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*Review*

REVIEW

# The modifications of bovine *β*-lactoglobulin – effects on its structural and functional properties

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*Abstract*: Due to its excellent techno-functional properties, high nutritional value and low cost, *β*-lactoglobulin (BLG), the main protein in whey, is a frequently used additive in wide range of food products. It is also considered as an acid-resistant drug carrier for the delivery of pharmaceutical and nutraceutical agents. However, BLG is the main allergen of milk. A variety of methods has been explored for the modification of BLG in attempts to improve its functional properties and to decrease its allergenicity. Due to its compact globular structure, BLG is relatively resistant to modifications, especially under mild conditions. BLG can be modified by physical, chemical and enzymatic treatments. Although chemical modifications offer efficient routes to the alteration of the structural and functional properties of proteins, they are associated with safety concerns. In the last decade, there is a tendency for application of novel non-thermal physical processing methods, as well as enzymes in order to obtain BLG derivatives with desirable properties. The objective of this review is to overview the chemical, physical and enzymatic processing techniques utilized to modify BLG and their effects on the structural and functional properties of BLG.

*Keywords*: *β*-lactoglobulin; modification; chemical; physical; enzymatic; functional.

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## 1. INTRODUCTION

*β*-Lactoglobulin (BLG) is the major whey protein found in the milk of most mammalian species, representing 60 % of the total whey proteins. BLG (Fig. 1) is a globular protein consisting of 162 amino acid residues with a molecular weight (MW) of 18.3 kDa. BLG has two intramolecular disulfide bonds and one free thiol group, which plays an essential role in the antioxidant activities of BLG.[2] At different pH values, BLG preserves more or less the same secondary structure but it can adopt various tertiary structures.[3] BLG undergoes time- and temperature-dependent denaturation above 65 °C, which is accompanied by extensive conformational transitions that expose highly reactive thiol and amino groups. Bovine BLG belongs to the lipocalin protein family. It binds a wide range of small hydrophobic ligands, acting as a transporter. The binding of retinol and fatty acids to BLG have been widely implicated in its proposed physiological functions.[4] BLG and its peptide fragments have various bioactivities. It was reported that the various peptides derived from the proteolytic digestion of BLG have inhibitory activity against the angiotensin-converting enzyme.[5] Antimicrobial, immunomodulating, opioid and hypocholesterolemic activities have also been documented.[5]

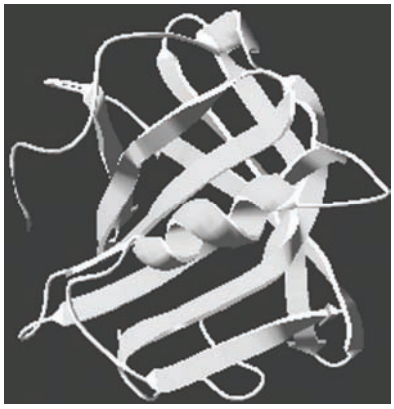

Fig. 1. Schematic representation of the structure of bovine *β*-lactoglobulin drawn using Swiss Pdb Viewer (http://www.expasy.org/spdbv/).[1]

BLG has desirable techno-functional properties determined by its physicochemical properties, such as viscosity, gelation, foaming, solubility and emulsification. This protein has a high nutritional value and may serve as a carrier for lipophilic nutrients and drugs. It is frequently used as an additive in a wide range of food products. However, BLG is the main allergen of milk and presents a significant health risk for patients allergic to milk. Due to its compact globular structure, BLG, as many other food allergens, is resistant to digestion.[6]

In the last decade, the requirements of food technologists, nutritional scientists and consumers for techno-functional, tropho-functional and hypoallergenic attributes of food products have been continuously rising. Different methods for

BLG modifications were studied in attempt to broaden its application in food and pharmaceutical industry by improving its techno-functional properties, as well as to increase its digestibility and reduce its allergenic potential. This review summarizes the effects of chemical, physical and enzymatic modifications of BLG on its structural and functional characteristics.

## 2. CHEMICAL MODIFICATIONS

Chemical modifications of proteins are not welcome in the food industry despite the application of non-toxic reagents, because of the complex procedures required to remove unreacted chemicals and the products of side reactions. However, chemical modifications offer efficient ways of imparting desirable properties to proteins.

*Acylation and alkylation.* Acylation by anhydrides of monocarboxylic acids replaces ammonium cations with neutral acylamino groups, resulting in electrostatically neutral groups. Acylation by anhydrides of di- or tricarboxylic acids leads to a conversion of cationic into anionic residues, which alters the net charge of a protein. A large degree of acylation can affect the secondary and tertiary structure of the protein.

It was demonstrated that acetylation and succinylation of BLG influenced not only its tertiary structure, but also its secondary structure. These modifications resulted in decreased hydrophobicity and increased content of beta sheet structures and random coils. Both acetylated and succinylated BLG were less stable against guanidine hydrochloride and urea denaturation.[7] The surface pressure of low-extent succinylated BLG was higher than that of native BLG, but higher levels of succinylation reduced the surface pressure.[8] Foam formation and stability of BLG were reduced following its succinylation.[8] Succinylated BLG was found to be a suitable natural excipient for the formation of delayed release tablets or tablets containing probiotic bacteria. Furthermore, succinylation of BLG improved its survival under gastric conditions.[9] In addition, succinylation of BLG decreased its solubility and its charge density at acidic pH values, resulting in decreased tablet erosion (protein loss) at pH 1.2. Solubility of succinylated BLG compared to unmodified protein increased at pH 7.5, making it suitable for the protection and intestinal release of gastro-sensitive compounds. Many studies have shown that acylated milk proteins with increased negative charge are potent antiviral compounds, having a therapeutic potential for local administration. Modification of BLG by 3-hydroxyphthalic anhydride (3HP) yielded compounds that exerted antiviral activity *in vitro* against herpes simplex virus type 2, influenza virus, human immunodeficiency virus 1 (HIV-1) and simian immunodeficiency virus (SIV).[10]

BLG modified with vanillin and benzaldehyde exhibited very low solubility near the isoelectric point and possessed improved emulsifying activity in the aci-

dic pH range, but lower emulsion stability in the alkaline pH range.[11] Alkylation of BLG enhanced its binding affinity for retinoids.[12] BLG alkylated by allyl isothiocyanate (AITC) gave BLG–AITC conjugates, which at pH 7.1 possessed loose and partially unfolded structures, whereas at pH 4.0, they exhibited features typical for a molten globule. BLG–AITC conjugates showed better emulsifying and foaming properties than those of unmodified BLG.[13] The immunogenicity of chitosan-conjugated BLG was reduced compared to native BLG. The linear epitope profiles of these conjugates were similar to those of BLG, but the antibody response to each epitope was dramatically reduced due to the masking of B cell epitopes.[14] The anti-BLG antibody response in mice was markedly decreased following immunization of the animals with BLG–carboxymethyl dextran (CMD) conjugates. Although linear epitope profiles of BLG–CMD conjugates were similar to those of BLG, the antibody response for each epitope was dramatically reduced as a consequence of the effective shielding of epitopes by CMD. BLG conjugated with poly(ethylene glycol) (PEG) had decreased antigenicity, while the allergenicity of the conjugate was weaker than that of the native protein.[15] Oxidative sulfitolysis combined with covalent binding of PEG to BLG resulted in a conjugate with improved emulsifying activity and emulsion stability at acidic as well as at neutral pH values.[16]

It is known that covalent linking of lipids to proteins results in increased hydrophobicity of proteins, which then display an improved capacity to form and stabilize emulsions and foams. Stearic acid-modified BLG, thus, possesses an increased hydrophobicity, while the solubility of the conjugate depends on its content of stearic acid. The emulsifying and foaming properties of these conjugates were improved at low and medium levels of stearic acid incorporation.[17] BLG with a low content of stearic acid demonstrated increased binding of IgE and IgG when compared to the native protein, which was due to the increased exposure of antigenic sites. However, the ability of BLG with medium content of stearic acid to bind antibodies was reduced. Finally, a high level of fatty acid attachment to BLG resulted in its denaturation and inability to bind to antibodies, due to the reduction or destruction of the available allergenic sites. A decrease in the digestibility of the modified BLG accompanied by an increased lipophilization was also observed.[18]

*Esterification.* Proteins modified by esterification gain a more positive charge as the number of ionizable carboxyl groups becomes reduced. Moderate esterification of BLG induces not only slight changes in its secondary structure, but also its tertiary structure acquires properties that are characteristic of the molten globule state. This leads to opening of the BLG molecule to the cleavage of the peptide bond. Esterified BLG is hydrolyzed rapidly by pepsin, which is due to 22 new sites of pepsin cleavage introduced by esterification.[19] Fourteen cleavage sites are pepsin-specific and their unveiling is due to the imposed ter-

tiary structure changes. Eight of the new cleavage sites are esterified carboxylates recognized by pepsin.[18] The methyl-, ethyl-, and butyl-esters of BLG showed enhanced surface activity, as well as hydrophobic probe binding activity, the most pronounced effect being that of methyl esters.[20]

The positive charges on the protein molecules enable them to interact with viral proteins or viral DNA, affecting viral replication, transcription or translation and, consequently, viral infectivity. Highly esterified BLG at pH 7 showed DNA-binding capacities comparable to those exhibited by native basic proteins, such as lysozymes and histones. Esterification of BLG enhanced its antiviral activity against the avian influenza A virus (H5N1), the influenza virus A subtype H1N1 and the HSV-1 virus.[21] Peptic hydrolyzates of esterified BLG also displayed antiviral activity.[22]

*Glycation by Maillard reaction.* Glycation by the Maillard reaction is a ubiquitous reaction of condensation of a reducing sugar with the amino groups of proteins. The products of Maillard reaction could improve the functional and/or biological properties of a protein, which could be utilized in the food industry. The glycosylation of BLG in Maillard reaction under mild conditions does not alter the protein structure, except a slight increase in its Stokes radius and an increase in the temperature of denaturation. Since the Stokes radius of denatured BLG is not significantly smaller than that of glycosylated BLG, it was proposed that its non-polar residues associate with the sugar moieties in the unfolded state, thereby preventing their solvent exposure and affecting the aggregation propensity and the type of aggregate formed.[23] Glycation of BLG in the Maillard reaction by several alimentary sugars induced oligomerization of BLG monomers.[23] Among the sugars used, pentoses (arabinose and ribose) induced the highest degree of modification, while hexoses (glucose, galactose and rhamnose) were less reactive and lactose generated the lowest degree of modification.[24] Proteins substituted with pentoses formed polymers stabilized by sugar-induced covalent bonds. When other sugars were used, a proportion of the aggregated proteins were stabilized only by hydrophobic interactions and disulfide bonds. In the presence of less reactive sugars, heating of BLG induced only minor structural modifications, in contrast to more reactive sugars such as pentoses. Dry-state glycation of BLG did not significantly alter the native-like BLG behavior, while treatment in solution led to important structural changes. Denatured BLG monomers associated covalently *via* their free thiol group and in a subsequent step, non-covalent polymerization of the unfolded homodimers and swollen monomers occurred through hydrophobic interactions.[25]

Moderate glycation of BLG in the early stages of the Maillard reaction had only a small effect on its recognition by IgE, whereas a high degree of glycation had a clear “masking” effect on the recognition of epitopes.[26] The antigenicity of BLG glycated by glucose and oligoisomaltose was reduced.[27] High levels of

BLG glycation in the Maillard reaction impaired BLG proteolysis by simulated gastrointestinal digestion and, consequently, increased the IgG- and IgE-reactivities of the hydrolysates, regardless of the employed carbohydrate. However, protein aggregation during the advanced stages of the Maillard reaction had a masking effect on the BLG epitopes, counteracting the negative effect of the lower digestibility of the glycated protein on its allergenicity.[28] After mice immunization with BLG modified by acidic oligosaccharides, the anti-BLG antibody response was markedly diminished in the vicinity of the carbohydrate-binding sites,[29] which was due to reduced T cell response because of reduced susceptibility of the conjugates to processing enzymes for antigen presentation. High levels of BLG glycation with galactose, tagatose, and dextran impaired its proteolysis and, consequently, increased the IgG- and IgE- reactivities of the hydrolysates, regardless of the used carbohydrate. The different increases in susceptibility of glycated BLG to pepsinolysis were related to the alteration of the conformation of the protein when glycation was performed with highly reactive sugars.[30]

Glycation of BLG with highly reactive pentose sugars, such as arabinose or ribose, improved BLG emulsifying properties, while its foaming properties were improved when glycation occurred with hexose sugars, such as glucose or galactose.[24] Glycation of BLG with different sugars induced an increased ability for scavenging radicals, with ribose and arabinose having the most pronounced effect.[31] Maillard reaction products, formed in the reaction of hydrolyzed BLG with glucose, exhibited increased radical scavenging activity, which reduced its iron chelating activity.[32] BLG modified by glucose-6-phosphate showed a greater thermal stability and a greater emulsifying activity than the native protein.[33]

*SH-group modifications.* BLG has two intramolecular disulfide bonds that may be responsible for its allergenicity. After reduction of one or both of its disulfide bonds by thioredoxin, BLG became strikingly sensitive to pepsin and lost its allergenicity.[34] Modification of the buried thiol group of BLG by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) destabilizes the rigid hydrophobic core and the dimer interface, thereby producing a monomeric state that is native-like at pH 2.0 but is molten globule-like at pH 7.5. Upon reducing the mixed disulfide of DTNB–BLG with dithiothreitol, intact BLG was regenerated.[35] In order to cleave the disulfide bonds, BLG was modified by sulfitolysis, but it was hydrolyzed by pepsin and trypsin more readily than the intact protein.[36]

*Phosphorylation.* Phosphorylation of BLG with $POCl_3$ in the presence of either triethylamine or hexylamine resulted in a slightly cross-linked BLG with increased negative charge. The phosphorylation resulted in disordering of the secondary structure of BLG and shifting BLG solubility minima toward lower values.[37] The addition of butylamine produced phosphobutylated BLG with excellent foaming and emulsifying properties, while the use of longer aliphatic pri-

mary amines (C8–C12) gave rise to reduced solubility, poor foaming and weak emulsifying properties.[38]

*Non-covalent chemical modifications.* Ligand binding to BLG may have a stabilizing effect on the protein. Susceptibility of BLG/retinol or BLG/retinoic acid complexes to trypsin was significantly lower than that of unconjugated BLG, due to the more compact structure.[39] When bound to gallic acid, BLG displays a lower degree of hydrolysis than native BLG, but gallic acid induces oxidation of the four methionine residues in BLG to methionine sulfoxides.[40] On the other hand, binding to BLG can protect some ligands from oxidation; this suggests that BLG could be employed as an effective carrier of oxidation-sensitive hydrophobic drugs and nutriceuticals. Binding of folic acid to BLG improves its photostability.[41] Binding of resveratrol to BLG, although partially disrupting its tertiary structure, provides a slight increase in the photostability of resveratrol and a significant increase in its hydrosolubility.[42] Epigallocatechin-3--gallate (EGCG) in thermally-induced BLG–EGCG conjugates are protected from oxidative degradation.[43] BLG/retinoid complexes exhibited an increased thermal stability and a higher resistance to UV light-induced oxidation.[44] It was reported that BLG degrades more rapidly in the presence of catechin-enriched polyphenols from green tea. However, laccase polymerized green tea catechins (GTC) adversely affected protein digestion of BLG and the protecting effect of polyphenols correlated well with the ability of proteins to form insoluble complexes with oxidized catechins.[45] In a study of non-covalent interactions between BLG and polyphenols from extracts of green and black teas, coffee and cocoa, it was demonstrated that stronger non-covalent interactions delayed pepsin and pancreatin digestion of BLG and induced the β-sheet to α-helix transition at neutral pH values. The positive correlation between the strength of protein–polyphenol interactions and the half time of protein decay under gastric conditions was found, as well as a masking of the total antioxidant capacity of protein–polyphenol complexes.[46] BLG saturated with palmitic acid possessed a higher gelling temperature and gelation time, implying that differences in the amount of bound fatty acids could be an important source of variability in the gelation behavior of BLG.[47] Phosphatidylcholine (PC) binding increased BLG thermostability, accelerated heat-induced gelation, and reinforced the mechanical strength of gels.[48] PC protected BLG from degradation under duodenal conditions and altered the pattern of the digestion products. PC binds to a secondary fatty acid binding site in BLG, thus blocking the action of proteases, rather than occupying the central calyx.[49] Interaction of flavor compounds with BLG have an influence on the release of flavor from food. BLG possesses at least two binding sites for aroma compounds. The longer the hydrophobic chain of the aroma compound, the greater is the affinity of BLG for the compound. This results in a decreased release rate and a lower final headspace aroma concentration.[50]

## 3. MODIFICATION BY PHYSICAL METHODS

During a physical treatment, an irreversible change occurs only above a given threshold of treatment intensity. The formation of covalent and non-covalent protein aggregates contributes to the irreversibility of the process. Below the threshold, a number of reversible modifications occur, but after the perturbation is over, the protein returns to a native-like form. Since comprehensive reviews that deal with the effects of thermal treatment on the structure and function of BLG already exist,[51] in this review, focus is directed on the modifications of BLG caused by non-thermal physical methods.

*Gamma irradiation*. γ-Irradiation of BLG in solution promotes the formation of linear oligomers, which consist of dimers of BLG (tetramers, hexamers, *etc*.), the oligomerization being partially caused by intermolecular cross-linking between tyrosyl radicals.[52] The changes in the secondary and tertiary structure of BLG induced by γ-irradiation are similar to the alterations observed in BLG that had been treated thermally under mild conditions.[53] Both changes result in the reduced solubility of the protein and increased agglomeration. Application of γ-radiation up to a dose level of 10 kGy did not affect the molecular-weight distribution of BLG, but reduced its solubility and increased its antigenicity.[54]

*Pulsed electric field (PEF) and electrolysis*. Application of high-intensity PEF to BLG resulted in the partial denaturation of the protein and its aggregation, including covalent cross-linking. PEF treatment increased the thermal stability of BLG by 4 to 5 °C, as well as its gelation rate.[55] After the treatment by electrolysis, BLG on the cathode showed markedly mitigated allergenic properties, attributed to the dislocation of the allergenic peptides from the protein surface.[56]

*Ultrasound*. High intensity ultrasound under uncontrolled temperature conditions induced changes in the secondary structure of BLG, which was accompanied by the formation of dimers, trimers and oligomers, and by an increased susceptibility to pepsin digestion. Although sonication under controlled temperature conditions induced changes in the secondary structure of BLG, it did not induce protein oligomerization or a change in its capacity to bind retinol.[57] The sonication-induced BLG forms had larger hydrophobic surfaces than native BLG and, thus, more easily underwent cross-linking with phenol oxidase. Sonication had only a minor effect on the ability of BLG to bind to IgE, both *in vitro* and *in vivo*.[57] The reactive thiol content and surface hydrophobicity of BLG increased continuously during sonication, suggesting an unfolding of the dimer structure, as an initiation step, with exposure of the thiol groups and the hydrophobic regions. Minor secondary and tertiary structural changes were also observed.[58] A noticeable synergism between ultrasound and heat on BLG denaturation was observed.[59] It was shown that even under conditions that were not very favorable for the Maillard reaction, such as neutral pH and reaction in solution, BLG could be glycated with the aid of high intensity ultrasound. The ultrasound-promoted

BLG glycation did not have a drastic impact on its secondary or tertiary structure. Forms of BLG obtained in the Maillard reaction forced by ultrasound exhibited radical scavenging ability and possessed a greater ferrous ion-chelating activity and better reducing power than the native protein.[60]

*UV irradiation*. UV irradiation of BLG induces changes in its molecular size distribution and a disruption of the ordered structure of BLG. This led to changes in the BLG antigenicity and to altered activity of BLG in the regulation of immunoglobulin production.[61] After 24 h of UV-irradiation, 18 % of the protein had been denatured with some protein aggregation. Changes in the secondary structure of BLG were similar to those that occurred in the early phase of heat-induced denaturation. The number of exposed sulfhydryl groups increased, but the number of total sulfhydryl groups decreased together with some photo-oxidation.[62]

*High hydrostatic pressure (HHP)*. Stapelfeldt *et al.*[63] proposed a three step pressure denaturation model for BLG in neutral solution at ambient temperature: an initial pressure-melted state (up to 50 MPa) with the partial collapse of the inner calyx and solvent exposure of the free thiol group, followed by a reversible denaturation with exposure of the hydrophobic regions (half-denaturation at 123 MPa) and irreversible denaturation with the thiol–disulfide exchange becoming increasingly important at higher pressures. In the initial stage of HHP-induced aggregation of BLG, only dimers and trimers arose due to SH/S–S interaction.[64] The high-pressure denaturation of BLG led to increased reactivity of the thiol group that was buried inside the native globule. This was a result of the exposure of the thiol group to the protein surface. The release of monomers seems to represent one of the earliest events, while association of the transiently modified monomers stabilized the denatured form of the protein.[65] Up to 200 MPa, a large number of monomeric BLG molecules was formed, which carried exposed thiol groups and which had an increased surface hydrophobicity. At pressures larger than 200 MPa, the hydrophobicity of the protein surface continued to increase, while the exposure of thiol groups decreased, due to the formation of covalently linked oligomers. Pressure-induced BLG oligomerization and formation of disulfide bonds resulted from SH/S–S interchange reactions rather than from oxidation of the thiol groups. At a pressure between 100 and 300 MPa, there was a significant increase in relative hydration of BLG due to structural changes of the protein. The number of water molecules associated with BLG molecule was also increased. Only a small decrease in the hydration of BLG was observed at higher pressures, due to the irreversible denaturation and aggregation.[66] Mazri *et al.*[67] found the reaction order for HHP-induced BLG denaturation to be $n = 1.5$, which is the same as that found for thermal denaturation of BLG. This unusual value has been attributed not only to a complex reaction mechanism, which involves not only many consecutive and/or concurrent steps, including the dissociation of

BLG dimer into monomers, but also to unfolding of monomers followed by the aggregation. Kolakowski *et al.*[68] demonstrated that the pressure-induced unfolding of BLG was significantly lower at 4 °C than at 25 °C and was without aggregation. They suggested the existence of different BLG conformations under different *p*/*T* conditions: the native conformation at 0.1 MPa/20 °C, a pressure-denatured state at 200 MPa/20 °C and a cold-denatured state at 200 MPa/–15 °C.

Although BLG exhibits a considerably enhanced susceptibility to proteolysis under HHP, the addition at ambient pressure of proteases to pressure-treated BLG gave only a modest increase in proteolysis. The products of chymotrypsin hydrolysis obtained under pressure were different from those obtained at atmospheric pressure without immunochemical reactivity, which indicated that chymotrypsin effectively hydrolyzed the hydrophobic regions of BLG that were transiently exposed during the pressure treatment.[69] The rate of pepsin hydrolysis of BLG (negligible at 0.1 MPa) increased considerably when a pressure up to 300 MPa was applied. However, this was not accompanied by qualitative changes in the profiles of the peptides obtained after hydrolysis. HHP treatment at 600 and 800 MPa resulted in a rapid digestion of BLG by pepsin.[70] HHP treatment increased the binding of BLG to rabbit anti-BLG IgG antibody, but it did not affect the binding of BLG to IgE from patients allergic to BLG. There was no apparent relationship between these responses and the degree of protein aggregation.[71]

The viscosity of BLG solutions increased with pressure due to the unfolding of BLG monomers and their aggregation. BLG modified by high-pressure displayed a reduced emulsifying capacity and a reduced foamability when compared to native BLG. This was attributed to an increase in both BLG hydrophobicity and its potential for aggregation.[72] Furthermore, a pressure-treated BLG showed a greater capacity for protein–protein interactions in the adsorbed layers of interfaces. The binding of retinol to BLG was enhanced by an increase in pressure up to 150 MPa, but at higher pressures, it decreased and disappeared altogether at 300 MPa. Once dissociated, the BLG–retinol complex did not re-associate after decompression at neutral pH.[73]

## 4. ENZYMATIC MODIFICATIONS

The employment of enzymes offers many advantages, including the ability to perform modifications under physiological conditions with great specificity and stereoselectivity, but without undesirable side reactions. Enzymatically modified proteins are well accepted by consumers, as enzymes are natural products. However, due to their labile activity and difficulties in their purification, enzymes are still underexploited as protein modifiers. Exposure to different pH conditions, heat treatments, and adsorption to interfaces are all potential ways to in-

duce conformational changes, which could then increase accessibility of BLG to enzymatic catalysis.

*Cross-linking enzymes*. A protein that undergoes cross-linking by modifying enzymes should have a flexible structure. As a globular protein with a compact structure, BLG is poorly accessible to cross-linking enzymes. Therefore, for an efficient cross-linking, it is necessary to partly unfold the BLG molecules, either prior to or during the cross-linking. The addition of mediators can help, for example, the addition of small phenolic compounds during the cross-linking of BLG by phenol oxidases.

*Transglutaminase (TG).* Proteins can be cross-linked using transglutaminase, which catalyzes an acyl group transfer between the γ-carboxamide group of glutaminyl residues in proteins and the ε-amino group of lysyl residues, forming an isopeptide bond. Moderate TG treatment of BLG in the presence of dithiothreitol (DTT) led to a decrease in the thermal stability of BLG. In contrast, treatment of BLG in an excess of TG resulted in a remarkable increase in the thermostability of the protein, regardless of the presence or absence of DTT. The modulation of the thermal stability of BLG by TG treatment may be attributed to partial unfolding of the protein molecule and subsequent re-arrangement of its conformation.[74] BLG modified by TG in the presence of cysteine possesses increased foamability and index of surface hydrophobicity, although less than heat-treated BLG.[75] Subjecting BLG to slightly alkaline (pH 7.5) and alkaline (pH 9.0) conditions results in a limited cross-linking by TG, which is due to a minor population shift towards a molten-globule state. However, upon heat treatment followed by cooling, the cross-linking of BLG was associated with a molten-globule-like conformation that showed a small change in the secondary structure and a significantly disturbed tertiary structure.[76]

*Tyrosinase.* In tyrosinase-induced cross-linking of a protein, the oxidation proceeds as a two-step reaction. Monophenols are first oxidized to diphenols, after which, quinones are formed. Thalmann *et al*.[77] reported that BLG can be cross-linked by tyrosinase from *Agaricus bisporus* only in the presence of a low molecular weight phenolic compound, which probably acted as a bridging agent between the protein subunits. It appears that the cross-linking of BLG by *Trichoderma reesei* tyrosinase (TrTyr) may occur even without the presence of phenolic mediators at pH 9, but not at pH 7.5. The increased accessibility of BLG to the tyrosinase at pH 9 was associated with small changes in the protein secondary structure and a loosening of the hydrophobic core, rather than with a major unfolding of BLG, which occurs in the higher pH range (between 10 and 12).[78] At neutral pH, BLG was susceptible to TrTyr only after heat treatment, as the BLG adopted a molten-globule-like conformation that enabled accessibility to TrTyr. Heat-treated BLG molecules adsorbed on an air/water interface and then enzymatically treated favored intra-molecular over inter-molecular cross-linking within

the packed BLG layer, which makes the adsorbed molecules even more rigid and less free to reorganize.[75]

*Laccase*. The application of laccase in the cross-linking of proteins is affected by the accessibility of their phenolic moieties, as proteins generally contain a small number of phenolic groups. In the presence of ferulic acid, laccase was able to form irreducible intermolecular cross-links in BLG, as well as to induce oxidative modifications. The latter included: dityrosine formation, formation of fluorescent tryptophan oxidation products and formation of carbonyl derivatives of histidine, tryptophan and methionine, which resulted in the protein molecules exhibiting a higher surface tension.[79] The allergenicity of BLG cross-linked by laccase in the presence of phenolics originating from sour cherry decreased, whereas the digestibility of the remaining BLG monomers increased under conditions that simulated the human gastrointestinal tract. The tryptic hydrolysates of cross-linked BLG showed a 57 % increase in radical-scavenging activity when compared to the control BLG.[44] The cross-linking of BLG in the presence of phenolics from apple (APE) rendered cross-linked BLG. Laccase treatment of BLG caused a bi-phasal pepsin–pancreatin digestibility of the monomeric and cross-linked protein to decrease, thus decreasing its nutritional value.[80] Cross-linking of BLG by laccase in the presence of polyphenols from green tea extended the half-life of BLG that was subjected to *in vitro* digestion by pepsin.[81]

*Proteolytic enzymes*. Whey protein hydrolysates can be practically used in the production of infant formulas for atopy. It is known that only mixtures of amino acids are completely non-allergenic products and that other hypoallergenic products available on the market still contain peptides, which may trigger allergic mechanisms. Hydrolysis could partly reduce the immunogenicity of native BLG, as it destroys the three-dimensional structure of BLG by removing some of its epitopes.[82] Proteolysis by trypsin resulted in extensive degradation of BLG, which then had a remarkable reduction in IgE binding capacity.[83] In addition, peptides obtained by tryptic hydrolysis of BLG induced a specific oral tolerance in mice.[84] The application of high pressure enhanced this proteolysis, which resulted in a decrease of BLG antigenicity and also a decrease in its ability to bind IgE from serum.[85] Furthermore, the application of a mixture of different proteases possessing different specificities proved to be more effective in enhancing the hydrolysis of BLG and also in reducing its residual antigenicity.[86] A contaminating proteolytic activity in the enzyme preparations used for cross-linking removed a peptide from the *N*-terminus of BLG, making it more susceptible to pepsin digestion and reducing its allergenic potential.[87]

Fragments of the $\beta$-barrel domain of BLG, obtained by proteolysis, have a lower surface hydrophobicity and slightly higher surface and interfacial tension. As a consequence, these proteolytically formed oligopeptides possess higher emulsifying activity index, higher emulsion stability and better foaming proper-

ties.[88] A limited tryptic proteolysate of BLG formed much stronger gels with a lower gel point and higher rate of gelation, which were due to a more efficient association of the fragments that had unfolded domains and also to the existence of structured domains with lower thermal stability.[89] An improvement of the functionality of BLG hydrolysates, compared to the intact protein, was caused by the presence of peptides, as higher concentrations of molecular species were present in the hydrolysates. However, BLG treated by alcalase first forms transparent gels, before the non-covalently linked protein aggregates appear.[90] BLG hydrolysate possesses a higher anti-oxidative capacity and iron-binding capacity than native BLG, mostly due to Met and Tyr residues.[91]

## 5. CONCLUSIONS

Research devoted to modifications of BLG is attracting more and more scientists who work in the food industry. This is mainly because BLG is the most abundant protein in whey, which is a by-product of cheese production and, thus, a cheap source of this highly valuable nutrient. In addition, the excellent inherent functional properties of BLG enable a wide range of applications of this protein in the food and pharmaceutical industries. However, there is an increasing prevalence of food allergies worldwide, which includes an allergy to milk. Having a compact globular structure, BLG is relatively resistant to modifications, especially under mild conditions. On the other hand, its resistance to modifications helps it retain its desirable functional characteristics after a controlled modification.

Alterations in the digestibility of BLG induced by modifications can have several consequences. On the one hand, the increased digestibility may reduce its allergenic potential. The alteration in the peptide profile, which is obtained after BLG digestion in gastrointestinal tract, could also lead to a loss of one set of health-promoting biopeptides and gaining another. On the other hand, some of BLG modifications reduce its digestibility and are, therefore, suitable for the application in weight-loss food products. Many methods for modifications of BLG target its lysine residues. As lysine is an essential amino acid, extensive modifications of lysines lead to a substantial loss of the nutritional quality of milk products. Any change in BLG structure induced by modifications may increase allergenicity of this molecule by exposing hidden epitopes or by creating new epitopes. Therefore, an allergenicity assessment is necessary to test the safety of modified BLG molecules.

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ИЗВОД

## МОДИФИКАЦИЈЕ БЕТА-ЛАКТОГЛОБУЛИНА – ЕФЕКТИ НА ЊЕГОВЕ СТРУКТУРНЕ И ФУНКЦИОНАЛНЕ ОСОБИНЕ

ДРАГАНА СТАНИЋ-ВУЧИНИЋ и ТАЊА ЋИРКОВИЋ ВЕЛИЧКОВИЋ

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Бета-лактоглобулин (БЛГ) је главни протеин сурутке који се често користи као адитив у великом броју прехрамбених производа захваљујући својим изванредним техничким и функционалним особинама, високој нутритивној вредности, као и ниској цени. Овај протеин се показао као погодан носач за лекове и хранљиве супстанце, јер је отпоран на киселу средину у гастро-интестиналном тракту. Међутим, БЛГ је и главни алерген млека. Са циљем унапређења функционалних особина и смањења алергености овог протеина до сада је развијен велики број метода за његову модификацију. Захваљујући својој компактној глобуларној структури БЛГ је релативно резистентан на модификације, нарочито оне под благим условима. БЛГ може бити модификован физичким, хемијским и ензимским методама. Мада су хемијске модификације ефикасан начин промене структурних и функционалних особина протеина, у вези са њима се често поставља питање безбедности. Током последње деценије постоји све већа тенденција ка примени нових третмана заснованих на физичким нетермалним методама, као и на примени ензима, како би се добио БЛГ са жељеним особинама. Циљ овог прегледног рада је приказ хемијских, физичких и ензимских техника које се користе за модификацију БЛГ, као и њихових ефеката на структуру и функцију БЛГ.

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