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Important role of methionine 145 in dimerization of bovine β-lactoglobulin

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β-Lactoglobulin (LG) contains nine β-strands (strands A–I) and one α-helix. Strands A–H form a β-barrel. At neutral pH, bovine LG (BLG) forms a dimer and the dimer interface consists of AB-loops and the I-strands of two subunits. On the other hand, equine LG (ELG) is monomeric. The residues 145–153 of BLG, which compose a dimer interface, are entirely different from those of ELG. The difference in the association states between BLG and ELG can be attributed to the residues 145–153. To confirm this, we constructed a chimeric LG, ImBLG (I-strand mutated BLG), in which the residues 145–153 were replaced with those of ELG. Gel-filtration chromatography and analytical ultracentrifugation revealed that ImBLG existed as a monomer. To identify the residues important for dimerization, we constructed several revertants and investigated their association. This experiment revealed that, in addition to the interface residues (Ile147, Leu149 and Phe151), Met145 is critical for dimerization. Although Met145 does not contact with the other protomer, it seems to be important in determining the backbone conformation of the I-strand. This was supported by the fact that all Met145-containing mutants showed circular dichroism spectra similar to BLG but different from ImBLG.

Keywords: analytical ultracentrifuge/β-strand/circular dichroism/gel filtration/site-directed mutagenesis.

Abbreviations: BLG, bovine β-lactoglobulin; CD, circular dichroism; ELG, equine β-lactoglobulin; LG, β-lactoglobulin; PLG, porcine β-lactoglobulin.

β-Lactoglobulin (LG) is an abundant protein in milk. LGs from ruminants are known to be dimeric, whereas those from non-ruminants are monomeric. Among ruminant LGs, bovine LG (BLG) has been investigated most extensively (*1*). BLG contains 162 amino acid residues including 5 cysteines (Cys66, Cys106, Cys119, Cys121 and Cys160), 4 of which form 2 disulphide bonds (Cys66–Cys160 and Cys106–Cys119) and Cys121 is free. The crystal structure of BLG dimer shows that each subunit contains nine β-strands (labelled A–I): eight of which form a β-barrel and an α-helix (*2*, *3*). The I-strand of one subunit forms an intermolecular β-sheet with that of another subunit (Fig. 1B). The salt bridges between Asp33 and Arg40 of the opposing AB-loops are also important to the stability of the dimer (*4*). Although BLG is dimeric at neutral pH, it dissociates into monomer at low pH and low ionic strength (*5*). At low pH, the structure of BLG has been determined by solution NMR. The global structure is similar to that of crystal structures at neutral pH (*6*, *7*).

Among non-ruminant LGs, equine LG (ELG) is monomeric at any pH (*8*). It contains the same number of disulphide bonds but no free thiol groups. Although a three-dimensional structure of ELG has not yet been determined, our previous NMR study showed that the secondary and tertiary structures of ELG are similar to those of BLG (*9*). The sequence identity between ELG and BLG is 57%. The most remarkable difference in the amino acid sequences is nine contiguous residues flanking (and including) the I-strand (145–153) (Fig. 1A). Previously, Kobayashi *et al.* (*10*) constructed a chimeric LG (mutant I) in which the residues that form the BLG dimer interface replaced the corresponding residues in ELG; *i.e.* the nine contiguous residues (145–153) were replaced with the corresponding BLG sequence. It was hypothesized that if the mutant I could assume the BLG conformation, then the dimer interface would be formed as a consequence of the BLG-specific I-strand sequences. The mutant I did not dimerize, however, and the authors concluded that the monomeric property of ELG cannot be attributed solely to the difference in the amino acid sequence of the I-strand.

Recently, we constructed another chimeric LG, Gyuba, in which the loop sequences of ELG and the secondary structure sequences of BLG were combined. Gyuba was able to form a dimer in a manner similar to BLG (*11*). This result suggested that the entire arrangement of the secondary structural elements is important for LG dimer formation.

As a next attempt, in this study, we constructed a chimeric LG, ImBLG (I-strand mutated BLG), in which the nine contiguous residues (145–153) of BLG were replaced with the corresponding ELG sequence. That is, ImBLG is a reversed version of the previously constructed mutant I of ELG (here, we rename the previous mutant ImELG). For technical reasons, Cys121 of ImBLG was also mutated to alanine. Because most of the sequence of ImBLG is identical to that of BLG, the entire arrangement of the

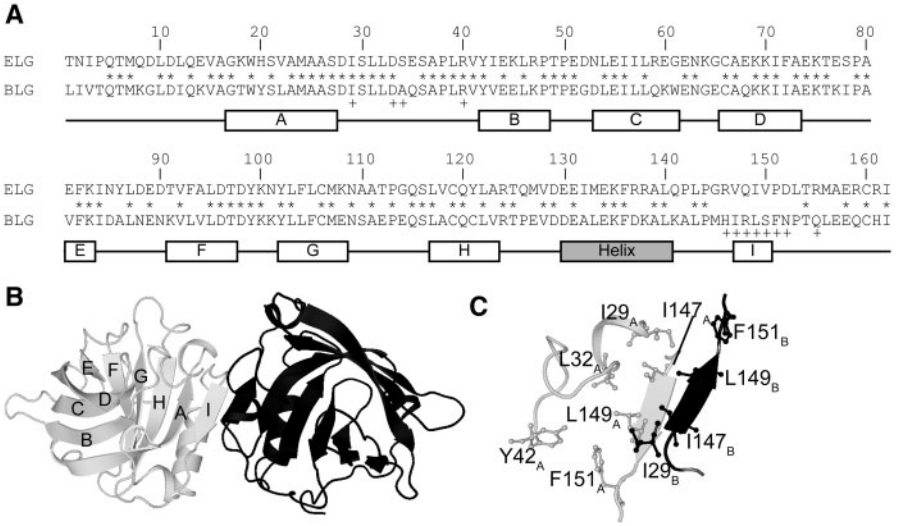

Fig. 1 The amino acid sequence and structure of LG. (A) Aligned amino acid sequences of BLG and ELG. Asterisks represent identical amino acids and symbols below the sequences show the side-chains of the residues that are contained in the dimer interface. The interface residues were defined by whether the solvent-accessible surface area (SASA) of side-chains changed between monomer and dimer. SASA was calculated by NACCESS (Hubbard, S.J. and Thornton, J.M., NACCESS Computer program, Department of Biochemistry and Molecular Biology, University College, London, 1993). (B) The crystal structure of BLG [1bsq (*2*)] is represented by a ribbon diagram. Two subunits are coloured black and light grey, respectively. (C) Hydrophobic residues on the I-strands (Ile147, Leu149 and Phe151) and Ile29, Leu32 and Tyr42 are shown by ball-and-stick representation. Subscripts indicate chain ID.

secondary structural elements (the scaffold for the I-strand) in BLG is expected to be maintained in ImBLG. Therefore, ImBLG was expected to form a dimer. However, ImBLG could not form a dimer,[1] suggesting that the sequence of the I-strand of BLG was also important for the dimerization of BLG. To identify key residues for dimerization, we constructed several revertants and examined whether they formed dimers.

Materials and Methods

Materials

The sources for the enzymes, chemicals and the kits for the molecular biological experiments have been described (*9*). The synthetic ImBLG gene and DNA oligomers for mutation were obtained from Operon Biotechnologies (Tokyo, Japan). The purified BLG variant B was obtained from Sigma-Aldrich (Tokyo, Japan). Other chemicals were analytical grade from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise noted.

Construction of the ImBLG and its revertant genes

The ImBLG gene was inserted between the NdeI and BamHI sites of the pET3c vector, which was used as an expression vector of ImBLG. Expression vectors of ImBLG revertants and BLG mutant were produced by site-directed mutagenesis using QuikChange (Stratagene).

Expression, refolding and purification of proteins

Each mutant protein was expressed in *Escherichia coli* strain BL21 (DE3) cells transformed with its expression vector. The procedures of expression and refolding were described previously (*9*). Refolded mutant proteins were purified by DEAE-Sepharose chromatography with a linear gradient of 0–400 mM NaCl in 50 mM Tris–HCl (pH 8.0). The fraction containing protein was further purified by gel-filtration chromatography [Sephacryl S-100 equilibrated with 50 mM Tris–HCl (pH 8.0)]. SDS–PAGE, native PAGE and reverse-phase HPLC were used to assess the purity of the protein. The pure fraction was then dialysed against distilled water and lyophilized.

Circular dichroism spectrum

Circular dichroism (CD) measurements were done using a J-720 (Jasco) or a Chirascan (Applied Photophysics) spectropolarimeter at 25°C. The proteins were dissolved in 50 mM sodium phosphate (pH 7.0) and their concentrations were determined spectrophotometrically. The extinction coefficients used for ELG and BLG were reported values of $12{,}000\,M^{-1}cm^{-1}$ at 280 nm (*8*) and $17{,}600\,M^{-1}cm^{-1}$ at 278 nm (*12*), respectively. The value for ImBLG was determined to be $17{,}000\,M^{-1}cm^{-1}$ at 280 nm by the method of Gill and von Hippel (*13*). The cuvette path length was 1 mm for far-UV CD measurements and 10 mm for near-UV CD measurements. A buffer of 20 mM PIPES (pH 7.0) was used for thermal denaturation experiments. Thermal denaturation curves were obtained by monitoring the changes in molar ellipticity at 293 nm. A scan rate of $2°C\,min^{-1}$ was used.

Gel-filtration chromatography

Samples were dissolved in 20 mM PIPES (pH 7.0) containing 200 mM NaCl and then loaded onto a column of Superdex 75 (GE Healthcare) at a flow rate of $0.5\,ml\,min^{-1}$. The elutions were monitored at 280 nm.

Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed using a Beckman XL-A or XL-I analytical ultracentrifuge at 22,000 or 27,000 rpm, 20°C. A Beckman Type 60 Ti rotor and charcoal-filled Epon centerpieces were used. Before ultracentrifugation, proteins were dissolved in 20 mM PIPES (pH 7.0) containing 200 mM NaCl and dialysed against the same buffer. Radial distributions were analysed as described (*10*).

[1] Whether a protein is monomeric or dimeric depends on the protein concentration used for observation because the monomeric and dimeric forms are in equilibrium. In this article, the term 'dimer' is used when a protein is observed as a dimer at the concentration used for gel filtration experiments (~1 mg/ml).

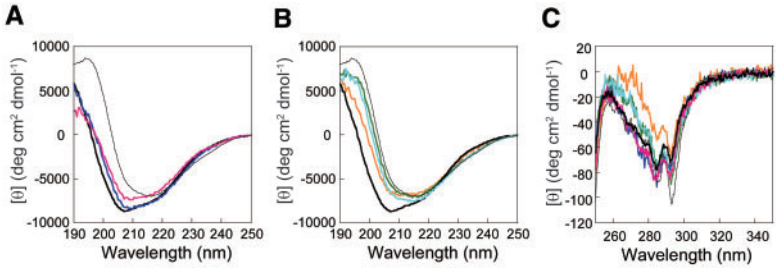

Fig. 2 Near- and far-UV CD spectra. (A and B) Far-UV CD spectra of BLG and constructed mutants. The spectra of BLG and ImBLG are represented in black thin and thick lines, respectively. (A) ImBLG-ILF (blue) and BLG C121A/M145G (magenta). (B) ImBLG/G145M (orange), ImBLG/G145M//P151F (sky blue), ImBLG-ILF/G145M (green). (C) Near-UV CD spectra of BLG and constructed mutants. Colours are equivalent to (A and B).

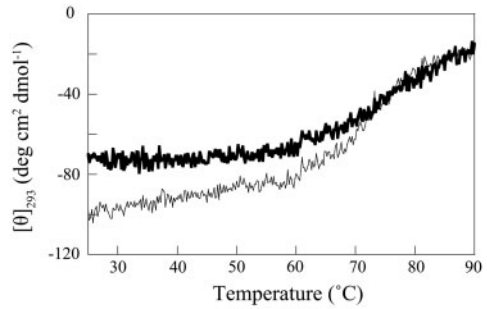

Fig. 3 Thermal denaturation curves of BLG (thin line) and ImBLG (thick line) monotored by near-UV CD at 293 nm.

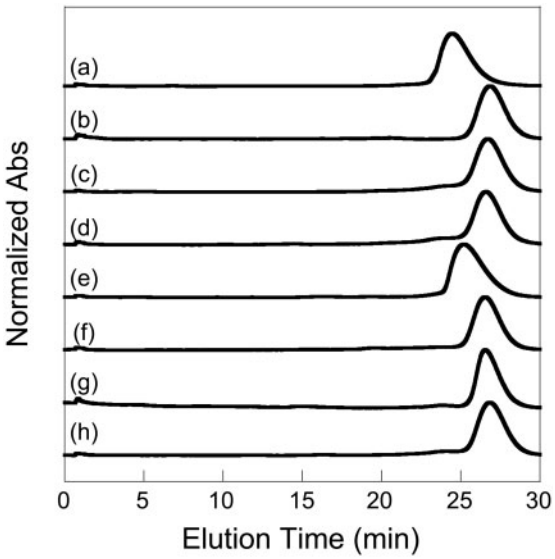

Fig. 4 Gel-filtration experiments of BLG, ELG and mutants. Elutions from Superdex 75 of (a) BLG, (b) ELG, (c) ImBLG, (d) ImBLG-ILF, (e) ImBLG-ILF/G145M, (f) ImBLG/G145M, (g) ImBLG/G145M/P151F and (h) BLG/C121A/M145G.

Results

Structure of ImBLG

ImBLG was constituted of the residues 145–153 of ELG and other residues of BLG, except for Ala121. To investigate the effect of mutations, the CD spectrum of ImBLG is measured (Fig. 2). Surprisingly, the far-UV CD spectrum of ImBLG is significantly different from that of BLG. However, the near-UV CD spectrum of ImBLG is similar to that of BLG, suggesting that ImBLG has a well-ordered tertiary structure and is not in a conformational state such as a molten globule. To confirm the rigid tertiary structure of ImBLG, thermal unfolding was measured by CD spectroscopy (Fig. 3). ImBLG was reversibly unfolded at 75°C, supporting the conclusion that ImBLG has a rigid tertiary structure. Gel-filtration experiments (Fig. 4) showed that ImBLG elutes at the same elution time as ELG elutes, suggesting that ImBLG is monomeric. Analytical ultracentrifuge experiments show that an association constant of ImBLG is 1/50 (Fig. 5). Therefore, the substitution of the nine residues 145–153 causes some change in the backbone conformation and loss of dimerization ability.

Revertants of ImBLG

As compared with BLG, ImBLG has nine contiguous mutations (M145G, H146R, I147L, R148Q, L149I, S150V, F151P, N152D and P153L) and an exceptional mutation (C121A). To identify which mutations affect dimerization, we investigated the crystal structure of BLG. In the structure of BLG, the side-chains of Ile147, Leu149 and Phe151 make a hydrophobic packing with Ile29, Leu32 and Tyr42 of the same protomer and Ile29, Ile147, Leu149 and Phe151 of the other protomer (Fig. 1C). Ile147, Leu149 and Phe151 were replaced with Val, Ile and Pro, respectively, in ImBLG. These substitutions may disturb the dimer formation. Thus, we constructed three residue revertants of ImBLG (ImBLG-ILF; Fig. 5), *i.e.* V147I/I149L/P151F mutants of ImBLG. Both gel-filtration experiments (Fig. 4) and analytical ultracentrifuge experiments (Fig. 5) show that ImBLG-ILF is monomeric. By inspecting the crystal structure of BLG, we found that the side-chain of Met145 inserts into the hydrophobic cavity composed of Ala25, Leu117, Cys119, Leu140 and Leu143 (Fig. 6). Thus, we mutated Gly145 of ImBLG-ILF to methionine (ImBLG-ILF/G145M, Fig. 5). The elution of ImBLG-ILF/G145M from the gel-filtration column is earlier than that of ImBLG and close to that of BLG (Fig. 4). This suggests that ImBLG-ILF/G145M exists as an equilibrium mixture of monomeric and dimeric forms, and that their dimerization affinities were weaker than that of BLG. Since ImBLG-ILF/G145M was eluted

	amino acid sequence of residues 145–153	association constant Ka (M^{-1})
BLG	M145 H146 I147 R148 L149 S150 F151 N152 P153	$(9.61 \pm 0.22)\times10^4$
BLG*	M145 H146 I147 R148 L149 S150 F151 N152 P153	$(2.46 \pm 1.07)\times10^4$
BLG/C121A*	M145 H146 I147 R148 L149 S150 F151 N152 P153	$(1.94 \pm 0.49)\times10^4$
ImBLG	G145 R146 V147 Q148 I149 V150 P151 D152 L153	$(1.84 \pm 0.02)\times10^3$
ImBLG-ILF	G145 R146 I147 Q148 L149 V150 F151 D152 L153	$(3.11 \pm 0.26)\times10^3$
ImBLG-ILF/G145M	M145 R146 I147 Q148 L149 V150 F151 D152 L153	$(2.01 \pm 0.07)\times10^4$
ImBLG/G145M	M145 R146 V147 Q148 I149 V150 P151 D152 L153	$(4.50 \pm 0.56)\times10^3$
ImBLG/G145M/P151F	M145 R146 V147 Q148 I149 V150 F151 D152 L153	$(4.58 \pm 0.48)\times10^3$
BLG C121A/M145G	G145 H146 I147 R148 L149 S150 F151 N152 P153	ND

Fig. 5 Association constants of LG variants. The amino acid sequence of residues 145–153 are schematically represented. The residues equivalent to ELG or BLG are coloured black or white, respectively. The interfacial residues and non-interfacial residues are represented by squares and circles, respectively. Asterisks indicate that the data taken from Yagi *et al.* (*14*). ND means that the association constant could not be obtained.

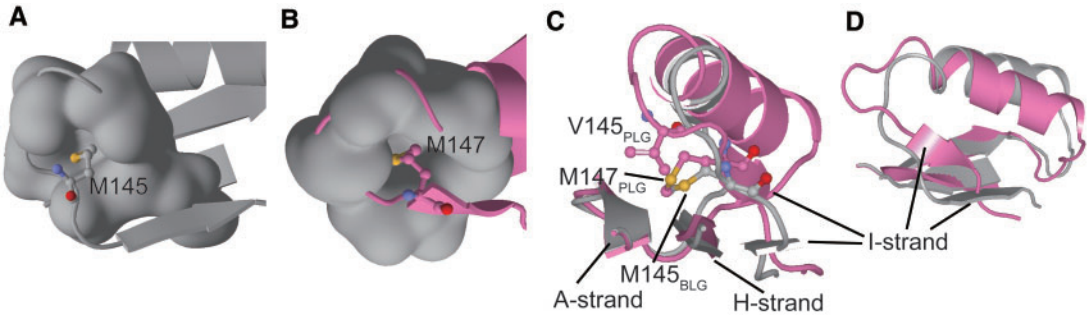

Fig. 6 The structural comparison of the I-strand and its upstream region between BLG [1bsq (2)] and PLG [1exs (15)]. The ribbon representations of BLG and PLG are coloured grey and pink, respectively. In the ball-and-stick representation, carbon of BLG (grey) and PLG (pink), nitrogen (blue), oxygen (red) and sulphur (yellow) are coloured as indicated. Met145 of BLG (A) and Met147 of PLG (B) are shown by ball-and-stick representations. The cavity-forming residues of BLG (25–27, 117–119, 140 and 143) are shown by grey surface representations. V145 of PLG is an additional cavity-forming residue. (C) The structures of BLG and PLG are superposed for the residues 23–27 and 117–124. Met145 of BLG, Val145 and Met147 of PLG are represented by balls and sticks. It shows that the position of Met147 of PLG is similar to that of Met145 of BLG. (D) The structural difference between BLG and PLG of the I-strand is shown.

as a single peak, the rate of dimer–monomer conversion seems to be fast. Analytical ultracentrifuge experiments also showed that ImBLG-ILF/G145M forms a dimer, although its association constant is one fifth that of BLG (Fig. 5). These results show that Met145 plays an important role in the dimerization of BLG.

To discover whether Met145 solely determines the dimerization ability, we constructed ImBLG/G145M

and ImBLG/G145M/P151F (Fig. 5). The side-chain of Phe151 makes a hydrophobic core as described above and its backbone NH makes a hydrogen bond with the backbone carbonyl oxygen of Leu22 on the A-strand. Therefore, Pro151 in ImBLG removes the hydrogen bond and may disrupt the I-strand. Gel-filtration chromatography (Fig. 4) and analytical centrifugation (Fig. 5) showed that both ImBLG/G145M and ImBLG/G145M/P151F exist as monomers. Therefore, the residues on the I-strand Ile147 and Leu149 are important for dimerization, as well as Met145.

Finally, we also constructed a BLG mutant, BLG/C121A/M145G (Fig. 5), and found that it exists as a monomer (Figs 4 and 5). Since it has been shown that C121A mutation does not disturb the dimer formation (*14*), the results clearly indicate that Met145 is critical for dimerization of BLG.

Conformational role of Met145

Far- and near-UV CD spectra were measured for all mutant proteins used in this study. As described above, the far-UV CD spectrum of ImBLG is significantly different from that of BLG. Interestingly, CD spectra of all mutant proteins can be categorized into two groups (Fig. 2A and B). The spectra of mutants with Met145 (ImBLG/G145M, ImBLG/G145M/P151F and ImBLG-ILF/G145M) are similar to that of BLG, whereas the spectra of mutants with Gly145 (ImBLG, ImBLG-ILF and BLG/C121A/M145G) are similar to that of ImBLG. It should be noted that BLG/C121A/M145G shows a CD spectrum similar to that of ImBLG. BLG/C121A/M145G has only two substitutions, C121A and M145G. Since it has been shown that C121A has a CD spectrum similar to that of BLG (*14*), M145G mutation clearly induces the change in CD spectrum, probably the change in the backbone structure. This must be the reason why Met145 is critical for dimerization.

Discussion

The present study suggested that Met145, Ile147, Leu149 and Phe151 were important for the dimerization of BLG. Ile147 and Leu149 are the residues in the I-strand and make hydrophobic contacts with Ile29, Leu32 and Tyr42 of the same protomer and Ile29, Ile147, Leu149 and Phe151 of the other protomer. One methyl group is lost by I147V mutation in ImBLG and a cavity is created beneath the AB-loop. The change in the side-chain shape by the mutation L149I does not seem to be accommodated by rotamer change. Intra- and inter-molecular interactions are disrupted by a mutation F151P as described above. These residues are located at the dimer interface and substitution of these residues directly affects the dimer formation. On the other hand, Met145 is not involved in the intermolecular contact. The side-chain of Met145 seems to lock the I-strand because the side-chain of Met145 is deeply buried in the cavity formed by the residues 25 (A-strand), 117, 119 (H-strand), 140 and 143 (upstream of the I-strand) (Fig. 6A). Intriguingly, a significant difference was observed in far-UV CD spectra between mutants with Met145 and with Gly145, suggesting that the conformational change around the I-strand is caused by that mutation.

To consider the conformational change, the structure around Met145 of BLG was compared with the corresponding residue of porcine LG (PLG), the only monomeric LG for which the crystal structure has been determined (*15*). Although the structure of PLG is similar to that of BLG, the α-helix and the I-strand of PLG are obviously different to those of BLG (Fig. 6D). Interestingly, Met147 of PLG was buried in the cavity instead of Val145, which is a component forming the cavity (Fig. 6B and C). The cavity of PLG was basically similar to that of BLG, except for the involvement of Val145. The cavity-anchored Met147 of PLG makes it difficult to form a BLG-like I-strand. The difference in the I-strand structure between BLG and PLG originates from the residue buried in the cavity. The I-strand of PLG seems to twist and the angle between the H-strand and the I-strand of PLG is larger than that of BLG (Fig. 6D), so that it is difficult to form the intermolecular β-sheet on the I-strand of PLG. Since the residues 145 of ELG and BLG/C121A/M145G are glycine, their I-strands must be different from a BLG-like I-strand. Since ImELG consist of the residues 145–153 of BLG and other residues of ELG, the residue 145 of ImELG is methionine. However, ImELG could not form a dimer (*10*). The fact that Gyuba forms a dimer in a manner similar to BLG (*11*), whereas ImELG cannot form a dimer (*10*), indicates that the scaffold for the I-strand is insufficient in ELG and that the proper scaffold is provided by the secondary structure sequence. The amino acid sequence around the cavity, which corresponds to the A- and H-strand and the α-helix may be important for its conformation. ImBLG-ILF/G145M was dimeric, but their dimerization affinities were lower than that of BLG. Therefore, the remaining residues His146, Arg148, Ser150, Asn152 and/or Pro153 contribute to the stabilization of the dimer. R148Q and N152D substitutions would affect the electrostatic interaction between the protomers. Recently, Bello *et al.* (*16*) suggested the importance of interfacial water for dimerization of BLG. The substitution of these four residues may affect water sequestering.

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Conflict of interest

None declared.

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