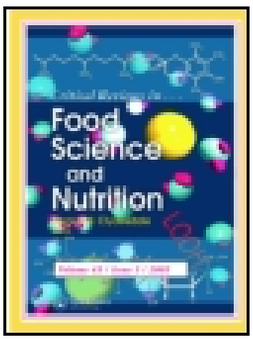

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Altering allergenicity of cow's milk by food processing for applications in infant formula

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Abstract

Cow's milk-based infant formulas have a long tradition in infant nutrition, although some infants are unable to use them due to presence of several known allergens. Various processing methods have been identified capable of reducing cow's milk protein allergenicity including thermal and non-thermal methods and their combinations. Heat treatment and enzymatic hydrolysis have been in production of hypoallergenic infant formulas. However, modulation of allergenic epitopes depends on the extent of heat treatment applied, which consequently may also reduce a nutritional value of these proteins. In addition, enzymatic hydrolysis may not target allergenic epitopes thus allergenicity may persist; however released peptides may have detrimental impact on taste and functional properties of final products. Modulation of allergenicity of milk proteins appears to require a concerted effort to minimize detrimental effects as clinical studies conducted on commercial hypoallergenic formulas demonstrated persistence of allergic symptoms. This article covers traditional and novel processing methods and their impact on reduction of cow's milk allergenicity in milk-based infant formulas.

Keywords

Cow's milk protein, Allergy, Infant formulas, Food processing, Heat treatment

INTRODUCTION

Breast milk is the best and recommended option for most infants (Lee et al., 2011). However, when exclusive breast-feeding is not possible during the first months of life, this practice is substituted usually with cow's milk based infant formula (Muraro et al., 2012). This substitution can lead to nutritional deficiencies and immunological conditions including cow's milk protein allergy (CMPA) (Crittenden and Bennett, 2005). Food allergy presents an important public health problem (Van Hengel, 2007) as it occurs in approximately 5--10% of the population of infants and children. Most food allergens are present in cow's milk, eggs, peanuts, nuts, soy, wheat and fish (Fritsché, 2003).

Allergy presents an abnormal immune reaction to the presence of a foreign antigen (El-Agamy, 2007). Food allergy is caused by a complex interplay between genetic and environmental factors, in addition to food allergens themselves. Breakdown of oral tolerance leads to food allergy (Weiner et al., 1994). In most cases of food allergy, referred to as type I food allergy, the response of immunoglobulin E (IgE) antibodies to a food protein is considered most important, which is a measure of the immunogenicity of food proteins. Other types of allergic diseases (Type II and IV) are associated with IgG antibodies and formation of certain immune complexes, which is a measure of the antigenicity of food proteins. The first step in IgE mediated allergic reactions is crosslinking between IgE on the surface of mast cells and basophils and a small epitope from the allergenic protein. The epitope, could either be linear (sequential) or conformational (non-sequential). The reactivity of these epitopes is dependent on their structure, some of which may be manipulated by processing (Rahaman et al., 2016).

Cow milk protein allergy (CMPA) is a complex disorder and follows the pattern described above and is usually classified as IgE-mediated and non-IgE-mediated allergies. Monocytes play an important role in initiation of the specific immune response by allergen and antigen presenting cells (APCs) (Figure 1). Upon stimulation by APCs, Th0 (T helper) cells, that are in naive status, can differentiate into two pathways. The pathway towards Th1 phenotype is stimulated by interleukin (IL) 12, with Th1 cells

producing characteristic cytokines including IL 2, IL-15 and interferon gamma (IFNγ). The pathway towards the Th2 phenotype is stimulated by IL-4, followed by release of characteristic cytokines, including IL-4, IL-5, IL-10, and IL-13. Most importantly, Th1 and Th2 responses are mutually inhibitory. For example, IL-4 and IL-10 inhibit Th1 responses, while IL-12 and IFNγ inhibit Th2 responses. This cross-regulation can generally result in a mixed phenotype, but could be biased towards one of the pathways in an individual, depending on genetic background, exposure to antigens, and causative environmental factors. In terms of immediate milk protein hypersensitivity, the Th2 pathway becomes dominant and IL-4 and IL-13 induce IgE production by plasma cells, which leads to IgE-mediated allergy. In the meantime, IL-5 secreted by Th2 may result in the non-IgE-mediated/cell mediated allergy due to accumulation and activation of eosinophils Milk protein sensitization (Bogahawaththa et al., 2017). CMPA is usually more severe in infancy (Crittenden and Bennett, 2005) and entails a spectrum of clinical symptoms involving the skin (hives, eczema, and swelling), gastrointestinal tract (nausea, vomiting, diarrhea, and stomach cramps), respiratory tract (runny nose, nasal congestion, wheezing, and coughing), and in more severe cases anaphylaxis (Nollet and Toldra, 2009). CMPA may also be developed in infants, who are exclusively or partially breast-fed by mothers whose diets included cow's milk proteins. Incidence of allergy to cow's milk proteins in infants fed only breast milk is low. In fact, 0.5% of these infants can show reproducible clinical response to cow's milk proteins and most of them are mild to moderate. This occurrence could be related to the lower level of (100000 times) cow's milk proteins present in breast milk than in cow's milk. Moreover, existence of immunomodulators in breast milk and important differences in the composition of the intestinal flora in breast-fed and that of the formula-fed infants appear to be important contributing factors associated with occurrence of CMPA (Vandenplas et al., 2007). As milk presents one of the staple foods in the human diet, research and reports in this area, especially when it comes to abnormal reactions to milk including both milk allergy and milk intolerances, have been voluminous (Crittenden and Bennett, 2005; Fox and Thomson, 2007; Caira et al., 2012).

Although several articles have been published reporting on the methods of reducing food allergenicity (Mills et al., 2009; Tammineedi and Choudhary, 2014; Verhoeckx et al., 2015 Jiménez-Saiz et al., 2015); none of them has addressed in particular reduction of antigenicity and immunogenicity of cow's milk proteins with emphasis on applications in infant formulas. Therefore, in this report we hypothesize that it may be possible by a process selection to reduce immunogenicity of milk allergens and potentially develop and manufacture a hypoallergenic infant formula. The important points of this paper include a discussion on the effects of heat treatment, currently the exclusive treatment in production of hypoallergenic infant formula, on the structure of allergens (proteins), interactions between various compounds in infant formula during heating and bioavailability of constituents. In addition, enzymatic hydrolysis of cow's milk protein is challenged. Finally, based on the tolerability of other animals, the non-cow's milk-based formula is recommended as a way of managing CMPA in allergenic infants. This study can be informative in the manufacture of hypoallergenic infant formula.

Prevalence of CMPA

Food allergies are listed as the sixth of the most common human health problems (Li et al., 2011). One of the most common food allergens in infants is associated with cow's milk as infants are usually first exposed to this food early in their lives and the prevalence of it varies with age (Høst, 2002). The CMPA has been confirmed clinically in 1--17% of infants, while the overall occurrence in the population has been reported at 2--7% in different countries (Høst, 2002; Monaci et al., 2006; El-Agamy, 2007; Shriver and Yang, 2011) and decreases in the adulthood to an incidence level of 0.1-0.5% (Crittenden and Bennett, 2005). In general, majority of CMPA children gradually becomes tolerant in adulthood; however, the mechanism(s) associated with this rise in tolerance rather remain sketchy and unidentified (Nowak-Wegrzyn et al., 2008). One of the assumptions is that, despite high acidity and enzymatic activity in the gastro-intestinal system, approximately 2% of ingested food is absorbed through the intestine in a form that is immunologically active enough to induce an immunological response leading to expression

as a food allergy. Since the intestinal mucosal gut barrier in infants and young children is still immature, this consequently results in incomplete digestion with a greater proportion of food absorbed in an immune active form leading to increased prevalence of food allergies (Kurowski and Boxer, 2008). In addition, the immune system is still in a development stage thus unable to differentiate among the presented antigens.

Allergenic milk proteins and their epitopes

Common allergens are usually proteins although a role of milk proteins in allergic reactions is not fairly well understood (Sharma et al., 2001). Cow's milk contains about 30--35 g/L proteins, composed of more than 25 different proteins but only a few are known to be allergenic (Hochwallner et al., 2014). In general, cow's milk proteins are composed of two main protein fractions, primarily based on their solubility at low pH. These include the caseins (80%) and whey proteins (20%) fractions. The casein (CN) fraction is insoluble at pH 4.6 and is primarily composed of four different proteins including α_{s1}, α_{s2}, β- and κ-CN in a respective ratio of 4:1:4:1. On the other hand, whey proteins, which are soluble at pH4.6, present a large group of mainly globular proteins including β-lactoglobulin (BLG) and α-lactalbumin (ALA) as the main proteins, and with some minor proteins such as bovine serum albumin (BSA), lactoferrin, lactoperoxidase, and immunoglobulins (Caira et al., 2012). Clinical studies revealed that CMPA children react to a specific protein fraction of cow's milk that contains specific epitopes widely spread along the protein molecules. The prevalent cow milk proteins involved in allergic responses in children include ALA, BLG, and α_{s1}-CN. In general, a study of the molecular characteristics of a known major allergen allows for identification of technological processes that may be capable of improving the tolerance of allergic subjects to cow's milk and can be used in the production of hypoallergenic formulas (Restani et al., 2004).

BLG is the major whey protein in milk of ruminants, comprising 50% of the total whey proteins. The immunereactive structures are regularly scattered across all 162 amino acid residues of the BLG molecule. Some of them are short linear sequences, whereas others are large fragments constituting

conformational epitopes. Three peptides from BLG have been identified as major allergenic epitopes including f41-60; f102-124; and f149-162. The C-terminus of f149-162 peptide forms a short α-helix and appears to be very mobile according to its crystalline structure (Brownlow et al., 1997). Jarvinen *et al*. (2001) identified seven (fragments: 1--16, 31--48, 47--60, 67--78, 75--86, and 127--144) IgE-binding as well as four (fragments: 49--60, 119--128, 129--138, and 143--152) IgG-binding regions on BLG molecule. Using alanine scanning analysis, amino acid sequences of f17-36, f72-86, f92-106, and f152-166 were identified for IgB-binding epitopes (Cong et al., 2012). Peptides f41-60 and f102-142 form loops on the surface of the molecule and hence are also accessible to antibodies (Luo and Bu, 2012). Microarray-based immunoassay technique showed that sequences of f58-77, f76-95, and f121-140 are recognized by more than 75% of patients (Cerecedo et al., 2008).

ALA is a monomeric globular protein consisting of 123 amino acid residue with a molecular weight of 14.4 kD and four disulfide bridges (Jarvinen et al., 2001). A sequence analysis showed a high degree of homology, approximately 75% similarity, between the amino acid sequences of cow's with human ALA. Despite this, bovine ALA has been identified as one of major cow's milk allergens (Hochwallner et al., 2014). Four (fragment: 1--16, 13--26, 47--58, and 93--102) IgE-binding as well as three (fragment: 7--18, 51--61, and 89--108) IgG-binding regions on ALA were identified (Jarvinen et al., 2001). Another study concerning the CMPA allergenicity demonstrated five IgE-binding sites including f1-15, f6-20, f46-60, f71-85, and f101-115 in ALA amino acid sequences. Moreover, four IgG binding epitopes were identified at f6-20, f21-35, f36-50, and f86-100 (Cong et al., 2016).

α_{s1}-CN, a single-chain linear phosphoprotein of 199 amino acids residues, is the most abundant protein in cow' milk and is thought to be the most potent among all casein proteins in inducing a specific allergic response. This protein has only a small amount of secondary structure (α-helices or β-sheets) and lacks disulfide bonds, resulting in reduced tertiary interactions (Cong et al., 2013). Using overlapping decapeptides, six major (fragment: 17--36, 83--102, 109--120, 139--154, 159--174, and 173--194) and

three minor (fragment: 39--48, 69--78, and 123--132) IgE-binding as well as five major (fragment: 15--36, 93--108, 143--160, 159--174, and 173--186) and one minor (fragment: 1--10) IgG-binding regions on α_{s1}- CN have been identified. Two unique IgE-binding epitopes (f69-78 and f173-194) identified could potentially be useful for predicting the natural history of CMPA in individual patients. Interestingly, epitope f69-78 was recognized by IgE antibodies from the majority of the patients in the group, but was not recognized by IgG from any of the patients. This difference could be explained by different affinities of IgE and IgG antibodies to this epitope, but it could also indicate that epitopes responsible for allergen-specific Furthermore, Spuergin *et al*. (1997) characterized three peptide sequences (fragment: 19--30, 86--103, and 141--150) of α_{s1}-CN, which reacted with the serum collected from 15 allergic patients. These peptides were localized in the hydrophobic regions of the molecules and became accessible to antibodies only after casein denaturation. Moreover, Cerecede *et al*. (2008) reported that f16-35, f28-50, and f73-92 were identified by more than 75% of patients using microarray-based immunoassay. Recently, other researchers revealed that IgE-binding epitopes (fragment: 6--20, 11--25, 76--90, 126--140, 171--185) and IgG-binding epitopes (fragment: 21--35, 56--70, 161--175) in α_{s1}-CN by alanine scanning analysis. Two amino acid overlapping regions were found to cross-react with IgE and IgG (Cong et al., 2013). Locations of epitopes are usually altered in several studies; this may due to different serology in different countries. Moreover, this discrepancy maybe due to the differences in the methods of studies. Generally, IgE and IgG recognition patterns of α_{s1}-CN support the significance of linear epitopes in persistent CMPA.

In contrast to α_{s1}-CN, there is very little data available on allergenic epitopes of β-CN and κ-CN in humans. Six major (fragment: 1--16, 55--70, 83--92, 107--120, 135--144, and 185--208) and three minor (fragment: 149--164, 167--178, 173--184) IgE-binding epitopes, as well as eight major (fragment: 1--14, 23--34, 55--68, 79--92, 107--120, 135--144, 149--160, and 183--208) and one minor (fragment: 169--184) IgG-binding regions were identified in β-CN. Similarly, eight major (fragment: 9--26, 21--44, 47--68, 67--78, 95--116, 111--126, 137--148, and 149--166) IgE-binding epitopes, as well as two major (fragment:

55--80 and 105--116) and two minor (fragment: 15--24 and 37--46) IgG-binding epitopes were detected in κ -CN (Chatchatee et al., 2001a, b). Cerecedo *et al.* (2008) recognized amino acid sequences of f1-20, f13-32, f67-86, and f181-207 in α_{s2}-CN; f25-50, f52-74, and f154-173 in β-CN; and f34-53 in κ-CN by more than 75% of patients with a peptide microarray-based immunoassay (Cerecedo et al., 2008).

Even today, there is still a great deal of controversy about the prevalence of IgE and IgG reactivity of cow's milk protein. One of the reasons for this might be that study groups were selected based on varying and different criteria and often a sample size was very small.

Human milk as a benchmark

Only breastfeeding is recommended for newborns during the first six months of life. Breast milk is the natural food for neonates as it provides all the energy and nutrients that the infant requires for appropriate development (Lee et al., 2011). However, if the mothers are unable to provide enough milk to feed babies or breast-fed babies were born premature with low birth weight, infant formula or breast milk substitutes may be used as a supplement (Nasirpour et al., 2004). Accordingly, the chemical composition and functionality of human milk is used as a guide for the preparation of infant formula or breast milk substitutes. This understanding and knowledge of constituents of human milk and their functionality and differences in relation to cow's milk are of outmost importance for development of an appropriate infant formula, especially that components of cow's milk are used as a base for most infant formulas (Armaforte et al., 2010). While scientific knowledge has expanded in this area, the following differences still remain to be resolved:

1) Composition of breast milk changes constantly with the changing needs of the baby

2) Human milk contains immunologically active living cells (macrophages, lymphocytes and neutrophils).

3) Human milk includes antibodies (secretory IgA, IgG and IgM) and many bioactive compounds.

4) Human milk contains human proteins.

5) Human milk contains more than 120 oligosaccharides with specific roles (Fisher, 2011).

In addition, the amount of casein and whey proteins varies greatly in early lactation. In fact, during the first days of lactation human milk contains higher concentrations of whey proteins than the caseins. Later in lactation, casein levels start to increase, with concomitant decline in the amount of whey proteins. As a result, the casein fraction increases in a larger proportion of human milk protein with a progression of lactation (after birth) without any fixed ratio of whey proteins to caseins. High level of plasmin in human milk appears to facilitate the protein digestion for immature babies (Armaforte et al., 2010). In general, the protein profiles of infant formula are very similar to cow's milk, since they are often formulated with dairy ingredients, i.e. skim milk powder and demineralized whey powder or whey protein concentrate. The main aim is to increase levels of whey proteins to achieve a casein: whey protein ratio similar to that of human milk. Higher ALA fraction of infant formula than that in skim milk powder is a step towards the humanization of infant formula (Armaforte et al., 2010).

General milk-based formulas

Most infants are provided these formulas in a powder, liquid concentrate or ready-to-eat form. They are obtained by modifying the cow's milk, such as removing the fat and adding vegetable oils. For the most appropriate flavor and nutritional quality, other carbohydrates are added, often in form of lactose while a number of other proteins are excluded. General milk-based formulas are divided into two groups: 1) casein-predominant and 2) whey protein-predominant formulas (Nasirpour et al., 2006). The casein-predominant formulas appear to be the most appropriate for infants; however this has not been confirmed by a randomized controlled trial. On the other hand, whey-predominant formulas have been shown to produce less metabolic stress in premature infants and for this reason they are recommended for low birth weight babies (Fisher, 2011). General milk-based formulas contain intact proteins, and if the infant cannot tolerate formula (CMPA infant), other formulas are introduced. Numerous infant formulas (conventional

and hypoallergenic) are currently available on the market (table 1); however not all of them are suitable for feeding or recommended by pediatricians.

Partially, extensively and fully protein hydrolyzed infant formulas

One of the approaches in the management of CMPA is avoiding intact cow's milk allergens in the diet. It has long been known that the allergic properties of many proteins are reduced by enzymatic hydrolysis with digestive enzyme. Therefore for fully diminishing allergenicity and hypersensitivity, cow's milk proteins should be hydrolyzed into small peptides and free amino acids as these are not allergenic (Nasirpour et al., 2006). However these hydrolysates often have poor flavor, bitter taste, low lipid emulsifiability, high osmolality, and high cost, which limit their use in the general infant formula (Exl, 2001). However, it is recommended as first alternative in CMPA children before using other formulas (El-Agamy, 2007).

The first of the partially/moderately hydrolyzed formulas (Beba HA, Good start, NAN HA, Nestle) was introduced in 1985 (Exl, 2001). Partially hydrolyzed formula (pHF) or eHF always contains residues of cow's milk and can induce allergic reactions (De Almeida, 2011). In general, a large difference exists in the amount of BLG between pHF and eHF. The BLG level of pHF is 40,000 times greater than that in eHF (Mäkinen-Kiljunen and Sorva, 1993). However, it is not clear which of hydrolysates is preferable in the allergy inhibition and for natural infant feeding; pHF appears as a better alternative than the general milk-based formula (Exl and Fritsché, 2001). Moreover, pHF is advantageous over most of eHF due to cost and taste preference (Exl, 2001). Although there is no clear definition of eHF and pHF, this group of infant formulas is now used widely. Modern hydrolyzed infant formulas differ due to protein source, a degree and type of hydrolysis (depending on the enzymes used), other processing techniques (e.g., heat processing), and finally peptide profiles, which may mean that various residual allergen may remain (Exl and Fritsché, 2001). Residual antigenicity in formulas depends on the degree of hydrolysis and filtration techniques applied during manufacturing. Therefore, it is suggested that before the introduction of

hydrolyzed formulas in nutrition of CMPA infants, their safety should be confirmed (Maldonado et al., 1998).

According to the American Academy of Pediatrics, it is important to confirm that these formulas are hypoallergenic by conducting appropriate pre-clinical testing. The main criterion for labeling an infant formula as hypoallergenic is that 90% of children or infants with confirmed CMPA does not show immunoreactive responses (in prospective randomized, double-blind, placebo-controlled trials) (Baker et al., 2000).

Based on several studies about allergencity of pHF and eHF, fully hydrolyzed or amino acid-derived formulas (AAF) are recommended. The studies showed that the use of AAF reduces antigenic effect (Isolauri et al., 1995). Provision of AAF to about 10% CMPA infants who are allergic to eHF is necessary (De Boissieu et al., 1997). It is also recommended to supplement AAF to infants with growth retardation or multiple food allergies (Isolauri et al., 1995). The main obstacle to a wider application of AAF is cost and fairly unpleasant taste (Restani et al., 2006). In some reports, eHFs and AAFs are considered hypoallergenic while pHFs has not been placed in this group as the required levels of immunoreactivity are not expected (Johnston, 2011).

Management of CMPA

Prevention of allergic diseases in infants by limiting or modifying diet is not a new topic and has long been proposed by many researchers. There are many reasons for preventing CMPA from the beginning. For example, the CMPA is a frequently occurring disease and cow's milk proteins often exist in various food products thus making their elimination from the diet difficult. Also, cow's milk is an important source of calcium, especially for babies during the period of bone growth up to the end of puberty (Exl and Fritsché, 2001). In other words, the management of CMPA should be based on modified bovine milk proteins (Isolauri et al., 1995). For this reason, much attention has been placed on applying various technological approaches that are common in the food industry in the reduction of milk protein

allergenicity. These mainly include thermal processing (Kleber et al., 2004; Meltretter et al., 2008; Nowak-Wegrzyn et al., 2008; Bu, et al., 2010a; Li et al., 2011), microwave heating (Grar et al., 2009; Kaddouri et al., 2009; Zellal et al., 2011), enzymatic hydrolysis (Svenning et al., 2000; Wróblewska et al., 2004; Liu et al., 2012), fermentation/or hydrolysis by lactic acid bacteria (Jedrychowski, 1999; Bu et al., 2010b; Pescuma et al., 2011), and high-pressure (Anema et al., 2005; Peñas et al., 2006; Kleber et al., 2007; Chicón et al., 2008). Also, the combined application of hydrolysis with high pressure and radiation has also been tested (Izquierdo et al., 2005; Izquierdo et al., 2007; Beran et al., 2009; El-Mecherfi et al., 2011). During recent years, several review articles have been published on technologies approaches to control cow's milk allergenicity, without specific emphasis on production of hypoallergenic infant formulas (Luo and Bu, 2012; Bu et al., 2013). An appropriate approach should impart a very small effect on the overall composition and sensory properties of milk with simultaneous reduction in milk protein allergenicity. In general, any process that modifies a structure of a protein might be expected to interfere with its ability to be recognized by antibodies. While most of studies focused on the impact of thermal treatments on milk protein allergenicity, some novel non-thermal techniques have been explored in regards to production of hypoallergenic foods, mainly due to minimal impact on the composition and sensory attributes of the foods that are substantially changed during a thermal treatment (Shriver and Yang, 2011).

Thermal processing methods

Thermal processes are one of the most common and oldest methods for processing of raw food materials. However the impact of this technique appears unresolved as studies have shown that measured bovine allergenicity may reduce, increase or remain unchanged.

Moist heating

Thermal processing is carried out with the aim to destroy pathogens that are dangerous to public health and reduce a microbial load in order to extend the shelf-life of milk (Monaci et al., 2006). It is often

assumed that the heat processing should reduce the allergenicity of milk proteins, since heating or cooking usually modify a protein structure. Some studies have reported findings that contrasted this expectation, especially when this processing took place in a complex food matrix. Because many other ingredients available to precipitate in complex physical and chemical reactions that resulted to the formation of variable amounts of numerous protein adducts and modify antigenicity (Davis et al., 2001).

In general, the main reason for the reduction of protein allergenicity is denaturation that alters spatial structure of a protein and destroys IgE-reactive epitopes (Paschke, 2009). Usually heating can destroy most conformational epitopes by unfolding native protein structure. However, linear epitopes remain unaffected by these structural changes and thus persist to be the allergenic problem after heating (Wróblewska and Jędrychowski, 2003). For example, caseins do not have highly structured configuration and they contain predominantly linear epitopes, which in turn render them highly resistant to heat treatment (Nollet and Toldra, 2009). Schematic representation of heat induced changes of linear and conformational epitopes is shown in Figure 2. However, allergenicity of proteins may also increase due to creation of new epitopes or a greater access of cryptic or hidden epitopes by denaturation of the native allergen (Ehn et al., 2004). Because the structure of the protein contain hidden epitopes that early buried in protein structure. After denaturation of protein, the protein structure is opened and hidden epitopes can be revealed, which would increase the antigenic response. However, at higher temperatures remarkable decrease in antigenicity occurs as a result of accompanying denaturation, aggregation and Maillard reaction with other molecules such as sugars that mask allergenic areas and reduce the binding antibodies to allergic areas (Kleber et al., 2007; Van Hengel, 2007).

Previous research demonstrated the relationship between the antigenic response, the temperature of denaturation, the degree of whey protein denaturation and the microstructure of heat-treated aggregates. The internal structure of heat-treated particles may develop differently during different heating conditions. Soft, open and loose structure is formed at 90 ºC, enabling antibodies to penetrate and react

with conformational or linear epitopes. However, at higher temperatures, particle collisions increase resulting in more compact and denser aggregates, thus limiting access to the interior and allowing contact with the epitopes located at the particle surfaces (Kleber et al., 2004). Accordingly, the authors were proposed a model for these two hypotheses (Figure 3):

Dry heating

The difference can be seen in dry versus moist methods of heating is related to the temperature. Moreover, the allergens (proteins) are modified by non-enzymatic browning reaction (Maillard browning) in dry heating or protein denaturation in wet heating (Shriver and Yang, 2011).

The Maillard reaction in dry conditions appears as an effective way to reduce antigenicity of cow's milk proteins. However, this approach still has several weaknesses as the reaction would proceed slowly during storage impacting functional behavior and nutritional value (Birlouez-Aragon et al., 2004; Thomas et al., 2004; Le et al., 2011; Wang and Ismail, 2012). The limited Maillard-induced glycosylation may preserve the nutritional quality of whey proteins (Wang and Ismail, 2012).Another study observed that the reaction of lactose with BLG enhances allergenicity (Paschke, 2009). The products of Maillard reaction are generally called advanced glycation end products (AGEs) which can play an important role in food allergy (Davis et al., 2001). Other covalent modifications, apart from the Maillard reaction, of proteins caused by heating or storage involve reactions with oxidised lipids, disulfide bond scrambling or deamination of the amino acid asparagine, all of which can contribute to changes in antigenicity (Monaci et al., 2006). The rate of Maillard reaction in milk products largely depends on the heat treatment conditions, storage temperature, humidity, pH and milk ingredients (such as sugar types) and protein involved in the reaction (Le et al., 2011).

Infant formula due to the amount of high carbohydrate content, lysine-rich proteins, relatively high temperatures during the manufacturing process, neutral pH and long-term storage (up to one year after production) is very sensitive to the Maillard reactions (Pereyra Gonzáles et al., 2003). As a consequence

of their specific formulation and processing, infant formulas containing much more of glycation markers than other milk products. For example, whey proteins contain more lysine groups than caseins and can be more extensively glycated. Particularly, the glycation rates are very high in hypoallergenic formulas that related to N-terminal α-amino acid groups resulted from hydrolysis (Pischetsrieder and Henle, 2012).

Also, it should be noted that all studies have been performed in model systems (specific protein-polysaccharide mixture) and not in complex matrices (formula and other cow's milk products). Some of the studies describing modifications of CMPA by thermal treatment (moist and dry) are presented in table 2.

In general, differences in allergenicity of cow's milk protein are not only dependent on the temperature and time of heating but also potential interactions with other cow's milk proteins and food matrix components (Monaci et al., 2006; Davis et al., 2001). Therefore, heat treatment can be part of a procedure for producing hypoallergenic food, but alone may not be as efficient (Lee, 1992). Despite the little advantage of heat treatment on antigenicity of whey proteins, this process has some other disadvantages such as the Maillard reaction, reaction of lysine with dehyroalanine resulting from cysteine degradation to form lysinoalanine, protein digestibility, protein solubility, and cooked flavor (Nasirpour et al., 2006).

Non-thermal processing methods

Heat treatment and enzymatic hydrolysis are two conventional methods frequently used to reduce milk proteins allergenicity. But each method has its own weaknesses and none is completely satisfactory. For example, thermal treatment and hydrolysis of milk proteins not only reduce the allergenicity, but adversely affect the nutritional and sensory properties of treated milk products. On the other hand, enzymatic hydrolysis requires specific enzymes that degrade antigenic epitopes and may require removal of bitter peptides. Also, in some cases heat treatment and enzymatic hydrolysis can increase the allergenicity of milk proteins by generating new epitopes (Jedrychowski, 1999).

Recently, new techniques including non-thermal processing methods for the production of hypoallergenic products are being explored. Non-thermal methods have advantages such as preservation of the organoleptic characteristics and nutritional content, which are usually altered during thermal processing (Shriver and Yang, 2011). Food technology aims to reduce allergenicity, either by irreversible removal of allergen, or by modifying the allergen structure in such a way that the allergenic epitopes are no longer recognized by the immune system.

High Pressure processing

Thermodynamic and kinetic parameters of proteins can be affected by pressure that is a basic physical state and such as is at least as important as temperature. But, experiments in the context of the characteristics of milk and milk products at different pressures have been very limited, especially in comparison to those involving heat treatment of milk. Perhaps this is due to the higher cost of high-pressure (HP) equipment and the lower potential for commercialization of this technology (Anema et al., 2005). Generally, HP can affect the ionic equilibrium in milk, casein micelles, whey proteins, fat globules and the natural enzymes of milk (Huppertz et al., 2006).

HP has been used to inactivate microorganisms and enzymes and improvement of the product texture (the result of protein modification and denaturation) in the food industry. Therefore, this technique provides an alternative to heat treatments. The effects of HP related to changes in non-covalent interaction such as hydrophobic and electrostatic, which causes conformational modification (Kleber et al., 2007; Korhonen et al., 1998; Shriver and Yang, 2011). Since it has been investigated that HP induce structural changes of proteins, this theory emerged that HP can alter allergens reactivity by restructuring of food allergens (Peñas et al., 2006; Chicón et al., 2008).

Recently, it has been reported that protein hydrolysis at the HP is a novel method for enzymatic hydrolysis of substrate that are hardly hydrolyzed at HP conditions. HPs act with changing in the balance of intra-molecular and protein/solvent interactions. The progress of pressure-induced changes in proteins

depends on the native structure of proteins, as well as factors such as ionic strength, temperature, solvent, pH and levels of applied pressure. Therefore, the pressure can be effective on the proteolysis of proteins by enzymes (Izquierdo et al., 2005).

Several studies about the increasing of enzymatic proteolysis under HP have been reported. Accordingly, the combined effect of HP treatment and enzymatic digestion with food grade enzymes to reduce of antigenic proteins is used (Peñas et al., 2006). Therefore, milk protein hydrolysates obtained by enzymes in combination with HHP treatment could be used for production of hypoallergenic infant formulas (Beran et al., 2009). There are significant differences in protein denaturation and aggregation during HP in comparison to that of heating effect. Researchers found that the antigenicity of whey protein hydrolysates treated with HP was found to be lower than of heat-treated hydrolysates (Korhonen et al., 1998). In addition of the positive effects of HP on milk proteins allergenicity, some studies have reported negative effects of this treatment (Kleber et al., 2007; Shriver and Yang, 2011). Kleber *et al*. (2007) showed that HHP had a negative effect on allergen reactivity of milk. In particular, they reported that the major milk allergen, BLG, was more reactive after HHP treatment at 200--600 MPa. The combination of HHP with heat treatment increases the allergic potential. It has been suggested that the structural changes may expose new linear epitopes and binding sites of immunoglobulin E, and agreement with greater allergen reactivity (Kleber et al., 2007).

Although the first food to be treated with HP was milk and many researches have focused on milk's modification during HP, but so far the HP-treated milk product has been not produced commercially. Several factors limiting the application of this technology include the cost as the HP treatment is an expensive technology and also benefits since these need to be clearly proven. Besides, HP-induced changes in milk than other foods are questionable and require further investigation (Huppertz et al., 2006). Nevertheless, combined hydrolysis and HP treatment can be one of the promising approaches for producing hypoallergenic infant formulas.

Microwave radiation

The effects of microwave irradiation on structural properties of milk proteins have been also studied. Microwaves are electromagnetic wavelengths and heating by microwave energy is different compared to conventional methods. In this system, heating is performed both by the absorption of microwave energy by rotation of the dipolar water molecules and by the translation of the ionic components of food. Microwave irradiation is shown to affect the kinetic of conformational change of milk proteins and to accelerate their denaturation (Izquierdo et al., 2005; Grar et al., 2009). Furthermore, it has been reported that the epitopes on the allergens were structurally altered by radiation treatment. So, irradiation technology can be applied to reduce allergenicity of proteins. In fact, the IgE of patients cannot be easily diagnosing the irradiated allergens (Arvanitoyannis and Tserkezou, 2010).

It was found that microwave irradiated whey proteins (ALA and BLG) lost a part of their IgG-binding ability, but their antigenic activities were not entirely abolished. Indeed, microwave irradiation on its own is very unlikely to be sufficient to substantially reduce or entirely eliminate the antigenicity of BLG and should, therefore, be combined with other treatment (i.e. enzymatic treatment) or intrinsic parameters (i.e. pH) (Kaddouri et al., 2009).

Recently, combined microwave irradiation (MWI) and enzymatic treatments on reducing antigenicity of whey proteins were assessed by several studies (Izquierdo et al., 2007; Izquierdo et al., 2008; El-Mecherfi et al., 2011;). These authors reported that MWI enhanced the hydrolysis rate of BLG and WPI as compared to the conventional conditions. Microwave irradiated BLG at 200 W enhanced the hydrolysis of this protein by pepsin in 3 min and decreased significantly its immunoreactivity. But, the extensive hydrolysis of the microwave-treated BLG and WPI with trypsin, chymotrypsin, and the mixture of them did not have an effect on the IgE binding of resultant products (El-Mecherfi et al., 2011).

Izquiedo *et al*. (2007) showed that the highest pronase and chymotrypsin activity occurred under MWI because smaller peptides were produced. Moreover, they suggested that MWI can be used efficiently to

accelerate BLG hydrolysis, depending upon the choice of enzymes (Izquierdo et al., 2007). One year later, these authors were analyzed the effect of MWI on the hydrolysis of a commercial bovine whey proteins concentrate by Pronase, Chymotrypsin and five food grade enzymes (Papsin, Corolases 7089 and PN-L 100, Alcalase and Neutrase). They found that proteolysis of dairy whey proteins by some enzymes (Pronase, Papain and Alcalase) in combination with MWI has the potential to produce more efficiently hypoallergenic dairy hydrolysates. In addition, it can render products less palatable (bitter) and can also reduce functional properties such as emulsion activity and stability (Izquierdo et al., 2008).

Hence, it is suggested that hydrolysates obtained by combined MWI and enzymatic treatments can be used as a source of peptides in eHFs. Nevertheless, though irradiation (especially MWI) is an effective method for limiting BLG allergenicity and production of hypoallergenic formulas. However, further researches to investigation MWI effects on allergenic properties of milk proteins (i.e. BLG and so on) should be carried out.

Fermentation

Fermentation is an oldest processing technology in the food industry. The hydrolysis of milk proteins upon fermentation by lactic acid bacteria (LAB) may have important effects on milk digestibility and the production of bioactive peptides. Moreover, the proteolysis can destroy some epitopes and thus reduce milk allergenicity (Bu et al., 2010b). Recently, a high interest concerning LAB and their immunomodulative properties has been noticed.

As previously mentioned, in addition to LAB, proteases from other microorganisms, animals or plants were also used to hydrolyse milk proteins and decrease their antigenicity. However, some bitter peptides were produced during the hydrolysis process, which greatly affected the development and application of the hypoallergenic milk products. Hence, hydrolysis during fermentation by LAB not only reduced the antigenicity of milk proteins, but also made the good taste hydrolysate with many bioactive substances (Wróblewska and Jędrychowski, 2003; Bu et al., 2010b).

LAB fermentation is suitable method for decreasing the antigenicity of whey proteins. Results showed that the antigenicity of whey proteins was reduced by over 99% in sterilized cow's milk after LAB fermentation compared to raw milk (Jedrychowski, 1999). Bu *et al*. (2010b) was demonstrated that antigenicity of whey proteins significantly decreased after the fermentation by LAB (inhibition rate of 53--87% for ALA and 86--95% for BLG as compared with unfermented milk). So, inhibition rate of BLG antigenicity was higher than ALA in the fermented milk. This result may be due to greater hydrolysing capacity of some LAB protease on BLG in comparison to ALA. Moreover, thermal stability of BLG is poorer than ALA and hence preheating treatment before fermentation can lead to the denaturation and then unfolding of the BLG conformational structure, thus making BLG easier to hydrolyse. They reported that the combined strains of *Lactobacillus helveticus* and *Streptococcus thermophilus* were the most effective in reducing the antigenicity both whey proteins in comparison of each of them alone. Synergistic effects were observed between the combined strains (Bu et al., 2010b).

In the recent, the use of hydrolyzed 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 though the hypoallergenic formulas (Monaci et al., 2006).

Lactobacillus rhamnosus GG (LGG) is the most studied probiotic, with demonstrated benefits when added to an eHF. LGG decreased severity of atopic dermatitis, reduced intestinal inflammation and faster induction of tolerance in infants with CMPA and improved recovery from allergic colitis (Muraro et al., 2012). In fact, it have been combined the benefits of a high hydrolysed formulas with probiotics. However, the presents are still not totally satisfying according to the European Society of Paediatric Gastroenterology and Nutrition (ESPGHAN) committee since the real safety of the probiotics incorporated in the formulae has not yet been sufficiently evaluated (Monaci and van Hengel, 2007).

Further, *in vitro* and *in vivo* studies are necessary to better understand the mechanism of allergenicity of dairy proteins and the methods can be to decrease it.

Some of studies about modification of CMPA by non-thermal treatment were demonstrated in table 3.

Hypoallergenicity of other animals milk (cow's milk alternations)

Cow's milk is widely used as an alternative to breast-feeding, but as previously mentioned that in the most cases, it can lead to abnormal immunological responses. De almeida *et al*. (2011) reported that there are some reasons not to consider hydrolysates formulas as ideal option. They are includes: 1) all hydrolyzates contain molecules larger than 970 kDa and the IgE can easily bind with them. 2) Peptides with 13--17 amino acids and molecular weight less than 1200 kDa may to simulate clones of T cells; those peptides are found in pHF and eHF. 3) Reduction of molecular weight does not necessarily mean a reduction of allergenic potential and there is no useful way to understand the exact moment at which to stop the hydrolysis. 4) Many processes of hydrolysis can be release biologically active peptides. Hence, in the Europe Union, hydrolysates are controlled by demanding that immune-reactive proteins amount to less than 1% of the total nitrogen compounds (De Almeida C, 2011). Moreover, AAFs have unpleasant taste, but should be considered in severe clinical manifestations of CMPA or when eHFs are not effective were proposed. Although, they are quite expensiv e (Exl, 2001; Restani et al., 2006). Therefore, due to the limitation of pHFs, eHFs and AFFs were expressed, several researchers have attempted to an alternative to cow's milk and seemingly hypoallergenic formulas with other animals milk (if being hypoallergenic). So, many research articles on the application of other species milk (such as goat, sheep, camel, donkey or horse) in CMPA children nutrition have been published (Martins et al., 2005; Shabo et al., 2005; Polidori et al., 2013; Swar, 2011).

For example, on the bases of findings in camel milk by El-Agamy *et al*. (2009), it can be concluded that camel milk might be a promising new protein source for CMPA children and camel milk infant formulas can be taken in to account (El-Agamy et al., 2009). Recent study showed that the possibility of using

donkey milk in feeding children with CMPA (Polidori and Vincenzetti, 2013). Moreover, Swar (2011) formulated donkey milk-based formula with added amount of sunflower oil (for compensation of low fat content and low caloric value) that to be closest to breast milk (Swar, 2011). However, the results of some animals milk as alternative to breast milk is slightly controversial.

It should be noted that infant formula based on the other animals milk without any modification to treat CMPA children may be show allergic cross-reactivity and insufficient nutritional value. In addition, most of them are low in energy and extremely low in protein compared with cow's milk. So, elemental supplementation such as calcium, phosphorus and vitamin D is necessary. Some of clinical studies on tolerability of other animal's milk in CMPA children were shown in table 4.

Generally in hypoallergenic products, firstly milk was treated with proteolytic enzymes which resulting in remaining traces of intact proteins in the final product. So, these residual intact allergenic proteins can be removed by ultrafiltration and suppresses the allergenicity of milk (Paschke, 2009). Therefore, this treatment is complementary to other processes.

CONCLUSION AND RECOMMENDATION

Finding new strategies for producing hypoallergenic formulas is still an important field and under investigation, especially that it has been reported that some of commercial infant formulas are not antigenically neutral and are awaking classic symptoms of cow's milk allergy. In general, the best way to management of CMPA is elimination of the allergens from the diet. However, since some food allergens (e.g. milk's proteins) have high nutritional value, they are often present in many processed foods and cannot be removed from the diet, so it should be looking for alternatives.

Therefore, a number of technological approaches aimed at reducing of milk allergies (one of the most valuable food) have been trialed. These methods (thermal and non-thermal treatment) have great potential to reduce the milk allergenicity, but in some cases (e.g., wet heat treatment) may fail to reduce allergenic potential and also may increase it. Because some epitopes which were masked in the native proteins, after

denaturation may become accessible and/or reactive. Several researchers believed that both thermal and non-thermal techniques modify allergen reactivity by changing protein structure and altering IgE binding sites. The ability to alter conformation of allergens with these techniques is an important concept that can be utilized by researchers dedicated to developing methods for food allergen elimination (Shriver and Yang, 2011). Therefore, firstly it is recommended that more research is conducted to reveal clearly how traditional processing methods affect food allergenicity (especially in milk processing), followed by looking for new ways to produce hypoallergenic foods and prevent the allergic responses. But, it is important to maintain the quality and origin of food whereas reducing food allergenicity due to altering food protein.

The choice of CMPA alternatives for children depends on two main factors, i.e., nutritional adequacy and allergenicity. Cost and taste must also be taken in to account. Heat treatment of milk may destroy heat-labile proteins, but in some studies have been reported that CMPA infants are still reactive to heat-denatured milk proteins. Moreover, hydrolytic treatment of milk proteins has resulted in products with unacceptable taste due to bitterness originating from the production of peptides and amino acids (Exl, 2001).

Nowadays among these methods, enzymatic hydrolysis is widely applied for the production of hypoallergenic formulas as it is economical in comparison to other techniques. Insufficient activity of used proteases may account for some of children being sensitized to hydrolysed formulas and also, extensive hydrolysis can deteriorate functional properties of milk proteins. Hence, great hopes are placed on development of new technological treatments in order to produce entirely hypoallergenic formulas.

It was shown that some technological processes can be used to reduce the allergenicity or/and antigenicity of cow’s milk proteins. We recommend that different technological approaches can be combined together in the future, such as fermentation and hydrolysis with proteases from animal and plant sources, heat treatment, high pressure, and microwave irradiation combined with enzymatic hydrolysis. As new

epitopes may appear during processing, a combination of these strategies may be very valuable. While further research on the specific effect of the various treatments is still required, other species milk (e.g. camel and donkey) also appears as a suitable option for developing new hypoallergenic formulas for applications in the future.

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Table 1: Examples of commercially standard and specialized formulas

Brand	Type	Producer
Novalac Premium	CMF	Novalac
Nutrilon Premium 1	CMF-C	Nutricia
Infacare® Gold 1	CMF-W	Aspen Nutritionals
NAN 1 Protect Start	CMF-W	Nestle
Karicare Sensikare HA	pHF-C	Nutricia
Gentlease	pHF-C	Mead Johnson
Good Start Supreme	pHF-C	Nestle
Humana HA 1, 2 and 3	pHF-W	Humana
Beba HA	pHF-W	Nestle
Good Start	pHF-W	Nestle
Similac® Advance HA	pHF-W	Abbott
Novalac® HA	pHF-W	Novalac
Infacare® Nuture HA Comfort	pHF-W	Aspen Nutritionals
Nutramigen	eHF-C	Mead Johnson
Similac® Alimentum	eHF-C	Abbott
Aalfa-Re®	eHF-W	Nestle
Nutrilon Pepti Junior HE	eHF-W	Nutricia
Hypolac Alimento	eHF-W	ALK
Hipp HA	eHF-W	AGRANA
Pepticate	eHF-W	Nutricia
Allernova	eHF-W	Novalac
Peptidi-Tutteli	eHF-W	Valio
Neocate Infant	AAF	Nutricia
Elecare	AAF	Ross
Nutri-Junior	AAF	Nutricia
Neocate	AAF	SHS International
Isomil	SF	Abbott
Prosobee	SF	Mead Johnson
Alsoy	SF	Nestle
Nursoy	SF	Gerber
Infacare® Soya 1 and 2	SF	Aspen Nutritionals
Similac® Isomil Advance	SF	Abbott

CMF: Standard cow's milk formula
CMF-C: Cow's milk formula/ casein-predominant
CMF-W: Cow's milk formula/ whey-predominant
pHF-C: Partially hydrolysed casein formula

pHF-W: Partially hydrolysed whey formula
eHF-C: Extensively hydrolysed casein formula
eHF-W: Extensively hydrolysed whey formula
SF: Soy formula

Table 2: List of studies about modification of CMPA by thermal methods

Type	Objectives	Highlights	References
Moist heat	ALA, BLG, α-CN, and β-CN antigenicity	- Reduction of the antigenicity and allergenicity of ALA	(Xu et al., 2016)
	(in the range of 65--100 °C and 10--30 min)	- Increasing the antigenicity of BLG; but allergenicity decreased only at 85 and 100 °C for 25 min.	
		- The antigenicity of α-CN and β-CN were lower than unreacted sample.	
		- The allergenicity of α-CN decreased at 65 and 70 °C for 25 min.	
		- The allergenicity β-CN was higher than unreacted sample.	
Moist heat	Allergencity of extensively heated milk in CMPA children	- The majority (75%) of children with milk allergy tolerate heated milk	(Nowak-Wegrzyn et al., 2008)
Moist heat	Modification of IgE binding of the cow's milk allergen BLG	- A significant decreased IgE binding observed between unheated and heat-treated BLG solution at 74 °C.	(Ehn et al., 2004)
		- The inhibition of IgE binding of milk after heat treatment at 90 °C was decreased.	
		- There was a small difference of IgE binding between the native forms of genetic variants A and B.	
Dry heat	Antigenicity of BLG and fructo-oligosaccharides (FOS) conjugates	- BLG at a biopolymer ratio of 1:4 (BLG:FOS) had a lowest antigenicity than other ratios (1:0, 1:1, 1:2, 1:6, 1:8, and 1:10), which was about 7 time lower than that of the native BLG	(Zhong et al., 2013)
Dry heat	Effect of BLG-carbohydrate (galactose, tagatose, and dextran of 10 or 20 kDa onjugation) conjugations on the gastrointestinal digestibility and immunoreactivity of BLG	- Protein aggregation had a masking effect on BLG epitopes, counteracting the negative effect of the lower digestibility of glycated protein on its allergenicity.	(Corzo-Martínez et al., 2010)
		- High levels of glycation reduced the susceptibility of BLG to proteolysis resulting in increased IgG- and IgE-reactivities of hydrolysates, regardless of the type of carbohydrates used.	

Dry heat	Antigenicity of ALA and BLG in conjugation with maltose	- Conjugation whey protein isolate wit maltose is an effective way to reduce the antigenicity of ALA and BLG.	(Li et al., 2011)
		- Temperature had the greatest effect on the antigenicity of ALA.	
		- Ratio of whey protein isolate to maltose is the most significant factor on BLG antigenicity.	

Table 3: List of some studies about modification of CMPA by non-thermal methods

Type	Objectives	Highlights	References
Enzymatic hydrolysis	Effects of pH (7-11), temperature (30-60 °C), and enzyme-to-substrate ratio (4000-8000 units g^{-1} protein) on the residual antigenicity of whey protein concentrate hydrolysates prepared by Alcalase	- Enzymatic hydrolysis reduced the antigenicity of ALA and BLG effectively. - Reduction of antigenicity could be controlled by regulation of pH, temperature, and enzyme-to-substrate ratio.	(Zheng et al., 2008)
Enzymatic hydrolysis	Effects of pH, temperature, enzyme-to-substrate ratio and reaction time on the antigenicity of casein hydrolysates prepared by Papain	- Enzymatic hydrolysis reduced the antigenicity of α-CN and β-CN effectively. - Reduction of antigenicity could be controlled by the reaction conditions. - Enzyme-to-substrate ratio had the most significant effects on the antigenicity of α-CN and β-CN.	(Liu et al., 2012)
Enzymatic hydrolysis	Immunoreactive properties of whey protein concentrate after enzymatic hydrolysis by Alcalase, Papain, and with together	- The "two-step" process is the most effective but allergenic epitopes was still present, as it was found by ELISA. - Alcalase-Papain partially hydrolysated whey protein concentrate is a promising base for production of hypoallergic infant formulas.	(Wróblewska et al., 2004)
UHP and HHP	Effect of HP on *in-vitro* digestibility of BLG	- HP-treatment of BLG at 400 MPa for 10 min slightly increased pepsin hydrolysis - HP treatment at 600 and 800 MPa resulted in rapid BLG digestion - Digestion HP-treated BLG was performed at IgE epitope regions	(Zeece et al., 2008)
UHP and HHP	Effect of combined HP and enzymatic treatments on the antigenicity of bovine whey protein hydrolysates	- HP treatment enhanced the hydrolysis of bovine whey proteins. - The optimum pressure was 300 MPa.	(Peñas et al., 2006)

UHP and HHP	Immunoreactivity and digestibility of HP-treated whey proteins	- Treatment of BLG at 200 and 400 MPa increased binding to BLG-specific rabit IgG and did not affect binding to IgE from patients. - HP treatment at 400 MPa promoted the hydrolysis of BLG by pepsin.	(Chicón et al., 2008)
Microwave radiation	Peptic hydrolysis of BLG during microwave treatment and assessment allergencity of BLG hydrolysates	- Microwave treatments combined with peptic hydrolysis can generate peptides with low IgE immunoreactivities in a short time (3 min of hydrolysis)	(El-Mecherfi et al., 2014)
Microwave radiation	Effect of microwave heating on cow's milk protein antigenicity	- Microwave heating of entire cow's milk diminish its whey proteins reactivity towards the specific antibodies (IgG).	(Kaddouri et al., 2009)
Microwave radiation	Allergencity of BLG induced by microwave irradiation under different pHs	- Microwave irradiation of whey proteins at the natural milk pH (pH 6.8) or pH 4.6 induces a significant decrease in the BLG allergencity.	(Zellal et al., 2011)
Fermentation	Proteolytic action of *Lactobacillus delbrueckii subsp. bulgaricus* CRL 656 on bovine BLG antigenicity	- *L. delbrueckii subsp. bulgaricus* CRL 656 degraded three main BLG epitopes (V41-K60; Y102-R124; L149-I162) - The BLG hydrolysate had less immune-reactive (32%) than the native BLG	(Pescuma et al., 2011)
Fermentation	Effects of fermentation, refrigeration at 4 °C and simulated gastrointestinal digestion (SGD) on the antigenicity of ALA, BLG, α-CN and β-CN	- Antigenicity of ALA and BLG reduced - Antigenicity of α-CN increased - No effect on β-CN antigenicity - Refrigeration had no significant effect above mentioned protein antigenicity	(Zheng et al., 2013)

		- SGD was effectively reduced ALA, BLG, α-CN and β-CN antigenicity	

Table 4: Main published clinical studies on tolerability of other animal's milk in children with CMPA

Species	Experimental conditions	Results	References
Donkey	- Application of donkey's milk in a population of 46 selected children with CMPA, for whom it was not possible to use any cow's milk substitute	- 38 children (82.6%) tolerated donkey's milk.	(Monti et al., 2007)
Goat and sheep	- 28 children with severe allergic reactions to goat and sheep (cow's milk tolerated)	- Skin prick testing and Ig E-binding studies confirmed the diagnosis goat and milk allergy without associated cow's milk allergy.	(Ah-Leung et al., 2006)
	- Casein and whey protein fractions were isolated and EAST inhibition studies performed with sera of the allergic children	- Goat and sheep allergy involves the casein fraction and not whey proteins.	
		- αS_1-CN, αS_1-CN, and β-CN from goat and sheep had high affinity by the patient's IgE.	
Camel	- Investigation of camel milk effect in 8 children (4 months to 10 years) with severe food allergies (mainly milk).	- All eight children reacted well to the milk and recovered fully from their allergies.	(Shabo et al., 2005)

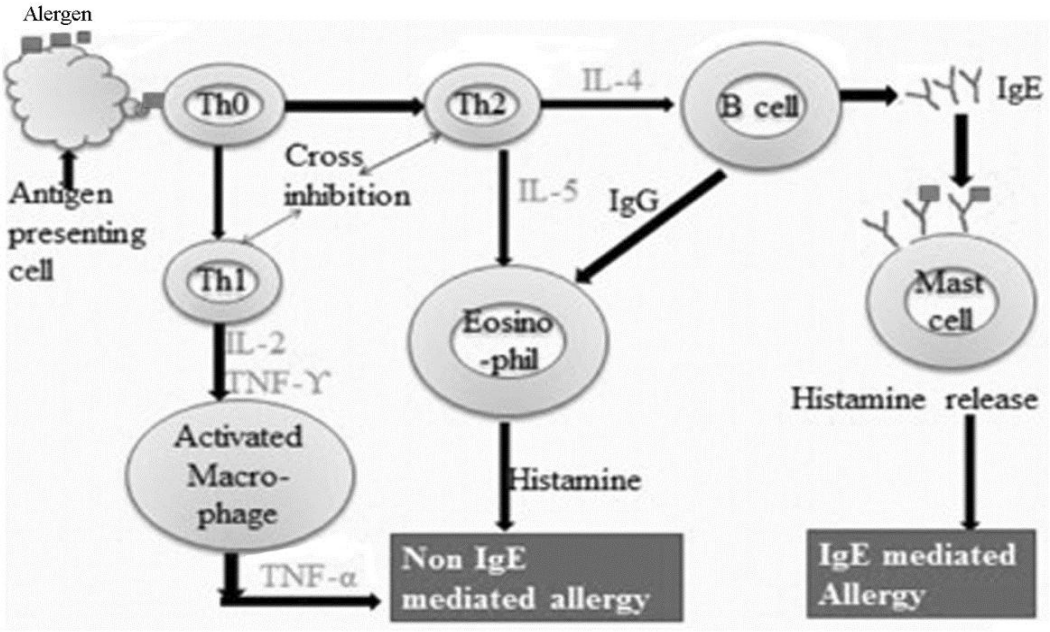

Figure 1. The immunological profile during an allergic response

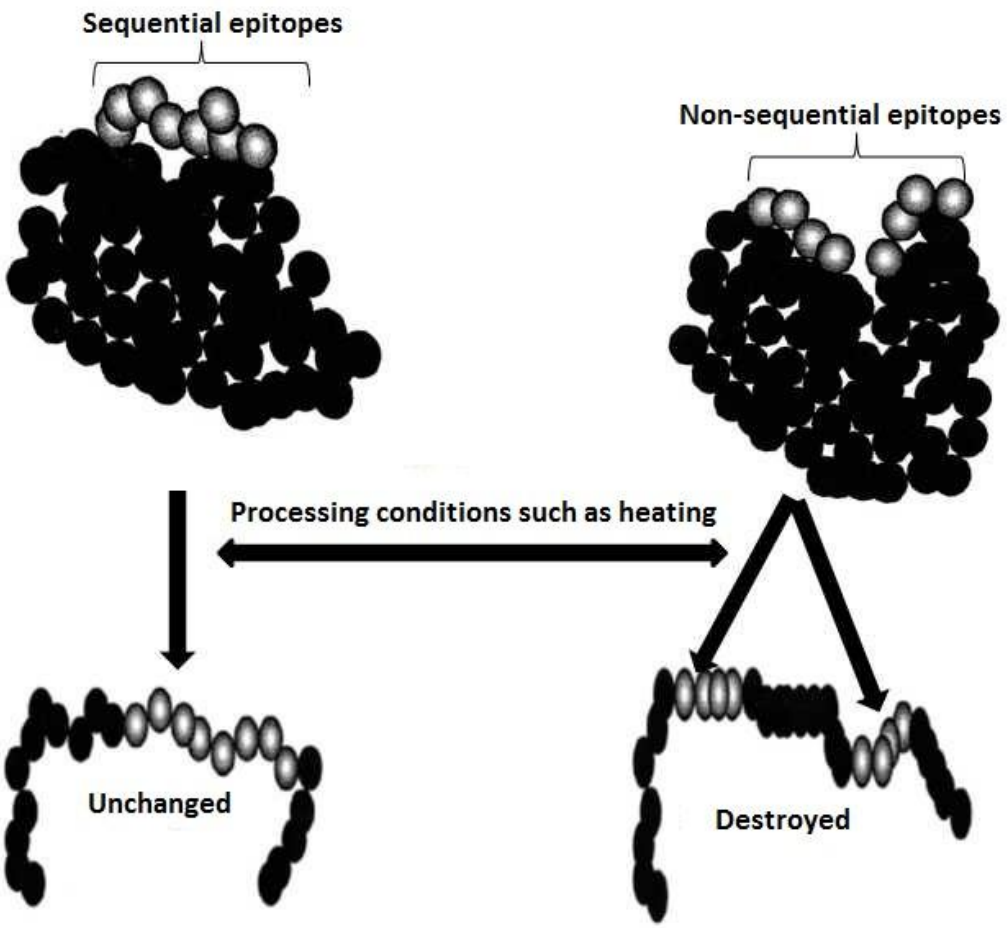

Figure 2. Schematic representation of linear and conformational epitopes after heat treatment (Rahaman et al., 2016)

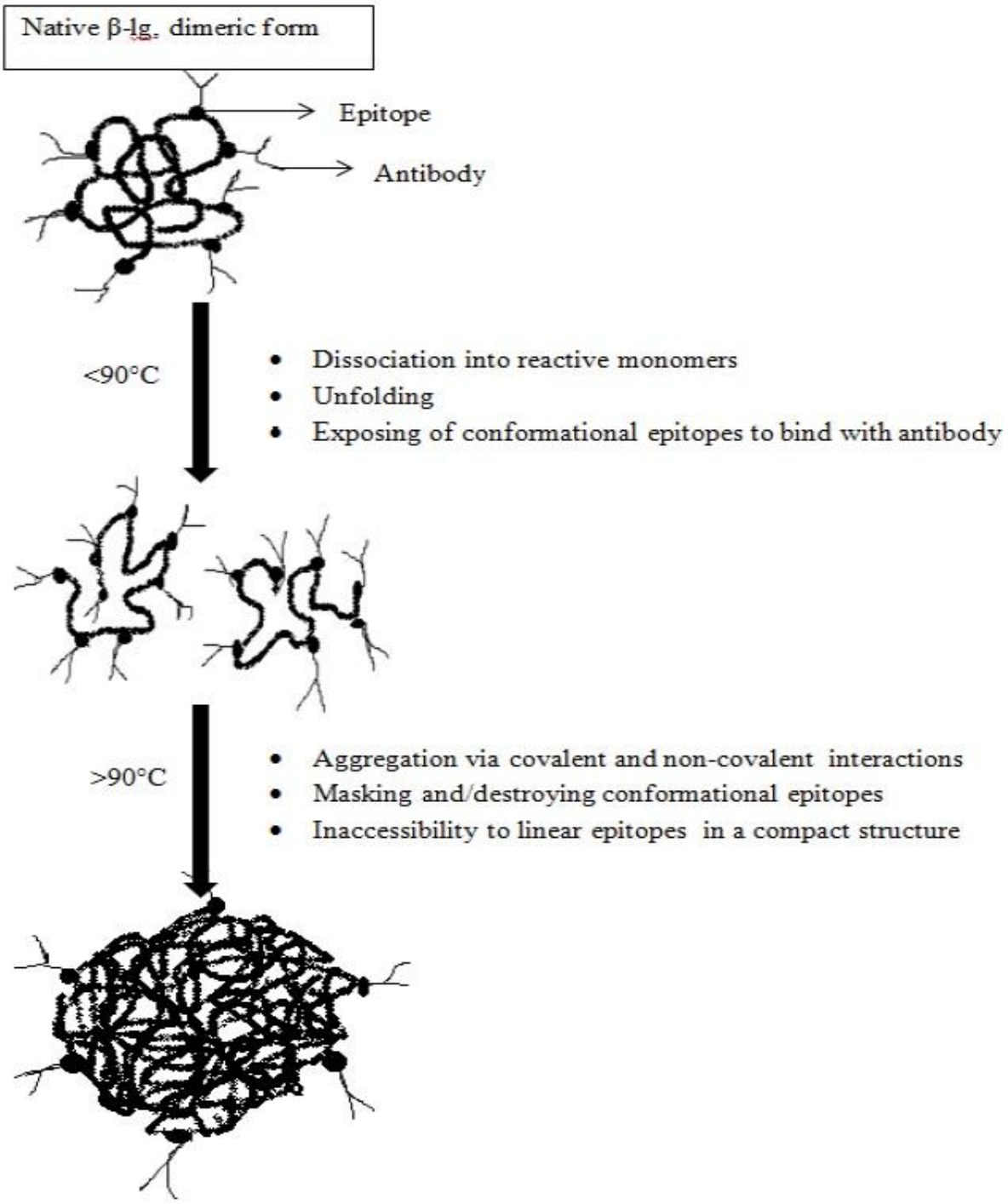

Figure 3. Schematic model for the binding of antibodies to open and compact structured of whey protein aggregates over different temperatures (Rahaman et al., 2016).