Manchester Institute of Biotechnology

In vitro protein digestibility tests for allergenicity risk assessment

Clare Mills
Institute of Inflammation and Repair
The University of Manchester

Declaration of interests

Funding:

UK Food Standards Agency

UK Biological and Biotechnological Sciences Research Council

UK Technology Strategy Board

European Union

European Food Safety Authority

DBV Technology

North West Lung Centre charity

In-kind sponsorship of students and collaborations

Waters Corporation, Romer Laboratories Ltd, Genon Laboratories, R-Biopharm, Campden BRI, Leatherhead Food Research, LGC Ltd, DSM.

Spin-out company

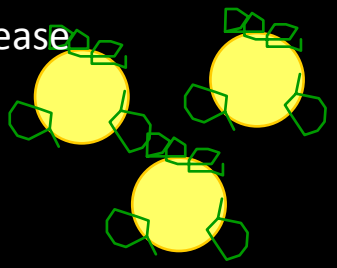
ReactaBiotech Ltd

MANCHESTER 1824

Why is digestibility important for food allergy?

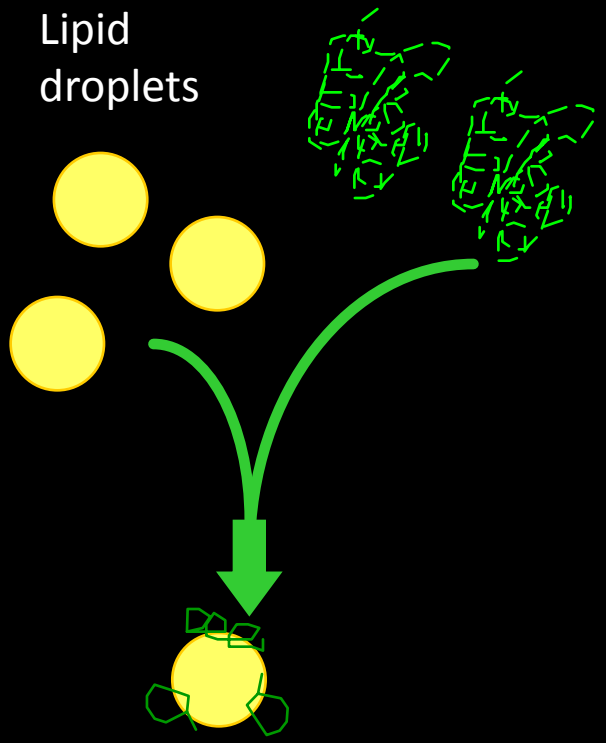
Events in the gut lumen affecting protein release and breakdown

Impact on release from intact cells



Protein stabilised emulsions

Lipid droplets



Proteolysed protein aggregates

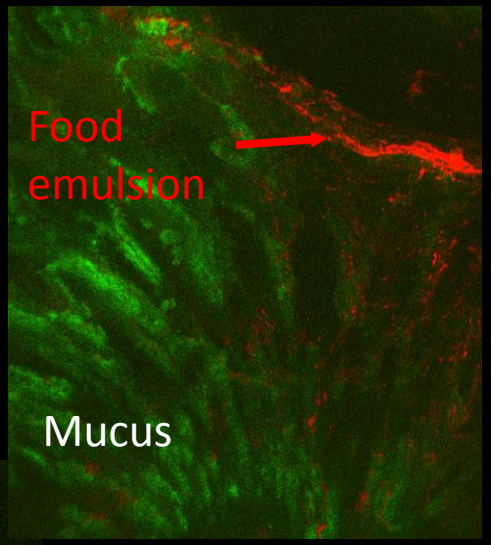
Resistant allergen

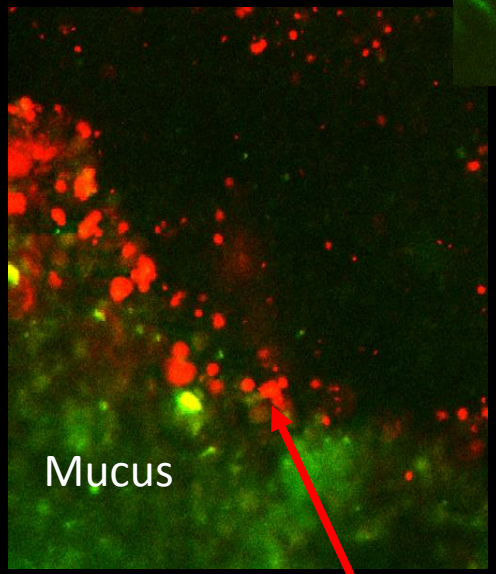
Lipid-adsorbed allergen

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

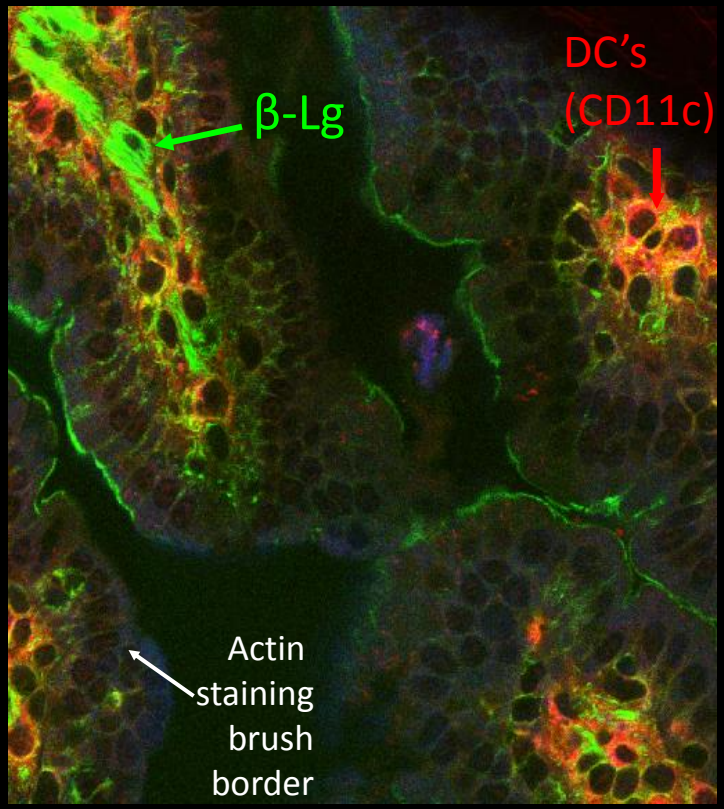
Uptake and intracellular processing of allergens…….

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

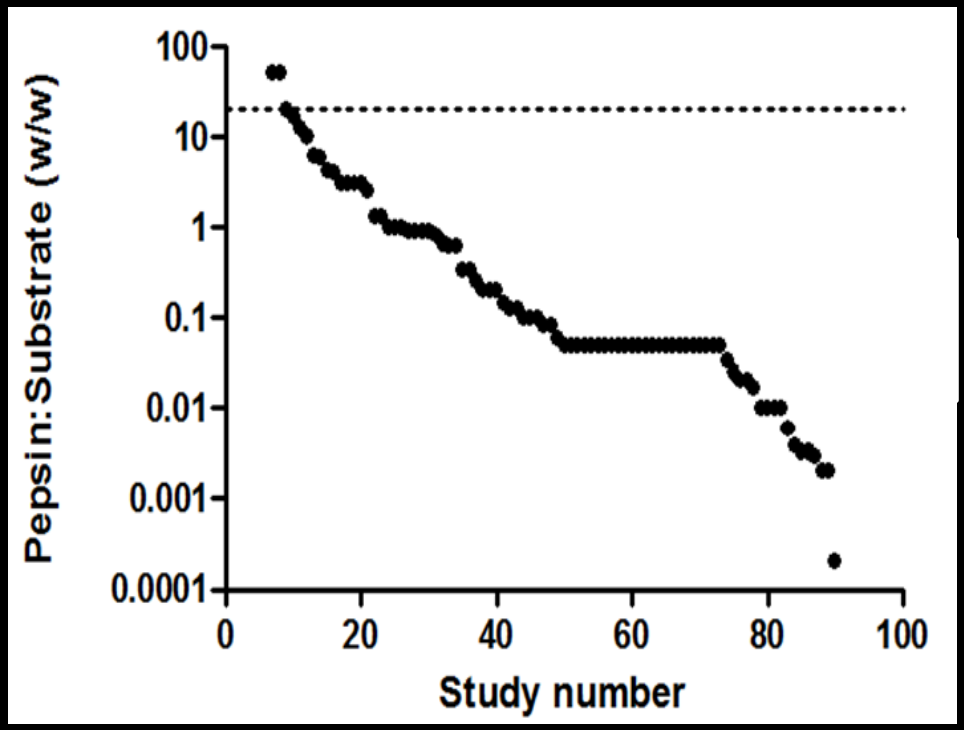
1996: Astwood et al identify a correlation between resistance to pepsin digestion and allergenicity [Nat Biotechnol. 1996 Oct;14(10):1269-73.]. This

- 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!



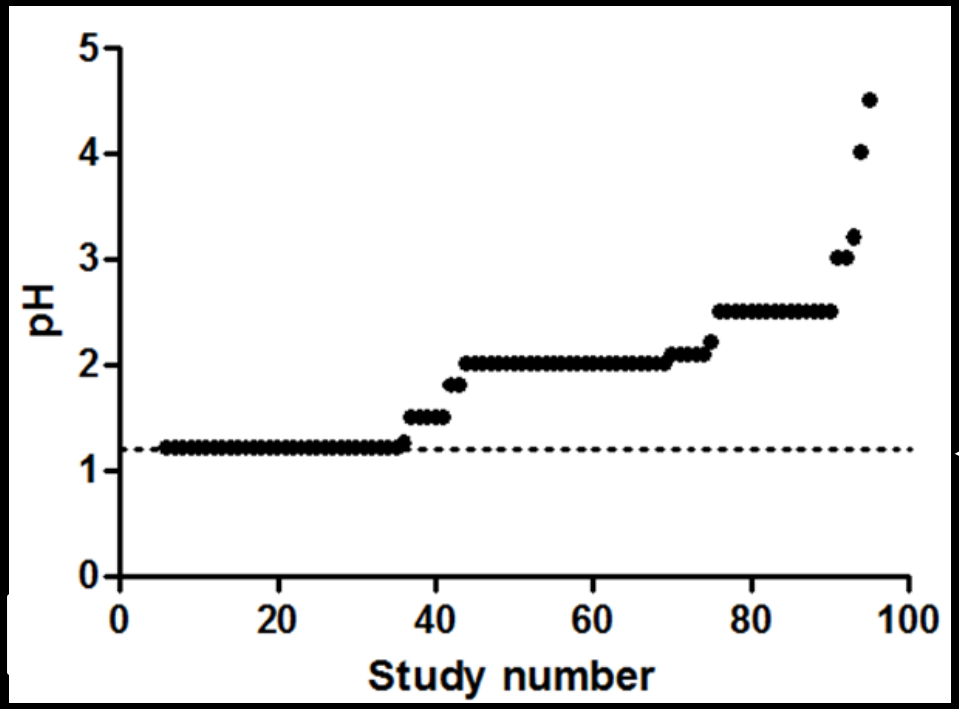
← Astwood et al conditions

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



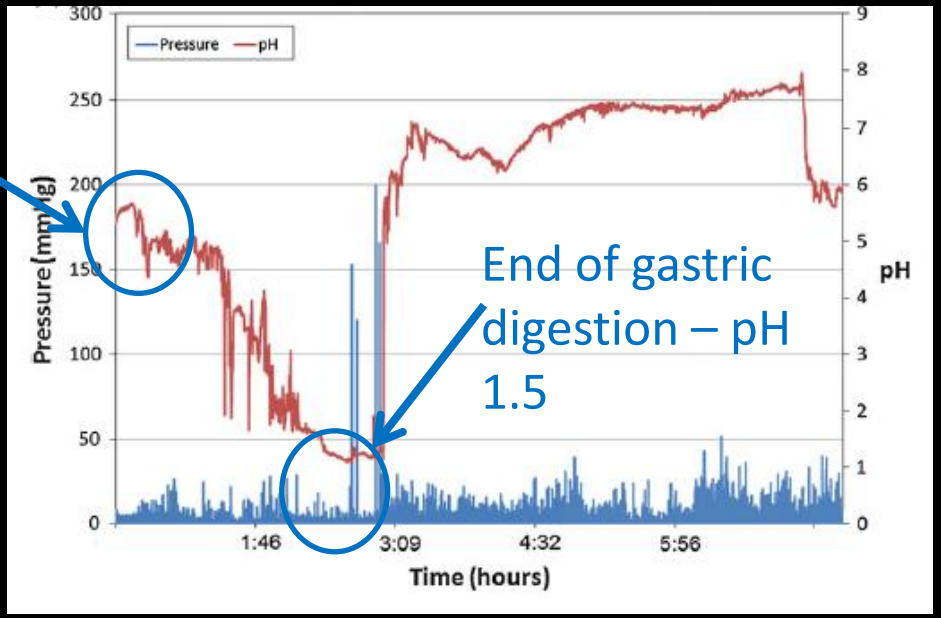
Astwood et al conditions

These will need to take account of changes

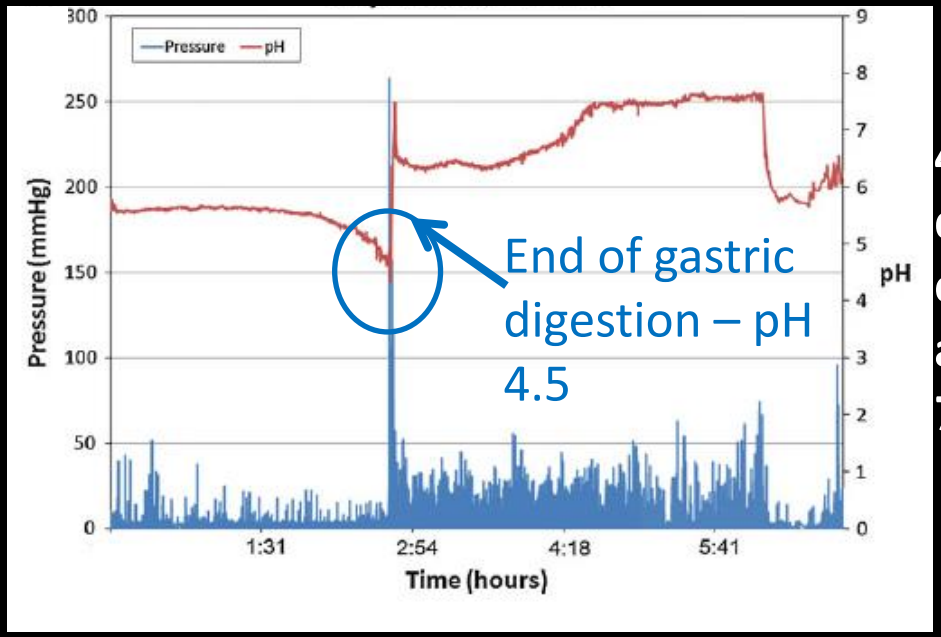
- In rates of secretion during digestion
- The effects of food composition on secretion
- Differences in digestive process in infancy
- Effects of antacids (taken by 25-30% population!)

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



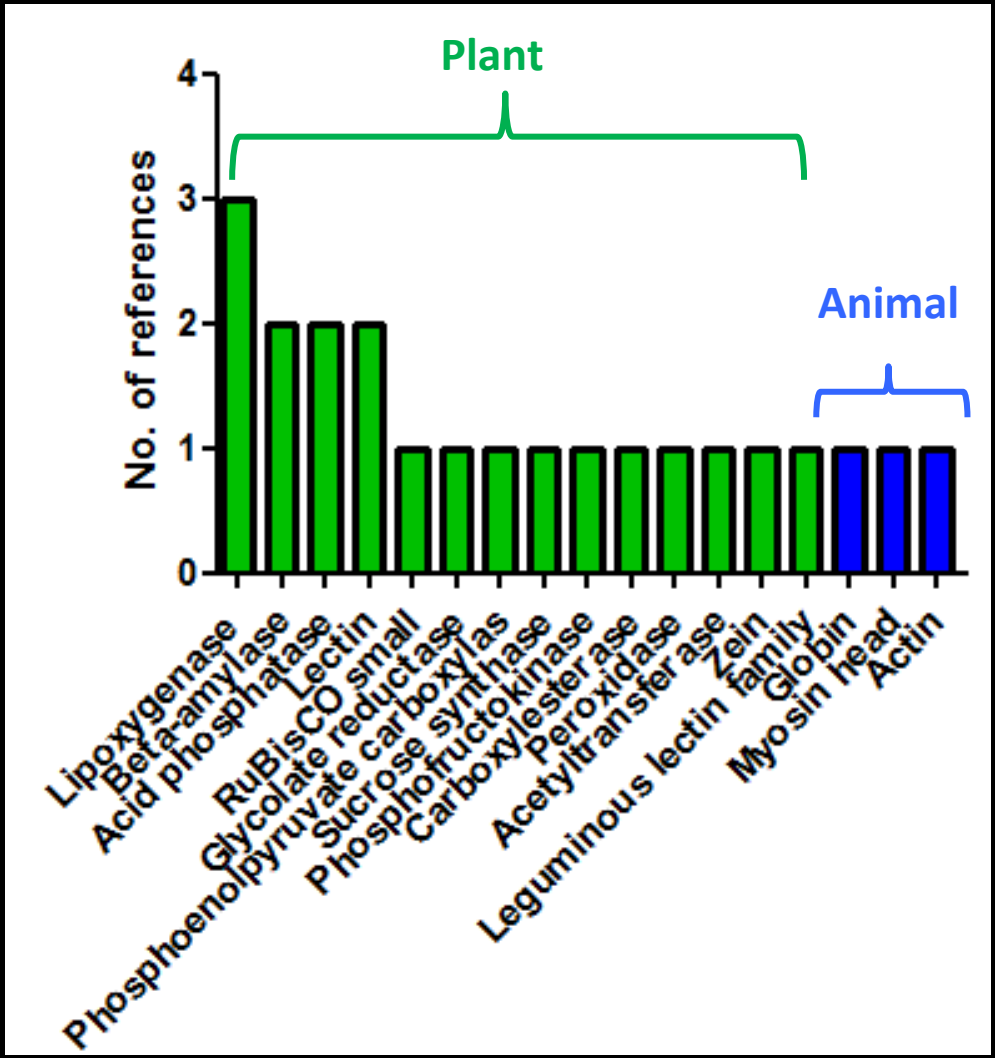
Healthy subject



40mgx2/ day esomepr-azole for 7 days

Michalek et al Dig Dis Sci (2011) 56:1735-1742

Use of comparator (non-allergenic) proteins

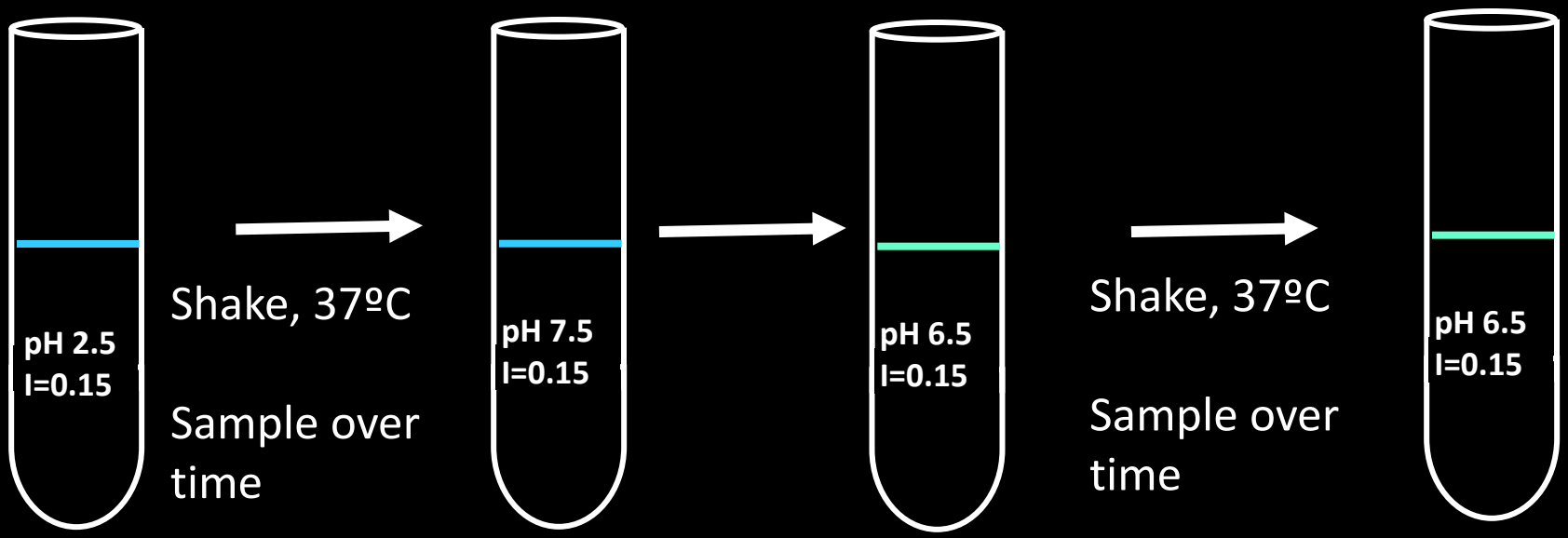


- Very few papers used comparator proteins
- This reflects the focus of research
- Access to purified, well-defined comparators
- Lack of consensus on choice of comparators
- This aspect also needs a consensus workshop

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
Phosphatidyl choline vesicles

Stop gastric digestion by addition of NaOH

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

Stop duodenal digestion by addition ofSBTI 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]

XNot well suited to analysis of foods [sampling, soluble versus insoluble phases]

Gastroduodenal digestion and the gut as a biological processing plant

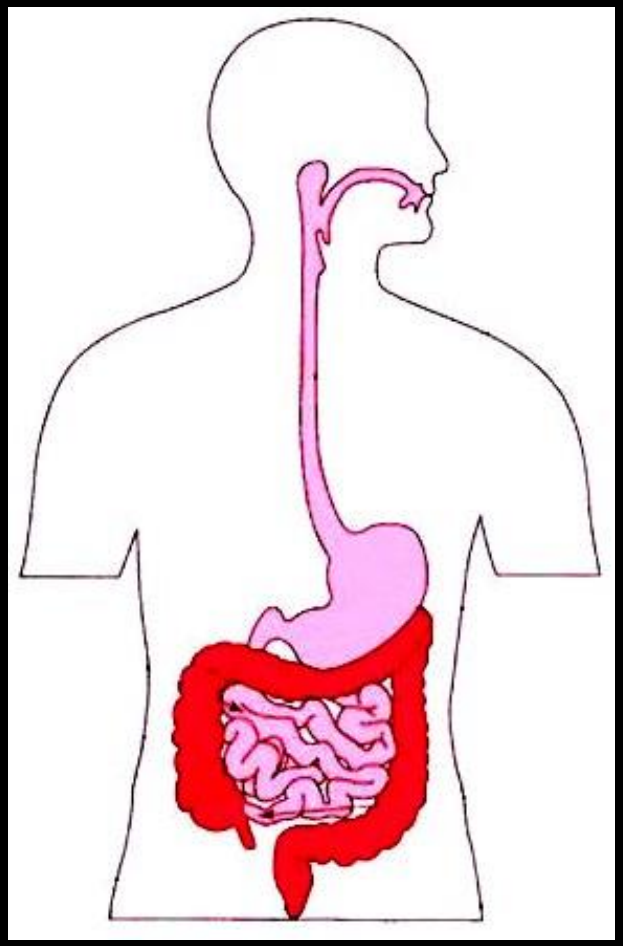
Biomechanics and motility determine luminal flow and mixing behaviour

+

Variation in activity, content and secretion of digestive enzymes

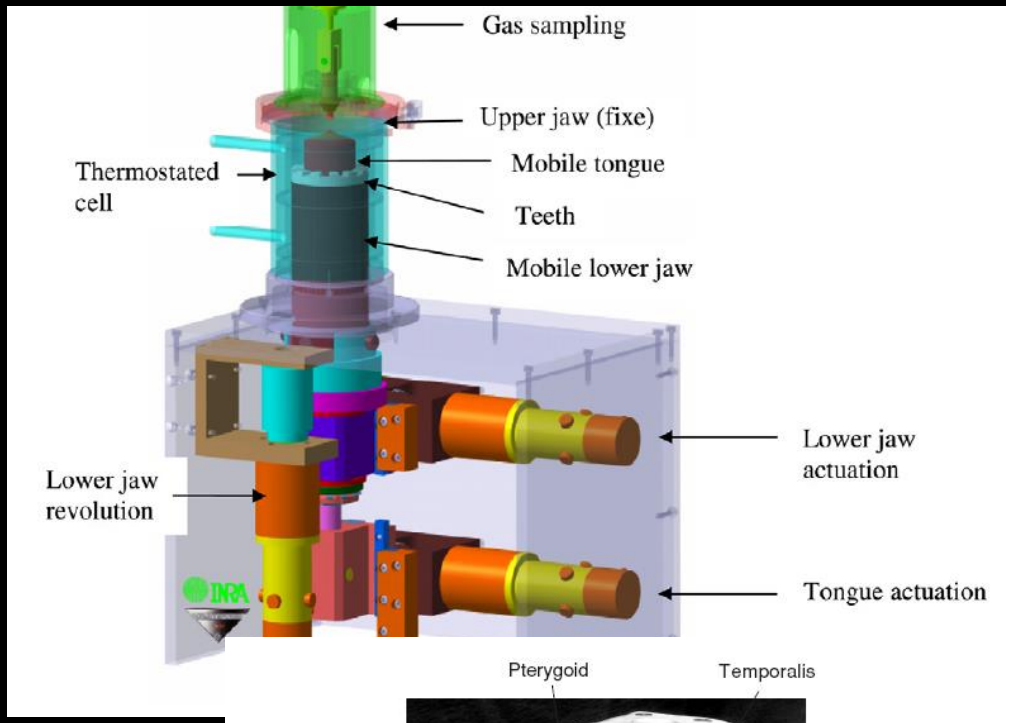
↓

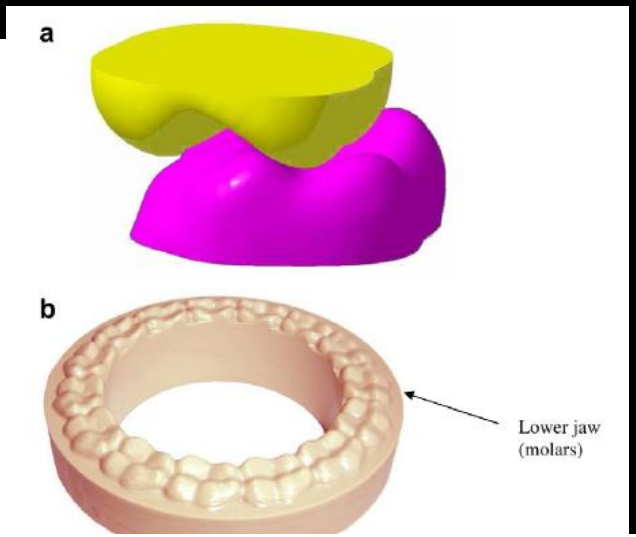
Determine the rate of delivery of absorbable species to the gut wall

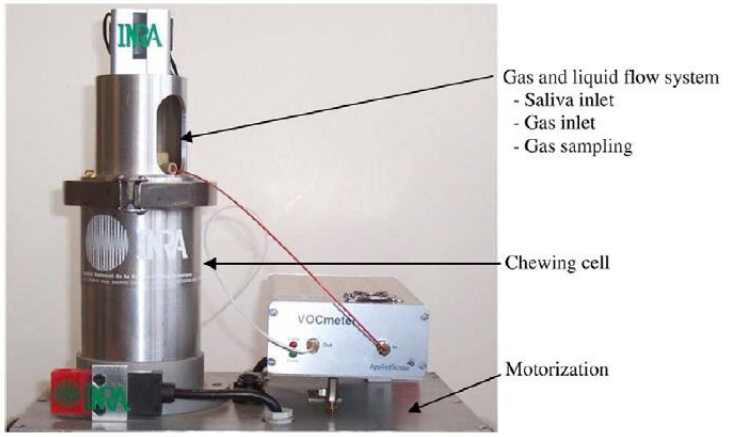


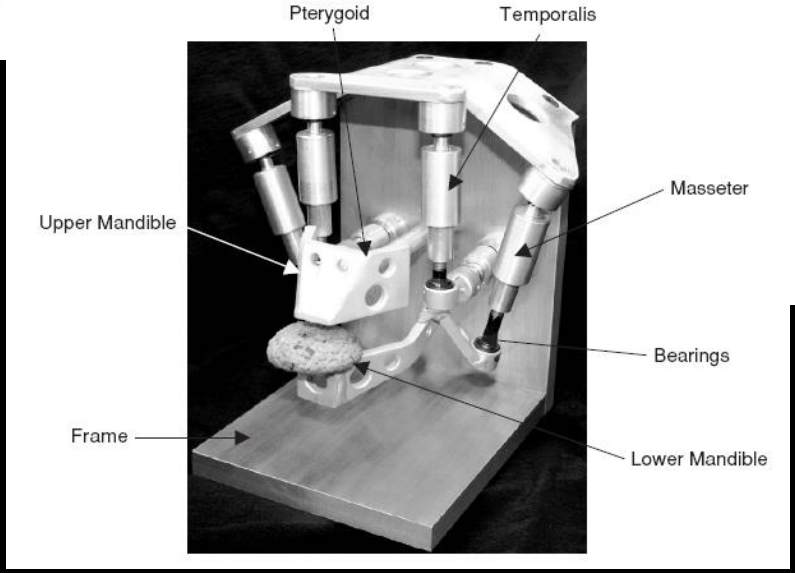
All of this is under tight biological control, including gut-brain signalling

Modelling chewing



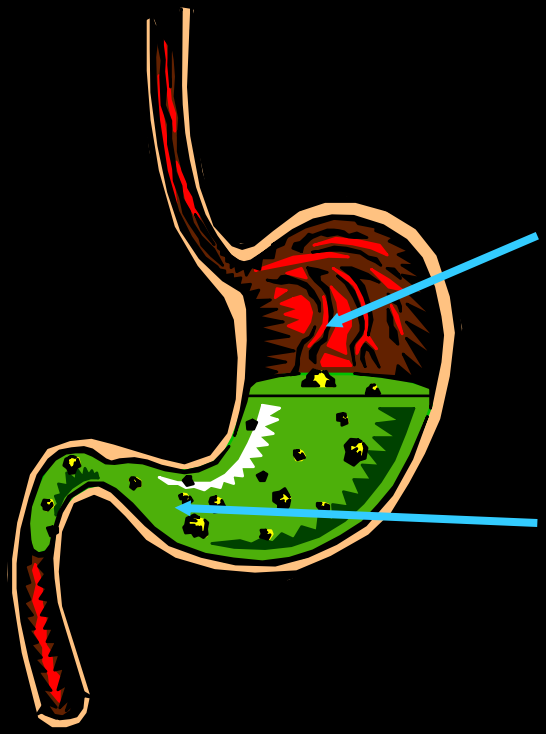






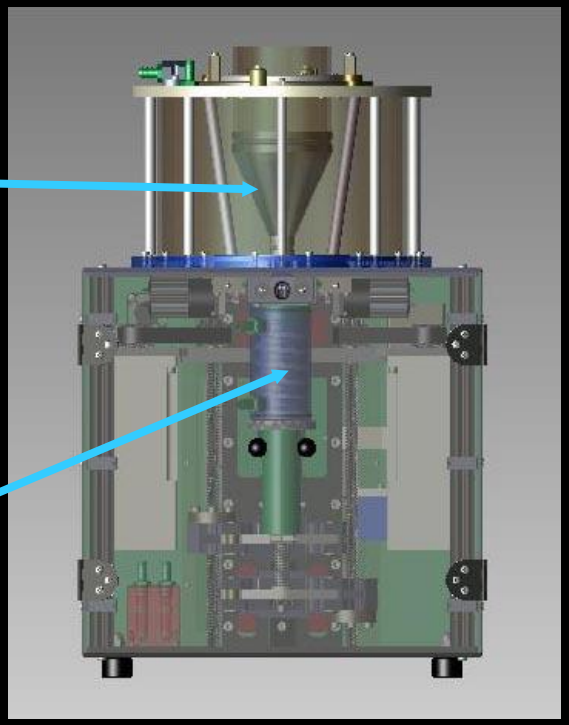
Diverse mechanical models are being developed – especially for studying flavour release

Dynamic Gastric Model (DGM): Full simulation of gastric forces and motility



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

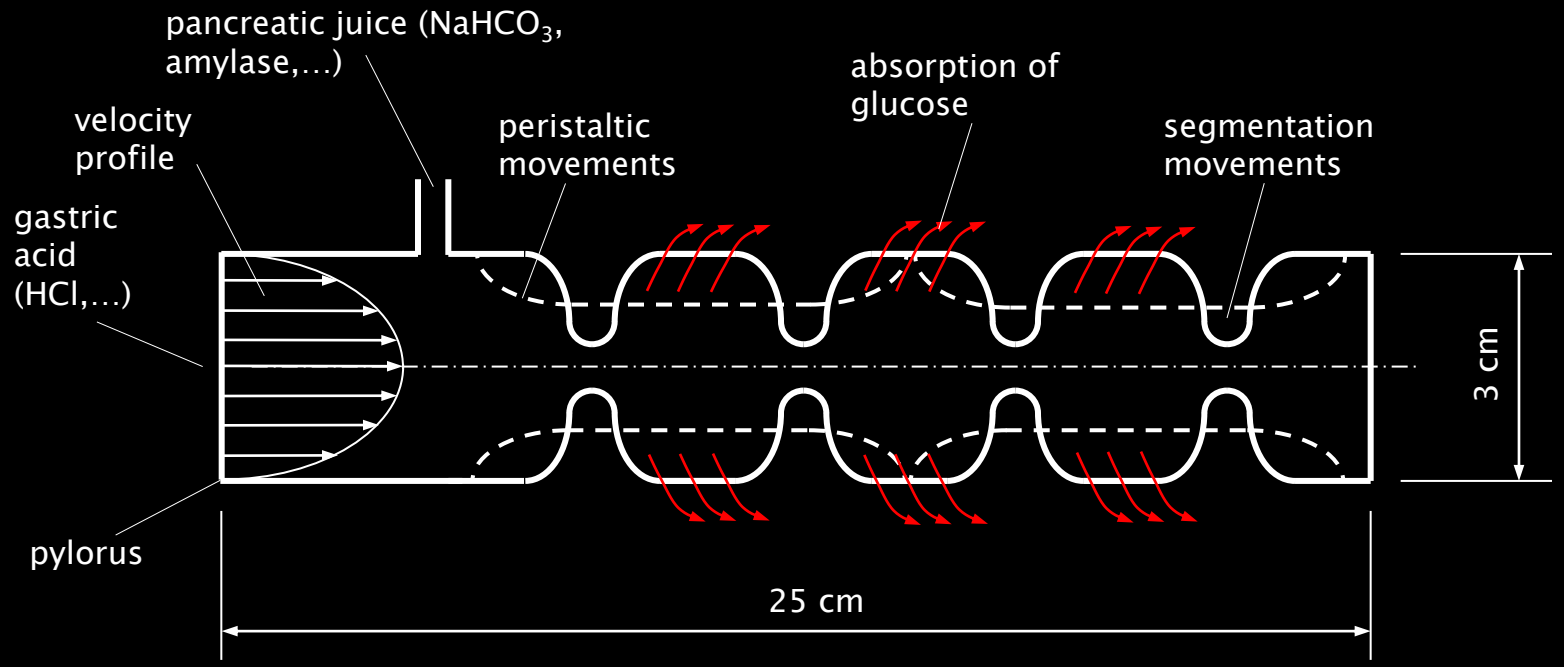
Antrum:
High shear well mixed environment
Shear at 10-100 sec^{-1}
Phase II contraction waves



Inventors: Martin Wickham, Richard Faulks
Available from Bioneer;

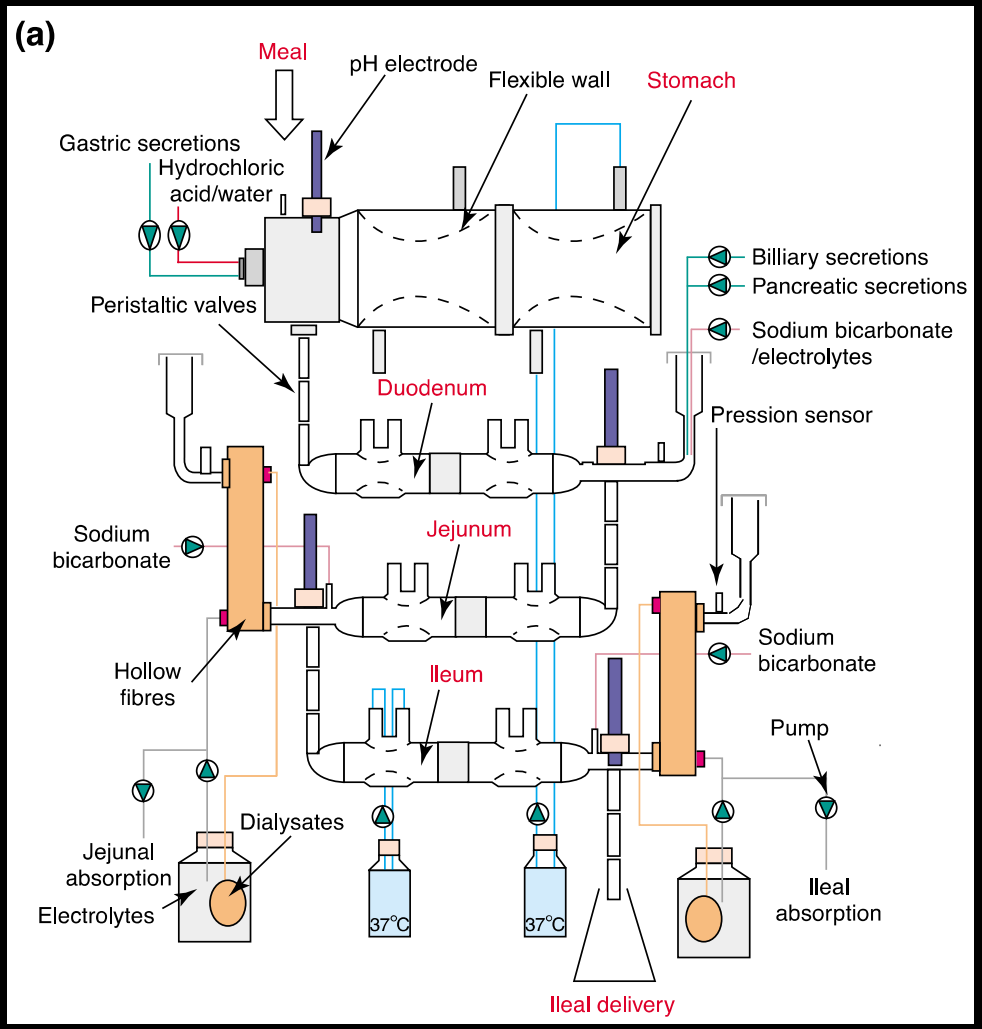
bioneer
your partner in biotechnology

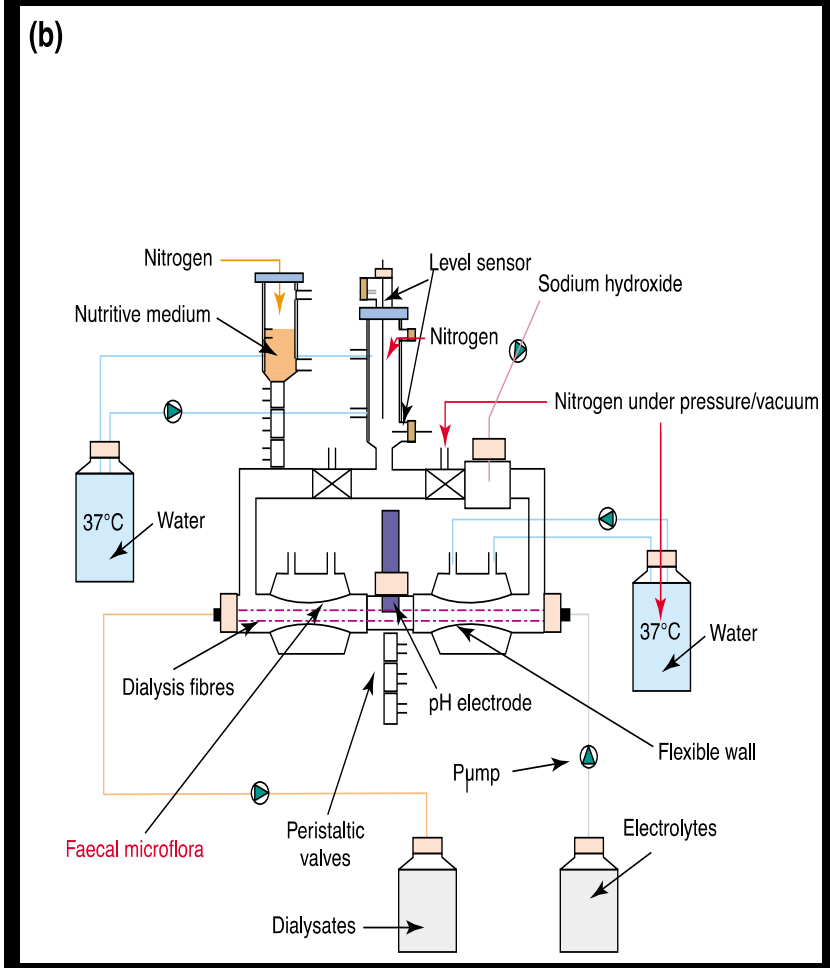
Dynamic Duodenal Model: combining segmented and peristaltic flow



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

X Not necessarily validated against the human situation

XNot adapted to analysis of small amounts of material or purified protein

Dynamic models of digestion are

- The only 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

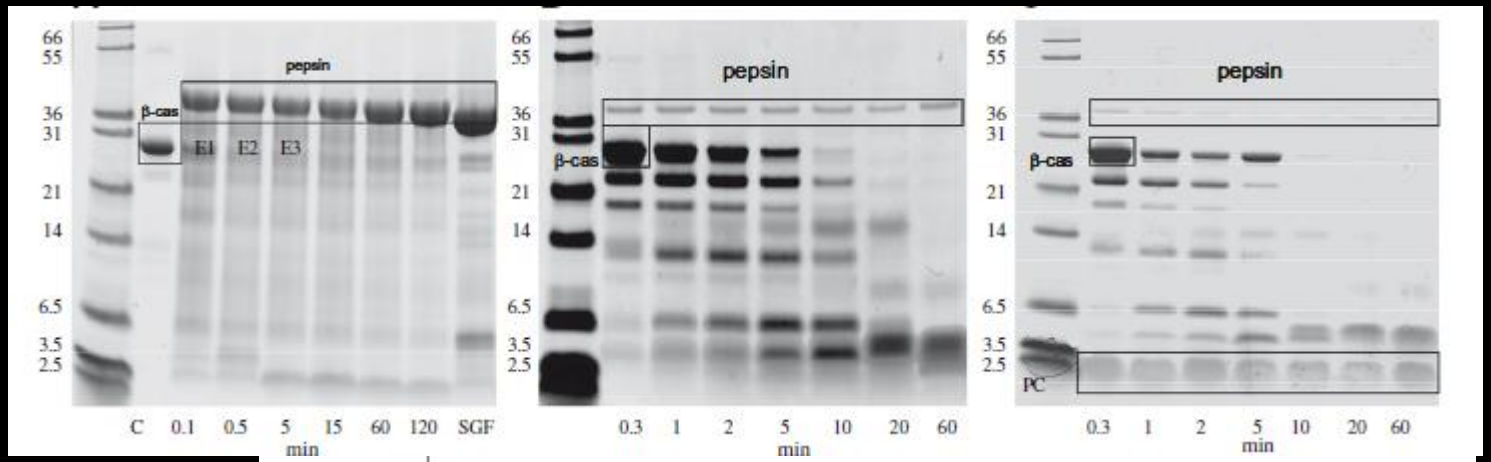
Validation is needed regarding

- 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)

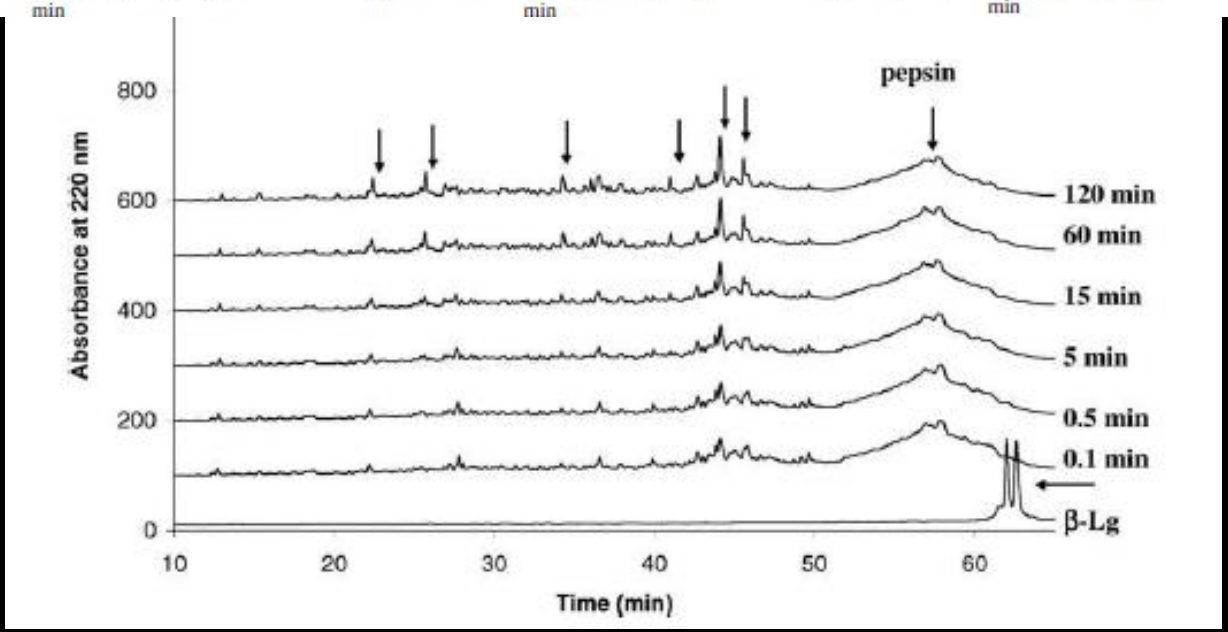
NB – variation in outcomes of interlaboratory trials maybe determined more by measurement and sampling than the protocol per se!

How to measure digestiblity?

SDS-PAGE and chromatography

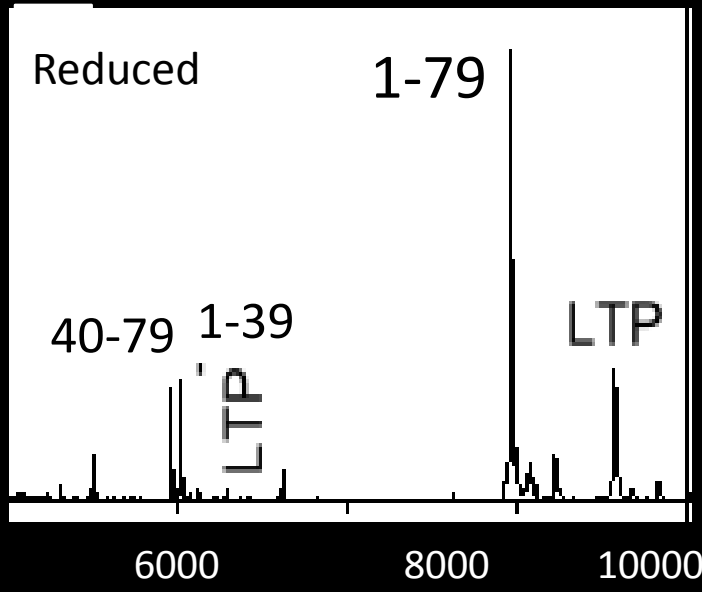


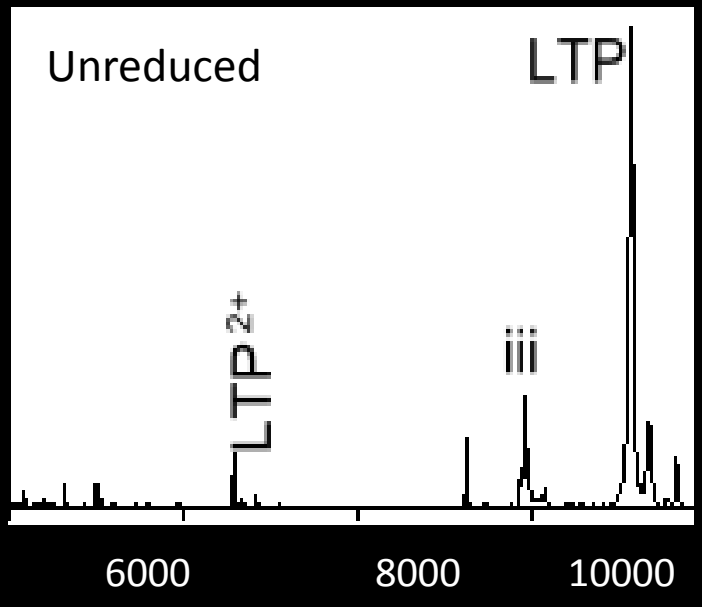
- How do you asses digestibility?
- What constitutes a resistant fragment?



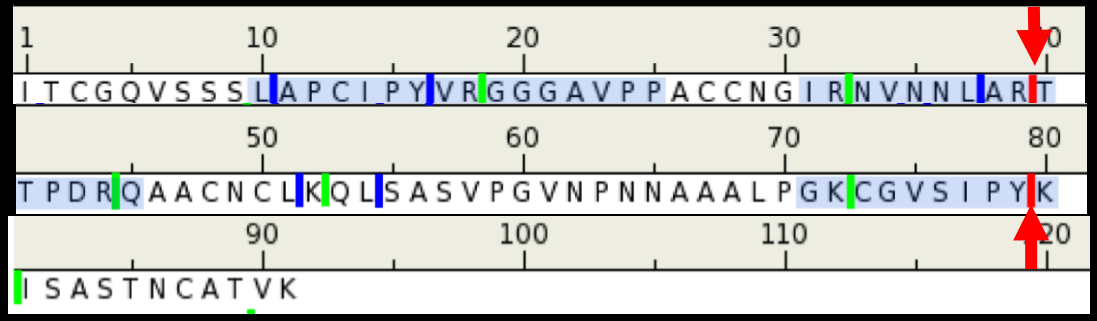
Mandalari et al Regul Toxicol Pharmacol. 2009 Dec;55(3):372-81.

Mass spectrometry for profiling peptides and large fragments?





Chymotrypsin — Trypsin

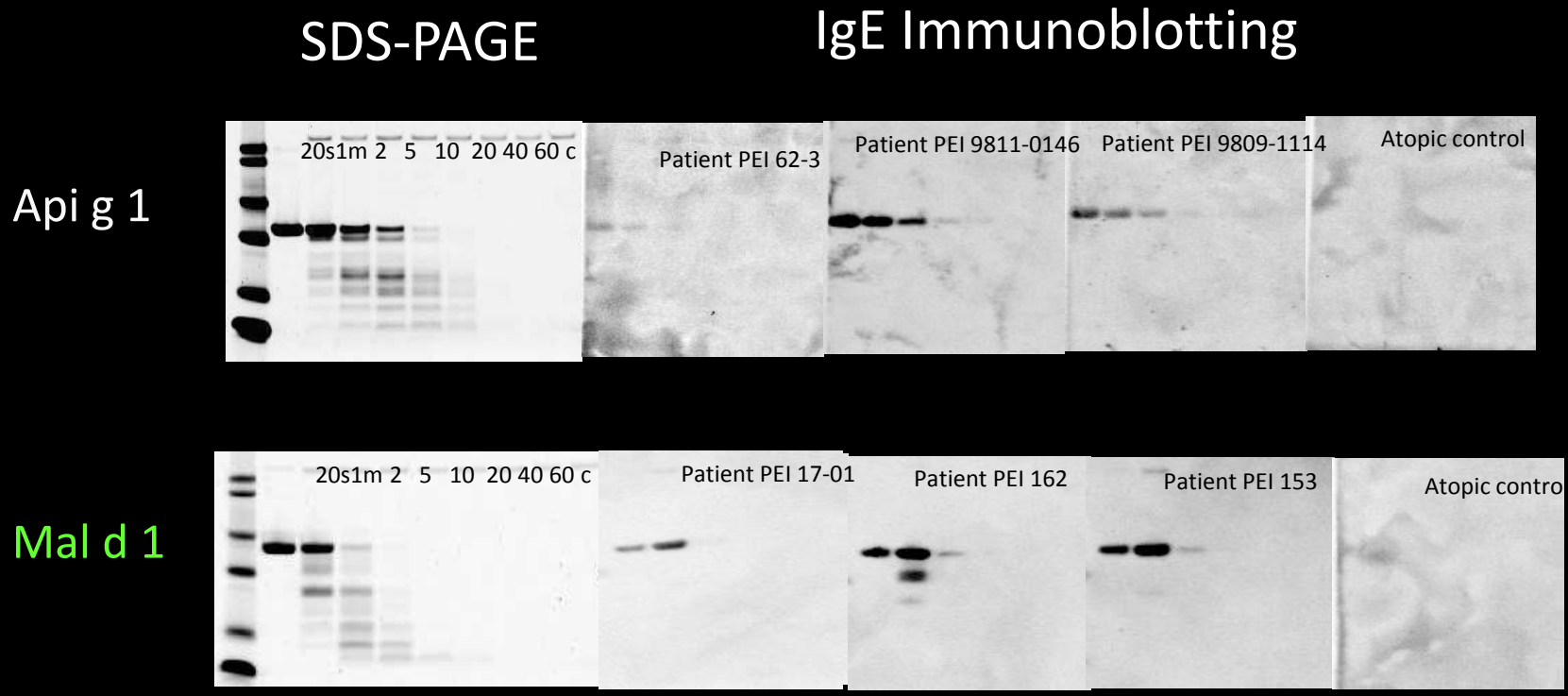


Duodenal digestion - only 2 out of 14 tryptic and chymotryptic are hydrolysed!

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

Wijesinha-Bettoni et al Biochemistry. 2010 Mar 16;49(10):2130-9

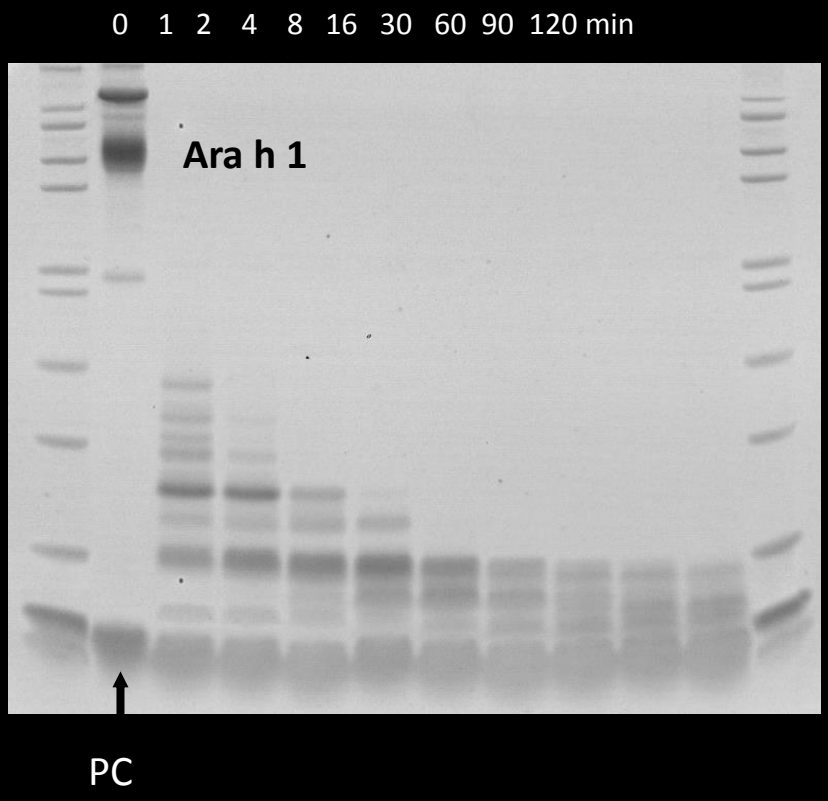
Biological activity of digestion products - IgE immunoblotting – effect of digestion on Bet v 1 homologues

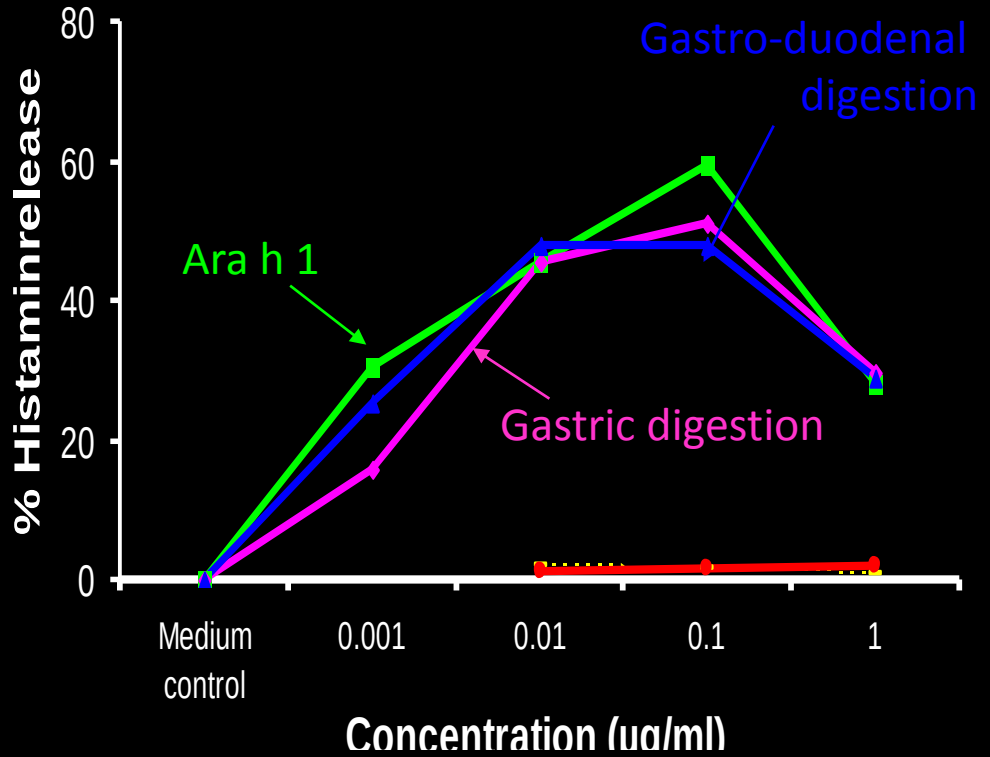


Sancho, et al 2011 *Mol Nutr Food Res.* 55(11):1690-9.

EuroPrevall

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

Allergest
The effect of gastrointestinal digestion on the allergenicity of foods

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!)

The Team

University of Manchester

Phil Johnson, Justin Marsh, Ivona Baricevic-Jones, Aafke Tekema, Anuradha Balasundaram, Rebekah Sayers, Frances Smith, Sophie Bromilow, Anya-May Hope, Carol-Ann Costello, Matt Sperrin, Angela Simpson, Adnan Custovic, Aida Semic-Jusufagic, Marina Themis.

EuroPrevall and iFAAM Partners

Barbara Ballmer-Weber – Zurich
Kirsten Beyer – Berlin
Montserrat Fernandez-Rivas - Madrid
Jonathan Hourihane – Cork
Rene Crevel – Unilever

IFR Team

Marianne Defernez, Jack Dainty, Neil Rigby, Alan Mackie

MANCHESTER 1824