Chapter

Characteristics of Cow Milk Proteins and the Effect of Processing on Their Allergenicity

Roua Lajnaf, Sawsan Feki, Hamadi Attia, Mohamed Ali Ayadi and Hatem Masmoudi

Abstract

Milk proteins are well known for their nutritional and functional properties. However, they are also members of the Big-8 food allergens including egg, fish, shellfish, soy, peanuts, wheat and tree nuts, in terms of prevalence. The most common milk allergens are casein fractions and β-lactoglobulin naturally not present in human breast milk. Thus, the examination of cow's milk proteins as potential allergens that may cause food allergies and the identification of methods of reducing their immunogenicity are of great interest. The main objective of this chapter is to review the physico-chemical characteristics cow milk proteins as well as their studied allergenicity and immunogenicity as a function of some denatured dairy processes such as heating, high pressure, enzymatic hydrolysis and lactic acid fermentation.

Keywords: cow's milk proteins allergy, protein allergenicity, immunoreactivity, milk processing, β-lactoglobulin, caseins

1. Introduction

Food allergy is a major public health which has been estimated to affect around 1–2% of the adult population and 5–8% of pediatric population at the age below 3 years [1–3]. It is thought to result from disorders of the immune response to food allergens proteins and develop due to the defect in oral tolerance. Food allergens are contained in eight common foods including animal-based foods (cow's milk, eggs and fish) and plant-based foods (crustacean/shellfish, peanuts, soy, nuts and wheat). These allergens account for over 90% of the occurrence of all serious allergic reactions to foods worldwide [4]. Epidemiological studies have reported that animal food allergens, especially cow milk proteins allergy, was the most prevalent allergy for infants or young children, meanwhile, plant based food allergens was more encountered for adults [5].

Thus, Cow's Milk Allergy (CMA) represents 10–40% of the total food allergies. As an animal proteins allergy, it concerns mostly young children and less frequently adults. CMA is reported to affect approximately 3–8% of the total

pediatric population worldwide with symptoms at different levels of severity, which can endanger the patient's life [6]. Indeed, CMA is considered as the most common food allergy responsible for anaphylaxis reactions in young children as it is the first food eaten since birth. CMA is ranked third among all food allergies responsible for serious anaphylactic reactions to adults representing 15% from all allergic cases [7].

CMA disappears spontaneously at the age of 5 years in the majority of patients representing approximately 80% [8]. However, it seems that a minority of patients remains allergic in adulthood [9]. In most cases, the food allergy manifests itself as an immediate hypersensitivity reaction induced after recognition of food antigens by specific immunoglobulins type E (IgE). Other forms of allergy can also involve mechanisms not mediated by IgE. Their frequency is increasing but the immune responses involved are still poorly defined.

Like all food allergies, CMA involves both immunological reactions: immunoglobulin E (IgE) which is encountered in most allergic cases and non-IgE mediated reactions [10, 11]. The immunological reactions of IgE-mediated reactions occur immediately after proteins ingestion because of the interaction between allergens and immune mechanisms. This allergenic reaction is characterized by the production of IgE antibodies in allergic patients resulting in the degranulation phenomena of mast cells, the release of inflammatory mediators including histamine, 5-hydroxytryptamine (5HT) and prostaglandin E2 (PGE2) (**Figure 1**). These mediators induced the resulting allergy symptoms (hives, diarrhea, vomiting, and breathing difficulty). On the other hand, non-IgE mediated immunological reactions take up between 1hour and several days after ingestion of milk to develop involving the immune system as the IgE-mediated reactions [12–14].

The complete exclusion of cow's milk protein from the diet is still the only safe treatment that can be offered to patients today. In this case, infant formulations

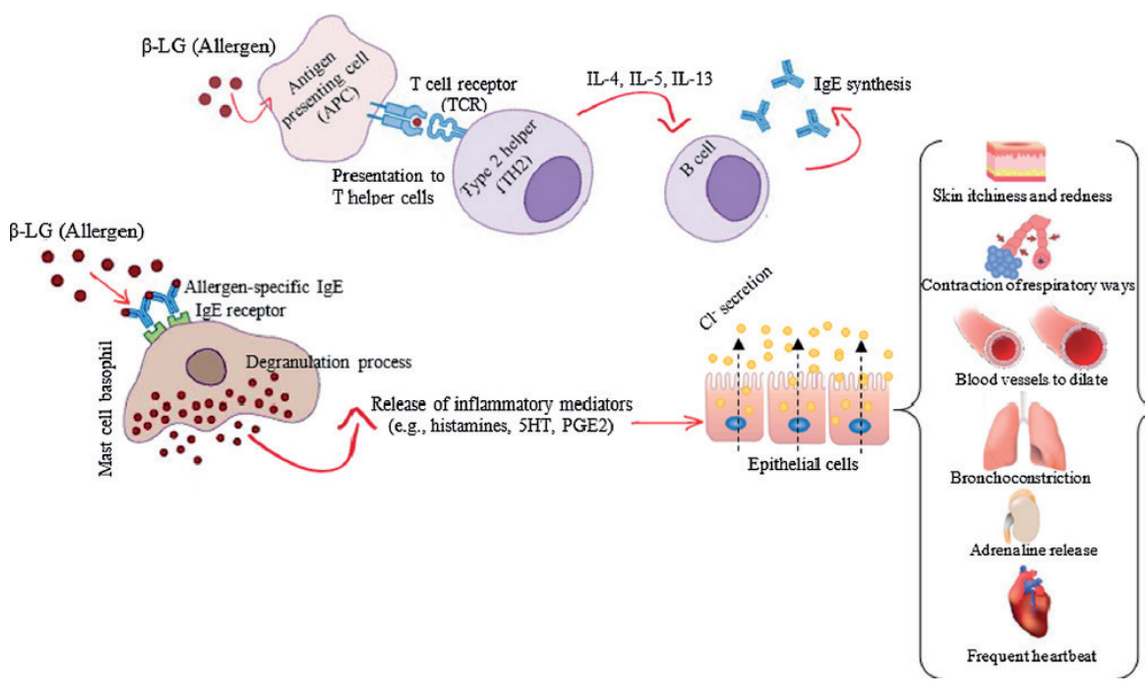

Figure 1.
A schematic representation of allergenicity mechanism of bovine milk in the body (abbreviations: β-Lg: β-lactoglobulin; IL-4, IL-5 and IL-13, inflammatory cytokines; IgE, immunoglobulin E; 5HT, 5-hydroxytryptamine; PGE2, prostaglandin E2) [12].

containing cow's milk proteins are replaced by milks which are designed from more or less extensive hydrolysates of bovine proteins from whey or from the casein fraction in order to limit allergenicity as much as possible residual product. On the hand, researchers, scientists and industrials keep searching for new potential milk alternatives including hydrolyzed milk formulae, plant-based formulae and other milks from different mammalian species such as goat, sheep, donkey, mare and camel milks [11, 12, 15, 16].

2. Characteristics of cow's milk proteins

Milk proteins represent an important nutritional source due to their high biological value and the presence of essential amino acids. They are also the source of various dairy products due to the important techno-functional properties of its proteins. Cow milk is a heterogeneous mixture of proteins with different structural and physico-chemical properties. As milks from all mammalian species, cow milk proteins are divided according to their solubility into two fractions: caseins (insoluble in acidic conditions) and whey proteins (soluble proteins). Indeed, caseins precipitate at their isoelectric pH which is located at 4.6, while whey proteins remain soluble in this pH level [17].

2.1 Caseins

Caseins are phosphoproteins which represent the most abundant protein fraction in milk. They represent approximately 80% of the total milk protein.

Caseins consist of 4 proteins which differ in contents of phosphorus, concentration, amino acid composition, isoelectric point (pI) and molecular weight (MW): alpha S1, alpha S2, beta, and kappa (α_{S1}, α_{S2}, β and κ). The α and β caseins are calcium sensitive caseins as they precipitate at a calcium concentration at 30 mM while the κ-casein remains in solution under these conditions. The β-casein represent 39% of total caseins, followed by α_{S1}, α_{S2} and κ caseins which represent 38%, 10% and 13% of total amounts of caseins, respectively (**Figure 2**) [19].

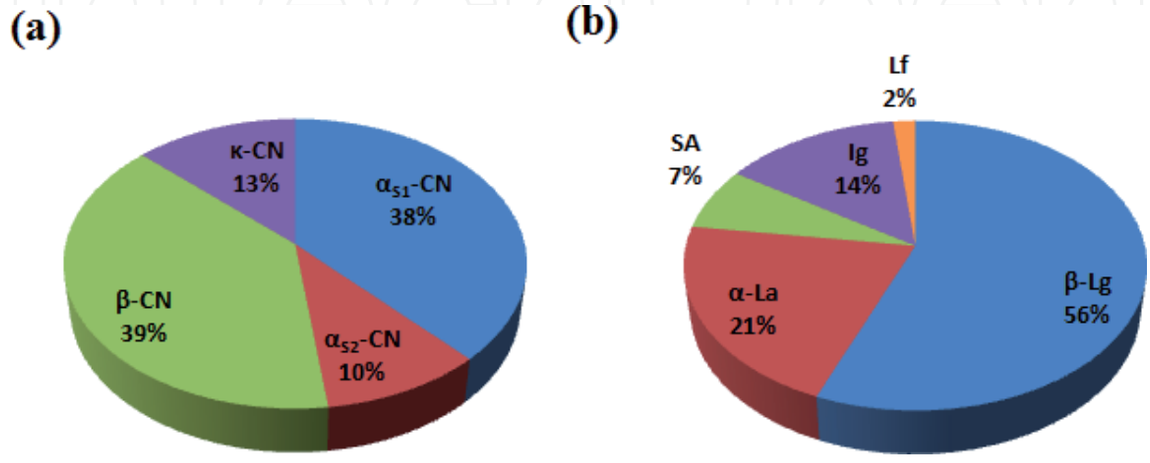

Figure 2.
Proportions of the different caseins (a) and whey proteins (b) in cow's milk (abbreviations: β-CN: β-casein; α_{S1}-CN: α_{S1}-casein; α_{S2}-CN: α_{S2}-casein; κ-CN: κ-casein, β-Lg: β-lactoglobulin; α-La: α-lactalbumin; SA: serum albumin; Ig: immunoglobulins; Lf: lactoferrin [18].

The 4 different caseins are associated with minerals forming colloids called casein micelles (concentration of minerals 80mg/g of caseins) with a diameter ranging between 100 and 140 nm. Bovine casein micelles and their characteristics have been the subject of much research and different micellar models have followed one another over the years (models by Horne, Holt, Bouchoux, etc.) [20, 21].

- **α_{S1}-Casein**: is a phosphoprotein with a MW of 22.9 kDa (199 amino acid residues) and is present in milk in the amount of 19.5 g/L and with pI of 4.46 [22]. Bovine α_{S1}-casein is characterized by the absence of cysteine residues in its molecular structure. Furthermore, no structural and functional homolog of animal α_{S1}-casein was observed in human milk. This is a major cause of the immunogenicity of this protein for humans and occurrence of CMA [23].

- **α_{S2}-Casein:** its concentration in milk is relatively low (3 g/L). It is composed of 207 amino acid residues and has a MW of 24.4 kDa and pI of 4.78. α_{S2}-Casein is the most hydrophilic of the caseins: it has 11 phosphorylated serine residues and is characterized by the presence of two cysteine residues (residues 36 and 40) creating intramolecular disulfide bridges. Hence, this casein is found in milk partly in dimeric form: two polypeptides which are linked by two disulfide bridges [24]. Four genetic variants were observed including variants A, B, C and D, while the A variant is the most common [23].

- **β-Casein**: is a phosphoprotein with a MW of 23.5 kDa, composed of 209 amino acid residues with a pI of 4.49. The concentration of this protein in cow milk is 11.7 g/L. 12 genetic variants were found for β-casein while the most common variants are A1, A2 and B. Homolog protein with similar structure and physico-chemical properties as bovine β-casein was found in human milk suggesting that this casein is the least allergenic casein in cow milk [23, 25].

- **κ-Casein:** is found in cow's milk at a concentration of 4.4 ± 0.3 g/L; thus, representing 13% of bovine caseins [26]. It is the least phosphorylated and the only glycosylated casein in milk from all mammalian species. The κ-casein has 169 amino acid residues with a MW of 18,974 kDa and a pI of 3.97. Furthermore, κ-casein has a particular amphipolar structure with a C-terminal which contains carbohydrate residues with a hydrophilic character and a hydrophobic N-terminal. It is also characterized by low calcium binding ability due to the presence of a single phosphorylation site at the residue 149. The κ-casein exhibits several biological functionalities such as anticoagulant properties as well as the prevention of platelet agglomeration and serotonin secretion [18].

2.2 Whey proteins

Soluble protein fraction or whey protein, is the second main protein fraction in milk (20–25% (w/w) of total protein). Overall, the protein composition of whey varies depending on the mammalian species. For cow's milk whey, the protein composition is as follows: β-lactoglobulin is the main protein (~56%), followed by α-lactalbumin (~21%), immunoglobulins (14%), bovine serum albumin (BSA) (7%) and lactoferrin (2%) (**Table 1**, **Figure 2**).

- **β-Lactoglobulin (β-Lg)** is a globular protein, present in the milk of all mammalian species except camelids, rodents and humans. The biological function of this

	Proteins	Allergen name	Molecular mass (kDa)	pI	Relative amount [a,b]	Amino acid residues	Allergenic activity (% of patients)[c]
Caseins, 80% (w/w) of total protein	α_{S1}-Casein	Bos d9	22.9	4.46	38%	199	57%
	α_{S2}-Casein	Bos d10	24.4	4.78	10%	207	
	β-Casein	Bos d11	23.5	4.49	39%	209	
	κ-Casein	Bos d12	18.9	3.97	13%	169	
Whey proteins, 20–25% (w/w) of total protein	β-Lg	Bos d5	18.28	5.2	56%	162	66%
	α-La	Bos d4	14.18	4.65	21%	123	18%
	BSA	Bos d6	66.4	4.7	7%	583	
	Lf	Bos d lactoferrin	76.1	8.18	2%	689	—
	Ig	Bos d7	150–800	5.5–7.5	14%	240–250 amino acids of the heavy chain	—

Abbreviations: pI: isoelectric point, α-La: α-lactalbumin, β-Lg: β-lactoglobulin, BSA: bovine serum albumin, Lf: lactoferrin, Ig: immunoglobulins.
[a]Proportion of individual caseins in the whole casein fraction of milk.
[b]Percentage of globular whey protein in the soluble fraction of milk.
[c]The allergenicity of the main proteins as reported by El-Agamy and Peñas et al. [13, 27].

Table 1.
Physico-chemical characteristics of the main cow's milk proteins and their allergenic activity (% of patients) [18].

protein is to transport the fatty acids, retinol and vitamins (A, D), binding Cu^{2+} and Fe^{2+} ions and inhibiting autooxidation of fats during digestion [28]. The β-Lg is the major protein in the soluble fraction of cow's milk with a concentration ranging between 2 and 4 g/L representing approximately 56% of the total bovine whey proteins [29]. The secondary structure of β-Lg consists of 10% α-helices, 45% β-sheets. It has two disulfide bonds at the cysteine residues (Cys106-Cys119 and Cys66-Cys160) and one free cysteine (Cys121) [30]. This protein is characterized by different quaternary structures depending on the environmental conditions of the protein (pH, temperature, ionic strength). The β-Lg, the primary structure comprises 162 amino acid residues with a MW of 18.281 kDa and a pI of 5.2. The β-Lg is an allergenic protein present due to its highest proportion among whey proteins (56% of total whey proteins) and due to the fact that this protein is totally absent in human milk [11, 31]. Food allergies associated with this allergenic protein may be present even in 80% of the total population [32].

- **α-Lactalbumin** (α-La): The α-La is a less allergenic protein than β-Lg and constitutes 21% of total whey protein [25]. Furthermore, chemical composition of bovine and human *α*-La bears a strong resemblance. This protein is a small protein of 123 amino acid residues (14.186 kDa, pI 4.65) known for its high content in the essential amino acids and for its important role in the biosynthesis of lactose with lactose synthetase and UDP galactosyl-transferase [33]. The α-La is a metalloprotein that contains one Ca^{2+} atom per mole of protein molecule, a divalent cation that plays an important role in stabilizing its spatial structure. The binding of this calcium ion is affected by the acid functions of the aspartic acid residues located

in position 82, 87 and 88. There is also a second calcium binding site occupied by the zinc, but which has an affinity 105 times lower than that of calcium. The α-La contains 4 disulfide bridges (Cys6/Cys120, Cys28/Cys111, Cys61/Cys77 and Cys73/Cys91) but no free thiol groups. This configuration makes it more resistant to the phenomenon of protein aggregations caused by heat treatment, even though its denaturation temperature is relatively low (~64°C) [34].

The tertiary structure of this protein contains

- a β domain formed by β sheets. This domain has 10 Asp residues: it is acidic and represents the binding site of the Ca^{2+} ion, its pI is 3.37.

- an α domain consisting of four α helices forming a hydrophobic core. This domain is basic, containing 9 Lys residues with a pI 9.6

- **Lactoferrin** (Lf) is a protein synthesized by secretory epithelial cells of the mammary gland. It is a glycoprotein that belongs to the transferrin family containing two iron cation binding sites and more preferably the ferric ion (Fe^{3+}). This ability to scavenge for iron ions persists even at low pH values in the stomach and intestines, in order to deplete free iron which could slow bacterial growth in the intestine [35, 36]. The concentration of Lf in milk varies according to the producing animal species and according to the stage of lactation. The main function of this protein is binding iron and transporting it to the intestinal vascular system. Lf supports immune systems functionality, detoxification processes, as well as antineoplastic effect by inhibiting the attachment of tumor growth factors [37].

- **Bovine Serum Albumin** (BSA)—similarly, to caseins, β-Lg and α-La, this protein may also be a milk allergen. BSA is a whey protein characterized by its relatively high molecular mass. Indeed, bovine serum albumin (BSA) consists of 583 amino acids residues with a molecular mass of 66.4 kDa, its primary sequence has been determined by Hirayama et al. [38]. It has 17 intramolecular disulfide bridges and one free thiol group. This protein is present with w relative low concentration of 0.36 g/L in cow milk. This protein is inactivated at a temperature of 70–80°C. Among all cow's milk proteins probably only bovine serum albumin remains immunoreactive after heat treatment [25].

- **Lactoperoxidase** and **lysozyme** are active enzymes with antibiotic-like activity. For the lactoperoxidase, it is an oxidoreductase with antibacterial function, antineoplastic agent and viral growth inhibitor. On the other hand, lysozyme in milk has antiviral and anti-inflammatory properties.

3. Allergenicity of cow's milk proteins

Cow's milk contains approximately 30–35 g/L of proteins divided into 30 proteins, some of them are potentially allergenic and called "Bos d" and numbered according to the protein type [39].

The main cow milk allergens in are caseins (Bos d8) including β-casein (Bos d11), α_{S1}-casein (Bos d9), α_{S2}-casein (Bos d10) and κ-casein (Bos d12). On the other hand, whey consists of high allergenic proteins including α-La (Bos d 4), β-Lg (Bos d 5), immunoglobulins (Bos d7), BSA (Bos d6) and traces of Lf (Bos d Lf) [31, 40].

Scientists confirmed that the most commonly allergens which are usually detected in cow milk allergic patients are whole caseins especially the α_{S1}-casein (Bos d9), β-Lg (Bos d5) and α-La (Bos d4). Indeed, 66% of CMA is caused by the main cow milk allergen which is the β-Lg, followed by caseins (Bos d8) and significantly less by α-La and BSA (18%) [13, 25]. The high allergenicity of the β-Lg is attributed to the fact that this protein is totally deficient in human milk. Indeed, IgE response against β-Lg precedes those against the other allergens including caseins and α-La since birth. Afterwards, before the age of 1 year, IgE response toward caseins becomes predominant, whereas, the IgE response to α-La appears later after the age of 1 year [41].

However, the major problem of CMA is the fact that that only 27% of total patients with CMA are allergenic to only one allergen, meanwhile, the other patients present sensitization to two and more cow milk allergens leading to conclude that none of the main milk proteins allergens can be considered as the only responsible for the allergenicity of this food [11]. IgE do not react entirely with the antigenic protein but only with its allergenic part which is called epitope. Hence, one allergenic protein may have several epitopes, which might be the same or different depending on its quaternary structure and its exposed allergenic peptides. Epitopes of proteins molecules include immunodominant epitopes, which are the high allergenic epitopes and the main targets of immune response system. Allergy can not only be caused through bloodstream by the absorption of allergen but also by direct skin contact with the allergen [13].

The allergenicity of proteins, as well as the IgE epitopes of milk proteins, can be mapped and carried out using various bioinformatic tools through an *in silico* analysis. Overall, the primary protein sequences were taken from the UniProtKB protein Blast database, while the three-dimensional structures (downloadable as a pdb file) are listed in the PDB Protein Data base.

PD index, Bepipred, AlgPred, Discotope-2.0, Ellipro (prediction of linear and discontinuous epitopes) are some bioinformatic tools to study the allergenicity of proteins:

1. *Measuring the PD index (PD index)* using the physico-chemical properties of amino acids rather than their substitution frequencies in related proteins. Peptides or proteins with PD values less than 10 are considered to have significant physico-chemical similarities [42].

2. *BepiPred* predicts the location of linear B cell epitopes using a combination of a hidden Markov model and a propensity scale method. Peptides or proteins with a score greater that a value of 0.35 are suggested to be part of an epitope and stained yellow on the graph (where the Y axes represent residue scores and residue positions on the X axes in the sequence) [43].

3. *AlgPred* can be used for the prediction of the binding between the antigenic determinant and IgE. AlgPred can predict allergens by amino acid sequences by citing representative peptide sequences of allergens and this is based on the similarity of the known epitope to any region of protein. The SVM (Support vector

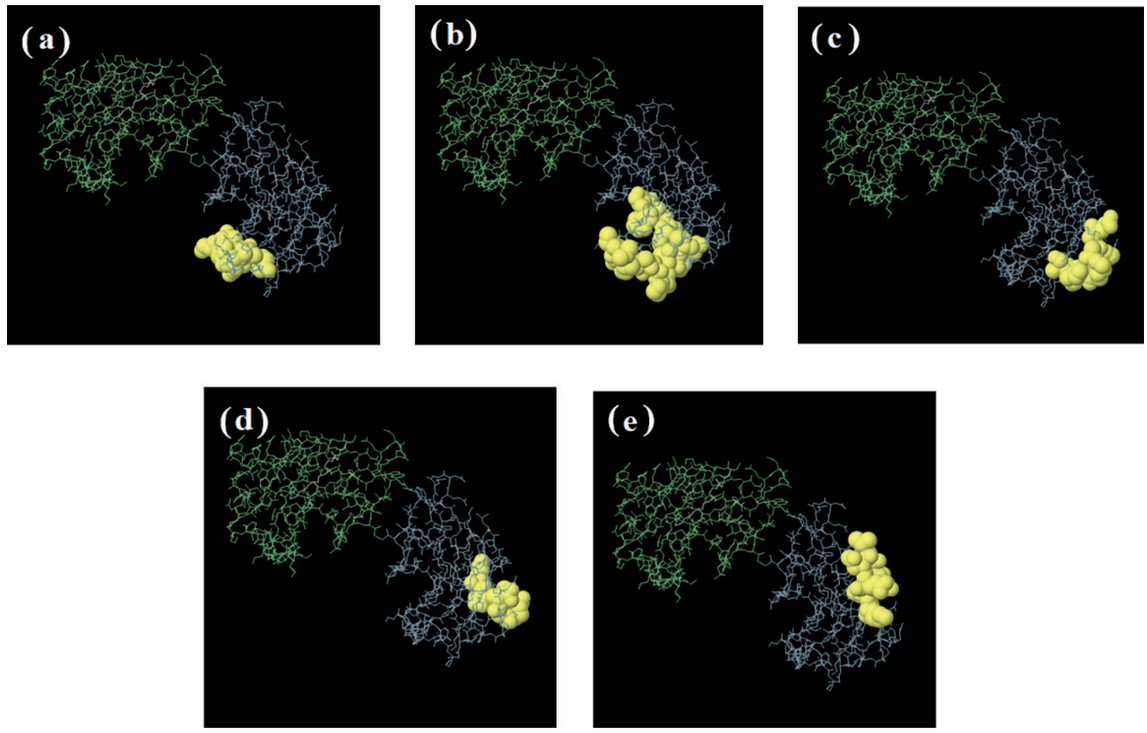

Figure 3.
An example of discontinuous B-cell epitopes predicted by the ElliPro. (a–e) Three-dimensional representation of conformational or discontinuous epitopes of bovine β-Lg. The epitopes are represented by yellow surface, and the bulk of the protein is represented in gray sticks.

machine) method of AlgPred calculates the allergenicity score of the protein that qualifies as "Allergen" for a score ≥ −0.5 [44].

4. *Discotope-2.0* is used for the prediction of discontinuous B cell epitopes from the 3D structure of the protein (pdb file). The method uses the calculation of surface accessibility and presented in terms of contact numbers. Final scores are calculated by combining the propensity scores of the spatial proximity residuals and the contact numbers [45].

5. *Ellipro* can also be used for the prediction of epitopes with a risk of cross-reaction between proteins. Ellipro can predict the protein's epitope based on peptides with a high allergenicity score as shown in **Figure 3**. Ellipro also makes it possible to present the potential epitopes on a 3D structure of the protein [46].

4. The effect of different processes on the allergenicity of cow's milk proteins

Food processing and additional ingredients cause changes in immunodominant epitopes and hence, the allergenic properties of proteins. Food processing may lead to the destruction of epitopes structures and/or the formation of new epitopes which are called neo-allergens. On the other hand, food processing can be associated with the reduction of allergenic properties of proteins or/and can have no influence on their allergenicity, it can even increase the immunogenicity of the treated proteins by the appearance of new epitopes [47].

Type of technological process	Operating conditions	The effect of process of the allergenicity of cow milk protein	The reference
Heat treatment	Pasteurization 90°C during 15 s	Low decrease on the immunoreactivity of whey proteins such as α-La and β-Lg	Wróblewska and Jędrychowski [50]
	Pasteurization 90°C during 15 min		
	Ultrasound at 52°C during 60°C	A significant reduction of the immunorectivity of α-La and β-Lg	
	Heat treatment at 80°C and 90°C during 30 min	The reduction of the allergenicity of immunoglobulins in milk	Jost et al. [51]
	Heat treatment at 120°C for 20 min	The reduction of the allergenicity of α-La by 25% compared to the native protein	Kleber and Hinrichs [52]
	Heat treatment of the cow milk proteins powder at 500°F (260°C) for 3 min	• 68% of children (n = 100, mean age, 7.5 years; range, 2.1–17.3 years) tolerated heated milk. • Smaller skin prick test wheals for heated milk-tolerant subjects • Lower milk-specific and casein specific IgE and lower IgE/IgG4 ratios to both of caseins and β-Lg (compared subjects with allergy to heated milk)	Nowak-Wegrzyn et al. [53]
High-pressure-processing	High-pressure treatment at 200–600 MPa and (temperature between 30°C and 68°C)	An increase of the antigenicity of the treated β-Lg in the WPI solution, sweet whey and skim milk	Kleber and Hinrichs [52]
	High-pressure treatment at 200 and 400 MPa	• The increase of the binding to β-Lg specific IgG from rabbit, • No effects on the IgE from allergic patients	Chicón et al. [54]
	High-pressure treatment at 600 MPa	The distribution of the structure of casein micelles and the decrease of the immunogenic capacity of milk proteins	Bogahawaththa et al. [55]
	High-pressure treatment at 400 MPa during 50 min	The loss of the allergenicity of the β-Lg hydrolysates with chymotrypsin by the absence of anaphylactic symptoms	López-Expósito et al. [56]

Type of technological process	Operating conditions	The effect of process of the allergenicity of cow milk protein	The reference
Enzymatic hydrolysis	Trypsin alone or in combination with both of chymotrypsin and pepsin	The reduction of the allergenicity of β-Lg without eliminating it	Bonomi et al. and Monaci et al. [57, 58]
	The combination of pepsin and *α*-chymotrypsin	The reduction of allergenicity by selective proteolysis of both *α*-La and β-Lg with a degree of hydrolysis of 1–20% and depending and incubation time	Monaci et al. [58]
	Trypsin	Only 4/10 patients (n = 10) had IgE antibodies to undigested β-Lg, while all 10 patients had IgE antibodies to β-Lg hydrolysates	Haddad et al. [59]
		The increase of the allergenicity of β-Lg: the derived peptides showed a specificity to bind human IgE by ELISA assays.	Selo et al. [60]
	Pepsin in the pH range 2–4	No differences were found in the antigenic properties of the hydrolysates of α-La, β-Lg, BSA and immunoglobulin G at pH 2 or 3, An enhancement of antigenicity of all proteins at pH 4 except β-Lg	Schmidt et al. [61]
	Corolase 7092	The increase of the antigenicity of proteins including BSA and immunoglobulin G	Ena et al. [62]
The lactic acid fermentation	*Lactococcus lactis* ssp. *lactis* 136	Immunoreactivity of raw milk: 0.10% for α-La and 3.36% for β-Lg	Wróblewska and Jędrychowski [50] and Miciński et al. [25]
	Lactobacillus casei 2	Immunoreactivity of raw milk: 0.56% for α-La and 2.18% for β-Lg	
	Lactobacillus acisophilus 67L	Immunoreactivity of raw milk: 0.09% for α-La and 1.46% for β-Lg	
	Lactobacillus delbruecki ssp. *bulgaricus* S11	Immunoreactivity of raw milk: 0.09% for α-La and 1.46% for β-Lg	

Abbreviations: α-La: α-lactalbumin, β-Lg: β-lactoglobulin, BSA: bovine serum albumin, WPI: whey protein isolate.

Table 2.
The effect of food processes (heating, high pressure, enzymatic hydrolysis and lactic acid fermentation) on the allergenicity of cow milk proteins.

4.1 The effect of heat treatments

Heating is an important process in the manufacturing of dairy products in order to obtain bacteriologically safe products leading to extend their shelf life. During the heating process, various structural modifications occur in the milk proteins depending on temperature, heating time, and heating exchanger. The structural and chemical changes in heating milk proteins such as denaturation, aggregation and "Maillard reaction" may have significant impacts on the antigenicity level of milk proteins [3, 48]. Among cow's milk proteins, caseins are the most heat stable proteins contrary to globular whey proteins which are sensitive to heat treatment and start to denaturate at temperatures above 60°C in the following order: BSA (denaturation temperature 94.9°C) < β-Lg (denaturation temperature 79.6°C) < α-La (denaturation temperature 70.5°C) [49].

Sterilization and pasteurization, which are the major categories of thermal processes have a significant impact on structural and functional properties of milk proteins leading to the increased, reduced or similar allergenicity. of allergenicity [50]. Wróblewska and Jędrychowski [50] noted that pasteurization of milk at 90°C resulted in a low decrease on the immunoreactivity of whey proteins such as α-La and β-Lg, while ultrasound treatments at 52°C during 60 min reduced greatly the immunorectivity of these proteins (**Table 2**). On the other hand, Jost et al. [51] reported that heating whey proteins at a temperature ranging between 80°C and 90°C during 30 minutes reduces the immunoglobulins contents as well as their immunogenicity.

Other researches carried out with bovine whey proteins confirmed that the antigenicity of β-Lg and α-La increases when heating temperature rose from 50 to 90°C because of the exposure of allergenic epitopes buried inside the native molecule due to the unfolding of conformational structure during heat denaturation. However, the antigenicity of these proteins decreased significantly above 90°C. Furthermore, the antigenicity of α-La decreased by 25% compared with its native state when it is treated at 120°C for 20 min [52, 63].

Other researches have evaluated whether children (n = 100) with CMA can tolerate extensively heated milk proteins and they found that approximately 68% tolerated the extensively heated milk. Furthermore, Heated milk-tolerant subjects showed significantly smaller skin prick test wheals, lower milk-specific and casein specific IgE as well as lower IgE/IgG4 ratios to both of caseins and β-Lg when compared subjects with allergy to heated milk [53]. Hence, some manufacturers use denatured whey proteins for the production of hypoallergenic infant formulae [25].

4.2 The effect of high-pressure processing

High-pressure processing is considered as a suitable nonthermal alternative method for milk pasteurization when it is in the range of 300–600 MPa [64]. High-pressure processing can even preserve the organoleptic and nutritional properties of the treated foods. However, this process can also alter structural and physico-chemical characteristics of proteins and result in their denaturation of native milk proteins. Indeed, high-pressure leads to the denaturation of whey proteins as the β-Lg and the changes of the casein micelles structures by their disassociation [55]. These changes may also influence the allergenicity of milk proteins. For instance, high-pressure treatment (200–600 MPa) at a temperature ranging between 30 and 68°C increased the antigenicity of β-Lg in the WPI (whey protein isolate) solution, sweet whey and skim milk [52]. Another study indicated that the high-pressure processing caused a

severe whey protein denaturation especially the β-Lg and the minor whey proteins (Immunoglobulins) but no effect was observed for the α-La. Indeed, a high-pressure processing at 600 MPa induced the formation of large protein aggregates involving both of β-Lg and κ-casein through the thiol/disulphide interchange reactions. Furthermore, this treatment can disturb the structure of casein micelles leading the alteration of the immunogenic capacity of milk proteins diminished at 600 MPa [55]. Chicón et al. [54] found that the high pressure treatment of the pure β-Lg and whey protein isolate solution at 200 and 400 MPa resulted in an increase of the binding to β-Lg specific IgG from rabbit, without any effect on the IgE from allergic patients (**Table 2**). This behavior can be explained by the exposure of the buried epitopes in the unfolded protein molecules becoming more accessible for the antibodies.

Several researches focused on the effect of high-pressure on milk proteins hydrolysates. For instance, it was reported that a significant high degree of hydrolysis was levels obtained in high pressure (600 MPa), in comparison to atmospheric pressure depending upon the used enzyme. This behavior is attributed to the increased enzyme availability of immunogenic hydrophobic areas which, as a result, intensifies hydrolysis [25, 57].

On the other hand, hydrolysates obtained via the enzymatic treatment of main allergen in cow milk: β-Lg under high-pressure may result in a lower antigenicity and IgE binding ability [3, 57]. Indeed, the evaluated *in vivo* allergenicity of the β-Lg hydrolysates with chymotrypsin indicated that the tested hydrolysates with high-pressure treatment at 400 MPa during 50 min resulted in the loss of the allergenicity of the studied protein by the absence of anaphylactic symptoms. These results demonstrate the safety of hydrolysates produced under high-pressure conditions for manufacturing of novel milk formulae [56].

Other studies carried out with milk protein hydrolysates have also reported that the application of high-pressure treatment during enzymatic hydrolysis can significantly reduce the antigenicity of the treated proteins due to the increase of accessibility of the potentially immunogenic regions to the enzyme [3, 27, 54, 57].

4.3 The effect of enzymatic hydrolysis

Proteolysis have been usually considered as an efficient process to reduce allergenicity of milk proteins by destroying their allergenic epitopes [65]. The enzymatic hydrolysates were prepared with the use of digestive enzymes including pepsin, trypsin and chymotrypsin in order to imitate potential digestion processes and to reduce intestinal activity and the activity of enzymatic system in children [58]. However, the differences in the types of enzymes in this process as well as hydrolysis model and the hydrolysis degree may result in some discrepancies in the composition of the resulted peptide and a residual antigenicity of the hydrolysates as well as their taste [3]. Previous researches showed that the overall antigenicity of whey protein can be reduced by hydrolysis with trypsin alone. Caseins including α-casein and β-casein also show sensitivity to trypsin (unlike immunoglobulins and BSA). However, Nakamura et al. [66] noted that using many enzymes at the same hydrolysis process including papain, neutrase, alcalase and protease is more efficient in reducing the allergenicity of whey proteins when compared to those treated with a single enzyme. Thus, the hydrolysis of β-Lg by trypsin alone or in combination with chymotrypsin and pepsin.

It was proved that hydrolysis of β-Lg (Bos d5) by trypsin alone or in combination with both of chymotrypsin and pepsin reduces its allergenicity without eliminating

it, while the combination of both of enzymatic hydrolysis and heat treatment was reported to reduce greatly the allergenicity of β-Lg [57, 58].

The combination of pepsin and *α*-chymotrypsin is considered as the most effective combination of enzymes used for the reduction of allergenicity and act by a selective proteolysis of both allergens *α*-La (Bos d4) and β-Lg (Bos d5) with a degree of hydrolysis of 1–20% and depending and incubation time [58].

An innovative technique of preparing hypoallergenic formulae for newborns involves the combination of hydrolysates and probiotics, which reduces allergic symptoms. Probiotics, including *Lactococcus lactis*, *Lactobacillus rhamnosus* and *Bifidobacterium lactis* significantly reduced the severity of atopic dermatitis in breast-fed infants after 2 months of treatment. Indeed, probiotics probably participate in mucosal degradation of macromolecules, leading to reduced allergenicity of milk proteins [25, 67–69].

Despite all these advantages of the hydrolysis of milk proteins for the reduction of their allergenicity, some researches confirmed the increase of the allergenicity of proteins by the exposure of new epitopes that appeared upon hydrolysis treatment. For instance, Haddad et al. [59] detected serum IgE from allergic patients using radioallergosorbent tests with a total tryptic hydrolysate of β-Lg (Bos d5) even when no IgE response was detected with the native protein of β-Lg (**Table 2**). Schmidt et al. [61] reported that no differences were found in the antigenic properties of the whey protein hydrolysates including α-La, β-Lg, BSA and bovine immunoglobulin G at pH 2 or 3, whereas, at pH 4 a further decrease in pepsin hydrolysis resulted in enhancement of antigenicity of all these proteins except the β-Lg. In the same way, the enzymatic proteolysis with Corolase 7092 was reported to increase the antigenicity of proteins including BSA and immunoglobulin G by exposing more antigenic sites during hydrolysis [62]. *In vitro* tests of Selo et al. [60] showed that that tryptic hydrolysis retained and even enhanced the allergenicity of β-Lg. In fact, the derived peptides showed a specificity to bind human IgE by ELISA assays. These authors also noted that numerous epitopes are widely scattered all along the β-LG molecule. They may be located in hydrophobic parts of the protein molecule, inaccessible for IgE antibodies in the native conformation of the protein but become bio-available after hydrolysis processes [60].

4.4 The effect of fermentation

The lactic acid fermentation process may have a potential influence on the allergenicity of cow milk proteins. Thus, researches were conducted with the use of several mesophilic and thermophilic bacterial strains which are already used in the production of fermented dairy products [25]. This process did not show a significant influence on the allergenic properties; indeed, the *in vitro* studies were not consistent with those *in vivo*.

Many studies have reported that Lactobacillus fermentation can induce degradation of milk allergens. For instance, lactic acid bacteria *Lactobacillus casei*, which are defined as probiotics [58]. In the same way, *Lactonacillus rhamnosus* GG has the ability to reduce phagocytosis which is stimulated by milk allergens by blocking receptors involved in phagocytosis on neutrophils and monocytes. It can even modify clinical symptoms in children with dermatitis and eczema [25, 70].

Clinical investigations have noted that dietary consumption of fermented foods, such as yogurt, can alleviate some of the symptoms of atopy and might also reduce the development of allergies through a mechanism of immune regulation. The

consumption of fermented milk cultures containing lactic acid bacteria can enhance the production of both Type I and Type II interferons at the systemic level [71]. However, changes of cow milk protein antigenicity and allergenicity depend on the species of lactic bacteria as well as the conditions of fermentation (**Table 2**). Lactic acid fermentation can reduce 90% of the antigenicity of the β-Lg in skim milk and 70% of this protein in sweet whey compared with untreated samples [52].

Finally, the reduction in antigenicity suggested that during the fermentation process with *Lactobacillus*, some epitopes of proteins were destroyed. These results are very useful for the preparation of new fermented milk products with reduced antigenic properties [3].

5. Conclusion

Cow's milk is a high nutritious food. However, it should be noticed that it contains many proteins which are considered as major food allergens leading to induce allergic reactions especially in infants.

The challenge for the food scientist, nutritionists and physicians is to resolve the problem of the CMA by searching new cow milk alternatives and/or new dairy processes that may reduce the allergenicity of cow milk proteins. Some processing technologies (heating, high pressure, enzymatic hydrolysis and lactic acid fermentation) can be used to effectively reduce the allergenicity of milk proteins by optimizing and controlling the processing conditions. However, attention should be paid during modification of milk proteins upon the used processes in order to prevent the appearance of some new epitopes during processing which are buried inside the native molecule. On the other hand, *in vitro* tests should be carried out to further detect the residual allergenicity of proteins and ensure the edible safety of milk products obtained by processing technologies. These strategies should provide valuable support for the development of the hypoallergenic milk formulae especially to infants.

Author details

Roua Lajnaf[1,2*], Sawsan Feki[2], Hamadi Attia[1], Mohamed Ali Ayadi[1] and Hatem Masmoudi[2]

1 Alimentary Analysis Unit, National Engineering School of Sfax, Sfax, Tunisia

2 Immunology Department, Habib Bourguiba University Hospital, Sfax, Tunisia

*Address all correspondence to: roua_lajnaf@yahoo.fr

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