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Static and dynamic *in vitro* digestion models to study proteins stability in the gastrointestinal tract

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Abstract

Food protein allergenicity has been linked to the survival of the allergen in the gastrointestinal tract. Therefore, *in vitro* digestion models have been widely used as tools to help predicting allergenicity. A huge diversity of static *in vitro* digestion models based on different experimental conditions have been proposed in the literature making the comparison between studies impossible. For this reason, an internationally harmonized static model has recently been developed. Dynamic *in vitro* digestion models are complex but more physiologically relevant and could represent an excellent alternative to study allergenic food digestion. Overall, these models have shown that the ability of a protein to survive in the gastrointestinal tract highly depends on whether the protein is pure or embedded into a complex food matrix.

Introduction

Introducing new protein sources to our daily diet is not easy and requires making sure that these proteins will not generate adverse reactions like allergy. However, there is a current lack of methods that could allow prediction of the allergenic properties of a food protein and the features that make a protein an allergen are still under investigation. Nevertheless, it has been hypothesized that for eliciting an allergic reaction, a protein has to partly persist in the gastrointestinal tract and pass through the epithelial barrier to come into contact with immune cells. The present paper aims to review the different types of *in vitro* digestion models available and discuss their physiological relevance to investigating food protein hydrolysis in the gastrointestinal tract.

Is there a link between digestibility and allergenicity?

A possible connection between the ability of a protein to resist the digestive process and its ability to raise an allergic reaction is still highly controversial. The protein does not have to be intact when reaching the epithelial cells and peptides generated by the digestion process and long enough to contain at least 2 epitopes could be responsible for sensitization [1]. The general opinion appears to be that the lower limit for allergenicity of peptides is a Mw of approximately 3.5 kDa [2]. Astwood et al. (1996), using a rather basic incubation test with pepsin, compared the resistance to pepsin digestion of 16 known food allergens i.e. ovalbumin, β -lactoglobulin, Ara h2, β -conglycinin etc and 9 common plant proteins considered to be non-allergens like Rubisco LSU and SSU from spinach leaf, lipoxygenase from soybean seed, sucrose synthetase from wheat kernel, β -amylase from barley kernel or acid phosphatase and phosphofructokinase from potato tuber. They showed that while major food allergens in general resisted the digestion process, non-allergenic proteins (mainly enzymes) were in contrast rapidly digested [3]. Using sturgeon caviar and parvalbumin, the major fish allergen, as examples, impairment of the digestion process was shown to increase allergenicity of the proteins under investigation in a Balb/c mouse model further supporting the hypothesis of a link between resistance to digestion and allergenicity [4]. These results were confirmed in human adults a few years later by the same group [5]. When reviewing all the literature available on digestibility studies of pure allergens, Bøgh and Madsen did not find clear evidence of such a link [6] but this could be due to the wide range of digestion methods employed in the studies reviewed, many of which were not physiologically relevant. Studies assessing the allergenicity of digestion products, by either IgE-binding, elicitation or

sensitizing capacity shows that digestion may abolish, decrease, have no effect, or even increase the allergenicity of food allergens. Fu et al. tested a number of similar allergenic and nominally non-allergenic proteins with similar cellular functions for their ability to survive *in vitro* digestion. They selected 23 allergens including 15 storage proteins (casein, β -lactoglobulin, ovalbumin, conalbumin, Ara h1, Ara h2 etc), 2 plant lectins from soybean and peanut, 5 enzymes (lysozyme, lactoperoxidase, papain, bromelain and actinidin) and 1 contractile protein i.e. tropomyosin from shrimp. They compared the resistance of these known allergens to 16 proteins with similar functions but unproven allergenicity: 4 storage proteins (α -lactalbumin, zein, and 2 trypsin inhibitors), 5 plant lectins from pea, lentil, lima bean, jack bean and red kidney bean, 4 enzymes (cytochrome c, rubisco, phosphofructokinase and sucrose synthetase) and 3 contractile proteins i.e. tropomyosin from bovine, chicken and pork. They found there was no clear relationship between digestibility measured *in vitro* and protein allergenicity [7]. The overall controversy can certainly be explained by the different experimental conditions (enzyme: substrate ratio, pH and duration of the gastric phase etc) that were used in those different studies and also by differences in analytical techniques that were used to characterize the digested product. There are a number of structural families of allergens that are more resistant to proteolysis than others. For example, so called lipid transfer proteins have been shown to be a pan-allergen with a degree of cross-reactivity comparable to profilin. It shows significant resistance to pepsin digestion [8]. Similarly, the IgE binding capacity of thaumatin-like protein Act d 2 from kiwi was found to be largely unaffected by low pH and simulated digestion [9]. In contrast, protein families such as patatin, zein, chlorophyll binding or flavodoxin contain few or no known allergens [10].

Another important aspect to consider is that allergens are not consumed as pure proteins but are embedded into complex food matrices. Interactions with other food constituents or differences in the propensity of proteases to interact with different proteins might dramatically modify the hydrolysis of an allergen in the gastrointestinal tract. Furthermore, the pH of a food is usually between 4 and 7 and its buffering capacity will significantly increase the pH of the stomach during the first stages of digestion consequently limiting the activity of the main gastric protease i.e. pepsin whose optimal activity is around pH 2 [11]. This will strongly reduce the proteolysis and intact proteins have been shown to reach the small intestine even after 20 min of gastric digestion [12]. Finally, protein structure can be significantly affected by the physico-chemical conditions found in the gastrointestinal tract, affecting the rate of proteolysis. One of the best examples to emphasize the importance of these structural modifications is the case of milk caseins. Caseins consist of 4 individual proteins (α _{s1}, α _{s2}, β and κ) that are organized in milk into a supramolecular structure called the casein micelle. Submitted individually as pure proteins to an *in vitro* digestion model, caseins will be cleaved and reduced into short peptides within a few minutes [13]. However, when ingested in the form of milk, caseins will clot in the stomach due to the acid conditions and form a curd that will be retained in the stomach and slowly released as curd particles in the small intestine. For this reason, caseins have been called “slow proteins” [14] and it is therefore not surprising that caseins are considered as major allergens for the pediatric population. In contrast, the whey protein, β -lactoglobulin, is generally highly resistant to gastric proteolysis. However, when it becomes adsorbed to the surface of oil droplets its digestibility is altered radically and a significant proportion, most likely the population of molecules directly adsorbed to the oil droplet surface, becomes highly digested, probably as a consequence of denaturation [15]. In addition, the whey portion of milk remains in solution under gastric conditions and so is emptied from the gastric compartment relatively quickly and is subsequently hydrolyzed by duodenal proteases and has thus been designated as a “fast protein” [16].

Finally other routes for generating allergic reactions to food have been described like the respiratory mucosa [17] or the skin [18]. For example, inhalant allergens are able to sensitize subjects that will exhibit an allergic reaction when cross-reacting food allergens are ingested [19-21].

The pepsin resistance test

In vitro testing has a central place in the risk assessment process for allergenicity evaluation. *In vitro* digestion tests, cell-based assays and IgE-binding tests are among the tools that can be combined to have a rough idea of the allergenic potential of a protein source. One of the first tests to assess protein digestibility as a way to predict allergenicity was the pepsin resistance test formerly proposed by Astwood et al. [3]. It consists of hydrolyzing food proteins with 0.32% pepsin at pH 1.2. Three patterns of stability of the allergens included in the study were observed:

1. Complete stability resisting pepsinolysis for 60 min
2. Intermediate stability, proteins resisting digestion for at least 30 s but being digested within 60 min
3. Protein completely susceptible to proteolysis with no intact protein remaining after the first time point sampled (15 s), with stable fragments being observed for at least 8 min.

This study concluded that resistance to pepsinolysis was indicative of allergenic potential, and as a consequence it was proposed to include the pepsin resistance test in the decision tree approach to allergenicity risk assessment by Metcalfe et al. [22] which was then taken up by FAO/WHO Codex Alimentarius Commission [23].

***In vitro* gastrointestinal digestion models for predicting allergenicity**

The pepsin resistance test is based on drastic conditions that exacerbate the hydrolytic action of pepsin. The pH is extremely low (1.2) and the enzyme: substrate ratio is high, quite far from the physiological reality [24]. Furthermore, this test takes only the gastric phase into account whereas it has been shown that a protein can be highly resistant to gastric digestion but be completely hydrolyzed within a few minutes when entering the small intestine [25]. Therefore, other groups have developed gastroduodenal or gastrointestinal models taking intestinal proteolysis into account and dozens of *in vitro* digestion models have been developed and published. Among these models, some have been specifically used for assessing protein allergenicity. For example, a simulated gastrointestinal digestion has been carried out on purified peach lipid transfer protein, one of the main allergens among the population of the Mediterranean area and the major allergen of peach allergic patients [26]. About two thirds of the proteins were hydrolyzed during digestion and the peptides formed essentially derived from trypsin action, whereas the protein appeared to be resistant to pepsin and chymotrypsin. The intact protein and some high Mw peptides were found to be recognized by patients' sera. More recently, three edible mealworm species (*Tenebrio molitor*, *Zophobas atratus* and *Alphitobius diaperinus*) subjected to processing and *in vitro* digestion were analyzed for IgE cross-reactivity [27]. IgE from crustaceans or house dust mite allergic patients showed cross-reactivity to mealworm tropomyosin or alpha-amylase, hexamerin 1B precursor and muscle myosin, respectively. Heat processing as well as *in vitro* digestion did diminish, but not eliminate, house dust mite or tropomyosin IgE cross-reactivity. These two examples selected among many others show the interest of *in vitro* digestion protocols as first screening tools to assess the allergenicity of food proteins or new protein

sources. However, whereas the outcome of digestion studies is sometimes clear and easy to interpret for proteins that are either highly resistant to digestion or rapidly and fully hydrolyzed, it is more difficult for proteins that show an intermediate behavior. How should a protein that needs a long time to be fully digested be assessed? More data are needed for a better guidance to interpret digestion outcomes. Another difficulty is that all these models differ in their physicochemical conditions (pH, enzyme: substrate ratio, ionic strength of the medium) and their duration making a comparison of data between different studies impossible.

The Infogest consensus *in vitro* digestion protocol

Infogest was a COST Action (www.cost-infogest.eu) that took place between May 2011 and May 2015. The objective of this international network was to gather scientists from different disciplines (food science, nutrition, gastroenterology etc) in order to improve health properties of food by sharing our knowledge on the digestive process. It involved 340 scientists from 130 institutes in 37 countries (Europe but also New Zealand, Australia, USA, Argentina etc). One aim of the network was to consolidate conditions for simulated digestion of food and find a consensus, if possible, for a digestion model. A frameset of parameters including the oral, gastric and small intestinal digestion were outlined and their relevance discussed in relation to available *in vivo* data and enzymes. A consensus paper was released [24] giving a detailed protocol and line-by-line guidance, recommendations and justifications but also limitation of the proposed simple static model. A YouTube channel was created with videos showing how to run the model, calibrate the digestive enzymes and quantify the bile salts allowing the new comers to conduct experiments in the proper way (https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg). To validate this protocol, an inter-laboratory trial on the *in vitro* digestion of skimmed milk was conducted within the Infogest network [28]. The degree of consistency in protein hydrolysis was investigated. Analysis of the hydrolyzed proteins, after the gastric and intestinal phases, showed that caseins were mainly hydrolyzed during the gastric phase, whereas β -lactoglobulin was, as previously shown, resistant to pepsin. Moreover, generation of free amino acids occurred mainly during the intestinal phase. The study also showed that a few critical steps were responsible for the remaining inter-laboratory variability. The largest deviations arose from the determination of pepsin activity. Therefore, this step was further clarified, standardized, and implemented in a third inter-laboratory study. The “harmonized” static, *in vitro* digestion method for food which will aid the production of more comparable data in the future and has started to be used all around the world. It has been used to study the digestion of major allergens of egg [29], milk [30] and pasta [31]. It has been recently compared with *in vivo* data obtained in pigs for the digestion of skimmed milk showing an excellent correlation with the extent of proteolysis observed with the animal model used (manuscript in preparation). Since the model has been detailed in an open access publication and media, challenged in inter-laboratory trials, validated towards *in vivo* data and is currently widely used, it represents an excellent tool for assessing the resistance of new protein sources to digestion including processed foods containing these proteins.

Would dynamic *in vitro* digestion models be relevant?

Digestion is a dynamic process. Food entering the gastrointestinal tract will be transferred from one compartment to another at variable rates depending on its structure, caloric content, osmolarity and rheological properties. Physico-chemical conditions (pH, ionic

strength, digestive enzyme concentrations etc) occurring in the different compartments will evolve with time. Static *in vitro* digestion models do not take these evolutions with time into account. In contrast, several dynamic multi-compartmental models have been developed during the last decades and recently reviewed [32]. One of the most well-known is the TIM model that was developed at TNO (the Netherlands) in the nineties [33] and is commercially available. The model has been used to study the fate of gluten [34] and milk allergens [35] in the digestive tract. Another multi-compartmental dynamic model is the SHIME[®] that was developed at Ghent University (Belgium), representing the gastrointestinal tract (GIT) of the adult human, as described by Molly *et al.* [36]. It consists of a succession of five reactors (stomach, small intestine, ascending, transverse and descending colon) simulating the different parts of the gastrointestinal tract. More recently, new dynamic models have been developed like the DIDGI[®] at INRA (France) [37] and the SIMGI[®] at CSIC (Spain) [38] and mainly used for studying the digestion of milk and dairy products [39]. When relevant physiological parameters are available for setting up these systems, they have been shown to be able to closely mimic the fate of food in the gastrointestinal tract and have been validated against *in vivo* data [37,40,41].

To be physiologically relevant, *in vitro* dynamic models need to be properly programmed. For most of the existing systems, key information needs to be entered in the software. For instance, the gastric emptying half-time is one of these key parameters and will be highly dependent on the properties of the food (caloric charge, viscosity, structure, osmolarity) that contains the allergens. Also the evolution of pH in the stomach is of crucial importance and will also highly depend on the buffering capacity of the food itself. For these reasons, it is rather difficult to use dynamic models to study the digestion of pure allergens in aqueous solution but these models are extremely relevant to study the digestion of allergens in real foods. Harmonizing at the international level the physiological parameters that would be relevant to digest different families of foods in dynamic conditions is one of the future objectives of the Infogest network.

Conclusion and perspectives

Resistance to digestion is one of the criteria to distinguish allergenic from non-allergenic proteins/foods. This criteria will be properly assessed only if physiologically-relevant *in vitro* digestion models are used. The Infogest consortium have developed a simple static model that can be used in a consistent manner and gives results that appear to mimic the situation *in vivo*. Nevertheless, interpretation of digestion data is sometimes difficult especially for allergens not showing a strong resistance or a rapid hydrolysis and more guidance on digestion output is needed. Recently, the model has been applied to food allergens but more evidence is needed to make sure that, for allergens, it would correlate with *in vivo* data. Dynamic models are more complex but much more physiologically-relevant than static ones. They would be of great interest in the future to study the persistence in the gastro-intestinal tract of allergens embedded in their foods. Research effort is urgently needed to validate these models for their ability to predict allergenicity. Microsystems are currently being developed [42] and would help in limiting the quantity of pure allergens to digest. *In silico* models [43] could also be of interest for simulating food digestion, but have not been applied so far to food allergens to our knowledge. Finally more models simulating the digestive process of specific populations like infant [44] or the elderly [45] will need to be tested for their ability to predict protein allergenicity.

Conflict of interest

The author(s) have no conflict of interest to declare

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