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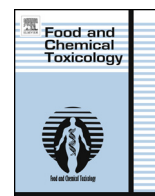Part of the Food Science Commons

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Review

Food processing and allergenicity

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ABSTRACT

Food processing can have many beneficial effects. However, processing may also alter the allergenic properties of food proteins. A wide variety of processing methods is available and their use depends largely on the food to be processed.

In this review the impact of processing (heat and non-heat treatment) on the allergenic potential of proteins, and on the antigenic (IgG-binding) and allergenic (IgE-binding) properties of proteins has been considered. A variety of allergenic foods (peanuts, tree nuts, cows' milk, hens' eggs, soy, wheat and mustard) have been reviewed.

The overall conclusion drawn is that processing does not completely abolish the allergenic potential of allergens. Currently, only fermentation and hydrolysis may have potential to reduce allergenicity to such an extent that symptoms will not be elicited, while other methods might be promising but need more data. Literature on the effect of processing on allergenic potential and the ability to induce sensitisation is scarce. This is an important issue since processing may impact on the ability of proteins to cause the acquisition of allergic sensitisation, and the subject should be a focus of future research. Also, there remains a need to develop robust and integrated methods for the risk assessment of food allergenicity.

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1. Introduction

Food allergy describes the adverse health effects in which immunological mechanisms are involved (allergic reactions) that can be induced in sensitised subjects following dietary exposure to relevant allergens in food. Food allergy is an important health problem (Sampson, 2004), and estimates of its prevalence in Europe are commonly in the region of 0.1–3.2% for adults and 0.1–5.7% for children. However, the extent to which the prevalence of food allergy has increased in line with other forms of atopic disease is not clear (Nwaru et al., 2014).

Most cases of food allergy are associated with a limited range of products. Previously the most commonly allergenic foods were considered to be cows' milk, hens' eggs, peanuts, tree nuts, soy, wheat, shellfish and fish (the 'big 8') (Hefle et al., 1996; Young et al., 1994). More recently, in Europe, that list has been expanded in number to 14: cereals containing gluten, crustaceans, molluscs, eggs, fish, peanuts, tree nuts, soybeans, milk, celery, mustard, sesame, lupin and sulphur dioxide (Commission-Directive 2006/142/EC). It is apparent, however, that the extent to which allergy is associated with particular foods varies with time and geography, with changing dietary habits and preferences, the introduction of new

foods, the way in which food is prepared, and the age at which foodstuffs are first introduced into the diet (Hourihane, 1998; Lucas et al., 2004).

In common with other forms of allergic disease, food allergy develops in two phases. In the first phase susceptible subjects are immunologically primed to specific food proteins resulting in allergic sensitisation. Such sensitisation may be acquired following dietary exposure to food proteins, or possibly via other routes of exposure (including inhalation and skin contact). If sensitised subjects subsequently encounter sufficient levels of the inducing allergen(s) in the diet then an allergic reaction may be elicited. The symptoms of such reactions vary considerably and can range from mild, local and transient effects to systemic anaphylaxis that is potentially fatal (Perry and Pesek, 2013; Sicherer and Sampson, 2014; Sicherer and Wood, 2013).

By definition, allergy results from the elicitation of a specific immune response. The most common immunological mechanism implicated in the acquisition of sensitisation to food proteins is the elaboration of IgE antibodies. However, non-IgE-mediated cellular immune responses are also important in some forms of food allergy (Johnston et al., 2014; Kimber and Dearman, 2002; Sicherer and Sampson, 2014). The primary focus of this article is on IgE-mediated food allergy.

Although many uncertainties remain, there have been important advances in characterisation of some of the factors that influence the acquisition of sensitisation to food proteins and the development of food allergy. Among the important variables are the inherent allergenic potential of food proteins, the timing, duration, extent and route of exposure to food allergens, and heritable and acquired susceptibility factors (Kimber and Dearman, 2002; Sicherer and Sampson, 2014). Some intriguing questions that remain unanswered are the factors that maintain operational tolerance to foods and food proteins, and the events and immunological processes through which tolerance is broken and sensitisation is acquired.

There remains a need to understand in greater detail differences between proteins with respect to their inherent allergenic potential, and the properties that confer on proteins the ability to induce sensitisation. It is known, for instance, that protein function (including enzymatic activity), stability (including resistance to proteolytic digestion) and glycosylation patterns can affect both immunogenic and allergenic potential (Huby et al., 2000). However, in the case of food allergy there is an additional dimension that must be considered; the impact of food processing, and of the food matrix, on allergenic potential (Jiménez-Saiz et al., 2014; Lepski and Brockmeyer, 2013; Mills et al., 2009; Nowak-Wegrzyn and Fiocchi, 2009). The types of processing that have been implicated in influencing allergenic properties are: heating (thermal processing), fermentation including endogenous enzymatic hydrolysis, enzymatic and acid hydrolysis, physical treatments (such as high pressure processing or extrusion), the use of preservatives, changes in pH, or combinations of any two or more of these (EFSA, 2014, Mills and Mackie, 2008, Thomas et al., 2007).

In the context of this report it is important to appreciate that food processing can potentially impact on different aspects of food allergy, and it is necessary to distinguish clearly between these. A draft scientific opinion on the evaluation of allergenic foods and food ingredients for labelling purposes published recently by EFSA made the point as follows: '*Most studies available report on the IgE-binding capacity of processed foods rather than on their allergenicity, whereas systematic investigations on the effects of food processing on allergenicity are scarce*' (EFSA, 2014).

While it is clear that consideration of the influence of processing on not only the antigenic integrity/IgE-binding capacity of allergenic proteins, but also the ability to induce sensitisation is important, it must be acknowledged that addressing the latter is not without difficulty. Currently what is required is the use of well conducted and controlled animal studies in which the inherent allergenic potential of processed and unprocessed foods can be compared (Kroghsbo et al., 2014b).

In this report the impact of processing on antigenic and allergenic integrity of proteins (IgG and IgE antibody binding, respectively), and the ability of foods to elicit allergic reactions will be considered, together with potential effects on sensitisation where relevant data for the latter are available. For the purposes of this article we have chosen not to base our review solely on a consideration of what are normally considered to be the most common allergenic foods, although most are included. The foods evaluated were selected on the basis of the availability of relevant literature and the various forms of processing to which they are normally subjected. Those reviewed are: peanuts, tree nuts, cows' milk, hens' eggs, soy, wheat and mustard.

2. Influence of processing on the antigenic integrity and allergenicity of food proteins

For the purposes of this article it is necessary to clarify definitions and terminology. This is because food processing can potentially affect two aspects of the allergenic properties of proteins, as follows:

(a) In most investigations it is the impact of processing on the integrity of epitopes recognised by IgG antibodies or IgE antibodies that has been reported. Such changes are of potential importance because they will influence the ability of antibodies to bind to the modified protein, and in the case of IgE antibody binding this may result in an altered capacity to elicit an allergic reaction.

(b) Much less commonly the impact of processing on the ability of food proteins to induce allergic sensitisation has been investigated. Here, in the case of IgE-mediated food allergy, the question addressed is whether processing has impacted on the capacity of a protein to stimulate the production of IgE antibody.

To distinguish effectively between these two types of effects it is important to adopt for this article clear definitions that will avoid confusion. The definitions summarised below are not necessarily intended to be universally applicable, or to take the place of definitions that are commonly employed elsewhere. Rather, the intention is to adopt working definitions that will provide clarity in considering the influence of processing on the allergenic properties of food proteins. These are as follows:

2.1. General definitions

Food allergy: an adverse reaction to food that is mediated through immunological mechanisms. Such reactions can be provoked in sensitised subjects following dietary exposure to relevant allergens in food.

Allergic sensitisation: the process of specific immunological priming through which heightened sensitivity (sensitisation) to food proteins is acquired.

Allergenicity or allergenic potential: the potential of a material to cause sensitisation and allergic reactions, frequently associated with IgE antibody.

IgG or IgE antibody binding capacity: an altered ability of IgG antibody (also antigenic integrity) or IgE antibody (also allergenic integrity) to bind to epitopes, respectively.

Immunogenicity: the ability of a material to elicit an immune response.

3. Milk and milk allergens

3.1. Introduction to milk and milk allergens

Milk is an excellent source of fat, proteins, minerals and vitamins. On average, cow's milk consists of 90% water, 5% carbohydrates (48 g/L lactose, and minor to trace amounts of resp. glucose and galactose), and 4–5% proteins (40–50 g/L). The main cow's milk allergens are caseins, β-lactoglobulin, and α-lactalbumin (Bu et al., 2013). Less common antigens in cow's milk are serum albumin (BSA) and immunoglobulins. Most proteins in milk, including the allergens, are glycoproteins (Besler et al., 2002; O'Riordan et al., 2014). Caseins account for 80% and whey proteins for 20% of the total amount of proteins present in cow's milk. The group of caseins (MW 20–30 kDa) includes alpha-S1 (12–15 g/L), alpha-S2 (3–4 g/L), beta (9–11 g/L), kappa (6–8 g/L) and gamma (3–4 g/L) caseins. Major whey proteins comprise β-lactoglobulin (MW 18.3 kDa; 3–4 g/L), α-lactalbumin (MW 14.2 kDa; 1–1.15 g/L), immunoglobulins (MW ca. 160 kDa; 0.6–1 g/L), bovine serum albumin (MW 67 kDa; 0.1–0.4 g/L) and lactoferrin (MW 76–80 kDa; 0.09 g/L) (Besler et al., 2002).

In developed countries cow's milk is extensively processed before consumption, and rarely consumed in its raw form. Upon harvesting, milk is cooled to 4 °C, stored and transported in stainless steel tanks at 4 °C. Then the milk is centrifuged in order to separate the milk fat from the skimmed milk residue. The next step is standardising, which is the industrial addition of milk fat to the milk in a specified ratio. This ratio determines the type of milk produced: skimmed milk (1% or less fat), semi-skimmed milk (2%) or whole-milk (>3.25%) (Chandan and Kilara, 2011). Finally, the milk is heated. Most commonly used heat treatment conditions are: pasteurisation (heating milk to 70–80 °C for 15–20 seconds), sterilisation (110–120 °C for 10–20 minutes) and ultra-high temperature (UHT) processing (135–145 °C for 0.5–4 seconds) (Claeys et al., 2013, EFSA, 2014). UHT processing method dominates in Europe because during UHT processing both the pathogens and spores are destroyed but no/little Maillard reaction occurs; this minimises the impact on colour and taste of the milk (Tamime, 2009). After heating, the milk is immediately cooled down to a temperature below 4 °C and packaged.

To produce cow's milk-containing products, the milk is further processed (condensation, spray drying, filtration, hydrolysis and/ or fermentation). Liquid concentrates such as coffee milk and powders such as infant formulas, are two common delivery forms of milk solids. To obtain liquid concentrates and milk powders, the milk has to be condensed by vacuum evaporation. This process ensures that most of the liquid evaporates and a four-fold concentrated liquid remains. To produce milk powders, the concentrate is spray dried with a controlled flow of hot air (200 °C for 20–60 seconds to minutes) (Chandan and Kilara, 2011; Kasinos et al., 2014; Schuck, 2013; Schuck et al., 2013).

3.2. Effect of processing on allergenicity

Human studies that analyse the effect of processing on milk and its allergens are very scarce. The experimental design in almost all studies involving humans is not targeted at analysing processing effects, which would require, e.g. comparing a challenge with raw milk vs. challenge with milk that has been treated in a strictly defined way, but rather serves a clinical setting, to test, e.g. diagnostic procedures, establish eliciting doses, or to test a therapeutic approach. Most data presented here refer to *in vitro* test results.

3.2.1. Effect of homogenisation

Homogenisation of milk is the process in which fat globules are pressed at high pressure through small holes. During this process the fat globules are broken down into smaller fat globules with a greater surface area. This increase in surface area causes proteins to adsorb to it, forming fat globules loaded with protein.

Little *in vivo* research is done on the effect of homogenisation on the allergenicity of milk proteins. Poulsen et al. (1987) showed, in a murine model, that homogenised milk and pasteurised milk triggered anaphylactic reaction at a dose of 340 µg, whereas this was not the case for raw milk. In addition, they found that the potential of homogenised milk to trigger anaphylactic reactions increased with increased fat content. On the other hand, Pelto et al. (2000), performing a randomised, double-blind, cross-over study on humans, found no significant differences in allergenicity between the groups treated with and without homogenised milk. This finding was supported by DBPCFC performed by Host and Samuelsson (1988), stating that homogenisation had no effect on the allergenicity of milk.

3.2.2. Effect of thermal processing

The degree of structural changes of proteins occurring during heating depends on both the type of protein and the thermal load. Whey proteins denature progressively upon heat treatment (Chandan and Kilara, 2011). Caseins are heat stable because they do not have secondary, tertiary and quaternary structures that can be disrupted by heating, implicating that heating of milk can only partly reduce its allergenicity (Bu et al., 2013; Michalski and Januel, 2006). The effects of the different heating methods on the allergenicity of the whey proteins are analysed in the following sub-sections.

Bu et al. (2009b) found that IgE-binding by α-lactalbumin and β-lactoglobulin increased significantly after pasteurisation at temperatures between 50 and 90 °C, compared to non-heated milk, by means of indirect competitive enzyme-linked immunosorbent assays (ELISA). These findings are backed up by epidemiological studies showing a lower prevalence of milk allergy upon ingestion of raw milk compared to consumption of commercially available milk products (Loss et al., 2011; Waser et al., 2007). However, there were no published controlled human intervention studies examining the allergenicity of pasteurised milk compared to non-heated (raw) milk (Van Neerven et al., 2012).

Sterilisation causes denaturation of 75% of the whey proteins and Maillard reaction to occur (Chandan and Kilara, 2011; Porter, 1978). Ehn et al. (2004) and Taheri-Kafrani et al. (2009) showed, by means of indirect competitive ELISA, that denaturation and aggregation of β-lactoglobulin caused by heating of whole milk and β-lactoglobulin solutions at 90–95 °C decreased the IgE-binding capacity of this whey protein slightly but significantly. Their research indicated that heating of milk was insufficient to eliminate/destroy all epitopes but that the affinity of the remaining epitopes might be lower or that epitopes may have been destroyed after heating. Bu et al. (2009b), using an indirect ELISA with rabbit serum, found a decrease in IgG-binding by both α-lactalbumin and β-lactoglobulin after sterilisation compared to pasteurisation. IgG-binding by β-lactoglobulin that has been heated at 120 °C was still higher than the IgG-binding by unheated β-lactoglobulin. IgG-binding by α-lactalbumin heated to 120 °C is lower than the IgG-binding by the pasteurised α-lactalbumin and the non-heated α-lactalbumin samples. Heating may expose IgE-binding epitopes that previously were hidden inside α-lactalbumin (Maynard et al., 1999).

In addition, Bu et al. (2009a) investigated the effect of the Maillard reaction on IgG-binding by α-lactalbumin. They found that conjugation of α-lactalbumin with reducing sugars decreased the IgG-binding capacity of this whey protein. Complementary, Taheri-Kafrani et al. (2009) found that conjugation of β-lactoglobulin with lactose (reducing sugar) led to reduced IgE recognition.

There were no reports found on the effect of UHT processing on the allergenicity of milk. All studies examined the effect of thermal treatment on the allergenicity of milk up to 120 °C. This is problematic as UHT processing is the most common thermal processing

method used within Europe (Tamime, 2009). No conclusion can be drawn from the thermal effects of pasteurisation and sterilisation on the allergenicity of UHT processed milk, because the thermal load applied during UHT processing differs from pasteurisation and sterilisation.

Also the effect of vacuum evaporation on the allergenicity of cow's milk is not reported in the literature. Vacuum evaporation comprises heating of the milk to its boiling point under vacuum (80 °C for 15 minutes) (Schuck et al., 2013). Vacuum exerts no influence on the structural properties of proteins. Therefore, temperature is the parameter that is the most relevant for protein structure during vacuum evaporation. The effect of temperature increases until 80 °C and is discussed under the section pasteurisation. However, one should keep in mind that pasteurisation takes only seconds whereas vacuum evaporation takes 15 minutes and that, before vacuum evaporation, milk is pasteurised, sterilised or UHT processed. Therefore, vacuum evaporation should be further examined as it may cause some additional denaturation, which thereby may affect the allergenicity of milk.

There is no literature that directly examines the effect of spray drying on the allergenicity of cow's milk. During spray drying, the milk is placed in a dry air stream that causes both a temperature and a water gradient, leading to evaporation of the water from the milk to the air stream and heat transfer from the air stream to the milk particles. Maillard reactions may occur during spray drying, possibly affecting the allergenicity of milk (Schuck et al., 2013). It should be kept in mind that prior to spray drying, milk is pasteurised, sterilised or UHT processed and possibly subjected to vacuum evaporation.

3.2.3. Effect of fermentation and hydrolysis

Fermentation of milk and milk allergens (such as α-lactalbumin, β-lactoglobulin, α-casein and β-casein), either via simulated gastric fluid or fermentation with *Lactobacilli*, seems to strongly reduce allergenicity, as shown in IgG-binding or IgE-binding assays (Yao et al., 2014). Currently the only available preparations that are considered hypoallergenic are extensively hydrolysed milk formulas, which are primarily prepared by enzymatic hydrolysis, and elemental formulas, which are prepared from synthesised free amino acids (see e.g. (Bahna, 2008; Baker et al., 2000; Niggemann et al., 2008; Oldæus et al., 1991)).

For allergy to cheese, few and conflicting reports were found. Alessandri et al. (2012) reported that 45 out of 66 cows' milk-allergic patients could tolerate 3 y old Parmigiano-Reggiano (PR) cheese, although all PR-preparations still inhibited IgE-binding to milk proteins. Stoger and Wulhrich (1993) described cheese-allergic patients, but not the cheese, apart from that is was dried. Williams et al. (2007) report contact urticaria in people who handle Parmesan cheese. In this case, a reaction to enzymes used for cheese production could not be excluded.

No reports on allergenicity of yogurt were found.

3.3. Concluding comments

- Pasteurisation increases allergenicity of milk as measured by IgE binding studies, possibly due to aggregation, enhanced binding to and activation of mast cells; however, no human studies have been performed to confirm this.
- The decrease in IgE-, resp. IgG-binding capacity caused by sterilisation can be explained by denaturation and Maillard reaction of existing epitopes of both β-lactoglobulin and α-lactalbumin.
- Denaturation and non-enzymatic glycation lead to destruction of already existing epitopes or renders them inaccessible.
- No effects of UHT processing, vacuum condensing and spray drying on allergenicity of milk were found in the literature.

4. Egg and egg allergens

4.1. Introduction to egg and egg allergens

Egg is one of the foods whose allergenicity is most altered by cooking or processing. Egg white contains proteins with considerably higher allergenic potential than the egg yolk. The four major allergens in egg white are ovalbumin (OVA; Gal d 2, 54% of the total protein content), ovotransferrin (OVT; conalbumin, Gal d 3, 12%), ovomucoid (OVO; Gal d 1, 11%), and lysozyme (LYS; Gal d 4, 3.5%). Two yolk proteins, α-livetin (chicken serum albumin, Gal d 5) and lipoprotein YGP42 (Gal d 6), have been identified as egg allergens (Martos et al., 2013; Mine and Rupa, 2004).

Ovalbumin possesses 4 sulphydryl groups with a single disulphide bridge (Jacobsen et al., 2008) and is found to be sensitive to heat denaturation. Ovomucoid consists of 3 sub-domains, each being internally linked by disulphide bonds, which makes ovomucoid resistant to heat denaturation and proteolytic digestion (Julià et al., 2007; Maeno et al., 2013). Domain 3 is very stable, has most dominant IgE and IgG-binding epitopes, and is considered the major determinant of the strong allergenicity of the protein (Jiménez-Saiz et al., 2011b; Maeno et al., 2013). Gal d 5 is partially heat-labile and can cause both respiratory and food allergy symptoms in patients with bird-egg syndrome (Quirce et al., 2001), while Gal d 6 is a heat-stable allergen (Amo et al., 2010).

Eggs are universally used as nutrient and food additive. Common products in which egg is extensively heated are baked products and starches containing egg such as cakes, waffles, muffins, pancakes, egg noodles, egg pasta and bread. Examples of products in which egg is less extensively heated are custard, French toast, fresh mayonnaise, quiche and Caesar salad dressing. Furthermore, specific ways to use egg are seen for example in China where tea-boiled and spiced eggs are commonly consumed as Chinese savoury snacks (Liu et al., 2013).

4.2. Effect of processing on allergenicity

4.2.1. Effect of thermal processing

Several human studies have been performed in which egg allergic patients were challenged with heated and/or unheated eggs. In general, 50–85% of children with egg allergy are able to tolerate baked egg (Bartnikas and Phipatanakul, 2013; Cortot et al., 2012; Lemon-Mulé et al., 2008; Turner et al., 2013). This percentage varies depending on the characteristics of the allergic patients (age, severity of the allergy, etc.), heating procedure, matrix used, etc. A major drawback of these studies is that the individuals are often not challenged to unheated egg to establish clinical reactivity to egg prior to the oral food challenge (OFC) to the heated egg. This can result in an overestimation of the percentage of allergic subjects tolerating extensively heated egg products.

Urisu et al. performed a food challenge with 38 subjects with high levels of IgE antibodies for egg white to compare the allergenicity of heated egg white, freeze-dried egg white, and heated egg white depleted of OVO. Twenty-one subjects (55%) with a positive challenge to freeze-dried egg white had a negative challenge to heated egg white. Sixteen out of 17 (94%) with a positive response to heated egg white did not respond to the heated and OVO-depleted egg white (Urisu et al., 1997). This might indicate that OVO is the major determinant of causing egg induced allergies and that heating partially reduces the allergenicity of egg white. Another study compared the allergenicity of dehydrated egg white (DEW), a product that undergoes a double heat treatment (heating to 59 °C for 6 minutes and spray drying with hot air at 80 °C for 1 minute) with raw egg white (REW). Ten out of 40 egg-allergic patients (25%) had a positive OFC to both DEW and REW. The other 30 patients had a negative OFC to both forms.

The allergenicity of commercially available DEW was therefore shown to be equivalent to raw egg whites and the processing of DEW did not affect the allergenicity of the egg proteins (Escudero et al., 2013). Overall, from these studies, it can be concluded that the majority (50–85%) of children with egg allergy could tolerate extensively heated egg. However, milder forms of treatment (heating <80 °C) might still retain, to a great extent, the allergenic properties of the egg proteins.

To look more into the protein allergenicity mechanisms, mouse models were used to study unheated and heated purified egg white allergens. Mice sensitised and challenged with heated OVA (70 °C for 10 minutes) showed decreased clinical symptoms and a shift towards a Th1 response compared to mice sensitised and challenged with unheated OVA (Golias et al., 2012). In addition, C3H/HeJ mice orally sensitised with native OVA and OVO were challenged with native and heated (30 minutes in boiling water) OVA or OVO. The native forms did induce symptoms of anaphylaxis; this in contrast to the unheated counterparts (Martos et al., 2011). This decrease in allergenicity of heated OVA was shown to be partially the result of an enhanced gastrointestinal digestibility after heating (Jiménez-Saiz et al., 2011a; Martos et al., 2011) and a reduced intestinal absorption of OVA and OVO molecules that are capable of triggering basophils and T cells (Martos et al., 2011).

In vitro studies assessing IgE-binding capacity showed that heating of OVA clearly decreased the IgE-binding capacity compared to the unheated OVA (Ma et al., 2013; Shin et al., 2013). Heat treatment of OVO (95 °C, 15 min) lowered the IgE-binding activity of OVO (Jiménez-Saiz et al., 2011a; Mine and Zhang, 2002). However, glycation by Maillard reaction increased the IgE-binding (Jiménez-Saiz et al., 2011a). Ovotransferrin and lysozyme are less well studied but generally regarded as heat-labile proteins. One study showed a decrease in IgE-binding after heating for 15 minutes at 95 °C (Mine and Zhang, 2002).

4.2.2. Effect of non-thermal processing

Limited studies looked at combined processing methods. One study reported on the influence of combining various heat treatments with enzymatic hydrolyses on the structure and allergenicity of pasteurised liquid whole egg. The remaining IgE-binding capacity of the end product, which underwent three heating and two enzymatic treatments, was more than 100-fold reduced compared to untreated liquid whole egg (Hildebrandt et al., 2008).

UV-C exposure (1.6 to 29.1 W m^{-2}) of an egg white protein solution reduced the IgG-binding capacity of egg white proteins (ELISA), which was attributed to denaturation (Manzocco and Nicoli, 2012), while no difference in IgE-binding capacity (ELISA) was observed between egg white exposed to UV-C light (10.6 and 63.7 kJ m^{-2}) and untreated egg white (Manzocco et al., 2012).

Gamma and electron beam radiation decreased both IgE and IgG-binding capacity of OVO (Lee et al., 2007b).

Intense pulsed light (from 1.75 to 31.5 J cm^{-2}) increased IgG-binding capacity of egg white proteins in one study (Manzocco et al., 2013), while another study using diluted isolated egg proteins showed the opposite effect (Anugu et al., 2010).

4.3. Concluding comments

- Extensive heating diminishes the allergenicity of egg white proteins and the majority (50–85%) of egg allergic patients are tolerant to heated egg products.
- Other treatment methods, such as irradiation, might modulate the allergenic properties of eggs; however, more investigation is needed.

5. Tree nuts and tree nut allergens

5.1. Introduction to tree nut allergens

Tree nuts are beneficial food sources. They are rich in unsaturated fats and have comparatively high levels of phenolics, phytosterols, tocopherols, minerals and fibre. This may explain why the consumption of tree nuts is increasing, including the consumption of both raw and processed nuts. A variety of dietary tree nuts are associated with commonly severe food allergy, and sometimes fatal reactions are observed (Bock et al., 2007). Among those that are well documented food allergens are: pistachio (Pis v), hazelnut (Cor a), pecan (Car i), walnut (Jug r), Brazil nut (Ber e), cashew nut (Ana o) and almond (Pru du).

In general terms tree nut allergy can adopt two forms: allergic sensitisation to tree nut proteins or immunologic cross-reactivity between the structurally-related Bet v 1 (a major birch pollen allergen) and members of the pathogenesis-related protein 10 (PR-10) family expressed in tree nuts, e.g. Cor a 1 and Pru du 1 (Geroldinger-Simic et al., 2011). Other birch pollen-related allergens in tree nuts are profilins (e.g. Cor a 2, Pru du 4 and Jug r 5); these are expressed widely in fruits and pollens (Hirschwehr et al., 1992; Masthoff et al., 2013). The symptoms associated with cross-reactive allergy to tree nuts are generally relatively mild (Hirschwehr et al., 1992). The tree nut allergens that are not associated with birch pollen sensitisation (direct allergens) are lipid transfer proteins (Cor a 8, Pru du 3 and Jug r 3) and seed storage proteins, such as vicilins or 7S globulins (Cor a 11, Jug r 2, Ana 01, Pis v 3), 11S globulins (Cor a 9, Ber e 2, Jug r 4, Pru du 6, Ana o 2, Car i 4,Pis v 2) and 2S albumins (Jug r 1, Ber e 1, Pru du 2S, Ana o 3, Car i 1, Pis v 1) (Masthoff et al., 2013). Here reactions are often more severe (Flinterman et al., 2008).

5.2. Effect of processing on allergenicity

Commercially available tree nuts are mostly heat processed (dry and moist) or processed into butters. There are a variety of published reports that have described the influence of heat processing on the allergenic and antigenic integrity of tree nut extracts. However, these reports are mainly focusing on *in vitro* assays.

5.2.1. Effect of thermal processing

Solely two studies reported on the influence of roasting on the allergenicity of **hazelnut** in a DBPCFC. In both studies a decreased allergenicity was observed after roasting. In the study of Hansen et al. (2003), 17 patients with a proven birch pollen and hazelnut allergy experienced oral symptoms and three of them reported additional symptoms (e.g. asthma and rhinitis) after eating raw hazelnut. However, when eating roasted hazelnut, only five patients (29%) experienced oral symptoms and one of them also experienced rhinoconjunctivitis. Moreover, eliciting doses were elevated after roasting and >50% of the patients lost skin reactivity in prick-to-prick skin prick test (SPT) and IgE-binding with roasted hazelnut compared to raw hazelnut (Hansen et al., 2003). The DBPCFC with 20 birch pollen and hazelnut allergic patients performed by Worm et al. (2009) confirmed these results. Eliciting doses were also elevated compared to raw hazelnut in the majority of patients and the reactivity in the SPT and basophil activation test (BAT) was reduced (Worm et al., 2009). A thorough component resolved evaluation of the patients against other hazelnut allergens was not conducted. For other tree nuts, no DBPCFC with processed forms were found in the open access databases.

The decrease in allergenicity of hazelnut is probably caused by the decreased IgE-binding of the birch pollen related allergens, Cor a 1 and Cor a 2. Some papers showed this effect after roasting at temperatures >140 °C in EAST and/or BAT (Müller et al., 2000; Pastorello et al., 2002; Schocker et al., 2000; Wigotzki et al., 2000)

using sera from birch pollen and hazelnut allergic patients. At temperatures below 100 °C, Cor a 1 was heat-resistant. Furthermore, Wigotzki et al. reported that the IgE-binding of hazelnut in the EAST inhibition assay and immunoblot was decreased for different commercial products (e.g. hazelnut chocolates, nougat products, hazelnut cake, hazelnut cookies and hazelnut croquants) compared to unprocessed (native) hazelnut extracts (Wigotzki et al., 2001). The effect of heat processing on birch pollen related allergens (PR-10 and profilin) in other tree nuts was not described in the literature except for almond. Venkatachalam et al. (2002) and Bargman et al. (1992) both showed, by using immunoblotting with sera from almond allergic patients, that blanching and roasting reduces the IgE-binding of a 15–17 kD band, which might be Pru du 1 (Bargman et al., 1992; Venkatachalam et al., 2002). However, de Leon et al. (2003) found no difference in IgE-binding (ELISA) between roasted (180 °C) and unroasted almond using serum from one almond allergic patient (de Leon et al., 2003). The difference might be due to the small amount of sera tested in both studies.

Allergens belonging to the lipid transfer proteins and seed storage protein family are more stable to heat treatment. This is demonstrated in a few *in vitro* studies where serum from patients with a hazelnut allergy without birch pollinosis was used. No significant effect was seen in the EAST after roasting hazelnut for 40 min at 140 °C (Pastorello et al., 2002; Schocker et al., 2000). Other studies demonstrated the heat stability of Cor a 8, Cor a 9, Cor a 11 and Cor a 14, after heating hazelnut with temperatures above 140–185 °C using ELISA, immunoblot or EAST (de Leon et al., 2003; Dooper et al., 2008; Müller et al., 2000; Pastorello et al., 2002; Wigotzki et al., 2000). Autoclaving (138 °C, 15–30 min) of hazelnut showed a decreased IgE-binding on Western blot but this was most likely due to decreased solubility (Lopez et al., 2012).

Glycation between reducing sugars and free amino groups on proteins, also called Maillard reaction (37, 60 and 145 °C), decreased IgG/IgE-binding of Cor a 11 on immuno blot and on ELISA, while RBL activity was increased (Iwan et al., 2011). In addition, Cor a 9 was unaffected and appeared stable, while Cor a 1 showed only some decrease after glycation with glucose and heating for 48 hr at 70 °C (Cucu et al., 2011). In a BAT using blood from six hazelnut allergic patients, Cucu et al. (2012) showed that glycation of hazelnut (same conditions as above) enhanced (2/6) or decreased (3/6) the IgE functionality of hazelnut (Cucu et al., 2012). These results showed that the effect of glycation on allergenicity is not unequivocal.

In **almond** the effect on the allergenicity of 11S globulin (Pru du 6), which shows different protein bands between 37 and 66 kD on a Western blot, was investigated. Blanching and roasting did not have any effect on IgE-binding of 11S globulin bands, except for two bands between 55 and 65 kD. Other *in vitro* studies confirmed the heat stability of Pru du 6 (Bargman et al., 1992; de Leon et al., 2003; Roux et al., 2001; Venkatachalam et al., 2002).

Roasting (180 °C for 15 min) of **cashew nuts** did not result in loss of IgE-binding using sera from one allergic and one sensitised patient (de Leon et al., 2003). Immunoreactivity studies showed a minor effect on Ana o 1 and Ana o 3 after roasting and no effect on Ana o 2 after roasting or frying (191 °C for 1 min). However, extreme roasting conditions (160 °C for 30 min or 200 °C for 15 min) resulted in a decrease in Ana o 2 IgG-binding capacity. Conflicting data were found for microwaving and autoclaving. The minor effect of blanching was subscribed to leaking of the proteins in the water (Su et al., 2004; Venkatachalam et al., 2008).

For **Brazil nut**, only one paper showed that roasting at 180 °C for 15 min did not have any effect on the IgE-binding of Brazil nut with serum from two patients (one allergic and one sensitised) (de Leon et al., 2003). Sharma et al. (2009) also showed that different heating methods had minor effect on the IgG-binding capacity. However, the results were not consistent with the different methods (ELISA, dot blot and Western blot). No effect or only a slight decrease

was seen after blanching, roasting, autoclaving and frying, while for microwaving, the IgG-binding capacity was elevated (Sharma et al., 2009).

There are two papers on the effect of heat processing on IgE and IgG binding capacity of **walnut** proteins. One paper reported that heating (blanching, roasting, microwaving and frying at 191 °C) had no effect on the IgG-binding capacity of Jug r 2 and Jug r 4. However, autoclaving did reveal a decrease in recognition of 42–45 kD proteins (Jug r 2) and 45–66 kD (Jug r 4) bands, which was not shown after blanching and roasting (Su et al., 2004). Cabanillas et al. (2014) recently showed on an immunoblot that after autoclaving at 138 °C and 256 kPa, IgE binding capacity for walnut proteins and Jug r 4 recognition was diminished. Hardly any effect on IgE binding capacity was seen with high hydrostatic pressure treatment (300–600 MPa, 15 min, 15 °C). The results were confirmed by rat basophil leukaemia cell line and by skin prick testing.

The effect of heat processing on the allergenicity of **pistachio nut** was assessed in only one study. A small reduced effect on IgE-binding in an immunoblot and ELISA inhibition with two human serum pools was seen for dry roasting. The IgE-binding of steam-roasting was more reduced; however, this was most probably caused by protein aggregation (Noorbakhsh et al., 2010).

Minor effects of heating (blanching, 10 min, roasting 148 °C for 30 min or 172 °C for 12 min or autoclaving for 5 min) of **pecan nut** were detected by Western blot using pooled patient sera. Most protein bands of Car i 1 and Car i 4 seemed very heat stable. However, some subunits of Car i 4 almost disappeared; this was most likely due to irreversible loss of protein solubility rather than protein epitope destruction. These results were confirmed by immunoreactivity studies. However, a significant decrease of IgG-binding capacity was seen after applying extreme conditions, i.e. roasting at 160 °C, 20 and 30 min and autoclaving (Venkatachalam et al., 2006) or microwaving for 15 min (Polenta et al., 2012). Again this decrease could be due to the loss of protein solubility.

5.2.2. Effect of non-thermal processing

Processing of raw almond into almond butter (no extreme heat required) did not influence IgE-binding capacity. Bargman et al. (1992) showed similar IgE-binding patterns of almond butter compared to raw almond on an immunoblot with sera obtained from eight almond allergic patients (Bargman et al., 1992).

5.3. Concluding comments

- Heat processing has mainly an effect on the allergenicity of allergens related to Bet v 1 (PR-10 and profilins) by lowering the IgE-binding.
- Hardly any effects on allergenicity of allergens from the seed storage protein and lipid transfer protein family were observed.
- Solely two DPBCFC were performed with raw and roasted hazelnut. In other studies *in vitro* techniques were used with a limited amount of sera from allergic patients. In none of the studies, the allergenicity was completely abolished.

6. Peanut and peanut allergens

6.1. Introduction of peanut allergy

Peanut is one of the common causes of food allergy in Western countries. In North America and Europe, around 1% of the population has diagnosed peanut allergy (Ben-Shoshan et al., 2010; Sicherer et al., 2010). One birth cohort study found that approximately 12% of children at age 8 in the United Kingdom were sensitised to peanut (Nicolaou et al., 2010). The prevalence of peanut allergy is relatively low in the Asian population born and raised in Asian countries (0.47%) as compared with the Western-born Asian population (1.62%)

of similar genetic decent (Shek et al., 2010). In Western countries, peanuts are primarily consumed in a roasted form. In contrast, peanuts are often consumed after boiling in Asia. This difference in peanut processing in different regions may have an impact on the differences in the prevalence of peanut allergy.

As of 2014, 13 peanut proteins (Ara h 1 to Ara h 13) have been officially confirmed and characterised as allergenic molecules and registered by the International Union of Immunological Societies Allergen Nomenclature Sub-Committee (www.allergen.org). Despite the identification and characterisation of various peanut allergens, little is known about how food processing may affect allergic sensitisation and subsequent elicitation of adverse reactions to peanut proteins. To date, most studies have evaluated the influence of processing as a function of IgE reactivity. Only a limited number of *in vivo* studies have been conducted to evaluate whether food processing is associated with a reduced potential to induce sensitisation and/or elicitation (Kroghsbo et al., 2014b). Two human trials were reported; one was a randomised double blind cross-over challenge with peanut oil, the other was an OFC with boiled peanut (Hourihane et al., 1997; Turner et al., 2014).

6.2. Effect of processing on allergenicity

6.2.1. Effect of thermal processing

Several studies have demonstrated that boiling reduces the IgE-binding capacity of peanut proteins *in vitro*. Beyer et al. (2001) boiled peanuts for 20 minutes in water (100 °C) and IgE binding was evaluated by immunoblotting with sera from 8 peanut allergic patients. Boiling decreased the IgE-binding capacity of all tested allergens (Ara h 1, 2 and 3) compared with roasted peanut. Mondoulet et al. (2005) obtained whole peanut protein extracts and purified Ara h 1, Ara h 2 from boiled peanut (100 °C, 30 min) and evaluated them by enzyme allergosorbent test (EAST) with sera from 37 peanut allergic patients. They concluded that IgE-binding capacity to boiled peanut extracts was significantly lower than that of raw peanut extracts. They also reported that low-molecular-weight (LMW) proteins were transferred from peanut seeds into cooking water. Similar results were reported by Turner et al. (2014), who demonstrated that oral food challenge with boiled peanut to four peanut allergy patients was able to induce desensitisation of peanut allergy. They found that boiling for 6 hours in a closed vessel resulted in loss of proteins, particularly Ara h 2, Ara h 6 and Ara h 7 from peanut seeds and these LMW proteins could be found in cooking water. A decrease of allergenicity by boiling was also demonstrated by mediator release assays (MRA) (Blanc et al., 2011; Vissers et al., 2011a). The boiling treatment (100 °C, 15 min) of purified Ara h 1 impaired the IgE-binding capacity of Ara h 1 by MRA with utilising rat basophilic leukaemia cells and boiling (110 °C, 15 min) of purified Ara h 2/6 from raw peanut induced denaturation and caused a significant decrease of mediator releasing functionality of these proteins. Another study evaluated the influence on mediator release by SPT with the whole peanut protein extract of these processed peanuts. Although boiled peanut extract (100 °C, 60 min) had a similar protein profile to raw peanut extract on SDS-PAGE, only one patient out of seven elicited positive reactions (Cabanillas et al., 2012a).

Several studies reported that roasting of whole peanuts enhanced IgE-binding capacity of proteins (Beyer et al., 2001; Maleki et al., 2000; Mondoulet et al., 2005). Maleki et al. (2000) reported that roasting of purified Ara h 1 and Ara h 2 enhanced the IgE-binding capacity up to 90-fold. The high temperature during the roasting process increased advanced glycation end (AGE) adducts, which could explain this enhanced binding (Chung and Champagne, 2001). They concluded that the level of AGE adducts correlated with the level of IgE binding.

Recent studies have compared the results obtained by IgE reactivity with the results obtained by cell-based assay, such as MRA

and the BAT (Maleki et al., 2014; Sabato et al., 2011; Vissers et al., 2011b). These studies found that the results of IgE reactivity could give only limited power of prediction to the allergenicity of processed peanut proteins. Vissers et al. (2011b) demonstrated that roasting of purified Ara h 1 and 2S albumin (Ara h 2/6) reduced the degranulation capacity of Ara h 2/6, but significantly enhanced the degranulation capacity of Ara h 1 with MRA. Maleki et al. (2014) compared the ability of T-cell stimulation of Ara h 1 and Ara h 2 that was purified from roasted peanuts with those proteins purified from raw peanuts. Interestingly Ara h 1 purified from roasted peanut had a higher IgE reactivity than raw Ara h 1, but T-cell proliferation tested with five patients sera was reduced. Conversely, Ara h 2 had higher IgE reactivity and T-cell stimulation property than raw Ara h 2. It indicates that Ara h 2 is more immunogenic than Ara h 1. Sabato et al. (2011) pointed out the influence of air and oil roasting on the IgE-binding capacity and functionality of peanut, tested in immunoblotting and BAT with sera from ten individual peanut allergic patients. Although different responses (increased, reduced, abolished and unaltered) were observed in BAT towards air-roasted peanut, these patients' sera showed similar patterns, enhancing IgE-binding at the Ara h 2 doublet, in immunoblotting. So far, two studies have assessed sensitisation of roasted peanut extract animal models. Kroghsbo et al. (2014b) observed that an extract from roasted peanut increased the elicitation capacity of peanut allergens compared to an extract from blanched peanuts, although roasted peanut did not significantly impact on sensitisation potential in Brown Norway rats. Moghaddam et al. (2014) observed that dry roasting enhanced peanut-induced sensitisation across mucosal and cutaneous routes in mice. They concluded this enhanced sensitisation of dry roasted peanut is probably mediated by oxidation-driven generation of AGE-related adducts under the high-temperature of dry roasting.

Only one paper evaluated the influence of autoclaving on the allergenicity of roasted peanut. Cabanillas et al. (2012a) reported that intensive autoclaving on roasted peanut (2.56 atm, 30 min) decreased IgE-binding capacity and also reduced mediator release by SPT. All seven patients tested failed to elicit reactions to autoclaved roasted peanut extract, whereas extracts from peanuts that had only been roasted caused SPT reactions in 6 out of 7 subjects. It was also reported that autoclaving caused the unfolding of some basic protein structures (α-helix and β-strand) and increased the formation of random coils. These structural changes increased digestibility of a number of peanut proteins. However, it has been documented that the peanut allergens, Ara h 2 and Ara h 6, were extremely resistant to proteolytic digestion, partially due to the conserved pattern of 8 cysteine residues that form 4 disulphide bonds, which aid in stabilising the structure of these allergens.

Only limited data were available on the effects of frying on the allergenicity of peanut. Beyer et al. (2001) demonstrated that frying reduced the IgE-binding capacity of Ara h 1, Ara h 2 and Ara h 3. However, Cabanillas et al. (2012a) showed that the frying process did not reduce IgE-binding capacity and mediator release by SPT.

6.2.2. Effect of hydrolysis

Several studies demonstrated that hydrolysis reduced IgE-binding capacity of some peanut proteins. Two studies (Chung et al., 2004; Yu et al., 2011) demonstrated that peroxidase or digestive enzymes (α-chymotrypsin and trypsin) could hydrolyse and reduce Ara h 1 and Ara h 2 in roasted peanut, but not in raw peanut. Yu et al. (2011) also reported that blanching (5 min in boiling water) before hydrolysis enhanced the effectiveness of enzyme treatment in roasted peanut, but not in raw peanut. Cabanillas et al. (2012b) demonstrated how the choice of enzymes affected the IgE-binding capacity by comparing endoprotease and exoprotease. They concluded that endoprotease more effectively decreased IgE-binding capacity in the soluble fraction of roasted peanut compared

to hydrolysis with exoprotease. Pre-treatment before hydrolysis and the choice of proper enzyme(s) highly influences the degree of reduction in IgE-binding capacity. Interestingly, Shi et al. (2013) reported that although enzymatic hydrolysis could significantly reduce IgE-binding capacity in ELISA, IgE cross-linking capacity was still retained in the BAT. This indicates that reduction of IgE-binding capacity by hydrolysis does not give a clear prediction for the reduction of allergenicity.

6.3. Concluding comments

- Boiling of peanuts could reduce allergenicity in two ways: denaturation of allergenic proteins and transferring LMW proteins into cooking water. The decrease of allergenic potential might vary depending on the intensity of boiling.
- Roasting of peanuts seems to aggravate the elicitation property of peanut proteins by generation of AGEs. Limited data are available on whether roasting also aggravates sensitisation property.

7. Soybean and soybean allergens

7.1. Introduction to soybean

A recent EFSA supporting publication reviews the prevalence of soy allergy according to clinical history and challenge data; the prevalence ranges between 0% and 2.7% (University of Portsmouth, 2013).

Soybean seeds contain approximately 37% of protein, of which eight allergenic proteins (Gly m 1 to Gly m 8) have so far been registered by the International Union of Immunological Societies Allergen Nomenclature Sub-Committee (www.allergen.org). The major storage proteins β-conglycinin (Gly m 5) and glycinin (Gly m 6) represent 70% of the whole soybean protein and have been related to severe allergic reactions in European soy allergic subjects (Holzhauser et al., 2009). Recently, Gly m 8, a 2S-albumin, was reported with high diagnostic value in soy allergic children in Japan (Ebisawa et al., 2013). The allergens Gly m 5 through Gly m 8 have been associated with primary sensitisation to the allergenic food. In addition, soybean allergy can result from association to birch pollinosis. Clinical cross-reactivity between the major birch pollen allergen Bet v 1 and the homologous soybean allergen Gly m 4 has been described and sometimes associated with anaphylaxis (Kleine-Tebbe et al., 2002). The soybean hull allergens Gly m 1 and Gly m 2 have been identified as aeroallergens in isolated asthma outbreaks (Gonzalez et al., 1991) and are not considered as food allergens (Ladics et al., 2014). Various additional IgE-binding soy proteins, thus potential soy allergens, have been described. Of these, especially Gly m Bd30k (also named P34), a thiol-protease, might be a major allergen that could affect more than 50% of soy allergic subjects (Ogawa et al., 2000). However, for some of these IgE-binding proteins, the clinical relevance is unclear, since the underlying studies were based merely on soy sensitised subjects with unclear clinical reactivity.

Soybeans undergo various processing steps (see Fig. 1) to obtain many different soy products such as soy flour, texturised soy protein (TSP), soy protein concentrates (SPC), soy protein isolates (SPI), protein hydrolysates, as well as fermented products in which the soy proteins and their structures may undergo various modifications. Allergenicity assessment of soy products has been primarily done by using antibody-based *in vitro* techniques and hardly by means of the more predictive tests such as the DBPCFC (University of Portsmouth, 2013).

Comprehensive allergenicity assessment was done with only a few soy products, as for example soybean oil and lecithin. By means of immunoblotting and EAST inhibition experiments, the level of protein found in soy oil was low in comparison to that in soy lecithin.

Extracts from soy lecithin and non-refined oil still contained IgE-binding proteins, while refined soybean oil did not (Paschke et al., 2001). No allergic reactions were observed with commercially available soybean oils in a double blind crossover study with seven subjects having a history of soy related systemic allergic reactions (Bush et al., 1985). The panel on dietetic products, nutrition and allergies (NDA) of the European Food Safety Authority (EFSA) considers that it is not very likely that fully refined soybean oil and fat will trigger a severe allergic reaction in susceptible individuals (EFSA, 2007). Using sandwich ELISA with plasma from soy allergic patients, soy protein isolate (SPI) and concentrate (SPC) showed less IgE-binding capacity than soy flour. The IgE-binding capacity of tofu was about 20-fold higher than that of soymilk using sandwich ELISA (Song et al., 2008). In one case study of exercise-induced anaphylaxis to tofu, the soy allergic individual showed allergic reactions only after the ingestion of tofu but not of soy milk (Adachi et al., 2009).

7.2. Effect of processing on allergenicity

7.2.1. Effect of thermal processing

A few studies have investigated the effect of thermal processing on the IgE and IgG-binding capacity of soy and soy protein fractions. Burks et al. (1991) heated crude soy and its 7S and 11S protein fractions (80 °C or 120 °C, 60 min). Using sera from children with positive DBPCFC to soy, heating significantly reduced the IgE-binding capacity in ELISA. Some other studies reported on a varying IgG-binding capacity in ELISA or concentration of soy protein in differently processed soy based foodstuffs or after increased baking time in cookie matrix (Amigo-Benavent et al., 2008; Gomaa and Boye, 2013). Twin-screw extrusion of soybean meal with temperature higher than 66 °C was effective to decrease the binding capacity of specific IgG from calve, as assessed by ELISA analysis, to 0.1% of the original activity (Ohishi et al., 1994). However, the effect was analysed with animal antibodies and it may not be related to the process of heating only.

7.2.2. Effect of fermentation and hydrolysis

Hydrolysis with trypsin, pepsin and chymotrypsin is frequently used to prepare hypoallergenic formulas, but other enzymes of bacterial and fungal origin are also investigated. Artificial digestion of soy protein by pepsin, trypsin, chymotrypsin and intestinal mucosal peptidases was found to reduce the IgE-binding capacity by 10,000-fold using an ELISA inhibition assay (Burks et al., 1991). Other studies investigated the degradation of individual soy allergens depending on the selection of enzyme, temperature, and pH. For example, Yamanishi et al. (1996) found the hydrolysis of Gly m Bd 30 K was enzyme dependent and most successful for Proleather FG-F (protease from Bacillus subtilis) and Protease N. Tsumura et al. (1999) confirmed elimination of Gly m Bd 30K with Pro leather FG-F using immunoblot. In the same study it was demonstrated that the presence of β-conglycinin was almost reduced, but no such effect on glycinin was seen, based on SDS-PAGE analysis. The enzymatic hydrolysis of glycinin and β-conglycinin with tryptic and peptic enzymes was also investigated (Lee et al., 2007a; Tsumura, 2009; Zhao et al., 2010). The hydrolysis of both proteins depended on temperature and pH (Tsumura, 2009). At low pH, glycinin was denatured and more susceptible for hydrolysis, while β-conglycinin was denatured at higher temperature and became more hydrolysed in contrast to glycinin which was not affected. The IgG-binding capacity was never completely removed. Others combined enzymatic hydrolysis with heat treatment or high pressure (Tsumura et al., 1999; van Boxtel et al., 2008). For example, in a study from van Boxtel et al. (2008), the combined effect of heating at 100 °C and pepsin hydrolysis for 10 min reduced the IgE-binding capacity of glycinin to non-detectable in immunoblot analysis. Fermentation of cracked
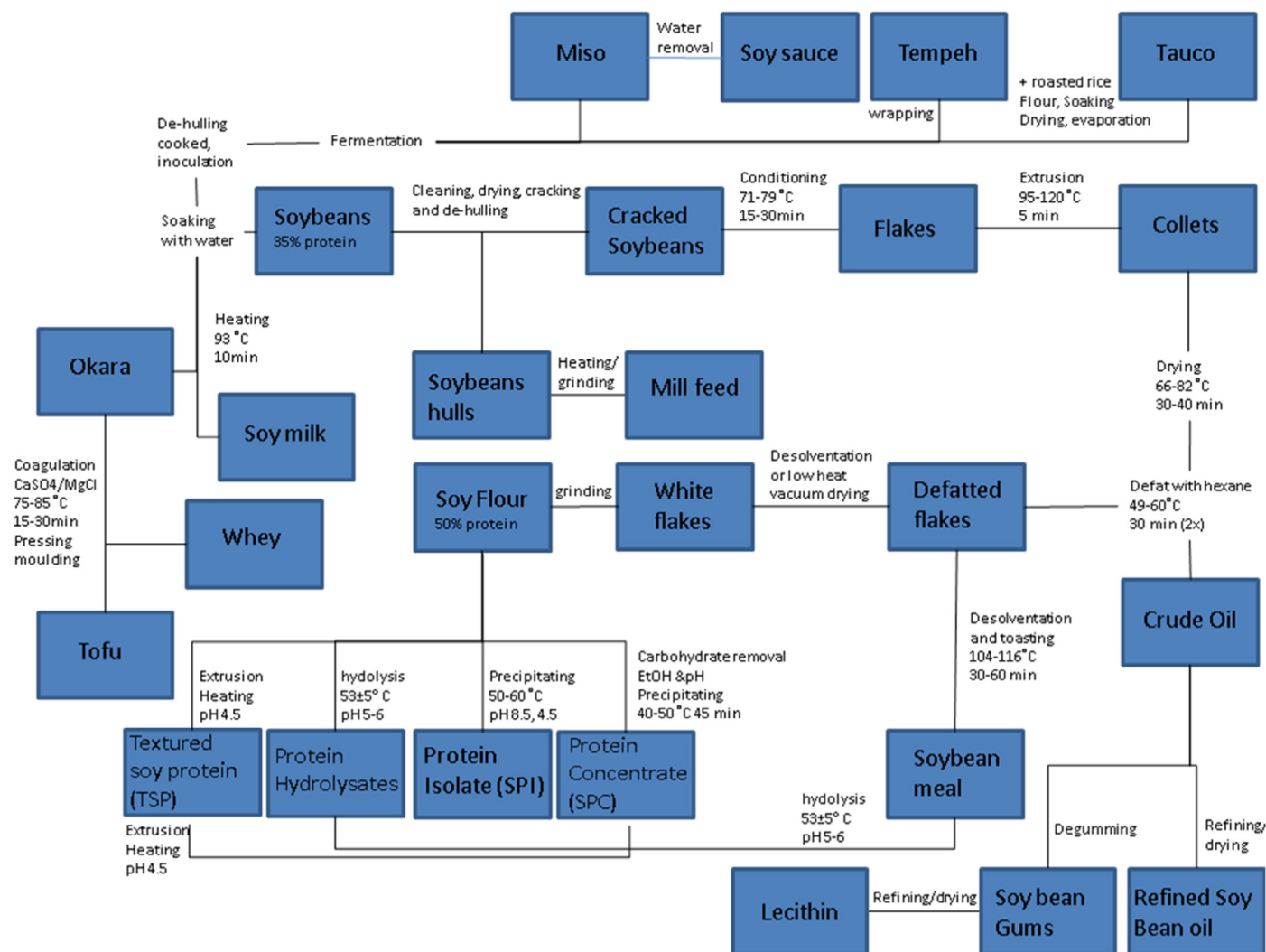
Fig. 1. Processing scheme of soy products adapted from B.G. Hammond et al. (Hammond and Jez, 2011), http://www.nsrl.uiuc.edu/aboutsoy/soyprocessing.html and http://www.agnet.org/library.

soybean seeds and soybean flour by various mould strains and bacteria have been shown to reduce the IgE-binding capacity by 65 to 99%, as was investigated using indirect ELISA with human serum (Frias et al., 2008).

7.2.3. Effect of non-thermal processing

The application of chemical treatments, such as Maillard-type carbohydrate conjugation or transglutaminase treatment, showed some evidence of reducing the IgE production in mice (Arita et al., 2001) or IgG-binding to soy protein (Babiker et al., 1998).

L'Hocine et al. (2007) investigated the effect of ionic strength and pH on the IgG-binding capacity of purified glycinin. Changes in IgG-binding were related to changes in the secondary and tertiary packing of this soy protein. High IgG-binding capacity was shown at low pH (2.2) and at neutral pH (7.2). Between pH 3 and 6, low IgG-binding capacity was related to low protein solubility.

For the purpose of preservation and structural modification, foods can be treated with high hydrostatic pressure (HHP). During HHP treatment, non-covalent bonds (hydrogen, ionic and hydrophobic bonds) are broken. A slight reduction in IgG-binding was observed after 15 min HPP treatment at 300 MPa (Li et al., 2012). Pulsed ultraviolet light (PUV) that was applied to soy extracts from raw dehulled and de-fatted soybean led to a decrease in the level of glycinin

and β-conglycinin using SDS-PAGE analysis (Yang et al., 2010). The IgE-binding was also reduced by 44% using ELISA. Another non-thermal processing step is controlled pressure drop (DIC), in which food is subjected to a short (1–3 min) drop in pressure (Cuadrado et al., 2011; Takács et al., 2013). DIC treatment at 6 bar for 3 min almost abolished the IgE-binding capacity of soybean proteins according to immunoblot analysis (Cuadrado et al., 2011). However, aqueous extracts were investigated but potentially low extractability of soy proteins after DIC treatment was not controlled.

7.3. Concluding comments

- The prediction of allergenicity of soybean and products thereof is limited because of a very limited number of high-quality studies performed in soy allergic humans or done with sera from clinically confirmed soy allergic donors.
- Some evidence exists that soy allergenicity may be reduced or retained by food processing, but yet there has been no indication for increased allergenicity due to food processing.
- Apart from highly refined soybean oil and other soybean products in which the level of soybean proteins are reduced below clinically relevant levels, one-step processing may not fully abolish soy allergenicity.

8. Wheat and wheat allergens

8.1. Introduction to wheat

Wheat is a staple food for most of the world's population. Wheat contains 10–14% protein. Based on the solubility in various solvents, wheat proteins are classified as albumins (water), globulins (diluted salt), gliadins (aqueous alcohol) and glutenins (diluted acid). Albumins and globulins are mainly structural and metabolic proteins. They include, amongst others, α- and β-amylases and their inhibitors, and lipid transfer proteins (LTPs). Gliadins and glutenins, known also as prolamins or gluten proteins, are the storage proteins of the wheat grain. They account for approximately 80% of the total wheat protein. Gliadins are monomeric proteins that interact by non-covalent forces (hydrogen bonds). They are classified into three groups on the basis of their electrophoretic mobility in acid PAGE: α/β-gliadins, γ-gliadins and ω-gliadins. Glutenins are polymeric proteins linked by intramolecular disulphide bonds. On reduction and separation in SDS-PAGE glutenins are classified into high molecular weight (HMW) and low molecular weight (LMW) subunits. Gliadins and glutenins are rich in glutamine and proline amino acid residues that are found in highly repetitive sequence motifs, but they differ in the arrangement, size and sequence of the repeated regions. α/β-gliadins, γ-gliadins and LMW glutenins are sulphur-rich proteins, and are composed of an N-terminal domain that contains glutamine- and proline-rich repeats and a C-terminal more structured non-repetitive domain with intramolecular disulphide bonds. ω-gliadins lack cysteine and repetitive motives cover almost the entire sequence (Shewry and Halford, 2002; Tatham, 1995).

Allergens involved in wheat food allergy were identified among the different wheat protein fractions. The IgE-binding profile correlates to age and symptoms that manifest. Sera from patients with atopic eczema/dermatitis syndrome show binding mainly with conformational epitopes. Patients with urticaria, anaphylaxis and wheat-dependent exercise-induced anaphylaxis (WDEIA), mainly adults, show strong IgE-binding to linear epitopes of the repetitive domains of gliadins and LMW-glutenins. Different IgE-binding sequences have been identified along these repetitive domains, with a.o. immunodominant epitopes on ω5-gliadin related to WDEIA (Bouchez-Mahiout et al., 2010; Denery-Papini et al., 2011; Inomata, 2009; Morita et al., 2007; Palosuo, 2003).

8.2. Effect of processing on allergenicity

8.2.1. Effect of thermal processing

In a DBPCFC study on wheat allergy in adults, Scibilia et al. (2006) showed that the clinical reactivity after consumption of raw and cooked (heated in water until boiling and immediately cooled) meals did not differ significantly: nearly all patients with a positive raw wheat challenge were also positive to the cooked wheat test meal and manifested the same symptoms. Starting from the same raw and cooked wheat samples Pastorello et al. (2007) confirmed that several IgE-binding proteins in the different wheat protein fractions maintained their IgE-binding capacity after cooking. Proteins of the α-amylase/trypsin inhibitor family which were present in the different fractions maintained their IgE-binding capacity after heating. Wheat LTP which was found only in the albumin/globulin fraction showed a lower structural stability upon heating and was not recognised by IgE in the cooked samples.

Wheat is mainly used in baking and pasta. This is largely due to the unique properties of the gluten proteins in wheat to form a viscoelastic dough that can be processed into leavened products and pasta.

Pasini et al. (2001) and Simonato et al. (2001) studied the effect of bread baking on the in vitro digestion and allergenicity of wheat proteins relative to the unheated bread dough. The binding of IgE from pooled sera of wheat allergic patients to the digestion products of the bread dough was strongly modified by the heat treatment. Differently from the unheated bread dough where a rapid breakdown of the IgE-binding components was detected, thermally induced protein aggregates prevented a complete proteolytic degradation of the allergenic proteins in baked wheat products, potentially allowing the passage of large IgE-binding protein fragments through the GI tract, where they could elicit an allergic reaction.

Pasta is mainly produced with durum wheat. The proteins of durum wheat are very similar to those of bread wheat in terms of molecular characteristics and allergenic properties. Pasta production involves extrusion and drying at temperatures up to 110 °C to reach about 12.5% of moisture. Pasta products are also boiled before consumption. Using in vitro digestion on model pasta samples dried at temperatures up to 110 °C used in industrial drying, complete degradation of IgE-binding components (SDS-PAGE with pooled sera from wheat (including durum wheat) allergic patients) was observed (De Zorzi et al., 2007). This was found also with a commercial pasta sample by Simonato et al. (2004). A very different IgE-binding pattern was evident in the pasta sample dried at 180 °C: heat induced aggregates with allergenic potential were resistant to the action of the digestive enzymes. However, for the pasta samples dried at temperatures up to 110 °C, IgE-binding was still detected with the unfractionated samples after digestion. It seems therefore that the digestion process, although sufficient to degrade the wheat protein to peptides, does not completely abolish the presence of allergenic structures.

8.2.2. Effect of fermentation and hydrolysis

Acid hydrolysed wheat gluten can be used as emulsifier in foods and cosmetics. In addition to partial hydrolysis of peptide bonds, acid hydrolysis also results in partial de-amidation of glutamine and asparagine residues, thus increasing negative charges in the protein. Partial acid hydrolysis of wheat gluten (combining low pH and heat) has been reported to cause allergic reactions in wheat tolerant people both by skin and oral contact. Most reported cases of allergenicity are related to the use of acid hydrolysed gluten in cosmetics (facial soap, shampoo), but cases of food allergy to different type of prepared foods were also reported. Sensitisation to acid hydrolysed wheat gluten through skin contact can result in allergenicity to the same product in food, and also to traditional wheat food products. Symptoms observed are generally severe: angioedema with generalised urticaria, anaphylaxis and WDEIA. (Chinuki and Morita, 2012; Denery-Papini et al., 2012; Pelkonen et al., 2011; Shinoda et al., 2012). IgE from patients allergic to acid hydrolysed wheat gluten preferentially binds to the native γ- and ω2-gliadin, have high IgE levels specifically to de-amidated γ- and ω2-gliadin and show some reactivity to other wheat proteins. Despite ω5-gliadin being known as a major allergen in WDEIA, no IgE specific for ω5-gliadin could be detected in patients allergic to acid hydrolysed wheat gluten showing symptoms of exercise induced anaphylaxis (Battais et al., 2008; Denery-Papini et al., 2012).

An in vivo study with non-gluten tolerant Brown Norway rats compared the sensitising capacity of acid hydrolysed gluten to that of native and enzyme hydrolysed gluten. ELISA inhibition tests were identical for rats sensitised to native and enzyme hydrolysed gluten, but IgE from rats sensitised to acid hydrolysed gluten bound remarkably stronger to acid hydrolysed gluten than to the other gluten products. The results indicated that sensitisation to acid hydrolysed gluten was possible in wheat tolerant subjects due to the presence of new epitopes, while enzyme hydrolysed gluten had an epitope pattern similar to native gluten (Kroghsbo et al., 2014a).

While processing could elicit an unintended effect with allergenic foods, it has also been used as a tool to reduce allergenicity

in the production of allergenic foods suitable for wheat allergic people. Watanabe et al. (2000) developed a two-step procedure for the production of hypoallergenic flour, using a cellulase to decompose glycoprotein allergens, and actinase as a proteolytic enzyme able to hydrolyse peptide bonds near the essential proline residues of the repeated sequence Gln-X-Y-Pro-Pro in major wheat allergens. ELISA testing with individual sera of wheat allergic patients showed no IgE-binding capacity in most cases. In a food challenge with 15 children with a history of severe urticaria after ingestion of wheat-based products, only 2 patients showed an immediate reaction to the hypoallergenic cake (Tanabe, 2008).

The proteolytic system of lactic acid bacteria of sourdough origin has been studied for its ability to hydrolyse toxic peptides. Lactobacilli have a complex peptidase system. Rizzello et al. (2006) and De Angelis et al. (2007) evaluated the potential of selected lactic acid bacteria and of a mixture of probiotic lactic acid bacteria and bifidobacteria in the production of sourdough bread to hydrolyse wheat flour allergens. Based on immunoblotting with pooled sera of wheat allergic patients, it was shown that compared to baker's yeast bread, selected lactic acid bacteria favoured the degradation by digestive enzymes of the IgE-reactive epitopes which persisted after baking wheat bread. The selected lactic acid bacteria possess a complementary protease and peptidase activity against polypeptides extremely resistant to proteolysis by digestive enzymes.

8.3. Concluding comments

- Heating at high temperature in the presence of carbohydrates may induce the formation of protein aggregates with allergenic potential that are resistant to digestibility.
- Acid hydrolysed wheat gluten may induce allergenicity in wheat tolerant people, including also allergenicity to traditional wheat products.
- In order for enzymatic hydrolysis to reduce the allergenicity of wheat, specific proteolytic properties are needed.

9. Mustard and mustard allergens

9.1. Introduction, including processed foods derived from oilseed rape

Many *Brassica* species are used for producing mustard. This paper will focus primarily on *Brassica juncea* and *Sinapis alba*, the most important species used for producing mustard worldwide (Food and Agriculture Organization of the United Nations, 2014). Both species are used in many diverse processed and prepackaged foods, as seasoning or flavouring agents, emulsifiers, and water-binding agents for texture control, for infants, children and adults (Lee et al., 2008). Mustard allergy is probably the most common among allergy to spices (Niinimaki et al., 1989, 1995; Rancé, 2003). Although the overall prevalence is relatively low, it has been found in several studies that the symptoms could be severe, e.g. anaphylactic reactions (André et al., 1994; Rancé, 2003). Interestingly, *Brassica napus* (also called rapeseed or canola) seeds are used for producing oil but not mustard. Although canola oil has not been shown to be allergenic, it is relevant to include it in this review as canola seeds contain cross-reactive allergens to those found in mustard seeds. However, the different species follow different food processing pathways (i.e. mustard vs. oil). In addition, very large productions of *Brassica* are recorded, especially in Canada (2007), and exposure to this species-derived product can be high (OECD, 2011). Canola oil is used for salad dressing and baking, and is also acceptable in hydrogenated products such as margarine and shortenings (OECD, 2011).

Several studies have investigated the potential for *B. napus* to cause food allergy. In general, sensitisation in children was associated with multiple allergies to other foods and pollen (Health Canada,

2010, Monsalve et al., 2001, Poikonen et al., 2009). There were a small number of DBPCFC with adults or children (Figueroa et al., 2005; Morisset et al., 2003) and SBPCFC studies with children (Rancé, 2003), due mainly to the difficulty of masking the mustard taste and the unethical health risk to conduct clinical studies with highly sensitive patients.

Because the protein was either at very low levels or absent in canola oil, the significance of the results of these allergenicity studies in determining the safety of consumption of canola oil by the general population was considered to be low (Gylling, 2006). In addition, the food allergy to canola oil in adults has not been reported in the scientific literature.

In adults, the mean cumulative dose–response was circa 125 mg of mustard seed (*S. alba*) (Figueroa et al., 2005), equivalent to circa 32 mg of proteins. The lowest dose of mustard seed eliciting a response was 45 mg, equivalent to circa 11 mg of protein. In children, the lowest dose of mustard seed (*B. juncea*) eliciting a response was 14 mg, equivalent to circa 1 mg of protein (Morisset et al., 2003). Furthermore, most of the case reports describe that the food eliciting the reaction was mustard sauce or mustard hidden in other sauces, or due to cross-contamination. Only one case report (Kanny et al., 1995) estimated the concentration of mustard in the dip responsible for causing the reaction as 0.15 mg/100 mg. Other case reports only indicated that the amount of mustard associated with the allergic response was small or present in trace amounts.

Many allergens in the *Brassica* and *Sinapis* species were sequenced and described in the AllergenOnline database (www.allergenonline.org). A high degree of sequence similarity exists between various *Brassica* species. Known allergens associated with food allergic reactions are Bra j 1, Bra n 1, Sin a 1, Sin a 2, Sin a 3, Sin a 4. The major food allergens of the mustard seeds (Bra j 1 in *B. juncea* and Bra n 1 in *B. napus*) are storage proteins of the 2S albumin class, which are abundant seed proteins (Puumalainen et al., 2006).

9.2. Effect of processing on allergenicity

9.2.1. Effects of thermal processing and hydrolysis

Interestingly, all previously mentioned allergens are described as quite resistant to heat. The heat stability is variable for each protein but all are above 80 °C. For example, Bra j 1 is denatured at 82 °C (Jyothi et al., 2007). The proteins can also interact with other food matrix constituents like the phenolic compounds forming again a more stable structure, which hence would lead to increased temperature required for the denaturation process (González De La Peña et al., 1996; Monsalve et al., 1993; Palomares et al., 2005). The disulphide bonds may explain this heat stability as they form a more compact structure (D'Hond et al., 1993; Gehrig and Biemann, 1996; Menendez-Arias et al., 1988; Schmidt et al., 2004).

Based on the Thomas et al. (2004) approach, the results also showed that the proteins were resistant to gastric and intestinal degradation throughout a 60 min digestibility period (pH 2 with pepsin and pH 6.8 with pancreatin, respectively). The *in vitro* digestibility study of Savoie et al. (1988) showed that digestibility of rapeseed proteins by pepsin and pancreatin was 83%, which was a lower value than for casein (97%). Again, this low digestibility was explained by the compact nature of the proteins, as a result of the high number of disulphide bounds. Overall, these characteristics suggested that the allergenic proteins in mustard remained intact throughout food processing (i.e. heating) and digestion and could elicit an allergic reaction in susceptible individuals.

Although *B. napus* contains a 2S-albumin, which is known to be strongly allergenic, the lack of reported allergenicity records is very likely due to the harsh nature of the combined heat and mechanical processing for producing oil. According to the OECD (2011), canola seed is traditionally crushed and solvent extracted in order

to separate the oil from the meal. The process usually includes seed cleaning, seed pre-conditioning and flaking (i.e. preheating of the seeds to approx. 35 °C), seed cooking/conditioning (including a steam-heating with a temperature, which is rapidly increased and which ranges between 80 and 105 °C, for 15–20 min), pressing the flake to mechanically remove a portion of the oil, solvent extraction of the press-cake to remove the remainder of the oil, oil and meal desolventising (with final stripping and drying at a temperature of 103–107 °C), degumming and refining of the oil. The most probable hypothesis is that the proteins are removed from the oil by extrusion. All these steps extract the potential allergens from oil.

9.3. Concluding comments

- Thermal processing and enzyme digestion are not sufficient for abolishing the allergenic potential of mustard seed allergens.
- A combination of physical- and thermal treatment (e.g. extrusion) can suppress allergenicity of mustard seed allergens by extracting the potential allergens from oil. Edible oils that are bleached and deodorised are devoid of allergenicity.

10. Influence of processing on sensitisation

It is generally appreciated that food allergy, in common with other forms of allergic disease, develops in two phases: a sensitisation phase and an elicitation phase, the latter resulting in clinical symptoms of allergic disease. Sensitisation is the phase during which immunological priming to the inducing allergen occurs, associated with the evolution of a Th2 biased immune response and the production of IgE antibody. If the sensitised subject is exposed subsequently to a sufficient amount of the inducing food allergen, then an allergic reaction may be elicited, resulting in a variety of clinical symptoms ranging from minor oral irritation to anaphylaxis (Perry and Pesek, 2013; Sicherer and Sampson, 2014; Sicherer and Wood, 2013).

Much of this review has focused on how food processing is able to impact on the IgG- and IgE-binding properties of food proteins and their ability to elicit allergic reactions, i.e. the effects of food processing on the recognition of epitopes on food allergens by IgE antibodies. This may impact on the capacity of the processed allergens to cross-link receptor-bound IgE, hence impacting on mast cell degranulation and the elicitation of an inflammatory reaction. Much less information is available with regard to the ability of food processing to impact on the potential of a protein to induce the immunological priming required for the induction of IgE antibody responses and the acquisition of allergic sensitisation.

Skewing of adaptive immune response to a Th2-type phenotype and IgE antibody production necessarily are preceded by an innate immune response to the allergen. Activation of receptors on cells of the innate arm of the immune system is a prerequisite for the initiation of adaptive immune responses. Allergens have been shown to have properties that allow interactions with various types of pathogen recognition receptors, favouring Th2-biased immune responses (see below for details). Allergen-bound lipids and glycans might mimic pathogen-associated microbial patterns, favouring receptor binding (Thomas, 2013). As most foods that are contained in the 'big 8' are often processed before being consumed, the question emerges as to whether and to what extent such processing can affect recognition of allergens by innate immune cells, in particular by dendritic cells (DCs) as most relevant antigen presenting cell type that are pivotal in directing Th1 and Th2-responses. Molecular characteristics of antigens (allergens) are crucial for biasing this Th-response (Jankovic et al., 2001). It has now been shown that several allergens, including some from foods, can directly activate DCs to induce Th2-skewing (Ruiter and Shreffler, 2012). Next to this there might also be a role for the epithelial cells. For instance it has been shown that heat- or enzymatic induced aggregation of (milk) proteins affected their transport across intestinal epithelia; as aggregation appeared to induce a shift from transcytosis to uptake via Peyer's patches (Roth-Walter et al., 2008; Stojadinovic et al., 2014), this may lead to a different immune response.

Processing changes the structural and chemical properties of proteins, and hence of allergens. Proteins denature, aggregate, bind to lipid structures, and undergo glycosylation and or glycation (Maillard reaction). Clearly, these processing-related structural and chemical changes will have the potential to influence the allergenicity of proteins as reflected by their propensity to bind to their specific IgE antibodies.

Although most steps in the development of IgE-mediated allergic sensitisation have been studied in some detail, much less is known about the roles played by the innate immune system in this process. Central events in the initiation of adaptive immune responses are the recognition, internalisation, processing and presentation of antigen by specialised antigen presenting cells (APC) (Kean et al., 2006). Here we consider briefly how food processing, and the changes that result from it, may impact on the recognition by APC of antigens, and their interaction with them. In this context probably the most important processing-related changes are aggregation, denaturation, lipid binding, glycosylation and/or glycation) (Thomas, 2014).

10.1. Glycosylation and glycation (Maillard reaction)

Analysis of the surface area of known allergens has shown that many antigens are glycosylated (Jiménez-Saiz et al., 2014; Kean et al., 2006). The glycosylated/carbohydrate structures can be recognised by the C-type lectin receptors on dendritic cells redirecting a Th2 response. For example, the mannose structure of Der p 1 (house dust mite allergen) is recognised by the mannose receptor (CD206) on dendritic cells. Cross-linking of its ligand to the mannose receptor leads to Th2 type polarisation of the dendritic cell (Chieppa et al., 2003). Referring to food allergens, native Ara h 1 (a glycoprotein) was found to stimulate monocyte-derived DCs, via binding to DC-SIGN, to induce Th2-differentiation in naïve T-cells, whereas de-glycosylated Ara h 1 did not (Shreffler et al., 2006). Analogously, heat-induced glycation (Maillard reaction, i.e. formation of advanced glycation end products, AGEs) may as well lead to allergic sensitisation, as stimulation of DCs with AGE-modified protein induced Th2-polarisation (Buttari et al., 2011; Mueller et al., 2013), in this case possibly via the RAGE-receptor. A very recent elegant study by Moghaddam et al. (2014) provides further support for the role of glycation in allergic sensitisation to peanut. Exposure of BALB/c strain mice, via either mucosal or cutaneous routes, to protein extracts from dry roasted peanuts resulted in significantly enhanced IgG and IgE antibody responses to raw peanut. The authors reported an involvement of AGE-receptors (CD36 and RAGE) in this process (Moghaddam et al., 2014). Ilchmann and her colleagues stimulated murine myeloid dendritic cells (mDCs) with either native OVA (hens' egg white allergen), heat treated OVA without glucose or OVA heated in the presence of glucose (AGE-OVA). Increased levels of IL-2 in the AGE-OVA stimulated mDCs compared to the two controls indicated enhanced T-cell activation by AGE structures. In addition, increased IL-4 and IFN-γ secretion have been observed in the AGE-OVA stimulated mDCs, indicating Th2-type polarisation of the mDCs by AGE structures. Finally AGE-OVA was better taken up by mDCS compared to non-glycated or native OVA (Ilchmann et al., 2010). Hilmenyuk et al. (2010) observed that AGE-OVA, but not non-modified OVA, stimulated the production of Th2-skewing cytokines in mDCs.

Following this line of reasoning, the glycation products formed by the Maillard reaction during processing might activate innate receptors to trigger allergic sensitisation by redirecting a Th2 response.

10.2. Lipid binding

Allergen-lipid complexes may be formed endogenously from naturally occurring lipids, but also be generated upon processing or storage of foods (Bublin et al., 2014). Most allergens contain patches with high proportions of surface exposed hydrophobic residues. Seong and Matzinger (2004) state that such hydrophobic patches are the main epitopes recognised by APCs triggering Th2 responses. In addition, the hydrophobic patches make allergens bind lipids by electrostatic or hydrophobic interactions (Bublin et al., 2014). It is found that the main allergens are often not covalently bound to lipids but have structures mimicking lipids, glycans or glycolipids. The lipid structures can mimic pathogen-associated microbial patterns and thereby cause binding of the antigens to TLR2, TLR4 or C-type lectins, initiating a Th2 response (Thomas, 2013, 2014). For example, the milk protein β-lactoglobulin is a member of the lipocalin family. Lipocalins have a hydrophobic ligand binding site that is enclosed with a fold (Thomas, 2014). This hydrophobic binding site allows lipid ligand binding, leading to enhanced Th2 skewing of the immune system (Bublin et al., 2014). Also, β-lactoglobulin was found to be more thermo-stable when bound to lipids (Considine et al., 2005). In addition, lipids can act as dietary adjuvants and skew the immune response towards a Th2 response, make food allergens more stable to proteolytic degradation in the GI tract and enhance the uptake of allergens by epithelial cells of the GI tract (Bublin et al., 2014).

10.3. Aggregation

Roth-Walter et al. (2008) have shown that during pasteurisation aggregation of both β-lactoglobulin and α-lactalbumin occurs. Aggregation is a consequence of denaturation. Whey proteins denature above 65 °C, resulting in the exposure of hidden hydrophobic groups (Croguennec et al., 2004). Hydrophobic residues of denatured whey proteins interact and form aggregates. Aggregation is driven by thermodynamics, favouring mutual interaction of hydrophobic groups over interactions with the aqueous environment (Raikos, 2010). Roth-Walter et al. (2008) showed that aggregation due to pasteurisation of both β-lactoglobulin and α-lactalbumin redirects the site from epithelial cells towards Peyer's Patches and that enhanced uptake by the Peyer's patches leads to sensitisation by inducing enhanced secretion of IgE and Th2 cytokines. From this it can be concluded that, during aggregation, already existing epitopes and/or new epitopes are formed and expressed on the cell surface of those aggregates, facilitating binding to APC present in the Peyer's Patches and redirecting a Th2 response.

Enzymatic cross-linking (with laccase) of β-lactoglobulin was found to lead to a shift in uptake from epithelia to Peyer's patches and to increase its allergic sensitising capacity in BALB/c-mice (higher levels of IgE, IgG1 and 2a) (Stojadinovic et al., 2014), corroborating earlier observations by Roth-Walter et al. (2008). Also, exposure of DCs *in vitro* to cross-linked β-lactoglobulin induced a higher Th2-response when co-cultured with T-cells (Stojadinovic et al., 2014).

10.4. Concluding remarks

- Allergens, especially if aggregated, glycated or lipid-associated, can interact with a variety of pathogen recognition receptors, an interaction that is often facilitated by 'adjuvant' structures, such as e.g. lipids or glycans, that are bound to the allergens, possibly mimicking pathogen-associated molecular pattern-like structures.
- Molecular characteristics of allergens are important for the type of and magnitude of the subsequent innate response. Some of such molecular characteristics that eventually lead to Th2-type skewing, such as glycation, interactions with lipids and aggregation, are influenced by processing.

11. Discussion

In this article the impact of processing (heat and non-heat treatment) on the allergenic and antigenic integrity (IgE binding and IgG binding, respectively) and allergenicity of food proteins has been reviewed. The foods considered were peanuts, tree nuts, cows' milk, hens' eggs, soy, wheat and mustard.

Much of the available literature has focused exclusively on the impact of processing on the integrity, including the antigenic integrity, of food allergens. In such cases the effects of processing are measured commonly as changes in the IgE-binding properties of protein allergens. In many instances the effects of processing on the IgE-binding activity of proteins have been (somewhat loosely) described as changes in allergenicity, or in allergenic potential. However, changes in the ability of a food protein to bind IgE antibody does not necessarily translate into altered allergenic functionality. To analyse allergenic functionality, methods such as mediator release assays (MRA), basophil activation tests (BAT), skin prick tests, and oral food challenges are required. Of these the double blind placebo-controlled food challenge (DBPCFC) is widely regarded as being the 'gold standard' and providing the most definitive diagnosis of food allergy. These tests are more relevant for predicting the effect of processing on the ability of a protein to elicit an allergic reaction in a previously sensitised subject. However, these tests are not always readily available, and are usually found only in specialised clinical centres.

A second important point is that changes induced by processing on the ability of IgE antibody to bind to a food protein do not necessarily indicate a change in the allergenicity of that protein and its ability to cause the acquisition of sensitisation. Processing may not only alter epitopes (changes in IgE antibody-binding properties), but may also create new epitopes, that might have the potential to induce sensitisation and food allergy. For this reason it is important to consider whether processing has had an impact on the inherent allergenicity of a food protein. However, it is not currently possible to make such assessments using *in vitro* methods and the most suitable approach involves the use of animal models. Unfortunately, few such models are available, and as yet none has been validated for the assessment of the allergenicity of proteins.

In addition, there are other issues that arise from food processing that are rarely addressed in the literature. For instance, it is commonly assumed that food allergens are relatively stable proteins and are resistant to proteolysis by pepsin in simulated gastric fluid. In fact this is part of the mandated safety assessment package required by WHO/FAO (FAO/WHO, 2001). It is possible that processing, and also the matrix may have a significant impact on the digestibility of a protein by altering susceptibility to gastrointestinal enzymes (as demonstrated for example with ovalbumin from egg (Takagi et al., 2003)). For this reason, a combination of processing and digestion has to be taken into account in the assessment of allergenicity and the resulting protein and peptide fragments should be tested in functional assays like BAT or MRA.

Another issue is the solubility of proteins after processing. In some cases solubility improves after heat processing, but in other instances proteins will aggregate and display reduced solubility in simple salt buffers (Lopez et al., 2012; Polenta et al., 2012). It is important, therefore, to monitor carefully the influence of processing on the solubility of proteins as this could impact on the integrity of assay systems. In most studies only tris or phosphate buffers are used. As a consequence, only readily soluble proteins will be extracted from the food product and in this way an incomplete protein panel is tested for allergenicity. The use of different buffers is therefore recommended, for instance a sequential extraction procedure using in succession 20 mM tris buffer, 6 M urea, and 2% SDS/1% DTT buffer. A more physiological approach would be to take into account

the capacity of the digestion system to solubilise the processed proteins and thus extraction methods that mimic the human digestive system should be considered. In addition, processing and the matrix can have a considerable impact on the detectability of proteins, since both may affect allergen conformation and extractability (Khuda et al., 2012). Due to changes in the immuno-reactivity of proteins after heat treatment, the amount of residual proteins detected using ELISA may also be variable. Direct detection methods like mass spectrometry may offer improved detectability (Azarnia et al., 2013; Popping, 2013).

Finally it is important to consider the number of patient sera that should be used to assess IgE binding capacity or functionality. In most studies only a few individual sera, or a serum pool, is used. Both options have their advantages and limitations. Using pooled sera will rule out the identification of inter-individual differences; however, it might also average IgE reactivity and in this way reduce detectability. Using individual sera would therefore be the preferred option, but an appropriate number of serum samples would be required for the identification of significant differences between individuals.

The overall conclusion drawn is that processing may influence, but does not abolish, the allergenic potential of proteins. Only microbial fermentation and enzymatic or acid hydrolysis may have the potential to reduce allergenic integrity and allergenicity to such an extent that reactions will not be elicited. The combination of heat processing and the aforementioned methods can improve this hypoallergenic potency. Other processing methods such as pressure treatments show promising results, though further studies are needed to clarify the effects. Literature on the effect of processing on allergenicity and the ability to induce sensitisation is scarce. This is an important issue since processing may impact, in various ways, on the ability of proteins to cause the acquisition of allergic sensitisation, and this subject should therefore be an important focus of future research. In addition, there remains a need to develop robust, thoroughly evaluated and validated methods for the risk assessment of food allergenicity where there is consideration of both protein digestion and protein analysis.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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