

AN OVERVIEW OF BOVINE α -LACTALBUMIN STRUCTURE AND FUNCTIONALITY

NICOLETA STĂNCIUC, GABRIELA RÂPEANU

“Dunărea de Jos” University of Galați, Faculty of Food Science and Engineering, 111 Domneasca Street, 800201, Galati, Romania,
nicoleta.sava@ugal.ro

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α -Lactalbumin is the second major protein in bovine milk (2-5% of the total protein in bovine milk). The human variant has several physiologic functions in the neonatal period. In the mammary gland, it participates in lactose synthesis and facilitates milk production and secretion. α -Lactalbumin binds divalent cations (Ca^{2+} , Zn^{2+}) and may facilitate the absorption of essential minerals. Also, it provides a well-balanced supply of essential amino acids for the growing infant. During its digestion, peptides with antibacterial and immunostimulatory properties are formed, thereby possibly helping in the protection against infection. The protein fractions enriched with α -lactalbumin may be added to infant formula to provide some of the benefits of this protein.

This article reviews a number of studies which may contribute to a more coherent understanding of the structure and some functional properties of α -lactalbumin.

Keywords: α -lactalbumin, structure, function

1. Introduction

Whey proteins (WP) have an exceptional biological value that exceeds that of egg protein by about 15% and a range of other common edible proteins. WP is a rich source of essential amino acids when compared with other typical food proteins, and it is also rich in branched chain amino acids (leucine, isoleucine, and valine) (>20%, w/w) (Smithers, 2008). These latter amino acids are thought to play a role as metabolic regulators in protein and glucose homeostasis, and in lipid metabolism, and as such, may play a role in weight control (Smilowitz *et al.*, 2005). Also, WP is a rich and balanced source of sulphur amino acids (methionine, cysteine). These amino acids serve a critical anti-oxidants role, as precursors to the potent intracellular anti-oxidant glutathione, and in one-carbon metabolism (Shoveller *et al.*, 2005).

WP and peptides derived from the enzymatic proteolysis modulate a variety of immune functions, including lymphocyte activation and proliferation, cytokine secretion, antibody production, phagocytic activity, and granulocyte and natural killer cell activity (Saint-Sauveur *et al.*, 2007).

WP are widely used as food ingredients on account of their good functional and nutritional properties (Ye and Taylor, 2009) and consist principally of β -lactoglobulin, α -lactalbumin (LA) and bovine serum albumin which have globular structures.

LA is a small, acidic, cations binding milk protein, which is very important from several points of view (lactose synthesis, model Ca^{2+} binding protein, classic *molten globule*, important biological and functional properties).

It has been reported that LA and its hydrolysate have many physiological functions, such as reduction of stress (Markus *et al.*, 2000), antimicrobial activity (Pellegrini *et al.*, 1999), opioid activity (Teschemacher *et al.*, 1998), antihypertensive action (FitzGerald *et al.*, 2004), regulation of cells growth (Sternhagen and Allen, 2001), antiulcer activity (Matsumoto *et al.*, 2001), and immunomodulation (Cross and Gill, 2000).

The objective of this paper is to review a number of studies which may contribute to a more coherent understanding of the structure and some functional properties of LA.

Structural and Physical properties

LA consists of 123 amino acid residues forming a compact globular structure stabilized by four disulfide bonds (Cys⁶–Cys¹²⁰, Cys⁶¹–Cys⁷⁷, Cys⁷³–Cys⁹¹, and Cys²⁸–Cys¹¹¹). LA is a globular, calcium metalloprotein with an isoelectric point of 4.6, a molecular mass of 14 200 Da and has no free thiol groups (Brew and Grobler, 1992). It is genetically and structurally homologous to c-type lysozyme (McKenzie and White, 1991).

The LA protein has two predominant genetic variants (A and B) (Farrell *et al.*, 2004). The B variant is present in the milk of most *Bos taurus* cattle, and both the A and B variants are found in the milk of *Bos indicus* cattle (Jenness, 1974). LA a variant is present at a low frequency in the milk of some Italian and Eastern European *Bos taurus* breeds (Mariani and Russo, 1977). The A variant contains a Glu at position 10 of the mature protein, and the B variant has an Arg substitution at that position (Gordon, 1971) (Figure 1). A third genetic variant, C, has also been reported but not confirmed yet.

The B variant is the reference protein for the family and is composed of the following amino acid residues: Ala₃, Arg₁, Asn₈, Asp₁₃, Cys₈, Gln₆, Glu₇, Gly₆, His₃, Ile₈, Leu₁₃, Lys₁₂, Met₁, Phe₄, Pro₂, Ser₇, Thr₇, Trp₄, Tyr₄, Val₁₆ (Farrell *et al.*, 2004). Both A and B variant contain four disulfide bonds and no phosphate groups.

1																				
	Gln	Leu	Thr	Lys	Cys	Glu	Val	Phe	Arg in Variant B	Glu	Leu	Lys	Asp	Leu	Lys	Gly	Tyr	Gly	Gly	
21									Gln	31										
	Val	Ser	Leu	Pro	Glu	Trp	Val	Cys	Thr	Thr	Phe	His	Thr	Ser	Gly	Tyr	Asp	Thr	Glu	Ala
41										51										
	Ile	Val	Glu	Asn	Asn	Gln	Ser	Thr	Asp	Tyr	Gly	Leu	Phe	Gln	Ile	Asn	Asn	Lys	Ile	Trp
61										71										
	Cys	Lys	Asn	Asp	Gln	Asp	Pro	His	Ser	Ser	Asn	Ile	Cys	Asn	Ile	Ser	Cys	Asp	Lys	Thr
81										91										
	Leu	Asn	Asn	Asp	Leu	Thr	Asn	Asn	Ile	Met	Cys	Val	Lys	Lys	Ile	Leu	Asp	Lys	Val	Gly
101										111										
	Ile	Asn	Tyr	Trp	Leu	Ala	His	Lys	Ala	Leu	Cys	Ser	Glu	Lys	Leu	Asp	Gln	Trp	Leu	Cys
121			123																	
	Glu	Lys	Leu	OH																

Figure 1. Primary structure of bovine LA (Mangino, 2007)

The position of the amino acid substitution that occurs in genetic variant B is indicated.

A small percentage of the LA found in the milk of cattle is glycosylated on an Asn residue (Barman, 1970). Under native conditions, the tertiary structure of LA is composed of a large domain (α) and a small domain (β) divided by a cleft. The α -domain (1–34 and 86–123) contains three *pH*-stable α -helices: H1 (5–11), H2 (23–34), H3 (86–98), a *pH* dependent α -helix (H4 - 105–110), and two short 3_{10} helices: h1 (18–20), h3 (115–118). The flexible 105–110 loop region adopts a helical conformation (H4) for *pH* values ranging between 6.5 and 8.0 (Pike *et al.*, 1996). The 35–85 β -domain is composed of a small three-stranded antiparallel β -pleated sheet (strand S1, 41–44; S2, 47–50; S3, 55–56) and of a short 3_{10} helix (h1b, 18-20, h2, 77–80 and h3c, 115-118) (Chrysina *et al.*, 2000).

LA possesses a single strong Ca²⁺ binding site (Permyakov and Berliner, 2000) and for this reason it is frequently used as a simple, model Ca²⁺ binding protein. It is very convenient for studies of calcium binding effects on interactions of the protein with proteins, peptides, membranes and low molecular weight organic compounds, which frequently have physiological significance.

LA has several partially folded intermediate states, which are being studied by many researchers interested in protein folding problems. It is very attractive for studies of the properties and structure of intermediate molten globule like states since at acidic *pH* and in the apo-state at elevated temperatures LA is the classic *molten globule*.

A remarkable property of LA as a model of protein folding studies is the high stability of its molten globule state, which is observed in the following conditions (Kuwajima, 1996):

- an equilibrium unfolding intermediate at a moderate concentration of a strong denaturant (guanidine hydrochloride or urea);

- the acid-denatured state, and
- a partially unfolded state produced by removal of the bound Ca^{2+} at neutral pH and low salt concentration.

The known structural characteristics of the molten globule state are (Kuwajima, 1989):

- native-like secondary structure;
- compact structure with a radius only 10-20% larger than that of the native molecule, and,
- the absence of the specific tertiary packing interactions of amino acid side chains.

Calcium binding strongly influences the molecular stability of LA and is required for refolding and native disulfide bond formation in the reduced, denatured protein (Permyakov and Berliner, 2000). The calcium-binding loop is located at the junction of the α - and β -domains and partly contains the α -helix H3 and 3_{10} helix h2. The calcium ion is coordinated to β -carboxyl groups of three aspartic acid residues (Asp⁸², Asp⁸⁷ and Asp⁸⁸), two backbone carbonyl oxygens (Lys⁷⁹ and Asp⁸⁴), and two water molecules (Acharya *et al.*, 1991).

The calcium binding site is presented in Figure 2.

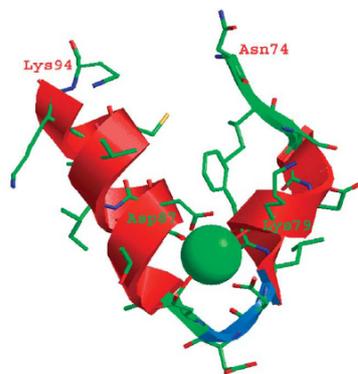


Figure 2. Calcium-binding site in LA from Asn⁷⁴ to Lys⁹⁴ (Farkas *et al.*, 2005)

In Figure 3 a ribbon model is given for Ca^{2+} -bound and Ca^{2+} -free protein. Calcium binding stabilizes the native state of LA in such a way that, for instance, the temperature induced unfolding of an LA shifts from near 20^o C for the apo-protein to nearly 70^o C for the Ca^{2+} -bound protein. Both Ca^{2+} -bound and free forms of LA can adopt practically the same folded conformation at low temperature (Permyakov, 2005). Moreover, correct refolding and correct disulfide repairing of the reduced LA requires the presence of Ca^{2+} (Farkas *et al.*, 2005).

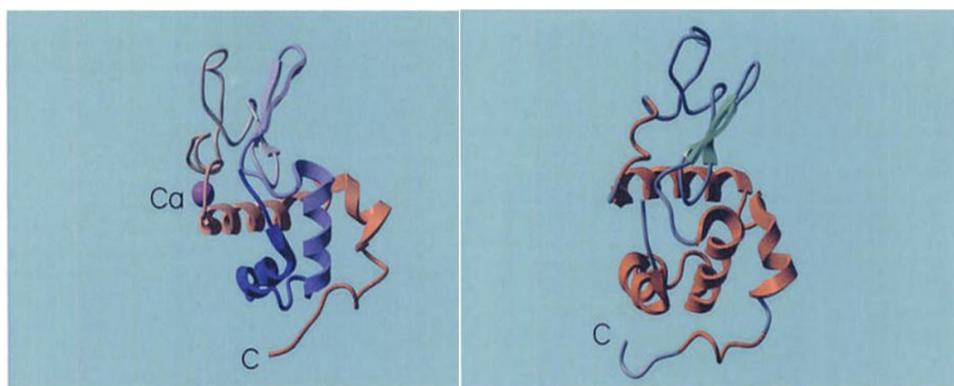


Figure 3. Ribbon model of Ca^{2+} -loaded and Ca^{2+} -free bovine LA (Permyakov, 2005)

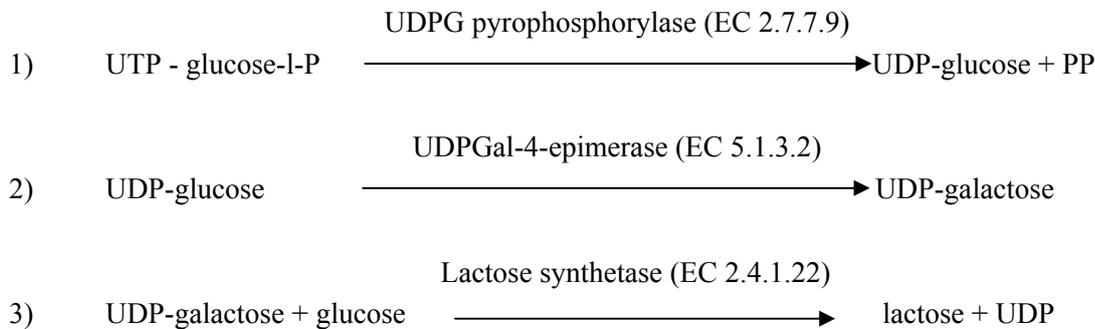
Under a variety of conditions (calcium removal, high temperature, strong acid conditions, or presence of denaturing agents), LA can adopt the *molten globule* conformation, which is described as a compact state keeping the secondary structures, but having a poorly defined tertiary structure (Ptitsyn, 1995).

Removal of Ca^{2+} from the protein enhances its sensitivity to pH and ionic conditions due to noncompensated negative charge-charge interactions at the cation binding site, which significantly reduces its overall stability (Griko and Remeta, 1999).

At neutral pH and low ionic strength, the native structure of apo-LA is stable below 14°C and undergoes a conformational change to a native-like molten globule intermediate at temperatures above 25°C (Permyakov, 2005).

Physiological properties

Its content LA in bovine whey is about 1.2-1.5 g/L, and is also present in the milk of many other mammalian species. In primates, LA expression is up-regulated in response to the hormone prolactin and increases the production of lactose (Kleinberg *et al.*, 1983). Three enzymes are involved in the biosynthesis of lactose and they catalyze the following reactions:



The reaction takes place in the Golgi lumen and requires Mn^{2+} ions.

Lactose synthetase is considered to be an enzyme that has naturally occurring subunits (A and B proteins), and enzymatic activity is presumably dependent upon the association of subunits to form a complex which is enzymatically active. Kinetic evidence is available to support complex formation between the A and B proteins in the presence of substrates and metal. LA forms the regulatory subunit of the lactose synthase heterodimer and β -1,4-galactosyltransferase forms the catalytic component. Together, these proteins enable lactogenesis to produce lactose by transferring galactose moieties to glucose.

When formed into a complex with β 1,4-galactosyltransferase, LA enhances the enzyme's affinity for glucose by about 1000 times, and inhibits the ability to polymerize multiple galactose units. This gives rise to a pathway for forming lactose by converting galactosyltransferase to lactose synthase.

Although calcium is not a requisite for the formation and enzyme activity of the LA-galactosyltransferase complex (Heine *et al.*, 1991), the calcium is complexed with aspartyl residues in a highly conserved region of the protein molecule and has significant effects on the tertiary structure of the LA.

LA from human and bovine milk binds calcium, zinc, manganese and cobalt, and the native protein in milk contains tightly bound calcium in a 1:1 molar ratio. FitzGerald and Swaisgood (1989) showed that the LA molecule contains one tight binding site for Ca^{2+} and a weaker binding site for Zn^{2+} . Although LA is a major calcium binding protein in milk (Lonnerdal and Glazier, 1985), only 0.1% to 0.15% of milk calcium is bound to LA.

Separation from whey

In the literature, the fractionation of LA was carried out using the following procedures:

- the salting-out procedure (Mailliart and Ribadeau-Dumas, 1988),
- selective thermal stability of LA in acidic conditions (Alomirah and Alli, 2004; Bramaud *et al.*, 1997; Gesan-Guiziu *et al.* 1999);
- separation by ion-exchange chromatography (Outinen *et al.*, 1996);
- or chromatographic methods (Gambero *et al.*, 1997; Heddleson *et al.*, 1995; Noppe *et al.*, 1999);
- membrane techniques in combination with enzymatic hydrolysis (Konrad and Kleinschmidt, 2008).

For the processes described above, the purity of LA was reported in the range 50–83%. Kiesner *et al.* (2000) and Tolkach *et al.* (2005) developed a method for LA isolation by selective denaturation of β -lactoglobulin at pH 7.5 and heating at 97^o C for 30 seconds. If caseinomacropptide is absent, the purity of LA can reach up to 98% (Konrad and Kleinschmidt, 2008). The disadvantage of this scheme is the irreversible denaturation of all other whey proteins except LA. Membrane filtration results in a permeate rich in LA but with relatively low purity. Mehra and Donnelly (1993) suggested that ultrafiltration of whey using 100 kDa membranes at pH 8.0 gives the best results. Muller *et al.* (1999, 2003) used a two step cascade to separate LA from acid casein. The method consisted of an UF step using 300 kDa ceramic membranes and a selective thermal precipitation by heating permeate at 55^o C for 5 minutes at pH 3.9. They obtained a precipitate in which LA was a calcium-free apo-protein. Cheang and Zydney (2004) developed a two-stage UF process for the isolation of LA from whey protein isolate, using diafiltration of whey protein isolate at pH 7 with a 100 kDa membrane in the first step. The permeate was separated into LA and β -lactoglobulin fractions, in a second step, using a 30 kDa membrane. Konrad *et al.* (2000) proposed an enzymatic method to isolate native β -lactoglobulin from whey as a pure compound. This method is based on a peptic hydrolysis of whey protein and selective membrane separation of the low molecular mass fractions.

Functionality

Whey proteins are used as food ingredients due to their important functional properties such as solubility, viscosity, water-holding capacity, gelation, adhesion, emulsification and foaming (Stănciuc, 2009). As foodstuffs they are applied not only for their functional properties, but also for their high nutritive value, reasonable cost and GRAS status.

As it is the main protein of human milk, there is considerable technical interest in its isolation if costs can be kept low. Besides this, owing to its high content in tryptophane it is applicable as a nutraceutical (Maubois and Ollivier, 1997) and due to its high cytotoxicity (Otani and Mizumoto, 1998) it possesses therapeutic uses. Matsumoto *et al.*, (2001) reported protective properties of LA against mucosal injury.

LA is rich in essential and conditionally essential amino acids and is a dominant protein in human milk (Brew, 1992). Since the amino acids content is strongly correlated with the nutritional value and with the functional properties of whey and whey products, a comparative analysis between human milk and bovine LA is presented in Table 1.

As it can be seen, LA has a high content of lysine and cysteine and a particularly high content of tryptophan (5.9% of the total amino acid content). The most striking differences between bovine and human milks are the lower concentrations of tryptophan and cysteine in the latter. As a single component of the diet, LA does not have a very high biological value.

The amino acid composition of milk may be a critical factor in the nutrition of neonates in general and premature neonates in particular (Stănciuc, 2009). Both are growing rapidly and are undergoing a series of important maturational processes. A successful outcome of these processes may depend upon the presence of specific amino acids in the milk consumed. Taking into consideration the high content

in essential amino acids, LA is an invaluable supplement for infant formulas. The high content of cysteine in LA is also valuable in boosting the immune system and promoting wound healing. LA also has a high level of tryptophan, which may help improve mood, sleep and cognitive performance (Heine *et al.*, 1991).

As a monomer, LA may possess bactericidal or antitumor activity. The active form of the protein, called “human LA made lethal to tumor cells” (HAMLET), was described as a complex formed by apo-LA and oleic acid (Svensson *et al.*, 2000). The HAMLET induces apoptosis (programmed cell death) in tumor cells but spares mature cells and has received much attention due to its potential use as a new therapeutic agent against tumor cells (Gustafsson *et al.*, 2005).

Table 1. Comparative analysis of percentages of amino acids between human milk protein and bovine LA (Heine *et al.*, 1991)

Amino acids	Human milk	Bovine LA
Tryptophan	1.8	5.9
Phenylalanine	4.4	4.0
Leucine	10.1	10.5
Isoleucine	5.8	6.1
Threonine	4.6	5.0
Methionine	1.8	0.9
Lysine	6.2	10.3
Valine	6.0	4.3
Histidine	2.3	2.6
Arginine	4.0	1.0
Cystine	1.7	5.3
Proline	8.6	1.4
Alanine	4.0	1.9
Aspartic acid	8.3	16.8
Serine	5.1	4.5
Glutamic acid	17.8	11.5
Glycine	2.6	3.3
Tyrosine	4.7	4.7

Pellegrini *et al.* (1999) suggested that some polypeptides derivated from proteolytic digestion of LA by trypsin (f1-5, and f (17-31) S-S(109-114)) and chymotrypsin (f(61-68)S-S(75-80)) had bactericidal properties, mostly against Gram-positive bacteria, while only a weak bactericidal activity was revealed against Gram-negative strains.

Hakansson *et al.*, (2000) have found an LA folding variant from human milk with bactericidal activity against antibiotic-resistant and susceptible strains of *Streptococcus pneumoniae*, but this had little or no activity against other bacterial species.

At physiological pH of bovine and human milk, LA associates with lysozym and this complex exhibits a higher antibacterial activity against both Gram-positive and Gram-negative bacteria (López Expósito and Recio, 2006). α -lactorphin, a peptide from LA behaves like opioid receptor agonists (Teschmacher *et al.*, 1998).

It was also demonstrated that whey protein or α -LA had a marked suppressive effect against the increased release of proinflammatory cytokines, such as IL-1, IL-6, and tumor necrosis factor- α (TNF- α), from the D-galactosamine induced liver injury rat model or ischemia/reperfusion induced intestinal injury rat model (Yamaguchi and Uchida, 2007).

Two synthetic peptides corresponding to the sequences f50-51 (Tyr-Gly) and f18-20 (Tyr-Gly-Gly) of LA also enhance both the *in vitro* proliferation and protein synthesis of concanavalin A-stimulated human peripheral blood lymphocytes (Gauthier *et al.*, 2006).

Improvements in functional properties may be achieved by modifying the protein structure by chemical, enzymatic or physical treatments (Faergemand *et al.*, 1998, Herceg *et al.*, 2005, Hudson *et al.*, 2000, Ibanoglu and Karataş, 2001, Kato *et al.*, 1994, Kresik *et al.*, 2006, Mleko and Foegeding, 2000). Functional properties of whey proteins such as emulsification, foaming and gelation are affected by their structure. In most dairy processes, thermal treatment of milk is an essential operation aiming at increasing shelf life and improving food safety of the final product (Jean *et al.*, 2006).

LA has the lowest denaturation temperature ($\sim 62^{\circ}\text{C}$) of the whey proteins, but this process is $>90\%$ reversible (de Wit and Klarenbeek, 1984). Minimal aggregation occurs when heating at 70 or 80°C and at neutral pH , caused by irreversible changes in the secondary structure (McGuffey *et al.*, 2005).

McGuffey *et al.*, (2007) investigated the aggregation behavior of two commercial samples of LA heated at 95°C at neutral pH in a complex mineral salt environment and they postulated that this process is complex and depends on a number of variables. A significant increase in aggregation occurred when LA was held at temperature $> 90^{\circ}\text{C}$ or was heated in the presence of thiol groups. The size, shape and thermal-reversibility of aggregates were altered by the relative amounts of LA and β -lactoglobulin. It is probable that the molecular structure of LA is more stable than that of β -lactoglobulin, and oligomerisation takes place only if free SH-groups are available during unfolding from other molecules, (Hinrichs and Rademacher, 2005).

Bertrand-Harb *et al.*, (2002) showed that when the protein is heated to temperatures $\geq 90^{\circ}\text{C}$ at $\text{pH} \geq 7.0$, pure forms aggregates linked in part by intermolecular disulfide bonds. Electrophoresis reveals multiple distinct monomer and dimer bands that are products of intramolecular disulfide bond shuffling (Hong and Creamer, 2002), mainly due to the highly relative reactivity of the cysteine residues Cys¹¹¹ and Cys¹²⁰ (Livney *et al.*, 2003).

Bernal and Jenel (1984) studied the role of Ca^{2+} on thermal denaturation of LA in the pH range of 2.5 to 6.5. They concluded that binding of calcium is an essential factor for both heat stability and renaturation of LA. Thermal denaturation of a protein may be reversible or irreversible, depending on the specific conditions under which denaturation takes place (Sava, 2005). The native tertiary structure of LA appears to be regained upon cooling down, due to its high calcium-binding affinity, as long as the calcium molecules are available to be bound again by the protein (de la Fuente *et al.*, 2002). This does not occur in the presence of EDTA or a high concentration of hydrogen ions in the medium.

Under combined exposure to pressure and temperature, LA was more resistant to denaturation than β -lactoglobulin (Hinrichs and Rademacher, 2005). The kinetic parameters (**Table 2**) were determined by these authors using a non-linear regression procedure in a single-step with an overall fit of the measured data. The formal reaction order for denaturation was not constant in the examined temperature range.

Table 2. Kinetic parameters of temperature-dependent denaturation of LA in skim milk under isobaric conditions, determined by non-linear regression with fixed order of reaction (reference temperature $T_{\text{ref}} = 303\text{ K}$) (Hinrichs and Rademacher, 2005)

Order of reaction	Temperature, $^{\circ}\text{C}$	p, MPa	E_A , kJ mol^{-1}	$k_{p,303}$, s^{-1}	r^2
2.5	60 - 70	100	192 ± 2	$(1.6 \pm 19) \times 10^{-3}$	0.945
	60 - 70	200	298 ± 4	$(1.3 \pm 3.4) \times 10^{-4}$	0.968
	1 - 60	400	501 ± 28	1.3 ± 23	0.835
	1 - 60	500	233 ± 2	$(4.8 \pm 8.0) \times 10^{-2}$	0.989
	1 - 60	600	154 ± 4	0.9 ± 1.6	0.972
	1 - 60	700	71.5 ± 1.0	$(3.1 \pm 2.4) \times 10$	0.998
	1 - 40	800	80.1 ± 1.3	$(1.5 \pm 2.7) \times 10$	0.982

E_A : activation energy; $k_{p,303}$, - reaction rate constant at pressure p and $T_{\text{ref}} = 303\text{ K}$; r^2 : coefficient of correlation.

The authors mentioned above suggested that the denaturation reaction was hardly influenced by pressure in the lower temperature range (1–10⁰ C). Increasing temperature caused a decrease in the activation volume from $-4.2 \pm 2.3 \text{ mLmol}^{-1}$ at 1⁰C to $-59.8 \pm 4.8 \text{ mLmol}^{-1}$ at 70⁰C.

This indicates that pressure dependence of LA denaturation in milk increases with increasing temperature and pressure, and that temperature and pressure act synergistically.

Susceptibility to proteolysis

Hydrolysis of LA is difficult to perform, as the compact globular structure is relatively resistant toward enzymatic proteolysis. Schmidt and Poll (1991) showed that native bovine LA is highly resistant to tryptic digestion.

The limited proteolysis experiments are useful for probing protein structure and dynamics, thus complementing the results that can be obtained by using other common physicochemical methods and approaches (Polverino de Laureto *et al.*, 1999). These authors have used three enzymes (pepsin, chymotrypsin and proteinase K). For the pepsin experiments, proteolysis was conducted at 4⁰C, because at lower reaction temperature it is more suitable to keep folded the initially formed proteolytic fragments being thus more resistant to further degradation. The initial cleavages at the level of the β -sheet region of native LA indicate that this region is highly mobile or even unfolded in the LA molten globule(s), while the rest of the protein chain maintains sufficient structure and rigidity to prevent extensive proteolysis. The subsequent cleavages at chain segment 95-105 indicate that also this region is somewhat mobile in the A-state or apo form of the protein. It is concluded that the overall domain topology of native LA is maintained in acid or at neutral pH upon calcium depletion.

Proteolysis of apo-LA by proteinase K at 4⁰C occurs slowly, leading to formation of small peptides only. At 37⁰C, proteinase K preferentially cleaves at bonds Ser³⁴ – Gly³⁵, Gln³⁹ – Asn⁴⁴, Phe⁵³ – Gln⁵⁴ and Asn⁵⁶ – Asn⁵⁷ (Permyakov, 2005).

LA is also very stable to transglutaminase catalyzed-polymerization in the native state. The polymerization reaction is enhanced if the protein is transformed from native to the molten globule-like state by Ca²⁺ removal.

To increase its susceptibility to proteolysis, the LA structure can be modified by different methods. For example, lowering the pH to 2.0 in the hydrolysis experiments with pepsin (El-Zahar *et al.*, 2005), esterification of LA followed by tryptic hydrolysis at 37⁰ C (Sitohy *et al.*, 2001), binding of zinc ions (Permyakov *et al.*, 1991). Polverino de Laureto *et al.*, (1995) suggested that the limited proteolysis with thermolysin at room temperature is enhanced in the presence of 50% trifluoroethanol.

An interesting approach was reported by N'Negue *et al.*, (2006). These authors used thermolysin in hydrolysis experiments at 70⁰C. The metalloendopeptidase thermolysin is stable up to 80⁰C, whereas the digestive proteases, that are usually used, rapidly lose their activity at high temperatures. LA was entirely hydrolyzed by thermolysin at 70⁰C for approximately 5 minutes, in particular its N-terminal 1–58 and C-terminal 95–123 regions. More efficient proteolytic activity of thermolysin at 70⁰ C was only due to the substrate unfolding at this high temperature leading to a better accessibility of LA peptide bonds to the solvent.

Barros and Malcata (2006) used two aspartic proteases from *Cynara cardunculus*, named cardosin A and B to evaluate the susceptibility of LA to hydrolysis at 55⁰C and pH 5.2. Both enzymes hydrolyzed the protein to a similar extent, low and medium peptide pattern (between 3 and 8 kDa) being detected after 24 hours of reaction.

Cardosin B had broader specificity than cardosin A toward LA, and the major cleavage sites are next to apolar and aromatic residues (e.g., Ala¹⁹-Glu²⁰, Phe²⁸-Arg²⁹, Glu³⁰-Leu³¹, Tyr³⁷-Gly³⁸, Trp⁴⁵-Val⁴⁶, Phe⁵⁰-His⁵¹, Ala⁵⁹-Ile⁶⁰, Ser⁶⁶-Thr⁶⁷, Leu⁷¹-Phe⁷², Phe⁷²-Gln⁷³, Gln⁷³-Ile⁷⁴, Ile⁷⁸-Trp⁷⁹, Leu¹¹⁵-Asp¹¹⁶, and Leu¹²⁴-Ala¹²⁵). These authors identified five complete peptide sequences released from LA: f29–30, f29–37, f46–50, f60–66, and f67–71. Conversely, cardosin A exhibited limited activity on LA, and cleaved the peptide bonds Phe²⁸-Arg²⁹, Ala⁵⁹-Ile⁶⁰, and Leu⁷¹-Phe⁷² (which were already found to be labile to cardosin B); in addition, Gly⁵⁴-Tyr⁵⁵ and Leu¹⁰⁵-Thr¹⁰⁶ were cleaved. One complete di-

peptide sequence (f29–30) was also determined. These results are very important as an approach to obtain peptides with functional properties and also products free of LA, in case of allergic reactions.

Ipsen and Otte (2007) used a specific serin protease from *Bacillus licheniformis* for the limited hydrolysis of LA in order to obtain nanotubes with possible applications in food industry (as viscosifier and gelling agent), pharmaceutical (encapsulated drugs), and nanotechnology (as templates for nanowire synthesis or scaffolding in tissue engineering).

2. Conclusions

This review demonstrates that LA has some very specific physiologic, functional and structural properties. The strong and selective binding of Ca^{2+} to LA is closely related to the regulation of many physiological and functional properties.

The protein can be isolated from whey through various methods. The most reproducible and easy to perform are the methods based on the transmission of LA through a UF membrane and a selective enzymatic purification.

The potential health benefits of LA and biological and functional peptides have been a subject of growing scientific interest in the context of health-promoting functional foods. LA and LA-peptides can be used as supplements in food in order to supply the host with essential amino acids, to improve/maintain the immune system, to reduce the stress, for its opioid activity, antihypertensive action, regulation of cell growth, immunomodulation etc.

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References

- Alomirah, H. F. and Alli, I. 2004. Separation and characterization of β -lactoglobulin and α -lactalbumin from whey and whey protein preparations. *International Dairy Journal*, **14**, 411–419.
- Barman, T. E., 1970. Purification and properties of bovine milk glyco- α -lactalbumin. *Biochimica et Biophysica Acta*, **214**:242–244.
- Barros, R. M. and Malcata, F.X. 2006. Molecular Characterization of Peptides Released from β -Lactoglobulin and α -Lactalbumin via Cardosins A and B. *Journal of Dairy Science*, **89**:483–494.
- Bernal, V. and Jenel, P. 1984. Effect of calcium binding on thermal denaturation of bovine α -Lactalbumin. *Journal of Dairy Science*, **67** (10), 2452-2454.
- Bramaud, C., Aimar, P., Daufin, G. 1997. Optimisation of a whey protein fractionation process based on the selective precipitation of α -lactalbumin. *Le Lait*, **77**, 411–423.
- Brew, K. and Grobler, J. A. 1992. α -lactalbumin. In P. F. Fox (Ed.), *Advanced dairy chemistry*, vol. 1 (Proteins) (pp. 191–229). New York, NY, USA: Elsevier Applied Science.
- Cheang, B. and Zydney, A. L. 2004. A two-stage ultrafiltration process for fractionation of whey protein isolate. *Journal of Membrane Science*, **231**:159–167.
- Chrysin, E. D., Brew, K., Acharya, K. R. 2000. Crystal structures of apo- and holo-bovine α -lactalbumin at 2.2-Å resolution reveal an effect of calcium on inter-lobe interactions. *Journal of Biological Chemistry*, **275**:37021–37029.
- Cross, M. L. and Gill, H. S. 2000. Immunomodulatory properties of milk. *British Journal of Nutrition*, **84**:S81–S89.
- De la Fuente, M., Singh, H., Hemar, Y. 2002. Recent advances in the characterisation of heat-induced aggregates and intermediates of whey proteins. *Trends in Food Science and Technology*, **13**, 262-274.
- El-Zahar, K., Sitohy, M., Choiset, Y., Metro, F., Haertle, T., Chobert, J.-M. 2005. Peptic hydrolysis of ovine β -lactoglobulin and α -lactalbumin. Exceptional susceptibility of native ovine β -lactoglobulin to pepsinolysis. *International Dairy Journal*, **15**:17–27.
- Faergemand M., Otte J., Qvist K.B. 1998. Crosslinking of whey proteins by enzymic oxidation. *Journal of Agriculture and Food Chemistry*, **46**: 1326–1333.
- Farkas, V., Vass, E., Hanssens, I., Majer, Z., Hollosi, M. 2005. Cyclic peptide models of the Ca^{2+} -binding loop of α -lactalbumin. *Bioorganic and Medicinal Chemistry*, **13**: 5310–5320.

- Farrell, Jr., H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., Hicks, C. L., Hollar, C. M., Ng-Kwai-Hang, K. F., Swaisgood, H. E. 2004. Nomenclature of the Proteins of Cows' Milk - Sixth Revision. *Journal of Dairy Science*, **87**:1641–1674.
- FitzGerald, R. J., Murray, B. A., Walsh, D. J. 2004. Hypotensive peptides from milk proteins. *Journal of Nutrition*, **134**:980S–988S.
- Fitzgerald, R. J. and Swaisgood, H. 1989. Bindings of ions and hydrophobia probes to α -lactalbumin and k -casein as determined by analytical affinity chromatography. *Archives of Biochemistry and Biophysics*, **268**: 239-248.
- Gamero, A., Kubota, L. T., Gushikem, Y., Airoidi, C., Granjeiro, J. M., Taga, E. M., et al. 1997. Use of chemically modified silica with β -diketoamine groups for separation of α -lactalbumin from bovine milk whey by affinity chromatography. *Journal of Colloid Interface Science*, **85**:313–316.
- Gauthier, S.F., Pouliot, Y., Saint-Sauveur, D. 2006. Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins – Review. *International Dairy Journal*, **16**: 1315–1323.
- Gesan-Guiziu, G., Daufin, G., Timmer, M., Allersma, D., Van der Horst, C. 1999. Process steps for the preparation of purified fractions of α -lactalbumin and β -lactoglobulin. *Journal of Dairy Science*, **78**:225–236.
- Gordon, W. G. 1971. α -Lactalbumin, Pages 331–363 in *Milk Proteins*: Vol. II, H. A. McKenzie, ed. Academic Press, New York, NY.
- Gustafsson, L., Hallgren, O., Mossberg, A. K., Pettersson, J., Fischer, W., Aronsson, A., et al. 2005. HAMLET kills tumour cells by apoptosis: Structure, cellular mechanisms and therapy. *Journal of Nutrition*, **135**:1299–1303.
- Hakansson, A., Malin, S., Mossberg, A. K., Sabharwal, H., Linse, S., Lazou, I., et al. 2000. A folding variant of α -lactalbumin with bactericidal activity against *Streptococcus pneumoniae*. *Molecular Microbiology*, **35**:589–600.
- Heine, W.E., Klein, P.D., Reeds, P.J. 1991. The importance of α -lactalbumin in infant nutrition- Critical Review. *The Journal of Nutrition*, **121**: 277-283.
- Herceg, Z., Lelas V., Krešić G. 2005. Influence of tribomechanical micronization on the physical and functional properties of whey proteins. *International Journal of Dairy Technology*, **58**: 225–232.
- Hinrichs, J., Rademacher, B. 2005. Kinetics of combined thermal and pressure-induced whey protein denaturation in bovine skim milk. *International Dairy Journal*, **15**: 315–323.
- Hudson, H.M., Daubert C.R., Foegeding, E.A. 2000. Rheological and physical properties of derivatized whey protein isolate powders. *Journal of Agriculture and Food Chemistry*, **48**: 3112–3119.
- Ibanoğlu, E. and Karataş, S. 2001. High pressure effect on foaming behaviour of whey protein isolate. *Journal of Food Engineering*, **47**: 31–36.
- Jenness, R. 1974. The composition of milk. Pages 3–107 in *Lactation*, Vol. III. B. L. Larson and V. R. Smith, ed. Academic Press, New York, NY.
- Kato, A., Osako Y., Matsudomi N., Kobayashi K. 1994. Changes in the emulsifying and foaming properties of proteins during heat denaturation. *Agricultural and Biological Chemistry*, **47**:33–37.
- Kiesner, C., Clawin-Radecker, I., Meisel, H., Buchheim, H. 2000. Manufacturing of α -lactalbumin-enriched whey systems by selective thermal treatment in combination with membrane processes. *Le Lait*, **80**: 99–111.
- Kleinberg J.L., Todd J., Babitsky G. 1983. Inhibition by estradiol of the lactogenic effect of prolactin in primate mammary tissue: reversal by antiestrogens LY 156758 and tamoxifen. *Proceedings of the National Academy of Sciences*, **80** (13): 4144–4148.
- Konrad, G. and Kleinschmidt, T. 2008. A new method for isolation of native α -lactalbumin from sweet whey. *International Dairy Journal*, **18**:47–54.
- Krešić, G., Lelas, V., Herceg, Z., Režek, A. 2006. Effects of high pressure on functionality of whey protein concentrate and whey protein isolate. *Le Lait*, **86**: 303–315.
- Kuwajima, K. 1996. The molten globule state of α -lactalbumin – a review. *The Federation of American Societies for Experimental Biology Journal*, **10**:102-109.
- Kuwajima, K. 1989. The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. *Proteins*, **6**:87-103.
- Lonnerdal, B. and Glazier, C. 1985. Calcium binding by α -lactalbumin in human milk and bovine milk. *Journal of Nutrition*, **115**: 1209-1216.
- López Expósito, I. and Recio, I. 2006. Antibacterial activity of peptides and folding variants from milk proteins. *International Dairy Journal*, **16**: 1294–1305.
- Mailliar, P. and Ribadeau-Dumas, B. 1988. Preparation of α -lactalbumin and β -lactoglobulin free proteins from whey retentate by NaCl salting out at low pH. *Journal of Food Science*, **53**:743–745.

- Mangino, M. 2007. Properties of whey proteins: α -lactalbumin, β -lactoglobulin, bovine serum albumin, proteose peptone and immune globulins. *Lecture Notes*, The Ohio State University.
- Mariani, P. and Russo, V. 1977. Polmorfismo genetico della α -lattalbumina nelle razze bovine. *Rivista di Zootecnia e Veterinaria*, **6**: 603–610.
- Markus, C. R., Olivier, B., Panhuysen, G. E. M., Gugten, J. V. D., Alles, M. S., Truitem, A., Westenberg, H. G. M., Fekkes, D., Koppeschaar, H. F., de Haan, E. E. H. F. 2000. The bovine protein α -lactalbumin increases the plasma ratio of tryptophan to the other large neutral amino acids, and in vulnerable subjects raises brain serotonin activity, reduces cortisol concentration, and improves mood under stress. *American Journal of Clinical Nutrition*, **71**:1536–1544.
- Matsumoto, H., Shimokawa, Y., Ushida, Y., Toida, T., Hayasawa, H. 2001. New biological function of bovine α -lactalbumin: Protective effect against ethanol- and stress-induced gastric mucosal injury in rats. *Bioscience, Biotechnology and Biochemistry*, **65**:1104–1111.
- Maubois, J.-L. and Ollivier, G. 1997. Extraction of milk proteins. In S. Damodaran, A. Paraf (Eds.), *Food proteins and their applications* (pp. 225–256). New York, NY, USA: Marcel Dekker.
- McKenzie, A. and White, F. H. Jr. 1991. Lysozyme and α -lactalbumin: Structure, function, and interrelationships. *Advanced in Protein Chemistry*, **41**:173-315.
- Mehra, R. K. and Donnelly, W. J. 1993. Fractionation of whey protein components through a large pore size, hydrophilic cellulose membrane. *Journal of Dairy Research*, **60**:89–97.
- Muller, A., Chaufer, B., Merin, U., Daufin, G. 2003. Prepurification of α -lactalbumin with ultrafiltration ceramic membranes from acid casein whey: Study of operating conditions. *Le Lait*, **83**:111–129.
- Muller, A., Daufin, G., Chaufer, B. 1999. Ultrafiltration modes of operation for the separation of α -lactalbumin from acid casein whey. *Journal of Membrane Science*, **153**:9–21.
- N'Negue, M.-A, Miclo, L., Girardet, J.-M., Campagna, S., Molle, D., Gaillard, J.-L. 2006. Proteolysis of bovine α -lactalbumin by thermolysin during thermal denaturation. *International Dairy Journal*, **16**:1157–1167.
- Otani, H. and Mizumoto, G. 1998. Suppressive effect of lysozymes and α -lactalbumins on mitogen-induced proliferative responses of mouse lymphocytes. *Animal Science Technology*, **11**:1029–1039.
- Outinen, M., Tossavainen, O., Syvaioja, E.-L. 1996. Chromatographic fractionation of α -lactalbumin and β -lactoglobulin with polystyrenic strongly basic anion exchange resins. *Lebensmittel-Wissenschaft und Technologie*, **29**:340–343.
- Pellegrini, A., Thomas, U., Bramaz, N., Hunziker, P., von Fellenberg, R. 1999, Isolation and identification of three bactericidal domains in the bovine α -lactalbumin molecule. *Biochimica et Biophysica Acta - General Subjects*, **1426** (3):439-448.
- Permyakov, E.A. 2005. *α -lactalbumin*. Nova Science Publishers, Inc., New York, ISBN 1-59454-107-8.
- Permyakov, E.A. and Berliner, L.J. 2000. α -Lactalbumin: structure and function. *FEBS Letters*, **473**:269-274.
- Permyakov, E. A., Shnyrov, V. L., Kalinichenko, L. P., Kuchar, A., Reyzer, I. L., Berliner, L. J. 1991. Binding of Zn(II) ions to α -lactalbumin. *Journal of Protein Chemistry*, **10**:577–584.
- Pike, A. C. W., Brew, K., Acharya, K. R. 1996. Crystal structures of guinea-pig, goat, and bovine α -lactalbumin highlight the enhanced conformational flexibility of regions that are significant for its action in lactose synthesis. *Structure*, **4**:691–703.
- Polverino De Laureto, P., Scaramella, E., Frigo, M., Geftter Wondrich, F., De Filippis, V., Zamboni, M., Fontana, A. 1999. Limited proteolysis of bovine α -lactalbumin: Isolation and characterization of protein domains. *Protein Science*, **8**: 2290-2303.
- Polverino de Laureto, P., De Filippis, V., Di Bello, M., Zamboni, M., Fontana, A. 1995. Probing the molten globule state of α -lactalbumin by limited proteolysis. *Biochemistry*, **34**:12596–12604.
- Saint-Sauveur, D., Gauthier, S.F., Boutin, Y., Montoni, A. 2007. Immunomodulating properties of a whey protein isolate, its enzymatic digest and peptide fractions. *International Dairy Journal*, **18**: 260–270.
- Sava, N. 2005. Research regarding the influence of heat treatment on functional properties of whey proteins, PhD Thesis, University of Dunarea de Jos, Galati.
- Schmidt, D. G. and Poll, J. K. 1991. Enzymatic hydrolysis of whey proteins. Hydrolysis of α -lactalbumin and β -lactoglobulin in buffer solutions by proteolytic enzymes. *Netherlands Milk and Dairy Journal*, **45**:225–240.
- Sitohy, M., Chobert, J. M., Haertle, T. 2001. Susceptibility to trypsinolysis of esterified milk proteins. *International Journal of Biological Macromolecules*, **28**: 263–271.
- Shoveller, A. K., Stoll, B., Ball, R. O., Burrin, D. G. 2005. Nutritional and functional importance of intestinal sulphur amino acid metabolism. *Journal of Nutrition*, **135**:1609–1612.
- Smilowitz, J. T., Dillard, C. J., German, J. B. 2005. Milk beyond essential nutrients: The metabolic food. *Australian Journal of Dairy Technology*, **60**:77–83.

- Smithers, G.W. 2008. Whey and whey proteins—From ‘gutter-to-gold’ – Review. *International Dairy Journal*, **18**: 695–704.
- Stănciuc, N. 2009. *Milk proteins. The structure-function relationship*. Ed. Academica, Galați.
- Sternhagen, L. G., Allen, J.C. 2001. Growth rates of a human colon adenocarcinoma cell line are regulated by the milk protein α -lactalbumin. *Advances in Experimental Medicine and Biology*, **501**:115–120.
- Svensson, M., Hakansson, A., Mossberg, A.K., Linse, S., Svanborg, S. 2000. Conversion of alpha-lactalbumin to a protein inducing apoptosis. *Proceedings of the National Academy of Sciences*, **97**:4221–4226.
- Teschemacher, H., Koch, G., Brantl, V. 1998. Milk protein-derived opioid receptor ligands. *Peptide Science*, **43** (2): 99 – 117.
- Tolkach, A., Steinle, S., Kulozik, U. 2005. Optimization of thermal pre-treatment conditions for the separation of native α -lactalbumin from whey protein concentrates by means of selective denaturation of β -lactoglobulin. *Journal of Food Science*, **70**: E557–E566.
- Yamaguchi, M. and Uchida, M. 2007. α -Lactalbumin suppresses interleukin-6 release after intestinal ischemia/reperfusion via nitric oxide in rats. *Inflammopharmacology*, **15**:43–47.
- Ye, A. and Taylor, S. 2009. Characterization of cold-set gels produced from heated emulsions stabilized by whey protein. *International Dairy Journal*, **19**: 721–727.