Agricultural Biotech Protein Allergy Assessments

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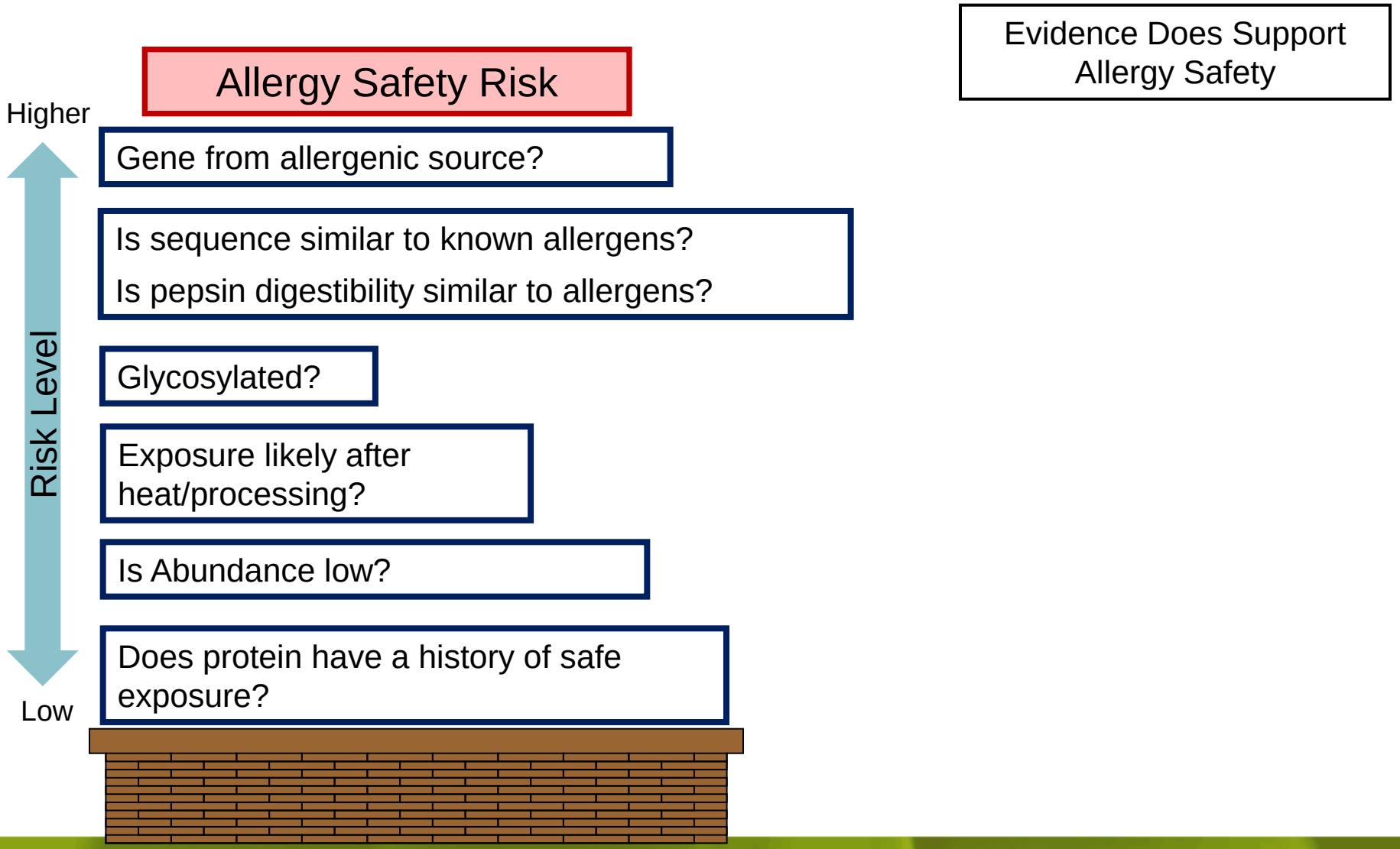
Allergy Health Risks – Biotech Proteins

- Has the protein been **unintentionally transferred** from a known allergen source and is the protein itself a known allergen?
- Has the transformation **process increased the normal expression of endogenous allergens** in such a way to increase the risk to allergic patients?
- Will the novel protein expressed in the biotech product become an allergen once exposed to workers/consumers? I.E., **is there risk of a *de novo* allergen**?

Building an Assessment of Allergy Safety for a Protein
A Weight-of-Evidence Approach



Allergy and Biotechnology: Safety Guidance

Evolving allergy strategy to manage health risks

- CODEX
  - Intergovernmental body (168 member states)
  - Implements joint FAO/WHO Food standards programs
  - Protects health of consumers and facilitates trade by setting international safety standards

Allergy and Biotechnology

Evolving allergy strategy to manage health risks

- CODEX recommended allergy assessment includes:
  - Source of the introduced protein
  - Similarity of the introduced protein to known allergens
  - Susceptibility to enzymatic digestion and/or heat stability

No single test can predict human allergenicity

Allergy and Biotechnology

Evolving allergy strategy to manage health risks

- CODEX recommended allergy assessment
  - ***If introduced protein from a non-allergenic source***
    - Assess sequence similarity to known allergens
    - Assess pepsin resistance
  - ***If introduced protein from an allergenic source***
    - Assess sequence similarity to known allergens
    - Assess pepsin resistance
    - Assess specific IgE binding
    - Assess skin prick testing on appropriate individuals

Allergy and Biotechnology

Evolving allergy strategy to manage health risks

- CODEX recommended allergy assessment
  - ***Other considerations***
    - Exposure level of the introduced protein
    - As science and technology evolves other methods may be considered
      - **Targeted serum screens**
      - **Animal models**
      - **T-cell epitopes, structural motifs associated with allergens**

Allergy and Biotechnology

Summary

- Science of allergy is still evolving
- Current assessment process utilizes best science available
- Current assessment process is very good at preventing the introduction of known or cross-reactive allergens into the food supply
- Harmonization of the allergy assessment process is underway
- No single test can predict protein allergenicity
- **Safety process utilizes a "weight of evidence" approach;**
  - the goal is to add to this approach with scientifically justified methods, when appropriate.

Summary

The current allergy assessment process is useful and robust for novel protein allergy assessments

- New methods are encouraged if they:
  - Are scientifically justified
  - Lend value to the allergy safety assessment process
- Methods such as bioinformatics are made more useful by careful curating of databases further characterization; However, they are not predictive.
- In vitro cell assays, animal models, and proteomics are evolving techniques that require validation in allergy assessments.

Case Study; Identifying the Transfer of an Allergen into a GM Crop

- Mid - 1990s, Soybean product was genetically modified with the 2S albumin (now known as Ber e1) to enhance the nutritional content – soybean are deficient in methionine, so sulfur-rich proteins from nuts make a good additive.

- Company proactively studied the risk of allergic reactivity to the GM soybean containing the Ber e1 gene and protein using

  - serum IgE reactivity and

  - reactivity with skin prick testing (Nordlee, et al., 1996).

- Product (the soybean crop seed) was never released to the public.

Questions for the GM Crop Industry…

- What is the risk that transformation issues such as the 2S albumin in soybean can happen and proceed past the development phase?

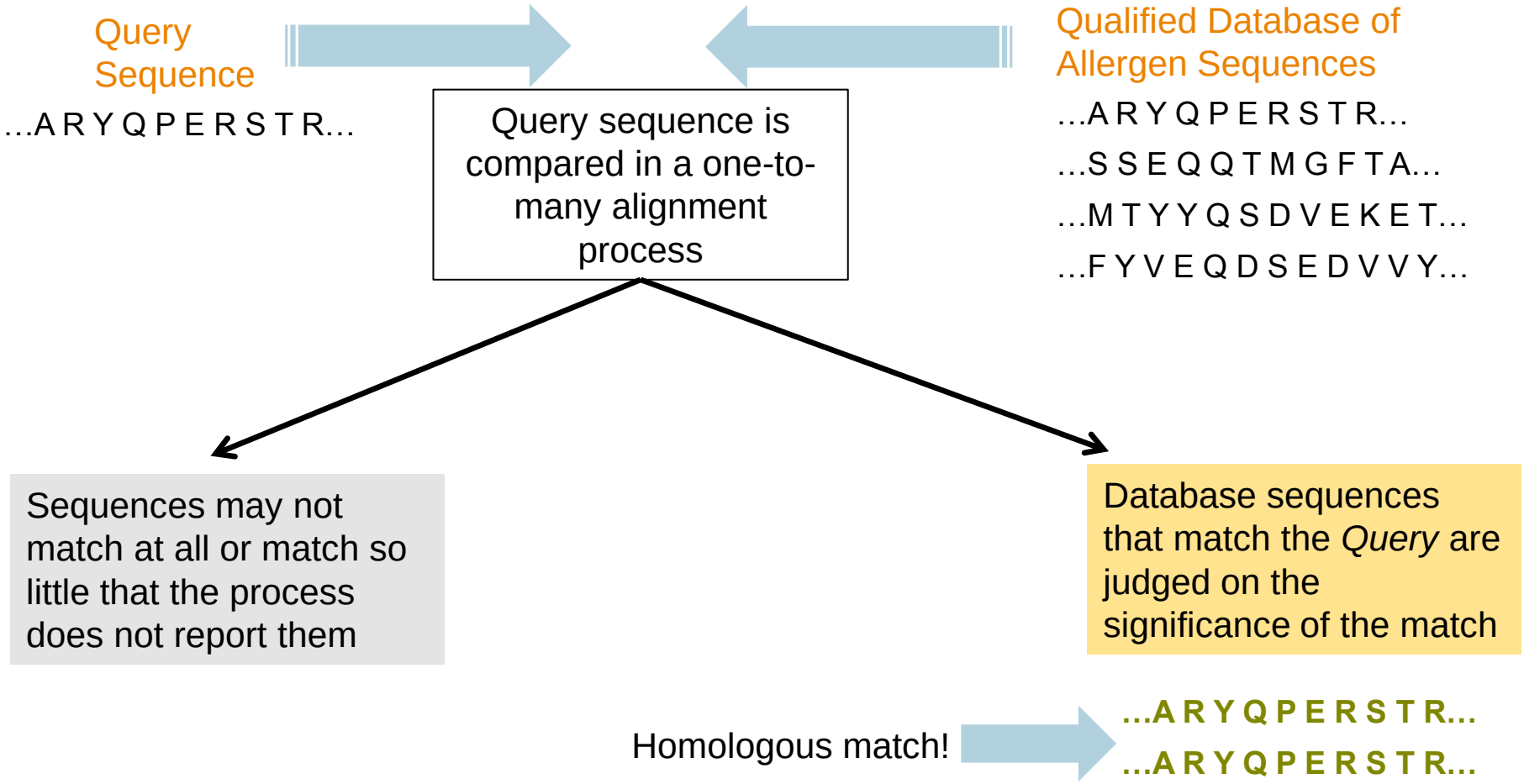
- More to the point, what has improved in the last 15 years to reduce allergy risk with GM food crops? What does the allergy safety assessment process actually have for “process” and allergy science that it did not have back in 1995?

Standardized Approaches to Characterizing Allergy Risk

Bioinformatics

- Allows one *primary* question to be asked:
  - Is the query (novel) protein similar to an existing allergen?
  - Can also use the results to identify source organism of a trait protein.
- Screening is possible because hundreds of food and respiratory protein allergens have been sequenced and characterized
- Significant similarity and possible homology with known allergens means that a novel protein may cross-react with an existing allergen.
- However, bioinformatics is not intended to answer whether a protein will *"become"* an allergen.
- Bioinformatics is never an answer in and of itself.

Bioinformatics -*comparing one-to-many-* Is there a match?



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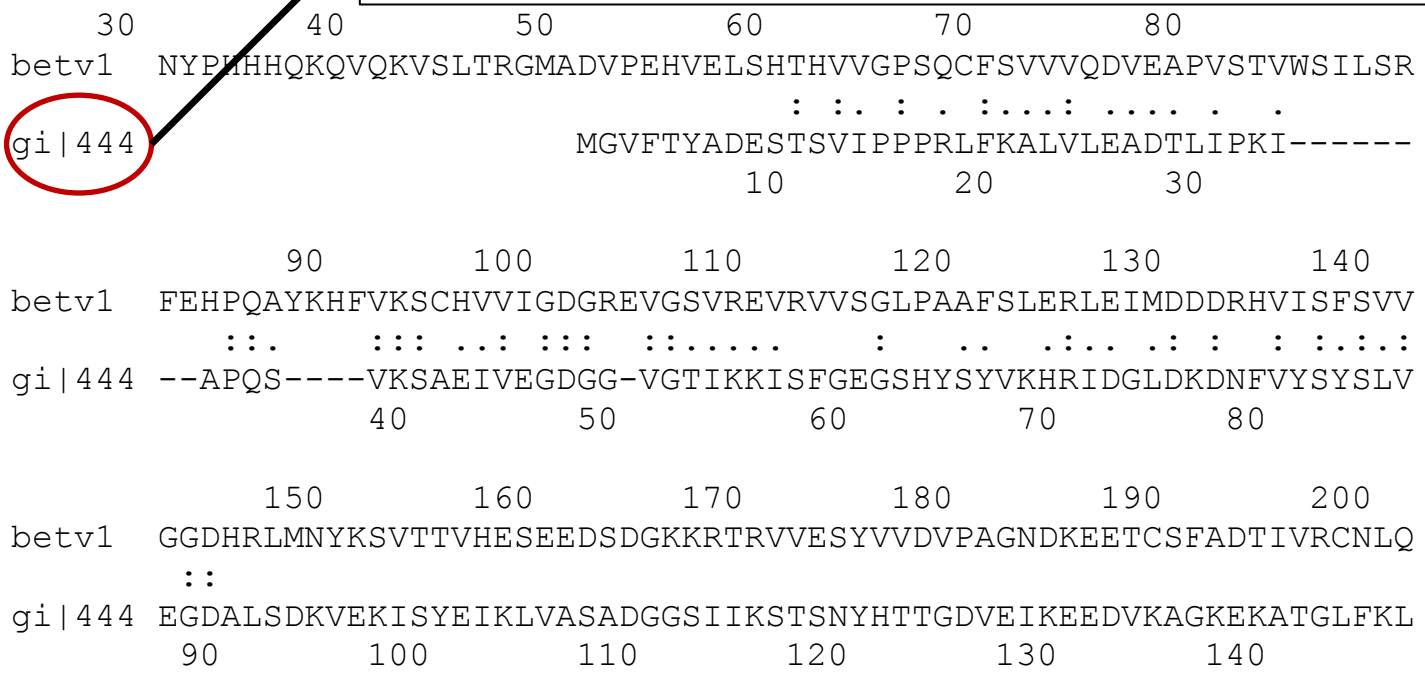
Bioinformatics - Alignment Scoring. A Challenge in Communicating what the Results Mean

>>gi|444 – QUERY SEQ - (160 aa) initn: 44 init1: 44 opt: 116 Z-score: 155.0 bits: 35.3. **E-Score = 0.001,** Smith-Waterman score: 119; **27.95% identity** (56.989% similar) in **93 aa overlap**

My search (query) sequence – Question: Has it significantly aligned with a known allergen?



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Bioinformatics has some Key Consensus Components

- Internationally recognized guidance, Codex 2009:
  - similarity is significant if shared identity is >35% for an alignment of at least 80 amino acids
  - Small exact matches (epitopes) of justified length (Silvanovich et al, 2006)
- Standardized use of algorithms for comparing sequences
  - BLAST and FASTA used mostly with default settings/filters
- Independent database of known allergens that is curated for accuracy by a panel of internationally recognized allergy scientists.
  - Food Allergy Research and Resource Program, U of Nebraska; Steve Taylor and Rick Goodman.
- Updates are annual and recognize new allergens
- Relevance is reviewed periodically
  - (Thomas et al, 2005; Ladics et al, 2011)

Standardized Approaches to Characterizing Allergy Risk

Simulated Gastric Fluid: protein stability to pepsin enzyme proteolysis

- Characterizes the rate at which a protein is degraded:
  - Premise: if a novel protein is “stable”, i.e., as stable as some allergens, then risk of exposure and potentially allergy is greater.
- Method standardization has been key to being able to characterize novel food proteins (Thomas et al, 2004); regulators are able to compare results from one company to the next for similar proteins.
- The characterization of simulated gastrointestinal stability for novel proteins has expanded to include additional characterization.
  - Simulated Intestinal Fluid (pancreatic enzyme mix) can be included.

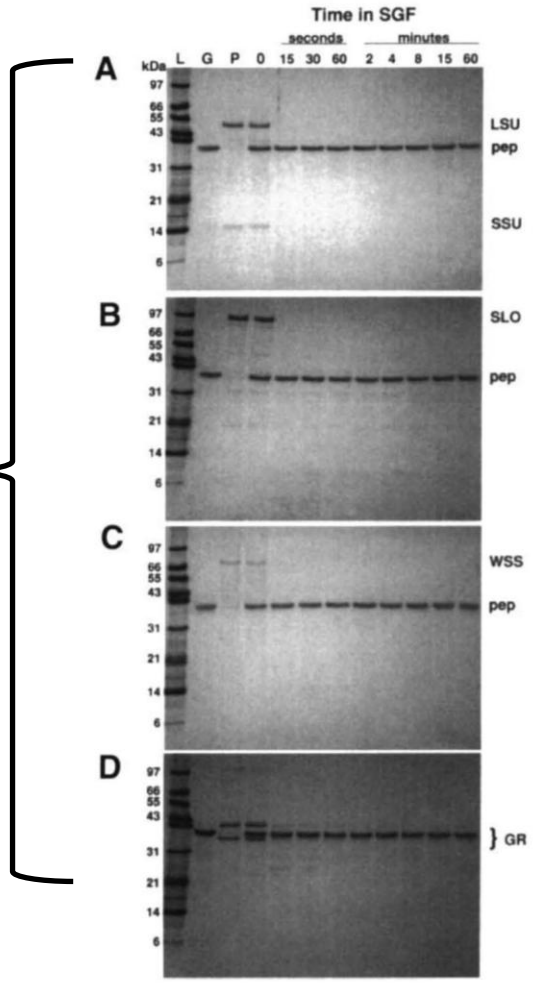
Allergens Compared to non-Allergen Plant Proteins

Expectation for Novel GM Crop Proteins is ~ 2 min

Astwood, Leach and Fuchs, 1996

Table 1. Summary of allergen and protein stability in SGF.

Protein	Stability (min) Whole Protein	Fragments
Egg allergens		
Ovalbumin	60	—
Phosvitin	60	—
Ovomucoid	8	—
Conalbumin	0	15
Milk allergens		
ß-lactoglobulin	60	—
Casein	2	15
BSA	0.5	15
Soybean allergens		
ß-conglycinin (ß-subunit)	60	—
SKTI	60	—
Soy lectin	15	—
ß-conglycinin (α-subunit)	2	60
Gly m 1	0.5	8
Mustard allergens		
Sin a 1	60	—
Bra j IE	60	—
Peanut allergens		
Ara h2	60	—
Peanut lectin	8	—
Common plant proteins		
Glycolate reductase (spinach leaf)	0.25 (15 sec)	—
Rubisco LSU (spinach leaf)	0 (<15 sec)	—
Rubisco SSU (spinach leaf)	0 (<15 sec)	—
Lipoxygenase (soybean seed)	0 (<15 sec)	—
PEP carboxylase (corn kernel)	0 (<15 sec)	—
Sucrose synthetase (wheat kernel)	0 (<15 sec)	—
ß-amylase (barley kernel)	0 (<15 sec)	—
Acid phospatase (potato tuber)	0 (<15 sec)	—
Phosphofructokinase (potato tuber)	0 (<15 sec)	—



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Some Aspects of Characterizing Allergens are Best Done at the Clinical Level

Glycosylation

- Is not consistently associated with clinically relevant allergenic potential

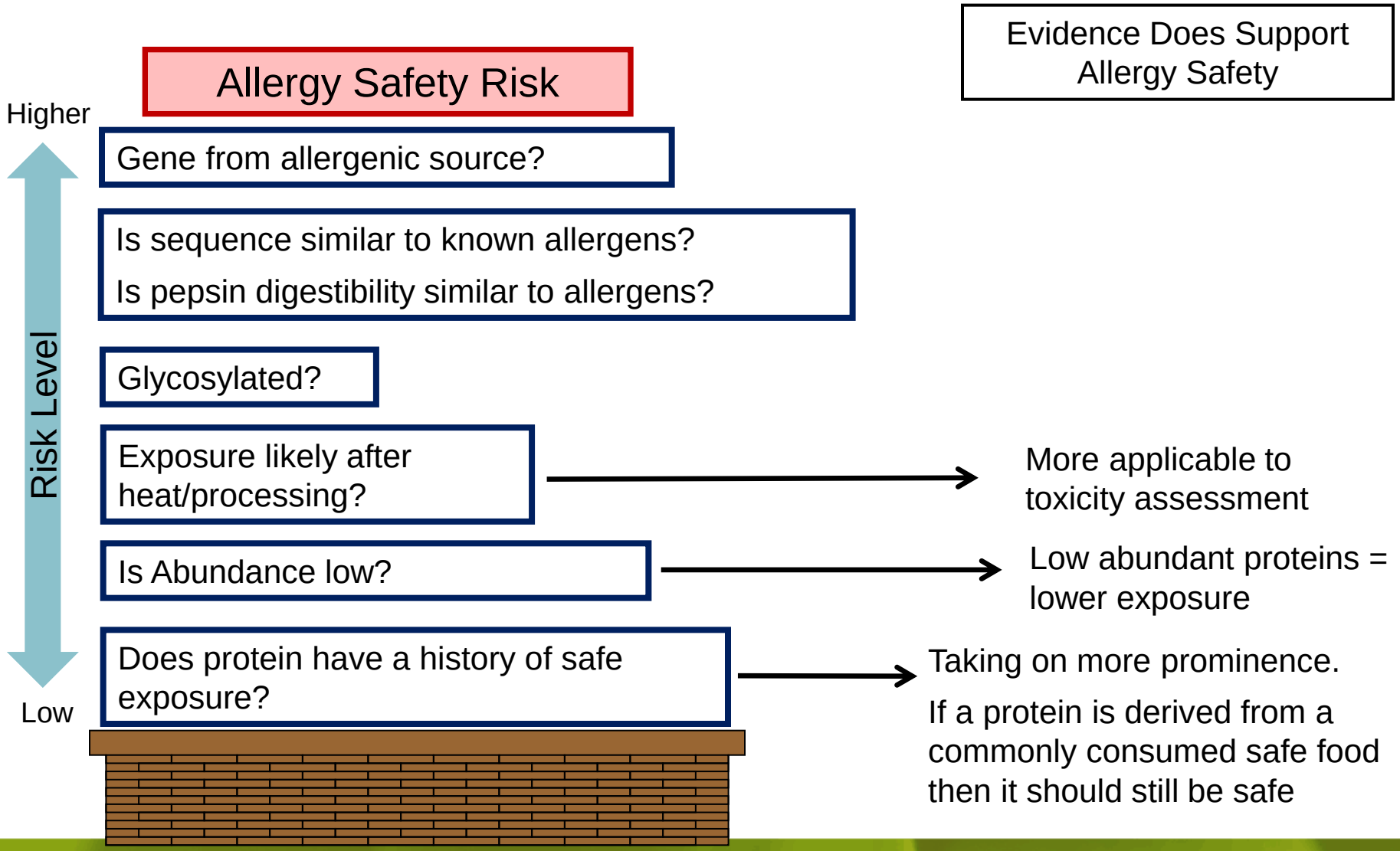
  - Mari A, et al., Evaluation by double-blind placebo-controlled oral challenge of the clinical relevance of IgE antibodies against plant glycans. *Allergy 63: 891-896.*

- Industry: characterization is by a **standardized in vitro kit** to measure presence of carbohydrate moieties on plant-extracted novel proteins.

- Does not have the priority status of SGF and bioinformatics, but is unlikely to be removed from registrations (and expectations).

  - *Does provide a conservative approach and does add to the weight of evidence for novel protein safety*.

Building an Assessment of Allergy Safety for a Protein
A Weight-of-Evidence Approach



Areas of Advancement: Allergy Assessment Methods

How can we measure allergy potential of novel proteins and how can we measure and better characterize known allergens?

- *Bioinformatics*: *to address novel protein similarity to known allergens*
- Serum screening, when appropriate; *measure cross-reactivity*
- Animal Models; *measure novel proteins or known allergens*
- Proteomics; *measure known allergens*
- *New approaches to determining epitopes*: *in-depth characterization of known allergens with potential application to novel proteins*
- *Cell-based in vitro assays*; *measure novel proteins or known allergens*

Bioinformatics

Epitope and Complex Structure Analysis

- Small sequence length searches (e.g. 8 amino acid) provide no added value to the novel biotech protein safety assessment process (Silvanovich et al., 2006; Goodman et al., 2008 Nature Biotech)

- A higher level analysis using the 3-dimensional analysis of protein structure may have value, but more research is needed to construct 3-D databases (Thomas et al., 2005).

  - known allergens must be modeled for their structure

  - software must be validated for use in aligning 3-D allergen structures.

Applications for using Data on Protein/Peptide Antigenicity

1. Antigen Selection - Can be used to predict valuable (better chance of initiating a response in vivo) peptide selections when considering animal antibody for developing detection reagents (ELISA or Western).
2. Product Safety - Useful for comparing proteins: Two proteins that may look similar using basic bioinformatic alignments may be compared. The more in depth epitope comparison may highlight and clarify difference/similarities
   - Data may be used as convincing evidence of a safety or a concern
3. May be useful in modeling proteins to limit their immunogenicity or allergenicity profile. Peptides could be modified and re-assessed in assays to observe best candidates (i.e., lowest potential for allergy) .

Protein Immunogens

- Only certain parts of a protein sequence interact with the cellular immune system to 1) present peptides to immune cells 2) stimulate peptide/antigen specific antibody development.

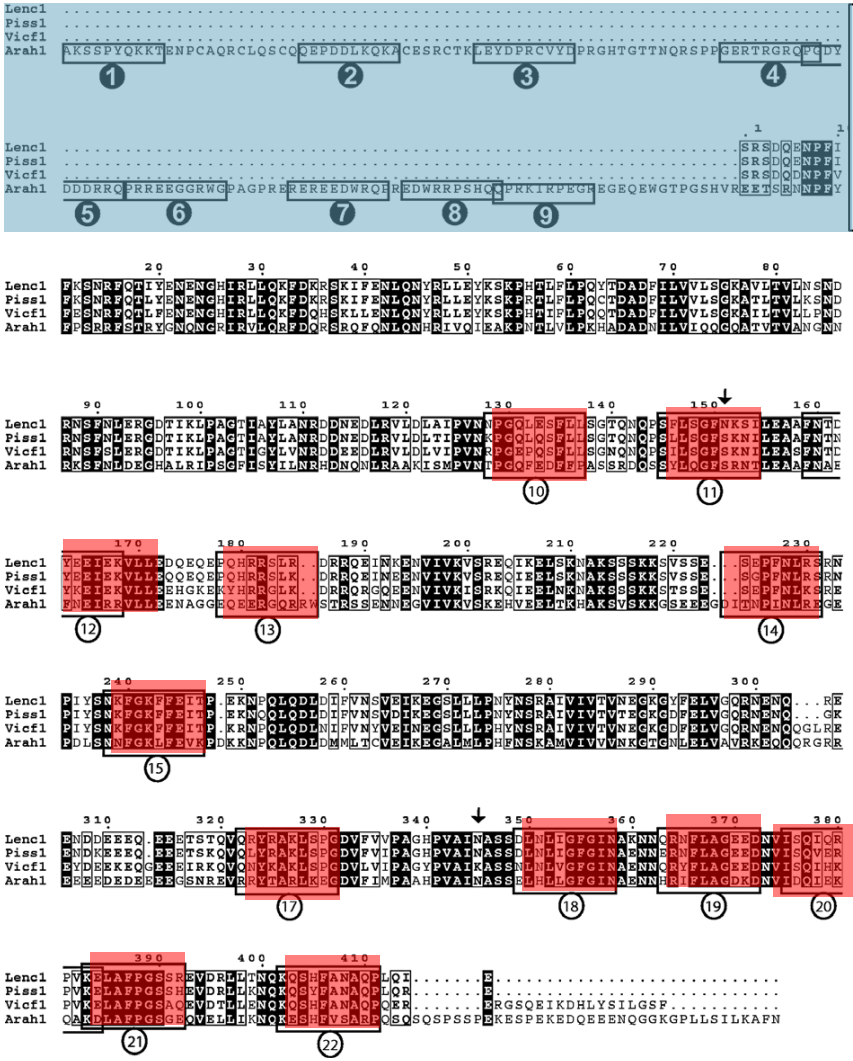
- Peptides can be short (8 – 30) amino acids in length that are exposed as small independent peptides. Or, peptide(s) can be part of an intact 3D structure that function as immunogen only within the context of the whole/part structure (discontinuous epitopes).

  - Example: a denatured and reduced protein allergen that only has functional discontinuous epitopes would theoretically NOT stimulate an immune reaction

Example: Using Epitope Prediction for Analytical Use of Antibodies with Biotech Proteins

- Trait Proteins are typically in short supply in early phase projects
  - Difficult to handle (solubility) proteins may only be available as denatured/linearized proteins (no discontinuous epitopes)
  - Typically an advantage to assessing protein in the form of synthetic peptides which can be synthesized easily/rapidly and assessed for epitopes.
- Predicting location and level of antigenicity for peptides and synthesizing those peptides may help develop robust antibodies that can detect the intact (properly folded full length protein) when it is time to develop a solution phase detection assay (ELISA).

Ever Increasing Value in Fully Characterizing Known Allergens



Peanut allergen, Ara h 1, specific b-cell epitopes; 1 – 9. Not present on the three other proteins

Barre, et al., 2005

Shared epitopes: Open boxes numbered 10–22 delineate the B-cell epitopes of Ara h 1 conserved in other vicilins Len c 1, Pis s 1 and Vic s 1.

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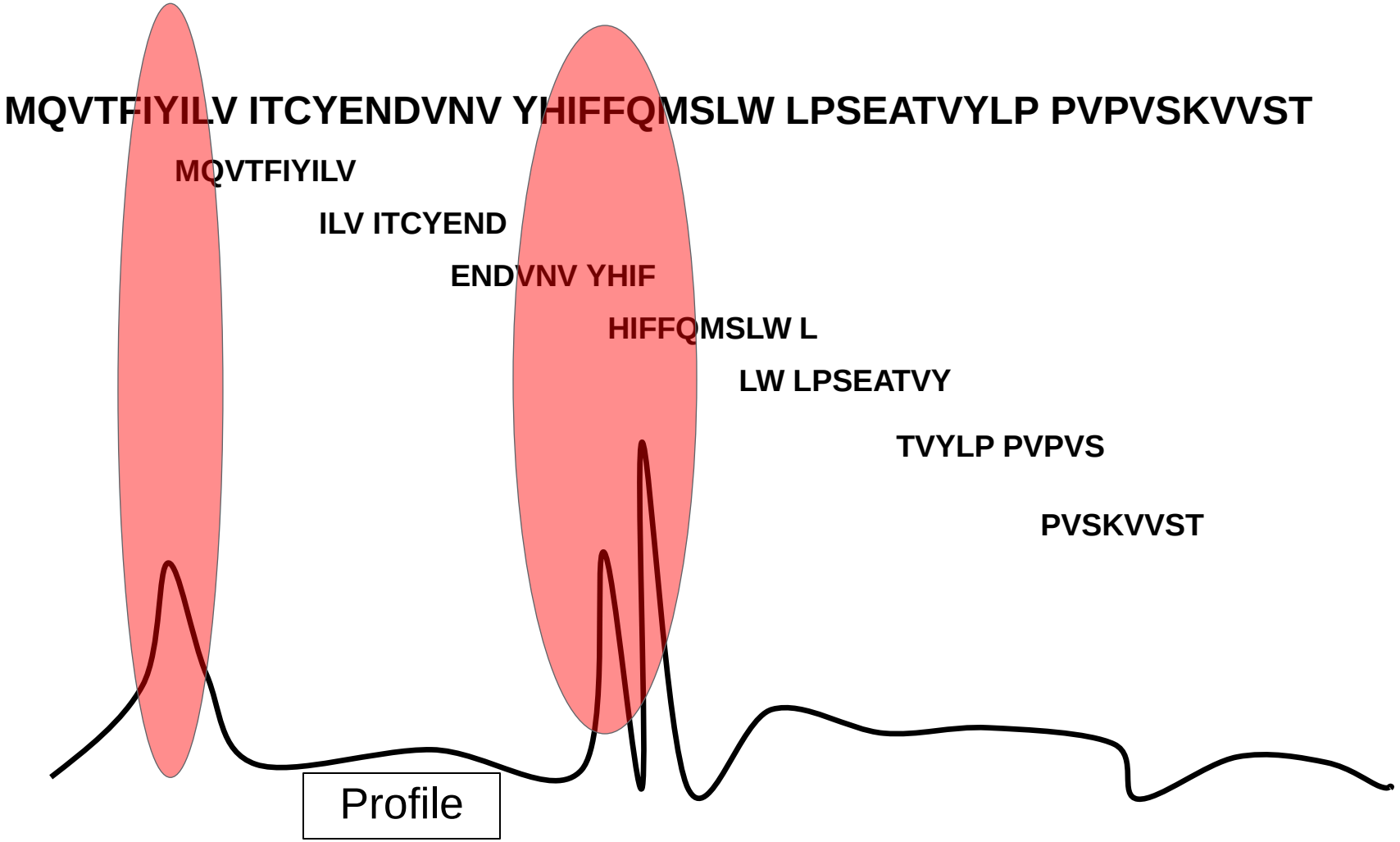
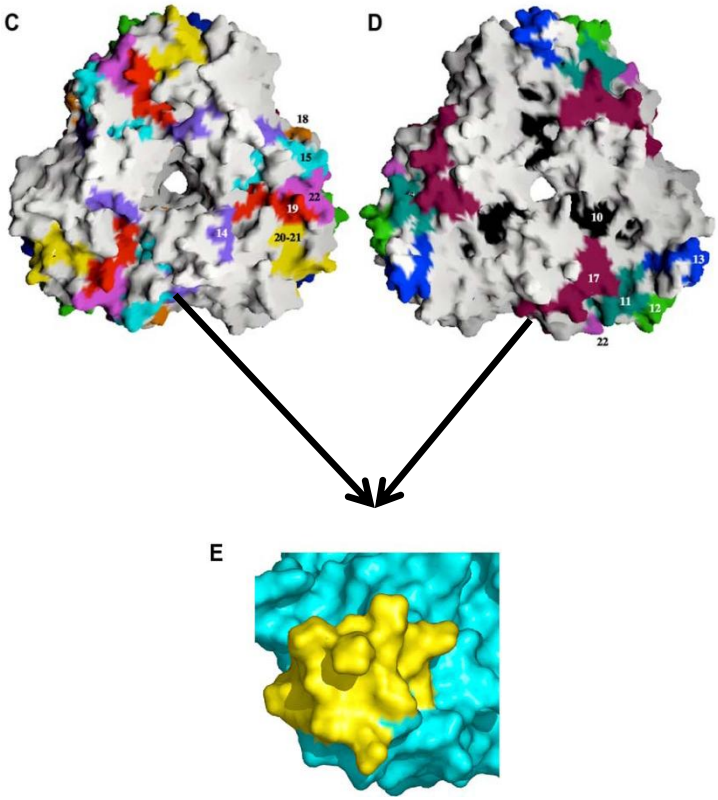
Individual peptide assessments can be summed to produce a Summary Profile of the Antigenicity/Immunogenicity of the Entire Protein Length



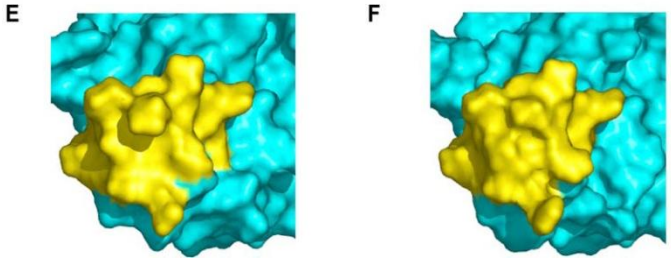
Modeling Epitope location onto 3-D Models

Front and Back models of the structure of Ara h 1 allergen are used to for the final space-filling 3-D model with epitopes.



Barre, et al., 2005

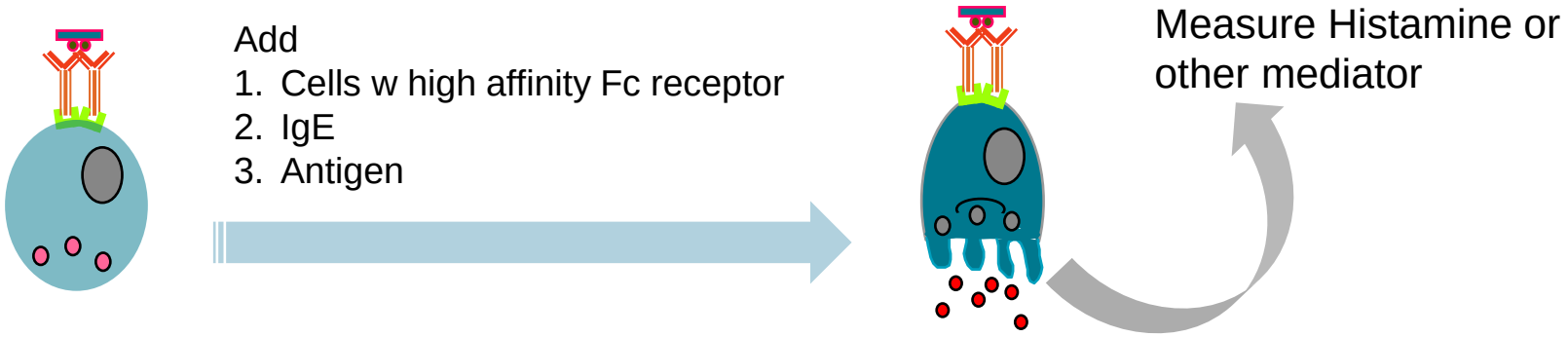
Once models are made and epitopes plotted, proteins can be compared. Shared epitopes are compared (Ara h 1 and Len c 1) in a final analysis.



Important attributes of an epitope (charge, position within the protein, and shape) can be assessed using an integrated approach.

In Vitro, Cell-Based Techniques for Assessing Allergenic Potential of Trait Proteins

- In vitro measures of allergenicity quantify markers of the allergenic response, e.g., histamine, CD markers (CD63, CD203), leukotrienes.
  - May offer a live-cell testing bridge between human clinical testing (food challenges) and in vitro, IgE-binding assays.
- Basophils taken from blood can be reacted *in vitro* with allergens.
- Basophilic cell-line (murine) may be used as a surrogate for human basophils and reacted with protein allergens



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