Protein Safety Assessments – Toxicity and Allergenicity

Laura Privalle, Ph.D.
BAYER CropScience
HESI PATC
ILSI IFBiC

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Biotechnology is an Extension of Traditional Plant Breeding

TRADITIONAL PLANT BREEDING

Many genes are transferred

PLANT BIOTECHNOLOGY

A single gene is transferred
Safety for humans, animals and the environment

- Safety assessment for plant biotech products is mandatory worldwide
- Considers human + animal health as well as environmental safety
- Approval only if authorities conclude:
  ➔ Genetically optimized plant is as safe as a conventionally bred plant
Assessment includes

- Molecular analysis
- Agronomic performance
- Compositional studies
- **Protein safety**
- Environmental safety studies
- Monitoring and traceability
Protein Safety

- Weight of evidence approach
- Toxicity assessment
- Allergenicity assessment
Protein Safety Studies Include

- Protein level in plant tissues – Expression study
- Protein characterization - Test substances
- Equivalence – Plant produced compared to test
- Toxicity assessment – Using test substance
- Allergenicity assessment – Weight of evidence
Why proteins do not typically represent a hazard

- Proteins are an essential part of the diet (avg. consumption 100 g/day).

- Digestive systems have evolved to convert the protein to its building blocks for incorporation (very efficient only 6 – 12 g protein lost/day). The human body synthesizes approx. 300 g protein/day.

- Protein consumption is not inherently associated with adverse effects.

- A small number of proteins are known to be toxic.
**Protein Toxins**

- Vast majority are from bacterial sources
  - Cholera, *E. coli*, anthrax, botulism
  - Small number of plant toxins or antinutrients
    - Ricin, lectins, protease inhibitors
- Primary structures are known and included in sequence databases
- Act acutely
- Extremely low levels cause toxicity
  - ng or µg /kg body weight
Weight of Evidence Approach

Two Tiers:

- Basic Assessment
- Supplementary Assessment

Basic Assessment

- History of safe use
- Bioinformatics sequence homology assessment
- Source of gene
- Mode of action
- Range and pattern of expression levels
- Stability of protein to temperature, pH, and digestive enzymes

REQUIRE SMALL AMOUNTS OF PROTEIN
CAN BE PERFORMED EARLY IN THE DISCOVERY PROCESS
Toxin Homology Searches

- No publicly available toxin/antinutrient database.
- GenBank non-redundant peptide sequence database used.
- Manual visual inspection of results generally employed.
- Currently the E-score used as the cut-off is highly conservative
- Task Force 13 is being convened to address issues here
Supplementary Assessment

PROTEIN -BASED STUDIES
Acute oral mouse study, single dose (2000 mg/kg bw), 14 day observation
Repeat dose (28 day) using mice
- diet incorporated or daily gavage
- limit dose or several doses with 100 – 1000 x safety margins

Hypothesis-based testing to be determined on a case-by-case basis

REQUIRES LARGE AMOUNTS OF PROTEIN
Proteins are tested at levels equal to a 75 kg man eating 50 tons of corn for lunch.
Allergenicity Assessments

• Introduction to allergenicity
• Risks
• Weight of evidence approach
• Specific examples
Adverse Reactions to Food

Adverse Reactions to food

May occur in all individuals
- Toxic
- Microbiological
- Pharmacological

Occurrences only in some susceptible individuals
- Food hypersensitivity
  - Non-allergic food hypersensitivity
    - Unknown mechanism
    - Metabolic abnormality
  - Aversion, avoidance and psychological intolerance
- Food allergy
  - IgE-mediated food allergy
  - Non-IgE mediated Food allergy
Prevalence of Food Allergy

Prevalence of IgE antibody-mediated food allergies among the general population-

1-2\% of adults
4-6\% of children

6-7 million (U.S)

Public Perception: 30\%
Common Allergenic Foods

Eight foods or food groups account for over 90% of food allergies (peanuts, soybeans, cow’s milk, hen’s egg, fish, crustacean, wheat, and tree nuts)

*Prevalence to allergy varies geographically*

- Buckwheat and rice allergy: Asia
- Fish allergy: Scandinavia
- Walnut/pecan: U.S.
- Hazelnut: Europe
- Fruit allergy: Spain

“Emerging”: avocado/kiwi; sesame seeds; spices

Disease management by avoidance (elimination diets)
Food Allergies: Key Points

- Affect a small percentage of the population
- Of the tens of thousands of proteins in food, few (approximately 200) are actually food allergens.
- Reactions can occasionally be severe
- Treatment for true food allergies
  - Specific avoidance diets
What Are The Protein Allergenicity Concerns with Agricultural Biotechnology?
Categories of Potential Health Risks Relative to Allergenicity

- Transfer an existing allergen or cross-reactive protein into another crop.
- Creation of food allergens de novo (i.e., potential to become a new allergen.)
- Alteration or quantitative increase of endogenous (existing) allergens (i.e., increasing the hazard of currently allergenic foods)
What steps can be taken to minimize these risks?

• Historical perspective
  – 1996 ILSI Decision Tree
  – 2001 FAO/WHO Expert team decision tree
  – 2003 Codex Weight of evidence approach
# Categories of Potential Health Risks Relative to Allergenicity

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<thead>
<tr>
<th>Risk:</th>
<th>Technology to reduce risk per CODEX (2003):</th>
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<tbody>
<tr>
<td>Transfer an existing allergen or cross-reactive protein into another crop</td>
<td>Bioinformatics/Immunological methods</td>
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<tr>
<td>Creation of food allergens <em>de novo</em></td>
<td>Physical properties of protein (e.g., stability in SGF)</td>
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<tr>
<td>Alteration or quantitative increase of endogenous (existing) allergens</td>
<td>Immunological methods, proteomic approaches</td>
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Allergenicity Assessment

- Sequence homology comparison with known allergens
- Source of gene
- Digestive fate study
- Stability to heat and processing
- Examination of alteration of endogenous allergen levels (case-by-case basis)
- Glycosylation analysis
- Sera screening (case-by-case basis)
Amino Acid Sequence Analysis (Bioinformatics)

- Necessary for all introduced proteins
- Amino acid sequences of allergens present in public domain databases (GenBank; SwissProt) utilized to construct allergen databases
  - Industry sponsored ‘common’ allergen database at Univ. Nebraska
- Evaluate sequence homology for structural relationships and potential epitope matches using local alignment programs, such as FASTA.
  - >35% similarity over an 80 amino acid window; 8 contiguous identical amino acids
Welcome to AllergenOnline.org

AllergenOnline provides access to a peer reviewed allergen list and sequence searchable database intended for the identification of proteins that may present a potential risk of allergic cross-reactivity. This website was designed to help in assessing the safety of proteins that may be introduced into foods through genetic engineering or through food processing methods. The objective is to identify proteins that may require additional tests, such as serum IgE binding, basophil histamine release or in vivo challenge to evaluate potential cross-reactivity.

The database is updated annually. Version 4 was released on a public website in 2004. The database is freely accessible with the intent of providing a simple and useful tool that may be useful in food safety evaluations.

Features and Tools Available.

Sequence search routines for food safety

- We continue to provide simple amino acid search routines to allow you to compare a protein sequence with the sequences in the current AllergenOnline database, which is updated on an annual basis. This is intended primarily for evaluating new proteins in Genetically Modified crops or in Novel Foods.
- Search for full-length alignments by FASTA: The most predictive search is the overall FASTA alignment (see FASTA Help Page), with identity matches greater than 50% indicating possible cross-reactivity (Aalberse, 2000).
- Search for 80 amino acid alignments by FASTA: A precautionary search using a sliding window of 80 amino acid segments of each protein to find identities greater than 35% (according to CIARB/FAO guidelines, 2003).
- Search for 8 amino acid exact match: An 8-amino acid short-sequence identity search is provided since some regulatory authorities demand results of this extremely precautionary search. Our scientific opinion is that there is no evidence that an 8 amino acid match will identify a protein that is likely to be cross-reactive and could be missed by the conservative 80 amino acid match (35%). In our experience, isolated identity matches of 8 contiguous amino acids occur by chance alone at some modest rate, matches of 7 and 6 occur more commonly. Experience (published and unpublished) demonstrates that two proteins sharing only a single short identity match of from 6 to 8 contiguous amino acids do not share IgE binding in the absence of more extensive identity alignments (at least >35% identity over 80 or more amino acids). And that sequences sharing less than 50% identity over their full-lengths are rarely cross-reactive. Thus we recommend not using these short identity matches as there is no scientific evidence that they predict IgE cross-reactivity and they do not predict shared clinical activities.
Amino Acid Sequence Analysis (Bioinformatics)

What are some issues?

- Lack of a comprehensive list of IgE binding epitopes
- Need to establish validation criteria and minimum positive cutoff values
  - Current recommendation: > 35% identity to a known allergen
- 8 contiguous identical amino acids
- Actually need two epitopes to cause the response cascade – potentially could be the case that the protein forms a dimer or multimer – then one is sufficient.
Specific IgE Sera Screening

- For proteins originating from an allergenic source, or having significant homology with a known allergen, specific serum screening is conducted.
- Sera screening may also be conducted if sequence homology to a known allergen was determined during the bioinformatics analysis.
- An issue of critical importance to sera screening is the availability of well characterized, quality human sera from a sufficient number of patients.
Areas of Consensus- Sera Screening

- For IgE sera screening studies, should strive to use individual sera.
- Use well-characterized sera, establishing a link between the sera sample and a DBPCFC is desired.
- To demonstrate a protein is an allergen, recommend at least two assays: 1) IgE binding and 2) biological activity (i.e., BHR or SPT).
Sera Screening
What are Some Issues?

- Sources of positive sera are limited (most likely to obtain sera from individuals allergic to major allergenic plants)
- Criteria for selection of positive sera needs to be outlined and standardized.
- Differentiating between meaningless apparent antigen specific IgE and IgE binding that could provoke a response.
- False positive and false negative rate
**In Vitro** Pepsin Resistance

- Protein resistance to pepsin evaluated in simulated gastric fluid (pH 1.2) containing 10 units pepsin activity/µg protein.
- Digestions performed for time intervals 0, 30 seconds, 2, 5, 10, 30, and 60 minutes at 37°C.
- Samples (each protein at each time point) then analyzed by SDS polyacrylamide gel electrophoresis and/or western blot analysis.
- A standardized protocol for evaluating the *in vitro* pepsin resistance of proteins was established (Thomas *et al.*, Regulatory Toxicology Pharmacology, 39:87-98, 2004).
- Although a correlation between pepsin resistance and allergenic potential has been proposed, the relationship is not absolute.
Can Animal Models Identify Allergenic Food Proteins?

- Very active area of research, but no consensus (rodents, dogs, pigs)

- Definite need for further evaluation
  - Assay selectivity
  - Assay sensitivity
  - Broad testing with a range of proteins

- Presently, no animal models (rodent or nonrodent) have been well validated or are widely accepted.
Animal Models
What are the issues?

- No validated model available
- What is the most appropriate endpoint or design for an animal model?
- What constitutes a positive allergic response?
- Route of exposure?
- Form of protein to be tested?
Conclusions

- Probability of an introduced protein being an allergen is extremely low

- Definitive methods are in place to detect the transfer of known allergens

- Currently, a combination of genetic and physiochemical criteria are available to evaluate the allergenic potential of novel proteins
Conclusions continued

- Use “weight of evidence approach” in determining the allergenic potential of foods derived from biotechnology

- Other endpoints are under development (e.g., animal models) or require further evaluation (targeted serum screening)
  - Positive/negative predictive value; clinical relevance
  - Availability of well-characterized patient sera

(Acknowledgement: Many of these slides or portions of these slides were originally prepared by members of the HESI Protein Allergenicity Technical Committee)
No scientific evidence that a biotech protein or a GM crop increased allergenic risk to the susceptible public
Thank you