AN OVERVIEW OF BOVINE α-LACTALBUMIN STRUCTURE AND FUNCTIONALITY

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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 homoeostasis, 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.
An overview of bovine α-albumin structure and functionality

LA consists of 123 amino acid residues forming a compact globular structure stabilized by four disulfide bonds (Cys\textsuperscript{6}–Cys\textsuperscript{120}, Cys\textsuperscript{61}–Cys\textsuperscript{77}, Cy\textsuperscript{71}–Cys\textsuperscript{91}, and Cys\textsuperscript{28}–Cys\textsuperscript{111}). 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: Ala\textsubscript{3}, Arg\textsubscript{1}, Asn\textsubscript{8}, Asp\textsubscript{13}, Cys\textsubscript{8}, Gln\textsubscript{6}, Glu\textsubscript{7}, Gly\textsubscript{6}, His\textsubscript{3}, Ile\textsubscript{8}, Leu\textsubscript{13}, Lys\textsubscript{12}, Met\textsubscript{1}, Phe\textsubscript{4}, Pro\textsubscript{2}, Ser\textsubscript{5}, Thr\textsubscript{7}, Trp\textsubscript{4}, Tyr\textsubscript{4}, Va\textsubscript{16} (Farrell et al., 2004). Both A and B variant contain four disulfide bonds and no phosphate groups.

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\textsuperscript{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\textsuperscript{10} helix (h1b, 18–20, h2, 77–80 and h3c, 115-118) (Chrysina et al., 2000).

LA possesses a single strong Ca\textsuperscript{2+} binding site (Permyakov and Berliner, 2000) and for this reason it is frequently used as a simple, model Ca\textsuperscript{2+} 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 Ca2+ 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 3₁₀ 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 Ca²⁺ - bounded and Ca²⁺ 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°C for the apo-protein to nearly 70°C for the Ca²⁺-bound protein. Both Ca²⁺ - 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 Ca²⁺ (Farkas et al., 2005).

*Figure 3. Ribbon model of Ca²⁺- loaded and Ca²⁺- free bovine LA* (Permyakov, 2005)
An overview of bovine $\alpha$-albumin structure and functionality

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:

1) $\text{UTP - glucose-1-P} \rightarrow \text{UDP-glucose} + \text{PP}$

2) $\text{UDP-glucose} \rightarrow \text{UDP-galactose}$

3) $\text{UDP-galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP}$

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 $\beta$-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 $\beta$ 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-Guiziou 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°C for 30 seconds. If caseinomacropeptide 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°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

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

Pellegrini et al. (1999) suggested that some polypeptides derivate 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 (Teschemacher 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 Karatas, 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 (~62°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°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 ≥ 90°C at pH ≥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 Ca²⁺ 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_ref= 303 K) (Hinrichs and Rademacher, 2005)

<table>
<thead>
<tr>
<th>Order of reaction</th>
<th>Temperature, ºC</th>
<th>p, MPa</th>
<th>E_A, kJ mol⁻¹</th>
<th>k_p,303, s⁻¹</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>60 - 70</td>
<td>100</td>
<td>192 ± 2</td>
<td>(1.6±19) x 10⁻⁷</td>
<td>0.945</td>
</tr>
<tr>
<td></td>
<td>60 - 70</td>
<td>200</td>
<td>298 ± 4</td>
<td>(1.3±3.4) x 10⁻⁴</td>
<td>0.968</td>
</tr>
<tr>
<td>1 - 60</td>
<td>400</td>
<td>501 ± 28</td>
<td>1.3±23</td>
<td></td>
<td>0.835</td>
</tr>
<tr>
<td>1 - 60</td>
<td>500</td>
<td>233 ± 2</td>
<td>(4.8±8.0) x 10⁻²</td>
<td></td>
<td>0.989</td>
</tr>
<tr>
<td>1 - 60</td>
<td>600</td>
<td>154 ± 4</td>
<td>0.9±1.6</td>
<td></td>
<td>0.972</td>
</tr>
<tr>
<td>1 - 60</td>
<td>700</td>
<td>71.5 ± 1.0</td>
<td>(3.1±2.4) x 10⁻¹</td>
<td></td>
<td>0.998</td>
</tr>
<tr>
<td>1 - 40</td>
<td>800</td>
<td>80.1 ± 1.3</td>
<td>(1.5±2.7) x 10⁻¹</td>
<td></td>
<td>0.982</td>
</tr>
</tbody>
</table>

EA: activation energy; k_p,303, - reaction rate constant at pressure p and T_ref= 303 K; r²: coefficient of correlation.
The authors mentioned above suggested that the denaturation reaction was hardly influenced by pressure in the lower temperature range (1–10°C). Increasing temperature caused a decrease in the activation volume from \(-4.2\pm2.3 \text{ mLmol}^{-1}\) at 10°C to \(-59.8\pm4.8 \text{ mLmol}^{-1}\) at 70°C. 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 trypsin 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 4°C, 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 \(\beta\)-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 4°C occurs slowly, leading to formation of small peptides only. At 37°C, proteinase K preferentially cleaves at bonds Ser\(^{34}\) – Gly\(^{35}\), Gln\(^{39}\) – Asn\(^{44}\), Phe\(^{53}\) – Gln\(^{54}\) and Asn\(^{56}\) – Asn\(^{57}\) (Permyakov, 2005).

LA is also very stable to transglutaminase catalyzed-polimerization in the native state. The polymerization reaction is enhanced if the protein is transformed from native to the molten globule-like state by Ca\(^{2+}\) 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 37°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 70°C. The metalloendopeptidase thermolysin is stable up to 80°C, whereas the digestive proteases, that are usually used, rapidly lose their activity at high temperatures. LA was entirely hydrolyzed by thermolysin at 70°C for approximately 5 minutes, in particular its N-terminal 1–58 and C-terminal 95–123 regions. More efficient proteolytic activity of thermolysin at 70°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 55°C 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., Ala\(^{19}\)-Glu\(^{20}\), Phe\(^{28}\)-Arg\(^{29}\), Glu\(^{30}\)-Leu\(^{31}\), Tyr\(^{37}\)-Gly\(^{38}\), Trp\(^{45}\)-Val\(^{46}\), Phe\(^{50}\)-His\(^{51}\), Ala\(^{59}\)-Ile\(^{60}\), Ser\(^{66}\)-Thr\(^{67}\), Leu\(^{1}\)-Phe\(^{2}\), Phe\(^{72}\)-Gln\(^{3}\), Gln\(^{73}\)-Ile\(^{74}\), Ile\(^{84}\)-Trp\(^{75}\), Leu\(^{115}\)-Asp\(^{116}\), and Leu\(^{124}\)-Ala\(^{125}\)). 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 Phe\(^{28}\)-Arg\(^{29}\), Ala\(^{59}\)-Ile\(^{60}\), and Leu\(^{71}\)-Phe\(^{72}\) (which were already found to be labile to cardosin B); in addition, Gln\(^{53}\)-Tyr\(^{55}\) and Leu\(^{106}\)-Thr\(^{106}\) 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 allergenic reactions. Ipsen and Otte (2007) used a specific serine 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


An overview of bovine α-lactalbumin structure and functionality


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