



Review

Scientific advancement of novel protein allergenicity evaluation: An overview of work from the HESI Protein Allergenicity Technical Committee (2000–2008)

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ABSTRACT

The safety assessment of genetically modified crops includes the evaluation for potential allergenicity. The current 'state-of-the-science' utilizes a weight of evidence approach, as outlined by the Codex Alimentarius commission (Alinorm 03/34A), recognizing no single endpoint is predictive of the allergenic potential of a novel protein. This approach evaluates: whether the gene source is allergenic, sequence similarity to known allergens, and protein resistance to pepsin *in vitro*. If concerns are identified, serological studies may be necessary to determine if a protein has IgE binding similar to known allergens. Since there was a lack of standardized/validated methods to conduct the allergenicity assessment, a committee was assembled under the International Life Sciences Institute Health and Environmental Sciences Institute to address this issue.

Over the last eight years, the Protein Allergenicity Technical Committee has convened workshops and symposia with allergy experts and government authorities to refine methods that underpin the assessment for potential protein allergenicity. This publication outlines this ongoing effort, summarizing workshops and formal meetings, referencing publications, and highlighting outreach activities. The purpose is to (1) outline 'the state-of-the-science' in predicting protein allergenicity in the context of current international recommendations for novel protein safety assessment, and (2) identify approaches that can be improved and future research needs.

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1. Protein Allergenicity Technical Committee background – Goals/Mandate

The ILSI HESI PATC was established to define the research areas of critical importance to advancing the science of conducting, interpreting and applying risk assessments for the allergic potential of agricultural biotechnology products and their constituent introduced proteins. This group took as its mission the goal of addressing areas where there were clear deficiencies in assessing protein allergenicity, either because of ambiguity in methodology or the science was not well understood. Each of these focus areas were addressed through a series of workshops, symposia and roundtable discussions with recognized experts from academia, government, and industry to better understand the basic science and/or technology needs for developing improved allergen assessment methodologies for regulatory purposes, product development, and public health goals.

The work of the committee was initiated by a scoping meeting held jointly with the United States Food and Drug Administration's (US FDA) National Center for Food Safety and Toxicology (NCFST) in December 2000. The specific areas that were initially identified included: (1) assessment of molecular characteristics of proteins, (2) allergen database and bioinformatic tool development, (3) development of serum banks, (4) evaluation of animal models, (5) identifying the prevalence and/or abundance of allergens in non-biotechnology derived food. A panel of international experts in allergy reported the current status of the science, identified the data gaps, and assisted in the development of a strategy to improve the science utilized in protein allergenicity assessments. Each topic area was developed into working strategies through a collation of the experts' opinions and is described in further detail below with regard to the scientific strategy and the impact of the PATC on safety assessment strategy for food and feed safety.

1.1. Assessment of molecular (physio-chemical) characteristics

The basis of the overall risk assessment process for potential allergenicity rests on the comparison of a candidate protein identified for crop improvement with the properties of known allergenic proteins using a characterization strategy. Evaluating characteristics such as protein source, stability to heat and enzymatic digestion, glycosylation site modification, and other physio-chemical properties are used to support an assessment that underpins the safe introduction of candidate proteins into animal and human foods. Of these different characteristics, resistance to digestion in human simulated gastric fluid (SGF) was chosen as one [characteristic] where a standardized method was required to deliver reproducible results. This was identified as a critical assessment methodology because many major food allergens tend to be relatively stable to digestion (Astwood et al., 1996) and found in abundance in their host organisms. To achieve the milestone of identifying a method that could reliably show the level of comparative stability of novel proteins, a set of standard test proteins with differing stabilities were tested using a common SGF protocol, adapted from the US *Pharmacopoeia* (USP, 1990), in an international ring study organized by the ILSI PATC (Thomas et al.,

2004). This standard protocol has now been accepted by many regulatory agencies around the world and is one of the characterization studies that can be performed reproducibly across different laboratories. However, interpretation of the protein digestibility findings as a part of a weight-of-evidence approach to allergy safety assessments is an aspect that has not been completely addressed and it remains a key area of study. Further details are provided in Section 3.

1.2. Bioinformatics allergen database and tool development

Regardless of the source of a novel protein, amino acid sequence similarities to other known proteins can provide valuable information, such as the level of similarity to proteins with a known history of safe use, or to proteins that are known to be allergens or toxins. A bioinformatics comparison of a novel protein to known allergens is another component used in the novel protein safety assessment process (Alinorm 03/34A, 2003). The bioinformatics analysis provides guidance on how to approach and when to apply additional safety assessment techniques (e.g., IgE serum screening *in vitro* studies; see Section 5).

Many different genomes have been sequenced over the last decade providing a vast resource of protein sequences and tools for analyzing sequence data. Several different databases are publicly available that contain hundreds of thousands of protein sequences (e.g., Uniprot-Swissprot, <http://www.expasy.org/sprot/>; National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>; Protein Informatics Resource, <http://pir.georgetown.edu/>). Specific allergen databases continue to be strengthened by the support of clinical evidence for inclusion of relevant sequences and a review process that can include peer-review networks of allergy experts (e.g., Allergenonline, <http://www.allergenonline.com>). In conjunction with specific protein allergen databases, the tools and criteria used for bioinformatics analysis are equally important. For instance, bioinformatic algorithms, such as FASTA and BLAST, were designed to evaluate the global percentage of identity and an estimate of structural similarity between protein sequences. To prevent the introduction of a cross-reactive protein allergen into a non-allergenic food, other bioinformatics algorithms (e.g., FindPatterns) can also help identify short sequences that may serve as 'theoretical' IgE epitopes that could be potentially cross-reactive. It was determined that a conservative approach to bioinformatics analyses would utilize searches of both short stretches of amino acids (8 or greater contiguous, identical amino acids residues) and larger windows, such as 80 or greater amino acids (Alinorm 03/34A, 2003). Further details are provided in Section 4.

1.3. Development of serum banks

Specific *in vitro* screening utilizing sera from subjects allergic to the source organism, with supporting clinical documentation of food allergy to a relevant allergen source material, should be performed under certain circumstances. As an example, the characterization of IgE binding sera can be useful to quantify levels of endogenous allergens in allergenic foods or identify potential cross-reactivity between two or more proteins that share a signif-

icant level of homology. A broader approach, termed “targeted *in vitro* testing” using sera from subjects allergic to materials that are broadly related by taxonomy to the source organism of the original gene has also been proposed for addressing safety issues on a case-by-case basis (FAO/WHO, 2001). However, the ‘targeted approach’ has not been validated for its utility in predicting potential protein allergenicity (Alinorm 03/34A, 2003).

The development of a commonly available human serum bank would be of great benefit in facilitating appropriate immunoassays (e.g., Enzyme AllergoSorbent Test [EAST], EAST Inhibition, western blot, Enzyme-Linked ImmunoSorbent Assay [ELISA] assays) due to the difficulties in acquiring appropriately characterized sera on a case-by-case basis. While developing and housing such a sera bank was considered outside the capabilities of ILSI HESI PATC, the Committee noted that the ILSI Allergy and Immunology Institute has extensive expertise that should be utilized to evaluate this need. It would be advantageous to define the ideal features of a sera bank, determine the appropriate requirements for individual sera included in the sera bank, and to document the optimal management strategy for such a sera bank. Further details are provided in Sections 4 and 5.

1.4. Development of animal models

For a holistic and more definitive assessment of the potential allergenicity of novel proteins, proposals have existed for some time regarding the potential to acquire informative data from animal models. Compared to other methods, *in vivo* models could potentially distinguish commonly allergenic foods from rarely allergenic or non-allergenic foods (Aldemir et al., 2009).

If method validation is possible, such models may help identify intrinsic allergenic hazards of novel proteins as part of the weight of evidence approach, with the next steps in the risk assessment process being to determine the characteristics of that hazard and to define the likely conditions and extent of exposure that could affect human health.

Development of a consensus set of animal model criteria, appropriate protocols for model assessment, and an adequate and broad range of standard proteins to evaluate the models were identified as critical elements to support the research needed for better characterizing the potential of a validated animal model in providing value to the safety assessment of a novel protein. Further details are provided in Section 6.

1.5. Exposure to proteins in food

Currently, it is unclear what role protein abundance in a food product should play in the assessment of allergic potential. Metcalfe et al. (1996) indicated that many allergenic proteins were abundant, frequently in excess of 1% of the total protein found in the allergenic food. Focused research on protein levels that lead to sensitization, as well as the levels that lead to elicitation of a clinical response or immune tolerance and how they should be evaluated were identified as important issues.

In parallel, the 2006 ILSI PATC Food Processing Workshop participants recommended that the traditional deterministic risk assessment that is usually applied and which leads to inconsequential conclusions such as ‘an allergic reaction cannot be excluded’, be progressively replaced with probabilistic models (Spanjersberg et al., 2007) (see Section 7). The elaboration of probabilistic models was also supported by another ILSI Task Force (ILSI Europe Food Allergy task Force, Expert Group on Determination of Eliciting Dose).

In order to validate such probabilistic models for the safety evaluation of biotechnology-based products, the level of human allergen exposure data in different foods needs to be determined. Currently, no database that contains this information exists and

the quantitative determination of specific allergenic protein levels in finished foods is often problematic or is reported in an inconsistent manner. To partly address this issue, a network of scientists (Taylor et al., 2002, 2004) has initiated a project to determine threshold doses of elicitation for major allergenic foods. These data, however, are of limited applicability to the assessment of the potential allergenicity of novel proteins since it only involves thresholds of elicitation and not sensitization. In addition, the impact of food processing on food allergens has started to be explored. Further details are provided in Section 7.

2. Protein stability: pepsin resistance and the SGF method – ring trial evaluation

The pepsin resistance assay was first developed as a means to determine the relative stability of a protein in the presence of pepsin protease under pH conditions similar to that encountered in the mammalian stomach and was originally developed and utilized as a method to assess amino acid bioavailability (Marquez and Lajolo, 1981; Nielson, 1988; Zikakis et al., 1977). Proteins that are rapidly digested would be expected to have less opportunity to be bioavailable as intact and functional proteins as they would be passing the gut mucosa in a degraded form.

The first published application of an *in vitro* pepsin resistance assay to address the question of food allergen stability was by Astwood et al. (1996). The stability of some of the major allergens found in peanut, soybean, egg, and milk relative to the stability of common non-allergen food proteins was determined in a pepsin resistance assay using simulated gastric fluid (SGF) prepared according to the USP (1990). Since this initial report, there have been numerous studies repeating the pepsin resistance assay for a variety of proteins (Buchanan et al., 1997; Kenna and Evans, 2000; Okunuki et al., 2001; Besler et al., 2001; Takagi et al., 2003). However in the early 2000s, there was no common protocol available to assess the digestibility of proteins using SGF and a number of variations in SGF assay protocols had been reported. Protocol variables included differences with respect to pH, the purity of the pepsin, the pepsin-to-target protein ratio, the target protein purity, and finally, the method of detection. Therefore, the HESI PATC organized an international laboratory ring trial to establish a standard SGF protocol (Thomas et al., 2004). The objective of this study was to assess the digestibility of a standard set of proteins in nine separate laboratories in order to determine if reproducibility of the assay was possible when performed using a common protocol. The laboratories participating in this test represented industry (Aventis CropScience [now Bayer CropScience], Dow AgroSciences, DuPont Company, Monsanto Company, Syngenta Biotechnology), federal researchers (National Center for Food Safety and Technology, USA, National Institute of Health Sciences, Japan) and a private research company (Central Laboratory of the Netherlands [now Sanquin Research]), and were located in the USA, Europe, and Japan.

Single production lots of pepsin and test proteins were supplied to each laboratory. These proteins included: Ara h 2, β -lactoglobulin (BLG), bovine serum albumin (BSA), concanavalin A (ConA), horseradish peroxidase (HRP), ovalbumin (Ova), ovomucoid (Ovm), phosphinothricin acetyltransferase (PAT), ribulose biphosphate carboxylase (RubisCO), and soybean trypsin inhibitor (STI). The proteins were selected to include potent human food allergens (e.g., Ara h 2), proteins consumed in abundant amounts without any evidence of human allergenicity (e.g., RubisCO), and a biotechnology protein not suspected to be allergenic (e.g., PAT) (Hefle et al., 1996). The proteins selected were expected to be stable, intermediately stable, and unstable to pepsin exposure. A ratio of 10 units of pepsin activity/ μ g test protein was selected, which represented a weight-to-weight ratio of 3:1 (pepsin:test protein).

The stability results of the ten examined proteins were consistent between laboratories for full-length proteins (Thomas et al., 2004). For proteolytic fragments, however, some of the results were less consistent in positively identifying the presence of fragments, which was likely due to slight variations in gel fixing/staining procedures. In comparing pH 2.0 vs. pH 1.2, protein digestion at pH 2.0 resulted in slightly slower rates of full-length protein and fragment degradation, but did not alter the overall sensitivity of a protein to digestion. Results at pH 1.2 were more consistent than at pH 2.0, with 91% and 77% agreement between laboratories, respectively. This investigation was not intended to rank the allergenic potential of these proteins; therefore statements comparing the profile of digestibility with relative estimates of allergenicity were not discussed as the SGF testing is not a mimic of mammalian digestion, but a characterization to support one of several allergenicity assessments. Overall, the publication of the SGF protocol has led to a robust *in vitro* characterization assay that is utilized internationally across industry. The protocol has served to provide industry with a consistent approach to regulators in assessing novel protein safety.

Recent findings continue to question the value and applicability of digestibility in the overall risk assessment of GM crops (Fu et al., 2002; Fu, 2002; Herman et al., 2006; Moreno, 2007). Stability is not a feature shared by all known allergens and therefore, it is not a determinative feature that can be used alone to predict the allergic potential of novel foods. Some researchers have recommended a more realistic assessment using sequential digestion, first with SGF followed by simulated intestinal fluid, as would be found in a mammalian digestive system (Delaney et al., 2008; Verwei et al., 2003). It has also been recommended that a rate of digestion technique, using SGF, may be useful for certain proteins that do not readily digest (Herman et al., 2003). Generally, the role of digestion-resistant fragments derived from some full-length proteins, as well as the role of the food matrix in protein absorption and potential stimulation of the immune system cannot be inferred using the standardized *in vitro* SGF test (Moreno, 2007). Consideration of a SGF test protocol in combination with other physiologically relevant *in vitro* digestion model(s) and possibly utilizing immunological (IgE recognition) assays may increase the value of stability data in the overall risk assessment of GM crops. Improved testing protocols may lead to a better understanding of stability as it relates to the relative potency of known allergens, which in turn may lead to a better characterization of enzyme stability in safety assessments.

3. Bioinformatics workshop

The ILSI HESI PATC hosted an expert workshop in February 2005 in Spain that produced a review of the state-of-the-science for conducting an amino acid sequence homology/bioinformatics evaluation for novel proteins utilized for GM crops. This workshop focused not only on the specifics associated with bioinformatic searches, but also on the utility and methods of allergen specific IgE testing. Both the executive summary (Thomas et al., 2005a) and individual manuscripts of each presentation topic were published (Gibson, 2006).

For proteins to be considered for allergen databases, participants agreed that the protein must have clear evidence of IgE-binding and minimum criteria for numbers of publications as well as use of appropriate sera to ensure that only sequences with the adequate clinical evidence for allergenic potential are included. The workshop participants also agreed that standards for database maintenance, management, archival, retrieval, and updating procedures should be designed prior to the development of any database. Additionally, it was agreed that a diversity of database approaches (such as exists now) is desirable and that allergen databases should be publicly accessible.

The main purpose of a bioinformatics screen of novel proteins for use in food crops is to determine if the introduced protein shares any amino acid sequence similarities with known allergens (as well as with known toxins or other health-compromising proteins). Shared similarity with a known allergen may be indicative that the introduced protein would be cross-reactive and could elicit a clinical reaction in a sensitized population. Today there are no established guidelines by which to conduct bioinformatics searches for comparisons of transgenic proteins with protein toxins, only with protein allergens. Bioinformatics alone, however, cannot be used to predict whether a protein will become an allergen *de novo* and therefore, a weight-of-the-evidence approach is utilized that incorporates bioinformatics with other *in vitro* characterization approaches. If a novel protein demonstrates similarity to an allergen or is from an allergenic source, it is critical to consider the need for further testing such as specific IgE binding studies using sera from well-characterized allergic subjects, to confirm the biological relevance of the bioinformatic match (Poulsen et al., 2006).

The current bioinformatics evaluation practice for allergenicity assessments of a novel protein sequence consists of: (1) a full-length sequence similarity search with the novel protein against an allergen database (e.g., <http://www.allergenonline.com>), and (2) a sliding window search designed to identify small peptides (i.e., theoretical IgE epitopes) of a novel protein that may have exact matching identity with known allergens.

The full-length sequence similarity search is performed using publicly available search programs such as FASTA or BLAST. A cutoff value of 35% or greater shared identity over at least 80 amino acids was proposed by a FAO/WHO scientific advisory panel (2001) and is used for determining whether a novel protein has the potential to cross-react with IgE directed against known allergens. Conference participants were in general agreement that this type of search utilizing the 35% threshold was useful for identifying allergic cross-reactivity. The sequence analysis is generally performed either with the full sequence or portions of the sequence prepared as 80 amino acid sequence windows. Although there are various strategies to preparing a bioinformatic analysis, investigators have reported that the use of a full-length sequence FASTA search (i.e., where the focus is on overall sequence alignment vs. 80 amino acid search windows) produced fewer false positive and equivalent false negative rates compared to the 80 amino acid sliding window FASTA search (Ladics et al., 2007; Cressman and Ladics, 2009).

The current strategy of using bioinformatics is one of analysis of similarity, with implications for shared structure and function, rather than one of prediction. The use of a 35% shared identity threshold over an 80 amino acid window may not be very accurate in identifying biologically relevant cross-reactivity with allergenic proteins i.e., a high rate of false positive alignments are consistently identified. Others have reported that for cross-reactivity to occur, a higher degree of similarity is needed, likely in excess of 50%, across shared sequence spans between the target protein and allergen (Aalberse, 2000; Radauer and Breiteneder, 2006). New computer assisted algorithms for identifying specific protein structures or motifs specific to allergens were also presented and may show promise for identifying potential cross-reactive allergens with further development and validation (Goodman et al., 2002; Ivanciuc et al., 2002).

An epitope-focused sliding window search was first recommended by Metcalfe et al. (1996) to determine if discrete regions of proteins that represented potential IgE-binding epitopes would share identity with known allergens. The recommendation of a sliding window search size of 8 contiguous identical amino acids was considered a conservative approach that was an attempt to consider the known sizes of IgE epitopes, while acknowledging the very limited experimental data regarding the size and structure

of confirmed epitopes on known food allergens. In 2001, an FAO/WHO scientific advisory panel recommended that the size of the sliding window be decreased due to experimental evidence that IgE binding epitopes could be as small as 6 amino acids. However, publications have since demonstrated that a match of 6 amino acids with any allergen occurs too frequently by chance when assessing shared sequence with bioinformatics, which severely limits the utility of this criteria with bioinformatics for assessing allergenicity (Hileman et al., 2002; Stadler and Stadler, 2003; Silvanovich et al., 2006; Ladics et al., 2006). The workshop participants agreed that the 6-mer sliding amino acid window searches do not provide any value for the bioinformatics evaluation of novel proteins in a protein allergenicity safety assessment. This was in general agreement with the *Codex Ad Hoc* Inter-governmental Task Force on Foods Derived from Biotechnology Report (Alinorm 03/34A, 2003) that stated any sliding window search should be performed with a scientifically justified search window size.

Information presented at the 2005 meeting also indicated that more than 150 linear IgE-binding epitopes had been determined and published for numerous allergens. More recently, Bannon and Ogawa (2006) showed that sizes of these epitopes ranged from 4 amino acids in length to as much as 23 amino acids long with the vast majority of epitopes consisting of at least 8 amino acids. Even with the expanding library of known allergen epitopes, the utility of assessing these is not yet proven. The variable quality of the IgE epitope data, the variability in the methods used to identify the epitopes, the limited databases of known epitopes, and the recognition that there is very little information available regarding conformational epitopes, are the reasons for the reluctance in adapting a strategy that uses an IgE epitope library at this point in time.

The workshop group also discussed the value of targeted serum screening as recommended by FAO/WHO (2001). The consensus of the participants was that the utility of targeted serum screening for predicting novel protein allergenicity has not been demonstrated, nor have standard methods been established and validated that would make this type of *in vitro* screening possible. There was recognition that bioinformatics methods associated with allergy assessment were evolving and new methods, once they are tested and validated, may lead to increased levels of confidence in identifying potentially allergenic novel proteins.

4. Serum bank workshop

ILSI HESI PATC hosted a workshop in April 2006 in South Korea to discuss the potential utility of an international serum bank. The specific objectives of the workshop were to review the state-of-the-science regarding patient diagnostic criteria for inclusion of material/samples in a serum bank, discuss a rational basis for conducting *in vitro* studies that utilize these sera to evaluate the allergenicity of novel proteins, and to identify appropriate methods for performing serological studies. As part of the effort to identify methods for serum studies, the workshop participants also addressed limits of detection, limits of quantitation, and accept/reject criteria for serum evaluation assays. A summary of the discussions from the Sera Bank Workshop has been published (Thomas et al., 2007a), as have the individual manuscripts based on the expert presentations (Ballmer-Weber et al., 2008; Barber et al., 2008; Goodman et al., 2008; Holzhauser et al., 2008; Ladics, 2008; Lee et al., 2008; Vieths et al., 2008).

There was agreement that serum screens may play a role in the allergy assessment of novel proteins, particularly when these studies utilize well-characterized patient sera to investigate potentially cross-reactive proteins identified through bioinformatics analyses. Criteria for sera selection for use in evaluating a novel protein's potential for cross-reactivity were discussed in detail. Attendees

agreed that 'well-characterized' sera should ideally include confirmation of clinical reactivity of the donors by double blind, placebo-controlled food challenge (DBPCFC) and a detailed clinical history, but if not possible, then the sera should be characterized using IgE binding studies and biological activity assays (e.g., skin prick testing or basophil mediator release tests). Assays employed to assess biological activity, however, can be technically challenging and require method validation in laboratories that would use them to further evaluate whether a protein with a bioinformatics match with an allergen is likely to cause clinical cross-reactivity.

IgE binding methods for diagnostic purposes and their potential utility and weaknesses for use in the safety assessment were also discussed during the February 2005 ILSI HESI PATC workshop (Thomas et al., 2005a; Gibson, 2006). Participants recognized the constraints around the availability of individual patient serum, which may limit the ability to conduct such studies. There was also general agreement that in most cases, IgE binding to carbohydrate domains is irrelevant to clinical allergy, unless there are multiple carbohydrates on the potentially allergenic protein (Van Ree et al., 2006). The need for careful clinical characterization of serum donors was also noted for the purpose of reducing potential for false negative or false positive findings.

It was also recognized that serum banks might be useful for tracking the development and prevalence of allergies over time. The most advanced regional sera banks from Europe and South Korea were described and differences, especially with regard to the criteria for sera inclusion, were noted. However, the consensus among participants was that while having an international sera bank could be very useful, from a practical point of view it created a number of obstacles involving the technical and ethical considerations associated with the use of human samples for research purposes. Instead, participants agreed that regional networks of clinicians would be a resource for identifying sources of well-characterized sera for testing novel proteins. These networks might also be utilized to conduct serum studies. However, the practical and ethical considerations would have to be more fully explored to develop a functional management paradigm and administrative infrastructure for such regional networks to exist.

As with all new testing methodology, vigorous validation processes must be utilized to ensure that the method is reliable, specific, and sensitive using appropriate positive and negative control proteins and extracts. While determining total IgE and allergen-specific IgE is relatively easy, it is much more difficult to distinguish the level of IgE binding at which there is an indication of clinically relevant allergic potential. Indeed, IgE binding is the basis for many of the false positive results incurred from the diagnostic testing of food allergy. The use of more physiologically relevant methods, such as basophil activation tests may help to identify the clinical relevance of the IgE reactivity of a particular serum. However, these tests are also well known to lack robustness, reliable outcomes, and accuracy (Ladics et al., 2008). Additional standardization and validation of such biological assays is necessary before using them in the safety evaluation approach.

5. Animal model studies

Historically, animal models have been valuable for characterizing adverse effects of various substances. Many have highlighted the need for an animal model that will characterize more definitively and holistically the allergenic potential of novel proteins expressed in agricultural biotechnology crop products (FAO/WHO, 2001; Alinorm 03/34A, 2003). During a workshop held in February 2001, experts shared their latest data on a wide range of animal models developed to evaluate the allergenic potential of proteins and foods.

While many animal models, such as the mouse, rat, pig and dog, have been used to better understand the mechanism of food allergy, only a few *in vivo* models have focused on the identification of intrinsic allergenic hazard of proteins in order to use them as a predictive tool for assessing the allergenicity of novel proteins (Helm, 2002; Ermel 1997; Buchanan 1997; Knippels and Penninks, 2002; Ladics et al., 2003; Betts et al., 2004; Birmingham et al., 2005; Dearman and Kimber, 2007; Herouet-Guicheney et al., 2008; Aldemir et al., 2009).

Although dogs and pigs demonstrate symptomology consistent with humans, practical issues limit their value. Specifically, these hurdles include the high cost of maintaining colonies of large animals and the lengthy testing time to gain meaningful results. However, rodents are relatively easy to rear, require lower quantities of test material, and shorter timing for results leading several research teams to further explore testing protocols for measuring both the sensitization and elicitation phases of allergic responses. Rodents have also been widely used to identify and characterize the toxicological impact of a variety of substances; therefore, there is a considerable body of data on rodent testing procedures and responses. However, this body of data on rodent responses to toxicants was, with some notable exceptions, generated in outbred species, not the inbred species used in immunological research. Mouse models offer considerable advantages compared to other models, particularly with availability of several inbred high IgE responder strains, and of various immunological and molecular reagents (Dearman et al., 2003). In addition, although some rat species have been shown to be high IgE reactors, e.g., Brown Norway rats, mice are also favored due to their smaller size and thus lower quantities of materials needed for analysis. Moreover, epitope similarities between mice and humans suggest that the mouse represents a valid model for evaluating human food allergy (Halay, 2003; McClain and Bannon, 2006).

Nevertheless, determining the characteristics of potential hazards, in addition to defining the likely conditions and extent of exposure, are essential for conducting human risk assessments. To date, none of the existing animal models that have been employed to identify potential protein allergenicity have been validated for use as a safety assessment tool. An evaluation of the Balb/c model was conducted between two laboratories (Dearman et al., 2003). Although the results demonstrated that this approach was accurate in identifying and ranking a small set of allergenic and non-allergenic proteins, further evaluation and validation with a larger set of test proteins and additional laboratories is needed. Moreover, it is generally considered that one model cannot likely predict all aspects of a food allergic response among various human populations exposed to potential protein allergens in the diet. For instance, a lack of symptomology that matches human clinical responses might limit the utility of the Balb/c or other murine models.

The ILSI HESI PATC organized a study involving four different laboratories to evaluate several mouse models. Several different protocols were evaluated to determine whether known protein allergens could induce a reproducible allergic reaction in four different strains of mice (A/J, Balb/c, BDF1, C3H/HeJ) and whether proteins consumed in abundant amounts without any evidence of human allergenicity did not induce a reaction. The various protocols differed in the routes of exposure (oral, intraperitoneal), use of adjuvant (presence or absence), number of exposures and doses, and the types of methods (homologous or heterologous Passive Cutaneous Anaphylaxis responses) and measured endpoints (number of responders per group, levels of IgG and IgE, clinical endpoints, mortality) (Thomas et al., 2005b).

The ILSI HESI PATC obtained purified major allergenic and putative non-allergenic proteins from bacteria or plant sources. The isolation and purification of this set of proteins were performed by

TNO Nutrition and Food Research Institute (Zeist, The Netherlands; <http://www.tno.nl/index.cfm>), which provided quantities of each to participating laboratories. The major allergens were from peanut, Ara h 1 and Ara h 2, and milk, β -lactoglobulin. The putative non-allergenic proteins were from soybean, soy lipoxygenase (which cross-reacts with the major allergen of egg, ovalbumin) and from spinach leaves, RubisCO.

It was not possible to differentiate between the major allergens and the putative non-allergens in any laboratory using the strains and endpoints evaluated (Thomas et al., 2005b). For example, all the laboratories demonstrated very high passive cutaneous anaphylaxis (PCA) responses to RubisCO from spinach in the absence of adjuvant. In two laboratories, the PCA response to RubisCO was significantly greater than the response to Ara h 1, a very potent allergen to humans. In addition, the nature and magnitude of the responses varied considerably between the different mouse strains. After examination of the data and the certificates of analyses of the tested proteins, there were discrepancies observed with regard to the purity of several proteins. One of the major findings was the presence of a high concentration of endotoxin, [lipopolysaccharides (LPS)], contamination in some of the proteins, which may have contributed to the poor differentiation between allergens and non-allergens. The contamination by LPS of the RubisCO batch was higher compared to other proteins (~2200 EU/ml LPS).

There was also a potential problem of protein conformation arising from the purification method for one of the two major human peanut allergens. Ara h 2 failed to induce a positive IgG response. Thus in absence of immunization, one laboratory decided not to measure the IgE response. Other laboratories, except one that measured clinical endpoints, observed a negative IgE response.

In summary, the comparison of these different protocols in the ILSI HESI PATC study was not conclusive for the ability of the murine model to identify an allergenic hazard and further characterize it. The key learning from this study was that well-characterized test material should be utilized. In particular, the presence of endotoxins may dramatically impact and skew the immune response (Eisenbarth et al., 2002; Delayre-Orthez et al., 2004).

Further validation work is necessary before mouse models may be considered for the evaluation of the potential allergenicity of proteins. In addition, putative non-allergenic proteins or rare allergens may possess some allergenic properties that have not been fully described in humans, suggesting an in depth understanding of the immune tolerance system is required in order to develop a model. An additional challenge in developing any animal model is the relatively poor characterization of allergenic potency in humans for most allergens with most, if not all, data being related to elicitation potency rather than sensitization potency. Furthermore, a better understanding of the etiology of food allergy would be helpful to establish reliable *in vivo* animal models for predicting protein allergenicity and human food allergy.

6. Food processing workshop

Allergenic proteins are typically present at relatively high levels in foods, some in amounts of 1% or higher of the total protein (Metcalfe et al., 1996). However, it is not clear how this high concentration impacts the allergenic potential of the final food product. It also is unclear how exposure can cause an allergic reaction in some individuals while others, with the same exposure, demonstrate no adverse responses. Some research has found that processing can reduce exposure and therefore the risk of allergic reaction, but others have shown that processing can enhance allergenicity. A better understanding of the impacts of food processing on proteins, especially the allergenic potential of food proteins, could be helpful for the safety assessment process.

It is well established that food and food ingredients have many different forms and are subjected to a wide variety of processing conditions that not only produce food products (e.g., oil, flours, etc) improving digestibility and palatability, but also preserve them by inactivation or removal of toxins and microbes. Processing of foods and food ingredients can have a dramatic effect on all components within the food, such as carbohydrates, oils, flavors, and proteins, including allergenic proteins. The nature of these changes to allergenic proteins was considered in an ILSI HESI PATC international workshop of experts in June 2006 in Portugal. The workshop focused on the impact of food processing on the sensitization/induction phases of food allergy and the bioavailability of allergens to the immune system. A report summarizing the expert discussion has been published (Thomas et al., 2007b).

The experts provided data on the stability, digestibility and allergenicity of food allergens during a variety of differing food processing techniques and their individual manuscripts based on their presentations have been submitted for publication. Some of the methods discussed included separation, isolation, purification, mechanical, thermal and biochemical processes, high pressure and electric field treatments, and irradiation. It was generally recognized that these effects are both complex and that evaluations are limited by assay methodology and a lack of basic data for allergens. Thermal processing by moist or dry heat (i.e. Maillard reaction) and enzymatic browning likely have the largest impact on the allergenic effects of foods. More emphasis on a search for processing methods that may reduce allergenicity without affecting functionality of known allergenic proteins would be helpful.

For some types of food processing, the IgE epitopes for allergenic proteins were destroyed and the IgE reactivity was reduced or lost, but under other conditions the reactivity was enhanced. Thus, the ability to predict how processing will impact the allergenicity potential of proteins requires more study before it can be utilized for the risk assessment process. Even thermal stability, which is included in some risk assessments, is of limited value. Specifically, improved testing protocols for investigating proteins contained in allergenic foods along with a better understanding of how individuals and populations react to modified allergenic foods are needed.

Nevertheless, known processing methods can totally eliminate allergenic risk by simply decreasing exposure to the protein. For instance, edible oils that are refined, bleached and deodorized do not pose a risk to allergic individuals. This is consistent with the very low levels of detectable proteins, including allergenic proteins, in the oil (Hefle and Taylor, 1999; Crevel et al., 2008).

7. Participation in Codex meetings

The *Codex Alimentarius* Commission initiated an international *Ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnology that met for the first session in March 2000 to develop “a set of broad general principles for risk analysis of foods derived from biotechnology” (Alinorm 01/34). After a joint FAO/WHO expert consultation on the allergenicity of foods derived from biotechnology (FAO/WHO, 2001), this Task Force agreed to develop a separate annex containing detailed procedures for allergenicity assessment and established an open-ended Working Group that met in September 2001.

Prior to the Working Group meeting, the HESI PATC group hosted a meeting to discuss the FAO/WHO allergy expert report with the goal of presenting information as well as soliciting input to the assembled experts on a harmonized approach that would serve as a global allergy safety strategy. Several members of the FAO/WHO expert consultation participated along with representatives from Health Canada, the United States Environmental

Protection Agency (US EPA), the US FDA and the member companies. Health Canada had the leading role in integrating the essence of the FAO/WHO report into the draft guidelines that were issued by Codex in September 2001. The representatives from the various regulatory agencies, in particular Health Canada, urged PATC to provide their perspective on a scientific-based path forward. The PATC prepared formal comments that were submitted to Codex on behalf of the ILSI HESI organization which then led to an invitation to PATC representatives to the Working Group meeting in September and subsequent Codex meetings (Alinorm 03/34; Alinorm 03/34A). A final proposed draft annex on the Assessment of Possible Allergenicity of the Draft Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants was developed and eventually accepted (Alinorm 03/34A) in 2003.

8. Harmonizing allergy safety testing

An integral aspect of the ILSI mission is to partner with government and academic scientists to proactively participate in both the advancement of science and information sharing. Part of the PATC tactic in harmonizing global acceptance of a unified approach to allergy safety has been to solicit concerns and organize strategies to address concerns. Usually published workshops serve best to bring together a consensus view and in some cases (Thomas et al., 2004) validation studies are organized for addressing the need for standardized protocols for use in regulatory safety studies. In various regions around the world, government authorities have teamed up with experts from academia and industry scientists to discuss and work through many difficult scientific issues, some of which have been highlighted in this article. The PATC members have convened meetings with government authorities (USA, Japan, Brazil, Argentina, South Korea; Philippines, Thailand – see Table 1) in order to share knowledge regarding current state-of-the-science around protein allergenicity assessments and to improve communications with regulators who face unique regional challenges in conducting risk assessments of novel proteins, especially those in genetically modified (GM) crops. The PATC reaches out internationally by both publishing in the peer-reviewed literature (Thomas et al., 2004, 2005a,b, 2007a, b) and presenting results at scientific meetings (Table 1., American Academy of Allergy, Asthma, and Immunology, Society of Toxicology, Eurotox, ICT meetings). Moreover, the PATC participates in the Codex discussions (Section 8) which ultimately become the driving force for establishing consistent approaches for the regulatory review of biotechnology products. A full listing of the outreach activities of the HESI PATC are provided in Table 1.

9. New initiatives in allergy safety science

A group of international experts who participated in the Sera Bank workshop and whom have agreed to participate further have recently assembled to establish a regional network of clinicians. The goal of this group is to identify sources of well-characterized sera, setting science-based criteria for inclusion of such sera for regulatory or research studies, and in some cases, perform sera studies. A functional management paradigm and administrative infrastructure for this regional network is currently under development. The organizational goal of this group is to have regional clinicians that can help address regionally specific concerns regarding potential sera studies.

A workshop discussing both current and new applications of methods for characterizing potential protein allergenicity was organized in October 2007. Publications from this workshop are currently in review and the executive overview has been published

Table 1
Outreach activities of the ILSI HESI PATC.

Date	Location	Participants (aside from HESI)	Topic(s)
March 2002	Tokyo, Japan	Japanese Teachers Association	Overall safety assessment process for biotechnology derived crops
June 2002	Washington DC, USA	National Academy of Sciences	HESI PATC activities
August 2002	Washington DC, USA	US FDA Food Advisory Committee	Potential allergenicity assessment for biotechnology crops
October 2002	Washington DC, USA	North American Regulatory Authorities: Mexico, USA, Canada and IFBiC	Recent activities related to Crop biotechnology
June 2004			
April 2008			
March 2003	New Orleans, USA	American Academy of Allergy, Asthma, and Immunology (AAAAI) Meeting	Pepsin digestibility protocol
September 2003	Florence, Italy	European Societies of Toxicology (EuroTox)	Pepsin digestibility protocol
September 2003	Tokyo, Japan	Japanese Regulatory Authorities and Allergy Researchers	HESI PATC activities with a special focus on bioinformatics
January 2004	Miami, Florida, USA	ILSI Annual Meeting Participants and Scientific and Regulatory Experts in the Areas of Food Allergy	Assessment of allergenic potential of crop biotechnology-based products
May 2004	Washington DC, USA	US FDA	HESI PATC activities
July 2004	Tampere, Finland	International Congress of Toxicology	Recent updates on aspects of allergenicity assessment for crop biotechnology based products
March 2005	San Antonio, USA	AAAAI Meeting	Mouse models for predicting protein allergenicity
March 2005	New Orleans, USA	Society of Toxicology Meeting	Mouse models for predicting protein allergenicity
April 2006	Seoul, South Korea	Meeting with Korean FDA Officials	Weight-of-evidence allergenicity evaluation; sera bank discussion
November 2006	Buenos Aires, Argentina		
	Sao Paulo, Brazil	Local industry representatives	Weight-of-evidence allergenicity evaluation
May 2007	Washington DC, USA	US FDA	Protein allergenicity assessment
November 2007	Sao Paulo, Brazil	Workshop on the safety evaluation of protein	Weight-of-evidence allergenicity evaluation
October 2008	Washington DC, USA	US EPA Workshop: Research to Improve Safety Assessment of Biotechnology Products	New tools for assessing the allergenic potential, with a focus on proteomics, animal models, bioinformatics
February 2008	Tokyo, Japan	HESI Member Stewardship and Development Meeting	New methods for evaluating allergenicity
February 2008	Manilla, Philippines	Seminar on Novel Protein Safety Evaluation	Weight-of-evidence allergenicity evaluation
November 2008	Cebu, Philippines	Symposium on Biotechnology and Nutritionally Enhanced Food and Crops	Recent HESI PATC activities and research
November 2008	Bangkok, Thailand	Seminar on protein allergenicity, Weight-of-evidence (WOE) allergenicity evaluation	Recent HESI PATC activities and research

(Thomas et al., 2008) The goals of this workshop were; (1) to discuss the current safety assessment approach and methods commonly used (e.g., bioinformatics), (2) to discuss endpoints being evaluated in various research programs and to assess their potential to be used in the safety assessment process of novel proteins (e.g., animal models), (3) proteomics applications that could be useful for allergenicity evaluation, and (4) to review and discuss *in vitro* assays that may impact the safety assessment process (e.g., histamine release assays). The PATC continues to lead this effort in addressing the ever-expanding scientific understanding of allergy by developing studies and workshops centered on agro-nomic crop proteomics.

Gaining interest in the allergy community within the last several years is the potential application of “omics” technology. The emergence of advanced analytical profiling techniques (i.e., genomics and proteomics) in combination with bioinformatics offers a relatively new and powerful tool for identifying the protein profile of organisms. They have already been identified as a potential tool for risk assessment (Kuiper et al., 2003). However, in order to interpret proteomic data generated on GM crops, it is essential that background data be first generated on non-GM crops. To use proteomics in allergy, there must first be a concerted effort to understand the natural variation of expression of specific allergenic proteins in non-GM crop varieties, similar to work that has progressed for years in compositional assessments of foods. Crop varieties are developed for many different growing conditions and therefore, they possess widely divergent characteristics as observed in the classical composition and nutrition evaluation. Initial proteomics protocols and studies should determine the natural variation of the protein profile of several non-GM crop varieties grown across different geographies under different environmental conditions and pest loads to identify the background profiles of crops of interest prior to specifically focusing on allergenic assessments for regulatory safety review.

The PATC remains committed to partner with government and academia to discuss scientific issues addressing the potential allergenicity assessment of novel proteins and is prepared to openly evaluate solutions and communicate results with the goal of driving consensus in allergy safety using the best scientific principles and procedures.

10. Summary

Over the last 15 years, approaches to identifying potential food allergens for purposes of safety assessment of GM crops have been developed and modified, and research continues to better understand what characteristics make a protein an allergen and provide additional predictive tools. As noted above, the PATC has taken a dynamic role in these activities. Despite these efforts, however, no single factor has yet to be recognized as predicting food allergy in humans, and therefore, a weight-of-evidence approach as recommended by the *Codex Alimentarius* Commission (Alinorm 03/34A) remains the best approach for allergy assessment of novel proteins in food or feed. These assessments are currently based on what is known about food allergens such as; history of exposure and safety of the gene product(s) (i.e., is the source of the gene for the protein known to induce allergy), protein sequence (*in silico* amino acid homology comparisons to known human allergens) and physico-chemical properties (resistance to pepsin digestion *in vitro*, glycosylation status, heat stability). When appropriate (i.e., an amino acid sequence match is observed to a known allergen or the novel protein is derived from a known allergenic source), specific IgE binding studies using well-characterized serum from individuals allergic to the identified source or skin-prick testing with relevant subjects are also conducted.

Importantly, the current weight-of-evidence approach has been successful. There have been no reported cases of human allergic

responses to any approved agriculture biotechnology product. However, as science and technology evolves, additional endpoints or refinements to current ones may add to the scientific basis for safety assessment. One important need regarding the current assessment parameters is the development of standard procedures for conducting the bioinformatic and serum screening analyses, similar to that already developed for pepsin resistance (Thomas et al., 2004). In addition, the current bioinformatic analysis could be improved by utilizing statistical calculations measured by *E* score, which are already embedded in the FASTA algorithm (Cressman and Ladics, 2009; Silvanovich et al., 2009). By switching to a “significance” threshold measured by *E* score rather than predefined length and identity thresholds; the number of false positives may be reduced. Data with additional proteins are needed to confirm this hypothesis.

Structural bioinformatics and proteomic analysis are two emerging areas in evaluating potential protein allergenicity. While such endpoints look promising, additional research and validation are needed to determine what utility they may have in the safety assessment process. Significant progress has been made since 2005, when *in silico* methods were the topic of a HESI workshop (Thomas et al., 2005). Using structural bioinformatics, a tiered or weight-of-evidence approach could potentially be developed within the protein structure analysis. This could include assessing potential allergens according to Pfam, property distance (PD) values, and 3-D structure (Ivanciuc et al., 2008a), in addition to conducting the > 35% shared sequence identity over 80 or greater amino acids using FASTA. This evaluation may provide a more accurate identification of potential allergens versus non-allergenic proteins.

Proteomics is a rapidly progressing technology used for a wide range of applications (Canovas et al., 2004; Fiehn et al., 2001; Jorin et al., 2007). It has been suggested that proteomics could be utilized to characterize the possibility that biotechnology may alter the amount of an existing (endogenous) protein allergen(s) in food crops, thus potentially increasing exposure doses. The natural variation range of protein allergen levels in non-GM crop species, however, must be determined before any of these methods can be utilized to evaluate and interpret potential safety issues with protein levels in GM crops. Research on the biological variation of allergen expression in non-transgenic crops would be critical, especially those food crops that are most commonly the focus of biotechnology, such as soybean, rice and perhaps in the future, wheat.

Animal models have proven value for the assessment of toxicological effects, but so far an animal model has not been validated to assess potential allergenicity of novel proteins or GM crops. Most animal models focused on allergy are able to evaluate allergenic mechanisms; however, their value as screening tools to differentiate between allergenic and non-allergenic proteins is limited at best. Once there is a better understanding of the etiology of food allergy, it will undoubtedly assist in the development of a useful *in vivo* animal model for predicting protein allergenicity.

Finally, since IgE responses may occur in the absence of an adverse clinical reaction in both humans and animal models, basic research to better understand co-factors that contribute to anaphylactic/systemic reactions are needed to better understand and to refine our approaches to safety assessment in the future. Likewise, research to improve our knowledge of oral tolerance will help to refine future safety assessments and add value to the existing process for ensuring food safety.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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