In vitro digestibility: application of a sequential protocol (gastric/duodenal) to pairs of proteins from the same protein family but with different allergenicity.

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HESI Protein Allergenicity Technical Committee

- HESI = Health and Environmental Sciences Institute
- International non-profit organization, science not advocacy

Mission Statement
Collaboratively identify and help to resolve global health and environment challenges through the engagement of scientists from academia, government, industry, NGOs, and other strategic partners.
HESI PATC: Support the development and identification of safe, nutritious food sources by...

- Developing science to better define what makes a protein allergenic;

- Establishing processes useful in a weight-of-evidence approach to the evaluation of novel proteins

- Communicating scientific findings and best practices to the academic, industry, research, and regulatory communities.
HESI Protein Allergenicity Technical Committee

Academic Medical Center, University of Amsterdam (Netherlands)
BASF Plant Science
Bayer SAS
Copenhagen University Hospital, Gentofte (Denmark)
Dow AgroSciences
DuPont Pioneer
Guangzhou Medical University (China)
Monsanto Corporation
Syngenta USA
US Environmental Protection Agency
US Food and Drug Administration
Background of the project

Considerations from EFSA concerning pepsin digestion protocol

"It is recognized that the pepsin resistance test does not reflect the physiological conditions of the digestion. The digestibility of the newly expressed proteins [...] may be assessed using in vitro digestibility tests using different conditions [than low pH and high pepsin:protein ratio]."

EFSA, 2011

"Recommendations

• In addition to the pepsin resistance test, other in vitro digestibility tests on newly expressed proteins are recommended to be performed in more physiological conditions"

EFSA, 2010

HESI PATC has taken up the challenge to evaluate different conditions during gastric digestion, i.e. multiple pHs and pepsin/protein ratios, followed by duodenal digestion.
Can conditions be identified that provide support to the weight-evidence approach to assess the risk of introducing an allergen into a GM crop?

Before going into the details of the protocol:

What is an allergen?

How could digestibility be associated with allergenicity?
What is an allergen?

Most stringent definition:

“An antigen that sensitizes (induces IgE) and (usually) causes symptoms”

COMPLETE ALLERGEN

Cross-reactive allergen:

“An antigen that does/can not sensitize itself but can cause symptoms”

INCOMPLETE ALLERGEN

Ergo, there are two potential risks when introducing a transgene:

- Introducing a risk for de novo sensitization (induction of IgE)
- Introducing a risk for inducing symptoms in already sensitized subjects
What is the origin of food allergy?

There are essentially two ways to become food allergic:

1. Exposure to foods such as egg, milk, fish, peanut or hazelnut
2. Exposure to respiratory allergens such as pollen or mites

   cross-reactivity to foods
How could digestibility be associated with allergenicity?

- Sensitization process
- Elicitation of symptoms

How does sensitization to food occur?

Intuitively the obvious answer is: by eating food.

(Additionally: by cross-reactivity with inhalant allergens)

How does sensitization to egg or peanut before first exposure fit in this picture?
Timing and routes for sensitization to food?

**ROUTE**
- **Respiratory tract**
  - indoor allergens and subsequently more and more outdoor allergens
  - inhalation food in house dust
- **Skin**
  - skin contact with food or food in e.g. ointments
- **Gut**
  - bottle-feeding
  - breast-feeding
  - solid food of increasing diversity
- **Blood**
  - trans-placental

**TIMING**
- pregnancy
- pre-weaning
- weaning
- pre-puberty
- puberty/adolescence

*Only here digestibility may be of relevance*
For elicitation of symptoms of food allergy, the importance of digestibility is quite well established.
A European study on apple allergy

CRD using four purified apple allergens:

1. Birch-pollen cross-reactive allergen
2. Mal d 2
3. Lipid transfer protein (LTP): true food allergen
4. Mal d 4

CRD reveals a clear geographic difference. So what?
Only patients with IgE antibodies against the COMPLETE ALLERGEN (green) have severe systemic symptoms (U: generalized urticaria / AX: anaphylaxis). The risk is for severe food allergy increased by around 8-fold!

Likely explanation: resistance to gastric digestion

IgE to cross-reactive proteins with homology to a protease-resistant allergen bears more risk than to a protease-sensitive allergen.
In summary,

- Digestibility **may be relevant** for assessing the risk of a protein to be a sensitization (complete allergen). Confirmation of the oral route as an important route for sensitization will be decisive for its relevance.

- Digestibility **is relevant** to assess the risk of a protein to induce systemic (severe) symptoms, but only in the presence of existing (cross-reactive) IgE, which in itself is a no-go for a transgene to go into a GM crop.

- Including digestibility in the weight of evidence approach is a **conservative strategy** which is 1) possibly relevant but not really evidence-based for sensitization and 2) relevant for symptom elicitation but only for proven allergens.
Does the degree of susceptibility to gastro-intestinal digestion separate allergens from non-allergens?

DIGESTION PROTOCOL
Combined Gastric + Duodenal phases

Method development based on the paper from Mandalari et al., 2009

**Gastric phase**
- **Gastric mix:**
  - pH 1.2
  - pH 2.5
  - pH 4.0
- Reaction Mix adjusted to pH 7.5 using l-con buffer (50 mM $\text{KH}_2\text{PO}_4$/$\text{K}_2\text{HPO}_4$) and NaOH
- final protein conc: 50 µg/ml

**Duodenal phase**
- **Duodenal mix:**
  - l-con buffer pH 7.5
  - bile salts (taurocholate and glycodeoxycholate)
- pepsin/protein ratio: $10 - 1 - 0.1$ (U/µg)
- Incubation times: G0; G5; G10; G60 min

**Protein (1 mg/ml)**
- HRP
- OVA
- βLG
- 5 pairs

**pancreatin solution**
- Incubation times: D0; D10; D60 min

SDS PAGE

WB

final protein conc: 50 µg/ml
Some considerations with respect to “more physiological conditions”:

**pH**

- Normal gastric pH lies between 1.5 and 3.5 (circadian rhythm)
- Food intake influences gastric pH
- The use of PPIs increases pH to 4.0 - 5.0

**Pepsin**

- pepsin concentration in healthy volunteers is probably around a few hundred units/ml
- in the ratio 10 (over 50 µg/ml protein) we use 500 U/ml
SECTION II—Experimental Physiology

Daily Variations in the Concentrations of Acid and Pepsin in the Gastric Juice of Three Persons Observed for Two Months

By

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For fifty years and more physicians have been depending on diagnostic acid on a single gastric analysis, seldom stopping to find out how different the figures might be if a second or a third analysis were to be made on successive days. In 1901 we tried to fill this gap in medical knowledge by studying the variations in acidity which took place in the gastric juice of two women observed daily for a month (1).

One of these women, who was nervous and stable, had a fairly constant gastric acidity except at the end of the period of observation, when she became excited over the prospect of her approaching vacation trip. Then there was a big variation from her normal. The other young woman, who was less stable emotionally, reacted to a severe disappointment with large swings up and down in the curve representing gastric solility. As a result, there were many days during the month when a single gastric analysis would have been worse than useless as an index to the normal activity of her gastric mucous.

Recently, while studying the effect of a diet deficient in vitamin B, the senior writer had the opportunity of studying for two months the concentrations of acid and pepsin in gastric juice removed almost every day from the stomach of two women and one man. The nutritional aspects of this experiment will be described elsewhere, and in this place we will record only the extent of daily variations in the gastric juice, together with a few other data. Because the deficient diet did not seem to have any effect on gastric secretion, it seems probable that the daily variations observed in those three persons closely resemble those that might be found in anyone living on a normal diet.

In Figure 1, the three sets of curves represent concentrations of acid and pepsin in gastric juice obtained from the three persons studied. It will be seen that with both acid and pepsin there was least variation in the case of the man, Dr. E. Interesting in the fact...
Combined Gastric + Duodenal phases: sampling

Digestion & sampling in gastric phase:
3 pHs (pH 1.2, 2.5 and 4.0) & 3 pepsin/protein ratios (10, 1 and 0.1)

Digestion & sampling in duodenal phase (pH 7.5)
## Pairs of proteins to compare in the GDD

<table>
<thead>
<tr>
<th>Protein family</th>
<th>Allergenic proteins</th>
<th>Non-/Weakly Allergenic proteins</th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2S albumins</td>
<td>Peanut Ara h 2</td>
<td>Pea Pis s albumin</td>
<td>5.2</td>
</tr>
<tr>
<td>Tropomyosins</td>
<td>Shrimp Pen a 1</td>
<td>Porcine “Sus d/s ?”</td>
<td>55.0</td>
</tr>
<tr>
<td>Parvalbumins</td>
<td>Carp Cyp c 1</td>
<td>Swordfish Xyp g 1</td>
<td>77.8</td>
</tr>
<tr>
<td>Collagens</td>
<td>Fish collagen</td>
<td>Bovine collagen</td>
<td>55-75</td>
</tr>
<tr>
<td>lipid transfer proteins</td>
<td>Peach Pru p 3</td>
<td>Strawberry Fra a 3</td>
<td>66.6</td>
</tr>
</tbody>
</table>

**Rationale:**

- **Ara h 2**: strong allergen (common allergy) – **Pea albumin**: weak allergen (rare allergy)
- **Pen a 1**: strong allergen (common allergy) – **Porcine trop**: no allergen (rare allergy)
- **Cyp c 1**: strong allergen (common allergy) – **Xyp g 1**: allergen (more often tolerated)
- **Collagens**: pair of proteins with weakest evidence base for allergenicity (some for fish)
- **Pru p 3**: strong allergen (common allergy) – **Fra a 3**: weak allergen (rare allergy)
Some first preliminary observations for the first two pairs:

2S albumins from peanut and green pea

Tropomyosins from shrimp and pig
Preliminary concluding remarks

- For first two protein pairs the allergenic one is more resistant to pepsin than the non-/weak allergenic one
- Optimal conditions for gastric digestion may vary per molecule
- At pH 4.0 gastric digestion is impaired but facilitates very efficient subsequent duodenal digestion
- Duodenal digestion may be different between allergen and non-/weak allergen if preceded by gastric digestion at low pH

Cave! These observations should be seen as preliminary. Comprehensive analysis still needs to be done.
Work in progress:

- Evaluation of three additional protein pairs
- Purification of swordfish parvalbumin

Future plans:

- Evaluation of susceptibility of protein pairs to endo-lysozomal proteases from DCs (sensitization)
- Evaluation of impact of matrix
Acknowledgements

Funding/facilitation: ILSI-HESI PATC – Nancy Doerrer, Syril Pettit
Cyndi Nobles

“Digestibility Working Group” PATC

Annabelle Capt, Muriel Totis (protocol development)

Jaap Akkerdaas (performance of experiments)