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REVIEW ARTICLE

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Milk allergens, their characteristics and their detection in food: A review

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Abstract Cow's milk allergy (CMA) is one of the most common food allergies in childhood. This allergy is normally outgrown in the first year of life, however 15% of allergic children remain allergic. Many studies have been carried out to define and characterise the allergens involved in CMA and described two major allergens: casein (αs1-CN) and β-lactoglobulin. In addition to this, many other milk proteins are antigenic and capable of inducing immune responses. Milk from sheep or goats differs from cow's milk (CM) in terms of composition and allergenic properties. Food processing such as heating affects the stability, structure and intermolecular interactions of CM proteins, thereby changing the allergenic capacity. Chemical and proteolytic treatments of milk to obtain milk hydrolysates have been developed to reduce allergic reactions. Prevention of CMA largely relies on avoidance of all food products containing cow's milk. To achieve this, interest has focused on the development of various technologies for detecting and measuring the presence of milk allergens in food products by immunoassays or proteomic approaches. This review describes the technologies implemented for the analysis of milk allergens (allergenicity, biochemistry) as well as their potential detection in food matrices.

Keywords Milk allergy · Milk proteins · Processing · Analytical methods · Milk hydrolysates · Immunotherapy

Abbreviations AA: amino acid · AGE: advanced-glycation-end-products · ALA: α-lactalbumin · β-LG: β-lactoglobulins · BSA: bovine serum albumin · CE: capillary electrophoresis · C-ELISA: competitive ELISA · CM: cow's milk · CMA: cow's milk allergy · CMI: cow's milk intolerance · CML: carboxymethyl lysine · CNs: caseins; α_{S1}-, α_{S2}-, β-, κ-, γ-caseins · Da: dalton · DBPCFC: double blind placebo controlled food challenge · 2DE: 2 dimensional electrophoresis · 2D-PAGE: 2 dimensional-polyacrylamide gel electrophoresis · EAST: enzyme allergosorbent test · eHF: extensive hydrolysed formula · ELISA: enzyme-linked immunosorbent assay · ESI: electrospray-ionisation · HIC: hydrophobic interaction chromatography · HPLC: high performance liquid chromatography · Ig: immunoglobulins (IgG; IgE) · LAB: lactic acid bacteria · LC-MS: liquid chromatography-mass spectrometry · LF: lactoferrin · LOAEL: lowest observed adverse-effect levels · MALDI: matrix-assisted-laser-desorption-ionisation · NFDM: non-fat dry milk · NOAEL: no-observed-adverse-effect level · pHF: partial hydrolysed formula · PCR: polymerase chain reaction · mg/kg: part per million · RAST: radio-allergosorbent test · RIE: rocket immuno-electrophoresis · RP: reversed phase · S-ELISA: sandwich ELISA · SPT: skin-prick test

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Introduction

Food allergy is an abnormal immunological response due to a sensitisation to a food or food component. It represents an important health problem, especially in industrialised countries where it has been estimated to affect around 1–2% of the adult population and up to 8% of children below the age of 3 [1].

Clinical manifestations of food allergy consist of disorders in the digestive tract or in organs as a result of an immunologic reaction [2–4]. The "gold standard" for the diagnosis of food allergy remains the double-blind placebo-controlled oral food challenge (DBPCFC) and this should always be carried out in all patients suspected of having a food allergy [5].

The increasing interest in the field of food allergy reflects the wide spread of this pathology throughout the population. In fact, the ingestion of food antigens that is usually followed by the induction of oral tolerance fails more and

more frequently [6]. Normally, systemic humoral (IgG) and cellular responses to food proteins that preceded tolerisation leading to the abolition of responsiveness towards the corresponding proteins are maintained. But, in the case of food allergy there is no abolition of this responsiveness which leads to allergic reactions [7].

One of the reasons for the observed increase in the prevalence of food allergic disorders could lie in changes in modern eating habits and the increasing complexity of food ingredients and food manufacturing [8]. Many substances added to food for different technical functions ranging from colouring and flavouring to nutrient purposes may be implicated as causative factors in food allergies and intolerances. It endangers public health and can result in severe and even fatal reactions, e.g. for peanuts and tree nuts. This means that levels of some allergens in food for consumption by allergenic individuals should not exceed the lower parts per million (mg/kg) range [9]. Since to date specific avoidance diets are the only way to prevent allergic reactions to foods, the ingredient declaration even in trace amounts on food labels assumes paramount importance in the protection of food-allergic consumers.

Directive 2003/89/EC regarding food labelling must be fully implemented by the end of 2005 into national legislations. The new regulation reinforces the general rule that all substances that have been intentionally introduced in a foodstuff should be indicated under their specific name in the list of ingredients. In this way, the "25% rule" (which was unaffected by the last Directive 2000/13/EC) allowing the non-mandatory of labelling the components (wherein compound ingredients make up less than 25% of the final product) is definitely abolished [10, 11]. Both directives aim to provide more comprehensive information to consumers with allergies, by allowing them to identify those ingredients and foods that they must avoid. For this purpose the new legislation will introduce a mandatory labelling of all food products that contain ingredients that are considered the most common food allergens. Milk is one of the twelve allergenic ingredients whose presence has to be declared on the label of food products.

Milk allergy is an adverse reaction to proteins that are present in milk. Milk of all ruminant species (e.g. cow, goat, ewe) contains the same or very homologous proteins which share the same structural, functional and biological properties [12]. This review will focus on allergy to cow's milk (CMA) that can be considered one of the most common food allergies especially in early childhood with an incidence of 2–3% in the first year. From an immunological point of view, CMA is a IgE-mediated reaction to CM and may induce cutaneous (atopic dermatitis, urticaria, angioedema), respiratory (rhinitis, asthma, cough) and gastrointestinal (vomiting, diarrhea, colic, gastroesophageal reflux) reactions, and in some extreme cases even systemic anaphylaxis. Different food products could be responsible for this type of allergy since CM proteins are used as processing aids and therefore a large number of food products may contain residual amounts of CM protein. Breast milk from mothers who have consumed products containing CM might be another threat for the development of CMA due to the absorption of cow proteins, their passage through the gut mucosa and their release in human milk [12]. This allergy is normally outgrown in the first year of life; however, 15% of allergic children remain allergic. The exposure to food proteins, especially CM proteins during the neonatal period can also trigger some clinical and immunological effects correlated to immaturities of the immune and "digestive" systems of the newborn [13]. Factors such as low stomach pepsin activity at birth, an immature stomach acid generating mechanism (stomach pH is ~3.5) and malfunction of pancreatic and intestinal enzymes contribute to the stability of CM proteins (β-lactoglobulin and α-lactalbumin) by limiting their gastric proteolysis and therefore expose neonates to "allergic" responses [14].

Strategies to prevent children from developing allergies have recently been elaborated on the basis of all available studies reported in literature, to better clarify the relationship between the mode of infant feeding and food allergy. Most studies show that breastfeeding decreases the risk of recurrent wheezing and the development of atopic dermatitis. These protective effects increase with the duration of breastfeeding up to at least 4 months. It has also been reported that feeding of CM formula increases the risk of CMA but, on the other hand exclusive breast-feeding does not eliminate this risk [15–17].

CM contains many proteins that are considered antigenic and capable of inducing immune responses, and sensitivity to different CM proteins has proven to be widely distributed. Studies carried out on large populations of allergic patients have indicated that the most abundant proteins in CM especially lactoglobulins (β-LG), caseins (CN) and α-lactalbumin (ALA) are the major allergens; however, also proteins that are present in low quantities such as bovine serum albumin (BSA), lactoferrin (LF) and immunoglobulins (Ig) have shown to be of great importance in inducing milk allergies. But, while sensitisation to CNs, β-LG, and ALA is closely linked, sensitivity to BSA appears to be completely independent [18].

Heat treatment is the most common method to reduce pathogens but it remains controversial whether this method reduces the risk of allergies [19]. It has been reported that milk heat treatments could lead to the loss of tertiary protein structures which do not always result in a decreased allergenic potential. On the contrary, formation of aggregates may increase the allergenicity of heated milk [12] or create new stable neoantigens as occurs in complex food matrices as a consequence of chemical and physical reactions [20]. In some other cases, processing technology has been exploited successfully for the production of hypoallergenic infant formulae as milk substitutes in infant nutrition and thereby decreasing the allergenic potential [19].

Different analytical approaches have been developed for the analysis of CM protein allergens mostly based on separation and characterisation techniques. The two most popular methods for protein separation are two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and liquid phase separation which is becoming a more common method in proteomic studies [21]. A relatively new approach in proteomic analysis is the

multidimensional liquid chromatography combined to mass spectrometric detection (LC-MS) which has been successfully used in many applications [22, 23]. Fast methods based on immunochemical detection protocols such as radio-allergosorbent tests (RAST), enzyme allergosorbent tests (EAST), rocket immuno-electrophoresis (RIE), immunoblotting and enzyme-linked immunosorbent assays (ELISA) are available to check the presence of potential allergenic proteins [24].

The present paper aims to give an overview of all known potential allergens that are present in CM, encompassing well-characterised protein and peptide allergens as well as neoantigens elicited as a result of thermal processing. Aspects relevant to the potential allergenicity of hypoallergenic formulae will also be discussed. The need to monitor and verify the presence of trace amounts of protein allergens in milk and dairy products has generated a demand for analytical methods capable of detecting, identifying, and quantifying proteins at the lowest mg/kg levels. The second part of this review is therefore dedicated to the analytical methods that are currently available to monitor the presence of high and low abundant protein allergens of milk. The characteristics of the methods are discussed and compared in terms of sensitivity.

Milk intolerance versus milk allergy

Food allergy is often mistaken for food intolerance. In fact, food intolerance is much more common than food allergy. Cow's milk intolerance (CMI) should refer to non-immunologic reactions to CM, such as disorders of digestion, absorption, or metabolism of certain CM components [5] as described in Fig. 1.

The range of symptoms which can be induced by food intolerance is very similar to those caused by food allergy complicating the distinction between the two conditions. It manifests itself in several different ways, producing symptoms such as eczema, vomiting, diarrhoea and stomach cramps, but not hives or breathing problems (symptoms of milk allergy). Symptoms can be well ameliorated by reducing the intake of CM or using lactose-hydrolysing agents (lactase, lactic acid bacteria (LAB): *L. Bulgaricus, S. thermophilus*,. . .). Nevertheless, in comparison to food allergy, CMI is not dangerous. The onset of symptoms is often delayed, and this makes recognition of the causative food component quite difficult. This contrasts with food allergy, in which the reaction is usually immediate and often occurs early in life. The severity of symptoms in food intolerance is dose-dependent, and the dose can be cumulative over days of ingestion. This characteristic further increases the difficulty of diagnosis, as the symptom-inducing chemicals may be common to many foods, so that different foods may appear to cause symptoms on some occasions, but not on others. The most effective diagnostic test for food intolerance is to remove all potentially offending foods from the diet for a few weeks and notice the resolution of the symptoms. The diagnosis of food intolerance and identification of the food component involved are then confirmed by gradually re-introducing individual foods or food chemicals into the diet in increasing doses over several days.

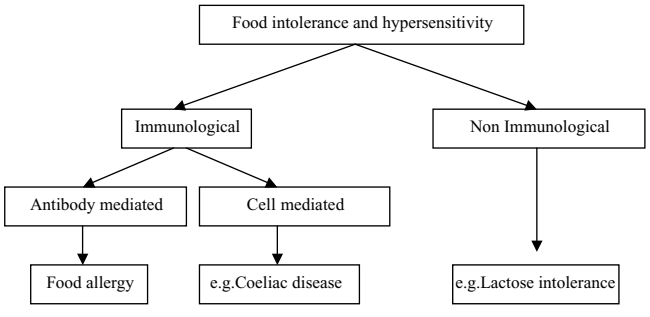

Fig. 1 Differences between food allergy and intolerance

The most common cause of CMI is malabsorption of lactose (carbohydrate) due to intestinal lactase deficiency, which is mostly acquired during late childhood or adulthood. It results from an inability to break down the main sugar present in dairy products (lactose) due to the lack of a specific digestive enzyme (lactase) by the small intestine. Adverse reactions to naturally occurring chemicals in food (salicylates, histamine, serotonin and tyramine), as well as reactions to food containing preservatives (sodium benzoate and sulphites), flavourings (monosodium glutamate, aspartame) and colorants (tartrazine) are also involved in food intolerance [25, 26].

Intolerance reactions to food or food components (especially lactose intolerance) can occur at any age but generally, babies born at term produce the enzyme (lactase) so they can digest milk and do not show signs of lactose intolerance until they are at least 3 years old. In Caucasians, it usually starts to affect children older than 5 years of age and it is very common in adults due to a gradual decrease in the production of lactase over time. However, lactose intolerance is sometimes seen in premature babies. Temporary lactase deficiency can result from viral and bacterial enteritis, especially in children, when the mucosal cells of the intestine are injured [27]. Additionally, this milk intolerance is a condition that is often genetically passed on from parent to child. In some rare cases a child is born without the ability to produce lactase at all. In this instance, a baby will be prescribed an infant formula based on soy protein, rather than cow's milk.

Lactose intolerance has been shown to have high racial predilection, being highest in dark-skinned populations and lowest in northern Europeans [28].

Composition of milk allergens

Bovine milk proteins

Bovine milk contains 3–3.5% protein which can be divided into two main classes: caseins (80%) and whey proteins (20%). The latter group of milk proteins remains soluble in milk serum after acidic precipitation of caseins at pH 4.6 that forms the coagulum [29]. Caseins and whey proteins show very different physico-chemical and allergic

Table 1 Main characteristics of major CM proteins

Proteins		Concentration (g/l)	Molecular weight (kDa)	Isoelectric point	No. ofamino acids	Function
Whole caseins (30 g/l)	αS1-CN	12–15	23.6	4.9–5	199	Calcium binding
	αS2-CN	3–4	25.2	5.2–5.4	207	Calcium binding
	β-CN	9–11	24.0	5.1–5.4	209	Calcium binding
	κ-CN	3–4	19.0	5.4–5.6	169	Stabilisation and coagulation of milk
	γ-CNs					
	γ_1 CN		20.5			
	γ_2 CN		11.8			
	γ_3 CN		11.6			
Whey proteins (5 g/l)	β-LG	3–4 g	18.3	5.3	162	Lipid binding protein
	ALA	1–1.5	14.2	4.8	123	Participates in synthesis of lactose
	Ig	0.6–1	<150			Defence scope
	BSA	0.1–0.4	66.4	4.9–5.1	582	Transport of ligands and protection from free radicals
	LF	0.09	76.2	8.7	703	Iron-binding protein, antimicrobial activity

properties and they will therefore be described independently. Their main characteristics are reported in Table 1.

Caseins

Casein (CN) forms the main fraction of milk proteins and is subdivided into a number of families of which the most important are α_{S1}-, α_{S2}-, β-, κ-, γ-caseins. The coagulum (solid fraction obtained after acidification of milk) consists of the whole casein fraction. In the coagulum, individual caseins are cross-linked to form aggregates so called nanoclusters [30] that assemble into larger structures to constitute casein micelles [31]. They are characterised by a central hydrophobic part and a peripheral hydrophilic layer containing major sites of phosphorylation mostly represented by phosphoserine residues [12].

The α_{S1}-caseins (α_{S1}-CN) represent up to 40% of the CN fraction in cow's milk. α_{S1}-CN consists of major and minor components, both are single-chain polypeptides with the same aminoacid sequence [32] differing only in their phosphorylation degree [33]. Three new variants (F, G, H) have been identified in addition to the former A, B, C, D variants characterising different cattle breeds [34–36].

The α_{S2}-CN family accounts for 12.5% of the CN fraction in CM and are the most hydrophilic of all caseins as a result of clusters of anionic groups. α_{S2}-CN consists of two major and several minor components exhibiting varying levels of post-translational phosphorylation. Another post-translational modification occurring in this protein is the formation of disulfide bonds that do not participate in the interaction with other caseins [29].

The β-casein (β-CN) family accounts for 35% of the CN fraction and is quite complex because of the action of the native milk protease plasmin. This protease cleaves the β-CN and thereby generates γ_1- γ_2- γ_3- CN fragments. β-CN is the most hydrophobic component of the total CN fraction. In addition to the existing seven genetic variants another three new variants have been identified called F [37], G [38] and H [39].

Clusters of α_{S1}-, α_{S2}- and β-CN, are due to the anionic regions present in the structures. Such clusters are able to chelate Ca^{+2} but also other metal ions including Zn^{+2} [40] and Fe^{+3} [41]. The significance of nanoclusters for determining the allergenic potential of caseins remains to be demonstrated although dephosphorylation was shown to reduce IgE binding to caseins [42].

The last family is represented by κ-CN which accounts for 12.5% of the total CN fraction. κ-CN consists of a major carbohydrate-free component and a minimum of six minor components. It was isolated from milk as a mixture of disulfide-bonded polymers ranging from dimers to octamers. The two common genetic variants are designated A and B. In addition to them another nine variants have been reported [29]. The κ-CN group plays an important role in the stability and coagulation properties of milk. Its hydrolysis by chymosin in rennet produces para-κ-CN and a caseinomacropeptide which participates actively in the first stage of the cheesemaking process [43].

CNs have hardly a clear three-dimensional structure [44] suggesting the presence of preferentially linear epitopes that have been described in several papers and are summarised in Table 2. A study carried out on sera of 15 milk-allergic children showed that six major and three minor Ig-E binding epitopes as well as eight major and one minor IgG-binding regions were identified on β-CN while two major and two minor IgG-binding epitopes were found for κ-CN [45]. In another study overlapping synthetic

Table 2 IgE binding epitopes from milk proteins in transient and persistent allergic patients

Milk proteins	B cell epitope				T cell epitope
	Sera 11 patients (4–18 years) persistent CMA		Pool sera 8 patients (<3 years) outgrow CMA		4 donors
β-LG [52, 53]	1–16	LIVTQTMKGLDIQKVA			1–21 LIVTQTMKGLDIQKVAGTWYS
					14–29 KVAGTWYSLAMAASDI
	31–48	LLDAQSAPLRVYVEELKP			30–47 SLLDAQSAPLRVYVEELK
	47–60	KPTPEGDLEILLQK	49–60	TPEGDLEILLQK	47–67 KPTPEGDLEILLQKWENGECA
	67–78	AQKKIIAEKTKI			
	75–86	KTKIPAVFKIDA			77–97 KIPAVFKIDALNENKVLVLDT-DYKKYLLFCM
			119–128	CQCLVRTPEV	97–117 TDYKKYLLFCMENSAEPEQSL
	127–144	EVDDEALEKFDKALKALP	129–138	DDEALEKFDKAL	
	141–152	KALPMHIRLSFN	143–152	LPMHIRLSFN	142–162 ALPMHIRLSFNPTQLEEQCHI
	Sera 11 patients (4–18 years) persistent CMA		8 patients (<3 years) outgrow CMA		
ALA [52]	1–16	EQLTKCEVFRELKDLK		–	
	13–26	KDLKGYGGVSLPEW		–	
	47–58	STEYGLFQINNK		–	
	93–102	KKILDKVGIN		–	
BSA [54]	107–123	DDSPDLPKLKPDPNTLC		107–123 DDSPDLP-KLKPDPNTLC	
	336–345	AKEYAVSVLL			
	364–382	PHACYTSVFDKLKHLVDEP		364–382 PHACYTSVFD-KLKHLVDEP	
	451–459	LSLILNRLC		451–459 LSLILNRLC	
	>3 sera 15 patients (4–8 years; median 11) persistent		<3 sera 8 patients less than 3 years transient		
β-CN [45]	1–16	RELEELNVPGEIVESL	1–16	RELEELNVPGEIVESL	
	45–54	LQDKIHPFAQ	45–54	LQDKIHPFAQ	
	55–70	TQSLVYPFPGPIPNSL	57–66	SLVYPFPGPI	
	83–92	VVPPFLQPEV	83–92	VVPPFLQPEV	
	107–120	KEMPFPKYPVEPFT	107–120	KEMPFPKYPVEPFT	
	135–144	LPLPLLQSWM	135–144	LPLPLLQSWM	
	149–164	QPLPPTVMFPPQSVLS	–		
	167–178	QSKVLPVPQKAV	–		
	173–184		QSKVLPVPQKAVPYPQRD		
	185–208	MPIQAFLLYQEPVLGPVRGPFPII			
	Sera 15 patients (4–8 years; median 11)		Sera 8 patients less than 3 years		
κ-CN [45]	9–26	IRCEKDERFFSDKIAKYI			
	21–44	KIAKYIPIQYVLSRYPSYGLNYYQ	21–44	KIAKYIPIQYVLSRYPSYGLNYYQ	
	47–68	PVALINNQFLPYPYYAKPAAVR	53–64	NQFLPYPYYAKP	
	67–78	VRSPAQILQWQV			
	95–116	MARHPHPHLSFMAIPPKKNQDK			

Table 2 Continued

Milk proteins	B cell epitope				T cell epitope
	111–126	KKNQDKTEIPTINTIA			
	137–148	137–148 EAVESTVATLED			
	149–166	149–166 SPEVIESPPEINTVQVTS			
	Sera 24 patients (1–17 years) 9 patients over 9 (9–17; median 12) persistent CMA		8 patients less than 3 years (1–3 years; median 2)		
αs1-CN [46, 51]					1–18 RPKHPIKHQGLPQEVLNE
	17–36	NENLLRFFVAPFPEVFGKEK	17–36	NENLLRFFVAPFPEVFGKEK	16–35 LNENLLRFFVAPFPEVFGKE
	39–48	EL SKDIGSES	39–48	ELSKDIGSES	31–50 VFGKEKVNELSKDIGSESTE
	69–78	EEIVPNSVEQ	–		
	83–102	KEDVPSERYLGYLEQLLRLK	83–102	KEDVPSERYLGYLEQLLRLK	76–95 VEQKHIQKEDVPSERYLGYL
					91–110 YLGYLEQLLRLKKYKVPQLE
	109–120	LE IVPNSAEERL	109–120	LE IVPNSAEERL	106–125 VPQLEIVPNSAEERLHSMKE
	123–132	MKEGIHAQQK	123–132	MKEGIHAQQK	
	139–154	NQELAYFYPELFRQFY	139–154	NQELAYFYPELFRQFY	136–155 GIHAQQKEPMIGVNQELAYF
	159–174	YPSGAWYYVPLGTQYT	159–174	YPSGAWYYVPLG TQYT	152–169 QFYQLDAYPSGAWYYVPL
	173–194	YTDAPSFSDIPNPIGSENSEKT	–		181–199 DIPNPIGSENSEKTTMPLW
					166–183 SSSEEIVPNSVEQKHIQK
	Sera 13 patients (4–15 years: median 8)				
αs2-CN [55]	31–44	SKENLCSTFCKEVV			
	43–56	VVRNANEEEYSIGS			
	83–100	NEINQFYQKFPQYLQYLY			
	93–106	PQYLQYLYQGPIVL			
	105–114	VLNPWDQ VK R			
	117–128	VPITPTLNREQL			
	143–158	STEVFTKKTKLTEEEK			
	157–172	EKNRLNFLKKISQRYQ			
	165–188	KKISQRYQKFALPQYLKTVYQHQK			
	191–200	KPWIQPKTKV			

peptides were used to identify major IgE and IgG-binding regions of α_{S1}-CN in patients with CMA which led to the identification of six major and three minor IgE-binding regions and five major and one minor IgG-binding epitopes [46]. Subsequently, it has been supposed that the majority of linear IgE epitopes in CNs could contribute to persistent allergy [44, 47]. Vila et al. (2001), indeed showed that milk-allergic children with persistent symptoms had a significantly higher levels of specific IgE antibodies to linear epitopes from α_{S1}- (AA69-78) CN and β-CN than children who have achieved tolerance [48]. Jarvinen et al. (2002) completed this statement by defining five IgE-binding discriminative epitopes (two on αs1-CN: AA123-132 and AA 69-78; one on αs2-CN: AA171-180; and two on κ-CN: AA155-164 and AA13-22) exclusively recognised by patients with persistent CMA [49]. Since some amino acids within the synthetic peptides were found to be critical for IgE binding, this could provide useful information for altering α_{S1}-CN cDNA to encode a protein with reduced IgE-binding capacity [50]. In contrast to IgE binding epitopes, T cell epitopes are poorly described in the literature likely due to the difficulty to obtain and generate T cell clones and/or lines from peripheral blood mononuclear cells (PBMC) of allergic patients. The lack of such information has hampered the pathophysiology of allergy and of development of potent a therapy since T cells have a crucial role linked to their involvement in oral tolerance induction as well as the allergy phenomenom. Recently data concerning T cell epitope mapping to the main allergenic proteins from milk is becoming available with the identification of T cell epitopes from αs1-CN [51] as seen in Table 2.

Whey proteins

The main allergic components within the whey fraction are the globular proteins β-lactoglobulin (β-LG) and α-lactalbumin (ALA) followed by minor constituents such as BSA, LF, Ig and proteosepeptone. The latter is derived from milk proteins by the action of indigenous enzymes the most significant of which are the hydrolases like lipoprotein lipase, plasmin and alkaline phosphatase.

In addition to the above mentioned proteins proteolytic fragments of CN and fat globule membrane proteins have been reported to occur in this fraction [56].

β-LG is the most abundant protein (MW=18.3 kDa) in whey, accounting for 50% of total protein in the whey fraction, This protein belongs to the lipocalin superfamily [57] and is one of the best characterised lipid-binding proteins and as such it is capable of binding a wide range of molecules including retinol, β-carotene, saturated and unsaturated fatty acids and aliphatic hydrocarbons [31]. It possesses three disulfide bridges and is present in several variants with the variants A and B widespread [29] and C only found in the Jersey breed. β-LG occurs naturally as a mixture of monomers and dimers, but the proportion of monomers increases after heating to 70°C [58].

Seló et al. (1999) used trypsin digestion to investigate the allergenicity of β-LG and have shown that there are many allergenic epitopes spread all over the β-LG structure [59]. The peptides recognised by 97, 92 and 89% of human sera were the fragments AA102-124, AA41-60 and AA149-162. A second group of peptides AA1-8, AA25-40, AA92-100 was recognised by 58–72% of human sera and the last group accounting for peptides AA9-14, AA84-91 and AA92-100 was recognised by 40%. Jarvinen (2001) [52] used synthetic peptides covering the amino acid sequence of β-LG and identified seven different IgE epitopes and six IgG binding regions (Table 2) that are largely in agreement with the human sera binding β-LG fragments described by Seló et al. (1999) [59]. A number of the β-LG epitopes were mentioned as a marker for persistent CMA. In addition to B cell epitopes, T cell epitopes of β-LG have also been described [53] and are reported in Table 2.

ALA is a monomeric globular calcium binding protein (MW=14.2 kDa) representing about 25% of whey proteins. Its high affinity site for calcium has an effect on the stabilisation of its secondary structure. It interacts with the enzyme β-1,4-galactosyltransferase to form the lactose synthase complex. ALA modifies the substrate specificity of β-1,4-galactosyltransferase allowing the formation of lactose from glucose and UDP-galactose. For its function in the production of lactose this protein plays a major role in regulating physiological functions in the mammary gland [60].

ALA is characterised by four disulfide bridges and is present in two variants. A third genetic variant has recently been reported but is not yet confirmed by protein sequencing [29]. The aminoacid composition of bovine ALA shows a 72% sequence identity to human ALA which makes it an ideal protein for the nutrition of human infants. As far as ALA allergenicity concerns, studies aimed to investigate the human IgE-binding to native ALA and large peptides confirm the importance of conformational epitopes [61]. However, in some patients reduced peptides exhibited a similar or even higher IgE-binding capacity than the native corresponding fragment suggesting the existence of linear epitopes located in hydrophobic regions and exposed as a consequence of protein denaturation [12]. Epitope mapping of ALA identified four different linear IgE-binding peptides overlapping with 3 IgG-binding regions [52]. However, none of these IgE epitopes was recognised in allergenic children suspected of persistent CMA. T cell epitopes for ALA have not yet been described.

BSA accounts for around 5% of the total whey proteins (MW=66.4 kDa). BSA is physically and immunologically very similar to human blood serum albumin. Its main role is the transport, metabolism and distribution of ligands and the protection from free radicals [29]. This protein is organised in three homologous domains and consists of nine loops connected by 17 disulfide bonds [62]. Most of the disulfide bonds are protected in the core of the protein and are therefore not easily accessible [63]. This may explain why its tertiary structure is quite stable, even under denaturing conditions.

The presence of disulfide bonds in the BSA molecule has been demonstrated to play an important role in maintaining the native antigenic determinants of this molecule.

Habeeb and Borella [64] showed that the reduction of disulfide bonds completely abolished the reaction between BSA with anti-BSA antibodies suggesting that a drastic modification occurred in the tertiary structure. Elsewhere it has been demonstrated that serum albumin antigenicity is only partially correlated with its native three dimensional structure [65]. Several studies have been performed in order to evaluate the antigenic potential of fragments derived from BSA by proteolysis and in these assays the formation of an immune complex was evaluated using sera derived from immunised animals. The epitopes identified in different studies are not always in agreement with each other, this is probably due to different experimental conditions [66–68]. Tanabe et al. (2002) tried to identify IgE binding epitopes as well as T cell epitopes from BSA and listed a few epitopes involved in beef allergy that are commonly T and B cell epitopes [54]. Restani et al. (1999) showed that IgEs specific for BSA from sera of allergic children, were able to cross-react with albumins from sheep and pig, but they did not recognise those of horse, rabbit and chicken [69]. It has been demonstrated that the most critical sequence seems to be AA524-542 even if this peptide should be included in a longer sequence to be recognized by human antibodies. Moreover, results showed that epitopic sites of an antigenic protein can change when different animal species antibodies are used [70].

LF (MW=76.1 kDa) is a protein of mammary origin and is a milk-specific iron-binding protein. It can be found in the milk of most species at levels lower than 1% [71]. LF consists of a single polypeptide chain folded into two globular lobes, each of them having high-affinity iron binding sites, connected by a 3-turn helix. It contains five potential glycosylation sites [72], and the molecular weight of this protein varies depending on the extent of its glycosylation [73]. The LF content is species dependent with significantly higher levels in human milk and colostrums compared to the bovine whereas the sequence homology and structure are very similar [12, 74]. Its main role is to defend the organism against infections and inflammations due to its ability to sequester iron from the environment and thereby removing this essential nutrient for bacterial growth [75]. Taylor et al. (2004) discussed the potential allergenicity of lactoferrin derived from bovine milk which is supported by the fact that some milk allergic individuals possess lactoferrin specific IgE [76]. But, the relevance of the allergenicity of lactoferrin is still under discussion since these patients also had IgE directed against one of the major milk allergens. So far no oral challenges have been performed in those patients presenting lactoferrin-specific IgE to confirm the allergenic capacity of lactoferrin. Wal et al. (1998) reported that milk allergic patients (41 out of 92) had detectable levels of IgE to bovine lactoferrin and concluded that lactoferrin is a milk allergen [77]. Sensitisation with lactoferrin was observed in mice models and rats with the production of high titers of lactoferrin specific IgE [78]. However, to date no lactoferrin IgE epitopes or T cell epitopes have been reported.

The Ig fraction accounts for about 1% of total milk protein and 6% of whey proteins. Different studies indicate that the basic structures of Ig in *Bos* species are very similar to those of human origin. Ig possesses a basic "Y shaped" unit composed of four polypeptide chains linked through intra and intermolecular disulfide bonds. The monomers are composed of heavy and light chains each of these composed by so called V and C domains. The V domains of H and L chains converge to form the antigen-binding site while the C regions characterise the isotype of the Ig in cow milk: IgG, IgA, IgM [79]. Within the IgG class findings demonstrated the existence of three IgG classes in cattle recognised as IgG1, IgG2, IgG3 [80, 81]. Data on the potential allergenicity of bovine immunoglobulins is very limited. However, some studies propose IgG as another milk allergen due to the observation that IgE from CMA patients specifically binds bovine IgG [82, 83]. Unfortunately neither B nor T cell epitopes of bovine IgG have been resolved. This is likely due to the fact that so far it has not been considered as an allergen, although Ayuso et al. (2000) identified bovine IgG as a major allergen in beef allergy [84].

The proteose-peptone fraction represents about 1.1% of the total milk protein and it is a heat-stable and acid-soluble protein fraction of milk with important functional properties. This milk component is mainly derived from the proteolysis of β-CN and the enzymatic activity of plasmin can increase its concentration in milk with time.

In conclusion, there is not just a single protein responsible for CMA. All milk proteins, encompassing whey proteins as well as CNs, can be considered potentially allergenic. The three dimensional structure of CM proteins plays an important role in maintaining conformational epitopes. Next to this, a large number of specific amino acid sequences present in CM proteins are recognized by B or T cells. Such linear epitopes are distributed throughout the cow milk proteome which contains a vast array of components that can be responsible for allergic reactions.

Comparative aspects of different types of milk

During the period of sensitisation to bovine milk, children with a high-risk of atopy or diagnosed CMA receive sometimes food substitutes. This can include other milk source proteins like formulae containing goat and/or soy milk, CM hydrolysates or amino-acid based formulae [85, 86]. It is therefore interesting to consider the adequacy of those alternatives by comparing their biophysical and allergenic properties to bovine milk.

The genetically determined polymorphism of milk proteins is of great importance in specifying the origin of milk and in animal breeding. Although the gross composition of cow's sheep's and goat's milk is very similar [87], slight differences in fat, solids (non-fat) and protein fractions result in changes in biological and physico-chemical properties. Table 3 shows the difference in protein content of different kinds of milk. Sheep's milk contains almost twice as many solids as cow's milk and has

Table 3 Protein content in different kinds of milk

Proteins	Protein (%)		
	Cow	Goat	Sheep
Total protein	3.1–3.9	2.9–3.1	5.5–6.2
Whole casein	2.3–3.3	2.1–3.3	3.8–5.2
αS1-CN	37.6–39.5	0–1.9	35
αS2-CN	7.8–12.1	14–19.3	8
β-CN	33.4–44.6	58.5–60.0	38
κ-CN	33.4–44.6	14.9–20.0	17
Whey proteins	0.6	0.4	0.8

Based on data from Jandal et al. (1996) [87]; Bramanti et al. (2003) [89]

a higher casein and fat content [87]. The higher proportion of medium/short chain saturated fatty acids is believed to lead to a higher lactose absorption which might benefit the mildly lactose intolerant [88].

Goat milk proteins have many significant differences in their amino acid composition compared to the milk proteins of other mammalian species, in addition to this the relative proportions of the various milk proteins differ in goat and cow's milk [87, 90, 91]. Goat's milk may contain only trace amounts of the allergenic casein protein, α_{S1}-CN, whereas β-CN represents the most abundant protein in goat's milk. This aspect can be exploited to detect possible adulteration of goat milk with cow milk. Compared to CN from cow's milk, goat's milk CN is more similar to human milk, although cow's milk and goat's milk contain similar levels of the other allergenic proteins.

Just like sheep's milk goat's milk differs from cow's milk in terms of fat content. Goat milk has a higher content of the essential fatty acids (linoleic and arachidonic) with a greater percentage of medium and short chain saturated fatty acids compared to cow milk. Moreover, it forms a softer curd by the action of the stomach acid on the protein. The lower curd tension of goat milk and the different chemical and physical composition of its fat, suggests greater digestibility of goat milk especially in infants [92].

Goat milk often appears as a substitute for cow's milk, particularly in diets administered by parents to children with atopic dermatitis and it has been demonstrated that many CMA children can tolerate goat's milk [93]. In a study of crossreactivity of the casein fractions among different species in fact the IgE response to sheep and goat's milk was weaker than that to cow and buffalo [94].

However, the true prevalence of goat's and sheep's milk allergy is not exactly known and is surrounded by controversy. IgE sensitisation to sheep and goat casein has been found to be as high as 93 to 98% in children with IgE-mediated cow's milk allergy, suggesting that children who are allergic to cow's milk are also allergic to sheep's and goat's milk [95]. This implies that for the prevention of CMA the use of milk from ruminant species other than cow is no adequate replacement. This is confirmed by the crossreactivity of goat's and cow's milk by RAST inhibition [94].

As for soy-based formulae, the reactivity noticed in CM allergic children is presumably attributed to cross-reactive allergens between soy and casein such as the suspected 30-kDa component from soybean that consists of two polypeptides (A5 and B3) linked by a disulfide bond [96].

Methods for evaluation of the immunogenicity of CM allergens

Enzyme-linked immunosorbent assay (ELISA) tests, as well as EAST for the determination of specific IgE binding to highly purified CM proteins such as β-LG variant B, CN, ALA, BSA, LF, have been used to study the variability of the affinity, specificity and magnitude of the human IgE response. Wal et al. (1995) performed a study aimed at estimating the IgE concentration in sera of 20 milk-allergic individuals [97]. The findings revealed that most CM proteins are involved to different extents and some patients may only be sensitised to minor proteins present in traces such as BSA and LF. But, despite the fact that the sensitivities to CN, ALA and β-LG appear to be closely related, the sensitivity to BSA was completely independent and is therefore not a good marker for CMA. Another work, on a larger population, demonstrated that only 26% of 92 allergic patients were monosensitised, whereas most of them were sensitised to several proteins. The proteins CNs, β-LG, ALA appeared to be major allergens since 65, 61 and 51% of patients were specifically sensitised to these proteins, respectively. Proteins present in very low quantities such as BSA, Igs, LF also appeared to be of great importance since 43, 36, 35% of patients were sensitised to these proteins, respectively [98]. However, there are indications that the pattern of allergenicity of different milk proteins is in continuing evolution [99].

In a recent study sera from 20 individuals, all proven to be CM allergic by oral provocation test and skin prick test, have been used for CM major allergen identification. All patients were positive to the skin prick test but five of them had negative RAST results. All patients were sensitised to one or more casein fractions, the prevalence of sensitisation being: β-CN (15%), κ-CN (50%), α_{S1}-CN (55%) and α_{S2}-CN (90%). Sensitisation to β- and κ-CN was always associated to α-CN sensitisation. Specific IgE to β-LG and BSA were detected in 45% of patients. However, specific IgE to ALA was not detected in any patient by 2-D immunoblotting [83].

From an allergenic point of view the casein fraction is well-known to contain the main allergenic proteins in CM [100]. Although the α_{S1}-CN linear epitopes have been correlated with persistent CMA the prevalence of sensitisation to each fraction is still controversial [50]. Most patients, shown to be allergic to specific casein, are sensitised to all four types of caseins. However, polysensitisation also appeared to be due to cross-sensitisation mechanisms [12]. On the other hand, the high IgE cross-reactivity between cow's, ewe's and goat's milk casein may also occur in many

patients with CMA and therefore adverse reactions can occur in patients allergic to CM who consume other types of milk [98].

Threshold doses

So far, very little information exists on threshold doses for specific allergens in food. Insufficient data are available to set lower confidence limits on doses that result in an allergic response in a given proportion of the studied population. Four different approaches have been defined in order to establish the thresholds namely approaches based on analytical methods, safety and risk assessments as well as statutorisation. The best estimates of threshold doses for various allergenic foods can be obtained from controlled clinical challenge trials. In just a few cases such trials were specifically intended to determine the threshold doses [101–105]. More often, these challenges have been performed for diagnostic purposes rather than for determining the lowest provoking dose. Typical protocols involve starting at doses that are one half or less of the amount of the offending food estimated to provoke symptoms in a patient [101]. Table 4 contains data relevant to the lowest provoking doses in DBPCFCs for CM. The results obtained are difficult to interpret because several different forms of CM products were used in the oral challenges such as CM, non-fat dry milk and infant formula. The nature of the challenge material is in fact another factor increasing the uncertainty; therefore, a standardization of the specific form of foods used in the challenge could be useful in achieving comparable data from different clinics.

As reported in a recent study, the lowest observed adverse-effect levels (LOAEL) have been estimated to be in the range of 1–2 mg of natural foods, representing a few hundred micrograms of protein. These minimal reactive doses have been demonstrated to characterise about 1% of people allergic to milk, egg, or peanut [106]. Such data emphasise the necessity of using detection tests with a sensitivity better than 10 parts per million. The modifications of allergenicity undergone by protein ingredients that are now commonly introduced into industrially made products are not yet sufficiently known. A better knowledge of the reactive doses of these proteins is needed.

However, clear results are available to conclude that the threshold doses for commonly allergenic food are measurable. Aiming at the estimation of threshold doses for commonly allergenic foods it has to be highlighted that some difficulties exist in designing experiments for its determination. It is probably due to the no-observed-adverse-effect level (NOAEL) not yet established for the vast majority of patient enrolled in these trials [101].

On the basis of published data, some authors have developed a statistical model using the actual allergen content in different food products. The lower confidence limit associated with one-part-per-million response rate considered the threshold value for food, was calculated to be for milk 0.046 mg. When the amount of food actually used was converted to calculate the allergen (protein) content, the allergenic protein threshold value found for CM was around 7×10^{-5} mg.

The model demonstrated that the threshold doses giving a reaction of one in a million susceptible patients were within the same order of magnitude for egg, milk and soy [107].

Effects of milk processing on allergenicity

Heating processes

Thermal processing is used to destroy pathogens that endanger public health and reduce microrganisms in order to prolong the shelf life of milk. A variable extent of inactivation of enzymes occurs, depending on temperature and treatment time. With the aim to retain as much as possible the good organoleptic and nutritive properties of the raw material, different heating methods have been developed. Heat treatments can be divided into two categories: pasteurisation treatments and sterilisation treatments, both aimed at a partial or total destruction of microgranisms. Pasteurisation could consist in different binary cycles temperature-time, respectively: *batch heating*

Table 4 Data relevant to the lowest provoking doses in food challenges for CM based on data from Taylor et al. (2002) [101]

Research group	No. of patients	Nature of challenge	Lowest provoking dose		From
			Amount of food	Amount of protein (mg)	
Bindslev-Jensen and Norgaard	3	DB	5000 mg	180	Milk
Hill et al.	100	Open	0.02 ml	0.6	Milk
Bock et al.	66	DB	2 ml	67	NFDM
Burks and Christie	21	DB	400 mg	140	NFDM
Rance et al.	31	DB	0.5 ml	15	Milk
Lack et al.	6	SB	5 ml	150	Milk
Moneret-Vautrin	6	DB or	1 ml	30	Milk
		SB	5 ml	150	
Zeiger et al.	56	DB	0.1 ml	1.5	Formulae
Host et al.	15 A∗	DB	5 ml	75	Formulae
	15 I**	DB	5 ml	75	

DB: double blind; SB: single blind; A* CMA; I** CMI; NFDM: non-fat dry milk (dry milk powder produced by extracting fat and water from pasteurized fresh CM); Formulae: estimated to contain 15 g of milk protein per liter.

(62–65°C for 30–32 min), *short time heating* (72–75°C for 15–30 s) or *high temperature for short time* (>85°C for 4 s). Besides, sterilisation treatments could be distinguished in *in-container sterilisation* (109–120°C for 20–40 min) and *UHT sterilisation* (135–150°C for 2–20 s).

For pasteurised products a slight binary cycle temperature-time is used in order to ensure the deactivation of the most dangerous pathogens. Pasteurised products have only a limited storage life since the number of microrganisms is reduced and not totally destroyed. Most enzymes that occur in raw milk can be inactivated by pasteurisation, the effects of heating time and temperature on enzyme inactivation vary for different enzymes. The shelf life of sterilised and ultra-high temperature-treated milk (UHT) is much more prolonged compared to pasteurised milk resulting from the high extent of destruction of all microrganisms and spores. Most enzymes in raw milk are inactivated under sterilisation conditions. Moreover, it has been reported that the same bactericidal effect of spore reduction by UHT-heating causes smaller losses of the vitamin thiamine and the amino acid lysine and other nutrients than in-container sterilisation [108].

Effects of heating on milk protein allergenicity

It is still unclear whether heat treatments decrease or enhance the allergenicity of CM proteins. Hence, the challenge for food scientist and manufacturers is to better understand the real effects of thermal processing on allergenicity to minimise the impact on allergic consumers. Heat processing has a different impact on the stability of the various individual CM proteins. Although pasteurisation is not expected to modify the protein structure significantly, a study carried out in allergic children and adults demonstrated that raw milk, pasteurised milk and pasteurised and homogenised milk caused allergenic symptoms with a higher allergenicity in the pasteurised samples [109].

Casein is reported to be more thermostable [12], whereas β-LG manifests a thermolabile behaviour. However, by interaction with caseins, β-LG could be protected against this behaviour. On a molecular level heat treatment causes a denaturation of proteins including unfolding and aggregation of the molecules. Depending on the conditions, partial, complete as well as reversible or irreversible denaturation can be observed. External factors acting on the native interactions of the protein structure (e.g. hydrogen bonds, electrostatic interactions, hydrophobic interactions and disulfide bonds) can affect protein denaturation. Some authors have used nuclear magnetic resonance (NMR) methods to study the thermal denaturation of selected proteins and found that the temperature of denaturation of β-LG and ALA was in the range of 70.5–81.5°C and 58.6–61.0°C, respectively [110].

Differences in allergenicity of CM proteins do not only depend on the temperature and time of heating but also on possible interaction with other CM proteins or components of the food matrix. It has been reported that heat denaturation can remove conformational epitopes as a result of the loss of tertiary protein structure and therefore leads to a decreased allergenic potential [111]. By contrast formation of aggregates generated by heat treatments may lead to an increasing allergenicity of the heated products [12].

Heat-induced changes of β-LG have been studied widely [112–116] and it was found that β-LG exhibits a complex mechanism of unfolding during denaturation as a result of heat processing [117]. It has been reported that initial β-LG denaturation leads to the formation of small well-defined clusters with a size independence of concentration, temperature and ionic strength [118]. In a second step these clusters aggregate in larger structures whose growth accelerates with increasing heating time. The aggregates are held together via disulfide bridges and non-covalent interactions, mainly hydrophobically driven associations [116, 119]. The formation of different sized aggregates has proven to be necessary intermediate stages in heat induced aggregation [120]. It is also well known that β-LG can interact with casein micelles involving κ-CN when milk is heated at temperatures above 80°C. Both sulphydryl disulfide interchange reactions and hydrophobic interactions are involved in this interaction [121].

When a mixture of ALA and β-LG is heated, aggregation between the two proteins takes place. One of the different models proposed to explain this phenomenon states that at the beginning different monomers of β-LG could react by way of a thiol-catalysed disulfide bond interchange to form dimers, trimers etc. At the same time reversible conformation changes occur in the ALA molecule so that an exposed group of β-LG could react via thiol/disulfide exchange with one of the disulfide bonds of ALA forming ALA/β-LG adducts. Subsequently the β-LG/ALA dimer with a free thiol group could react further with other ALA molecules or native β-LG driving to aggregates [122].

The heat induced changes in immunoreactivity of the main whey proteins have been analysed using inhibition ELISA for ALA and β-LG variant B [123]. Since the majority of epitopes recognised by anti-protein antibodies are conformational, it is expected that antibody binding may be perturbed by changes in the three-dimensional structure of a protein such as those that accompany thermal denaturation. The effect of heat-treatment on ALA consists of protein denaturation as well as protein aggregation which leads to a reduction in immunoreactivity as a result of a decreased accessibility of antibody recognition sites. In contrast to this, the antibodies raised to β-LG variants that preferentially recognise the thermally denatured protein show an increased immunoreactivity coinciding with the alterations in the secondary structure that are associated with thermal denaturation, as shown in Table 5 [123].

BSA that exists in dimeric and trimeric forms under native conditions was shown to be in its monomeric form after heat treatment at 100°C for 10 min with no apparent loss or change of allergenicity. When native or heat-treated BSA is denatured by treatment with SDS, the protein does not show a decrease of its antigenicity [65], despite the fact that this treatment drastically modifies the three-dimensional structure as a consequence of disulfide bridge reduction and the breaking of nine loops [63].

Maillard reactions result in the attachment of reducing sugars to the free amino groups of lysine in CM proteins which can occur as a consequence of milk heating [124].

These types of reactions that take place rapidly at temperatures above 100°C promote the interaction of the protein with a carbonyl group of a reducing sugar to form a reversible Schiff's base which rearranges to stable, covalently bond Amadori products for aldoses or Heyns products for ketoses. In the advanced phase of the reaction, Amadori products undergo further transformation to coloured substances used as indicators of the reaction rate and crosslinked polymers [125]. The end products generated in this way are advanced-glycation-end-products (AGE), which could exhibit an important role in food allergy. Another main Maillard reaction product is carboxymethyl lysine (CML) that is formed by a reaction of sugar breakdown-products with dicarbonyl intermediates [126].

Even sucrose can participate in the glycation process. It has been shown that, at the temperatures reached during normal heat processing, sucrose undergoes hydrolysis releasing fructose and glucose both of which are capable of modifying proteins [126]. But glycation is just one of a series of reactions occurring and resulting in the formation of numerous proteins adducts. Other covalent modifications of proteins caused by heating or storage such as reactions with oxidised lipids, disulfide bond scrambling or deamination of the amino acid asparagine can contribute to changes in antigenicity [20].

The ability of such neo antigens to work as allergens has been confirmed by cases reporting allergy exclusively against AGE modified proteins [20]. The β-LG heated at 50°C in the presence of lactose was described to increase 100 times its skin reactivity [127]. As far as AGE epitopes are concerned, *N*-ε-carboxymethyl lysine (CML) was found to be one of the major immunological epitopes among glycoxidation products [128]. In addition to this, AGE-proteins express at least two major non-CML epitopes [129].

In conclusion, various heat processing techniques used in the manufacture of food products can alter the allergenic potential of CM proteins. Findings have demonstrated that bovine allergens may be decreased, increased or unchanged by exposure to processing techniques such as heating, pasteurisation and homogenisation. More extensive research is needed to determine the precise effects of food processing on allergenicity.

Table 5 Immunoreactivity of the whey proteins ALA and β-LG in thermally-treated milks (after Karamonova et al. (2003) [123])

Milk treatment	Immunoreactivity of whey proteins (%)	
	ALA	β-LG
Raw	100	100
Pasteurised	100±19	1401±312
UHT	18±2	563±138
Sterilised	1.0±0.1	141±28

Alterations in immunoreactivity of the whey proteins in treated milks were calculated as percentage relative to the amounts of immunoreactive protein present in the raw milk samples

Hydrolysis

Protein hydrolysates or amino-acid based formulae constitute the best option for polyallergic or highly sensitive subjects who require high hypoallergenic alternatives since persistent sensitisation to food for a long period (above 1 year) in children with high-risk of allergy increase the susceptibility of those children to develop subsequent allergic airway diseases [130]. Many different protein hydrolysate-based infant formulae have been promoted as hypoallergenic [85]. Hydrolysates are currently also used to improve the functional properties of the proteins such as their solubility, the emulsifying and foaming as well as gel forming capacity and are therefore used in the manufacture of new products and/or in the innovation of the existing ones [131]. Differences in hydrolysates manufactured come from the protein source "used" for the production of those hydrolysed formulae. They can indeed be based either on exclusively casein or whey proteins or on a mixture of whey and casein (in a ratio 60/40 similar to the one found in mature human milk [132]) or even on a soy protein or a mixture of soy protein and bovine collagen. Rice hydrolysate formula seems to be well tolerated by children from 1 to 9 years and might be considered as an innovative product but its possible allergenicity is not yet fully addressed [133]. Whey protein hydrolysates are widely spread likely due to their better physical acceptance properties (palatability, taste. . .) combined with their hypoallergenic properties [134]. Those commercially available hydrolysates can also be distinguished in partially (pHF) and extensively (eHF) hydrolysed milk formulae according to their degree of protein hydrolysis [135]. For instance, pHF from CM whey or casein possess nearly 40,000-fold higher levels of β-LG than eHF [136].

While the former group, containing mainly large peptides (>8000 Da), is advised to high-risk atopic children, (the latter, characterised by a mixture of large and small peptides (<1500 Da) and free amino acids (∼100 Da) is destined for the consumption of diagnosed allergic neonates [137, 138].

Discussions and contradictions have arisen with regard to the use of pHF as standard feeding for infants with a high-risk of allergy. Studies on feeding babies with a high-risk of atopy, with pHF and eHF, revealed that only eHF, significantly decreased the prevalence of CMA [139, 140].

However, the efficiency of the use of pHFs in high-risk allergic infants has recently been proven in several clinical trials [141]. Their consumption has been accompanied by an adequate growth, a suppression of CM-specific cellular responses and a decrease in stimulation of specific IgG production compared to intact whey-predominant formula [142–144]. Furthermore, pHFs were suggested to be more effective in the prevention of CMA than eHFs, due to their ability to better induce tolerance as described in an animal model [145, 146]. To prevent any controversies, a strict legislation regarding the so-called hypoallergenic formulae has been implemented after recommendations from various health committees (American Academy of Pediatrics (AAP), European Society of Paediatric

Gastroenterology and Nutrition (ESPGAN) and European Society of Paediatric Allergy and Clinical Immunology (ESPACI)). This legislation defines criteria for the acceptance for hydrolysate formulae on the market [147]. It is stated that hypo-allergenic substitutes should be tolerated in DBPCFC by at least 90% of children with a diagnosed CMA and this tolerance should be confirmed within 2 months. Commercial extensive hydrolysates that were tolerated in the range of 94–97% in oral challenge test are therefore considered to be safe [148, 149]. It is not excluded nevertheless, that the remained percentage triggers some reactions with eHFs and even more with pHFs (tolerance 45–50%) [150]. In fact, despite the huge decrease in allergenicity of the hydrolysate formulae and the safety controls, both eHFs and pHFs may still induce severe reactions (anaphylaxis) in some allergic patients [151, 152]. In the most severe cases of allergy, multiple food allergy and/or reactivity against hydrolysed products, nutritionally complete amino-acid derived formulae, that are efficient in alleviating allergic symptoms and stabilising the infant growth, are preferentially purchased [153, 154]. Those reactions noticed towards hydrolysates suggest the persistence of allergenic molecules that might be either residual intact proteins or neo antigenic peptides (epitopes) induced during the hydrolysis process or even aggregates formed during the production or the reconstitution of the formula [138]. The formation of such aggregates (peptide–monosaccharide) has been limited by the elimination of lactose from hypoallergenic formula in order to avoid intolerance reactions but the effect of the presence of other sources of carbohydrates such as glucose, corn syrup or maltose, that are all already known to participate in Maillard reactions, or maltodextrine remains to be characterised.

Minute amounts of allergenic protein or insufficiently hydrolysed peptides with at least two epitopes could account for allergic reactions observed in individuals sensitised to CM [155].

Physico-chemical and immunological methods have been developed to confirm the safety of the hydrolysates and to evaluate their residual antigenicity [156]. Generally the allergenicity of hydrolysates is partially depending on their molecular weight [157]. The control of the lengths of peptides in hydrolysates is important for the development of physiologically optimal hydrolysates for use in peptide-based hypoallergenic infant formulae [158]. Determinations of the degree of hydrolysis and the molecular mass distribution (via gel permeation chromatography) illustrating the proportion and the size of the hydrolysed fragments were, however, not sufficient to define the residual allergenicity of the corresponding formula and required a complementary set of in-vitro as well as in-vivo tests. In fact, most of the tests described for the detection of milk proteins are applicable to hydrolysate formulae such as immuno-diffusion, immuno-rocket, electrophoresis, ELISA or radio-immunological (RIA) [159].

ELISA methods, based on allergen specific IgG or IgE detection, are the most conventional and sensitive in vitro techniques to estimate the residual antigenicity of commercial hydrolysed formulae that are available for infants with CMA [160, 161]. Detection of the interesting allergenic molecule in such assays can sometimes be hampered by the cross-reactivity of antibodies towards different proteins. As already stated, crossreactivity exists between β-LG and ALA (10%) Among milk proteins, but also different types of caseins seem to share common epitopes which explains somehow the co-sensitisation to the different caseins in CM allergic children [162, 163].

Such assays have proven the lower IgE and IgG residual antigenicity of individual milk proteins in hypoallergenic products compared to the one from intact milk protein (from 0.05 to 0.67% of total protein) [164]. Nevertheless, immunoreactive epitopes could still be detected in most of the products tested except in certain extensive casein hydrolysates or the amino-acid based formula [164, 165].

Hoffman et al. (1997) who looked at residual immunoreactive fragments in extensively hydrolysed formulae with inhibition-ELISA methods and sera from patients with IgE-mediated CMA, identified residual protein fractions of less than 20 kDa in several extensively hydrolysed CM-based formulae tested [166] but even protein fragments of 700–1400 Da (Dalton) have proved to be immunogenic [167].

Further studies evaluated the remaining risk of corresponding formulae by estimating the lower molecular weight limit of peptides able to elicit skin reactions and to bind IgE antibodies in vitro. Skin-prick tests were performed with an ultrafiltrated whey hydrolysate and its different molecular mass fractions in five milk allergic children. A positive skin reactivity was noted with peptides above 1400 Da while the minimal molecular mass for detecting a IgE binding in vitro ranged between 970 and 1400 Da [168]. Those peptide masses however should normally be absent in extensive hydrolysates. Wal concluded in the same way that peptides as short as 12–14 amino acids residues could be involved in a significant part of the allergenicity of CM molecules [18]. Such peptides might be used to develop a safe formula for patients reacting to milk hydrolysates or even for potential tolerance induction [168].

Hydrolysation processes

Various food manufacturing processes have been tested in order to alter the allergenicity of CM such as mechanical, thermal (dry heating, boiling, or cooking), biochemical, and chemical processes (enzymatic digestion) [111]. Heat treatment was previously believed to be a good way to reduce the allergenicity of milk proteins, but as discussed above the allergenicity of some CM proteins can even be increased as a result of heating. While initially evidence of a clear reduction of the proteins' antigenicity at high temperature and the absence of sensitisation of guinea-pigs participated to the interest in that process [169] but later it became clear that for decreasing allergenicity heating is not adequate. Heat denaturation was indeed not satisfying since it could not lead to an acceptable hypoallergenic formula due to a non-sufficient reduction of the antigenicity of some milk proteins, especially caseins, and to the

non-compliance of the hydrolysed protein formula in term of nutritional values [137].

The hydrolysation process has been seriously improved by the use of specific enzymes to prevent sensitivity to hypo-allergenic products [170]. Enzymatic hydrolysates were firstly generated with the use of gastrointestinal enzymes, e.g. trypsin, pepsin, chymotrypsin to potentially mimic physiological digestion and compensate the intestinal and enzymatic systems' immaturities of newborn babies [171, 172]. The use of those types of enzyme hydrolysed formulae also circumvents the low gastrointestinal enzyme activity of the newborn due to a buffered stomach. This is well illustrated in a study performed by Schmidt et al. where a poor and slow hydrolysis of whey protein epitopes was observed at a pH ranging from 2.0 to 4.0 supposed to simulate the pH conditions that prevail in the stomach of infants [173]. This way of abolishing the allergenicity of whey proteins seemed promising in studies carried out with guinea pigs. Those studies showed the absence of sensitisation after oral administration of trypsin-hydrolysed whey protein and an ultrafiltrated derived tryptic hydrolysate peptide in contrast to milk or untreated whey protein which led to anaphylactic reactions [170].

The effects of in vitro proteolysis on the allergenicity of major whey proteins with different proteases which simulate the human gastrointestinal conditions were evaluated for the reduction of whey protein antigenicity [174]. However, hydrolysation of β-LG by such enzymes (trypsin alone or associated to chymotrypsin or pepsin with chymotrypsin) significantly decreases the allergenicity of β-LG but it does not abolish it [174]. The combination of an enzymatic hydrolysis with a preceding heat treatment considerably enhanced tryptic and peptic hydrolysis of the major milk protein (β-LG) and the reduction of the allergenicity [175, 176].

Enzymatic hydrolysis became thereafter a standard procedure for the production of hydrolysates with hypoallergenic properties. However, as for heat treatment, milk proteins do not have the same sensitivity to endopeptidases used to manufacture hydrolysates. Among milk proteins, α- and β-CN as well as β-LG and α-ALA are very sensitive to trypsin in contrast to immunoglobulins and BSA [177]. Genetic variants of β-lactoglobulin also differ in their rate of hydrolysis with trypsin (β-LG A>β-LG B>β-LG C) [178].

Selective proteolysis of whey protein by pepsin and α-chymotrypsin was found to be the most efficient combination of enzymes to reduce allergenicity of both ALA and β-LG, the degree of hydrolysis ranged from 1 to 20% depending on the enzyme combination and time of incubation [179]. However, the conditions used to produce such hydrolysates led to problems. In particular the acidic pH conditions necessary for hydrolysation with pepsin required high salt levels to reach a neutral pH for the formulation of the hydrolysate [180].

A third generation of hypoallergenic formulae nowadays available is the result of a better understanding of the enzymatic processes involved in hydrolysis to yield hydrolysates with a low level of free amino acids and a main fraction of short peptides [179]. The whole range of physiochemical parameters defining the protein hydrolysates such as the purity of the protein source, the pre-treatment of the protein source, the specificity of the enzyme used for the proteolysis, the physicochemical conditions used during the hydrolysis (pH, temperature, ionic strength, activator), the degree of hydrolysis, the technique of inactivation of the enzyme (heat treatment, acidification, membrane filtration), the use of posthydrolysis treatments (adsorbents for free amino acids, membrane separation) are considered in order to optimise the hypoallergenic formulae [24, 181].

Indeed, more and more commercialised milk hydrolysates are produced with new types of enzymes from for instance bacterial or fungal origin which have a broader specificity [182]. Currently, endopeptidases and exopeptidases are combined for the production of hydrolysed formulae to improve at the same time the formulae taste by reduction of the bitterness as well as the allergenicity by hydrolysation of hydrophobic peptides suggested to be mainly responsible of the allergenic potency of the protein source [183] Innovation to increase the efficiency of enzymatic hydrolysis has been attempted with the use of reactor systems, characterised by continuous protein hydrolysation performed with immobilised enzymes (endopeptidase and exopeptidase) instead of conventional batch reactors [184, 185]. This procedure has been applied for instance for the obtention of purified CM derived bioactive peptides [186, 187]. But, despite the fact that this procedure is attractive due to the continuous hydrolysis with lower amounts of enzyme and is an easy way to exclude the enzyme from the final product without the need for further processing such as heat treatment, regeneration procedures are problematic or expensive and limit reactor expansion.

Progress has been made in the process of the production of hypoallergenic formulae with the introduction of a crucial additional step of ultrafiltration in order to remove large residual peptides (of molecular weight >2500 Da) [137, 188, 189]. The enzymatic hydrolysis in combination with ultrafiltration seems indeed the most efficient method to reduce the antigen content of hypo-allergenic infant formulae. This is proven in vitro, by immunoblotting, and in vivo, by the absence of sensitising capacity and anaphylaxis in orally sensitised animals, when submitted to hydrolysates previously ultrafiltered compared to those which were not [189, 190].

The description of more and more cases of allergic reactions elicited after the consumption of CM hydrolysates by children with CMA either transient or persistent, and soy allergy leads to research to find new food alternatives. Despite the fact that CMA often is a transient food allergy that is normally outgrown by the infants at the age of 3, 15–20% of them become permanently allergic with increased levels of IgE and more especially CM specific IgE [191]. Since monitoring of specific IgE concentration for milk and casein reflects the tolerance induction of the allergic patient, the sooner tolerance is detected, the earlier the substitute diets should be suspended [192].

Despite all the efforts given to processing methods, used to limit the allergenicity of formulae, the persistent occurrence of linear epitopes in hydrolysed formulae

rendered difficult their production. Knowledge about epitopes (mainly IgE) of the allergenic whey proteins and caseins as summarised in Table 2 can assist in looking for new processing methods that may lead to the destruction or the elimination of those offending sequences or the aggregates (peptide–peptide; peptide–sugar) generated during the hydrolysis process and detected by LC-MS with high sensitivity [193, 194].

Recently, the use of hydrolysed proteins has been extended by combining them with probiotics in the development of new functional hydrolysates. Based on several experiments that have demonstrated the beneficial effects of probiotics in the decrease of symptoms of allergic patients, probiotics appear one innovative mode of prevention and therapy of food allergy through the hypoallergenic formulae [195]. Probiotics such as *Lactococcus lactis*, other LAB or *Lactobacillus rhamnosus* GG or *Bifidobacterium lactis* Bb12 that have been shown to significantly reduce the severity of the symptoms of atopic eczema in breastfed infants after 2 months of treatment are good candidates for future hydrolysed protein formulae [196, 197]. In fact, those probiotics likely participate to the mucosal degradation of macromolecules leading to the reduction in the antigen load [198]. Thanks to their peptidases that hydrolyze tryptic-chymotryptic peptides from β-LG, they release numerous small peptides with immunomodulating properties which repress the lymphocyte stimulation [199, 200], up-regulate IL-10 production [201], and down-regulate IFN-γ and IL-4 secretion [202]. Those beneficial effects of probiotics have led to a great enthusiasm for the production of food supplements in which such bacteria are incorporated. Furthermore, *Lactobacillus paracasei* NCC2461 and *Lactobacillus rhamnosus* GG seem, according to results obtained from a mice model, to prevent CMA by stimulating and maintaining oral tolerance to β-LG [202] and are suggested to develop tolerance by several means such as the modification of the composition of the intestinal flora and the promotion of IgA immune responses. Therefore, the strain *Lactobacillus* LGG has been introduced in some commercially available hydrolysates with hypoallergenic properties in order to combine the benefits of a high hydrolysed formula with probiotics [203]. However, the present products are still not totally satisfying according to the ESPGHAN committee since the real safety of the probiotics incorporated in the formulae has not yet been sufficiently evaluated [204].

Other alternatives that are proposed are also dealing with the production of hydrolysates with therapeutic functionality. New hydrolysates might be carriers of tolerance inducing peptides either as modified allergens or peptide fragments [205]. Indeed, it has been proven that mutation of amino acids in the sequence of IgE binding allergenic epitopes of αs1 casein resulted in a reduction of the binding ability of those altered epitopes to milk-specific IgE antibodies from patients' sera [50]. This type of modification may also affect T cell responses by favoring the inhibition of Th2-dependent events (allergic) as noticed with an analogue peptide (AA142-149: LAYFYPEL) of αs1-CN in a mice model [206]. In the same way, introduction of short peptide sequences with a reduced IgE crosslinking ability but containing a dominant T cell epitope may be safer and more efficient to modulate human T cell responses [207]. For this purpose, peptides referring to the immunodominant T cell epitopes of the allergen are preferentially selected for oral tolerance induction. This type of immunotherapy was investigated in mice where a dominant T cell epitope (AA91-110) for αs1-CN has been successful [208]. However, toleration to peptides varies strongly depending on their dominance as shown in αs1-CN-fed mice that better tolerised T cell dominant determinants than cryptic determinants which escaped oral tolerance [209]. Those results found their application in the production of new patented hydrolysates with a tolerogenic property [210]. The corresponding hydrolysed formulae enriched in tolerogenic tryptic peptides from β-LG (AA84-91, AA92-99/100, AA125-135, AA125-138 et AA(61, 62–69):S-S:(AA149-162) offers a safe and non-allergic product with a potency of leading to a tolerance to milk [211].

Since the best food for the newborn remains to be human milk, which the infant formulae systematically try to copy, new alternatives of plant-based infant formulas, baby foods and health foods are under investigations in an attempt to reproduce the composition and health benefits of human milk. Advances in biotechnology resulted in the introduction of human genes into food plant species have led to the production of food plants producing human milk proteins. This may be an opportunity for CMA patients to have a highly nutritive food and to avoid CM proteins [212].

Effects of hydrolysis

Despite progress in the processing and improvement in the detection methods, the in vitro systems elaborated so far present drawbacks. Although most of these assays are based on antibody–antigen interaction (Ig binding), the existence of a signal resulting from this interaction does not always reflect the allergenic capacity. The detection of the presence of some fragments, or peptides from the potential harmful protein, reveals its antigenicity but does not predict its allergenic behaviour in humans. For those reasons, hydrolysates are usually tested in "in vivo" systems closer to the physiological conditions. Besides the common skin-prick tests (SPT), radioallergosorbent test (RAST) inhibition and more recently the patch test which have been described as suitable methods for determining the residual allergenicity of hydrolysed infant formulae, sensitisation of animal models is also included in the test stages preceding the clinical trial of DBPCFC in humans for the acceptance of the products [213, 214].

Animal models are indeed very helpful in predicting allergenicity and the tolerogenic potential of hypoallergenic infant formulae. The sensitising capacity of a formula can be examined by either the parenteral rat (IgE) also used for testing the oral tolerance inducing capacities of formulae, the guinea pig (IgG1a-mediated) or the oral mouse (IgE) models [215, 216].

Guinea pig has been the model of reference for testing new infant formulae due to their predisposed responsiveness [217]. Other suitable animal models for food allergy research are mice and rats [218]. The brown Norway (BN) rat is particularly interesting for its capacity to produce specific IgE, when exposed to β-LG or to semi-skimmed milk via the intraperitoneal route. This IgE is directed against dominant epitopes (AA21-40, AA41-60, AA107-117 and AA148-168) that are similar to those identified using sera from CMA patients. These studies provide evidence that the immune system of the BN rats and humans—at least in the case of milk allergens—is recognising similar protein allergens and similar peptide epitopes which may help in the elaboration of new immunoassays [216].

Cellular systems have been proposed as a close resemblance to biological conditions and are seen as alternatives to animal models for ethical reasons. Since T cells were found to participate and regulate allergic mechanisms, the measure of T cell proliferation and cytokine profiles has been thought to be a good diagnostic tool reflecting the in vivo allergic behavior. Despite discordant results regarding the relevance of T cell proliferation assessment in the diagnosis of food allergy, due to a lack of discrimination between non-allergic and CMA patients, this method seems to be suitable to evaluate the tolerance to a hydrolysate and the absence of immunogenicity of the product [219, 220]. It seems indeed, that through the proliferation test the immunogenicity of a hydrolysed formula can be detected via the induction of significant T-cell activation and cytokine secretion [221, 222].

For instance, whey partial hydrolysate displayed the same proliferative capacity as unmodified milk proteins in contrast to extensive hydrolysate which showed lower cellular responses. Surprisingly, no difference in cellular response was found between casein-based pHFs and casein-based eHFs. This type of assay can provide some information about the allergenic capacity of a hypoallergenic milk formula and could even be an alternative to the DBPCFC for some allergic patients. For instance, higher proliferative responses (PBMCs) from patients that do not tolerate a certain hypoallergenic formula (casein hydrolysate) correlated with the maintenance of symptoms of these patients when they ingest it. This suggests that an elimination diet with this casein hydrolysate formula may not be effective in those patients and may lead to a reaction in the worst case [223]. T cell clones were shown to proliferate to casein-derived hydrolysates with a higher response towards casein/whey-based pHF (14%) than to casein-based pHF (4%) or casein-based eHF (0%). This seemed promising but whey-based pHF was also found to induce proliferation in β-CN-specific TCCs (T cell clones) suggesting some crossreactivity of peptides [222]. This should be confirmed by using highly purified whey proteins, because casein contamination of the whey-based pHF is common since acidic precipitation of casein hardly results in a 100% removal of caseins.

New in vitro techniques with a functional aim attempt to mimic the mechanism of anaphylaxis and to replace the animal models. Degranulation assays by measurement of released "allergic" mediators (histamine, leukotriene etc.) are in progress and allow the comparison of milk hydrolysates [224]. Assays for histamine and leukotriene C4, released after in vitro basophil activation, are now more accurate and standardised [225, 226]. The development of basophil degranulation is assessed by flow cytometry methods that can quantify the presence of cell surface-bound allergen-specific IgE antibodies as well as the degranulation induced after incubation with the allergenic product via a marker of degranulation (CD63). This allows the confirmation of the allergenicity of a hydrolysate product. However, this technique presents some limitations since it requires the isolation of the PBMCs of the allergic donors and it can only be tested in a restricted time due to the short life time of basophils (1–2 days). Furthemore, the culturing procedures used are so far not adequate for the maintenance of basophils or mastocytes (other cells involved in degranulation mechanism).

Some innovations in this system are under investigations in order to decrease those limitations. Flow cytometry with IgE from allergic patients bound to microspheres incubated with the potential allergenic food might be a way to visualize the presence of allergenic proteins.

All these techniques can participate in the detection of the food allergen and/or its fragments (persistent reactive epitopes) obtained after processing. Although a strict avoidance of the offending food is accepted as the best way to prevent food allergy in the absence of a good treatment, a complete avoidance is impossible due to the high-risk of hidden, unexpected food allergens in food products and a lack of information on food labels or contamination in industrial plants. The presence of such hidden allergens has been traced with ELISA technics [227, 228]. Therefore, these techniques, in combination with knowledge of the reactive epitopes are indeed of great importance for the detection of peptides derived from milk hydrolysates, considering their diverse applications in pharmaceutical and nutritional products, cosmetics etc. [229, 230]. The high level of bioactive peptides released during milk hydrolysis (gastrointestinal digestion and/or food processing) and their diverse biological effects (immunomodulator, antimicrobial, antihypertensive, opioid, antioxydant, antithrombotic etc.), as listed in Table 6, brings indeed more and more industrial interests in this natural source of bioactive components [231, 232]. The attractive use of milk protein hydrolysates and/or peptides derived from them, for the production of new functional foods and nutraceutical products as well as cosmetics is exhibited by the high number of patents filed. These developments increase the potential risk for allergic reactions triggered by the use of new products in which the presence of food allergens seems unexpected [8, 233, 234]. For instance, the commercial potential as a toothpaste ingredient, or as food additives for the prevention of dental caries of the anticariogenic phosphopeptides β-CN-4P(f7-24), αs1-CN-5P(f61-78), and αs1-CN-5P(f59-78), that are released during the trypsin hydrolysis of casein, may present some risks for CMA consumers [235]. Allergic problems can emerge for persistent allergic patients with the presence of αs1-CN-5P(f59-78) in products which appeared antigenic and a marker of persistent allergy (Table 2)

Table 6 Applications of proteins, hydrolysates and bioactive peptides from bovine milk used as ingredients of nutraceutical, pharmaceutical and food products

Application	Origin	Matrix
Opacity/whitener	Calcium caseinate/acid casein	Nutritional beverages; coffee
Thickening/heat stability	Caseinates	Frozen desserts, canned soups
Gelation/viscosity/film formation, bio-plastic/texturization/extender	Caseinates (from which sodium caseinate); Rennet casein; WPC, whey	Salad dressings, soup, setting cheese, baked goods, gravies, meats, egg replacers, beverages, doughs, sausages, gel desserts, cheese, bakery glazes, edible films and coatings (snack peanuts, coating nuts used in confectionery), gloss coatings on confectionery (replace shellac), grease-barrier coatings on paper and paperboard (packaging for products: fast food and pet food), crunchy/textured products (combination whey protein/edible polysaccharide) for cereals, nutrition bars, trail mix, frozen dessert toppings and crunchy snacks, imitation cheese textured foods, meat
Flavour/aroma/browning	Whey	Confections, meats in microwave, sauces, breads, baked goods, soups, dairy products, crackers, caramels
Encapsulation	Whey	Aroma compounds, oleoresins, flavouring and colouring systems, vitamins, fats and oils could be encapsulated to extend their application, as well as pharmaceutical compounds
Foaming/whipping/stabilization/ aeration/water, fat binding/	Caseinates, WPC, whey and CN hydrolysates	Toppings (whipped or not), cakes, desserts, meringues, angel food cakes, chewy cookies, processed meats
Solubility (acid)/hydratation	WPC	Meat, beverage, bread, cake, sausages, fruit beverage
Emulsifying	Caseinates (sodium), WPC/β-LG Low hydrolysed CN and whey	Salad dressing, pancakes, soup, sausage, cakes, infant foods, coffee whiteners, processed cheeses, yogurts, meats
Antimicrobial	CN: casocidin-I (αs2-CN), isracidin (αs1-CN); casecidin (αs1-CN; κ-CN) Whey: lactoferricin B; LF	Food supplements or food additives or pharmaceutical products
Bactericidal	Whey: β-LG; ALA	Food supplements or food additives or pharmaceutical products
Anti-weight gain treatment of obesity (stimulation satiety)	CN (κ-CN)	Nutritional supplement of beverages: juice, sport beverages, milk UHT dairy, soy milk, shakes; milkshakes, smoothies, frappes. . .; Nutritional supplement foodstuffs (energetic bars), confectionery products (high calcium chews, chewing gum, chocolate, cookies), dairy products (yogurt, ice cream, milk, cheese, processed cheese, butter), farinaceous products (bread, muffins, biscuits, cereal or rolls)
Hypocholesterolemic	Whey: β-LG	Food supplements or food additives or pharmaceutical products
Metabolic: ACE inhibition	CN: α-casokinin-5 αs1-CN; β-casokinin-7 and 10 (β-CN) Whey (β-lactorphin β-LG; α-lactorphin ALA)	food supplements or food additives or pharmaceutical products
Opioid	CN: α-CN; exorphin αs1-CN; β-CN (casomorphin); Casoxins A and B (κ-CN)	Food supplements or food additives or pharmaceutical products
Agonists	Whey: β lactorphin: β-LG; α lactorphin (ALA); serorphin (BSA); lactoferroxin B and C (LF)	
Antagonists	CN: casoxins D (αs1-CN), and C (κ-CN) Whey: lactoferroxin A (LF)	Food supplements or food additives or pharmaceutical products

Table 6 Continued

Application	Origin	Matrix
Immunomodulatory/anti-inflammatory	CN: α-CN; β-casokinin-10 β-CN; GMP (κ-CN) Whey: ALA; lactoferricin B (LF)	Food supplements or food additives or pharmaceutical products
Antioxydant	CN	Food supplements or food additives
Anti-hypertensive	CN: αs1-CN; β-CN; κ-CN	Nutraceutical products (brands: Casein DP, Biozate, Evolus, Calpis, C12 peptide); Food for specified health uses
Muscle contraction	Whey: β-LG CN, whey hydrolysate; fermented or sour milk	
Anti-thrombotic	CN:casoplatelin κ-CN; GMP Whey: lactotransferrin	Food supplements or food additives or pharmaceutical products
Anti-cariogenic	CN: GMP; αs1-CN-P	Toothpastes, gels, mouth rinses, chewing gum
Biotransfer oligo elements (calcium binding/transport)	CN: αs1-CN-P; β-CN	Food supplements or food additives or pharmaceutical products

WPC: whey protein concentrate; ACE: angiotensin conversion enzyme; CN-P: caseinophosphopeptide; GMP: glycomacropeptide

[236]. The use of casein hydrolysates as "drug" carriers to control the dissolution rate and bioavailability of a variety of drugs can also bring allergenic risks depending on the peptide length of the casein fragments [237]. Numerous other applications of casein and whey proteins or peptides derived from them that take advantage of their properties like binding, emulsification, and film forming functionalities and tenderisation (coffee, beef, bread, sausages etc.) should be analysed for their allergic capacity and stress the need for analytical techniques capable of detecting CM proteins/peptides in (food) products.

Other processing techniques

In addition to heating and hydrolysis food irradiation technology is considered as a potential method for the reduction of milk allergy. It was suggested that epitopes on milk allergen proteins (α-CN and β-LG) were structurally altered by gamma irradiation according to competitive indirect ELISA and SDS-PAGE analysis. This effect was associated with a decrease of solubility of the proteins that might be caused by agglomeration [238]. High pressure treatment is another new technique being investigated for the improvement of an enzymatic hydrolysis. It has been shown for instance that the extent of protein enzymatic hydrolysation under pressure (600 MPa) is significantly higher than at atmospheric pressure. This was explained by an increase of accessibility of potentially immunogenic hydrophobic regions, to the enzyme resulting in an improved hydrolysis. Enzymatic hydrolysation under pressure triggered a different peptide pattern apparently non-immunogenic compared to the one obtained at normal pressure [176]. In another work the effect of high pressure treatment on non-hydrolysed ALA and β-LG was investigated. The results showed that both in milk and whey, β-LG was less baroresistant than ALA; notwithstanding that both proteins were considerably more resistant to high pressure-induced denaturation in whey than in milk. The higher level of HP-induced denaturation of ALA and β-LG in milk than in whey may be the result of the absence of the casein micelles and colloidal calciumphosphate from whey, which facilitate high pressure-induced denaturation of ALA and β-LG in milk [239].

Another processing method suggested to have an effect on allergenicity is based on lactic acid fermentation. This process has been investigated by utilizing many different species of bacteria (meso and thermophilic) that ferment milk products [240]. This process is, however, not adapted since it does not decrease potently the allergenicity of CM proteins. The antigenicity of the proteins (ALA and β-LG) is indeed drastically decreased as measured in in vitro system however the allergenicity of the corresponding proteins is persistent or only slightly diminished as illustrated by in vivo tests with allergic patients. This process can only be interesting in case of using lactic acid bacteria such as Lactobacillus casei defined as probiotics and potentially beneficial in the reduction of the allergenicity of the proteins as mentioned in the previous paragraph.

Analytical techniques for the analysis of milk proteins in food

Many techniques are available for the analysis of proteins and especially milk proteins. Despite this, literature referring to detection of specific milk proteins at low concentrations in food products is relatively rare. This might be due to the absence of standardisation of the techniques themselves. Therefore, the techniques reported in this review remain to be validated in order to provide relevant information and confidence in measurements concerning the presence and the quantification of an offending allergenic food component in a product and their implications for labelling purposes.

Table 7 Immunoassay methods for the detection of CM allergens in different food products

Method	Protein detected	Samples	Limit of detection	Analytical range	Reference
RIE	Casein	Ice cream, chocolate, lollipop, sausage, hot dog, meringue	(25–100)×10^3 µg/l		Yman et al. (1994) [246]
RAST inhibition	ALA	Baby food	1×10^3 µg/kg	(1–5)×10^3 µg/kg	Fremont et al. (1996) [247]
S-ELISA	β-LG	Infant formula, human milk	0.002 µg/l	0.1–10 µg/l breast milk	Makinen-Kiljunen (1992) [161]
S-ELISA	CN	Non-milk-containing products (juices, sorbets, chocolate)	500 µg/kg	n.a	Hefle (2004a) [227]
C-ELISA	β-CN	CM in ovine and caprine milk and cheese	5×10^3 µg/g	(5–250)×10^3 µg/g	Anguita (1997) [242]
C-ELISA	ALA, β-LG	Milk	13 µg/l, 27 µg/l, 20 µg/l	n.a	Karamanova (2003) [123]
C-ELISA rabbit polyclonal antiserum	β-LG	Milk hypoallergenic infant formulae	0.08 µg/l	(0.1-1)×10^3 µg/l	Mariager et al. (1994) [248]
Mouse monoclonal antiserum			3.2 µg/l	4–50 µg/l	
Sheep polyclonal antiserum			144 µg/l	(0.26–50)×10^3 µg/l	
SPR biosensor	κ-CN	Milk	450 µg/l	n.a	Muller-Renaud (2003) [249]
SPR biosensor	LF	Milk	20×10^3 µg/l	0-1×10^3 µg/l	Indyk et al. (2005) [74]

C-ELISA: competitive ELISA; S-ELISA: sandwich ELISA; SPR: Surface Plasmon Resonance

In addition to currently used methods new techniques have to be developed to improve the detectability and measurement of milk allergens in food products.

Immunoassay based methods for the detection of milk allergens

Antibodies play a major role in most allergen detection methods. The specific binding between antibodies and their recognised antigens has been exploited to create very sensitive and specific systems for the detection of proteins.

The majority of immunoassay methods use the ELISA format for the detection of milk allergens. Two approaches are available for the semi-quantification of milk proteins: sandwich and competitive ELISA. In the sandwich assay (S-ELISA) the protein is captured by an antibody bound on a solid phase support and is detected by a second protein-specific enzyme-labelled antibody which binds to the analyte forming a sandwich. A specific substrate is added for the reaction with the enzyme tagged antibody and a colorimetric reaction takes place that can be measured with a spectrophotometer.

In the competitive assay (C-ELISA) which is preferred for the detection of small proteins, the sera are incubated with the diluted sample before addition to the solid phase which contains the immobilised antigen. If the antigen is present in the sample it inhibits the binding to the immobilised antigen so that the absorbance is inversely proportional to the concentration of antigen in the sample. Purified anti-β-LG was used for the development of a very sensitive sandwich type ELISA for the determination of very low levels of CM β-LG in infant formulae and human milk with a limit of detection (LOD) around 0.002 ng/ml [161]. Later, two-site enzyme immunometric assays were developed which are based on monoclonal antibodies specific to either the native or denatured β-LG with the aim to detect any heat-denaturation in CM and milk products [241]. Monoclonal and polyclonal antibodies were also raised against casein components of CM with the aim to devise an ELISA method for the evaluation of residual antigenic activity in hypoallergenic infant formulae [165, 242]. More recently a sandwich-type ELISA has been developed for the detection of undeclared casein in different non-milk-containing products such as juices, sorbets and chocolate. The present assay offers a detection limit of less than 5 mg/kg [228].

Fast biosensor immunoassays with a good sensitivity have been developed for the detection of residual immunogenicity of food products [243]. These techniques are reported to be fast, repeatable, fully automated and able to discriminate between intact and degraded protein while little or no pretreatment of the sample is required [43, 244].

Recently, the development of an immunoprobe made of polyclonal antisera raised against the whey proteins ALA and β-LG A and B has been described. This immunoprobe can be used for the analysis of thermally-treated milks. The effect of thermal treatment was evaluated on the basis of

immunoreactivity changes observed by ELISA. The LOD was 13 ng/ml for ALA, 27 ng/ml and 20 ng/ml for β-LG A and B, respectively [123]. An inventarisation of immunoassays is shown in Table 7 which includes the relevant LOD and the type of food matrices analysed with the listed methods. Some multiplex enzyme immunoassay systems such as those described by Blais et al. (2003) designed for a simultaneous detection of multiple allergens in foods could be adapted for the detection of milk proteins [245]. This could allow determining the presence of different milk proteins used in food products (as single protein, casein or whey proteins) for their emulsifying properties. Furthermore, this might offer a possibility to detect those offending proteins as contaminants introduced in food products via some form of contact, such as the contamination of food by casein used in film for packaging. These assays may also provide information on the "size" of the interesting peptide and its potential anaphylaxis effect by the use of antibodies specific for two sites of the extremity of the reference peptide.

Although the majority of the techniques focuses on the presence of intact proteins and do not take into account that proteins can contain chemical modifications, some efforts have recently been made aiming at the discrimination of native, degraded or modified proteins as induced by processing. Docena et al. (2002) investigated the presence of residual antigenic and allergenic components in different milk substitutes employed in the prevention and treatment of CMA. By using a CM-specific antiserum and casein specific monoclonal antibodies in immunoblotting and ELISA, they could detect and identify residual components in CM moderate and extensive hydrolysates and cross-reactive proteins in different mammalian milks. The ability of these components to be bound by human IgE was evaluated by immunoblotting and EAST [159]. The specific IgE binding capacity of native bovine ALA and tryptic peptides derived from ALA was investigated by Maynard et al. (1997) by using 19 sera from patients with CMA. Out of the 19 sera 11 reacted exclusively with intact protein while 8 of them presented a specific IgE response to different tryptic peptides as was shown with ELISA. The existence of sequential epitopes exposed through protein denaturation was also demonstrated, in addition to conformational epitopes. Moreover, IgE binding sequences were also located within hydrophobic regions of ALA and within parts with high sequence homology to human ALA [61].

Currently, as a consequence of the increased demand in rapid and reliable tests for the detection of specific allergen markers in food products, various commercial ELISA test kits entered the market. Table 8, lists current commercially available ELISA-based test kits for allergen detection in milk products and shows the variety of targets with products detecting either total milk, CN, whey proteins or only β-LG or BSA. Unfortunately none of the test kits in Table 7 have been validated yet.

Few attempts have been reported on the development of in vitro assays for detection of milk-derived peptides. For such purposes adaptations have to be made considering the biochemical properties of the representative peptide of interest. Black et al. (1998) developed an ELISA assay specifically for multiphosphorylated peptides which present highly hydrophilic properties. For this purpose, the support was adapted which allowed a decrease in false negatives, and an improvement of the detection sensitivity (up from 10^6–10×10^6 ng/ml to 10^3 ng/ml) [250]. This is further supported by studies using sera from allergic patients in direct and indirect ELISA which showed that the IgE response to caseins may be affected by modifying or eliminating the major phosphorylation site [44].

In comparison to IgE epitopes that are largely defined for milk protein, T cell epitopes are rather unknown although some information on this is provided by using mice model systems. Because T cells are central in the allergy and tolerance induction, the determination of T cell epitopes in milk proteins may advance technical innovations in the in vitro assays. Indeed, antibodies directed to reference epitopes which should preferably be T and B determinants will allow the detection of the native protein as well as the peptide itself with a limited cross-reactivity. In a mice model, four peptides (AA91-110, AA106-125, AA136-155 or AA46-65) which contain both T and B cell determinants on αs1-CN were capable of eliciting a specific antibody that reacted with the protein as well as the peptide itself [251].

In addition to ELISA electrophoresis methods (SDS-PAGE) and immunoblotting techniques have been used widely for the visualisation of the molecular pattern of peptides contained in hypoallergenic formulae [252]. The presence/absence of the allergen and its size are easily observed showing for instance the lowest allergenicity in an amino acid-based product (absence of high molecular weight as well as intermediate signal) compared to native CM characterised with a multiple "bands" pattern. Many industrial companies use this technique to characterise their hypoallergenic product and to compare the new formula with existing hypoallergenic formulae and milk proteins [253].

Unfortunately those techniques generally show a decreased sensitivity compared to ELISA techniques which make them less powerful for the detection of low quantities of allergens due to the separation and/or transfer process.

DNA-based assays

A promising target for allergen detection is the DNA of specific food allergens that can be amplified using the polymerase chain reaction (PCR). The amplification of DNA takes place thanks to the presence of a specific enzyme (Taq polymerase) in the PCR mixture. This enzyme adds nucleotides to the primers that are bound selectively to the complementary part of the DNA. In 30 cycles up to 10^9 copies of DNA can be generated. The resulting products are separated onto an agarose gel and can be quantified using UV light. Nowadays, PCR techniques are widespread and are applied for the detection of a number of food allergens. It should be noted that this kind of assay detects DNA as a marker of the allergenic food and it does not detect the allergen itself [254]. Where ELISA methods detect the presence of (allergenic) proteins, PCR methods detect the DNA from a given source but not the presence of specific proteins in

Table 8 Commercially available DNA and ELISA-based test kits for allergen detection in CM products

Target allergen	Food	Type of immunoassay	Analytical range	Limit of detection	Specificity	Supplier
Milk and caseinate	Raw, cooked or processed food	DNA Qualitative identification of milk and caseinate	n.a	5 mg/kg	n.a	A
Total milk	Food products non containing milk (juice, wine, sauces)	Sandwich quantitative ELISA	2.5–25 mg/kg	<2.5 mg/kg	n.a	B
Milk	Bovine milk in ovine or caprine milk and cheese	Competitive quantitative ELISA	0.1%, 1%, 10% w/w	0.1% w/w	n.a	C
CN	Raw, cooked, processed food, Babyfood, raw materials	Competitive quantitative ELISA	1.6–25 mg/kg	<1 mg/kg	α-CN (110%), other CNs (58–103%), κ-CN (10%), β-CN (5%), whey proteins (1.7–7%)	A
CN	n.a	Sandwich Semi-quantitative ELISA	1–25	n.a	n.a	D
CN	CN (also for dairy speciation)	Quantitative ELISA	n.a	5 mg/kg	n.a	E
β−LG, whey proteins	Raw, cooked, processed food, Babyfood, raw materials	Competitive quantitative ELISA	25–500 mg/kg	<7.5 mg/kg	β-LG (58.4%), whey protein (29.1%), CNs (<1%)	A
β-LG	Whey protein, whey protein containing products	Competitive quantitative ELISA	0.010–0.8 mg/kg	5 mg/kg	Specific polyclonal antibodies to β-LG (100%)	C
β-LG	n.a	Sandwich Semi-quantitative ELISA	2.5–25	n.a	n.a	D
β-LG	Whey	Quantitative ELISA	n.a	5 mg/kg	n.a	E
β-LG	Food and swab samples	Competitive ELISA	n.a	1 mg/kg	Cross reactivity with other animal proteins	F
BSA	Raw, cooked, processed food, Babyfood, raw materials	Competitive quantitative ELISA	25–500 mg/kg	<10 mg/kg	BSA (96.9%), whey proteins (1.7%), CNs/caseinates (<1%)	A

Suppliers: A: Tepnel BIOSYSTEM (www.tepnel.com); B: Neogen VERATOX (www.neogen.com); C: R-Biopharm RIDASCREEN (www.r-biopharm.com); D: ELISA System (www.elisas.com.au); E: ELISA-TEK (www.elisa-tek.com); F: TRANSIA GMBH (www.transia.com)

that food. Consequently, a positive result by PCR does not prove the presence of allergenic protein. DNA-based detection has been reported to be less affected by food processing or cooking since DNA was shown to survive food processing better than proteins due to its higher chemical stability [254]. Disadvantages of this technique are mostly due to DNA matrix-dependent limits of detection [150].

So far a DNA-based kit is available on the market for the detection of milk and caseinate in raw, cooked or processed food as shown in Table 8.

Separation methods

Several analytical techniques have been developed for the analysis of proteins mostly based on 2D-PAGE and liquid phase separation. 2D-PAGE is a very useful tool for the separation of proteins from complex samples. It combines the electrophoretic technique of isoelectric focusing (IEF) and sodium dodecylsulphate (SDS) PAGE. This approach allows resolving proteins on the basis of their molecular weight and isoelectric point. Overall, the technique is a powerful tool for the separation of proteins with similar molecular weights, exploiting the second dimensional separation. Nonetheless 2D-PAGE suffers from a number of drawbacks such as the inability to separate polypeptide chains of masses larger than 150 kDa and smaller than 8 kDa and the difficulty to detect proteins with a low abundance [255]. Furthermore, proteins that are very basic or acidic are rarely resolved by 2DE requiring a prefractionation step prior to 2D-PAGE analysis.

2DE has been used for separation of proteins and peptides in commercial milk powder [256] and in bovine milk or colostrum [257]. Galvani et al. reported the separation of intact milk proteins by 2DE followed by MALDI/MS detection after passive extraction of proteins into a suitable solvent mixture. In another work, Natale et al. (2004) described the identification of milk allergens by immunoblotting following resolution of CMP components by two-dimensional electrophoresis [83].

The potential of capillary electrophoresis for the rapid separation of milk proteins has been widely demonstrated in recent years [258]. Different CE methods have been developed for the analysis of CNs and whey proteins in milk [259–262] milk powders [263] and cheese, mostly aimed at the characterisation of bovine milk in non-bovine and mixed cheeses [264–266]. A CE method for the determination of bovine whey proteins in soybean dairy-like products has been also described with detection limits for ALA and β-LG of 0.6 and 1.0 μg/g, respectively [267]. New improvements in capillary electrophoresis technics have been introduced by the use of fluorescence detection and capillary derivatisation. This allows a quantitative analysis of whey proteins at nanomolar levels as well as the detection of β-LG traces in hypoallergenic formulae [268, 269].

Concerning high performance liquid chromatography (HPLC) different methods have been described for the analysis of major milk proteins. Most of them are mainly based on anion-exchange chromatography [270, 271], cation-exchange chromatography [272], size exclusion chromatography [273], gel permeation and reversed-phase (RP) HPLC coupled to diode array detection [274, 275]. Bramanti (2003) [89] reported hydrophobic interaction chromatography as a method for the separation and quantification of CNs in milk. This method was used for a quantitative analysis of the casein fractions of unprocessed raw milk from cow, ewe, goat and buffalo. A simultaneous separation and quantification of major CM whey proteins including proteose peptone and caseinomacropeptide by reversed-phase HPLC on polystyrene-divinylbenzene has been reported by Elgar et al. (2000) [274]. The optimisation of all chromatographic parameters for the separation of β-LG and ALA in cow's, sheep's and goat's milk using the same copolymer-based packing has been described by Ferreira et al. (2003) [275], and this method was found to be suitable for the identification of homologues proteins in a mixture of milk from different species. Others have described a RP ion pair HPLC method using a C4 column for the simultaneous separation and quantification of caseins and whey proteins in cow's liquid and powder milk avoiding any sample pre-treatment. The overall optimisation has been achieved by using a statistical procedure and the identification of each protein was ascertained by peak area ratio and second derivative. The procedure was initially applied to various types of commercial milks [276]. A RP HPLC method using a copolymer column packed with polystyrene divinylbenzene beads has been developed by Garcia et al. (1997) [277] for the simultaneous separation of soybean and CM whey proteins. Detection of CM whey protein at low concentration amidst larger quantities of vegetable protein was achieved making this method of great interest for quality control in order to detect adulteration of soybean milks by addition of whey proteins. Moreover, the possibility of detecting up to 5% of α-LA and 24% β-Lg compared to soybean protein isolate is addressed [277]. Unfortunately, such limits of detection are by far exceeding limits required to protect CMA patients. In another paper the same authors developed a perfusion RP HPLC method that enables a very rapid and simultaneous separation of soybean and CM whey proteins. It represents an improvement in terms of sensitivity and with possible applications to soybean dairy-like products [117]. This method is able to detect adulterations of around 1% of CM whey proteins in powdered soybean milk. However, detection at such a high level is also not useful for preventing allergic reactions. Most of the above mentioned HPLC methods are listed in Table 9 which also shows their relevant LODs and linear concentration ranges of the application. As shown in Table 9, the most sensitive HPLC method allows the simultaneous detection of the highest number of milk proteins in various types of milk and is based on ion-pair RP chromatography using trifluoroacetic acid as ion pairing agent.

Peering at all the literature employed in the present review it turns out that from most methods suited for milk or dairy research only a few have been developed for detection of milk proteins in different foods. It has to be stressed that there is still a high demand for analytical methods

Table 9 LC methods for the analysis of milk proteins

LC Method	Sample	LOD (μg injected)	LOQ (μg injected)	Linear concentration Linear concentration range (μg)	References
RP-HPLC-UV (Polystyrene divinyl benzene column)	Soy bean and whey protein mixture	1.7	n.a	10.9–44.5	Garcia et al. (1997) [277]
		0.3		0.6–6.4	
Perfusion RP-HPLC-UV	Soy bean and whey protein mixture	0.9	n.a	3.5–16.7	Garcia et al. (1998) [117]
		0.09		0.3–2.0	
RP-HPLC-UV (C4 column)	Powdered milk, liquid milk	0.1	0.3	0.6–3.6	Bordin et al. (2001) [276]
i		0.3	1.0	1.3–8.0	
		0.2	0.7	0.9–5.6	
		0.2	0.7	0.3–1.8	
		0.1	0.3	0.5–2.8	
		0.1	0.5	0.5–2.8	
HIC-UV (Propyl column)	Bovin, ovin, caprin milk and cheese	0.9	n.a	12–120	Bramanti et al. (2003) [89]
		1.7		1.2–120	
		0.7		0.9–114	
RP-HPLC-UV (Polystyrene divinyl benzene column)	Milk and cheese mixture	2% (v/v)	5% (v/v)	1–95% (v/v)	Ferreira et al. 2003 [275]

aimed at the detection of milk allergens in potentially milk-containing products with detection limits low enough to ensure protection of allergic consumers.

MS-based technologies

Proteomics is aimed at the complex identification and characterisation of proteins including their post-translational modifications, protein conformations (native, denatured, folding intermediates), protein folding/unfolding and protein–protein interactions [278, 279]. A proteomic analysis is usually based on different steps such as sample prefractionation, protein solubilisation and separation prior to protein identification (Fig. 2). In this process mass spectrometry plays a pivotal role in protein characterisation which also includes the detection of postranslational modifications. A successful identification of proteins depends on the sensitivity of the mass spectrometer, the completeness of the database, post translational modifications and several other factors.

Soft ionisation techniques such as fast atom bombardment (FAB), electrospray ionisation (ESI) and matrix assisted laser desorption ionisation (MALDI) have made it possible to use this kind of mass spectrometers for the analysis of proteins and peptides due to the high sensitivity offered and the possibility to generate ions without significant chemical decomposition. The last two approaches have quickly become important tools for the detection and characterisation of large biomolecules. Besides this, collision-induced fragmentation can be performed by

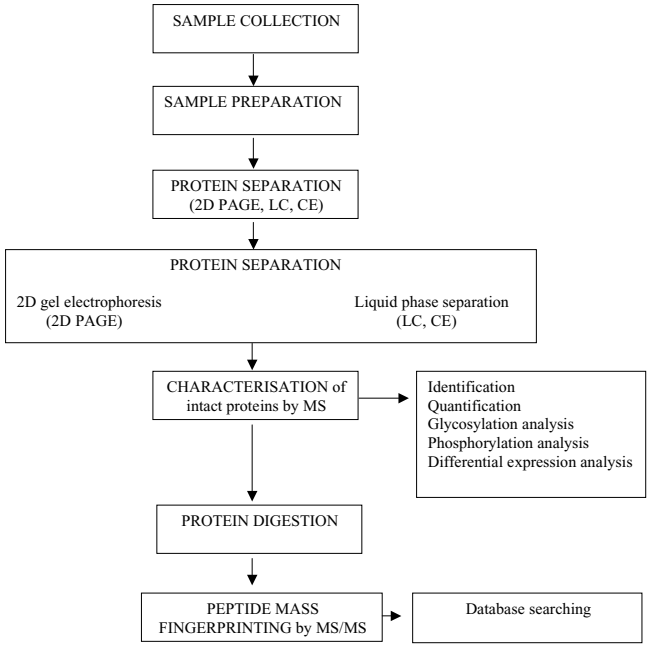

Fig. 2 Flowchart for proteomic analysis

tandem instruments (e.g. a triple quadrupole system) for structural elucidation and to characterise molecular events such as post-translational or chemically induced modifications. For instance, ESI mass spectrometry has been demonstrated to be a powerful tool for the characterisation of CM proteins providing spectra of multiply charged ions from which information about the molecular weight can be obtained by using a deconvolution algorithm [280, 281].

LC-MS is also a valid and accurate analytical technique for the identification of peptides, and it has been mostly exploited for the rapid identification of interesting compounds present in a complex food matrix such as biologically active peptides derived from a hydrolysate or a fermented product [282]. However, the interpretation of mass spectra can be difficult when a complex mixture or a complicated food matrix is analyses, due to limitations in the detection of the ions formed as well as formation of adducts affecting the m/z parameter which can occur. Identification of small peptides (di-tripeptides) is also limited exclusively to identification of the sequence by manual calculation relative to the m/z obtained from the spectra due to the uncertainty of the protein source in case of a mixture. This explains the restricted recovery of the peptide sequence even for purified protein [283]. Interest in developing new areas of technology based on the existing methods may lead to an improvement in the detection. For instance, the presence of peptides or proteins from a product involved in the potential allergenicity might be targeted via their detection as immune complexes in real-time with specific allergen fluorescent antibodies through separation by liquid chromatography with fluorescence and mass spectrometric detection [284].

Several authors have used ESI-MS systems interfaced with high performance liquid chromatography (HPLC) [280, 285–288]. Characterisation of modified whey proteins in milk ingredients ranging from the native proteins through different degrees of glycosylation and oxidation has been revealed using this approach leading to a better understanding of the impact of industrial processing on protein modification [287]. Evidence for the presence of numerous genetic variants of bovine β-CN has also been obtained by using ESI-MS based on the mass difference between the predicted and measured molecular weight combined with automated Edman degradation chemistry after proteolytic digestion [289]. The advantages of on-line LC-MS include an increase signal-to-noise ratio reducing background signals and a rapid mass determination which is feasible without the need of purification steps. Liquid chromatography techniques can be combined with tandem mass spectrometry (MS-MS) for enhancing the capacity of ESI to elucidate the structure of proteins by sequence assignments from peptide mapping. The applicability of on-line LC-ESI-MS to the study of covalent interactions between casein micelles and β-LG from goat milk has recently been explored [290]. Mass assignment accuracies of 0.01% are commonly obtained using a quadrupole mass spectrometer. Electrospray could also be interfaced with other mass analysers allowing higher resolving powers such as Fourier transform ion cyclotron resonance, magnetic sector, time-of-flight and ion trap mass spectrometers. In order to improve the resolution and mass accuracy, recent instrumental improvements of MALDI-TOF have been introduced such as time-lag focusing (delayed extraction mode) and reflectron, resulting in a gain in both resolution and accuracy thanks to the separated desorption process from the ion accelerating one. A mass resolving power better than 10000 and mass accuracy below 5 mg/kg could be achieved using this technology [289]. Nonetheless MALDI-TOF suffers from restrictions in detecting low molecular mass proteins which deliver few peptides [291]. For this purpose the application of MS/MS technologies like TOF-TOF, Qq-TOF could provide further advantages.

MALDI mass spectrometry has been employed in dairy science mainly to monitor milk protein modifications and to investigate the protein content of different milk samples [292, 293]. It has also been demonstrated that MALDI-based methods can be applied for the investigation of the milk protein composition from different breeds and at the various lactation stages of the same cow [294].

One of the limitations of MALDI-TOF based protein identification consists in the difficulty to identify multiple components in a mixture. For this reason, 2D gels are coupled to MALDI-TOF for the identification of individual protein spots. 2DE combined with MALDI-MS has recently been carried out in order to identify a number of proteins as well as certain protein modifications including phosphorylation and lactose-protein conjugates in milk powder. The mass spectrometric analysis has been performed in two steps: the analysis of intact proteins following the passive elution from the gel and the analysis in reflectron mode of in situ digests of certain gel spots [256]. The analysis of intact protein prior to digestion can provide additional details for protein identification and for a more efficient and reliable consultation of databases.

ESI combined with MALDI-MS spectra of tryptic digests has also been used widely to detect the extent of lactosylation and the modification sites induced by heat treatments in the most abundant whey proteins [295, 296] and caseins [297]. A detailed structural protein modification pointed out the occurrence of preferentially lactosylated sites in ALA and β-LG and in β- and α_{S1}-CN.

The above discussed studies, summarised in Table 10, prove the potential of MALDI-TOF-MS, ESI-MS and ESI-MS-MS techniques as valid tools for mass determination purposes and the detection of protein modification in the research field of allergenic milk proteins and peptides.

Emerging technologies for proteomics

Some of the most recent approaches in the instrumental technologies are aimed at developing automated multi-dimensional systems encompassing liquid-phase based protein separation, protein digestion and MS identification systems [302–304]. Other promising approaches describe micro and nano LC coupled to ESI Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS). By using microcapillary LC columns, on-line coupled with

Table 10 MS methods for the analysis of milk proteins

Target allergen	Food	Analytical method	Purpose of analysis	References
ALA, β-LG	Milk, milk powder	ESI-MS MALDI-TOF on the enzymatic digest	Investigation of the modification sites using tryptic digestion	Siciliano et al. (2000) [296]
ALA, β-LG, αS1-αS2-CN	Milk	MALDI-TOF	Monitoring of structural protein alteration in thermally treated products	Catinella et al. (1996) [294]
Milk proteins	Infant formulae	MALDI-TOF	Evaluation of intact protein content in infant formulas	Sabbadin et al. (1999) [293]
Milk proteins	Milk powder	2DE-MALDI-TOF	Identification of milk protein modifications	Galvani et al. (2000) [256]
β-LG	Milk powder	CE-ESI-MS	Detection of lactosylated LG	Jones et al. (1998) [298]
κ-CN macropeptide	Dairy products	HPLC-ESI-MS/MS	Determination of κ-CN macropeptide	Molle et al. (2005) [299]
β-LG	Milk	HPLC-ESI-MS	Quantification of CM adulteration in goat milk	Chen et al. (2004) [300]
ALA, β-LG, αS2-CN	Infant formulae	2DE-nano-ESI-MS	Characterization of lactosylated proteins using tryptic digestion	Marvin et al. (2002) [301]
αS1-CN, β-CN	Milk	MALDI-MS/Edman degradation on the enzymatic digest	Characterization of lactosylated CN and identification of modification sites	Scaloni et al. (2002) [297]

nano-ESIFTICR-MS, peptide sequence analysis at subfemtomole level has been achieved [305].

A potential more sensitive alternative to the 2DE approach for the multidimensional separation of proteins is the novel analytical technique surface-enhanced-laser-desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS), which has been introduced by Hutchens and Yip (1993) and represents a novel approach to biomarker discovery combining liquid chromatography and mass spectrometry in the same devise [21, 306]. This high-throughput, array-based technology coupled with mass spectrometry could provide spectra of complex protein mixtures based on the mass-to-charge ratio of the proteins based on their binding affinity to the chip surface. Different varieties of proteinchip arrays have been developed based on chemically (anionic, cationic, hydrophobic, hydrophilic, metal, etc.) or biochemically (antibody, receptor, DNA, enzyme, etc.) treated surfaces [307–309]. Optimised separation materials have been devised in order to retain different classes of proteins onto a solid phase chromatographic surface and are now available on the market. Any crude extract or sample can be applied directly to the chip surface to promote interactions with the bait molecule. After applying a few microliters of the sample onto the proteinchip surface, protein specificity is achieved through the application of a series of washes with an appropriate solvent or buffer designed to elute unbound proteins and interfering substances while retaining the protein of interest. The array is then inserted into the proteinchip reader that is a laser desorption ionisation TOF-MS instrument equipped with a pulsed UV nitrogen laser source [21].

Furthermore, a new ion source has been developed that allows the proteinchip arrays to be analysed using a hybrid triple Quadrupole/TOF MS providing additional sequencing capabilities [310]. With this new source, proteins can be tryptically digested directly on the arrays and the resulting fragments identified by tandem MS.

Subsequently, the enzymatic reactions could be directly performed on-spot prior to peptide mapping analysis and with no sample loss. This versatile instrumentation is presently being used for different purposes especially for the identification of disease biomarkers and study of biomolecular interactions, but in the future it may find applications in the detection and characterisation of milk proteins at low concentrations.

Conclusions

There is an enormous multiplicity and diversity of allergens in milk responsible for CMA. Due to the similar symptoms in patients, a clear distinction between CMA and CMI cannot be made. Proteins involved in CMA are numerous and heterogeneous and the discovery of new genetic variants has contributed to the establishment of a broader base of information on milk proteins.

Polysensitisation to several proteins is common and sensitisation to CN, β-LG and ALA has been demonstrated to be closely linked. The three dimensional structure of CM

proteins is an important feature in CMP allergenicity but IgE binding studies have also shown the existence of hidden linear epitopes that can be exposed through protein denaturation e.g. as a consequence of food processing. Therefore no definite relationship can be established between structure and allergenicity.

The effect of industrial treatments which might decrease allergenicity has been discussed but it remains controversial whether such treatments are capable of reducing the risk of allergies. There are many ways in which the antigenicity of proteins can be enhanced during thermal processing.

Since no real immunotherapy exists for CMA patients even with a lot of different innovations in development, CMA patients and mainly children that can not benefit of breastfeeding receive food substitutes like hydrolysed milk formulae.

Those alternatives helped considerably in the improvement of the situation for these children by reducing the symptoms and restoring harmonious growth. Nevertheless, they retain a potential capacity to trigger some adverse reactions in severe cases of CMA. Indeed, even if in most cases, children tolerate the hydrolysed formulae quiet well, some really sensitised children still react strongly to this type of substitutes. Diverse improvements are currently made to prevent the reactivities observed for some children and tested by several ways to insure the highest possible safety. It is still questionable if this capacity of the hydrolysates even when extensively processed can trigger such reactions and therefore some investigations are currently performed to solve this uncertainty.

One of the improvements of the hydrolysed formulae is the use of new commercially available enzymes from bacterial sources and the ultrafiltration process. Interest is also focusing on supplementing food substitutes with probiotics which should help to increase the tolerance of CMA patients for the formulae themselves as well as the potential development of oral tolerance.

As one of the most important measures for CM allergic patients is to avoid food products containing CM proteins, appropriate analytical methods to detect the latter in food are urgently needed. Amongst methods available to-date, the ELISA technique has demonstrated to be sensitive and specific for the detection of milk proteins in food products although none of the commercially available kits have been validated in a collaborative study yet and the majority suffer from the limitation that they can only detect intact proteins in raw food. Some efforts have been made aimed at the discrimination of native, degraded and modified proteins. Fast biosensor immunoassays that were recently developed have been described for the detection of residual immunogenicity of food products with a good sensitivity. Although such techniques have been described to be fast, repeatable and fully automated they do not allow a characterisation of the immuno-reactive compounds. Proteomic approaches in which mass spectrometry often plays a pivotal role do address this issue.

Mass spectrometry plays a key role in the complex identification and characterisation of post-translational modifications, protein conformations (native, denatured, folding intermediates), protein folding/unfolding and protein–protein interaction. Soft ionisation techniques such as ESI and MALDI have become important tools for the detection and characterisation of large biomolecules. MALDI-TOF-MS, ESI-MS and ESI-MS-MS techniques have proven their potential as valid tools for mass determination purposes and the detection of protein modification in the research field of allergenic milk proteins and peptides.

Other approaches use micro and nano LC coupled to ESI Fourier transform ion cyclotron resonance mass spectrometry. A potential promising approach for the multidimensional separation of proteins is the novel analytical technique SELDI-TOF-MS. Different proteinchip arrays characterised by chemically or biochemically treated surfaces capable of retaining on the same chip different classes of proteins are already available on the market.

In recent years many improvements have been reported for methods designed to detect food allergens. However, in parallel to detection method development the food industry is using novel food processing techniques and markets a large scale of products destined for human consumption that contain CM or CM-derived ingredients. It is therefore of utmost importance that (1) good production practices are followed by the food industry to prevent contamination with food allergens, (2) legislation aimed at the labelling of food products containing food allergens is introduced, adhered to and enforced, (3) the allergenicity of novel CM-derived food products is investigated, (4) detection methods are improved and validated according to internationally harmonised protocols.

It is not clear to what extend the current detection methods can protect allergenic consumers or even detect food allergens. The absence of threshold levels and the variability between individual allergenic patients complicates safeguarding CMA patients. So far the threshold is often limited by the detection limit of the current methods. The capability of methods to detect allergenic residues in food products depends on many factors including the food matrix, extraction of the allergen from the food material, the detection method used, food processing and the form in which the allergen is present (e.g. total milk or eHF).

Despite those limitations the currently available methods for the detection of CM or CM-derived products are playing a crucial role in the provision of information to the allergic consumer which is essential for an avoidance diet required to protect his or her health.

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