

Evaluating the effect of food processing on the potential human allergenicity of novel proteins: International workshop report

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Abstract

The ILSI Health and Environmental Sciences Institute Protein Allergenicity Technical Committee organized an international workshop in June 2006 in Estoril, Portugal, co-sponsored by the ILSI Research Foundation, ILSI International Food Biotechnology Committee and ILSI Europe. The objective was to discuss the effects of food processing on the allergenic potential of proteins and foods. The impact of food processing on the sensitization/induction phases of food allergy, and the bioavailability of allergens to the immune system were presented. Studies evaluating the stability, digestibility, and allergenicity of processed food allergens were identified, and their complexity and limitations discussed. Participants agreed that investigating food allergy mechanisms, validating appropriate methods for identifying allergenic proteins, and refining strategies to assess and manage the risks from food allergy were important before processing considerations are integrated into public-health decision-making for novel proteins. Other factors may also play a role in food allergy and include: food matrix; multiplicity of epitopes; geographic variation in patterns/prevalence of food allergies; and genetic factors, but required further exploration. Food processing may increase or decrease the intrinsic allergenicity of a protein, but current data do not facilitate the identification of specific variables that could be used to reliably determine how processing will influence protein allergenicity.

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Abbreviations: DC, dendritic cells; DBPCFC, double blind placebo controlled food challenge; FAO/WHO, Food and Agriculture Organization/World Health Organization; GPC, gel permeation chromatography; HESI, Health and Environmental Sciences Institute; OAS, oral allergy syndrome; PATC, Protein Allergenicity Technical Committee; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; UC, ultracentrifugation in sucrose gradients.

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1. Food allergy

For the purposes of this workshop, the participants were focused on food allergic reactions classified as type I hypersensitivity reactions that are mediated by IgE (also called Type I Anaphylactic Sensitivity). Food allergy affects up to 8% of children and 2–4% of adults (Zuberbier et al., 2004; Osterballe et al., 2006). Food allergic reactions occur typically in atopic individuals (i.e., those who are genetically predisposed to have allergic symptoms) and who have been previously sensitized to the allergenic protein (Sicherer, 2000). The classical symptoms of food allergy generally appear within minutes up to 2 h following ingestion of the offending substance. Skin, the gastrointestinal tract, respiratory tract and cardiovascular system are the main target organs. Symptoms vary from mild localized symptoms to severe, but rare, anaphylactic reactions. The oral allergy syndrome (OAS) is the most frequent clinical presentation of food allergy seen in adult patients (Mari et al., 2005). As peptides are believed to require a molecular weight of 3.5 kDa or greater in order to cross-link mast cell IgE and elicit a reaction, large stable fragments as well as intact proteins have the potential to elicit an allergic reaction (FAO/WHO, 2001; Bannon et al., 2002; Lack et al., 2002; Thomas et al., 2004).

In an effort to explore the events associated with the sensitization phase of food allergy, the relationship between dendritic cells (DCs) present in the digestive tract, lymphoid organs, and the antigens of the gut flora was discussed. These relationships are more dynamic than previously thought, with DCs reaching through into the lumen and capturing antigens. DCs are also thought to play a role in the homing of activated T cells to the gut lamina propria. Whether DCs present antigens in the gut to mucosal virgin T cells, thereby initiating cell-mediated immunity and T-cell dependent antibody responses (i.e., production of IgG, IgA and IgE), however, is not well understood. Moreover, it was noted by participants that the correlation between the level and nature of allergenic epitopes (including immunoreactive linear or conformational epitopes) in proteins, impact of the food matrix (interaction with other non-protein components in food like carbohydrates or lipids) on allergenicity, and the severity of the clinical reactions for most allergens is not well-defined.

2. Digestion process

The digestive process is likely to play a major role in the development of allergic sensitization, as well as in the clinical severity of food allergy symptoms since exposure of the immune system to proteins is required to initiate an allergic response. The gold standard for investigating food digestion is the use of *in vivo* approaches. Due to ethical concerns, sampling is very rare in humans, and animal studies are therefore performed. Development of *in vitro* models that mimic *in vivo* models are limited and currently are focused on three main stages: processing in the mouth,

stomach and duodenum. Two types of *in vitro* models are currently being investigated, static and dynamic. Static models (i.e., biochemical models) are defined as those where products of digestion are not removed and which do not mimic the physical processes that occur *in vivo* (e.g. shear, mixing, hydration, etc.). Dynamic models include both the physical processing and temporal changes in luminal conditions that mimic *in vivo* conditions. These holistic models can use more complex foods, and therefore integrate the effects of the food matrix that may impact the kinetics of allergen release. One dynamic model that was discussed during the workshop is the 'IFR model of human digestion' (i.e., the Model Gut) (Moreno et al., 2005). This model will be used in the EU-funded EUROPREVAL project to assess the effect of food structure on the release of allergens in the gastrointestinal tract and their subsequent stability to breakdown in the gut lumen. Further investigation of the interactions between allergens and food structure and the release and breakdown of allergenic proteins, digestive and metabolic processes, including adjuvant effects of the food structure, and the potential effects of processing in modulating allergenic properties of foods was encouraged by participants.

Alterations in protein absorption (i.e., bioavailability) are known to impact the potential for an allergic response. For example, a reduction of gastric acid by proton pump inhibitors resulted in a significant impact on the allergenic potential of foods by allowing absorption of larger, more immunologically active fragments (Untersmayr et al., 2005). This may involve a specific transcellular uptake of allergenic proteins, followed by a wider antigen non-specific increase in paracellular uptake by activated mast cells and/or a binding to the low affinity IgE receptor CD23 (FcεRII) expressed by activated enterocytes. In contrast to the facilitatory role played by IgE in allergen absorption, IgA may limit antigen penetration. It has been demonstrated that specific IgA can act as an antigen-excretory system by binding antigens and thus blocking apical to basal transcytosis. Moreover, several factors may alter epithelial permeability, and thus promote antigen presentation to the mucosal immune system. These factors include heat-stressed epithelium and chronic psychological stress. In addition, the presence of exogenous surfactants found in foodstuffs may induce mast cell degranulation or induce rearrangement of the cytoskeleton of enterocytes that permits excess penetration of proteins. Similarly, ingestion of alcohol may also permit excess penetration of proteins. Such factors may partially explain the observed variability in thresholds for food allergic responses.

3. Food processing

Food and food ingredients have many diverse forms and are subjected to a large variety of processing conditions in order to improve their sensory qualities (e.g. flavor, texture, taste, and appearance). Foods may undergo processing to preserve them by removing/inactivating toxins and/or

microbes (e.g., heating, pasteurization), modify their properties to suit the end use (e.g., gel network, improvement of digestibility), and/or derive a large diversity of additional food products (e.g., isolates, oil) from the initial food. There are several food processing methods that are used exclusively or in combination with others. Some of these methods include preparation, mechanical processes, separation, isolation and purification, thermal processes, biochemical processes, high pressure treatment, electric field treatment, and irradiation. These methods can be categorized into two processing types: thermal and non-thermal. In the first case, food may be thermally processed by using moist heat or dry heat. The Maillard reaction, enzymatic browning or roasting, and all dry heat processes are the most likely processes to impact the allergenic potential of foods. Non-thermal processing methods include germination, fermentation, proteolysis, ultrafiltration, storage, mechanical and enzymatic tissue disintegration, pulping, peeling, mashing and pasteurization treatment (Besler et al., 2001; Sathe et al., 2005). Distinction may also be made between proteins that are destroyed/removed (reduction of the allergen content) and those that are structurally altered (modification of conformational epitopes).

4. Methods for assessing the stability of allergens

The ability to elicit IgE responses declines with lower molecular masses, and polypeptides below 3.5 kDa lack this capacity (FAO/WHO, 2001; Bannon et al., 2002; Lack et al., 2002; Thomas et al., 2004). Therefore, evaluation of the stability of a protein structure is a key component for assessing the allergenic potential of proteins. Biophysical methods and strategies employed for assessing the stability of allergens were discussed and depend on the structural and biochemical properties of the tested proteins – a physicochemically-stable protein possesses a three-dimensional structure which can be difficult to disturb by altering temperature, pH or adding denaturants such as detergents or chaotropes such as urea.

Methods employed for studying secondary, tertiary, and quaternary structures, respectively include far-UV circular dichroism (far-UV CD) and Fourier-transform infra-red spectroscopy (FT-IR), fluorescence spectroscopy by near-UV CD, differential scanning calorimetry, and assessing how proteins can associate/dissociate under different conditions (i.e., high vs. low ionic strength, reducing vs. oxidative, with or without detergent) by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), ultracentrifugation in sucrose gradients (UC), and gel permeation chromatography (GPC). In all cases, the methods indicate the potential for processing to alter both linear and conformational epitopes.

In vitro digestive fate studies are commonly used as a part of the assessment of the allergenic potential of biotech proteins as recommended by the *Codex Alimentarius* (CAC, 2003; Thomas et al., 2004) in which pepsin digestion followed by SDS-PAGE with staining is utilized to monitor

the destruction of the primary structure of proteins. While the stability of a given protein to digestion and its physicochemical stability (as represented by stability to processing) are linked, the relationship between these two measures of stability has not been elucidated. Workshop participants agreed that protein stability was an important consideration in determining allergenic potential and should be used for assessing the allergenic potential in a weight of evidence approach. However, the correlation between stability to digestion and the allergenic potential of food proteins has not been conclusively established (Fu et al., 2002). Data indicate that some proteins resistant to digestion may be allergenic, while some stable proteins may be inert with regard to their respective allergenic potential. Therefore, a further investigation of this phenomenon was encouraged, particularly within structural families.

5. Methods for assessing IgE binding capacity

Specific IgE antibodies are implicated in the course of allergic diseases. Therefore, IgE binding capacity of proteins or foods is a prerequisite of clinical reactivity, i.e. ability to elicit an allergic reaction in sensitized populations. Tests based on the measure of IgE are recommended by FAO/WHO (2001); *Codex Alimentarius* (CAC, 2003), and EFSA (2006), if the test protein is derived from an allergenic source or demonstrates identity to a known allergen (Thomas et al., 2005). In all cases, the tests must be reliable, specific, sensitive and validated. While these assays are important for exploring the elicitation phase of food allergy, the example of roasted hazelnut discussed by the workshop participants demonstrates that such studies may contribute little to a quantitative estimate of allergenic potency *in vivo* (i.e., 100-fold reductions in IgE binding but an increase of the minimum eliciting dose by only 2-fold) (Skamstrup Hansen et al., 2003). Therefore, the diagnosis of food allergy, as well as the determination of the relative eliciting thresholds for processed and unprocessed whole food, requires the use of a double blind placebo controlled food challenge (DBPCFC) assay performed in the same group of well-characterized individuals.

It is important to note that the presence of specific IgE alone is not sufficient to trigger the allergic cascade (Poulsen et al., 2006). Participants agreed that although IgE binding methods were useful, particularly if coupled with functional assays (e.g., histamine release tests), to (1) investigate the allergenic potential of proteins; (2) permit the comparison of foods before and after processing, and (3) for better management of the allergenic risk from foods, they are not, by themselves, an absolute predictor of the allergenic potential of a protein. Data from DBPCFC assays with unprocessed and processed foods, however, are very limited and ethical considerations in addition to logistics may preclude their widespread use, even though they are considered the most reliable method for clinical evaluations. In addition to serological and clinical tests, animal models may be designed for predicting changes in

allergenic potential or evaluating mechanisms of allergenicity. *In vivo* models are frequently used for confirming the hypoallergenicity of foods (e.g., milk formulas for infants), or for investigating the allergenic potential of novel proteins). However, to date no animal model has been thoroughly evaluated or validated with pure proteins or unprocessed or processed foods (CAC, 2003). Consequently, the predictive value of animal models for identifying human allergenic proteins has not been sufficiently demonstrated. In addition, other biomarkers from the gut-associated lymphoid tissue (GALT) or other tissues may be identified (e.g., dendritic cells, T-cell characterization, cytokines) to predict sensitization/allergenicity potentials of food proteins.

6. Alteration of the allergenic potential of proteins by food processing

Any process that modifies the structure of a protein might be expected to interfere with its ability to be recognized by antibodies. Food processing induces several physical, chemical and biochemical changes that are known to potentially impact the allergenic potential of proteins. Certain methods of food processing may enhance, reduce, or eliminate the allergenic potential of a food. This impact is affected by the nature of the food process used (i.e., heat, pH, enzyme, presence of water, type of gaseous atmosphere, concentration of proteins, chaotropes, etc.) and the time and intensity of the process(es) (Wal, 2003). Examples are grouped below into two categories: heat-processed foods and non-thermal processed foods (chemical/biochemical or mechanical processes).

6.1. Impact of thermal processing

In general, heat-treatments have been found to significantly reduce the IgE reactivity of well known allergens, most likely as the result of unfolding mechanisms (Besler et al., 2001). Baking, cooking, roasting, grilling, drying, pasteurization, as well as, sterilization, denaturation (by unfolding and/or aggregation) and reaction with other molecules from the food matrix during thermal treatment could result in a modification to the allergenic potential. Alteration of the conformation of the heat-labile proteins, and thus loss of conformational epitope(s), may explain the reduction or abolition of allergenic potential for some of these proteins. Examples of proteins which are unfolded on heating are allergens belonging to the labile cross-reactive Bet v 1 family which are known to contain conformational epitopes. The loss of conformational epitopes from these proteins after heating abolishes the allergic reactivity of birch allergic patients towards Mal d 1 and Pru av 1, from apple and cherry (Scheurer et al., 2004; Bohle et al., 2006), respectively and Api g 1 from celery (Jankiewicz et al., 1997) after wet processing and for Cor a 1.04 from hazelnut after dry heating (e.g., roasting) (Pastorello et al., 2002; Skamstrup Hansen et al., 2003). In addition,

thermostability is not equivalent for all allergens, even from the same family. For instance, Gly m 4, which is also a Bet v 1 homologue retains its allergenic activity, even in a highly processed soya-based food supplement (Kleine-Tebbe et al., 2002) and has been detected in a variety of heat processed foods derived from soybean (Mittag et al., 2004). Moreover, depending on the severity of the treatment, a proportion of protein may remain in the native state, which may be sufficient for IgE-reactivity to be retained. In addition, the rheomorphic structure(s) of many proteins may prevent the alteration of their IgE binding capacity after thermal processing.

Aggregation mechanisms, induced by the formation of intermolecular disulfide bonds may also alter IgE binding as demonstrated by the heating of the beta-lactoglobulin (bLac) protein, which subsequently binds to other food proteins, making bLac less allergenic by affecting the forms in which the allergen is presented to the immune system. IgE binding studies have shown that while IgE binding is reduced towards both β Lg variants A and B following thermal treatments that would cause the protein to denature, some trace of IgE binding remained (Ehn et al., 2005). Thus, despite the presence of an allergen in a food, how it behaves in a food matrix may also impact its allergenicity. Therefore, the presence of other compounds, such as fats and sugars, is also important. The Maillard reaction between free amino acids and aldehyde or ketone groups of sugars is one of the main chemical reactions that may affect the allergenicity of food proteins by inducing the formation of aggregates which bind more effectively than unmodified allergens, and are also more resistant to gastric digestion. For instance, new IgE binding sites may be created by Maillard reactions on peanut allergens (Maleki et al., 2000; Gruber et al., 2005). For shellfish, such reactions may also increase the IgE binding capacity of the tropomyosin allergen by creating new epitopes (i.e., sensitization to the glycated tropomyosin). Nevertheless, glycation of other allergens does not appear to increase their allergenicity. For instance, Maillard reactions after pasteurization (Sancho et al., 2005) have also been shown to cause a drastic decrease of the IgE binding capacity of Pru av 1, the major cherry allergen (Gruber et al., 2004).

6.2. Impact of non-thermal processing

Other food processing treatments detailed below can influence the allergenic potential of some thermostable food products. Enzymatic hydrolysis is the most efficient process for disrupting sequential and conformational epitopes, and therefore allergenicity reduction is best achieved by this method. The proteolysis depends not only on the amino acid sequence of the protein but also on its secondary structure (e.g., presence of disulfide bonds) and post translational modifications (e.g., glycosylation – note: this is different from glycation; glycosylation is an enzyme driven reaction, while glycation, as in the Maillard reaction is a chemical, enzyme-independent reaction). Some examples

of enzymatic hydrolysis discussed include the reduction of the allergenic potential of rice with actinase, proteases that reduce the allergenic potential of soybean, and the use of trypsin and chymotrypsin for producing hypoallergenic formulas. Ultrafiltration of hypoallergenic infant formulas based on the enzymatic treatment of milk with proteases permits the removal of the remaining traces of intact proteins, and suppresses the allergenicity of milk. However, proteolytic treatments are not always able to destroy all epitopes due to incomplete hydrolysis (e.g., peanut or peach) (Asero et al. 2000; Lehmann et al., 2006). Peptides may also re-associate to form aggregates that may increase the allergenic potential of foods or such treatments may also potentially unmask existing allergens.

Removal of the part of the food that contains a high level of allergens permits the reduction or in some cases suppression of the exposure to allergens, preventing allergic reactions. For instance, peeling of a peach reduces the allergenic properties of the fruit juice, as the peach allergens are mainly concentrated in the peel of the fruit (Brenna et al., 2000). Additional examples would be, separating the starch from potatoes or highly refining an oil thus removing proteins that may be potentially allergenic.

7. EU-funded and other European research projects

Determination of intake thresholds and factors, including processing and digestion, which might modify the allergenicity of a food ingredient may be useful for risk analysis and risk management. Several research projects in the EU investigating various aspects of allergy to foods of plant or animal origin after processing were discussed. The German FEI-Project studied the alterations of the allergenicity of fruits and vegetables during technological processing and compared the allergenic potency of the raw, intermediate and final products by using immunoblotting and EAST inhibition assays. The EU ALLERGEST project involves understanding the role digestion plays in food allergenicity. This project was focused on assessing the extent that changes in allergen structure during processing and digestion impact the ability of a protein or allergenic food both to elicit a response in sensitized individuals and to sensitize *de novo*. The project used both *in vivo* and *in vitro* human digestion models, as well as an animal model for investigating changes in the allergenic potential of milk and peanut (Mondoulet et al., 2005). Currently, the EU project Reduced Allergenicity of Processed Foods Containing, Animal Allergens (REDALL) is investigating technological procedures to reduce the allergenicity of milk, egg and meat and their by-products as measured by reduction in their IgE binding capacity using clinical tests (i.e., skin prick and DBPCFC). The prevalence of food allergy and occurrence of severe food allergic reactions in ten European countries by survey is also an objective of REDALL. The EuroPrevall project (www.euoprevall.org) will obtain information on the patterns and prevalence of food allergies across Europe in infants and young children, with a

birth cohort covering children from birth to the age of 21/2 years, with complimentary studies in unselected populations being undertaken in school age children and adults. One aspect of these studies is that the food allergy diagnosis will be confirmed in at least a proportion of the individuals using DBPCFC. In addition a small scale investigation into the effect of the food matrix on allergenic potential will be undertaken, integrating information from *in vitro* digestion studies, assessments of sensitization potential in animal models and, where possible, *in vivo* studies in man.

8. Managing the risk from food allergens

Participants agreed that the effects of processing should be included in risk assessment as one variable that may impact thresholds of elicitation. Although limited data suggest the existence of thresholds below which allergic individuals will not react adversely to offending foods, these data are poorly documented even for well known allergens. (Taylor et al., 2004). Moreover, the range of protein levels provoking allergic symptoms is very wide (from a 10th of a milligram up to grams). Even less is known about thresholds of sensitization or the relationship between exposure to a protein and the frequency of sensitization (EFSA, 2004).

Probabilistic modeling may be used to validate established health based intake limits and to determine the statistical confidence and level of protection that such limits provide. Such models derive a probabilistic estimate of the frequency of allergic reactions in a population by integrating the distribution of a range of variables including the prevalence of food allergy, sub-populations (children; adults), thresholds for different allergens and severity of reactions, as well as consumption figures for the population of interest or subgroups. Such an experimental model may be a promising tool for assessing possible differences in risks in term of the incidence of allergic reactions in a given population, as well as its severity at certain exposure levels. However, such models need to be validated before they are implemented.

An altered immune response to processed foods may reflect a more complicated phenomenon than simply impaired recognition of allergenic proteins. Processing may also alter the immunogenicity of the processed protein compared to the native one. In addition, given that every protein in the human diet theoretically has the potential to induce allergic reactions in some individuals, assessing the risks of processed foods should be considered in the non-processed counterpart from which allergic reactions cannot be excluded. Participants agreed on the need for prevalence and epidemiological studies, which incorporate a collection of data about the nature of the foods consumed, local and specific processing techniques used, and the frequency and intensity of the clinical allergic reactions as well as the targeted population (genetic background, conditions of life, etc.). Mechanisms of sensitization were

also recommended to be further investigated. Participants further agreed that establishing thresholds of elicitation for even a small proportion of processing techniques will likely prove difficult, making a general requirement to assess such effects impracticable without consideration of the extent to which they would impact risk.

9. Conclusion

Overall, there was consensus among the international workshop participants from academia, government and industry that food processing may impact the allergenicity potential of proteins, and more generally of foods. However, there are no general rules regarding how different allergenic foods respond to physical (i.e., thermal; mechanical), chemical, or biochemical processing methods. For some, the epitopes are destroyed, but for others they are unaltered. For some proteins IgE reactivity is increased (i.e., appearance of new epitopes or unmasking of existing epitopes) subsequent to processing, which suggest a potential increase in allergenicity. The conditions and intensity of processing, the variability of the allergen composition in the whole food, the structural attributes and inherent stability of the allergen scaffold, the food matrix structure, or the multiplicity of conformational/linear epitopes on a given allergen can all influence immune responses to individual allergens and may impact the overall allergenicity of the food. Workshop participants stressed the need for investigating further how individuals and populations (i.e., in epidemiological studies) react against the modified allergenic foods, taking into account the geographic/genetic variability of the immune response in atopic individuals. Although processing may reduce or increase the allergenicity of foods, there was also agreement that the complete elimination of the allergenic potential of foods by using processing methods was unlikely, given what is currently known about the relationship between processing and allergenicity. In addition, the utility of the tests used for assessing the thermal stability and allergenicity of proteins/foods should be further investigated and validated before they are used for public-health decision-making. Participants agreed that the effects of processing might eventually be included in risk assessment as one variable that may impact thresholds of elicitation. Presently, however, there is a lack of an appropriate testing strategy, as well as basic data for various allergens, and this currently precludes including considerations associated with food processing into the risk assessment process.

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