In Vitro Digestion Methods for Assessing the Effect of Food Structure on Allergen Breakdown

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1. Introduction

Understanding the fate of proteins during digestion is of especial relevance to understanding the basis of food allergies. Little is known of the immunological mechanisms involved in the sensitisation of an individual towards a food and, with the exception of the fruit and vegetable allergies (which appear to be secondary responses to tree and weed pollen allergies), it is thought that food allergens (or fragments thereof) must cross the gastrointestinal mucosa in order to interact with the immune system. This is also a prerequisite for an allergen to elicit a reaction in an individual who has already become sensitised. Therefore understanding the physical behaviour of allergens under physiological conditions within the gut is fundamental to unravelling the complexities of food allergy.

2. Digestion of Allergens

For a food allergen to sensitise an individual via the gastrointestinal tract it must possess certain structural and biological attributes that preserve it from the denaturation and degradation conditions prevalent in the gastrointestinal tract. Two properties that seem, in general, to be shared by food allergens, are concentration in the diet and structural stability. This combination of properties may help to ensure that the allergen remains in a sufficiently intact form to be taken up by the gut and sensitise the mucosal immune system.

The human body has developed a complex system to breakdown foods in order to extract the nutrients required for the maintenance of health. When food is ingested it is first crushed and sheared in the mouth where it is mixed with saliva. It is then subjected to gastric processing for a variable period where the pH may fall to as low as 2 and finally on entering the small intestine it is neutralised and subjected to the duodenal, jejunal and ileal environments on its passage to the large intestine. During all of these phases it is mixed with enzymes (amylases, proteases and lipases) and in the duodenum to detergents (bile salts and phospholipids).

Proteins are released from food at all stages of ingestion/digestion, depending on solubility and accessibility, and undergo enzymic hydrolysis in the stomach (pepsin), duodenum (pancreatic proteases) and are further degraded as they pass through the unstirred layer covering the enterocytes (brush border enzymes) before being absorbed. Beyond these luminal events, proteins and protein fragments (peptides) which are absorbed by the enterocyte may undergo further intracellular degradation before the products of digestion reach the serosal side. Conventional nutritional wisdom states that nothing greater than di- and tri-peptides are exported to the serosal side of the enterocyte but this view is no longer tenable. It has been shown that bioactive and immunologically active peptides reach the serosal side in significant amounts either via the enterocyte, M-cells in the Peyer's patch and/or by paracellular diffusion.

In general the fate of proteins during the digestive process has been studied either *in vivo* in animal systems, using measures of digestibility such as nitrogen balance, or *in vitro* using proteases. As a consequence our detailed knowledge of the digestion of proteins relates either to its function as a macronutrient or from biochemical studies, such as peptide mapping. The latter technique exploits the specificity of proteolytic enzymes to study protein structure, but often under conditions far removed from those found in the lumen of the duodenum.

As peptides are believed to require a molecular weight of greater than 3,000 daltons (25 residues) in order to stimulate an immune response, large stable fragments, as well as intact proteins, have the potential to trigger initial sensitisation. Similarly, such large fragments may be able to cross-link mast cell immunoglobulin E (IgE), leading to histamine release. Whilst the bulk of food proteins are broken down into immunologically inactive fragments, very small proportions of material, which are still immunologically important, may escape digestion.

Circumstantial evidence that food proteins cross the gut barrier is offered by the presence in normal individuals of antibodies to a wide range of food proteins, such as β-lactoglobulin (β-Lg), in the circulation and the jejeunal mucosa. The presence of proteins, such as ovalbumin, in the blood of individuals after consumption of egg also supports the premise that intact proteins, albeit in small quantities, can enter the body. The route of such entry is not clear, and it may occur as a consequence of the active sampling of the gut contents by cells in the

Peyer's patches by pinocytosing material from the gut lumen. It has also been demonstrated that β-Lg from milk is taken up by the duodenal epithelium, possibly by a paracellular route. *In vitro* studies on confluent cultures of the enterocyte-like cell line Caco-2 have confirmed the endocytic nature of β-Lg uptake, and whilst the majority of the protein was degraded intracellularly, around a third was transported across the cells. The nature of the transported proteins has not been clearly defined, but circumstantial evidence, such as serum IgE, indicates that intact and degraded proteins cross the mucosal barrier. Thus it has been found that 10 out of 10 cow's milk allergic patients had specific IgE which recognised proteolysed β-Lg, with four of the patients IgE recognising the hydrolysed protein better than the native β-Lg.

Food allergy can have a major impact on everyday life, particularly in children, through trying to avoid allergenic foods or through the exclusion of foods. e.g. milk, which is a major source of nutrition. It is essential therefore, that individuals do not become sensitised in the first place, and, if sensitised, they are not subject to further exposure of the allergenic agent. In order to avoid sensitisation and re-exposure it is essential to identify the native allergenic proteins and the protein fragments (and aggregates) that are produced during the digestion process to see if they retain the allergenic properties. To date, no common features of protein molecules or protein molecule fragments have been identified as the major cause of allergy although some resistance to pepsin digestion is a common property[9].

3. *In vitro* models of digestion

The gold standard for investigating the human digestive process is the use of *in vivo* approaches. This normally involves a feeding study and the acquisition of serial samples of digesta from the stomach and upper small intestine, the rest of the small intestine being inaccessible. The next sample point is the terminal ileum (in ileostomy volunteers) or faeces. Samples taken from the upper GI tract need to be fluid so they can be aspirated through a naso-gastric or naso-jejunal tube so only fluid foods can be tested. Terminal ileal samples can be collected from ileostomy patients at regular intervals but only reveal the end point of upper GI tract processes while faeces are heavily degraded by the colonic microflora. Sampling from surgical patients or from sudden death victims is rare. Animal studies may offer an alternative but in addition to ethical considerations, concerns are frequently raised regarding their relevance to human systems, especially in the case of allergy studies.

Studies of the complex multistage process of digestion are therefore ethically and technically difficult, expensive to perform and make large numbers of studies impractical. There is therefore a strong case for the development and application of *in vitro* models which closely mirror the conditions and processes that actually occur *in vivo*. Such models have to be sufficiently refined to allow the process of digestion to be followed in some detail and have to be validated against *in vivo* data. Ideally, an *in vitro* model should offer the advantages of rapid representative sampling at any time point, testing the whole food matrix (or diet) instead of the isolated protein and be capable of handling solid foods which cannot easily be tested *in vivo*.

In vitro digestion models should consider 3 main stages: (i) processing in the mouth, (ii) processing in the stomach (cumulative to the mouth), and (iii) processing in the duodenum (cumulative of mouth and stomach). These three phases can be considered separately or in combination depending on the purpose of the study. Oral processing is perhaps the most difficult to simulate for solid foods so some standard process is normally used, e.g. homogenisation. Where liquid foods or isolated food components (e.g. purified protein allergens) are considered this particle size reduction phase is usually omitted although salivary amylase may be added.

For studies on the hydrolysis of proteins there is no significant enzyme action in the mouth but there is in the stomach and upper small intestine. These two zones differ with regard to the presence of enzymes, pH and surfactants and so both need to be simulated sequentially to explore the persistence of proteins and hydrolysed protein fragments.

Within this text we will describe two types of *in vitro* digestion models, Static and Dynamic:

3.1 Static Models:

Static models (also known as biochemical models) are defined as models where the products of digestion are not removed during the digestion process and which do not mimic the physical processes that occur *in vivo* (e.g. shear, mixing, hydration, changing conditions over time, etc). Good static models are particularly useful where there is limited digestion, e.g. stomach, but are less applicable for total digestion studies. These types of models are

predominately used for digestion studies on simple foods and isolated or purified nutrients, and are therefore ideal for assessments of the digestibility of isolated allergenic proteins.

Many of these models are quite crude and simply involve homogenisation of the food, acidification with hydrochloric acid, addition of gastric enzymes followed by a varying delay simulating gastric residence time, neutralisation with sodium carbonate or hydroxide and the addition of pancreatic enzymes and bile salts all the while stirring at 37^{o}C. The rate of loss of a component or the appearance of a component is used to measure the progress of the reactions but normally the system is allowed to run to completion to simulate 'total' digestion. Frequently the ratios of surfactants, enzymes and substrates are not physiological because the model is intended to cause exhaustive digestion in the belief that this is what occurs *in vivo*.

A static *in vitro* digestion model has been developed to assess the potential of a protein to become a food allergen using pepsinolysis and has subsequently been incorporated into the decision tree process proposed for assessing the allergenic risks posed by novel foods. Astwood *et al*[9] demonstrated that a number of allergenic proteins showed remarkable resistance to digestion by pepsin, including those from peanuts, soya and cow's milk, when compared with non-allergenic proteins. All the allergens remained either undigested, or were degraded to pepsin resistant fragments that persisted for up to an hour. All of the non-allergens were digested to low molecular weight peptides (<3,000Da) within 15 seconds. As peptides require a molecular weight of greater than 3,000 Daltons in order to stimulate an immune response, large stable fragments, as well as intact proteins, have the potential to act as sensitizers. Consequently resistance to pepsin digestion has become enshrined in the approaches used for assessing the allergenic potential of novel proteins.

However, there is much debate as to its validity as the apparent stability of a protein can be very dependent on the experimental conditions employed and later studies have thrown some doubt on the usefulness of pepsin resistance to predicting allergenicity. As with all enzyme assays the results are strongly dependent on the substrate concentration and it is evident that changes in the substrate: protease ratio alters the apparent susceptibility of allergens to proteolysis and depending on the ratio of enzyme used the same protein can be shown to be either stable or labile to pepsin[12]. The pepsin digestion protocols that have been employed typically involve pepsin: substrate ratios in the range 1:5 – 1:10. Such ratios may be

considered far in excess of those likely to be found in the stomach. For example, pepsin secretion in adults has been estimated between 20 – 30kUnits of enzyme activity/24h at 37^0C and from the activity of commercially available pepsin preparations used in digestion assays, this would be the equivalent of around 10mg pepsin secreted/24h. A typical adult dietary intake of protein around 75g/24h would give a ratio of ~ 3mg protein/unit pepsin secreted compared to ~ 3μg protein/unit pepsin used during digestion assays. Allowing for meal effects on secretion and gastric emptying and possible differences in pepsin activity under *in vivo* conditions, the ratios used during digestion assays are likely to remain orders of magnitude greater than ratios found *in vivo*.

3.2 Incorporation of gut lipid phases

Whilst resistance to pepsin digestion in standard, single-phase *in vitro* assays has previously been used as in indicator for protein allergenicity (as above) there has been little consideration of the effect of the multi-phase nature of the digestive system on protein digestibility. *In vitro* models currently in use to investigate allergen digestion do not generally incorporate the multi-phase nature of the gut where surface-active lipids originating either from ingested food, or from gut secretions, have been shown to form complex lipid structures, e.g. emulsions or liposomes within the gastric environment and liposomes or micellar phases in the duodenal environment. This is highly relevant to understanding mechanisms involved in developing food allergy since many plant food allergens have the ability to bind lipids and associate with membranes.

The presence of lipid phases within the digestion media can have a dramatic effect on the enzymic degradation of protein allergens. When investigating protein digestion it is normal for a single-phase system to be used or alternatively a lipid phase is added without control or quantification of its physicochemical characteristics. The physicochemical factors influencing the formation of lipid colloidal phases within the gastric and duodenal environment and the chemical characteristics of these phases have been extensively studied. The results of these studies have allowed physiologically relevant lipid phases to be prepared and incorporated within models of gastric digestion and duodenal digestion.

Burnett *et al* published data on the behaviour of a range of protein allergens in the presence of model emulsion systems under simulated gastric and simulated duodenal conditions. A

number of major allergens were found to adsorb to model stomach emulsions and subsequently desorb when duodenal conditions prevailed. Adsorbed protein may be more resistant to pepsin digestion than solubilised protein, indeed adsorption of proteins to an emulsion is more likely to decrease pepsinolysis and promote the delivery of allergens to the small intestine, where they are released. Decreased digestibility through adsorption would also help explain why proteins readily susceptible to pepsin digestion, like BSA, can be identified as food allergens. Furthermore, fluorescence and FTIR spectroscopy studies have shown proteins adsorbed to oil/water interfaces can be partly denatured. This is a recognised phenomenon associated with protein adsorption to oil/water interfaces. Thus at least part of the protein can be considered removed from the aqueous environment and therefore unavailable for digestion.

Whilst we acknowledge that static model systems lack the complexity of *in vivo* digestive processes, it is clear that the multi-phase nature of the gastric and duodenal environments could play an important role in the presentation of allergenic proteins to the immune system, and that current risk assessments for allergenicity that omit the gut colloidal phases could be oversimplified. Therefore it is advised that the gut colloidal phases should be included within the design of static digestion models used to assess the digestibility of protein allergens.

We have been systematically applying a more physiological model of digestion, not as a means of predicting possible allergenic potential, but to investigate the role digestion may have in the development and elicitation of food allergies. To do this we have used a biochemical version of the IFR gut model which allows the study of small quantities of proteins, but keeps the full range of physiological surfactants found in the gastric and the duodenal compartments, unlike any of the systems previously employed. To-date we have studied the role of surfactants on cow's milk allergens, including β-casein and β-lactoglobulin, α-lactalbumin, the cupin allergen from peanut, Ara h 1and plant food allergens from the prolamin superfamily including the 2S albumin allergens from Brazil nut and sesame, a ns LTP allergen from grape (Vassilopolou et al in press). From these studies several patterns of digestion are emerging.

Firstly there are proteins which undergo very rapid degradation, the patterns of degradation being largely unaltered by the inclusion of physiological surfactants. This includes the peanut allergen Ara h 1 and the cow's milk allergen β-casein. There are other allergens, notably the

very stable 2s albumin allergens, which resist digestion, residual structure being retained even after 2 hours gastric digestion, followed by 2 hours duodenal digestion, physiological surfactants again having no apparent effect on digestion profiles. The third group of allergens which includes the allergens β-lactoglobulin, α-lactalbumin and the ns LTPs do show changes in their digestion patterns as a consequence of interacting with the physiological surfactants, particularly the phosphatidylcholine vesicles secreted by the stomach. The digestion of both β-lactoglobulin, and the ns LTPs is negligible in the gastric compartment, but the presence of PC appears to confer some protection on these proteins to duodenal digestion. In contrast α-lactalbumin is more labile to gastric digestion but the presence of PC significantly slows the rate of breakdown. This is because α-lactalbumin is an amphitropic protein and when partially denatured to its molten globule state, as occurs at the low pH of the gastric compartment, it is able to interact and penetrate into the PC vesicles. This penetration effectively buries part of the protein structure, protecting it from the action of pepsin.

3.3 Dynamic Models

Anyone who has closely examined terminal ileal effluent collected from ileostomy patients will realise that digestion in the stomach and small intestine is far from complete. It is frequently possible to clearly identify what the patient has been eating. Because plant cell walls are not enzymically degraded in the upper GI tract, ileal effluent is found to contain quite substantial pieces of fruit, vegetable, mushroom, nuts and seeds. This is particularly true if these foods are eaten raw, whereas when cooked, the chewed fragments are more likely to disintegrate when subjected to shear forces in the antrum. The extent, rate and site within the GI tract where nutrient becomes available for absorption is not therefore just a function of the luminal environment, but also of the physical characteristics of the food. Certainly the structure of the food matrix can have a great impact on the elicitation of allergic reactions and may affect the kinetics of allergen release, potentiating the severity of allergic reactions.

It is becoming increasing clear that in order to understand the digestion of structured foods that it is insufficient to simply consider just the biochemistry of the gut as the gastro-intestinal processing plays an equally important role. This more holistic view of digestion will allow us to move away from the static models of digestion, which are only able to process simple model meals and isolated nutrients, to dynamic models, incorporating the physical processing of the gut, which can be used during digestibility studies on ‘structured’ meals (i.e. real foods or food materials).

Dynamic models may or may not remove the products of digestion but have the advantage that they include the physical processing and temporal changes in luminal conditions that mimic conditions *in vivo*. This is particularly useful where the physical condition of the digesta changes over time, e.g., viscosity, particle size reduction, and takes into account some temporal effects not otherwise considered, e.g., unstirred layers, diffusion, creation of colloidal phases, partitioning of nutrient between phases, etc.

An example of a dynamic model of digestion is the 'IFR Model of Human Digestion' (Model Gut) that was developed by the authors. In the first stage of developing the Model Gut, Echo Planar Magnetic Resonance Imaging (EPI) (in collaboration with the Sir Peter Mansfield Magnetic Resonance Centre at Nottingham University) was used to make *in situ* and non-invasive measurements of gastro-intestinal processing of complex meals in human volunteers. From these studies essential data was collected on the digestion of multiphase meals and the influence of structure, hydration, mixing, shear, transport and delivery within the gastrointestinal tract. These conditions are being replicated in the Model Gut. Parallel studies on the biochemistry of digestion have established protocols for enzyme and bile application that mimic organic micro-component digestion and release.

The Model Gut is built on a modular design of three stages. The first part simulates the main body of the stomach. This stage of the model mimics the mixing dynamics, diffusion profiles of both acid and enzymes and emptying cycles measured within the main body of the human stomach. This is followed by a unique emptying routine into a second module simulating the antrum (the lower part of the stomach). Here the digesta is subjected to high shear (as measured using EPI), forcing mechanical breakdown of the food structure. The final stage of the model provides a simulation of the small intestine. Here we have integrated intestinal mixing dynamics and diffusion with the addition of bicarbonate, phospholipids, bile, and digestive enzymes simulating the complex environment of the small intestine. All stages of the Model Gut have been validated against data collected from human digestion studies. The whole Model is computer controlled by state-of-the-art software that includes monitoring of all parts in real time.

The IFR model gut will be used in the EU-funded EuroPrevall project to assess the effect of food structure on the release of allergens in the gastro-intestinal tract and their subsequent stability to breakdown in the gut lumen.

With the advent of dynamic models of digestion we can for the first time understand the allergenic potential of real foods. These models can be used to investigate the interactions between allergen and food structure and the release and breakdown of allergenic material, the digestive and metabolic processes, including hitherto uncharacterised adjuvant effects of the food structure, and the effect of the food structure and the role of food processing in potentiating the allergenic properties of foods.

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