

# Whey Proteins

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Whey proteins have implications in different fields related to human life quality. The aim of this overview was to present the basic chemistry of  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, and lactotransferrin - main proteins of bovine whey.

whey

lactoglobulin

lactoferrin

lactoalbumin

## 1. $\beta$ -Lactoglobulin

$\beta$ -Lactoglobulin is the major bovine whey protein, accounting for approximately 10% of the total protein in bovine milk and approximately 50% in ruminants<sup>[1]</sup>, but it is not present in human milk.  $\beta$ -LG contains 162 amino acid residues, which form nine antiparallel  $\beta$ -sheets<sup>[2]</sup>. It belongs to the lipocalin family and has the ability to bind different hydrophobic molecules<sup>[3]</sup>, which can be useful for reducing allergenicity owing to its covalent conjugation to flavonoids because  $\beta$ -LG is one of the major milk allergens responsible for cow milk allergy<sup>[4]</sup>.

$\beta$ -LG has eleven genetic variants (A, B, C, D, E, F, G, H, W, I, and J). Genetic variants A and B are most common in bovine milk and differ in positions 64 and 118. Two bovine  $\beta$ -lactoglobulins I and J were isolated from bovine milk by isoelectric focusing by Godovac-Zimmermann et al.<sup>[3]</sup>. Moreover, various variants of  $\beta$ -LG translate into different metal affinities, e.g., to nickel or cobalt complexes<sup>[5]</sup>.

Additionally,  $\beta$ -LG has two disulphide bonds (Cys-106 to Cys-119; Cys-66 to Cys-160)<sup>[6]</sup> that maintain the structural integrity during hydrolysis and heat treatment and one free cysteine group (Cys-121) as the binding site for d-block metal ions, such as iron (II/III), copper (II), and silver (I)<sup>[7]</sup>. Due to the disulphide bonds and free sulphydryl group in its hydrophobic core,  $\beta$ -LG prevents oxidation by capturing reactive oxygen species (ROS)<sup>[8]</sup>.  $\beta$ -LG may be modified by phosphorylation<sup>[9]</sup> or glycation<sup>[10]</sup>, which are examples of post-translational modifications (PTMs) of the protein after its translation by proteolytic cuts or by adding a modifying group to one or more amino acids<sup>[11]</sup>. The molecular weight of  $\beta$ -LG and of other whey proteins is dependent on post-translational modifications (Table 1). It also can be observed that the number of significant figures in the value of molecular weight can be determined by the precision of the analytical method applied for its analysis.

Heating causes changes in a protein's structure, and subsequently, its properties, thus affecting the quality of food products. De Wit summarized the thermal behavior of  $\beta$ -LG up to 150 °C and concluded that thermal behavior of  $\beta$ -LG is dependent on pH, temperature, time of heating, and concentration<sup>[12]</sup>. Reversible conformational changes up to 60 °C, which are known as the Tanford transition (negligible between pH 6.5 and 7.8, accounts for 18% at pH 7.0), irreversible denaturation by unfolding and aggregation of monomers between 60 and 70 °C at pH  $\geq$  7.0 in the

presence of OH<sup>-</sup> ions) have been reported by several authors<sup>[12]</sup>. In addition, thiols oxidation between 65 and 75 °C, disulphide/thiol exchange reactions prevailing between 75 and 85 °C and induction of larger aggregates by specific non-covalent aggregation, and unfolding of the residual protein structures above 125 °C have been indicated in a number of publications<sup>[12]</sup>. Liu et al. reported a loss of antioxidant activity of β-lactoglobulin as a result of cross-linking free thiol groups upon heating (100 °C for 2 min)<sup>[13]</sup>. Wijayanti and co-authors evaluated the effect of lipoic acid in its acidic and reduced forms on heat-induced unfolding of β-LG and obtained results showed that the reduced form was more effective and its effects were similar to N-ethylmaleimide (NEM) and dithio(bis)-p-nitrobenzoate (DTNB)<sup>[14]</sup>. In contrast to heating, the antioxidant activity of β-lactoglobulin can be enhanced by ultrasound and enzymatic treatment, which modify its secondary structure and strengthen proteolysis<sup>[7]</sup>.

**Table 1.** Different whey protein molecular weights associated with post-translational modifications (PTMs).

Protein	Mol. Weight (kDa)	Theoretical mol. Weight (kDa)*	PTM	Method of Isolation/Purification	Identification	Ref.
β-LG	18			standard of β-LG (protein content > 90%)	SDS-PAGE	
	18.5	18.277	-		MALDI-TOF-MS	[4]
β-LG	18.3		monomeric and the dimeric forms at pH 7.4 glycated β-lactoglobulin	β-LG was dissolved in 9.1 mM glucose in water, and the pH was adjusted to 7 with 50 mM phosphate buffer	MALDI-TOF-MS	[15]
	36.6	18.277				
β-LG	17.4	18.277	-	anion-exchange chromatography (DEAE-Sepharose)	SDS-PAGE	[16]
β-LG	19.9	18.277	proteins appeared as strings of spots, indicating their different isoforms with different charges	precipitation via ammonium sulphate fractionation	2-DE	[17]
α-LA	16.2	16.247			MALDI-MS	

			as a result of PTMs occurring prior to secretion		
α-LA	14.1	16.247	small mass differences ruled out PTMs, such as phosphorylation and glycosylation	precipitation by ammonium sulphate	MALDI-TOF-MS <a href="#">[18]</a>
SA	67.7 (SA)	69.367	0.5 mL of raw milk was centrifuged at 4 °C for 30 min, fat and cellular layers were removed; residual lipids were removed by addition of three volumes (1.5 mL) of 2:1 chloroform/methanol, agitation, retaining of supernatant; protein was precipitated from supernatant with ethanol overnight at 4 °C, followed by centrifugation; precipitate was resuspended in 50 mM ammonium bicarbonate buffer (pH 7.5); glycans were separated by SDS-PAGE and extracted for MALDI-MS analysis		MALDI-MS <a href="#">[19]</a>
LTF	79.8 (LTF)				LC-MS/MS
LTF	69.0 (SA)	78.056			
LTF	80.002	78.056	-	milk was defatted by centrifugation, and the	RP-LC-MS/MS <a href="#">[20]</a>

pH was then adjusted to 4.6 using hydrochloric acid; precipitated casein was removed by centrifugation

\*values of theoretical molecular weight of the proteins from the Uniprot database (bovine, and for SA-human); Uniprot KB:  $\alpha$ -LA—P00711; LTF—P24627; SA—P02768, the value for  $\beta$ -LG accounts for form B from publication of Eigel et al.<sup>[21]</sup>.

Interestingly, Mercadante et al.<sup>[22]</sup> reported the ability of bovine  $\beta$ -LG to form dimers and studied the dissociation equilibrium and rate constant over the pH range of 2.5–7.5. The equilibrium constant increased with an increase in  $|\text{pH}-\text{pI}|$ , thus indicating the major role of the hydrophobic effect in the stabilization of the dimer and suggesting that electrostatic repulsion destabilizes the dimer, especially at low pH.

## 2. $\alpha$ -Lactalbumin

$\alpha$ -Lactalbumin consists of 123 amino acids, except for rat  $\alpha$ -lactalbumin, which contains 17 more amino acids and is an extension of the carboxyl end enriched with proline<sup>[23]</sup>.  $\alpha$ -LA constitutes approximately 22% of the total protein of human milk and approximately 36% of whey protein in human milk, and it constitutes approximately 3.5% of the total protein and approximately 17% of the whey protein in bovine milk<sup>[24]</sup>.

The native  $\alpha$ -LA consists of two domains: a large  $\alpha$ -helical domain and a small  $\beta$ -sheet domain connected by a calcium-binding loop.  $\alpha$ -LA possesses a strong calcium-binding site with residues of Lys79, Asp82, Asp84, Asp87, and Asp88<sup>[25]</sup>. Calcium-binding has a significant influence on the molecular stability of LA. Moreover, it is required for the refolding and formation of a native disulphide bond in the reduced, denatured protein<sup>[26]</sup>. The nuclear magnetic resonance (NMR) and circular dichroism (CD) pH titration studies reported by Kim et al. suggested that critical electrostatic interactions concentrated in the calcium-binding region contribute to the denaturation of the protein by determination of the  $\text{pK}_a$  values of individual functional ionizable groups<sup>[27]</sup>. When calcium ions dissociate from  $\alpha$ -LA at an acidic pH, the protein adopts the molten globule conformation, which has been described as a compact state with a significant degree of secondary structure in the native protein but with a fluctuating tertiary structure<sup>[28]</sup>. The molten globule has a weakly folded  $\alpha$ -helix domain and a domain with a disordered  $\beta$ -sheet domain<sup>[29]</sup>. The removal of calcium (II) ions resulted in conformational changes, as indicated by spectral (fluorescence and absorbance) changes<sup>[30]</sup>. Interestingly, the work of Noyelle and co-authors showed that magnesium (II) binding occurred more likely via interactions with the residues belonging to the zinc (II)-binding site in contrast to its expected binding to the calcium (II)-binding site<sup>[25]</sup>. A study by Wehbi et al. demonstrated that binding of calcium to bovine  $\alpha$ -LA increases the resistance of the protein structure to thermal treatment<sup>[31]</sup>.

$\alpha$ -LA is stabilized by four disulphide bonds between the cysteine residues (Cys-6 to Cys-120, Cys-61 to Cys-77, Cys-73 to Cys-91, and Cys-28 to Cys-111)<sup>[32]</sup>. The active molecular form of  $\alpha$ -LA may have various post-translational modifications in contrast to the native form<sup>[18]</sup>. Moreover, for structural reasons, the  $\alpha$ -LA has a metal affinity to ions of s-block elements, such as magnesium (II) and transition metal ions, e.g., zinc (II), which is especially promoted in the reaction with  $\beta$ 4-galactosyltransferase, according to immobilized metal-affinity chromatography (IMAC)<sup>[33]</sup>. In addition, calcium ions increase the stability of  $\alpha$ -LA in its native state<sup>[34]</sup>. Zinc ions may also bind to the calcium-binding site, thus increasing its absorption and bioavailability. In this way, the  $\alpha$ -LA complex of zinc can be used as a natural carrier for the supply of zinc in food systems<sup>[35]</sup>.

### 3. Lactoferrin

Lactoferrin (LTF) is a highly glycosylated protein of the transferrin family<sup>[36]</sup> that has a molecular weight of approximately 80 kDa, depending on its post-translational modifications<sup>[19][20]</sup>. Wei et al. suggested the presence of five N-glycosylated sites of bovine LTF-a (bLTF-a): -Asn-233, -281, -368, -476, and -545<sup>[37]</sup>. The degree of glycosylation may vary and thus determines the rate of resistance to proteases or to very low pH<sup>[38]</sup>.

Lactoferrin consists of a single polypeptide chain with approximately 700 amino acids folded into two symmetrical lobes: a N-lobe and C-lobe. These are homologues with respect to each other (33%–41% homology). Each lobe consists of two domains, such as C<sub>1</sub>, C<sub>2</sub>, N<sub>1</sub>, and N<sub>2</sub><sup>[36]</sup>. Both lobes contain approximately 345 residues, and their disposition in each lobe creates an interdomain pocket with a high affinity to iron; the binding is accompanied by synergistic binding of carbonate ions<sup>[38]</sup>. In more details, in each lobe, a single Fe atom is coordinated by amino acid side chains that are dispersed in each domain and connecting region because of the changes in conformations occur, causing domains to come together. A distorted octahedral coordination sphere is formed by coordinating ligands as carboxylate-O (Asp), two phenolate-O (Tyr), and imidazole-N (His), which is completed by bidentate binding of carbonate or bicarbonate ion. Carbonate is considered as synergistic since its presence is essential for iron binding. The stability constant for iron (III) complex is high ( $\log \beta$  28 at pH 7.4)<sup>[39][40]</sup>.

LTF can exist in two forms, apo-Lf and holo-Lf, depending on whether it binds iron (III) or not<sup>[36]</sup>. In addition to iron, LTF is capable of binding other ions, such as aluminum (III), gallium (III), manganese (III), cobalt (III), copper (II), and zinc (II), but with lower affinity<sup>[41]</sup>. It was reported that LTF releases iron in acidic conditions (pH below 4)<sup>[42]</sup>, and diferric transferrin readily loses iron at pH < 6.7<sup>[43]</sup>. It is also very important to consider the iron saturation and concentration at low pH, especially in places of infection and inflammation, where, as a result of metabolic activity of bacteria or stimulated leucocytes, the pH may be lower than 4.5<sup>[44]</sup>.

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