. Typically, at pressure up to 200–300 MPa, conformational changes are observed while few hundred MPa pressure can lead to protein denaturation. Several trials have been performed to identify structural consequences of high pressure on the structure of LGB, mostly in order to correlate them with LGB allergenicity and digestibility. Studies performed by Chicón, Belloque, Alonso, and López-Fandiño (2008) showed that high-pressure LGB treatment (200 and 400 MPa) did not affect its binding to IgE. Contrary to those findings, Kleber et al. indicated that high-pressure conditions (200–600 MPa) could enhance the antigenicity of LGB (Kleber, Maier, & Hinrichs, 2007). Other scientists suggest that allergenic activity of LGB depends on the pressure that was applied and can be higher (at mild conditions) or lower (at elevated pressure) (Zhong et al., 2012).

Previous studies that reported high-pressure LGB structure with dodecane have revealed interesting findings concerning the influence of pressure on LGB adsorption process (Kurpiewska et al., 2018). Structural changes in β -lactoglobulin molecule introduced by the pressure of 430 MPa were interpreted as early signs of dimer dissociation and conformational changes characteristic for partially unfolded intermediate. In this study, we have investigated the influence of pressure as high as 550 MPa on LGB molecule. Crystal structure of LGB complex with myristic acid (MYR) determined at high pressure allowed to observe unique and important structural response of the protein molecule to the pressurization. Detailed analysis revealed that at 550 MPa noticeable positional shifts of the digestive sites, as well as LGB epitopes can be identified. Present analysis supports our previous findings concerning the high-pressure modification of β -lactoglobulin conformation. Current results give a deeper insight into structural origins of changes in LGB biochemical features and allergenicity caused by elevated pressure.

2. Materials 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

Crystals were grown at room temperature using the hanging drop vapor diffusion method according to Wu, Dolores Pé Rez, Puyol, and Sawyer (1999). Lyophilized protein was dissolved in ultra-pure water to the final concentration of 20 mg/ml. A crystallization drop containing 2 μ l of the protein, 0.5 μ l of myristic acid (10 mM in EtOH) and 6 μ l of reservoir solution was equilibrated against 0.5 ml of reservoir solution containing 0.1 M Tris-HCl, 1.34 M sodium citrate, pH 7.5 (298 K, atmospheric pressure). An average size of LGB-MYR crystals was $0.25 \times 0.40 \times 0.50$ mm. Size and quality of obtained crystals were suitable for experiments using Merrill-Bassett type of diamond anvil cell (DAC).

2.3. Data collection and processing

X-ray diffraction data for LGB-MYR single crystal mounted inside the DAC were collected at ambient (ap) and high pressure (hp) conditions. Steel gaskets (0.3 mm thickness) with a hole diameter of 0.6 mm were used according to the procedure previously described in Kurpiewska and Lewiński (2010). Protein crystals were transferred into the chamber previously filled with the crystallization mother liquor (Fig. 2). Determination of pressure was performed at ambient temperature using ruby fluorescence method (Kurpiewska et al., 2018). Data were collected at room temperature using SuperNova (Rigaku-Oxford Diffraction) four-circle diffractometer with a microfocus MoK α radiation source ($\lambda = 0.7107$ Å) and multilayer X-ray optics. The data sets were processed with *CrysAlis^{Pro}* software (Oxford Diffraction (2006) *CrysAlis^{Pro}* Oxford Diffraction Ltd, Abingdon, England, Version 1.171.36.20) and *AIMLESS* (Evans & Murshudov, 2013). Preliminary experiments allowed to determine the unit cell parameters for crystals compressed to 0.1, 170, 370 and 650 MPa. Complete data collections were performed for LGB-MYR crystal at ambient pressure (ap LGB-MYR) and at 550 MPa (hp LGB-MYR).

For more detailed information of structure solving, refinement and analysis please see the Supplementary data.

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foodchem.2018.07.104>.

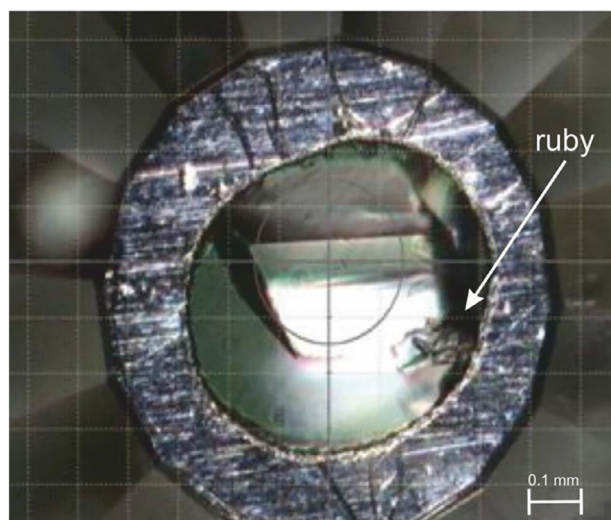


Fig. 2. Sample chamber with LGB-MYR crystal and ruby chip inside the DAC.

3. Results and discussion

3.1. Crystal structures of *ap* and *hp* LGB-MYR

At ambient and high pressure the LGB-MYR crystals present the same symmetry of the trigonal space group $P3_221$. Compression has improved the order in LGB-MYR crystal, diffraction data at ambient and high pressure were collected to maximum resolution 2.8 Å and 2.5 Å, respectively. Details of the data collection and refinement are summarized in the Table S1. Quality of the obtained data is lower than of those collected for LGB-MYR structure at low temperature (PDBid: 3UEV) (Loch et al., 2012) and refined to 1.9 Å resolution. However, both *ap* and *hp* LGB-MYR structures present a quality similar to the high-pressure structure of β -lactoglobulin complex with dodecane (PDBid: 5IO7; maximum resolution 2.65 Å) (Kurpiewska et al., 2018).

Unit cell parameters of LGB-MYR crystals were determined at room temperature at a pressure of 0.1, 170, 370, 550 and 650 MPa. The crystals diffracted to 2.5 Å at a pressure of 550 MPa and to 3.5 Å at 650 MPa. Similarly to LGB complex with dodecane, entire loss of the diffraction was observed for LGB-MYR at 740 MPa (Kurpiewska et al., 2018). The compressibility β of the unit cell parameters (Fig. S1) was calculated as $\beta = -1/V_u(\partial V_u/\partial p)$. For LGB-MYR crystal compressed to 550 MPa the calculated compressibilities of the unit cell parameters $a = b$, c and $V_{unit\ cell}$ are 0.047 GPa⁻¹, 0.007 GPa⁻¹ and 0.101 GPa⁻¹, respectively. Calculated values are slightly higher than those reported

for HP-LGB-D12 (Kurpiewska et al., 2018) but similar to the compressibilities reported for other proteins (Nagae et al., 2012).

3.2. Pressure promotes structural changes in β -lactoglobulin molecule

The overall architecture of LGB molecule before and after pressurization is similar. A dimer stabilized by hydrogen bonds between the main chain atoms of β -strands I from both subunits related by the crystallographic 2-fold axis is retained. The myristic acid is bound in the binding pocket located inside the β -barrel formed by eight antiparallel β -strands A to H. In both *ap* and *hp* structure EF loop adopts an open conformation that allows ligand to enter the β -barrel.

The r.m.s. deviation between the C α -atom positions in *hp* and *ap* LGB-MYR structure is 0.616 Å. Noticeable positional changes of the N-terminal fragment, as well as CD, EF and GH loop regions, are observed (Fig. 3A), however, for those regions the quality of the Fourier maps is relatively low. The similar fragmentation of Fourier maps was previously reported for structures of LGB complexes with different ligands determined at ambient conditions or cryo-conditions (Loch et al., 2012).

The intermolecular distances of hydrogen bonds that stabilize LGB dimer have slightly changed after pressurization. The hydrogen bonds N(Ser150)-O(His146') and N(Arg148)-O(Arg148') between β -strands I and I' are 2.82 Å and 2.88 Å long, respectively in the *ap* structure while the same distances in the *hp* structure were found to be 3.27 Å and 2.80 Å (Fig. 3B). Additionally, subtle subunits reorientation was also observed as a result of high-pressure treatment. Such reorientation was previously observed in *hp* structure of LGB with dodecane (Kurpiewska et al., 2018) where subunits were twisted by 2.5°. Pressurization of LGB-MYR to 550 MPa (pressure noticeably higher than one used during our previous studies) increased this twist up to 3.5°. Our findings are in agreement with previous reports that high pressure in the range of 200–400 MPa promotes dissociation of LGB dimer (Gebhardt, Toro-Sierra, & Kulozik, 2012), however, even at a pressure higher than 500 MPa for the protein in a crystalline state dissociation can be still restrained by the packing of molecules.

In *ap* and *hp* crystal structures of LGB-MYR, the ligand has been found in the β -barrel in an extended conformation. The most significant difference between *ap* and *hp* structure is the position of ligand polar fragment (Fig. 4A). In the *ap* structure, the carboxyl group is directed towards Lys69 side chain, but no hydrogen bond is formed. The similar conformation of myristic acid was reported in LGB-MYR structure determined at low temperature (Loch et al., 2012). In the *hp* structure, the carboxyl group of the myristic acid is shifted away from Lys69 and moved closer to Lys60 (Fig. 4B). As a result, a hydrogen bond has been formed between MYR carboxyl oxygen and Lys60 amine group. A water

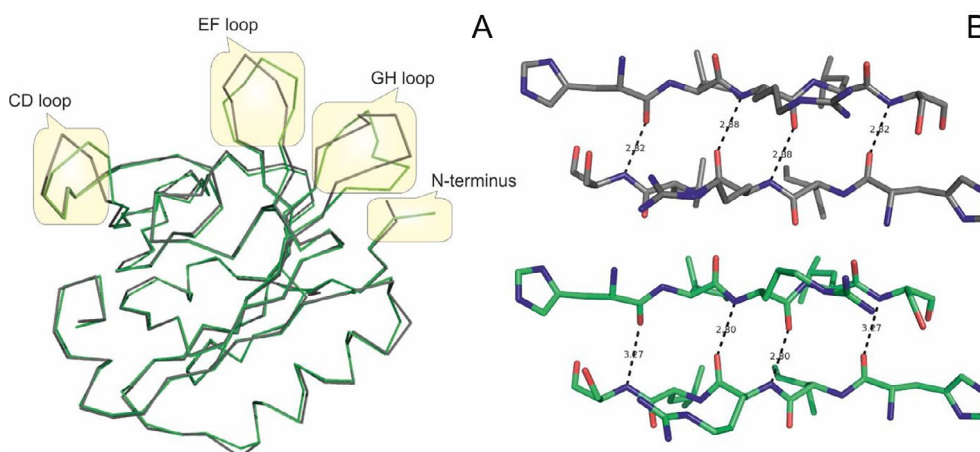


Fig. 3. Graphical representation of: (A) superimposed structures *ap* LGB-MYR (grey) and *hp* LGB-MYR (green); (B) stick representation of LGB dimer interface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

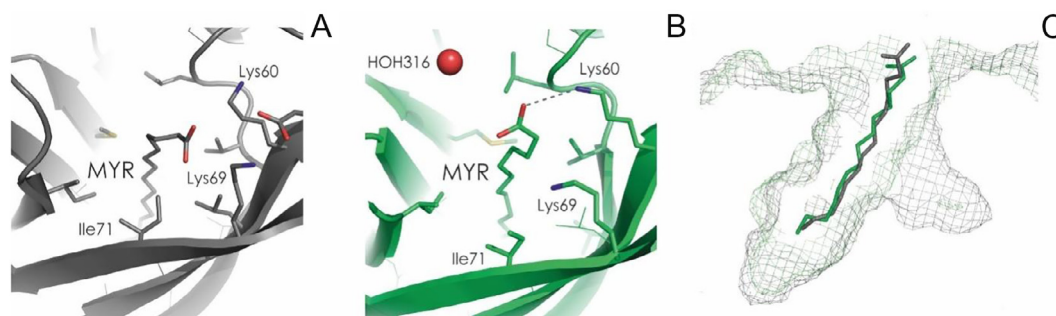


Fig. 4. Binding pocket in LGB and its surface in: (A) ap LGB-MYR; (B) hp LGB-MYR; (C) differences in shape and size of binding pockets in ap (grey) and hp (green) structures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecule (HOH316) was localized in the vicinity of the ligand's carboxyl group, but this solvent molecule is too distant to form an interaction with any protein atoms. An interesting observation is that HOH316 forms a 2.8 Å long hydrogen bond with the water molecule that is related by the symmetry. Those two water molecules occupy the central part of an empty space that is formed between LGB subunits. Previously reported high-pressure protein crystallography experiments showed that pressure induces water penetration into the protein interior (Nagae et al., 2012). In this case, water penetrates a hydrophobic space located between two LGB molecules, formed by two symmetry related protein molecules in which the binding pocket entrances are facing to each other.

As emphasized by the superimposition of the ap and hp structures, the residues involved in myristic acid binding generally retain their positions with small deviations. However, there is one exception. The Met107 side chain conformation differs in ap and hp structures and relevantly influences the binding pocket shape. Two Met107 conformations identified in ap and hp LGB-MYR structures were previously identified as alternative conformations in the LGB-MYR structure determined with higher resolution (1.9 Å) (Loch et al., 2012). A single conformation of Met107 observed in hp structure results from pressurization and has an important implication for volumetric properties of the LGB binding pocket. In the ap structure, the binding pocket expands to Leu10 and is occupied by the MYR molecule. Additionally, a large empty space that extends towards Leu32 is observed. As it can be seen from the Fig. 4C, in hp structure the binding pocket with myristic acid inside is also present, but now it is separated from the nearby cavity.

According to the Le Chatelier's principle, under high pressure, proteins adopt a state of the lower partial molar volume, which is expressed as the sum of the van der Waals volume, the void volume and the volume change caused by hydration. Structural and thermodynamic

data proved that responsible for the volumetric changes upon protein pressurization are mostly two latter components (Schweiker, Fitz, & Makhatadze, 2009). Thus, there are three possible ways for LGB to respond to pressure in order to reduce the overall system volume: the volume of the binding pocket can decrease by expelling the MYR molecule, an empty space may collapse or may be filled with water. Our observations prove that two of those scenarios are implemented. Protein responds to the compression reducing an empty space via subtle binding pocket deformation and by filling the void between two symmetry related protein molecules with water. High-pressure influence on cavity size was widely discussed for RNase A (Kurpiewska et al., 2016). The observation that water molecules can occupy cavities was also reported for other pressurized proteins. In particular, high pressure has promoted water penetration into a cavity in structures of T4 lysozyme (Collins, Quillin, Hummer, Matthews, & Gruner, 2007) compressed to 100, 150 and 200 MPa and structure of 3-isopropylmalate dehydrogenase (Nagae et al., 2012) under 160 MPa.

Even small local structural changes like side-chain rearrangement within the binding site can influence LGB binding properties. It was reported, that molten globule state of LGB induced by high pressure exhibits lower affinity for palmitic acid, capsaicin or carvacrol ligands than native LGB (Yang, Powers, Clark, Dunker, & Swanson, 2003). On the other hand, Yang, Dunker, Powers, Clark, and Swanson (2001) proved that pressure and temperature (600 MPa and 50 °C) induce molten globule state of LGB, which exhibits high affinity for the hydrophobic 8-anilino-1-naphthalenesulfonate, suggesting an increase in accessible hydrophobic surface originating from the aromatic residues. Moreover, Considine, Patel, Singh, and Creamer (2005, 2007) concluded that ligands protect LGB from denaturation and aggregation. Our structural analysis helps to explain these results. In particular, the crystal structure indicates that LGB molecule under pressure exhibits more tightly packed binding site that consequently can be less

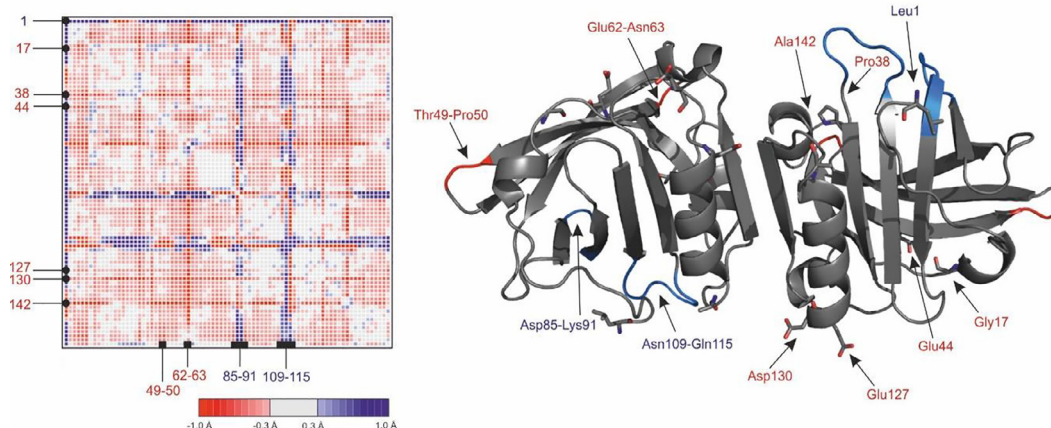


Fig. 5. Difference distance plot for hp LGB-MYR vs. ap LGB-MYR and a view of dimer structure with residues and fragments that shift upon pressurization.

accessible for the approaching ligands. Once the ligand is anchored in the binding pocket, the protein compressed conformation prevents dissociation by trapping ligand inside the β -barrel.

Subtle atoms rearrangement observed in hp LGB-MYR structure resulted in an overall energy change of 325 kJ/mol comparing to ap structure (Table S1). This energy change is equivalent to the formation of a few hydrogen bonds. The thermodynamic relation between the energy of a system and its volume can also be noticed. The difference in energy correlates with changes in β -LGB molecule volume, in agreement with previous reports (Kurpiewska et al., 2016; Nagae et al., 2012).

The difference distance matrix plot for ap and hp structures shows that significant positional shifts are observed not only for single amino acids or groups of few residues but also for larger protein fragments. The largest shifts are observed for Leu1, Gly17, Pro38, Glu44, Glu127 and Asp130 as well as for regions Thr49-Pro50, Glu62-Asn63, Asp85-Lys91, Asn109-Gln114 and Leu140-Ala142 (Fig. 5). The identified residues displaying largest displacements are related to digestibility and allergenicity of β -lactoglobulin.

3.3. The pressure influence β -lactoglobulin epitopes

Principally, two main classes of allergenic epitopes can be observed: conformational epitopes, which are expected to be more susceptible to processing-induced changes and linear epitopes, which are likely to be more resistant to physical treatments. Answering the question, whether the epitopes remain intact after pressurization is very essential. Thus, a considerable effort was made in order to compare the enzymatic digestion of the allergens before and after/during high-pressure treatment in general (Aouzelleg, 2014; Hoppe, Jung, Patnaik, & Zeece, 2013). Such experiments give also indirect structural information. A number of studies using biochemical and biophysical methods were undertaken, in order to determine the pressure needed for the unfolding of LGB, which is expected to destroy its epitopes. The second important issue that was investigated is the reversibility of the unfolding, i.e. whether the distortion of the epitope is irreversible. The experiments in solution showed that high pressure in the range from 200 up to 600 MPa clearly affects the antigenicity of LGB (Chicón, Belloque, Alonso, & López-Fandiño, 2008; Chicón, Belloque, Alonso, Martín-Alvarez, & López-Fandiño, 2008).

As it can be seen from the Fig. 6, in hp LGB most of the significantly shifted regions belong to the aforementioned functional and structural epitopes. For Glu44 and Glu127, residues which are involved in interactions with IgE, IgG and Fab (Clement et al., 2002; Järvinen et al., 2001; Niemi et al., 2007), the shift of C α position is 0.74 and 0.48 Å, respectively. Also, residues Pro38 and Asp130, as well as fragments

Thr49-Pro50, Glu62-Asn63 and Leu140-Ala142 correspond to epitopes of LGB as proposed by Järvinen et al. (2001). Only the Gly17 residue and loops EF and GH do not correspond to any identified LGB epitope. An increased mobility of loops, especially loop EF, was widely described and explained in terms of structural changes related to ligand binding.

It has been reported that pressure above 400 MPa modifies the native structure of LGB, leading to exposure of new antigenic sites, “hidden epitopes”, thus, inducing the IgE response towards non-conformational epitopes of LGB (Zeece, Huppertz, & Kelly, 2008). Furthermore, it has been noted that pressure forces unfolding of LGB molecule resulting in a better accessibility of epitopes for the antibodies (Orcajo, de Marañón, & Lavilla, 2015). Since we observe that in hp structure pressurization leads to the exposition of the loop fragment and to the burial of the epitope regions our results support the other possibility, that is widely discussed in the literature. Our direct structural observations prove that high-pressure treatment drives natively exposed epitopes to more buried positions and therefore epitopes became inaccessible to antibodies. In addition to the local rearrangements high pressure also triggers the changes in β -lactoglobulin's electrostatic potential distribution. Our previous studies revealed that such changes promote LGB aggregation (Kurpiewska et al., 2018). Hence both factors modulate the epitopes immunogenicity. Reduction in the IgE-binding capacity of LGB was observed after microfluidization by Chen et al. (2017). Similar findings were reported for a number of other allergens studied under high pressure e.g. α -casein (Hu, Zheng, Liu, Deng, & Zhao, 2016), ginkgo seed proteins (Zhou et al., 2016) or tropomyosin Tod p1 (Jin et al., 2015).

Comparison of ap and hp structures indicates that subtle conformational changes resulting from high-pressure treatment of LGB may influence LGB allergenicity. Furthermore, reported in this paper hp LGB-MYR structure solved at 550 MPa serves as an important step forward in the elucidation of the protein response to pressurization.

3.4. High-pressure structure and digestibility of β -lactoglobulin

Pressurization of β -lactoglobulin, before or during enzyme treatments, have been reported to enhance its enzymatic hydrolysis (Zeece et al., 2008). This effect can derive from the exposure of normally hindered cleavage sites on the protein and from changes in the protein conformation that facilitate binding to the proteolytic enzyme. Experimental support for pressure-influenced changes in β -lactoglobulin digestibility comes from the investigation of high pressure treated LGB digested with trypsin, chymotrypsin and pepsin (Chicón, Belloque, Alonso, & López-Fandiño, 2008; Zeece et al., 2008). The specificity of

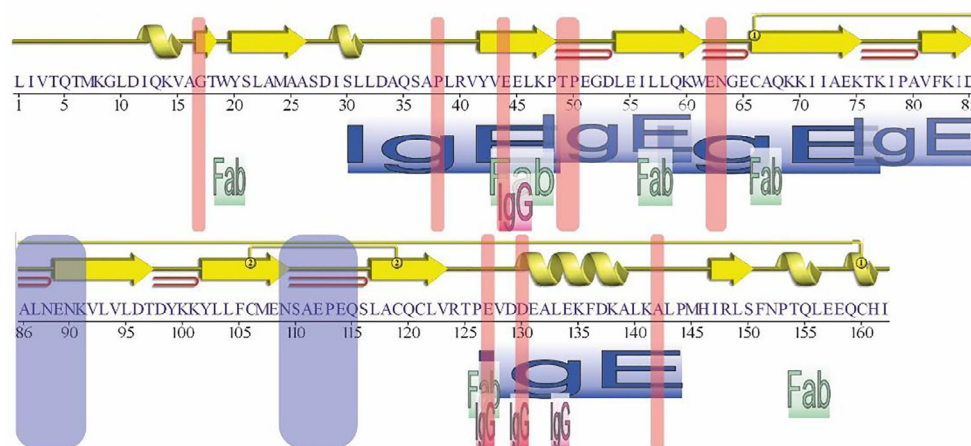


Fig. 6. Localization of epitopes (IgE, IgG and Fab) and regions influenced by high pressure (red and blue) in LGB structure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

those three enzymes is different. Trypsin is specific to Arg and Lys, mostly located on the surface of the protein molecule, and chymotrypsin acts on large hydrophobic residues such as tryptophan, tyrosine and phenylalanine. Pepsin action is not highly specific and it preferentially attacks the peptide bonds between Glu/Asp and Phe, Tyr or Trp. An increased sensitivity of LGB to proteases induced by the pressure reported by many authors is presented in Table S2. Most of such studies performed at a pressure up to 300–400 MPa with different incubation times, typically lead to extensive protein hydrolysis and/or enzyme inactivation (Chicón et al., 2006; Dufour, Hoa, & Haertlé, 1994).

Unexpectedly, there is no correlation between regions of pressure-induced positional shifts identified by us in LGB molecule and reported cleavage sites. With the present data, this requires the assumption that LGB high-pressure conformation observed in the crystal compressed to 550 MPa do not reflect the conformation present in solution under the same pressure. Further combined biochemical and high-pressure studies should be able to explain that phenomenon and make clearer structural origins that control changes in LGB digestibility after pressurization.

To conclude, high pressure is known to affect the structure of proteins. Typically, few hundred MPa pressure can cause structural destabilization of the polypeptide chain. A detailed comparison of hp and ap structure gave a comprehensive picture of high-pressure effects on different structural issues that can be related to allergic properties of β -lactoglobulin. Noticeable positional shifts of major IgE, IgG and Fab epitopes without changing global three-dimensional β -lactoglobulin structure prove that pressure can modulate allergenicity of LGB due to small rearrangements within particular regions of the protein molecule.

4. Conclusions

In summary, the structure of β -lactoglobulin was relevantly influenced by pressure, however, there is strong evidence that the protein core resists higher pressure. This core conditions LGB stability even if the applied pressure is higher than 500 MPa. The existing crystallographic studies of the proteins flexibility under high pressure shows that within a well packed crystal lattice the initial response to pressurization is observed for the outer fragments of the protein molecules while the inner parts are less affected (Kurpiewska et al., 2016, 2018; Nagae et al., 2012). In principle, in the crystalline state a significantly disordered structure cannot be reached. However, analysis of hp β -lactoglobulin structure details proved that pressurization influences number of structural and functional LGB epitopes. Antigenicity decrease may be associated with the fact that exposed and accessible for the antibodies epitopes are hidden due to pressure-induced structural changes and aggregation. In future, we will investigate interaction of β -lactoglobulin after pressurization (in solution and in crystal state) with IgE to understand the impact of epitope modification on LGB allergenicity as well as to determine pressure range that can effectively channeling its conformation. At the same time, we found no correlation between reported enhancement of enzymatic digestion caused by pressure treatment and observed structural changes in hp LGB-MYR. Altogether, our results demonstrate that high pressure protein crystallography can potentially identify the most pressure-sensitive fragments in allergens, serve as indicator during modulation of the protein allergenicity and can therefore support development of hypoallergenic food products.

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Conflicts of interest

The authors declare no conflict of interest.

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Supplementary Material

Towards understanding the effect of high pressure on food protein

allergenicity: -lactoglobulin structural studies

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Structure solving, refinement and analysis

The structures were solved by molecular replacement method with *PHASER* (McCoy et al., 2007) using the coordinates of β -lactoglobulin PDBid: 3UEV (Loch et al., 2012) as a starting model. Crystallographic refinement was carried out using maximum-likelihood target functions implemented in *PHENIX* (Adams et al., 2010). Each round of the refinement with automatic weighting was interspersed with a round of the model building using *WinCOOT* (Emsley & Cowtan, 2004). About 10-13% of the data were randomly excluded from the refinement and used as a test data set to monitor R_{free} . As phases improved, ligand molecule and ordered solvent molecules were added manually. Finally, the structures were validated and deposited to PDB as 5LKE (ap) and 5LKF (hp).

Superposition of all structures and calculation of the root-mean-square deviation for atomic coordinates (r.m.s. deviation) were performed using *LSQKAB* (Kabsch, 1976). Difference distance matrices (DDM) were calculated using *ESCET* program (Schneider & Schneider, 2000). Molecular volume using a radius equal 1.4 Å was calculated with server *PISA* (Krissinel & Henrick, 2007). The energy computation was done with *GROMOS96 43B1* parameters implemented in *SPDBV_4.1* (Guex & Peitsch, 1997). Figures were prepared using *PyMOL* (DeLano, 2002).

Tables

Table S1. Statistics of data collection, refinement and structural parameters. Values in parentheses are for the outer shell.

	ap LGB-MYR	hp LGB-MYR
<u>Data collection and refinement</u>		
PDBid	5LKE	5LKF

Maximum resolution (Å)	2.80	2.50
Pressure [MPa]	-	550
Number of reflections	6298	8247
Number of unique reflections	4458	5846
Mosaicity (°)	0.483	0.502
Resolution range (Å)	13.95-2.80	7.36-2.50
	(2.95-2.80)	(2.65-2.50)
R(I) _{merge}	0.283 (1.395)	0.480 (0.617)
Completeness (%)	98.5 (98.9)	91.0 (93.3)
Space group	P3 ₂ 21	
Unit-cell parameters		
<i>a</i> , <i>b</i> (Å)	54.18	52.76
<i>c</i> (Å)	112.92	110.45
	$\alpha = 90.00^\circ \beta = 90.00^\circ \gamma = 120.00^\circ$	
Overall <i>B</i> factor from	42.56	32.01
Wilson plot (Å ²)		
Refinement resolution range (Å)	13.95-2.80	7.36-2.50
R (%)	0.179	0.255
R _{free} (%)	0.312	0.397
Ligand	C ₁₄ H ₂₈ O ₂	C ₁₄ H ₂₈ O ₂
Number of solvent molecule	18	30
Stereochemical restraints, r.m.s.		

Bond distance (Å)	0.014	0.011
Bond angles (°)	1.664	1.554
Core Ramachandran (%)	89.5	82.6

Structural parameters

RMSD ^a C α (all)	-	0.616 (1.553)
Molecular volume (V), [Å ³]	41 840	41 120
Energy, [kJ/mol]	-4380	-4055

Table S2. The products obtained after action of trypsin, chymotrypsin and pepsin on pressure-treated LGB.

Enzyme	Pressure [MPa]	Fragments	Reference
Trypsin	0.1-400	Val15–Arg40 Val41–Lys69 and two disulfide linked fragments: Val41–Lys70 (S-S) Leu149–Ile162 Val41–Lys69 (S-S) Leu149–Ile162	(Chicón et al., 2006; Chicón et al., 2008)
Chymotrypsin	200-400	Val43–Leu57 Val43–Gln59 Val43–Leu58 and two disulfide linked fragments: Lys60–Phe82 (S-S) Ser150–Ile162 Glu62–Phe82 (S-S) Ser150–Ile162	(Chicón et al., 2008)
Pepsin	400-800	Leu1–Leu10	(Zeece et al., 2008)

	Leu32–Leu54	
	Phe82–Leu104	
	Ser150–Leu156	

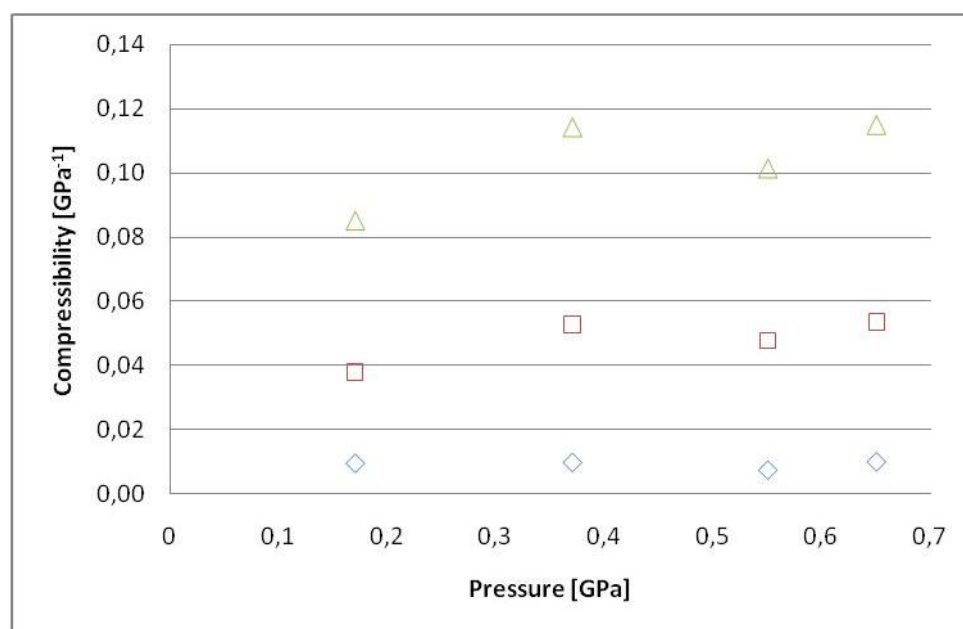


Fig. S1. Compressibility of the unit-cell parameters a, b (open squares), c (open diamonds) and the unit-cell volume (open triangles) as a function of pressure. Errors in the compressibilities variation of the parameters were smaller than the symbol sizes.

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