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REVIEW PAPER

BINDING PROPERTIES OF β-LACTOGLOBULIN WITH POLYPHENOLS – A REVIEW

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Bovine β-lactoglobulin is the most abundant whey protein secreted in the milk of most mammals but not in the human, rodents and lagomorphs milk. The biological function of this protein is still not completely understood, but it is believed to be related to its globular structure and the presence of an internal cavity called calyx or β-barrel, where small hydrophobic molecules can bind. Recent studies revealed that β-lactoglobulin has at least three binding sites, located in the internal core of the calyx, at the dimer interface and in the hydrophobic region between α-helix and β-barrel. In particular, this review focuses on the studies presenting β–lactoglobulin as potential carrier for polyphenolic compounds, molecules well-known for their beneficial health effects. Regarding the polyphenols binding site, several studies indicated that it is located outside the protein calyx.

Keywords: bovine β–lactoglobulin, binding affinity, Tanford transition, polyphenols

Introduction

β–Lactoglobulin (β-LG) is a globular protein belonging to the lipocalin family (Sawyer, 2013). It is present in the milk of ruminants and many other mammalian species such as sow, mare, kangaroo, dolphin, and manatee. β-LG is notably absent in the human, guinea pig, mouse, rat, camel, llama, and alpaca milk (O'Mahony and Fox, 2014). The protein was isolated for the first time from milk in 1934 by Palmer, being extensively studied afterwards, and used as model for trying new experimental and theoretical techniques because of its small dimensions and rapid separation and purification from the matrix (Palmer, 1934, Sawyer, 2013). So far, at least 12 genetic variants of β-LG are known, the most common in bovine milk being variants A and B (O'Mahony and Fox, 2014). The difference between these two variants is a mutation that occurs at positions 64 ($Asp_A \rightarrow Gly_B$) and 118 ($Val_A \rightarrow Ala_B$) in the amino-acids sequence. Even though the overall conformation

remains the same, these small differences affect properties like solubility, thermal stability, and pressure denaturation (Oliveira *et al.*, 2001, Botelho *et al.*, 2000, Keppler *et al.*, 2014).

In the beginning, whey was considered a dairy industry waste. Later, the beneficial value of whey components was recognized and whey protein concentrates and isolates started to be progressively used in the food industry (de Wit, 1998). In bovine whey, β-LG is the major protein accounting for approximately 50% of the total protein (O'Mahony and Fox, 2014).

β-LG molecules have good functional properties being able to stabilize emulsions and foams by forming interfacial films and to specifically interact with each other and to associate into networks by forming gels or edible films (Foegeding *et al.*, 2002).

Structure of β-lactoglobulin

In order to understand the protein properties, the comprehension of the molecular structure is required. Therefore, different technique, such as macromolecular (X-ray) crystallography and Nuclear Magnetic Resonance (NMR) spectroscopy, were applied to elucidate the structure of β-LG (Sawyer, 2013). Braunitzer *et al.* (1972) reported for the first time the correct amino acid sequence of β-LG. The primary structure of the β-LG consists of 162 amino acids, with a molecular weight of 18.4 kDa and an isoelectric point of 5.3 (Sawyer, 2013, Oliveira *et al.*, 2001). In solution, the secondary structure of β-LG consists of 8% α-helices, 45% β-sheets and 47% random coils, although some prediction methods indicate a greater helical content with implication in protein folding (Sawyer, 2003, Sawyer and Holt, 1993, Sakurai *et al.*, 2009). In addition, each β-LG monomer contains two disulphide bonds (Cys^{66}-Cys^{160} and Cys^{106}-Cys^{119}) highly responsible for protein secondary and tertiary structure stabilization, and one free thiol (Cys^{121}) (O'Mahony and Fox, 2014, Creamer *et al.*, 2011).

The β-LG monomer structure is made of nine antiparallel β-sheets strands (A – I), out of which eight (A – H) are wrapped around to form a conical barrel, called the central calyx (Kontopidis *et al.*, 2004). The β-strands A – D along with strands E – H are the building sheets of this calyx. The strand A is present in both sheets of the conical barrel because of a 90° midpoint bending at Ser^{21} (Li *et al.*, 2013). The bonding pattern specific to the antiparallel interaction between strands A and H that leads to a complete calyx shape, is favored by this bending. The calyx has a cylindrical shape. The cylinder has hydrophobic walls, a length of approximately 15Å and a volume of 315 Å (Sawyer, 2013). The dimer interface in the ovine and bovine β-LG is formed by the antiparallel interactions between 146-150 residues of the ninth strand (I) with the equivalent motif from the other subunit, along with three amino acids from the A-B loop (Asp_{33}, Ala_{34} and Arg_{40}) (Sakurai and Goto, 2002). Even though it is available in the porcine β-LG, strand I is not involved in the dimer formation at low pH values (Kontopidis *et al.*, 2004). A 3-10 helix is located on the outer surface of the calyx, between strands G and H. The BC, DE

and FG loops that connect the β-strands at the closed end of the β-barrel are shorter compared to those at the open end which are more flexible and longer (Kontopidis *et al.*, 2004). The EF loop acts as a gate to the calyx. At pH values lower than 7, the loop is closed, making binding impossible. Higher pH values lead to an opening of the loop, further allowing the ligands to bind into the hydrophobic core of the calyx. The Glu^{89} residue acts as a trigger for the opened / closed EF loop based on the fact that this residue is also implicated in the Tanford transition and has an extremely high pK_a (Qin *et al.*, 1998a, Li *et al.*, 2013). The free thiol group of Cys^{121} has a pH-dependent reactivity, and is involved in the denaturation and aggregation behaviour (Havea *et al.*, 2001). Cys^{121} is located on strand H on the outside of the β-barrel and under the α-helix. It has a low reactivity because of the limited exposure (Burova *et al.*, 1998). Increasing the pH values from 4 to 8.5, a raise in the reactivity of the thiol group is observed (Kehoe *et al.*, 2007). The reactivity and accessibility of the buried thiol group increases at pH values above 7.4 due to the Tanford transition (Qin *et al.*, 1998a).

Figure 1. Details on the bovine β-lactoglobulin structure. The protein model was taken from RCSB protein Data Bank (PDB entry 3NPO; Loch *et al.*, 2011) and is represented in magenta in New Cartoon style using VMD software (Humphrey *et al.*, 1996).

Conformational transitions of β-lactoglobulin

Circular dichroism and the related technique of optical rotatory dispersion, along with ultracentrifugation were initially used to show the conformational transition of β-LG between pH 2 and 10 (Sawyer, 2003). Reversible structural changes occur between pH 2.5 and 8 where the protein has an extremely well-conserved conformation. The structural changes that lead to protein unfolding take place at pH values higher than 9 (Uhrinova *et al.*, 2000, Sakurai and Goto, 2007). Taulier and Chalikian (2001) performed an extended analysis of pH-dependent conformational variations using ultrasonic, densimetric, and spectroscopic studies. Five structural changes were suggested, the two extra transitions being present at pH values lower than 2 and higher than 10 (Taulier and Chalikian, 2001). The structural transition at pH between pH 2 and 10 can be schematically represented as Q↔N↔R→S.

Acidic state (Q) ↔ Native form (N)

Between pH 4.5 and 6.0, variants A and B undergo a reversible Q↔N transition leading to very small changes in the protein conformation. The most important is the "closed" position of the EF loop which prevents the access of other molecules in the internal core of the calyx. The Glu^{89} residue is buried and any slight modification of the free thiol group of Cys^{121} leads to a disturbance in the monomer-dimer equilibrium (Kontopidis *et al.*, 2004).

Native form (N) ↔ Reversible denatured form (R)

A second reversible structural change that occurs between pH 6.5 – 7.8 (N↔R) is the so called Tanford transition (Tanford *et al.*, 1959). This change is observed either by the modification of optical rotation ($[\alpha]_D = -25°$ at pH 6 and $-48°$ at pH 8) or by the thermal denaturation peak (Qi *et al.*, 1997, Qi *et al.*, 1995). Upon increasing pH, the carboxyl of Glu^{89} becomes exposed and ionized due to the hydrogen bonding to the carbonyl of Ser^{116} (Brownlow *et al.*, 1997). A detailed explanation of the Tanford transition was provided by Sakurai *et al.*, 2009. Initially, Glu^{89} is deprotonated, causing an oscillation of the hydrogen bonding in Ile^{84}, Asn^{90}, and Glu^{108} residues. This fluctuation allows for the rearrangement of the EF loop, strand D, and GH loop, leading to accessibility to the internal binding site of the calyx (Sakurai *et al.*, 2009, Sakurai and Goto, 2007). In 2008, Vijayalakshmi *et al.*, produced a β-LG where one subunit had an opened EF loop and the other one had a closed loop, leading to the conclusion that the Tanford transition does not involve a co-operation between these subunits.

Reversible denatured form (R) → Base-induced denatured state (S)

The third structural change leads to an irreversible alkali denaturation of the protein conformation (Mercadé-Prieto *et al.*, 2008).

β-Lactoglobulin binding properties to polyphenols

The structure of the β-LG justifies its affiliation to the lipocalin family and calycin subclass, naturally involved in binding and transporting small hydrophobic bioactive compounds (Sakurai *et al.*, 2009). Nowadays, the lipocalin family consists of more than 40 constituents (Åkerström *et al.*, 2006). The majority of the

members have a molecular weight of 18 – 20 kDa, except for insecticyanin and crustacyanin, two larger proteins that adopt the lipocalin folding, and bind chromophores. The tertiary structure of the family is very well-conserved and is comprised of an antiparallel β-barrel or calyx (Sawyer, 2013). The biological functionality of these lipocalins relies on binding and transporting small hydrophobic molecules. *Bos* d 2 was identified as an allergen, even though it is a bovine lipocalin (Rouvinen *et al.*, 1999). It was suggested that the biological property of β-LG is the binding in the central calyx of retinol and fatty acids (Sawyer and Kontopidis, 2000). In the last decade, other different potential ligands were studied for the interaction with β-LG (Sawyer, 2013). Oleic acid was the first ligand reported for binding to β-LG (Davis and Dubos, 1947). X-ray crystallography of β-LG – ligand complexes revealed that the main binding site is the internal core of the calyx. Also, other external binding sites were suggested (Qin *et al.*, 1998b, Wang *et al.*, 1999).

Polyphenols are intensively studied due to their health-promoting properties. The most important physiological property of polyphenols is the antioxidant activity against reactive oxygen species associated with oxidative stress, cancer and other serious diseases (Shirai *et al.*, 2015). Molecular docking (MD) studies were used to evaluate the interaction of different flavonoids, such as quercetin, quercitrin, and rutin, with β-LG. The MD studies revealed that quercetin and quercitrin bind to the internal core of the β-barrel. Because of its high volume, rutin cannot enter the calyx, instead it binds at the entrance of the cavity with four hydrogen bonds interactions. Greater binding constants values were reported for quercetin and quercitrin (1.2×10^6 and 1.9×10^6 M^{-1} respectively) due to the binding of these molecules in the internal cavity of β-LG which is more hydrophobic than its entrance, where rutin binds (Sahihi *et al.*, 2012). Li *et al.* (2013) used Fourier transform infrared and fluorescence spectroscopy to investigate the binding of curcumin to bovine β-LG. At pH 6.0, curcumin binds on the external surface of the calyx, while at pH 7.0, due to a massive hydrophobicity, it binds into the central cavity. An improvement in the antioxidant activity was observed when curcumin had bound to β-LG (Li *et al.*, 2013). Riihimaki *et al*. (2008) studied the binding of different classes of polyphenols' aglycones to bovine and reindeer β-LG, using fluorescence quenching. Among flavonoids, the amount of bonded quercetin was higher compared to that of rutin (Riihimaki *et al.*, 2008). Rawel *et al.* (2003) reported that both quercetin and rutin bind to β-LG. Moreover, the rather good binding affinities of β-LG for caffeic, ferulic, sinapic, and rosmarinic acids were also found. Other phenolic acids, such as gallic, protocatechuic, and syringic acids and octyl and propyl gallate, had either a very small or nonexistent binding affinity for β-LG.

The effect of pH on the binding of polyphenols to β-LG was studied, revealing that the phenolic compounds were still attached to the protein at pH 2. Piperine, myricetin, and daidzein showed an affinity for binding to β-LG in alkaline conditions, when the protein exhibits a protective role toward polyphenols during non-heating food processing. The authors also established that during the thermal

treatment the complex between β-LG and polyphenol was greatly affected (Riihimaki *et al.*, 2008).

Table 1. Binding and apparent dissociation constants of polyphenolic ligands and β – lactoglobulin complexes

Ligand	Apparent dissociation constant, K_d, M ($\cdot 10^{-6}$)	Binding constant, k_a, M^{-1}	References
Quercetin	-	$1.2 \cdot 10^6$	Sahihi *et al.*, 2012
Quercitrin	-	$1.9 \cdot 10^6$	
Rutin	-	$7.4 \cdot 10^4$	
Curcumin	-	$(5.23 \div 8.9) \cdot 10^4$	Li *et al.*, 2013
(-)-Epigallocatechin gallate	0.86	-	Riihimaki *et al.*, 2008
Daphnetin	0.58	-	
Hesperidin	0.30	-	
Naringenin	0.24	-	
Luteolin	0.65	-	
Vitexin	0.39	-	
Morin	0.34	-	
Myricetin	0.23	-	
Myricitrin	0.33	-	
Daidzein	0.44	-	
Genistein	0.72	-	
Ferulic acid	0.60	-	
Sinapic acid	0.60	-	
Resveratrol	-	$10^4 \div 10^6$	Liang *et al.*, 2008
(-)-Epigallocatechin	-	$1.3 \cdot 10^3$	Wu *et al.*, 2011
Green tea catechins	-	$(3.82 \div 6.54) \cdot 10^3$	Stojadinovic *et al*., 2013
Coffee chlorogenic acid	-	$(3.11 \div 14.4) \cdot 10^4$	
Cocoa flavonols	-	$(8.15 \div 14.6) \cdot 10^4$	
Naringenin	-	$5.4 \cdot 10^4$	Shpigelman *et al.*, 2014
Malvidin-3-*O*-glucoside	-	$0.51 \cdot 10^3$	He *et al.*, 2016

Liang *et al.* (2008) studied the binding of resveratrol to bovine β-LG using circular dichroism and fluorescence spectroscopy. A blue shift of the fluorescence emission maxima was observed along with an increase of the emission intensity, suggesting that the environment surrounding the interaction surface is not as hydrophobic as the calyx internal cavity. Authors concluded that the binding site for resveratrol is on the surface of the protein (Liang *et al.*, 2008). Wu *et al.* (2011) studied the binding interaction between (–)-epigallocatechin (EGC) from green tea and β-LG using spectroscopic methods. Catechins are used in the food industry as additives

and have antioxidant and anticarcinogenic properties. A series of changes in the β-LG conformational structure suggested the binding between EGC and the protein (Wu *et al.*, 2011). Polyphenol – β-LG complexes were studied for their antioxidant effect and resistance to extremely acid pH. Polyphenol extracts from tea, cocoa, and coffee acted as a protective shield for the secondary structure of β-LG when exposed to pH values of the gastrointestinal tract. Polyphenols display a strong antioxidant activity but, when in complex with β-LG, a reduced radical activity was observed (Stojadinovic *et al.*, 2013).

Allicin is a labile, bioactive compound of garlic with outstanding health-properties. In order to be suitable to enriching the functional foods, a delivery system for this polyphenol that masks its flavour was proposed (Wilde *et al.*, 2016). Wilde *et al.* (2016) reported that allicin bound covalently to β-LG and the established complex modified the thiol group of β-LG that leads to a greater thermal stability compared to the native protein. The beverage enriched with allicin – β-LG complex was garlic odourless and tasteless (Wilde *et al.*, 2016). Shpigelman *et al.* (2014) studied the binding properties of β-LG with two citrus flavonoids. Naringin and its aglycone naringenin possess many health properties, both presenting low water-solubility. Additionally, naringin is very bitter. Naringenin bounded to both native and preheated β-LG, compared to naringin where no binding was detected. It was suggested that this lack of binding of naringin to β-LG might be due to the lower hydrophobicity and the steric obstruction of the sugar. The formation of β-LG–naringenin complex enabled solubilisation and prevented crystallization of the flavonoid. The nanocomplexes obtained after freeze-drying may be used to fortify different beverages and enrich the functional foods area (Shpigelman *et al.*, 2014). Anthocyanins were also reported to bind to β-LG (Oliveira *et al.*, 2015, He *et al.*, 2016). The binding of malvidin-3-O-glucoside to β-LG was studied using fluorescence, Fourier transform infrared and circular dichroism spectroscopy and the complex obtained had a positive effect on the thermal stability of anthocyanin, preventing the degradation of colour during heat treatments (He *et al.*, 2016). Usually, polyphenols interact with matrix components such as protein and polysaccharides from food systems (Oliveira *et al.*, 2015). Oliveira *et al.* (2015) studied the behaviour of nanoparticles consisting of cyanidin-3-glucoside, β-LG, and polysaccharides. The nanoparticles affected the antioxidant activity of the anthocyanin due to the interaction between polymers and cyanidin-3-glucoside. (Oliveira *et al.*, 2015). Oliveira *et al.* (2016) demonstrated that (+)-catechin interacts with β-LG and pectin/chitosan to form complexes. The mixture of each (+)-catechin with each polymer and β-LG had a stabilizing effect on the antioxidant activity (Oliveira *et al.*, 2016).

Conclusions

The data presented in this review focused on the structural and conformational particularities of β-LG as a functional ingredient, since it has a high nutritional value and GRAS (generally recognized as safe) status. It also has a remarkable stability against gastric condition and can protect the ligand from the stomach harsh

environment or from another compound present in the food matrix. These properties qualify the β-LG as a suitable transporter of bioactive compounds for the enrichment of different food matrixes.

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