In vitro protein digestibility tests for allergenicity risk assessment

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Declaration of interests

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Why is digestibility important for food allergy?
Events in the gut lumen affecting protein release and breakdown

How does the form of a protein determine its release from food and stability to digestion?

- Lipid droplets
- Protein stabilised emulsions
- Impact on release from intact cells
- Proteolysed protein aggregates
- Resistant allergen
- Lipid-adsorbed allergen
Uptake and intracellular processing of allergens......

How do allergens cross the mucus layer?

......and their uptake by the epithelium, including dendritic cells

...... making an antigen into an allergen

Adam Maciercenka, Alan Mackie, Claudio Nicoletti
This is important for

• Elicitation of allergic reactions in sensitised individuals
• Severity of reaction and conditions like exercise-induced anaphylaxis
• Affecting the balance between tolerance and sensitisation to dietary protein
Digestibility and allergenicity risk assessment

Digestibility studies provide useful data regarding the properties and characteristics of the novel protein affecting

- Gut luminal processing and uptake
- Intracellular processing and antigen presentation

These may influence properties such as tolerance induction or sensitisation in a host.

They may also provide data on the stability and molecular mobility of polypeptide chains

Mutschlechner et al. J Allergy Clin Immunol 2010;125:711-8
Pepsin resistance test

• Posed the hypothesis ‘that food allergens must exhibit sufficient gastric stability to reach the intestinal mucosa where absorption and sensitization (development of atopy) can occur.’

BUT

• The test is non physiological
  • the pH is lower than found in vivo and changes pepsin specificity,
  • Pepsin is present in a gross excess which affects kinetics of digestion
Metanalysis shows a continuum of substrate: protease ratios are used spanning 7 orders of magnitude!

Batch models will need to apply several conditions to cover those found physiologically.

Selected conditions need to take account of changes

- In rates of secretion during digestion
- The effects of food composition on secretion
- Differences in digestive process e.g. in infancy
Similarly a range of pH conditions have been used.

These will need to take account of changes:
- In rates of secretion during digestion
- The effects of food composition on secretion
- Differences in digestive process in infancy
- Effects of antacids (taken by 25-30% population!)
Test meal – pH is ~ 5.5

Any batch models will again need to apply several conditions to cover those found physiologically and to account for individuals on medication such as proton pump inhibitors.

Use of comparator (non-allergenic) proteins

- Very few papers used comparator proteins
- This reflects the focus of research
- Access to purified, well-defined comparators
- Lack of consensus on choice of comparators
- This aspect also needs a consensus workshop
Is the pepsin resistance test more a biochemical surrogate of “stability” than simulated gastric digestion?

- Pepsin, like other endoproteases (trypsin, chymotrypsin) cleaves mobile, surface accessible sites on a substrate
- Resistance to pepsinolysis is a function of
  - Resistance to low pH unfolding
  - Polypeptide mobility at the cleavage site
- Resistance to pepsin maybe a surrogate measure of endosomal processing involved in antigen presentation
- It may have validity as a correlative test defined using panels of “allergens” and “non-allergens” included in the integrative risk assessment approach
Modelling Digestion – Biochemical Batch Models

**Gastric mix:**
- Pepsin
- Phosphatidylcholine vesicles

Shake, 37°C
Sample over time

pH 2.5
I=0.15

Stop gastric digestion by addition of NaOH

**Duodenal mix:**
- Trypsin, chymotrypsin
- Lipase/colipase
- Amylase
- Bile salts

Shake, 37°C
Sample over time

pH 7.5
I=0.15

Stop duodenal digestion by addition of SBTI or PMSF
Biochemical batch models of digestion

✓ Can be scaled down to analyse small amounts of single proteins
✓ Some collaborative trials published showing inter-laboratory validation
✓ Homogeneously mixed so sampling is easier
✓ Can mimic the GI tract- a model system with assumptions and limitations [enzyme:substrate ratios, pH titration, homogeneous mixing]
X Not well suited to analysis of foods [sampling, soluble versus insoluble phases]
Gastroduodenal digestion and the gut as a biological processing plant

Biomechanics and motility determine luminal flow and mixing behaviour

+ Variation in activity, content and secretion of digestive enzymes

Determine the rate of delivery of absorbable species to the gut wall

All of this is under tight biological control, including gut-brain signalling
Diverse mechanical models are being developed – especially for studying flavour release.
Dynamic Gastric Model (DGM): Full simulation of gastric forces and motility

**Main Body:**
Gentle 3 contraction wave per min cycle
In-homogenously mixed

**Antrum:**
High shear well mixed environment
Shear at 10-100 sec\(^{-1}\)
Phase II contraction waves

Inventors: Martin Wickham, Richard Faulks
Available from Bioneer;
Dynamic Duodenal Model: combining segmented and peristaltic flow

pancreatic juice (NaHCO$_3$, amylase,...)

velocity profile

peristaltic movements

absorption of glucose

segmentation movements

gastric acid (HCl,...)
pylorus

Bostjan Hari, Serafim Bakalis, Peter Fryer, University of Birmingham
TIM 1 and TIM 2

Blanquet et al 2001 *Trends in Biotechnology*, 19(10), 393-400
Physical models of digestion are

- Mimics of flow and mixing behaviour in the GI tract
- Addition of digestive enzymes and pH adjustment more like a “real” gut
- Designed to digest real foods and meal-sized portions
- Easier to sample than human volunteers
- Not necessarily validated against the human situation
- Not adapted to analysis of small amounts of material or purified protein
Dynamic models of digestion are

• The only physiologically relevant models available
• Adapted for analysis of whole foods AND NOT individual purified proteins
• Analysis of individual protein targets in mixtures is technically difficult
• Development of models is in its infancy, validations against the human situation is often lacking
• Main drivers are pharmaceutical industry (dosage forms, interactions with foods) and nutrition
• Models have not been developed/adapted to suit the needs of GMO risk assessment
Tests for resistance to gastroduodenal digestion

Validation is needed regarding

• Levels of enzymes and biosurfactants [these change with age, food composition]
• Standardisation of mixing conditions
• Interlaboratory comparisons
• Agreed “outcome” measures (SDS-PAGE, mass spectrometry to monitor digestion of polypeptides, bioactivity measurements like IgE binding, T-cell reactivity)

NB – variation in outcomes of interlaboratory trials maybe determined more by measurement and sampling than the protocol per se!
How to measure digestibility?
SDS-PAGE and chromatography

- How do you assess digestibility?
- What constitutes a resistant fragment?

Mass spectrometry for profiling peptides and large fragments?

Duodenal digestion - only 2 out of 14 tryptic and chymotryptic are hydrolysed!

Three digestion products can be identified – residues 1-79, 1-39 and 40-79 which are only observed in unreduced protein.

Biological activity of digestion products - IgE immunoblotting – effect of digestion on Bet v 1 homologues

SDS-PAGE  IgE Immunoblotting

Api g 1

20s 1m 2 5 10 20 40 60 c  Patient PEI 62-3  Patient PEI 9811-0146  Patient PEI 9809-1114  Atopic control

Mal d 1

20s 1m 2 5 10 20 40 60 c  Patient PEI 17-01  Patient PEI 162  Patient PEI 153  Atopic control

Biological activity of digestion products – effector cell activation by gastric digestion products of peanut Ara h 1

Eiwegger, Rigby et al Clin Experimental Allergy 2006
**In vitro** protein digestibility assays and their relevance to the risk assessment

- **In vitro** Gastroduodenal digestion provides information relevant to understanding the context of how a protein is presented to the immune system in a physiologically relevant context
- **The Pepsin Resistance Test** is a distinctly different biochemical test which provides complimentary information on the biochemical stability of a protein which may be predictive of allergenic potential

(of course if we had an effective animal model we would not have to rely on these tests so much!)
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