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Review

In vitro digestion methods for assessing the effect of food structure on allergen breakdown

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This article reviews the *in vitro* digestion models developed to assess the stability of food allergens during digestion. It is hypothesised that food allergens must exhibit sufficient gastro-intestinal stability to reach the intestinal mucosa where absorption and sensitisation (development of atopy) can occur. The investigation of stability of proteins within the gastrointestinal tract may provide prospective testing for allergenicity and could be a significant and valid parameter that distinguishes food allergens from nonallergens. Systematic evaluation of the stability of food allergens that are active *via* the gastrointestinal tract is currently tested in traditional pepsin digestibility models. The human gastrointestinal tract however is very complex and this article points out the importance of using physiologically relevant *in vitro* digestion systems for evaluating digestibility of allergens. This would involve the simulation of the stomach/small intestine environment (multi-phase models) with sequential addition of digestive enzymes, surfactants such as phospholipids and bile salts under physiological conditions, as well as the consideration of the effect of the food matrices on the allergen digestion.

Keywords: Allergenicity / Dynamic / In vitro digestion model / Protease / Sensitisation

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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 about the immunological mechanisms involved in the sensitisation of an individual towards a food and, with the exception of those with fruit and vegetable allergies which for some appear to be secondary responses to tree and weed pollen allergies, it is thought that food allergens (or fragments thereof) must cross the gastrointestinal (GI) 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 and why some proteins do sensitise *via* the GI tract and others do not. This paper is focussed on events in the gut lumen during digestion, although other factors, such as the genetics of the host, play an important role in determining whether a particular individual becomes sensitised.

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Abbreviations: **β-Lg,** β-lactoglobulin; **DGM,** Dynamic Gastric Model; **GI,** gastrointestinal

2 Results and discussion

2.1 Digestion of allergens

For a food allergen to sensitise an individual *via* the GI tract, it must possess certain structural and biological attributes that preserve it from the degradative conditions prevalent in the GI tract. Two properties that seem, in general, to be shared by food allergens are concentration in the diet and structural stability. The combination of these 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. It may also play a role in elicitation, although the rapid nature of allergic reactions (frequently within minutes of exposure) probably means exposure *via* the oral cavity and the oesophagus is sufficient to trigger a response in sensitive individuals.

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 vary from less than 2 to 4

or more during digestion of meals (actual pH depends on the volume, meal content, presence or absence of antacids and the individual consumer) 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 food digesta is also mixed with enzymes (amylases, proteases and lipases) and detergents (bile salts and phospholipids), in the duodenum.

Proteins are released from food at all stages of ingestion/digestion, depending on solubility and accessibility, and undergo enzymic hydrolysis in the stomach (pepsin) and duodenum (pancreatic proteases). They are further degraded as they pass through the mucous layer (which contains additional proteases) covering the epithelium 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 either *via* the enterocyte, dendritic cells, M-cells in the Peyer's patch and/or by paracellular diffusion [1].

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 [2]. As a consequence, our detailed knowledge of the digestion of proteins either relates to its function as a macronutrient, or derives 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 from those found in the lumen of the duodenum [3].

Studies on the immunogeneicity of whey hydrolysates have shown that peptides smaller than 3500 Da (~25–30 residues) are not immunogenic (or at least, unable to elicit mast cell degranulation), unlike larger fragments, as well as intact proteins [4]. Similarly, such large fragments may be able to cross-link mast cell 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. These may retain their ability to both sensitise individuals and elicit allergic reactions.

Circumstantial evidence that intact food proteins or peptide fragments 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 [5] and the jejeunal mucosa [6]. 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 [7]. 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 [8]. *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 [9]. 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 ten out of ten 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 [10].

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 desirable therefore that individuals do not become sensitised in the first place, and, if sensitised, they are not subjected to further exposure of the allergenic agent. To avoid re-exposure, individuals who are allergenic to a given food, should learn what food(s) they are sensitised to and thus what they should avoid.

In order to understand sensitisation and re-exposure identified native allergenic proteins and the protein fragments (and aggregates) that are produced during the digestion process can be studied to see if they retain the allergenic properties during transit down the gastro-intestinal tract. 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]. Resistance to pepsinolysis has been identified using conditions that are far from physiological, as part of the allergenicity risk assessment process for target transgenes. Stability or lack of stability by themselves does not predict whether a dietary protein is allergenic or not. There is a reasonable correlation between stability and food allergenicity [11]. However, the relationship between allergenic potential and resistance to pepsin is not straightforward and there is considerable evidence that it is not predictive of allergenicity *per se* [12]. Further, a number of stable, but nonallergenic, food proteins listed in the citation by Fu *et al*. [12] are immunomodulatory lectins. Even though they are not allergens, they are immunologically important as they are thought to survive gastric digestion. Thus, it is not clear that there is considerable evidence that pepsin digestibility is not protective of food allergenicity.

Using pepsinolysis to assess stability and its relationship to allergenicity should not be confused with *in vitro* digestion systems which mimic the *in vivo* environment more closely and are an important tool for investigating and dis-

covering the rules governing the fate of food proteins and structures in the gut lumen. This is fundamental knowledge which is relevant to understanding the behaviour of foods and food components and their relationship with a host of physiological responses of which allergy is only one.

2.2 *In vitro* models of digestion

Investigation of the human digestive process normally involves a feeding study and the acquisition of serial samples of digesta from the stomach and upper small intestine *via* naso-gastric/ naso-duodenal aspiration, 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 three 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. *In vivo*, foods are chewed and formed into a soft bolus that can be swallowed. To replicate this, a 'chew and spit' process is used for food grade materials where bacterial contamination of the sample is not an issue. If chewing to the point of swallowing is not an option a standard process is normally used, *e.g.* homogenisation, although this does not create a bolus, a process that may be important, particularly if the bolus is cohesive and does not easily disperse/disintegrate in the gastric compartment. Where liquid foods or isolated food components (*e.g.* purified protein allergens) are considered this particle size reduction and bolus formation phase is 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.

3 Static and Dynamic *in vitro* digestion models

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 (*i.e.* no absorption) 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 whilst stirring at 37°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 [13] 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 [14]. Astwood *et al* [12] 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 nonallergenic proteins. All the allergens remained either undigested, or were degraded to pepsin resistant fragments that persisted for up to an hour. All of the nonallergens were digested to low Mr peptides (<3000 Da) within 15 s. As peptides require a Mr greater than 3000 Das in order to stimulate an immune response (at least in cross-linking mast cells and basophils), large stable fragments, as produced during the pepsin digestion of allergenic proteins, as well as intact

native proteins, have the potential to act as sensitizers. Consequently, resistance to pepsin digestion has become established as one of the methods used for assessing the allergenic potential of novel proteins.

However, there is debate over the validity of pepsin digestion studies, 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 [11, 15]. 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 activity in the range of 8 and 12 Units per mg of test protein. 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 and 30 k Units of enzyme activity in 24 h at 37°C [16] and from the activity of commercially available pepsin preparations used in digestion assays, this would be the equivalent of around 10 mg pepsin secreted in 24 h. A typical adult dietary intake of protein around 75 g in 24 h would give a ratio of ~3 mg protein/Unit pepsin secreted compared to ~3 μg 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 an 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 and micellar phases in the duodenal environment [17]. 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 [18]. The physicochemical factors influencing the formation of lipid colloidal phases within the gastric and duodenal environment [19] and the chemical characteristics of these phases have been extensively studied [20]. The results of these studies have allowed physiologically relevant lipid phases to be prepared and incorporated within models of gastric and duodenal digestion [21].

Burnett *et al* [22] 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 [23], 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 that proteins adsorbed to oil/water interfaces can be partly denatured [22]. This is a recognised phenomenon associated with protein adsorption to oil/water interfaces [24]. 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 [13] 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 [25]. To-date we have studied the role of surfactants on cow's milk allergens, including β-casein and β-Lg, 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 [23–26], a ns LTP allergen from grape [27]. From these studies, patterns of digestion are emerging which indicate that the presence of surfactants (lecithin) has a concentration-dependent impact on the survival (persistence) of some of the native allergenic

proteins, information that would not be obtained from a simple pepsin digestion model.

Firstly, there are proteins which undergo very rapid degradation, the patterns of degradation being largely unaltered by the inclusion of physiological surfactants. These include 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 h gastric digestion, followed by 2 h duodenal digestion, physiological surfactants again having no apparent effect on the digestion profiles. The third group of allergens which includes the allergens β-Lg, 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 (PC) vesicles secreted by the stomach. The digestion of both β-Lg 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, vegetables, mushrooms, 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 [28].

It is becoming increasingly clear that in order to understand the digestion of structured foods, it is insufficient to simply consider the biochemistry of the gut, as the gastrointestinal 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 dynamic gastric model (DGM) that was developed by the authors. In the first stage of developing the DGM, 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 were collected on the digestion of multiphase meals and the influence of structure, hydration, mixing, shear, transport and delivery within the GI tract [29–37]. These conditions are being replicated in the DGM. Parallel studies on the biochemistry of digestion have established protocols for enzyme and bile application that mimic organic micro-component digestion and release. As a general rule, where complex real foods are tested the oral phase is included by using a 'chew and spit' process.

The DGM is built on a modular design of two stages. The first part simulates the main body of the stomach (fundus). 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. All stages of the DGM 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. Material emptied from the DGM can be then processed within 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.

Because the Model works in real time, samples of simulated gastric and duodenal digesta can be sampled during processing when the relative ratios of food to gastric and duodenal secretions and pH are changing and the food is being broken down chemically, biochemically and mechanically. The persistence or rate of loss of potentially allergenic native proteins and digested fragments from those proteins can be monitored by separation with SDS–PAGE gel electrophoresis (SDS–PAGE) and by using specific

antibodies, preferably from sensitised individuals. When using antibodies raised in animal species, care should be taken to ensure that the antibodies are specific to proteins and peptides that are responsible for the allergic response in humans. In addition, bands from the SDS–PAGE gel can be subjected to proteomic techniques to obtain information on their origin, sequence and conformation.

To date, using SDS–PAGE, the authors have been able to compare the protein and peptide profiles obtained from samples derived from the DGM (gastric and duodenal phases) with those from a temporal series of gastric and duodenal aspirates from human volunteers fed full fat cow's milk and peanut 'milk' with an equivalent protein load. The gels from the Model and those obtained *in vivo* are remarkably similar. This work is in the process of being prepared for publication.

As yet, studies attempting to differentiate between allergenic and nonallergenic proteins have not been carried out to determine if there are any differences in digestibility as is commonly claimed for allergenic proteins.

The IFR DGM and small intestinal simulation are being 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.

4 Conclusions

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, as well as the effect of the food structure and the role of food processing in potentiating the allergenic properties of foods.

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