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Allergenicity of Milk Proteins

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1. Introduction

Adverse reactions to food are currently classified into toxic and non-toxic reactions. There is a normal range of concentrations of naturally occurring toxic compounds, which can easily increase during food processing. For example, thermal processing can cause the unintended and undesirable formation of toxic compounds, such as acrylamide in fried potato chips and furan in sterilized canned vegetables, together with losses of certain nutrients. The incidence of non-toxic reactions depends on individual susceptibility to a specific food or food ingredient, although these reactions are often dose-dependent. The non-toxic types may be divided further into immune- and non immune-mediated reactions. The term “hypersensitivity” is used for immune-mediated reactions, and the term 'intolerance' is used for non immune-mediated reactions (Figure 1). Immune-mediated reactions may be IgE-mediated (i.e., allergy or type I hypersensitivity) or non-IgE-mediated, whereas food intolerance may be enzymatic, pharmacologic or undefined. The incidence of immune-mediated adverse reactions to foods has increased in recent decades. In healthy subjects, orally ingested dietary proteins induce antigen-specific systemic hyporesponsiveness, termed oral tolerance. This phenomenon is well described in animal models, although the mechanisms remain unknown. Abrogation of oral tolerance or failure to induce oral tolerance may result in the development of food hypersensitivity. Immune reactions that cause tissue damage may be mediated by four reaction types that were defined by Coombs and Gell [1] (Figure 2). Type I, or anaphylactic hypersensitivity, is mediated by the reaction of an antigen with specific IgE antibodies that are strongly bound through their Fc receptor (CD23, IgεR) to the surface of the mast cell. Crosslinking of Igε receptors by divalent hapten leads immediately to the release of mediators, for example, some cytokines [primarily interleukin 4 (IL4)] and histamine, which are both activities of eosinophil chemotactic factor (ECF) and neutrophil chemotactic factor (NCF) (Figure 2A). This type of hypersensitivity occurs within minutes of antigen exposure. Some reaction mediators produce local skin,

gastrointestinal and respiratory tract manifestations, and a systemic allergic reaction to an allergen that is associated with a dangerously low blood pressure. Moreover, some of the mediators exhibit chemoattractant activity and induce the infiltration of neutrophils, eosinophils, macrophages, lymphocytes and basophils within 6-12 h after challenge. The localized late-phase inflammatory response may also be mediated partly by cytokines that are released from mast cells. In type II or antibody-dependent cytotoxic hypersensitivity, antibodies recognize antigens on the surface of specific cells or tissues. Once activated, the complement system can initiate a variety of responses that can lyse and destroy cells. Phagocytic and cytotoxic K cells, which have receptors for the Fc-part of IgG or an activation component of complement, i.e., C3b, may also destroy cells (Figure 2B). In type III, or immune complex-mediated hypersensitivity, the soluble antigen can activate the complement and deposited phagocytes. Leucocytes may release tissue-damaging mediators and activate phagocytes, culminating in tissue damage. The complex can also induce thrombin-mediated platelet aggregation and release a vasoactive amine (Figure 2C). Type IV reaction, or delayed-type hypersensitivity, arises more than 24 h after an encounter with the antigen. Type IV reactions are mediated by antigen-sensitized CD4+ T cells (T helper cells) that release inflammatory mediators [e.g., IL2 and interferon- γ (IFN- γ)], attract phagocytes to site of infection, activate an inflammation response and lyse invading cells (Figure 2D). Because lytic enzymes are secreted from the phagocytic cells into the surrounding tissue, localized tissue destruction can progress.

CLASSIFICATION AND TERMINOLOGY OF ADVERSE REACTION TO FOOD

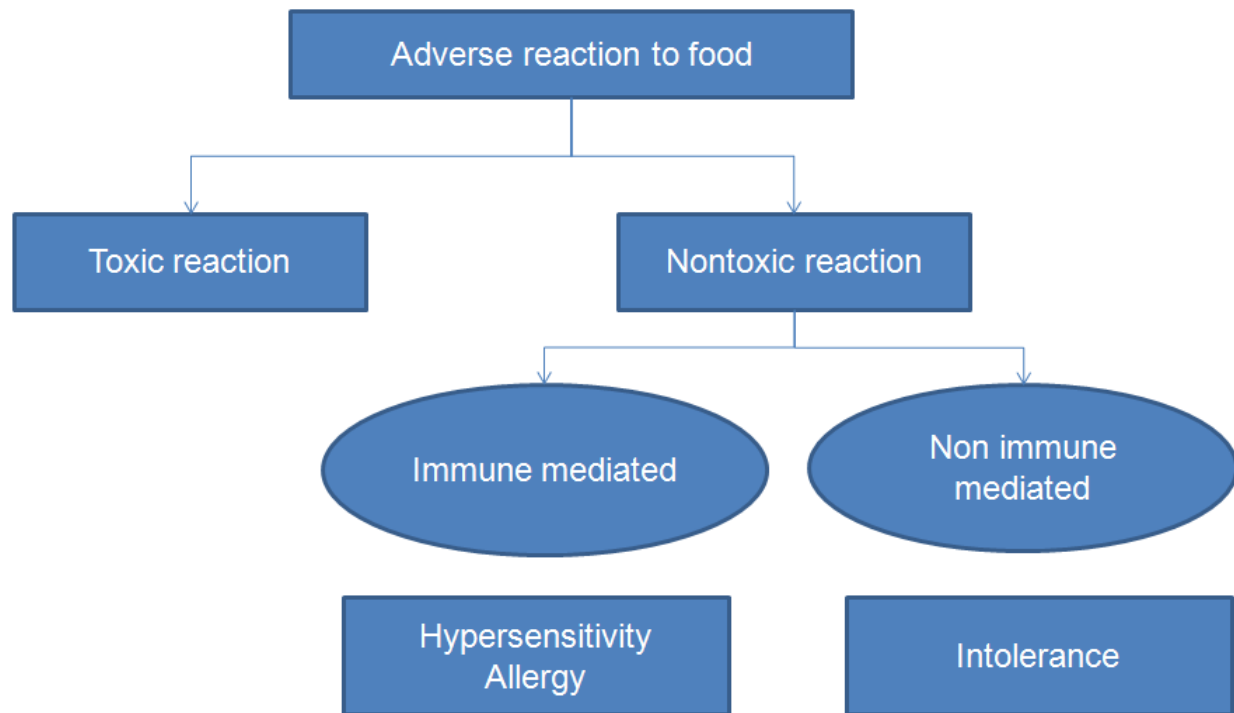


Figure 1.

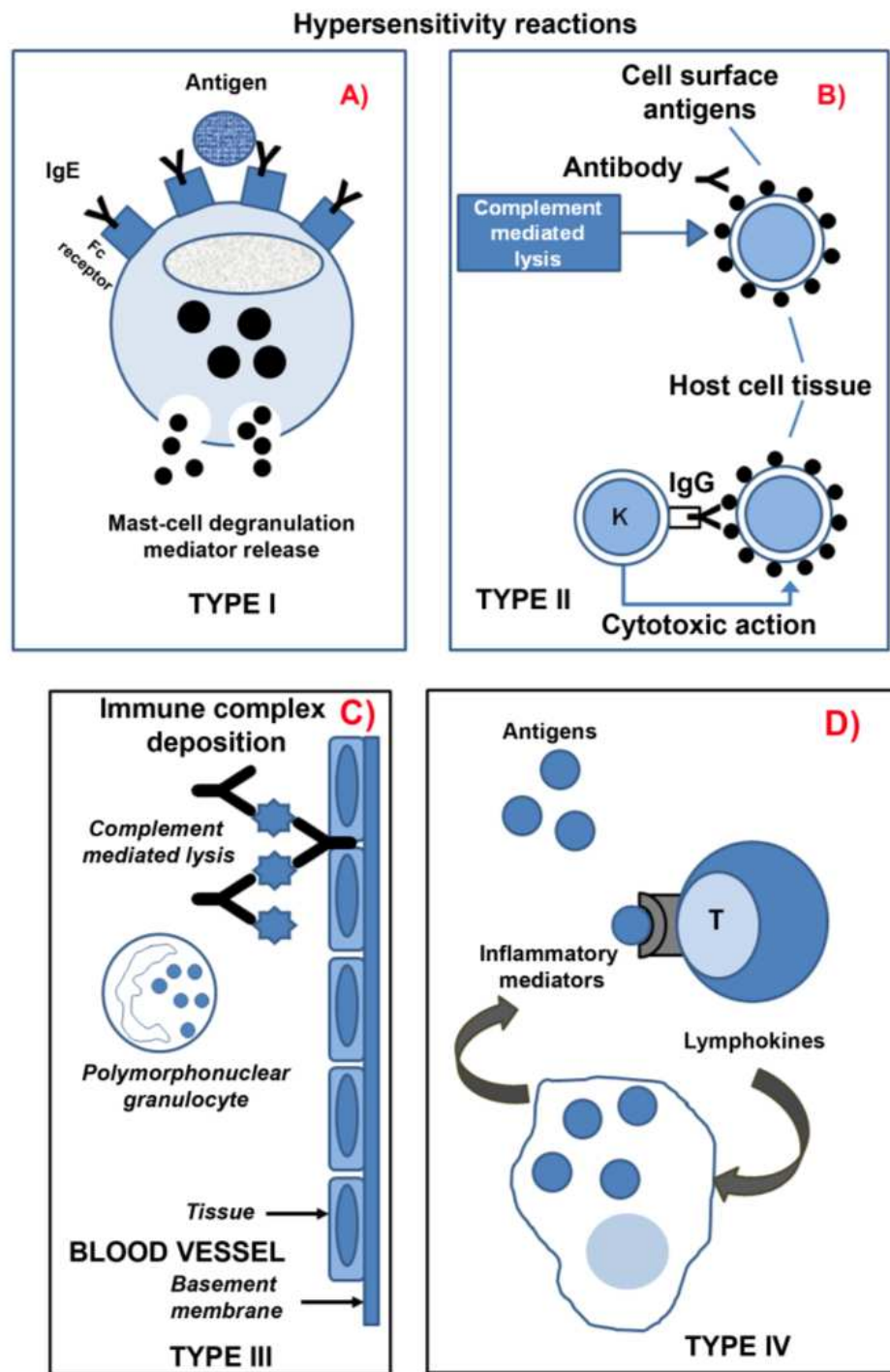


Figure 2. Types of Allergy Mechanisms according to Coombs and Gell [1].

The mechanisms underlying of allergic sensitization to food include genetic susceptibility, aberrant barrier functions of the skin epithelium and gut mucosa and dysregulation of immune functions. Despite a wide range of clinical manifestations, there are at least two common prerequisites for the development of a general food allergy (FA). First, intraluminal antigens must penetrate the mucosal barrier of the intestine. Second, the absorbed antigens must cause harmful immune responses [2].

2. Adverse reaction to milk

Non-toxic adverse reactions to milk are primarily caused by either lactose intolerance or milk allergy.

2.1. Lactose intolerance

Milk intolerance is due to the inherited lack of the specific enzyme, β -galactosidase that is required to hydrolyze lactose. For lactose malabsorption, the most common therapeutic approach excludes lactose-containing milk from the diet. To make yogurt edible, exogenous β -galactosidases that hydrolyze yogurt lactose or probiotics for their bacterial lactase activity are added. However, further studies are required to clarify the role probiotics play in lactose intolerance therapy, which includes considering their well-known beneficial effects on intestinal function, gas metabolism and motility [3]. A prolonged contact time between β -galactosidase and lactose delays the gastrointestinal transit time and chronic lactose ingestion to improve colonic adaptation. It is known that high concentrations of β -galactosidase are physiologically present in neonates, but a genetically programmed and irreversible decline of the activity occurs after weaning [4], which results in primary lactose malabsorption. The secondary hypolactasia because of intestinal mucosa brush border damage that increases the gastrointestinal transit time is a transient and reversible condition [5]. Bloating, flatulence, abdominal pain, the passage of loose and watery stools, excessive flatus and diarrhea are gastrointestinal symptoms of lactose intolerance [6]. However, lactose occurring in the colonic lumen does not necessarily produce gastrointestinal symptoms because of the variable amount of residual intestinal mucosal lactase activity that possibly digests lactose. The availability of recombinant β -galactosidase as an exogenous lactase has resolved problems concerning bacteria that release β -galactosidase during gastric passage. By this means, lactose is partially or fully degraded to glucose and galactose and is therefore easily eliminated by simple dietary adjustments that mediate the effects of lactose intolerance [7]. An accurate diagnosis of lactose intolerance can significantly reduce patient anxiety and avoid unnecessary examinations and treatments [8].

2.2. Milk allergy

Cow's milk allergy (CMA) is a complex disorder that implies an immunologically mediated hypersensitivity reaction with varying mechanisms and clinical presentations. The type I reactions appear to be the most common immune reaction to milk. However, the dominance of IgE reactions (Type I) may be an artifact as the reaction is easy to diagnose because of an immediate IgE measurement, whereas other reaction types are more difficult to diagnose. Non-IgE-mediated hypersensitivity has been increasingly diagnosed, and it is likely that several mechanisms operate in an individual patient. In clinical work, hypersensitivities are classified as IgE- and non-IgE-mediated or as immediate and late reactions based on the appearance of the first types of symptoms [9]. Cow's milk is a member of the "Big-8" food allergens that include egg, soy, wheat, peanuts, tree nuts, fish and shellfish in terms of prevalence [9]. The incidence of CMA varies with age. CMA is prevalent in early childhood

with reported incidences between 2 and 6% [10-12] and decreases into adulthood to an incidence of 0.1–0.5% [13-14]. It has been suggested that infants have milk allergies because milk is usually the first source of foreign antigens that they ingest in large quantities, and the infant intestinal system is insufficiently developed to digest and immunologically react to milk proteins. When milk is eliminated, the inflammation response is controlled. After several years, oral tolerance is developed, and milk can again be tolerated [15]. Most milk-allergic children are considered symptom-free by 3 years of age, but several studies have indicated that older children may also have immune reactions to milk. Children whose CMA has been diagnosed at an age older than 3 years do not tend to outgrow the problem. In adults, CMA is less common than lactose intolerance [16-17], even though it has been reported that approximately 1% of the adult population has milk-specific IgE antibodies. However, studies on CMA in adults are scarce. Little is known about the clinical symptoms, eliciting doses, and allergens involved. It has established that CMA in adults is rare but serious [18]. In a study by Stöger et al. [19], the main target organs in adult CMA were the skin and the respiratory tract. Gastrointestinal (mild to moderate) and cardiovascular (severe) symptoms were observed less often in adults compared with children. Milk allergies and hypersensitivity may be more common now than they were several decades ago. Further, the prevalence of atopic diseases has also increased in recent decades. Because genetic diversity has not changed over a short period, environmental factors are believed to have influenced the phenotype. Such factors may include increased air pollution, such as passive smoking, and dietary factors, such as the duration of breastfeeding, amount of antioxidants and the type of dietary fats (favoring saturated fat and n-6 fatty acids). Another approach is the hygiene hypothesis, which states that early exposure to microbial antigens may reduce the risk of having allergies [20]. For as long as milk allergy and hypersensitivity have been experienced, and still may be, these conditions may be misdiagnosed as a disease other than an allergy/hypersensitivity, particularly if symptoms are delayed. Classical IgE-mediated milk allergies with objectively recorded skin reactions may have been diagnosed easily, whereas hypersensitivity with subjective gastrointestinal reactions may have been diagnosed as lactose intolerance, irritable bowel syndrome or some other intestinal disorder. In adults, viral infection, antibiotic treatment or stress may alter intestinal integrity so that its balance is disturbed and the number of protecting agents, such as microflora and mucosal immunity, are altered [21]. This process may result in milk hypersensitivity. Over one-third of women with IgE-mediated reactions to milk proteins have been reported to exhibit their first symptoms of hypersensitivity during or shortly after pregnancy, and one-quarter reported the first symptoms during a period of severe emotional stress [22].

3. Milk allergens

No single major allergen has been identified in cow's milk according to either challenge tests or laboratory procedures; [23]. Indeed, clinical challenge tests demonstrate that most CMA patients react to several protein fractions of cow's milk and each allergenic protein may have several epitopes, which are widely spread along the molecules. The cow milk proteins prevalently implicated in allergic responses in children are the whey proteins α -

Lactalbumin (α -La)(Bos d 4) and β -Lactoglobulin (β -Lg) (Bos d 5), in addition to the casein (CN) fraction (Bos d 8) [24-26]. In adults, the predominant allergen is CN, whereas sensitization to whey proteins is rare. Biochemical characteristic of allergenic cow milk proteins are reported in Table 1. Currently, milk allergen analyses are generally based on immunoassay methods, such as enzyme-linked immunosorbent assay (ELISA) [27]. Commercial test kits are available for the determination of CN, β -Lg, or total milk proteins (CN and β -Lg) [28]. Interlaboratory studies were performed to evaluate the reliability and reproducibility of these kits [29]. Antibody cross-reactivity has been reported for some milk proteins [30]. Confirmatory tests are required to corroborate ELISA detection results and improve the detection specificity of undeclared milk allergens. For the last several decades, mass spectrometry has become the dominant technology for the identification of peptides and proteins. The primary current approaches used for protein identification are top-down [31-33] and bottom-up [34] sequencing. Top-down sequencing involves introducing the intact protein into the gas phase. The protein is identified by measuring either the protein molecular weight or its fragmentation pattern using various techniques [35]. The bottom-up approach is more common. The sample is usually digested with an enzyme, such as trypsin, followed by accurate sequence analysis by tandem mass spectrometry (MS/MS) of the proteolytic fragments. For protein identification, an algorithm is used for database searching based on amino acid sequence assignments.

Cow's Milk Proteins (100%)	Protein	Allergen Name	Allergenicity	Total Protein %	MW (kDa)	pI	Amino Acid Residues	Calcium sensitivity	Phosphate groups
Caseins (80%)	α_{s1} -Casein	Bos d 8	Major	32	26.6	4.9 - 5.0	199	+++	8-9
	α_{s2} -Casein	"	"	10	25.2	5.2 - 5.4	207	++++	10-13
	β -Casein	"	"	28	24.0	5.1 - 5.4		++	4-5
	$\gamma 1$ -Casein	"	"	Traces	20.5	5.5	181	+	1
	$\gamma 2$ -Casein	"	"	Traces	11.9	6.4	104		
	$\gamma 3$ -Casein	"	"	Traces	11.5	5.8	102		
	κ -Casein	"	"	10	19	5.4 - 5.6	169		
Whey proteins (20%)	α -Lactalbumin	Bos d 4	Major	5	14.2	4.8	123		
	β -Lactoglobulin	Bos d 5	Major	10	18.3	5.3	162		
	Immunoglobulins	Bos d 7	—	3	150	—	—	—	1-3
	BSA	Bos d 6	—	1	66.3	4.9 - 5.1	582		
	Lactoferrin	—	—	Traces	80	8.7	703		

Table 1. Chemical characteristics of cow's milk proteins and their inclusion in the official list of allergens

3.1. Casein

The CN fraction is composed of four proteins α_{s1} -, α_{s2} -, β -, and κ -CN, in approximate proportions of 40%, 10%, 40%, and 10%, respectively, and α_{s1} -CN is a major allergen according to IgE and T cell recognition data [36-39]. The specificity of the IgE response to the different purified CNs has been analyzed on 58 sera from patients allergic to whole-CN [40].

Multi-sensitization was observed, which most likely corresponded to a co-sensitization to the different CN components after the disruption of the CN micelles. The CN fractions cross-link to aggregates termed nanoclusters, which combine into micelles. In the new state of aggregation, there is a hydrophobic central part and peripheral hydrophilic one that include phosphorylation sites. Although the primary structure of CNs is known, the micelle structure remains relatively unknown. α s2-, α s1-, and β -CN bind calcium to form a core that is covered with a κ -CN layer. The κ -CN latter protects CN micelles from precipitation in the presence of milk constitutive calcium ions. During the last five decades, different models for the structure of the bovine CN micelle have been proposed. Walstra has summarized the common structural elements into a “ball-shaped model” [41]. According to Walstra, a well-accepted model considers the CN micelle as follows: (i) it is roughly spherical; although it does not have a smooth surface; (ii) it is built of smaller units, termed sub-micelles, which mainly contain CN and have a mixed composition; (iii) sub-micelles vary in composition and consist of two main types-one primarily consists of α s and β -CNs and the other primarily consists of α s- and κ -CNs; (iv) the sub-micelles can be linked together by small calcium phosphate clusters bridging them; (v) the sub-micelles aggregate until they have formed a micelle in which those with κ -CN are outside; and (vi) consequently, molecular chains of the C-terminal end of κ -CN protrude from the micelle surface to form a “hairy” layer that prevents any further aggregation of sub-micelles by steric and electrostatic repulsion. The hairy layer is also held responsible for the stability of the micelles against flocculation. Destabilization of CN micelles can be made by treating milk with milk clotting enzymes, which limitedly degrade κ -CN affording to CN micelle fusion and formation of a para- κ -CN aggregate. Finally, a variable proportion, up to 5% total, consists of a heterogeneous group of CNs termed γ -CNs (γ -CN) that result from the limited proteolysis of β -CN by plasmin, the native milk protease. Plasmin disperses in low amounts from blood to milk during secretion to generate CN fragments whose structure are shown in Figure 7 with peptides labeled according to the current nomenclature [42]. CNs are highly sensitive to proteolysis and do not maintain a unique folded conformation [43], which has led them to being termed rheomorphic. Because the folded structure is limited, heating does not generally change the structure and hence its IgE binding [44]. α s1-CN, α s2-CN, and β -CN can chelate Ca^{2+} , Zn^{2+} , and Fe^{3+} , respectively. The four CNs share little sequential homology, but, despite this lack of homology, simultaneous sensitization is often observed.

3.1.1. α s1-CN

α s1-CN consists of major and minor polypeptides that include the same amino acid sequence but have different degrees of phosphorylation [45]. Allergenic epitopes were identified by Spuergerin et al. [46] in α s1-CN regions 19–30, 93–98 and 141–150 as immunodominant epitopes. Some sequential IgE-binding regions were recognized at AA 17–36, 39–48, 69–78, 93–102, 109–120, 123–132, 139–154, 159–174, and 173–194 using sera from nine older children (> 9 years old), and the epitopes AA 69–78 and 175–192 were recognized by 60% and 80% of sera, respectively, from older children [47]. A later study by Elsayed et al. [48] has

demonstrated that the N- and C-terminal peptides, AA 16–35 and 136–155, respectively, have the highest human IgE-binding affinity, and AA 1–18 and 181–199 exhibited high binding to rabbit IgG. cDNA coding for α_{s1} -CN from a bovine mammary gland cDNA library with allergic patients' IgE Abs has been isolated. IgE epitopes of α_{s1} -CN were determined with recombinant fragments and synthetic peptides that spanned the α_{s1} -CN sequence using microarray components and sera from 66 cow's milk-sensitized patients. The allergenic activity of recombinant α_{s1} -CN and the α_{s1} -CN-derived peptides exhibited IgE reactivity, but mainly the intact recombinant α_{s1} -CN induced strong basophil degranulation. The results indicate that α_{s1} -CN contains several sequential IgE epitopes, but the isolated peptides were less potent than the complete allergen in initiating effector cell degranulation. These results suggest that primarily intact α_{s1} -CN or larger IgE-reactive portions thereof are responsible for IgE-mediated symptoms of FA [49].

3.1.2. α_{s2} -CN

α_{s2} -CN is the most hydrophilic of the four CNs because of an anionic group cluster. α_{s2} -CN consists of two major and several minor components that exhibit varying levels of phosphorylation. α_{s2} -CN contains two cysteines and forms disulfide-linked dimers. Using 99 synthetic decapeptides, 10 regions binding IgE from the sera were identified as allergenic, i.e., 31–44, 43–56, 83–100, 93–108, 105–114, 117–128, 143–158, 157–172, 165–188, and 191–200 [50]. Studies on the presence of α_{s2} -CN epitopes 87–96 and 159–168 with weak binding to 145–154 and 171–180 with originate from individuals with persistent cow's milk allergy. Patients with transient allergies exhibited only weak binding to α_{s2} -CN peptides [51].

3.1.3. β -CN

β -CN is the most hydrophobic component of total CN. Sequence variants are known because of both partial proteolysis and variant genes [42]. β -CN is less phosphorylated than α_{s1} -CN and α_{s2} -CN, with five potential phosphorylated sites located in the N-terminal region. Plasmin cleaves β -CN into γ_1 -, γ_2 -, and γ_3 -CN. Synthetic decapeptides were used to estimate the β -CN region that binds IgE of patients, and peptides 1–16, 45–54, 55–70, 83–92, 107–120, 135–144, 149–164, 167–184, and 185–208 were described as typical for patients with persistent CMA. Sera from eight young patients exhibited a simpler IgE pattern because the sera were bound to peptides 1–16, 45–54, 83–92, 107–120, and 135–144 with weak binding to residues 57–66 and the C-terminal region [52].

3.1.4. κ -CN

κ -CN consists of a major carbonate-free component and a mini-bonded polymer that ranges from dimers to octamers. κ -CN plays an important role in the stability and coagulation properties of milk [89]. κ -CN is most likely more structured than α_s - and β -CN and contains specific disulfide bonds [53–54]. The β -CN can rearrange on heating [55]. κ -CN is sensitive to proteolysis and is hydrolyzed by chymosin to produce *para*- κ -CN and caseinomacropeptide in the cheese-making process. Allergenic potential sequences remain in cooked cheeses. κ -

CN is essential to the stability of CN micelles [55]. Diagnosing patients with persistent cow's milk allergies with the use of 80 overlapping synthetic decapeptides helped to identify some regions that bind to IgE from sera, specifically sequences 15–24, 37–46, 55–80, 83–92, and 105–116 [37].

3.2. Whey proteins

Whey proteins contain two major allergens, β -Lg and α -La, and minor constituents, e.g., lactoferrin, bovine serum albumin (BSA), and immunoglobulins. In this fraction, proteolytic fragments of CN and fat globule membrane proteins can occur.

3.2.1. β -Lg

β -Lg (Bos d 5) is the major whey protein in ruminant milks, comprising 50% of the total whey protein. β -Lg is found in the milk of other mammals but is missing from the milk from rodents, lagomorphs and humans. Notably, 13–76% of patients are found to react with β -Lg. β -Lg has a molecular weight of approximately 18 kDa [37] and belongs to the lipocalin superfamily. The β -Lg (Bos d 5) allergen is capable of binding lipids, including retinol, β -carotene, saturated and unsaturated fatty acids, and aliphatic hydrocarbons, and transporting hydrophobic molecules, which is an important function [56–57]. Under physiological conditions, β -Lg is an equilibrium mixture of monomeric and dimeric forms. The proportion of monomers increases after heating to 70°C. β -Lg contains five cysteine residues, of which four are engaged in intra-chain disulfide bridges. Because of the single unpaired cysteine, β -Lg predominantly exists as a stable dimer that tends to dissociate into monomers at a pH between 2 and 3 [58]. β -Lg is present in several variants, i.e., A, B, and C, that are found in the Jersey breed. β -Lg is sensitive to thermal processes. Ehn et al. [59] reported that heating β -Lg to 74°C and 90°C reduced IgE binding significantly. Heating to 90 °C reduced IgE binding more extensively. Chen et al. [60] reported that nearly 90% loss and denaturation of β -Lg are observed in processed milk and that high heat is the major cause of protein aggregation. Circular dichroism demonstrated no significant conformational changes at temperatures below 70°C for as long as 480 s. The rapid changes of β -Lg occurred between 80°C and 95°C. Fifty percent of the maximal changes could be reached within 15 s. Guyomarc'h et al. [61] reported that large micellar aggregates, 4×10^6 Da, are formed upon heating milk that contained 3:1 ratios of β -Lg and α -La together with κ -CN and α_{s2} -CN. Proteolysis and use of monoclonal antibodies proved that β -Lg possesses many allergenic epitopes spread over the β -Lg structure [62]. Major human IgE epitopes for β -Lg amino acid fragments are composed of residues 41–60, 102–124, and 149–162; intermediate 1–8, 25–40, and 92–100; and minor 9–14, 84–91, 125–135, and 78–83 [63]. Similar IgE epitope regions (21–40, 40–60, 107–117, and 148–168) were reported for a rat model of β -Lg allergy [64,65].

3.2.2. α -La

α -La (Bos d 4) is a homologue of C-type lysozymes. It is a member of glycohydrolase family 22 and Pfam family and weighs 14,186 Da in the mature form and between 15,840 to

16,690 Da for the glycosylated forms. α -La is stabilized by binding to calcium. Polverino de Laureto et al. [66] reported that α -La is cleaved by pepsin at pH 2 in the region of residues 34–57, which produces large fragments. Veprintsev et al. [67] reported that differential scanning calorimetry of α -La at pH 8.1 exhibited transitions at 20°C–30°C with calcium chelator ethylene glycol tetra-acetic acid and near 70°C with the addition of calcium. McGuffey et al. [68] investigated the heating effects of purified α -La and demonstrated that the extent of irreversible aggregation varies at temperatures between 67°C and 95°C. When milk is heated to 95°C, α -La denatures more slowly than β -Lg. The folded α -La structure is destabilized at low pHs with the formation of a molten globule [69]. The stability to denaturation is also strongly lowered by the reduction of disulfides [70]. Disulfide exchange can occur during thermal denaturation, which leads to the formation of aggregates [71]. Although evolved from a lysozyme [72], the function of α -La is to form a complex with galactosyltransferase, which alters the substrate specificity and increase the lactose synthase rate in milk. The galactosyltransferase and α -La is termed lactose synthase [73]. α -La alone does not have any catalytic activity as a lysozyme or a synthase. Several other properties of α -La and possible additional functions have been described [74], which include binding of several ligands and antimicrobial activity, both as a complete molecule [75] or as peptides [76]. The cytotoxic effects against mammalian cells have also been investigated [77]. The major component of α -La is unglycosylated. However, a mass spectrum of α -La contains at least 15 distinct peaks [78], and a minor glycosylated form (approximately 10%) results from asparagine 45 glycosylation. A study concerning the allergenic properties of α -La demonstrated that in 60% of the study patients, allergic sera were specific for intact α -La with only 40% binding to peptides obtained after tryptic hydrolysis. Residue 17–58 was the most frequently recognized in the sequence 59–93, 99–108, and 109–123 [79]. The linear epitopes were identified by using sera of patients suffering from persistent allergies and IgE to cow's milk levels > 100 kU(A)/L. Serum IgE bound most strongly to peptides 1–16, 13–26, 47–58, and 93–102 [80].

3.2.3. Minor allergens

Bovine serum albumin (BSA) (Bos d 6), which is a heat-labile protein, is a major allergen in beef but a minor allergen in milk [81–84]. Accordingly, beef allergic individuals are at risk of being allergic to cow's milk and vice versa. BSA allergies account for 0–88% of sensitization events, whereas clinical symptoms occur in up to 20% of patients. BSA is one of the proteins most frequently involved in binding with circulating IgE [85–86]. Bovine immunoglobulins (Bos d 7) may be also responsible for clinical symptoms in CMA.

4. Post-translational modifications

In evaluating the allergenic potential of a protein, post-translational modifications of amino acid residues should be considered in addition to sequential and conformational IgE binding domains. Notably, such modifications may either generate additional IgE epitopes or induce changes in protein folding that affect IgE-protein interactions. Accordingly, recombinant allergens do not generally have the IgE-binding capacity of their natural

counterparts, most likely because of a deficiency in the post-translational events [87]. Regarding milk proteins, selective phosphorylation of serine residues in all of the four CNs, O-glycosylation of threonine residues in κ -CN and N-glycosylation of asparagine residues in α -La have been long described.

4.1. Phosphorylation

The removal of phosphate groups from CNs significantly reduces the CN-binding capacity of IgE from patients who suffer from milk allergies, which indicates that at least part of anti-CN IgE is directed against CN domains that comprise a major phosphorylation site [88, 89]. It has been suggested that currently observed co- and cross-sensitization to the different CNs that are encoded by different genes and display few amino acid sequence homologies can be caused by the occurrence of common highly conserved major sites of phosphorylation, i.e., the Ca^{2+} binding CN sequence SerP-SerP-SerP-Glu-Glu that corresponds to α_{s1} -CN 66-70, β -CN 17-21 and α_{s2} -CN 8-12 and 56-60 [87]. Most likely, sensitization to milk is caused by a large release of phosphopeptides that are resistant to further degradation by digestive enzymes [90] during intestinal proteolysis of milk proteins. However, serine phosphorylation poorly affects the overall antigenic potential of individual CNs. Notably, antisera raised against native β - and α_{s2} -CNs can recognize their targets after dephosphorylation or deletion of a major phosphorylation site [87]. Furthermore, polyclonal antisera that are produced in rabbits using a bovine β -CN 1-28 phosphorylated peptide as an antigen have been utilized to detect all of the tryptic phosphopeptides that originate exclusively from the 1-28 region of β -CN, regardless of the content of the phosphorylated Ser residues, and none of those generated by the other bovine CN fractions [91]. β -CN from human milk contains the phosphopeptide cluster $^5\text{Glu-Ser-Leu-SerP-SerP-SerP-Glu-Glu}^{12}$, also found in the bovine β -CN sequence 14-21; however, a lower level of phosphorylation has been generally observed. For example, according to the phosphopeptide analysis of human milk that is reported in Table 2 and Figure 3, the 2092.8 Da component, which corresponds to β -CN(f2-18)2P, caused the third peak in intensity order in combination with the fully phosphorylated components. This lower phosphorylation level is lacking in its bovine counterpart. The overall higher degree of phosphorylation of bovine CNs can play a role in sensitizing humans to bovine milk.

4.2. Glycosylation

The effect of glycosylation on the allergenic potential of milk proteins has been long disregarded despite efforts to identify the domains responsible for the allergenicity of milk proteins, mostly based on an epitope mapping approach. Notably, the role of carbohydrate epitopes in initiating an allergic reaction is still unclear [92]. Potential glycosylation sites have been identified in major milk proteins, i.e., N⁴⁵ and N⁷¹ of mature α -La [93] and T¹³¹, T¹³³, T¹³⁵, T¹³⁶, T¹⁴², and S¹⁴¹ of mature κ -CN [94]. Approximately 10% of α -La has been found glycosylated at N⁴⁵, giving rise to at least 14 distinct peaks by electrospray-ionization mass spectrometry analysis [95]. However, these glycosylated forms were not included among the IgE epitopes in a study because they were not detected by matrix-assisted laser

desorption/ionization time-of-flight (MALDI-TOF) analysis of α -La; notwithstanding in the same study, IgE reactivity of sera from patients allergic to α -La were proven to be sensitive to periodic acid treatment [93]. As reported in Table 3, the genetic variant A of water buffalo α -La that carries an N⁴⁵→D⁴⁵ substitution cannot be glycosylated. To assess the effect of glycosylation on the allergenicity of α -La, it might be used as substrate for IgE reactivity testing of sera from patients sensitized to α -La.

Molecular mass (Da)		CPP identification
Theoretical	Measured MH ⁺	
Human β-Casein		
2488.1	2489.1	β-CN (f1–18)5P
2408.1	2409.1	β-CN (f1–18)4P
2328.2	2329.1	β-CN (f1–18)3P
2248.2	2249.3	β-CN (f1–18)2P
2168.2	2169.2	β-CN (f1–18)1P
2252.0	2252.7	β-CN (f2–18)4P
2172.0	2173.0	β-CN (f2–18)3P
2092.0	2093.0	β-CN (f2–18)2P
2012.0	2013.0	β-CN (f2–18)1P
3100.8	3101.0	β-CN (f1–23)5P
3020.8	3021.8	β-CN (f1–23)4P
2940.9	2941.0	β-CN (f1–23)3P
2860.9	2861.9	β-CN (f1–23)2P
2780.9	2781.9	β-CN (1–23)1P
Human α _{s1} -Casein		
3077.0	3078.9	α _{s1} -CN (f15–38)3P
3068.1	3069.1	α _{s1} -CN (f12–36)2P
2488.5	2489.5	α _{s1} -CN(f8–27)2P
2408.5	2409.5	α _{s1} -CN (f8–27)2P
2118.8	2119.5	α _{s1} -CN (f68–83)5P

Table 2. Identification of human milk soluble TCA 12% peptide fractions enriched on hydroxyapatite after MALDI-TOF analysis by FindPept (<http://www.expasy.org/tools/findpept.html>) software.

Despite some indications that the allergenic character has been identified in the glycosidic moiety of native κ -CN [96], at present this issue remains to be settled. Glycated forms of κ -CN account for approximately 40% of the κ -CN that normally occurs in bovine milk, but glycans are not randomly distributed among potential glycosylation sites. The hierarchy of glycan addition proceeds according to the order T¹³¹, T¹⁴², T¹³³, whereas the other sites remain latent until these sites are occupied [97]. κ -CN is cleaved by chymosin during the primary stage of cheese making at the peptide bond F¹⁰⁵-M¹⁰⁶. The C-terminal 106-169 fragment, known as glycopeptide because all of the glucides originally present in κ -CN are

retained, is released and lost in the whey. Therefore, cheese is devoid of any glycosylated major component. Potential allergenicity of κ -CN glycoforms has been suggested by analyzing the IgE binding capacity of an individual human serum from an adult atopic patient who had outgrown a cow milk allergy in early childhood. Bovine κ -CN has been selectively recognized by IgE immunostaining of an electrophoretic profile of milk proteins. No additional IgE-reactive proteins other than bovine κ -CN have been found in either bovine cheese, regardless of the cheese making technology and time ripening, or in ewe, goat and water buffalo milk. Moreover, chemical removal of glucide chains from bovine κ -CN has not impaired IgE binding, thus proving a primary involvement of the glycoside moiety of the protein in IgE recognition. According to the specificity displayed by IgE, N-acetylneuraminic acid as a terminal unit of a tetrasaccharide chain has been argued to be an IgE epitope [98].

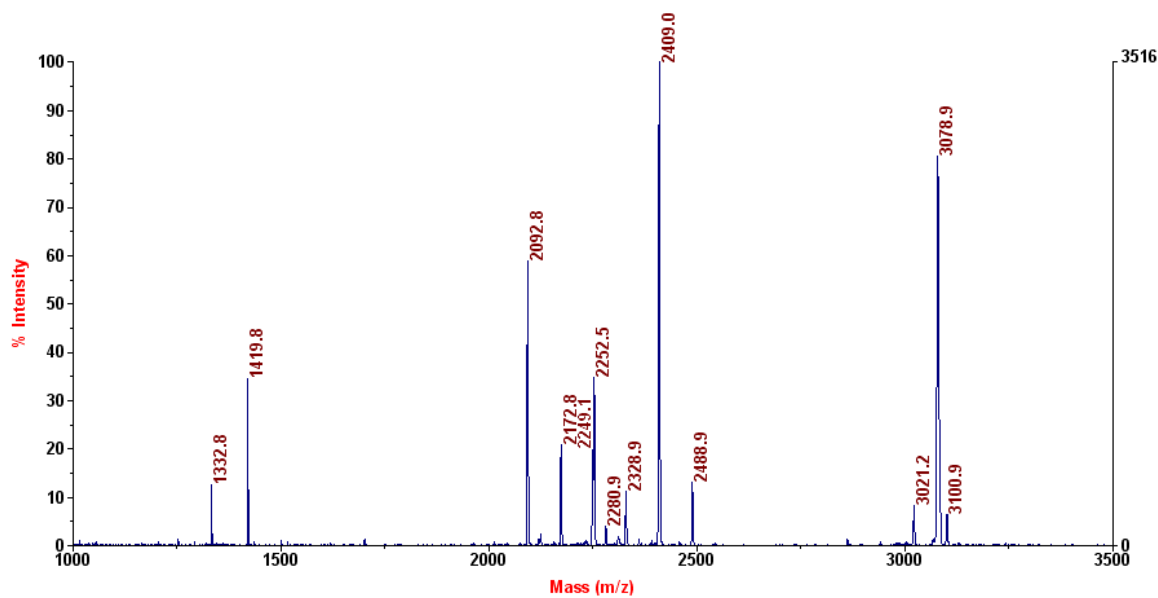


Figure 3. Mass spectrum of human milk soluble TCA 12% fraction enriched on hydroxyapatite by MALDI-TOF.

Site	10	17	45
Species			
Bovine A	Gln	Gly	Asn
Bovine B	Arg	Gly	Asn
Water buffalo A	Arg	Asp	Asp
Water buffalo B	Arg	Asp	Asn
Caprine	Gln	Asp	Asn
Ovine	Gln	Asp	Asn

Table 3. Position and amino acid differences among the genetic variant of α -La from four animal species.

5. “Allergenomics”

The application of proteomic methodologies for the analysis of food allergens has been termed “allergenomics” [99]. For “type I” FA, IgE-binding indicates that the target carries the risk to be an allergen. MS-based proteomic methods have identified many proteins and allergens. MALDI [100] and electrospray ionization (ESI) [101] and MS/MS sequencing are the techniques most widely used to produce high-quality spectra of post-translationally modified peptides [102-104] or intact proteins (see [105]). The characterization of glycosylated allergens has been partly overcome by specific enrichment using lectin or hydrophilic resins (HILIC) prior to MS analysis [106,107]. Native and de-glycosylated peptides are analyzed by MALDI or ESI-MS. Because of the difficulty of profiling oligosaccharides released by glycoprotein [108-110], glycan profiles are obtained after permethylation of the oligosaccharide chains according to the procedure of Das et al. [111]. By this means, glycosylated site(s) are identified together with the peptide backbone. Although widespread, several studies are dedicated to milk protein analysis for the detection of allergenic proteins or peptides in dairy products. This is determined by the concentration of allergenic compounds in food products that are often secondarily masked by dominant non-allergenic proteins. Among the various methods currently used to detect allergens in food products, immunochemical techniques that rely on antibody-binding properties have been developed. Commercially based kits are used for rapid screening, and enzyme-linked immunosorbent assays (ELISA) provide evaluations. Limits of detection (LOD) attained by ELISA tests are in the range of 1–5 ppm. Because the epitopes to be detected and their possible cross-reactivity with matrix components are unknown, detection reliability strongly depends on various factors that include the thermal changes of whey proteins, which are of primary importance. Furthermore, in several foods, linear epitopes can be released by parent protein hydrolysis, whereas retain their allergenic potential. As cited above, MS measurements can be finalized to evaluate the molecular mass of proteins and derived-peptides (MS1), determine the amino acid sequence and identify post-translational modifications (MS/MS or MSⁿ). Two-dimensional electrophoresis (2DE) separates proteins according to the pI (i.e., first dimension, isoelectric focusing, IEF) and subsequently molecular weight (SDS-PAGE) in an orthogonal dimension. By this means, the separation of thousands of proteins has been achieved using highly specific stains that visualize specific protein classes, i.e., phosphoproteins, or nonspecific stains that simultaneously target total proteins without particular functional groups. Protein spots are localized, excised from the gel and subjected to an in-gel tryptic digestion. Mass spectrometry analysis either by MALDI reflectron TOF or microcapillary liquid chromatography MS-MS detects the proteins based on the expected masses of peptides available in databases and other plant proteins in pollen diffusates. More directly, tandem mass spectrometry is used to identify the peptide sequence and search for allergens in databases. More elegantly, allergens are localized on the gels after a one- or two-dimensional electrophoretic separation followed by a nitrocellulose transfer of the proteins (i.e., western blotting), which is stained with sera from allergic patients as a source of specific IgE. Combined with the analysis by mass spectrometry of electrophoretically separated allergens, immunoblotting is useful for the rapid determination of allergen

identities. Allergen-IgE complexes are also detected using conjugated anti-human IgE as a secondary antibody. Once localized in a 2DE map, the allergen can be monitored using allergen specific antibodies [112,113]. Immune-reactive allergenic protein(s) are identified along the immunoblots by comparison with a reference electrophoretic map. All of the major milk proteins are allergen candidates because sera of allergic patients contain various percentages of immune-reactive proteins that are recognized by IgE [114]. The order of milk protein allergenicity is as follows: α_{s2} -CN > α_{s1} -CN > β -CN > κ -CN > β -Lg > serum albumin > IgG-heavy chain > Lf. This list contains allergenic proteins that have been identified by experiments on several MS platforms [115]. Thus far, it is quite difficult to find a separation method that can accommodate the diversity of proteins equally. Therefore, modern separation techniques have been performed off-line or by online ion-exchange/reversed phase liquid chromatography prior to MS analysis. ESI-MS is currently the interface most frequently used to perform an LC separation of intact proteins. The protein identification is most commonly achieved after a proteolytic digestion and molecular weight determination of the LC-separated peptides. "Shotgun" proteomics is the most effective LC/MS-based strategy because a trypsin-digested protein sample generates thousands of peptides that are subsequently separated by LC prior to MS/MS sequencing [103,116]. Proteins with at least one matching peptide are candidates to occur in the sample. However, with CNs, it is difficult to determine which fractions are present in the sample if they share the same set of phosphopeptides or have only one constituent peptide detected. LC-Q/TOF MS/MS has been used to detect wine CN as a fining agent. Two peptides were identified from α_{s1} -CN and four peptides from the tryptic digestion of α - and β -CNs [117]. A similar strategy could be applied to monitoring allergens in processed milk products. Signature peptides could be identified as CN or whey protein allergens by submitting protein concentrates to trypsinolysis. In this manner, information on the molecular weight of the intact allergen is lost, but cross-reactive immunogenic peptides can be discriminated. Because of the higher sensitivity of MS in the detection of peptides, MS expands the dynamic range of the protein species detected. MS is a method for discovering "hidden" or traces of allergens. Proteomics has become pivotal to the development of modern structural immunology and to the understanding of interacting systems that are involved in immune responses, regardless of FA status.

5.1. Allergen quantification methods

Difference gel electrophoresis (DIGE) is utilized to compare multiple proteins in samples migrating in parallel in the same chamber. The proteins are labeled with three distinct fluorescent dyes on the same 2D gel and differentially visualized via fluorescence at different wavelengths. This methodology enables the detection of a differential presence of proteins and small differences in protein abundance. Allergens can be quantified by LC-MS. An accurate evaluation of the protein/peptide requires a suitable standard. In the direct quantification of intact proteins, the intensity of multi-charged analyte ions is compared with that of an internal or external standard. For example, quantification of cow's milk allergens in fruit juice samples [118] and whey drink [119] was performed by simultaneously monitoring several multiple-charged ions of whey protein components.

With a similar approach, internal standard β -Lg was used to quantify non-bovine β -Lg in different milk-derived products [120]. The use of “bottom-up” methods, such as SILAC, ICAT, and iTRAQ, for quantitative analysis in proteomics has progressively increased [121,122]. Quantification of allergens in complex samples requires simple and precise methods of analysis, such as selected reaction monitoring (SRM) [123]. SRM is presently considered the gold standard for absolute quantification, whereas multiple reaction monitoring (MRM) can monitor the masses of selected signature peptides. For an allergen evaluation, internal reference peptides are required for food product monitoring by LC-SRM MS [124]. Hydrolyzed protein samples are spiked with known amounts of synthetic peptides and monitored by LC-MS in the SRM mode. Absolute amounts of peptide(s) are determined by the ratio of the ion intensities of natural and synthetic peptides (Figure 4).

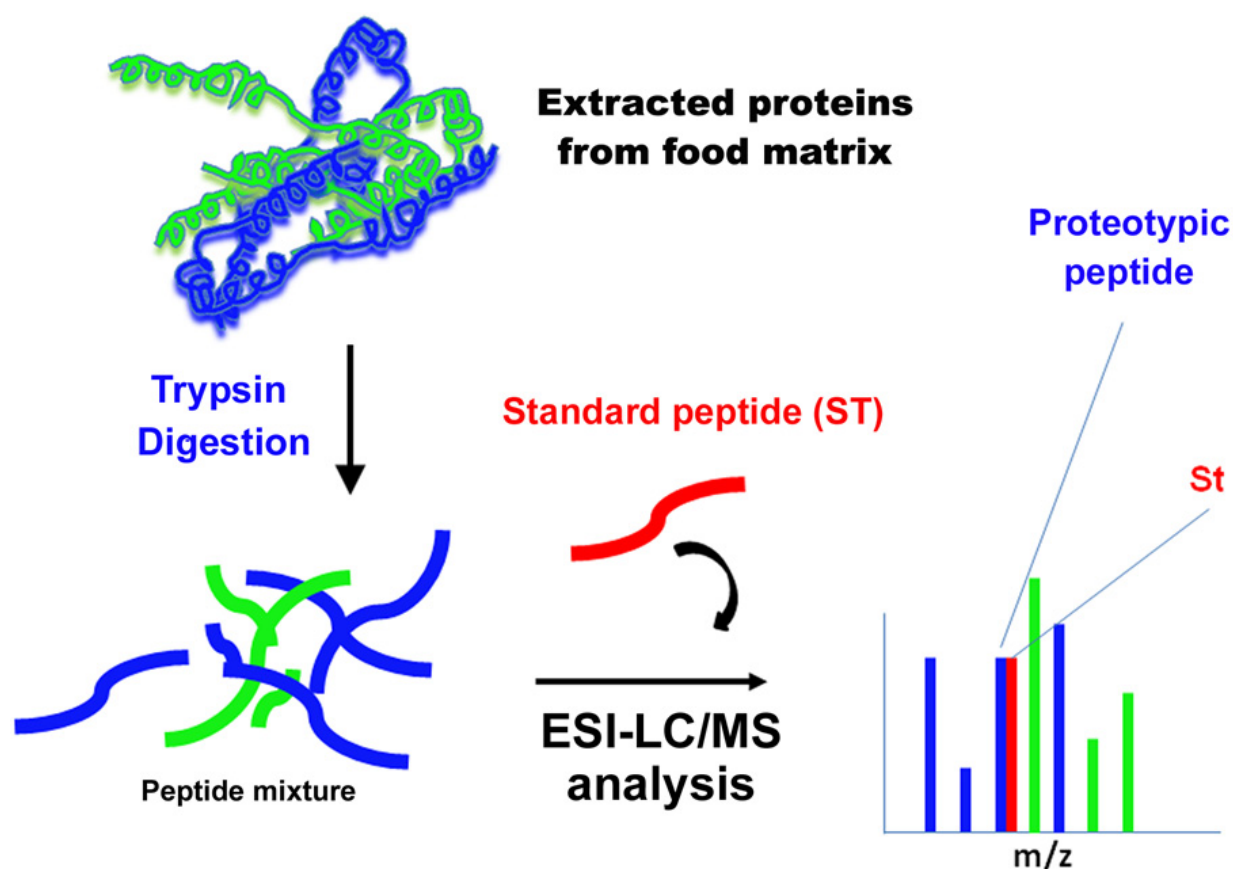


Figure 4. Mass Spectrometry procedure analysis for protein absolute quantification. Proteotypic peptides are selected with a preliminary fullscan. For quantitative analysis whole protein extracts are trypsinized and the peptide mixture is spiked with external standard peptide. Proteotypic peptides (blue colored) selected as analytical probes of the target protein(s), are quantified by comparing the ionic intensities.

This strategy has demonstrated its validity for using signature peptides as analytical surrogates to measure allergens in crude protein extracts. One advantage of the SRM procedure is the possibility of one-step monitoring of a variety of allergens. Recently LC-triple quadrupole MS operating in an MRM mode has been effectively demonstrated to simultaneously detect allergens from seven different potentially allergenic matrices, such as

milk, eggs, soy, hazelnuts, peanuts, walnuts and almonds. The detection limits were in the 10-1000 $\mu\text{g/g}$ range. However, prior knowledge of allergens was required to monitor the most suitable allergenic peptides [125]. Based on the above-specified considerations, allergen evaluation requires the following: (i) allergen extraction from the food; (ii) enzyme proteolysis, usually trypsinolysis; and (iii) identification of signature peptides that are characteristic of food proteins or food ingredients. The signature peptides should be determined experimentally by prior LC/MS analysis of food-derived digested protein extracts.

5.2. Standardization of allergen preparations

Although the search for clinically relevant allergens has progressed, the characterization of allergens still requires studies on milk proteins as starting material. Pure native and recombinant allergens are needed as reference materials to calibrate methods among different laboratories. Recently, a panel of 46 food plant and animal allergens [126,127,128] has been made available within an EU-funded research project. In a recent 2DE application, calibration has been utilized for microbial complex protein systems using data obtained by MS [129]. Developing more allergen standards could be realized in the near future. Moreover, the search for allergenic sequence stretches would comprise only those immunodominant produced during digestion that can translocate the gut barrier and reach the mucosal immune system. Among the digestion/adsorption models of food protein stability, pepsin digestion has been included in the Food and Agriculture Organization/World Health Organization to assess food safety [130]. A model study has established milk-derived peptide candidate-mediated resistance to proteases to display allergic effects. The survival of milk protein epitopes [131,114] requires structure determination. To this end, a simulated digestion of bovine milk proteins *in vitro* that includes the sequential use of pepsin, pancreatic proteases, and extracts of human intestinal brush border membranes, has allowed the identification of produced peptides by MS. The presence of characteristic β -Lg resistant peptides could implicate β -Lg in the case of a cow's milk allergy [132]. The identification by MS of peptides arising from simulated digestion is complicated by a lack of enzyme specificity. Currently, there is no treatment to fully resolve or provide long-term remission from FA allergies. The research for therapy is mainly focused on the introduction of anti-IgE antibodies and specific oral tolerance induction. Immunotherapy appears to be an attractive approach; however, the risk of anaphylaxis should be considered. To this purpose, engineered proteins have been designed, i.e., anaphylaxis-initiating epitopes have been removed within these proteins, while preserving the tolerance-inducing epitopes [133-135]. It appears clear that to successfully pursue similar strategies, the precise identification of epitopes is necessary. It is expected that such approaches will be extended to an increasing number of food sources, whereby MS will play a key role for characterizing novel protein entities. The accurate characterization of the offending sequences could also be the starting point for developing less allergenic food products through the use of enzymatic, microbiological and technological processes to effectively remove allergens [136-139].

6. Dairy research versus CMA

6.1. Milk and dairy products from mammals different from cow

According to the current clinical approach to FA and intolerance based on an elimination diet, the treatment of choice is complete avoidance of cow milk. Although of moderate importance in an adult diet, cow milk elimination has a significant nutritional significance in the infant diet, especially during early childhood. Milk from other mammals has been suggested as a possible alternative to cow milk. At first, goat milk had been proposed as a hypoallergenic infant food or cow milk substitute in human diet, but much of this thesis has no credible scientific evidence. Despite the immunological cross-reactivity between cow and goat milk proteins, due to the close biochemical similarity associated with the same phylogenetic origin [140], it has been estimated that from 40 to 100% of patients allergic to cow milk proteins can tolerate goat milk intake [141]. However, clinical and immunochemical studies aimed at evaluating goat milk safety for cow milk allergic subjects have demonstrated that goat milk cannot be a substitute for cow milk without risk of anaphylactic reactions [142,143]. It has been suggested that evidence for goat milk tolerance in clinical trials can be due, at least in part, to a higher number of genetic polymorphisms in goat CNs, especially for α_{s1} -CN [144]. Null or reduced expression of α_{s1} -CN in individual goats; consequently, the overall α_{s1} -CN content in goat bulk milk is lower than that found in cow bulk milk. According to this general finding, and taking into account that little β -Lg persists in cheese, fresh cheese produced from raw milk has been suggested to be a promising hypoallergenic protein source [145]. Unexpectedly, water buffalo milk yogurt has successfully been employed as an alternative food for children with cow milk allergies [146] despite homologous proteins from cow and water buffalo milk [147]. In contrast, several studies have reported the existence of allergies to goat and sheep milk [148-151] and cheese [152,153] in patients with tolerance to cow milk proteins. Overall, this type of allergy is less common and occurs later than that initiated by cow milk proteins, which is likely because goat and sheep dairy products are not usually included in an infant diet. Moreover, IgE epitopes have been widely recognized in the CN components of goat and sheep milk. Differences in the degree of CN phosphorylation, on average lower in goat and sheep milk than in cow milk, rather than differences in IgE epitope sequences, may be involved in initiating selective allergies to goat and sheep milk, as observed in recent cases. In addition to the four ruminant species of dairy interest (i.e., cow, water buffalo, sheep and goat), other monogastric mammals produce milk for human consumption, such as mares and donkeys. Mare milk, which is more similar to human than cow milk, has been proven to be an acceptable substitute of cow milk for children with severe IgE-mediated cow milk allergy; although the evidence of its tolerability by a supervised oral challenge test is recommended [154]. However, mare milk availability is limited, and its collection is difficult. Donkey milk provides nutritional adequacy and excellent palatability similar to that of mare milk but is more readily available. The composition of donkey milk is more similar to human milk than cow milk because of the higher lactose content (6.5 vs. 5 g/100 mL), lower protein content (1.2 vs. 3.2 g/100 mL), lower CN/whey protein ratio (approximately 1 vs. 4) and a higher non-protein nitrogen fraction level (0.29 vs. 0.18%) [155]. These features have prompted clinicians to propose donkey milk as a valuable breast milk substitute. Additionally, donkey

milk intake has demonstrated positive effects in the diet therapy of patients allergic to cow milk proteins [156]. Although the mechanism of this tolerance is unclear, the reduced allergenic properties of donkey milk can be related to the structural differences compared with bovine milk. Because of scientific and clinical interest in donkey milk, characterization of the whey protein fraction [157], caseome [158], and the minor protein components [159] of donkey milk have been recently provided. Presently, milk from mammals with a geographically restricted distribution area, such as reindeer living in Northern Europe, has been utilized to overcome immunological cross-reactivity among proteins from mammals other than cows. In particular, β -Lg from reindeer milk, although belonging to the lipocalin family and similar to its homologous bovine protein, lacks several IgE epitopes of bovine β -Lg that are involved in CMA [160]. Recently, camel milk, mainly available in the Gulf area and Mauritania, is of growing interest to both nutritionists and pediatricians because of its high nutritive value and unique electrophoretic protein patterns, which strongly suggest a different immunological reactivity of camel milk proteins with respect to the bovine counterparts [161].

6.2. Gut microflora

It has been suggested that gut flora may be involved in the etiology of atopic diseases. It has been demonstrated that the gut microflora differs in children with high or low rates of allergy. Commensal gut flora play a role in inducing an oral tolerance, and the importance of the intestinal microbiota in developing food allergies is essential at early ages when the mucosal barrier and immune system are still immature. Probiotics interact with the mucosal immune system by the same pathways as commensal bacteria. A recent study has demonstrated that probiotic bacteria induced in vivo increased plasma levels of IL-10 and total IgA in children with allergic predisposition. Many clinical studies have reported significant benefits by probiotics supplementation in FA prevention and management. However, not everyone agrees on the effectiveness of probiotics supplementation. The differences are most likely related to the selected populations and probiotic strains used. The hygiene hypothesis proposes that disturbances in the gastrointestinal microbiota are associated with increased prevalence of allergic and autoimmune diseases [162]. Changes in the establishment of gut microbiota have been observed in Western infants [163,164]. This is most likely because of improved hygiene and cleanliness in Western countries and excessive use of antibiotics, which causes a reduced bacterial stimulus. Several clinical studies have reported differences in the composition of bacterial communities in the feces of children with and without allergic diseases. Many of those studies have highlighted the involvement of *Bifidobacterium* and *Bacteroides* in the protection against the development of atopy [165-168], but this observation remains a matter of debate [162]. Moreover, the mechanisms underlying such protective effects remain elusive. There is increasing evidence that T-regulatory cells derived from the thymus or induced in the periphery including the gut mucosa [169,170] are key players of immune regulation [171-173]. Using a single strain mouse model and defined bacterial communities and conventional mice, it has been recently demonstrated that the gut microbiota plays a protective role against allergen sensitization and allergic response in a mouse model of FA [174]. The difference between healthy and

allergic children may be in their microflora. At 3 weeks of age, infants in whom atopy developed then had more *Clostridia* and fewer *bifidobacteria* in their feces compared with infants who remained healthy. Moreover, fecal *bifidobacteria* microflorae were different between healthy and allergic children; the healthy infants' microflora was mainly *Bifidobacterium bifidum*, whereas the microflora was mainly *Bifidobacteria adolescentis* in the allergic infants. It can be hypothesized that individual species, rather than an entire genus, can affect the manifestation of allergy. In a recent study, the microflorae of milk-hypersensitive and control adults before and after a 4-week supplementation with probiotic bacteria (*Lactobacillus rhamnosus* GG, ATCC 53103) have been studied. The anaerobic microflora before supplementation was comparable between the healthy and hypersensitive subjects, whereas the response after supplementation was different. The number of *bifidobacteria* in the healthy subjects increased significantly after supplementation. However, this did not occur with the supplementation in milk-hypersensitive subjects; this may be because of altered intestinal integrity. However, other studies have suggested a beneficial effect of probiotic bacteria in milk-hypersensitive subjects. In one study, symptoms of hypersensitivity abated along with an elimination diet in 28% of the patients in 4 years. It can be hypothesized that with milk elimination and long-lasting probiotic treatment, the intestinal severity of IgE-mediated hypersensitivity reactions may increase the intestinal microflora or even eliminate them.

6.3. Reduction of allergenicity of milk proteins by hydrolysis

The main objective of the milk industry is to supply products while preserving both the nutritive value and safety against developing allergies. Nutritional value is preserved by exposing liquid or powdered milk to low heat treatments to reduce heat susceptible amino acid side chain modifications and preserve the integrity of triacylglycerols, native vitamins and other milk components. As noted above, infants can develop milk allergies because of increased gut permeability to large molecules, in addition to other causes [175]. This result is supported by measurements of unmodified proteins or partially modified proteins in the sera of infants and adults [176]. Milk proteins have a molecular mass between 14 and 80 kDa. To reduce allergenicity, milk proteins can be submitted to different hydrolysis procedures. Attempts to classify products by protein hydrolysis include "extensive" or "high degree" hydrolysis and "partial" or "low degree" hydrolysis. The rationale of such a classification is the spectrum of peptide molecular weights or the ratio of α amino acids to total nitrogen. For quality assurance, *in vitro* product characterization requires size measurements of the peptides that are generated by protein hydrolysis and then an *in vivo* allergenicity determination. The *in vivo* step would include evaluating immunogenic or allergenic effects in a recipient infant. The European Union regulates that infant formulas contain immunoreactive proteins in quantities lower than 1% of nitrogen compounds [177] to reduce allergenicity knowing that only pure amino acids are strictly non-allergenic. This criterion could be encountered by milk proteins that have undergone extensive hydrolysis partially to cleave amino acids [178-180]. In contrast, formulas with moderately reduced allergenicity (partially hydrolyzed) are not recommended for the treatment of allergies because of the high amounts of residual allergens [181]. The low quantity of native proteins

or residual high molecular mass peptides may produce adverse effects in highly sensitive patients. Therefore, a milk hydrolysate can be considered safe and non-allergenic if the nitrogen fraction does not contain unmodified milk proteins or high molecular mass peptides [178-180]. In the latter case, the product could be classified in the "low degree" protein hydrolysate category. However, the antigenic properties of protein hydrolysates may not be dependent on the molecular size of the peptide components alone [182]. By comparing protein structures with known allergens and allergen epitopes, protein allergenicity has been predicted [183]. Although this is true for crystallized proteins, such as α -La, β -Lg and Lf, this procedure cannot be applied to uncrystallized CNs. Because infants who are diagnosed for milk protein allergies must ingest foods that exclude the causal protein, including those ingested by the mother and filtered in breast milk, extensively hydrolyzed milk formulas are used for the development of appropriate dietary and management strategies. Preclinical testing of infant formulas is necessary to characterize the molecular properties and residual antigenicity of proteins [184-186]. Stringent criteria specify that extensively hydrolyzed CN with a molecular weight below 5000 Da should be reduced by at least 99.99%. There is a need for accurate diagnostic methods to confirm the amount of extensively hydrolyzed CN. Milk for allergic infants would consist of extensively hydrolyzed CN and whey proteins of which at least 99.99% of the hydrolysis products have molecular weights below 5000 Da. The crucial criterion is for the level of allergens to be sufficiently low as to cause no significant reaction, even in infants who are highly allergic to cow's milk. There have been no reports of adverse reactions because of whey [187, 188] and CN hydrolysates [189-191]. Therefore, caution must be maintained that milk formulas destined to infants with milk allergies contain correctly hydrolyzed proteins. This generic indication requires that molecular properties and residual antigenicity of proteins would be characterized [184-186]. In vitro incubation of milk proteins with pepsin, trypsin, and chymotrypsin causes the cleavage of numerous peptides of various sizes. Bacterial, fungal and plant proteases may also act as hydrolyzing agents. Various enzyme combinations, such as alcalase, pancreatin and enzymes from fungal sources, have been utilized to produce protein hydrolysates. Commercial hypoallergenic products are currently characterized by an average degree of hydrolysis (DH) of the protein components. The DH 19 milk protein value is calculated from the increase of the number of primary amino groups compared to that of native proteins. In practice, the DH value could vary from 1 to 100% in the case of total hydrolysis of the proteins. In the case of partly hydrolyzed proteins, intact and partly hydrolyzed proteins are visible bands along an electrophoretic pattern of the products. Two commercial formula preparations with DH values of 6.3 and 1.3% contained some intact β -Lg and peptides with an Mr between 6000 and 8000 Da [192]. Using gel permeation chromatography, quantitative results on peptides with an Mr larger than 10 kDa were obtained [192]. Regardless of the technique used, descriptive information was obtained on either the molecular mass or the origin of the peptides. As a result, consumption of infant formulas by allergic patients cannot be attributed to one specific protein or high molecular mass peptide. To suppress or reduce the antigenicity of peptides, natural enzyme cleaving

of many or most of the peptide bonds is required. In this manner, epitopes that determine the antigenicity of the protein molecules are destroyed. This result proves that evaluation of the adequacy of infant formula composition in preventing or delaying antigenicity is not based solely on DH or Mr determination. In highly sensitized infants with IgE-mediated cow's milk allergies, life-threatening anaphylactic shock usually develops shortly after the consumption of claimed hypoallergenic milk products in which a number of epitopes would have survived in the highly proteolytic environment. The possibility of using well-characterized monoclonal antibodies in ELISA tests can be used for assessing the origin of immunoreactive bovine milk proteins. Because the clinical significance of residual antigenicity requires prior molecular approaches, hypoallergenic products may first be screened for peptide identification in hydrolyzed milk products. Although protein hydrolysates can provide a positive effect, they can contain undefined peptide components, which are undesirable for pharmaceutical production purposes. In many cases, hydrolysates are produced by methods that are not well-controlled. Other complications arise from the raw starting material and differences in processing that lead to lot-to-lot hydrolysate composition variability. For these reasons, constant chemically defined products are needed. The data presented here represent the initial steps that have been taken to identify peptides treated with pepsin (P) and trypsin (T) and were used in succession to hydrolyze commercial milk protein powder (PT hydrolysate). To mimic commercial milk hydrolysates, the protein powder was treated with enzymes after a thermal shock deactivation treatment. Subsequently, the peptides are identified. RP-HPLC fractionation was used to aid with the peptide separation. In Figure 5, the hydrolysate was analyzed to demonstrate a correlation between proteolytic enzymes and the presence of peptides. Commercial milk protein hydrolysates may contain trace amounts of allergenic proteins whose molecular weights were determined by MALDI-TOF analysis. Among the number of peptides present in the hydrolyzed sample, the proteins/peptides exhibited molecular masses less than or equal to 2431 Da (Figure 6). This means that the CNs and whey proteins were digested by pepsin and trypsin into peptides with masses less than 3000 Da. This type of hydrolysate is not expected to elicit allergic reactions in already sensitized allergic patients (neither anaphylactic shock nor positive passive cutaneous anaphylaxis), as verified in experimental animals [193]. LC-ESI-MS/MS analysis was performed on the hydrolysate to identify peptides occurring herein. No sequence peptides with 3 or 4 residues were detected because the MALDI signals were acquired at a mass gate of m/z 400. Because some short peptides were in the hydrolysate, milk proteins were hydrolyzed by P and T into oligopeptides with different biological activities. In Figure 7, the amino acid sequence of the four bovine CN fractions and β -Lg are reported with a subscript that indicates the number of amino acid residues in MS/MS-identified fragment.

The proteins in the milk powder sample, which contained modified amino acid residues that may indicate the quality of the protein in milk powder, were not examined within the present work.

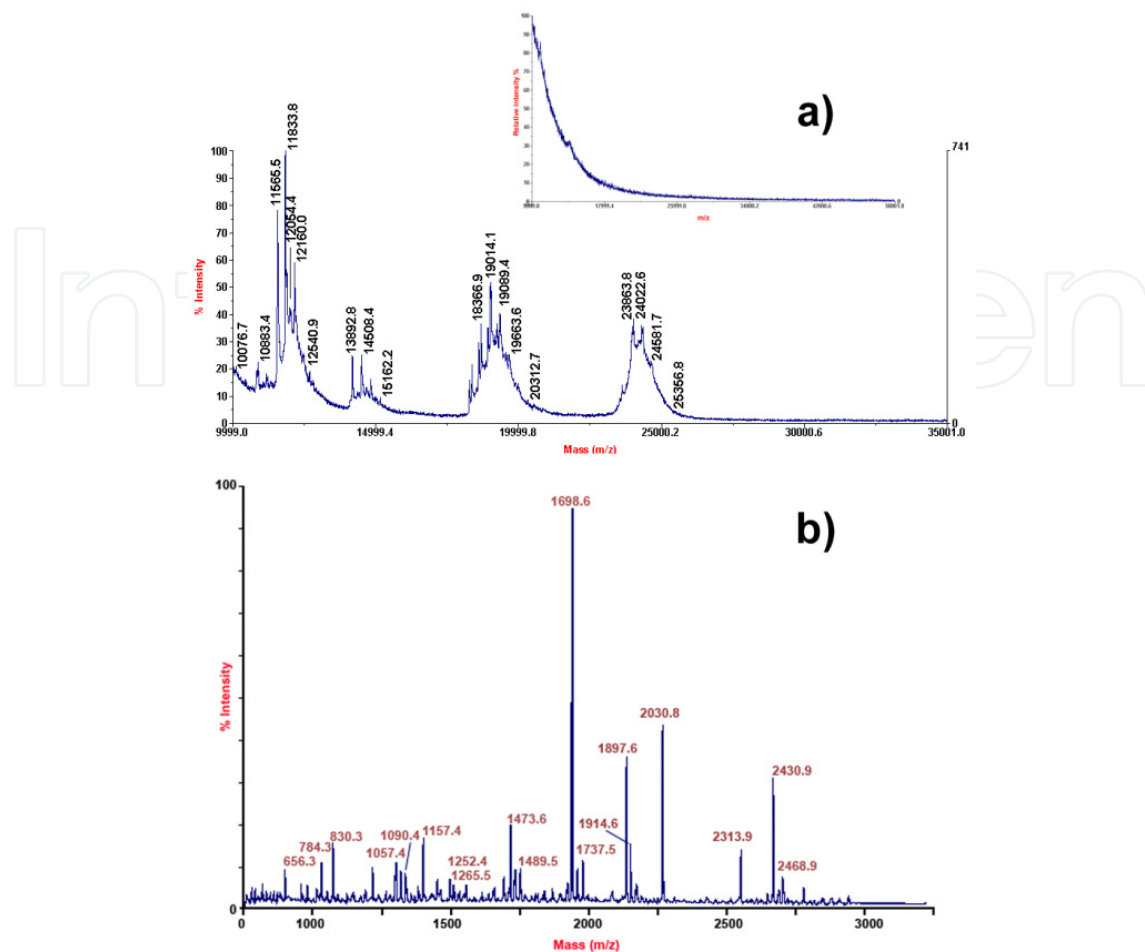


Figure 5. MALDI spectra of a sample of milk powder before and after sequential hydrolysis with pepsin and trypsin. A search for residual intact proteins and high molecular derived peptides (a) and measurements of molecular mass value of oligopeptides in the mixture (b). No peptide at a molecular mass higher than 3430.97 Da was observed in the MALDI spectrum.

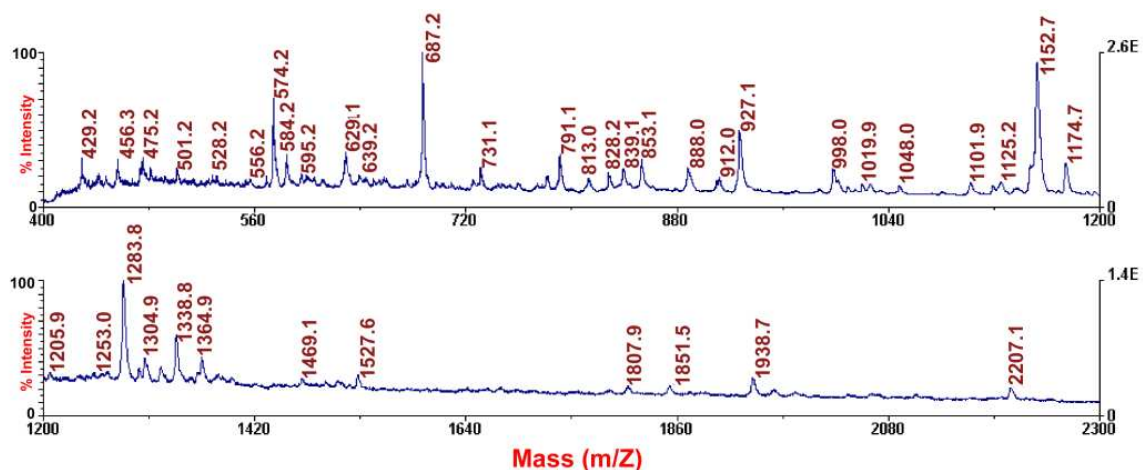
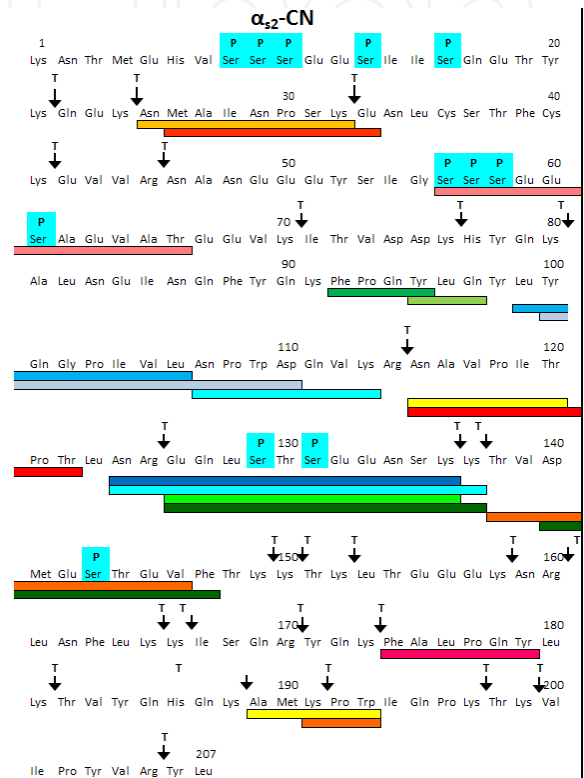
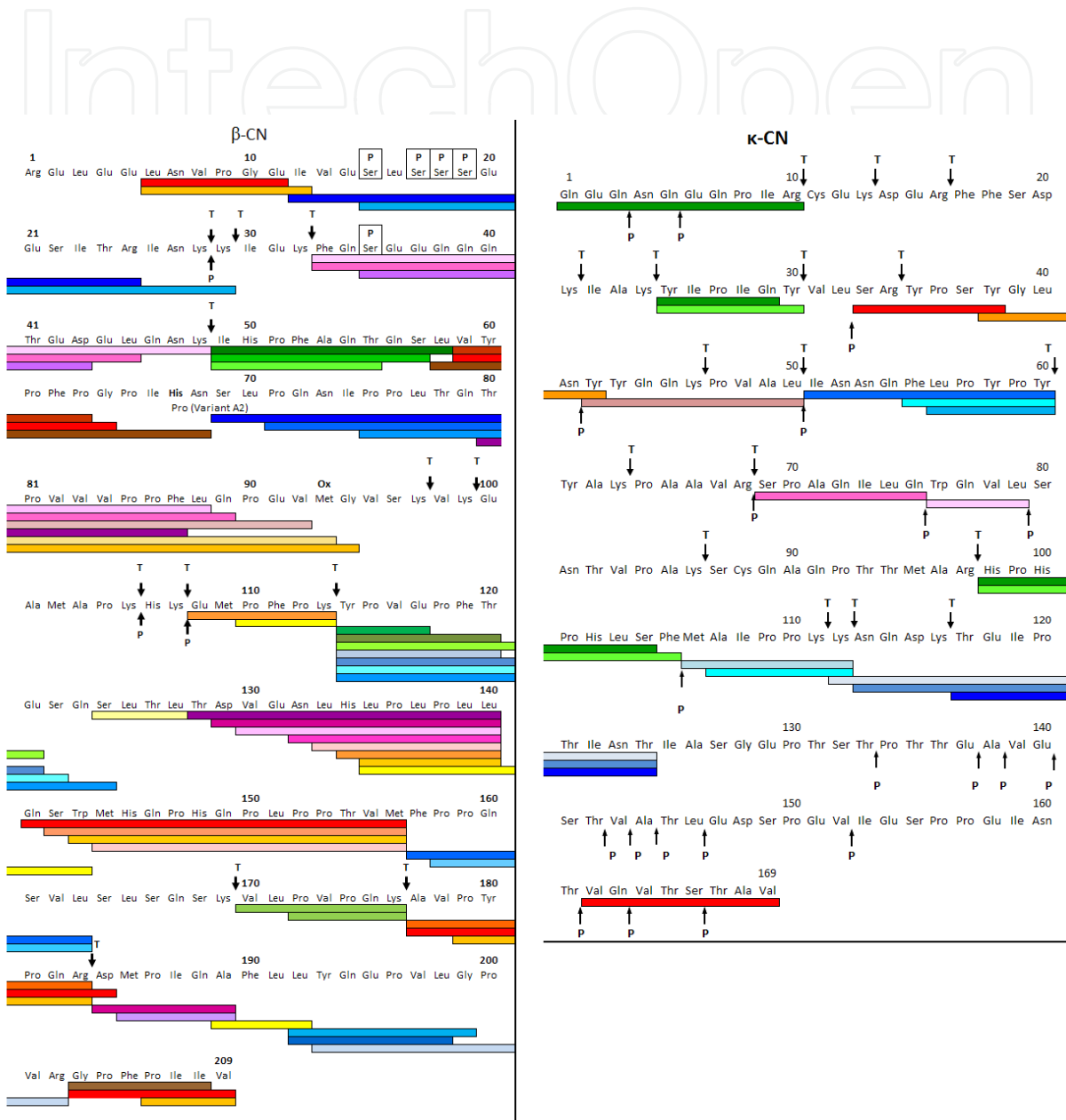


Figure 6. MALDI analysis of a milk sample after sequential hydrolysis with pepsin and trypsin. A molecular mass value corresponds to that of oligopeptides in mixture. No peptide at a molecular mass higher than 2207.1 Da was observed in the MALDI spectrum.





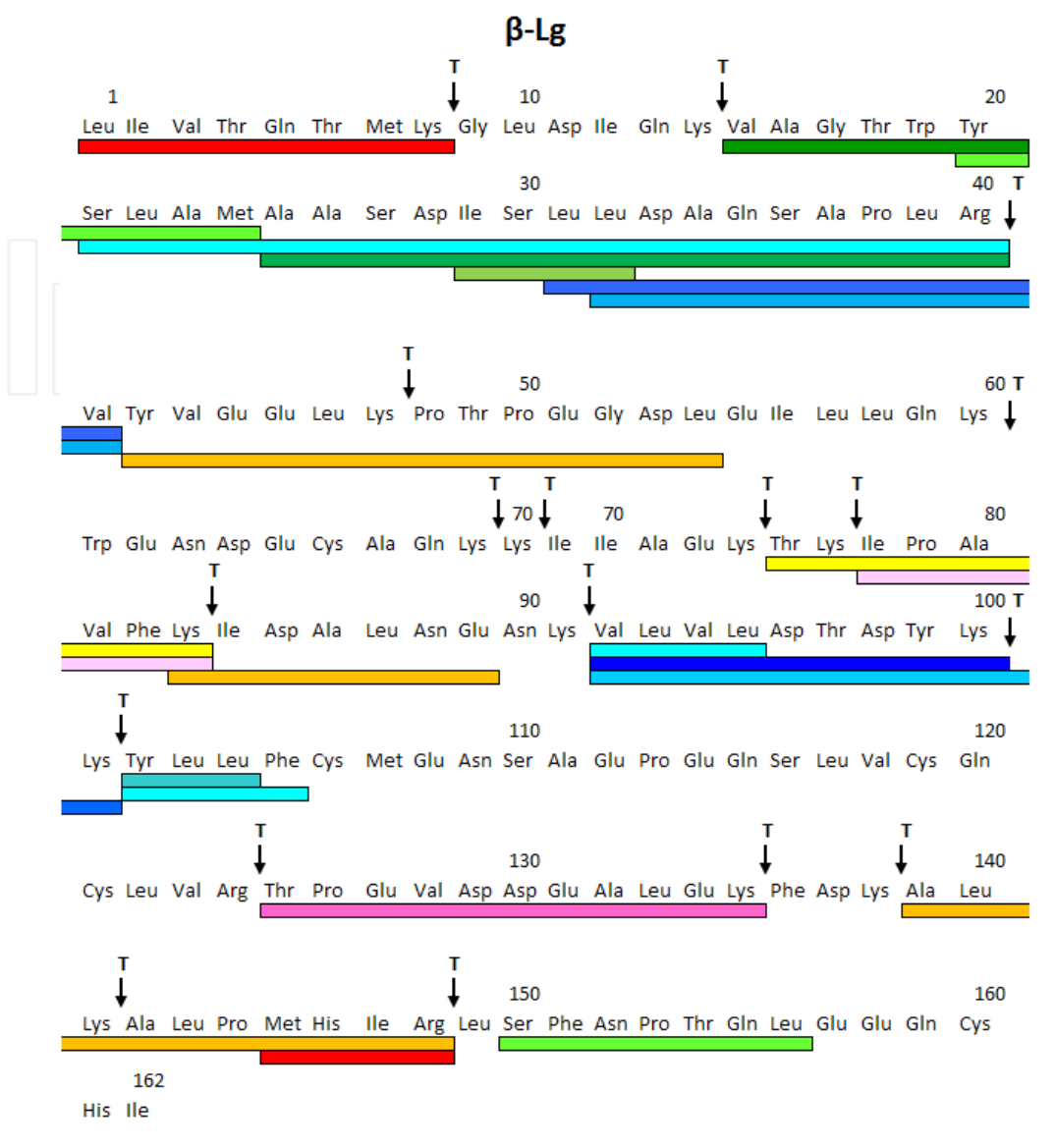


Figure 7. Primary structure of bovine β -, α_{s1} -, α_{s2} -, κ -CN and β -Lg together with identified peptides in commercial milk protein powder sample hydrolysed by sequential action of pepsin and trypsin. Protein/enzyme ratio 100/1 (w/w). Incubation with each enzyme was carried out for 16h at appropriate pH. Ends of horizontal bars indicate first and last amino acid residue of peptides isolated within this work. Arrows indicate casein peptide bonds broken by pepsin (P) and trypsin (T) as reported by Pelissier [194].

7. Oral immunotherapy as future perspective in CMA management

The primary treatment for managing food allergies is eliminating the offending food from the diet. In the case of milk, it is extremely difficult to achieve complete elimination because milk can be masked in any number of foods, which may lead to unwanted severe reactions. The natural course of a cow's milk protein allergy is the acquisition of tolerance spontaneously through an elimination diet, and 85% of patients overcome CMA by the time they are 4-5 years old [195-198]. In recent years, a number of studies have been published

regarding desensitization or oral tolerance to food antigens, particularly to cow's milk. Major advances in understanding the immunological processes involved in the development of CMA have revealed a considerable number of allergenic epitopes and the heterogeneity of allergic responses. Importantly, an elimination diet of dairy foods has negative consequences in terms of inadequate calcium and vitamin intake. In the literature, several conflicting studies have reported on possible desensitizing therapies in the treatment of FA allergies. The possibility to obtain an oral desensitization is now gaining acceptance widely, even if the mechanism is still unclear. Oral tolerance or desensitization is the active non-response of the immune system to an antigen through sublingual, oral administration. Tolerance or the long-term loss of allergic reactivity follows a desensitization treatment. In the literature, several studies have reported on possible physiopathogenetic mechanisms of oral desensitization, but the exact mechanism is still unknown. That tolerance may be involved in the mechanism of desensitization is still uncertain [199-201]. A growing understanding of the molecular and cellular mechanisms of oral tolerance is reinforcing advances in potential therapies for food allergies and is pivotal to eventually curing allergies in sensitized individuals. Oral desensitization should be taken into consideration in the management of food-allergic patients even if the physiopathogenetic mechanisms are still unexplained. Moreover, this treatment should be considered, particularly for children, because elimination from the diet of some foods (e.g., milk and eggs) for these patients could cause psychological and/or nutritional problems. Oral immunotherapy (oral desensitization) may be a promising treatment strategy for cow's milk allergy in children and valuable for other foods, such as eggs or peanuts. Although the mechanisms of IgE-mediated allergies are fairly well understood, the immunology and variety of non-IgE-mediated reactions remains largely unknown. A better understanding of these allergy mechanisms is a prerequisite to the development of improved diagnostics, which in turn will facilitate an improved understanding of the epidemiology of CMA, particularly for non-IgE-mediated reactions.

8. Conclusions

A better understanding will also aid the development of hypoallergenic dairy products, especially for adults with CMA for whom there is currently a dearth of suitable low-allergenic dairy products. Some of the risk factors for developing CMA have been identified; a familiar history of atopy is one of the main determinants. However, the mechanisms of allergic sensitization and the precise interactions between genetics and various environmental factors that lead to CMA remain unclear. The first few months of life, during which the immune system is still maturing, appear to be a critical risk period for allergic sensitization. For at-risk infants with at least one atopic parent, breastfeeding during this period is currently the best identified preventative strategy; the use of hydrolyzed formulas is recommended for babies who cannot be breastfed. The use of immunomodulatory dietary adjuvants, such as probiotics, is an emerging approach with considerable promise for primary prevention. For CMA sufferers, the avoidance of dietary milk proteins remains the only effective management strategy but carries with it nutritional implications, particularly for adequate vitamin and calcium intake as well as protein and energy where unorthodox

alternative diets are implemented. Increasing knowledge of the molecular and cellular mechanisms of oral tolerance reinforces the advances in potential FA therapies and is pivotal to eventually curing allergies in sensitized individuals. Unraveling the links between innate and adaptive immunity and characterizing the roles of dendritic cells and T cells in directing immune responses and homeostasis to environmental antigens are likely to remain a focus of fundamental FA research in the coming years.

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