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Food allergens: Is There a Correlation between Stability to Digestion and Allergenicity?

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ACCEPTED MANUSCRIPT

Food allergens: Is there a correlation between stability to digestion and allergenicity?

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ABSTRACT

Food allergy is a major health problem in the Western countries, affecting 3-8% of the population. It has not yet been established what makes a dietary protein a food allergen. Several characteristics have been proposed to be shared by food allergens. One of these is resistance to digestion. This paper reviews data from digestibility studies on purified food allergens and evaluate the predictive value of digestibility tests on the allergenic potential. We point out that food allergens do not necessarily resist digestion. We discuss how the choice of *in vitro* digestibility assay condition and the method used for detection of residual intact protein as well as fragments hereof, may greatly influence the outcome as well as the interpretation of results. The finding that digests from food allergens may retain allergenicity, stresses the importance of using immunological assays for evaluating the allergenic potential of food allergen digestion products. Studies assessing the allergenicity of digestion products, by either IgE-binding, elicitation or sensitising capacity shows that digestion may abolish, decrease, have no effect, or even increase the allergenicity of food allergens. Therefore, the predictive value of the pepsin resistance test for assessing the allergenic potential of novel proteins can be questioned.

Keywords: Food allergy, dietary allergens, degradation, proteolysis, sensitisation, elicitation, risk assessment

INTRODUCTION

Food allergy is defined as an immune mediated adverse reaction observed upon ingestion of an otherwise harmless food or food ingredient (Bruijnzeel-Koomen et al., 1995; Johansson et al., 2001; Sampson, 2004). Food allergy is a major health problem in Western countries, where it affects around 5-8% of young children and 2-4% of adults (Sicherer and Sampson, 2010; Cianferoni and Spergel, 2009), and appears to be an increasing problem (Grundy et al., 2002; Poulos et al., 2007; Branum and Lukacs, 2009; Branum and Lukacs, 2008). The focus of this review is immunoglobulin E (IgE) mediated food allergy (type I food allergy), which is divided into two phases; a sensitisation and an elicitation phase. Sensitisation usually occurs by a primary exposure to the given dietary protein, where oral tolerance induction fails or is abrogated and where naïve B cells are primed to become IgE secreting plasma cells. Allergen-specific IgE

antibodies then bind to the high affinity receptor Fc ϵ RI on tissue mast cells and blood basophils,

and upon reexposure to the offending dietary protein, cross-linking of Fc ϵ RI-bound IgE occurs.

This leads to degranulation of mast cells or basophils, releasing mediators, inducing the allergic symptoms characteristic of food allergy (Gould et al., 2003; Stone et al., 2010; Winter et al., 2000).

Among the large number of proteins, that humans eat, only a small number are allergens. This could suggest that certain dietary proteins possess specific intrinsic features of allergenicity. Yet there is no absolute answer to the question ‘what makes a dietary protein a food allergen?’ (Huby et al., 2000). Even though the mechanism by which dietary proteins sensitise an individual remains basically unresolved, many food allergens are thought to sensitise through the gastrointestinal (GI) tract. This is likewise the general entrance route of the eliciting food allergens. Thus, resistance to proteolysis in the GI tract has received much attention in recent years and has been proposed to be a prerequisite for a protein to sensitise via the mucosal immune system of the GI tract (Mills et al., 2004; Taylor and Hefle, 2001; Bannon, 2004; Astwood et al., 1996). Since the first systematically evaluation of proteolytic stability of allergenic as well as non-allergenic dietary proteins, by means of an *in vitro* digestibility (Astwood et al., 1996), many studies have been performed assessing the digestibility of known food allergens. Besides an assessment of the proteolytic susceptibility of food allergens, several studies have in addition evaluated the residual allergenicity of the emerging digestion product. By use of immunological assays, IgE-binding capacity and/or the eliciting capacity of the generated peptide fragments have been assessed. However, the sensitising capacity of digested food allergens is not well investigated.

This review presents data from allergen digestion studies and discusses the methods used in relation to their predictive value as well as relevance for food allergy elicitation and sensitisation.

DIGESTIVE SYSTEM

Proteins are broken down by hydrolytic enzymes originating in the stomach, pancreas, and small intestine (Erickson and Kim, 1990). In the stomach proteins are exposed to proteolysis by different pepsins (Erickson and Kim, 1990; Etherington and Taylor, 1970). Pepsins have a wide specificity, preferentially cleaving peptide bonds between hydrophobic and aromatic amino acid residues like, phenylalanine, tyrosine, and tryptophan (Tang, 1963; Inouye and Fruton, 1967). Pepsins have an activity optimum around pH 2 (Johnston et al., 2007; Piper and Fenton, 1965), though the exact optimum may vary depending on the protein being digested, because the impact that the acidic environment have on the tertiary structure differs from protein to protein (Sakai et al., 2000; Renard et al., 1995). The gastric pH is thought to be between 1.2 and 3.0 (Untersmayr and Jensen-Jarolim, 2008; Savarino et al., 1988; James et al., 1962), but may vary further during the ingestion of a meal, because of influence by the volume and meal content (Wickham et al., 2009; James et al., 1962). The period of time the food stays in the stomach also varies, but the average transit time is estimated to be 1 to 2 hours (Marciani et al., 2001). Subsequently, the gastric digests are released into the small intestine, where the pH is 6 to 6.5 (Rune and Viskum, 1969). Here the gastric digests are subjected to proteases and peptidases produced by the pancreas, such as trypsin and chymotrypsin, or produced by the brush border of the intestinal mucosa (Erickson and Kim, 1990; Untersmayr and Jensen-Jarolim, 2008). While trypsin cleaves peptide bonds at the carboxyl side of the basic amino acids arginine and lysine, chymotrypsin cleaves peptide bonds where the carbonyl group is aromatic, like phenylalanine, tyrosine, and

tryptophan (Erickson and Kim, 1990; Olsen et al., 2004), with an activity optimum at pH 8 and 7.8, respectively (Asgeirsson and Bjarnason, 1991; McLaren and Estermann, 1957; Sipos and Merkel, 1970). In the stomach and small intestine digests is mixed with surfactant such as phosphatidylcholine (PC) and bile salts, which may influence the digestibility of proteins (Dupont et al., 2010; Gass et al., 2007; Moreno et al., 2005a; Mandalari et al., 2009b; Vassilopoulou et al., 2006). The susceptibility of dietary proteins to digestion varies greatly, but the goal is to achieve a mixture of amino acids and small peptides that can rapidly and efficiently be absorbed over the intestinal mucosa and serve as nutrient for the body (Erickson and Kim, 1990). While the majority of proteins are digested to amino acids and small peptides, some larger immunologically active fragments may survive the digestion process, and be absorbed and presented to the immune system of e.g. the Peyer's patches (PPs). The approximately transit time down the duodenum to the site of the first PP, the first site of the inductive mucosal immune system, is approximately 15 min (Moreno et al. 2005a). Also small quantities of intact protein may escape the digestion process, which has been shown for the cow's milk allergens β -lactoglobulin (BLG) (Marcon-Genty et al., 1989; Husby et al., 1987; Kilshaw and Cant, 1984) and bovine serum albumin (BSA) (Curtis and Gall, 1992) and the hen's egg allergen ovalbumin (OVA) (Husby et al., 1986; Husby et al., 1985; Husby et al., 1987; Kilshaw and Cant, 1984). Several *in vivo* studies of human digestion have been performed (Polovic et al., 2007; Troost et al., 2001; Sandberg et al., 1981; Polovic et al., 2007). However, such studies are technically and ethically difficult to conduct and at the same time expensive (Wickham et al., 2009). Therefore, several attempts have been made to develop *in vitro* models simulating the human digestion process (Dupont et al., 2010; Vieths et al., 1999; Moreno et al., 2005c; Mouecoucou et al.,

2004b). Large differences in the model systems are evident on e.g. pH, enzyme to protein ratio, digestion time, or addition of surfactant. However it is important to bear in mind that developing a single *in vitro* digestion model mimicking digestion of all humans is probably impossible, because digestion significantly varies from person to person and are influenced by factors such as age, health and medication status (Untersmayr and Jensen-Jarolim, 2008; Untersmayr et al., 2005). Further, *in vitro* digestion models have been made which did not attempt to mimic the human digestion process, but instead were designed to evaluate the allergenic potency of novel proteins based on their resistance to pepsin proteolysis (Astwood et al., 1996; Thomas et al., 2004).

FOOD ALLERGENS

An allergen is defined as the antigenic molecule giving rise to an allergic response (Bruijnzeel-Koomen et al., 1995) and is virtually always proteinaceous in nature (Mills et al., 2004). The term allergen, in type I food allergy, is used to describe three distinct molecular properties; (1) the property to bind IgE antibodies, (2) the property to elicit an allergic reaction, and (3) the property to sensitise an individual (Aalberse, 2000). Aalberse (2000) state that for an allergen to be ‘complete’ it must possess all three distinct properties. Not all allergens are ‘complete’ allergens. Well-known examples of such ‘incomplete’ allergens are the dietary proteins homologous to the birch pollen allergen Bet v 1, such as Mal d 1 from apple, Pru av 1 from cherry, Cor a 1 from hazelnut and Api g 1 from celery, which are known to elicit allergic

reactions but do not usually sensitise (Scheurer et al., 2004; Schimek et al., 2005; Aalberse, 2000).

More than 700 different allergens are described and more than 200 of these are derived from foods (Informall Food Allergy Database; IUIS Allergen Nomenclature). The food allergens originate from approximately 90 different species, with around 70% from plants and around 30% from animals. Although humans eat a varied diet, only few foods account for nearly half of the identified allergens, which is milk, peanut, egg, tree nuts, shellfish, fish, wheat and soy. Among the large number of proteins that humans eat, very few are allergens. This could suggest that allergens hold special features of allergenicity. However, no definitive answer to the question ‘what makes a dietary protein a food allergen?’ exists, though it is clear that some dietary proteins are intrinsically more allergenic than others (Huby et al., 2000). In general food allergens have been suggested to be water-soluble glycoproteins with a molecular weight (MW) of 10 to 70 kDa, that are abundant in the food and are stable to treatment with heat, acid and proteases (Huby et al., 2000; Lehrer et al., 2002; Bannon, 2004). However, many food allergens are found not to share such characteristics (Fu et al., 2002; Lehrer et al., 2002; Deifl and Bohle, 2011; Bannon, 2004), while proteins considered not to cause food allergy can be identified with those characteristics (Lehrer et al., 2002; Deifl and Bohle, 2011). Overall, it is likely that many factors may contribute to the overall allergenicity of any given protein, none of which is unique, but that some characteristics are more common among proven allergens than among other proteins considered to be non-allergenic (Huby et al., 2000).

Since no single characteristic of a dietary protein is sufficient for predicting its allergenic potential, it is recommended that the risk assessment process of novel proteins in genetically modified foods should implement a stepwise case-by-case approach that takes into account several types of information, including; (1) evaluation of the source of the gene, (2) the sequence homology of the newly introduced protein to known allergens, (3) the expression level of the novel protein in the modified food, (4) serum screening for the reactivity with IgE from the serum of individual with allergy to the source of genetic material or (5) from the serum of individuals with allergy to materials that are related to the source material for the gene, (6) test for resistance to pepsin degradation (FAO/WHO, 2001; EFSA Panel on Genetically Modified Organisms (GMO), 2010; Codex Alimentarius Commission, 2003) and (7) cell based assays (EFSA Panel on Genetically Modified Organisms (GMO), 2010). In addition, animal models have been suggested for prediction of the sensitising capacity of novel proteins (FAO/WHO, 2001).

RESISTANCE TO DIGESTION AS PREDICTOR OF ALLERGENICITY

One of the features proposed to be a characteristic shared by food allergens, which is also one of those that have received most attention in recent years, is resistance to digestion. Stability to proteolysis is generally believed to be an important feature of ‘complete’ food allergens, because of the hypothesis that for a protein to sensitise the mucosal immune system of the GI tract, it must survive the digestion process as an intact protein or as large fragments (Mills et al., 2004; Taylor and Hefle, 2001; Bannon, 2004; Astwood et al., 1996). This hypothesis seems highly

reasonable, since the longer a significant portion of the protein resists digestion, the more likely it is to encounter the cells of the inductive mucosal immune system and further the more likely it is to retain adequate structure and size to be recognised by these cells. This increases the likelihood of sensitisation and upon reexposure to the allergen increases the likelihood for cross-linking of IgE molecules bound to the surface of effector cells, thereby eliciting allergic responses (Bannon, 2004; Mills et al., 2004). Studies where impairment of the digestion process led to increased allergenicity of the proteins under investigation further support the hypothesis (Untersmayr et al., 2005; Untersmayr et al., 2007; Yoshino et al., 2004). For examples, studies with antacid drugs, medications that hinder peptic digestion by raising pH, converted normally degradable dietary proteins into potentially allergens, promoting sensitisation to caviar proteins in mice (Untersmayr et al., 2003) and hazelnut proteins in mice and humans (Scholl et al., 2005).

There is no evidence for a specific MW above which peptides may behave as ‘complete’ allergens and below which they may not. Still many different suggestions, for a lower MW limit, representing the minimum size of a peptide with inherent allergenicity, have been presented. Overall, the general opinion appears to be that the lower limit for allergenicity of peptides is a MW of approximately 3.5 kDa (Lack et al., 2002; Huby et al., 2000; FAO/WHO, 2001; Poulsen and Hau, 1987). Such suggestion might, in addition to experimental evidence, be based on the assumption that there must be at least two IgE binding sites on a peptide (Huby et al., 2000), each constituting a minimum of 15 amino acids covered by the antibody paratope (Laver et al., 1990). This equals a minimum of 30 amino acid residues necessary for cross-linking of two IgE

molecules and elicitation of an allergic response, and corresponds to an average MW of around 3.5 kDa.

Astwood et al. (1996) compared resistance to pepsin digestion in simulated gastric fluid (SGF) for allergenic and non-allergenic proteins. This study was a systematically evaluation of the stability of known food allergens and proteins with no proven allergenicity using a simple *in vitro* model of gastric digestion. The study showed that while major food allergens in general resisted the digestion process, non-allergenic proteins were in contrast rapidly digested. These results were in agreement with the hypothesis that it is a prerequisite for food allergens to survive the GI digestion process to retain sufficient structure and size for uptake and sensitisation when reaching the inductive immune system of the intestinal mucosa. Thereby Astwood et al. (1996) concluded that stability to digestion may be a significant and valid parameter for distinguishing food allergens from non-allergenic dietary proteins and suggested that the ability of food allergens to reach the intestinal mucosa in intact form could be a necessity for allergenicity.

The study by Astwood et al. (1996) contributed to the inclusion of pepsin resistance as a test parameter in the safety assessment of novel proteins in genetically modified foods. In both the report of the Food and Agricultural Organisation (FAO)/World Health Organisation (WHO) from 2001 (FAO/WHO, 2001) and the report of Codex Alimentarius Commission from 2003 (Codex Alimentarius Commission, 2003), concerning evaluation of allergenic potential of genetically modified foods, it is recommended that resistance of the novel protein to degradation in the presence of pepsin should be incorporated in the test regime. FAO/WHO (2001)

recommends that the purified protein in non-heated and non-processed form, as well as the main edible form, should be subjected to pepsin degradation. The pepsin-protein mixture should be prepared using 0.5 mg of protein in 0.2 mL of 0.32% pepsin (w:v), in 30 mM/L NaCl, pH 2.0, and maintained shaking at 37 °C for 60 min. Aliquots from the digestion mixture should be taken at different time points, at 0, 15 and 30 sec and at 1, 2, 4, 8, 15 and 60 min. Analysis for protein stability and presence of intact protein or intact peptide fragments greater than 3.5 kDa should be evaluated using 10-20% gradient Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels or equivalent gel systems under both reducing and non-reducing conditions, followed by staining procedures (Figure 1) (FAO/WHO, 2001). It is well recognised that the described digestion protocol do not mimic the physiological conditions of the human gastric digestion (Codex Alimentarius Commission, 2003). The European Food Safety Authority (EFSA) report (EFSA Panel on Genetically Modified Organisms (GMO) 2010), concerning assessment of allergenicity of genetically modified organisms, recommends that resistance to digestion of novel proteins likewise should be evaluated using other *in vitro* digestibility methods, methods designed to mimic physiological conditions and thereby simulating the conditions of the human digestion process. However, the predictive value of such test system has not been evaluated.

Since the study by Astwood et al. (1996), further studies comparing the digestibility of allergens with non-allergenic dietary proteins have been performed (Fu et al., 2002; Kenna and Evens, 2000; Thomas et al., 2004; Takagi et al., 2003; Herman et al., 2007; Ofori-Anti et al., 2008). Collectively, these studies did not support an obvious correlation between resistance to digestion

and allergenicity. These studies all showed examples of dietary allergens that were highly susceptible to digestion as well as non-allergenic dietary proteins that resisted the digestion. However, many of the allergenic proteins that were shown to be labile are regarded as ‘incomplete’ allergens that are known to cause reaction before reaching the gut and many of the non-allergenic proteins that were shown to be resistant are lectins known to hold immunomodulatory activity.

In contrast to the study by Astwood et al. (1996), where most of the tested allergens were storage proteins, while all tested protein with no proven allergenicity were enzymes, Fu et al. (2002) compared allergens with non-allergenic proteins of similar functions. Fu et al. (2002) showed that some proteins, such as storage protein were inherently more stable to digestion than other proteins, such as enzymes, and concluded that food allergens were not always more resistant to digestion measured *in vitro* than non-allergenic proteins and that it could be difficult to rank the allergenic potential of proteins based on their susceptibility to pepsin digestion. Kenna and Evans (2000) likely concluded that allergens and proteins with no allergenicity display similar stability to pepsin digestion in SGF and that resistance to digestion is not a characteristic equal to food protein allergenic potential.

While the studies by Astwood et al. (1996), Fu et al. (2002), Kenna and Evans (2000) and Thomas et al. (2004) used the visual time disappearances of intact protein and/or large fragments in SDS-PAGE gels as the parameter to assess digestion (which may be a highly subjective parameter for evaluation (Ofori-Anti et al., 2008)), Herman et al. (2007) calculated the half-life of the test protein, assuming a negative exponential (first-order kinetics) decline and Ofori-Anti et al. (2008) estimated the time point achieving 90% digestion. Ofori-Anti et al. (2008) report

that all three methods have limitations; while the method by Astwood et al. (1996), Fu et al. (2002), Kenna and Evans (2000) and Thomas et al. (2004) rely on the reliable detection of bands with residual intact protein or fragments hereof, the methods by Herman et al. (2007) and Ofori-Anti et al. (2008) may be difficult to interpret for very labile proteins as well as very stable proteins.

STABILITY OF KNOWN FOOD ALLERGENS

Besides comparative studies of the susceptibility to pepsinolysis of known allergens with proteins of no proven allergenicity, many studies have been performed, selectively studying the resistance of known food allergens to pepsin degradation. Table 1 summarises the pepsin stability data of purified food allergens originating from plants and Table 2 summarises the pepsin stability data of purified food allergens originating from animals. While studies of the susceptibility to pepsinolysis is simply used as a model system for evaluation of the general biochemical stability of proteins, or is designed only to mimic the digestion process that takes place in the stomach, several studies have been performed mimicking the physiologic digestion process that takes place in the duodenum as well, as recommended by the EFSA panel (2010). Digestion products from such studies reflect to a greater extent the peptide profile to which the inductive mucosal immune system is exposed, and thereby the situation of oral sensitisation (EFSA Panel on Genetically Modified Organisms (GMO), 2010). Table 3 summarises the stability data of purified food allergens exposed to simulated gastro-duodenal digestion.

Digestion conditions, known to influence the outcome of the digestibility assay, such as enzyme to allergen ratios, pH and digestion time, are included in the Tables. Studies of food allergen stability have been performed based on either enzyme mass to allergen mass ratios or enzyme mass to allergen molarity. Only enzyme mass to allergen mass ratios are included in the Table 1-3, based on the statement of Ofori-Anti et al. (2008) that the use of a constant mass provides the most reliable comparison parameters. Enzyme activity unit per mg of allergen have also been included in the Tables, since different enzyme products can be purchased, and batch to batch variation of individual products occur, with varying activity units per mg of enzyme. In addition, studies have been performed using enzymes immobilised to agarose.

No clear definition seems to exist, defining an allergen as labile or stable to digestion. Yet, Goodman and Hefle (2005) describes that a protein which are no longer detectable after 2 min of digestion are unstable, proteins that are no longer detectable between 2 and 15 min of digestion are partially stable, while proteins still detectable after 60 min of digestion are stable. In Table 1-3, an allergen is defined as stable, under the given digestion conditions, when identifiable residual intact protein was left after termination of the digestion process. However, stability time varied greatly among the allergens as well as did the stability time for an individual allergen under different digestion conditions.

There is also no general consensus about the importance of the stability of peptide fragments emerging from the proteolysis processes. While all studies on food allergen digestibility evaluated the stability of intact allergen, far less considered the stability of peptide fragments generated during the digestion process, and even less identified the size of the largest fragments

(Table 1-3). Although the study of peptide fragments was not the intention of most studies, if the general assumption that any peptide fragments larger than 3.5 kDa may be of importance for the potential allergenicity of a food allergen holds true (Lack et al., 2002; Huby et al., 2000; FAO/WHO, 2001; Poulsen and Hau, 1987), greater effort for identifying such fragments should be done. Thomas et al. (2004), Herman et al. (2007), Takagi et al. (2003) as well as Ofori-Anti et al. (2008) all acknowledged the importance of considering stable peptide fragments derived from food allergens that are rapidly digested. Examples of this are the major peanut allergens Ara h 1 and Ara h 2 as well as the major hen's egg allergen ovomucoid (OVM), which form stable fragments upon digestion that retain significant IgE-binding capacities (Urisu et al., 1999; Yamada et al., 2000; Sen et al., 2002; Maleki et al., 2000). In addition, the EFSA panel (2010) express that the appearance of stable peptide fragment or aggregates hereof formed during the digestibility assay should be considered as a risk factor for allergenicity, and recommend that peptide fragments and aggregates should be further investigated. Thus in addition to the stability of the intact proteins, the stability of the peptide fragments formed during the digestion process, also needs to be evaluated (EFSA Panel on Genetically Modified Organisms (GMO), 2010).

More than half of the *in vitro* digested allergens were not stable to digestion (Table 1-3). For the plant derived food allergens, 18 were not stable to pepsinolysis, 6 were both stable and labile, depending on the digestibility conditions, while 11 were stable to the pepsin digestion (Table 1). For the animal derived food allergens, these numbers were, 8, 5 and 6, respectively (Table 2). This indicates that no strict correlation exist between resistance to digestion and allergenicity. This viewpoint is in agreement Fu et al., stating in 2002 that such correlation was not absolute.

By far most digestibility studies have been performed on allergens from only a few foodstuffs, namely peanuts, soy, cow's milk and hen's egg. Figure 2 is an illustration of digestibility results of allergens from these four foodstuffs using 'wordle'. 'Wordle' is a tool for creating 'word clouds' based on a text, here illustrating the number of studies showing the given food allergen to be either stable or labile to pepsinolysis based on Table 1 and 2.

Food allergens may sensitise via different routes. It is well known that dietary proteins may sensitise through the skin (Asero and Antonicelli, 2011; Lack, 2012) or via the respiratory tract (Asero and Antonicelli, 2011; Ramirez and Bahna, 2009), though most food allergens are thought to sensitise through the oral route, and consequently sensitise through the intestinal mucosal immune system (Moreno, 2007). In addition to 'complete' food allergens, having the capacity to both sensitise and elicit allergic reactions (Aalberse, 2000), some food protein, known to elicit allergic reactions, are thought not to induce allergic sensitisation (Moreno, 2007; van Ree, 2002). Patients suffering from e.g. pollen or latex allergies may experience immediate reactions upon ingestion of foods, mainly fruits and vegetable (Kondo and Urisu, 2009). Such association is due to cross-reacting IgE antibodies, recognising structures of the dietary protein homologous to IgE-binding sites of pollen or latex allergens (Kondo and Urisu, 2009). In Europe, more than 70% of patients suffering from pollen allergy may experience symptoms after food intake. Here the food allergens from e.g. the pathogenesis-related protein-10 (PR-10) family, Mal d 1 from apple, Cor a 1 from hazelnut, Pru av 1 from cherry and Api g 1 from celery are allergens that cross-react with IgE antibodies specific for the major birch pollen allergen Bet v 1 and e.g. the food allergens from the profilin family such as Pru av 4 from cherry is an

allergen cross-reacting with the birch pollen allergen Bet v 2 (Kondo and Urisu, 2009). All five allergens are proteins labile to pepsinolysis (Table 1) (Scheurer et al., 2004; Schimek et al., 2005) as well are other dietary proteins considered to be non-sensitising allergens (Yagami et al., 2000; Besler et al., 2001; Lucas et al., 2008). These non-sensitising allergens are usually associated with oral allergy syndrome (OAS), which are mild allergic reactions restricted to the oral cavity (Kondo and Urisu, 2009; van Ree, 2002). An assessment of the stability to digestion might therefore not be relevant for such ‘incomplete’ food allergens and indicates that an *in vitro* digestibility assay for assessment of potential allergenicity could be misleading for allergens of non-sensitising characteristic, eliciting allergic reactions based on cross-reaction with IgE antibodies raised against other non-food allergens.

Taking into account only food allergens believed to sensitise through the intestinal mucosa, data from digestibility assays, indicate that there are still a little more ‘complete’ food allergens that are susceptible to digestion, than those completely stable. For example the major peanut allergen Ara h 1 and the major cow’s milk allergen β -casein have been shown to be highly labile proteins, broken down to small peptide fragments within minutes (Koppelman et al., 2010; Eiwegger et al., 2006; Mandalari et al., 2009a; Morisawa et al., 2009). Hence for dietary proteins sensitising through the intestinal mucosal no significant correlation between resistance to digestion and allergenicity seems to exist.

Susceptibility of food allergens to proteolysis has mostly been tested by analysing the effects of pepsinolysis. Comparing Table 1 and 2 with Table 3 reveals that some allergens, such as the major allergen Ber e 1 from Brazil nut, may be resistant to pepsinolysis, but on the other hand, are broken down in a process simulating the gastro-duodenal digestion (Moreno et al., 2005c),

where the protein in addition to pepsinolysis also is proteolysed by trypsin and chymotrypsin. In addition, Takagi et al. (2003) showed that some food allergens, such as the major cow's milk allergen BLG was resistant to pepsinolysis in SGF and labile to digestion by pancreatin in simulated intestinal fluid (SIF). In contrast other known allergens, such as the cow's milk allergen BSA were labile to digestion in SGF, while resistant to digestion in SIF. Such results, in agreement with the perception of the EFSA panel (2010), could indicate the usefulness of not only implementing a degradation phase resembling the gastric digestion process but also a phase resembling the duodenal digestion process. This could potentially lead to more precise knowledge of the general stability of dietary proteins, and a more realistic picture of the peptides the intestinal immune system may be exposed to as pointed out by Moreno (2007). However, the value as a predictive tool in the risk assessment of potential allergenicity of novel proteins is unknown. To our knowledge a systematic evaluation of the stability of food allergens and dietary proteins with no proven allergenicity using an *in vitro* gastro-duodenal digestion system has not been performed.

FACTORS INFLUENCING THE OUTCOME OF THE DIGESTIBILITY ASSAY

The assay condition and hence the resulting outcomes vary extensively between the different digestibility studies (Table 1-3). For example the stability of Ara h 2 (Table 1) to pepsinolysis resulted in either Ara h 2 being an easily digestible protein (Fu et al., 2002; Thomas et al., 2004; Sen et al., 2002) or being a highly resistant protein (Astwood et al., 1996; Koppelman et al., 2010). Similar results were shown for the related peanut allergen Ara h 6 (Koppelman et al.,

2010; Suhr et al., 2004) and the Brazil nut allergen Ber e 1 (Moreno et al., 2005c; Murtagh et al., 2003; Koppelman et al., 2005; Murtagh et al., 2002). Also the milk allergen BLG, which is normally regarded as a protein highly resistant to pepsin digestion (Thomas et al., 2004; Dupont et al., 2010; Astwood et al., 1996; Fu et al., 2002; Takagi et al., 2003; Mandalari et al., 2009b) was in a single study found to be susceptible to pepsinolysis (Mandalari et al., 2009a) and in another study found to be susceptible to simulated gastro-duodenal digestion (Macierzanka et al., 2009). These results reveal the very importance of the assay condition used, and points to the necessity for using the same assay condition for comparability between studies and the prerequisite to decide the assay condition most suitable for the assessment of stability of novel proteins.

In addition, two separate studies using the same assay conditions, showed contradicting results. Mandalari et al. (2009a) found BLG to be degraded by pepsin digestion, and Fu et al. (2002) found BLG to be stable to the pepsinolysis. This indicates inter-laboratory differences, and point to the necessity of using extremely stable and standardised assay conditions.

Factors such as digestion time, enzyme to allergen ratio, pH, purity of allergen, allergen processing, presence of surfactant and presence of matrix may all have a great impact on the outcome of the digestion assay. The methods used for determination of residual intact protein as well as amount and sizes of emerging peptide fragments may on the other hand have a significant influence on the interpretation of the susceptibility of the given dietary protein to digestion.

Enzyme to allergen ratio

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There is a huge variation in protease to allergen ratios in the published studies (Table 1-3). Many studies were performed with an enzyme activity of approximately 10,000 unit per mg of protein, as originally presented by Thomas et al. (2004). These studies are far from physiological, based on a standard SGF used for preclinical assessment of pharmaceuticals, and only gives a biochemical measure of a protein's overall physiochemical stability (EFSA Panel on Genetically Modified Organisms (GMO), 2010; Mandalari et al., 2009a). Other studies were performed with an enzyme activity of approximately 180 units per mg of protein, as originally presented by Moreno et al. (2005c). These studies seek to elucidate the role of physiologically relevant digestion on the peptide profile of digestion products, and hence what the intestinal mucosal immune system is exposed to (EFSA Panel on Genetically Modified Organisms (GMO), 2010; Moreno et al., 2005a).

In a study by Takagi et al. (2005) digestion of OVM at different pepsin to allergen ratios showed that the stability of the intact protein as well as some of the generated peptide fragments increased markedly by lowering the pepsin concentration 10 and 100-fold. In addition, Mandalari et al. (2009a) showed that BLG was resistant to pepsinolysis under physiologically relevant pepsin to allergen ratio, while it was degraded by increasing the pepsin concentration 256-fold. The enzyme to allergens ratio also significantly affected the stability of the peanut allergens Ara h 2 and Ara h 6. They were both stable at an enzyme activity of 100 unit per mg of peanut protein, while completely labile after raising the enzyme activity 100-fold (Koppelman et al., 2010). These studies show that the digestibility of allergens is significantly influenced by the enzyme to protein ratio, so that an allergen which appears to be stable at one ratio is susceptible

at higher ratios. In addition the amount and sizes of stable fragment may significantly be affected by the enzyme to protein ratio (Fu et al., 2002).

pH

The pH under which the digestion assay was performed also varied (Table 1-3). In the pepsin studies the pH varied between 1 and 4. No single pH will ever mimic the condition in the stomach. While the pH of the fasted stomach is around 2 (Untersmayr and Jensen-Jarolim, 2008; Savarino et al., 1988; James et al., 1962), the pH rises after the ingestion of food to a pH of up to 5 (EFSA Panel on Genetically Modified Organisms (GMO), 2010).

Lucas et al. (2008) digested kiwi allergens at different pH values and showed that the stability of the allergens was significantly affected by the pH. An increase from pH 1.5 to 2.5 significantly reduced proteolytic break down of the allergens and a further increase in pH to 3 abolished the break down. In addition, studies of the digestibility of codfish proteins showed that proteins were degraded within 1 min at pH ranging from 1.25 to 2.5, while only a marginal shift in pH from 2.5 to 2.75 completely abrogated the digestion of the cod allergens (Untersmayr et al., 2005). Also studies of hen's egg allergen digestion showed that digestibility of both OVM, OVA, Ovotransferrin (OT) and hen's egg lysozyme C (HEL) were pH dependent, though to different degrees (Yoshino et al., 2004). These studies show that the digestibility of allergens may greatly be affected by variation in pH, probably because of the pH dependent activity of the enzymes as well as the pH dependent denaturation of proteins.

Surfactant

Also addition of surfactant, such as PC or bile salts (which is secreted by the gastric mucosa or being a constituent of the bile, respectively) may greatly affect the susceptibility of proteins to digestion (Table 1-3). For example, addition of PC altered the kinetics of the BLG degradation, protecting the allergen from digestion by slowing the digestion process as well as altered the pattern of the generated peptide fragments (Mandalari et al., 2009a; Mandalari et al., 2009b; Dupont et al., 2010; Macierzanka et al., 2009). A protective impact of PC was also seen on the degradation of the cow's milk allergen α -lactalbumin (ALA) (Moreno et al., 2005a) as well as the grape allergen Vit v 1 (Vassilopoulou et al., 2006). In contrast PC did not influence the digestibility of the cow's milk allergen β -casein (Mandalari et al., 2009a) as well as the Brazil nut allergen Ber e 1 (Moreno et al., 2005c) and the sesame allergen Ses i 1 (Moreno et al., 2005b). The presence of bile salts may also affect the digestion of proteins. Gass et al. (2007) showed that proteolysis with the pancreatic enzymes trypsin and chymotrypsin of the cow's milk allergens BLG and BSA was greatly accelerated by addition of bile salts, while the digestion rate of the hen's egg allergen OVA and the minor soy allergen soybean trypsin inhibitor (STI) were unaffected. These studies show that addition of surfactant may influence the susceptibility to digestion, by either raising or decreasing the digestibility.

Processing

Food processing may affect the susceptibility of allergens to digestion. For example heat treatment of proteins prior to digestion may affect the digestibility. Preheating of OVA before digestion by pepsin in SGF significantly accelerated the digestibility. While the OVA was stable without preheating, it was rapidly digested within 0.5 min following preheating (Takagi et al.,

2003). Also, heating of BLG significantly increased its susceptibility to digestion in a time and temperature dependent manner (Morisawa et al., 2009). In contrast heating of OVM, Bere 1 or Ses i 1 prior to digestion did not alter the digestion pattern (Takagi et al., 2005; Moreno et al., 2005c; Moreno et al., 2005b). Since allergenic food proteins are commonly eaten in a processed state, these studies imply the importance of assessing of digestibility of such proteins after processing.

Matrix

The food matrix may also influence digestibility. This have been shown by Macierzanka et al. (2009), who demonstrated that when β -casein was digested in an emulsified form, the digestion rate was increased, but on the other hand making some peptide fragments more resistant to digestion compared with the digestion of β -casein in no emulsion. In the same study it was shown that the susceptibility of BLG to digestion was increased if presented in an emulsified form. The conclusion was that emulsification led to adsorption-induced changes in either the conformation or the flexibility of the allergens, rendering them more easily digested in simulated gastro-duodenal digestion (Macierzanka et al., 2009). Mandalari et al. (2009b) suggested, based on a study concerning the impact of PC on the digestion pattern of BLG, that molecular interactions between proteins and lipids may influence the pattern of proteolysis. Polovic et al. (2007) studied the matrix effect of the plant polysaccharides pectin, and showed that the polysaccharides decreased the digestibility of the kiwi allergen Act c 2. Also for BLG, addition of polysaccharides significantly reduced the digestibility of simulated gastro-duodenal digestion (Mouecoucou et al., 2004b; Mouecoucou et al., 2003; Nacer et al., 2004). Polysaccharides were

also shown to modify the digestibility of peanut allergens, however, the pattern of digestion was dependent on the type of polysaccharides (Mouecoucou et al., 2004a). As also suggested by Ofori-Anti et al. (2008) these studies show that investigation of digestibility using purified proteins, not taking into account the possible effect of the food matrices, could potentially be misleading, providing result which may differ from those received with digestion of whole food extracts.

ASSESSMENT OF RESIDUAL INTACT PROTEIN AND PEPTIDES

Evaluation of residual intact protein and peptide fragments formed during the digestion process has most commonly been done by the use of SDS-PAGE and/or western blotting, but other methods have also been used (EFSA Panel on Genetically Modified Organisms (GMO), 2010). The methods used for detection of residual intact allergen and emerging peptide fragments may greatly influence the results and thereby the interpretation of the digestibility assay.

Several studies have shown the significant implication that the choice of detection method may have on the outcome of the assessment of resistance to digestion. For example Lucas et al. (2008) reported that immunoreactive peptide fragments of kiwi allergens could be detected by immunoblot but not with Coomassie staining. Diaz-Perales et al. (2003) further showed that neither protein staining nor IgE immunoblotting with sera from allergic patients resulted in the detection of any peptide fragment bands in SDS-PAGE from the digestion process of the avocado allergen Pers a 1, which was shown to be a food allergen highly susceptible to digestion. However, the peptide pool from the digestion of the Pers a 1 had similar inhibitory potency as

the intact Pers a 1 in both immunoblot- and enzyme-linked immunosorbent assay (ELISA)-inhibitory experiments. The peptides could also induce positive skin prick test (SPT) responses in 5 out of 8 allergic patients. In addition, when tested individually, single peptides showed a large inhibitory capacity in ELISA as well as an ability to induce responses in SPTs. The sizes of these peptides were 5.1+2.6, 2.5 or 1.4 kDa. Thomas et al. (2004) even reported that the type of electrophoresis gel and fixation techniques could influence the detectability of peptide fragments. These results indicate the importance of evaluating residual intact protein and peptide fragments hereof in assays with appropriate sensitivities, and that the use of more than one method could be worthwhile. Goodman and Hefle (2005) pointed to the importance of choosing suitable detection methods, since proteins may have different staining ability because of different amino acid composition. For these reasons Ofori-Anti et al. (2008) suggested the inclusion of a 10% diluted control sample of undigested protein in the assay gel to control for differences in staining between proteins and between experiments. Since evaluation of SDS-PAGE fractionation of peptide fragments resulting from the digestion process may in some situations only allows for detection of fully stable allergens and/or permanent peptide fragments of adequate large size (Diaz-Perales et al., 2003), Vieths et al. (1999) suggested the need for immunological assays to investigate the potential allergenicity of food proteins instead of monitoring the degradation of bands by analytical electrophoresis. In a study by Mandalari et al. (2009b) residual intact BLG after simulated gastro-duodenal digestion was evaluated in SDS-PAGE as well as with reverse phase high-performance liquid chromatography (RP-HPLC). While visualisation of bands in SDS-PAGE indicated that no intact BLG was left after the digestion process, RP-HPLC showed that around 10% intact BLG was left. Similar results was obtained for the kiwi allergen Act d 2

(Bublin et al., 2008). These studies imply that evaluation of residual intact protein with other protein-chemical methods in addition to SDS-PAGE could be very useful.

ASSESSMENT OF THE ALLERGENIC POTENTIAL OF DIGESTION PRODUCTS

As discussed in the previous sections the relationship between resistance to pepsinolysis as well as digestion in general and allergenicity is not straightforward. The inadequacy of the tests performed adds to the uncertainties. Given that some food allergens are easily digested, digestibility assays should be combined with immunological assays. This will allow for an elucidation of the role of peptide fragments emerging from the digestion process, by an assessment of their allergenic capacity. For proteins to which IgE from sera of individuals with allergy to the given protein or a related protein is available, the following questions could be addressed by conducting immunological assays in connection to the evaluation of susceptibility to digestion; do the peptide fragments generated during the digestion process retain IgE-binding and eliciting capacity equivalent to the parent allergen? Will the IgE-binding and eliciting capacity be reduced or even abolished, or could the peptide fragments be even more allergenic than the parent allergen? For proteins of true novelty, where human IgE is not available, implementation of animal studies could answer questions like; do the peptide fragments generated during the digestion process retain sensitising capacity? Will they simply be ignored by the immune system or will the peptide fragments be able to induce tolerance mechanisms? Based on knowledge from research in peptide vaccines, all three situations could occur, as studies have shown that mixtures of small peptides, intended as constituents in vaccines, may

either be ignored, induce tolerance or induce active immunity (Knittelfelder et al., 2009; Muller, 1999).

To evaluate the effect of digestion on the allergenicity of food allergens, three distinct molecular properties need to be addressed; (1) the ability to bind IgE antibodies, (2) the ability to elicit an allergic reaction, as well as (3) the ability to sensitise, as these are the features of ‘complete’ allergens (Aalberse, 2000).

IgE-binding

Several studies have evaluated the IgE-binding capacity of digests generated during *in vitro* proteolysis. A study on the effect of peptic digestion on the IgE-binding ability of the peanut allergen Ara h 3 and the soy allergen glycinin showed that these proteins did not retain any IgE-binding capacity after 2 h of digestion (van Boxtel et al., 2008). Likewise Untersmayr et al. (2003) showed that 5 sec of peptic digestion were sufficient to abrogate the IgE-binding capacity of caviar proteins. Other studies found that the IgE-binding capacity was retained after digestion, though in a reduced manner. For example, Takagi et al. (2005) found that one fifth of OVM allergic patients had serum IgE that could bind to the pepsin digested OVM which contained peptide fragments with sizes between 4.5 and 6.0 kDa. Such result was confirmed by studies of Urisu et al. (1999) and Yamada et al. (2000) who also found pepsin digested OVM to bind IgE, though it was reduced compared to the intact OVM. Likewise, the IgE-binding capacity of cod allergens were retained after pepsin digestion, though strongly reduced compared to the parent protein (Untersmayr et al., 2005). Again, other studies have shown that the IgE-binding capacity of digestion products equal the IgE-binding capacity of the intact allergen. For example, gastro-

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duodenal digestion products of the grape allergen Vit v 1 were found to have IgE-binding capacity similar to that of the intact Vit v 1 (Vassilopoulou et al., 2006) and peptic digestion products of the cow's milk allergen BLG had similar IgE binding reactivity as the intact BLG (Selo et al., 1999). At last, few studies have shown the digestion products of food allergens to be even more IgE reactive than their parent allergen. This has been shown for the cow's milk allergen BLG (Haddad et al., 1979) as well as for kiwi allergens (Lucas et al., 2008), and was suggested to be a result of the emerging of new epitopes, not accessible prior to digestion.

Only a minority of the studies, which have evaluated the IgE-binding capacity of digestion products, have made the effort to correlate the binding capacity to the sizes of the peptide fragments generated during the digestion process. However, a study with gastro-duodenal digests from Ara h 1, containing peptides of sizes less than 2.0 kDa, showed that peptides within this size range may retain their IgE-binding capacity (Bøgh et al., 2009). Results from peptide epitope mapping studies have additionally contributed to the knowledge of IgE-binding capacity of small peptides, collectively demonstrating that even very small peptides, consisting of down to 10 amino acids, corresponding to approximately 1.1 kDa, representing short amino acid sequences of the primary structure of food allergens may indeed contain the ability to bind IgE (Burks et al., 1997; Beyer et al., 2003; Jarvinen et al., 2002; Battais et al., 2005).

Studies have demonstrated that cautions should be taken when evaluating the IgE-binding capacity of peptide fragments and that the immunologically assay should be chosen with care, because of the great impact it may have one the resulting outcome. For example, Diaz-Perales et al. (2003) showed that immunoblotting with sera from avocado allergic patients did not reveal any IgE-binding of digestion products from the avocado allergen Pers a 1, a dietary protein

highly susceptible to peptic digestion. However, the digestion products were shown to have an inhibitory potency equally to that of the intact allergen in both immunoblot- and ELISA-inhibitory assays. Similar results were found in another study, revealing that while digestion products of hazelnut allergens showed no IgE-binding capacity when tested by immunoblotting, the digestion products had a very strong binding capacity in enzyme allergosorbent assay (EAST) assays (Vieths et al., 1999).

Elicitation

The eliciting capacity of digests generated during *in vitro* proteolysis has been studied, however, compared to IgE-binding these studies are very few. Elicitation studies demonstrate the capacity of proteins or peptides to cross-link receptor-bound IgE on the surface of mast cells or basophils, leading to mediator release from these effector cells. This shows the biological relevance of the IgE-peptide interaction.

Studies of elicitation capacity of digestion products show different results with different allergens. In a study assessing the allergenicity of the digestion products of the Bet v 1 homologues Mal d 1, Cor a 1 and Api g 1, all very labile proteins, being digested to peptides with sizes less than 2 kDa, it was shown that digestion abrogated their eliciting capacity of humanised rat basophilic leukemia (RBL) cells (Schimek et al., 2005). Diaz-Perales et al. (2003) investigated the influence of digestibility on the allergenicity of the avocado allergen Pers a 1, and showed that digested Pers a 1, containing peptides of sizes up to approximately 5-6 kDa retained their eliciting capacity in SPTs, though in a reduced manner compared to the intact Pers a 1. They further showed that individual peptides of sizes down to 1.4 kDa could induce positive

responses in SPTs. Similar results were seen after digestion of the cow's milk allergens BLG and α -casein (Morisawa et al., 2009). A study comparing histamine release (HR) from human basophils induced by intact or digested cod extract showed that the digested cod extract retained some eliciting capacity, though in a very reduced manner compared to the intact extract (Untersmayr et al., 2005; Untersmayr et al., 2007). Similar results were seen after digestion of cherry extract (Scheurer et al., 2004). Other studies, revealed the digestion products to retain eliciting capacity in a magnitude similar to the parent protein. For example, Eiwegger et al. (2006) showed that the gastric as well as gastro-duodenal digestion products of the labile peanut allergen Ara h 1, had a HR pattern from basophils identical to that of the intact Ara h 1. Also Vassilopoulou et al. (2006) showed that the digested grape allergen Vit v 1 contained similar capacity to induce responses in SPTs as the intact Vit v 1, as well as showing similar biological activity in HR from basophils. Likewise, gastro-duodenal digests from Ara h 1, containing peptides of sizes less than 2 kDa, could induce degranulation responses from RBL cells, with a similar magnitude as intact Ara h 1. These small peptides were shown to aggregate to larger complexes, which was hypothesized be the reason for their eliciting capacity (Bøgh et al., 2009). Only few studies have correlated elicitation results to the sizes of the peptide fragments.

Untersmayr et al. (2005), showed that digestion products of cod allergens retained IgE-binding capacity but not eliciting capacity. This demonstrates that IgE-binding capacity do not necessary correlate with a capacity to trigger allergic reactions and that there are additional requirements for peptides to have eliciting capacity compared to IgE-binding capacity. Similar results were found by Diaz-Perales et al. (2003), showing that some individual peptides down to sizes of approximately 0.8 kDa could bind IgE, but did not show positive responses in SPT.

Sensitisation

It is difficult to study sensitisation in humans because they are exposed to many allergens in an uncontrolled and unpredicted manner and it is unethical to perform experimental sensitisations studies. Animal models have been regarded as a good alternative (Fritsche, 2009; Fritsche, 2003). Although an evaluation of sensitising capacity has only been performed with a limited numbers of dietary proteins, several rodent models have been regarded as good candidates for animal models of food allergy (Aldemir et al., 2009; Bowman and Selgrade, 2008; Dearman et al., 2003b; Dearman et al., 2002; Dearman et al., 2003a; Knippels and Penninks, 2003; Jia et al., 2005).

Only a small numbers of studies have addressed residual sensitising capacity of degradation products from food allergens, by measure of specific IgE. Most of these have been performed on milk proteins. This is probably a result of the great interest in the design of safe hypoallergenic infant milk formulas as well as the requirement for an evaluation of residual sensitising capacity of such hypoallergenic formulas (Commission Directive 96/4/EC of February 1996 amending Directive 91/321/EEC on infant formulae and follow-on formulae, 1996). Historically, assessment of the residual sensitising capacity have been performed by oral sensitisation studies in guinea pigs (Fritsche, 2009; Boner et al., 1992; Anderson et al., 1979), however the reaginic antibody response assessed in these animals is of the IgG1a subtype, making this animal model less than perfect for studying IgE mediated allergic disease.

A sensitisation study in Sprague-Dawley rats immunised i.p. with cow's milk infant formulas based on either intact proteins, partially hydrolysed proteins (pHF) or extensively hydrolysed

proteins (eHF) with the use of Al(OH)_3 as adjuvant, showed that the pHF as well as eHF were able to induce specific IgE, though to a degree which were 100 and 10,000 times lower, respectively, than the level induced by the formula based on intact proteins (Fritsche and Bonzon, 1990). In another study, Balb/c mice were immunised i.p. with different pHFs and eHFs with Al(OH)_3 as adjuvant (Niggemann et al., 2001). These studies showed that all three tested pHFs were able to induce specific IgE antibodies, shown by a positive skin test after intradermal injection with intact BLG or the formula itself, while only one out of two eHFs were able to induce specific IgE antibodies. These hydrolysates could all induce specific IgG1 antibodies, though in varying amounts, signifying the different degree of immunogenic potency (Niggemann et al., 2001). Recently, sensitisation studies have been conducted in a mouse model of orally induced cow's milk allergy (Schouten et al., 2008), where C3H/HeOuJ mice were orally sensitised with whey proteins or partially hydrolysed products hereof with cholera toxin as an adjuvant (van Esch et al., 2011; van Esch et al., 2010). These studies showed that while intact whey protein could induce a specific IgE response, no detectable specific IgE antibodies were measured in sera from mice dosed with partially hydrolysed whey products. However, in one study an acute skin response was evident after whey skin challenge on the ear, suggesting some residual sensitising capacity of the partially hydrolysed whey not evident by ELISA analysis of the specific IgE (van Esch et al., 2010). In our own studies, it was not possible to demonstrate sensitising capacity of extensively hydrolysed whey proteins containing peptides up to 2.5 kDa when administered to BN rats by i.p. immunisation with or without Al(OH)_3 as adjuvant (unpublished results). Animal studies have also been performed with degradation products from the cow's milk allergen BSA (Michael, 1989; Dosa et al., 1979; Ferguson et al., 1983). A single

i.p. immunisation of BDF₁ mice with digestion products of BSA using Al(OH)₃ as adjuvant failed to sensitise the mice, in contrast to intact BSA, when degradation products were pepsin digested for more than 12 min. However by two i.p. immunisation of the mice only BSA digestion products digested for more than 40 min failed to induce IgE (Dosa et al., 1979). The MW of digestion products emerging from the digestion after 40 min was in the range of 8 to 16 kDa. In a BN rat model, the sensitising capacity of digestion products from the peanut allergen Ara h 1 as well as the cow's milk allergen BLG were investigated by i.p. immunisation with or without the use of adjuvant (Bøgh et al., 2009; Bøgh et al., 2012; Bøgh et al., 2013). Both gastric digests of Ara h 1, containing peptides with sizes up to 4 kDa (Bøgh et al., 2012) as well as gastro-duodenal digests of Ara h 1, containing peptides of sizes up to 2 kDa (Bøgh et al., 2009) had the ability to sensitise the BN rats, inducing specific IgE antibodies of statistically significant degree. On the other hand, gastro-duodenal digests from BLG, containing peptides of sizes up to 4.5 kDa, had no sensitising capacity (Bøgh et al., 2013). However, the sensitising capacity of intact BLG co-administered with digestion products from BLG was strongly reduced compared to the sensitising capacity of intact BLG alone, indicating that the digests have tolerogenic capacity (Bøgh et al., 2013).

CONCLUSION AND FUTURE PERSPECTIVES

What is the relationship between stability to digestion and allergenicity? This review demonstrates that stability to digestion is not a necessity for a dietary protein to act as an allergen. The data presented on susceptibility of food allergens to gastric (pepsin) as well as

gastro-duodenal digestion (pepsin, followed by trypsin and chymotrypsin) clearly illustrates, that even major allergens may be extremely labile proteins, being digested to small peptide fragments within few minutes.

The use of different *in vitro* digestion assay conditions are shown to have a significant influence on digestibility of food allergens, and the choice of method used to evaluate the digestibility are likewise shown to significantly influence the interpretation of results. This underlines the importance of not only the assay conditions, but also the requirement for appropriate ways to assess the digestibility data.

Historically, only few food allergens have been tested for their stability to digestion in combination with an assessment of the allergenicity of the digestion products including both a thorough analysis of the peptide profile as well as IgE-binding, eliciting and sensitising capacity. The existing data show no strict relationship between digestibility and allergenicity. So if the peptide profile of digestion products does not necessarily correlate with the associated risk of allergenic potency, caution should be taken when extrapolating allergenicity of proteins from digestibility studies alone. There is a need for further studies combining digestibility assays with an evaluation of the allergenicity of the generated peptide fragments and further correlating the resulting allergenicity to the peptide size distribution of these peptides. A better knowledge of the correlation between peptide sizes and sensitising capacity remains fundamental for an improved understanding of the influence of digestibility on the allergenic capacity and for achieving a better understanding of the mechanisms responsible for the direction of the immune system towards allergy.

Novel proteins in genetically modified food have for several decades been introduced into our food supply, and probably will continue to do so (Goodman and Hefle, 2005). The potential allergenicity of these ‘new’ proteins is of major concern. Therefore strategies for safety assessment of the potential allergenicity have been recommended. An evaluation of the stability to digestion with pepsin remains a central part of the allergenicity safety assessment of a novel protein, despite the fact that the relationship between resistance to digestion and allergenicity has been questioned (Fu et al., 2002; Fu, 2002; Kenna and Evens, 2000). This test is currently the only parameter contributing to a prediction of *de novo* sensitisation. Undoubtedly, survival in the GI tract plays an important role for the sensitising capacity of some food allergens, but as shown in the present review this is not true for all food allergens.

Pepsin resistance as a test parameter in the safety assessment of novel proteins in genetically modified foods needs a standard set of conditions, concerning not only the digestibility assay as such but also the assay used to evaluate residual intact protein as well as peptide fragment formed during the digestion process. Further, a revision on how to interpret the resulting outcome seems important. The digestibility assay should be combined with an assessment of the allergenicity of the digestion products. In addition, a supplementary test for *de novo* sensitisation of novel proteins could be of great value.

To predict allergenicity and in particular sensitising capacity by testing resistance to digestion, based on current knowledge, could be misleading.

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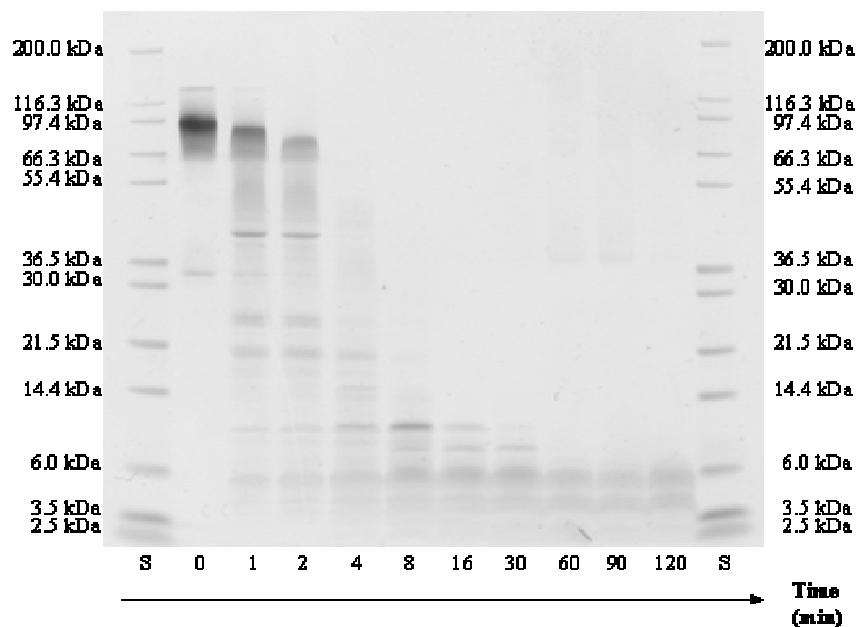
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ABBREVIATIONS

ALA, α -lactalbumin; BLG, β -lactoglobulin; BN, Brown Norway; BSA, bovine serum albumin; EAST, enzyme allergosorbent assay, EFSA, European Food Safety Authority; eHF, extensively hydrolysed formulas; ELISA, enzyme-linked immunosorbent assay; FAO, Food and Agricultural Organisation; GI, gastrointestinal; HEL, hen's egg lysozyme C; HR, histamine release; IgE, immunoglobulin E; IgG, immunoglobulin G; i.p., intraperitoneal, MW, molecular weight; nd, not described; ns-LTP, nonspecific-lipid transfer protein; OAS, oral allergy syndrome; OT, ovotransferrin; OVA, ovalbumin; OVM, ovomucoid; PC, phosphatidylcholine; pHF, partially hydrolysed formulas; PP, Peyer's patches; PR-10, pathogenesis-related protein-10; RP-HPLC, reverse phase high-performance liquid chromatography; RBL, rat basophilic leukemia; SPT, skin prick test; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; STI, soybean trypsin inhibitor; TLP, thaumatin-like protein; WHO, World Health Organisation

FIGURES**Figure 1. Example of the visualisation of the digestion process of the peanut allergen Ara h**

1. Ara h 1 was digested in an *in vitro* model simulating the human gastro-duodenal digestion. The digestion process was divided into 2 phases, resembling the gastric (**A** and **B**) and duodenal (**C**) digestion, respectively. Gastric digestion was performed with immobilised pepsin with an activity of 162 unit per mg of Ara h 1 (corresponding to a pepsin:Ara h 1 ratio of approximately 0.05 (w:w) for soluble pepsin), at pH 2.5 for 120 min at 37 °C, followed by duodenal digestion, performed with immobilised trypsin and chymotrypsin with an activity of 34.5 and 0.44 unit per mg of Ara h 1, respectively, (corresponding to a trypsin:chymotrypsin:Ara h 1 ratio of approximately 0.0025:0.01:1 (w:w:w) for soluble enzymes) at pH 6.5 for 16 min at 37 °C. Aliquots of 10 µL from the gastric digestion process were taken at the time points of 0, 1, 2, 4, 8, 16, 30, 60, 90 and 120 min and from the duodenal digestion process at the time points of 0, 2, 4, 8 and 16 min. SDS-PAGE was performed with 4-12% Bis-Tris Gel with MES running buffer under non- (**A** and **C**) as well as reducing (**B** and **C**) conditions. Gels were stained with SimpleBlue™ (SimpleBlue™ Safestain (Ready-to-use, fast, sensitive and safe Coomassie® G-250) Invitrogen, Carlsbad, CA, USA). As standard markers Mark 12™ was used (Unstained standard, Invitrogen, Carlsbad, CA, USA).



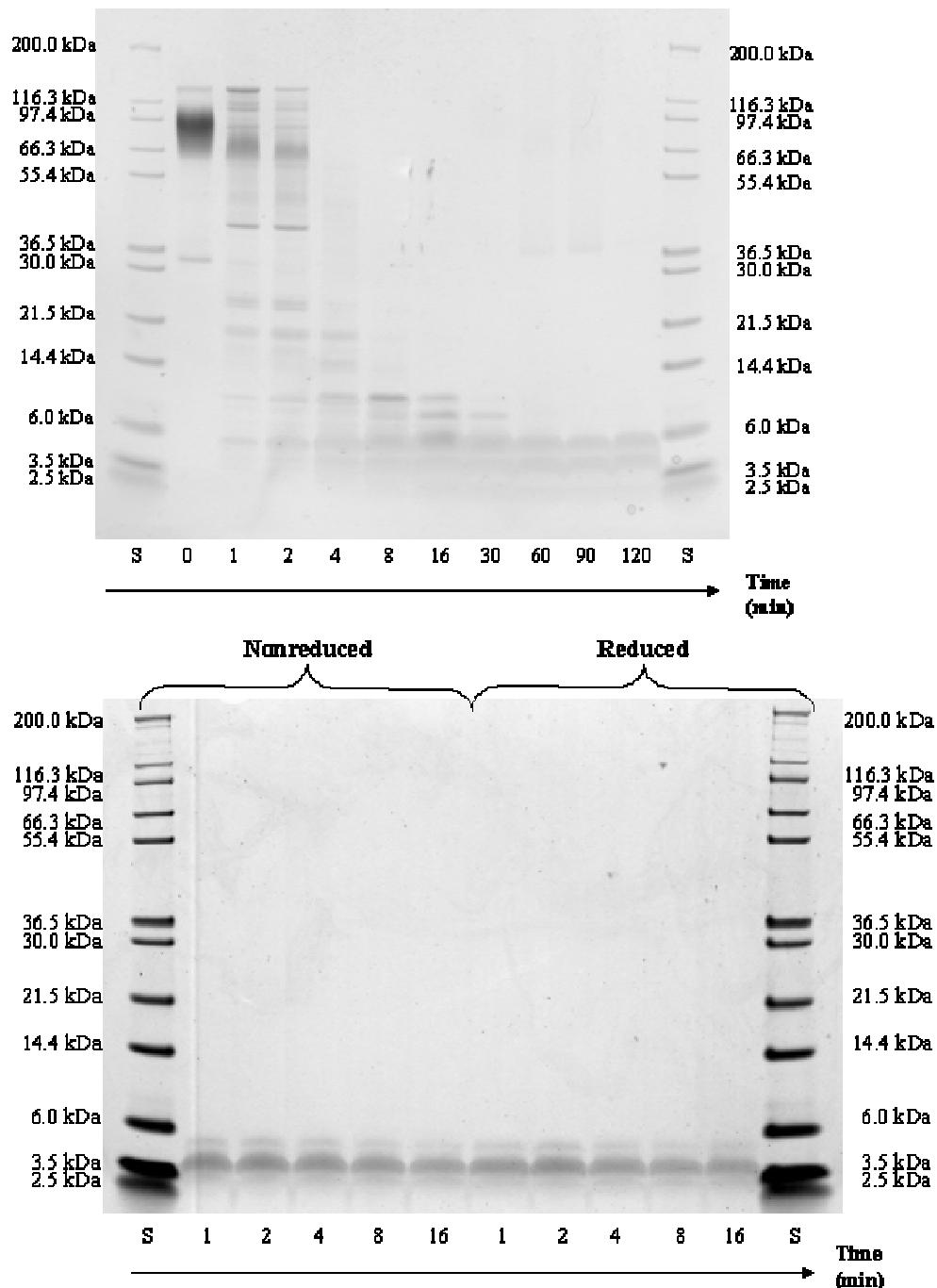
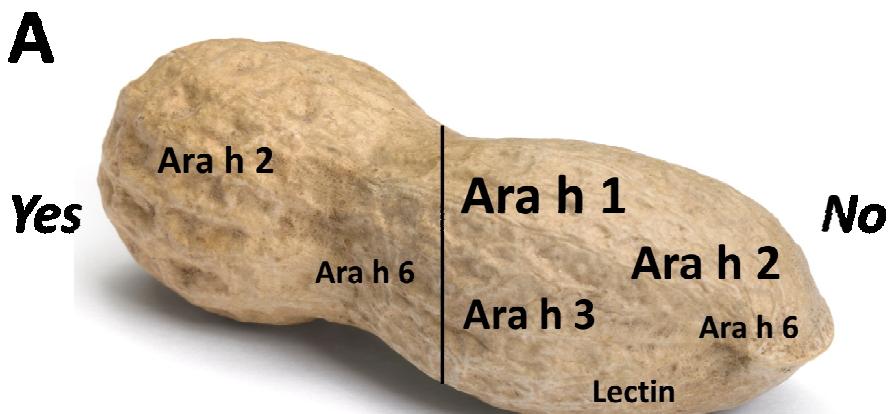
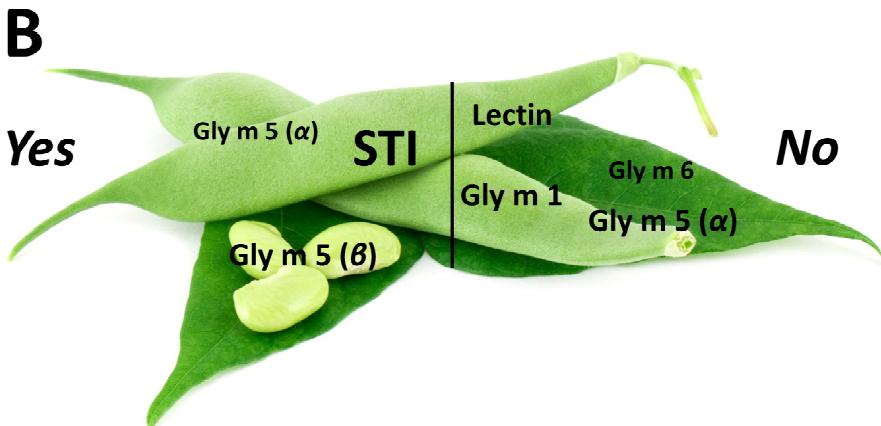


Figure 2. Digestibility results of allergens from peanut, soy, cow's milk and hen's egg. Data from Table 1 (peanut (A) and soy (B)) and Table 2 (cow's milk (C) and hen's egg (D)) are converted to a 'wordle' to illustrate the amount of data on stability to pepsin. The number of studies showing the allergen to be either stable (Yes) or labile (No) to digestion, is illustrated by the size of the name of the allergen. The larger the name the more studies support the statement. Photos are from Colourbox.







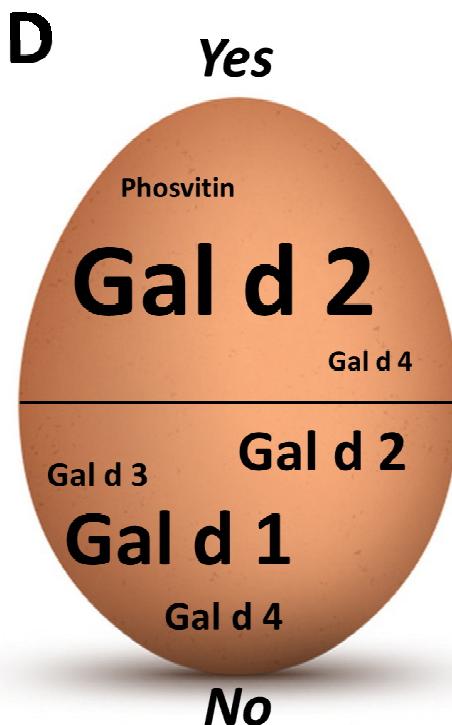


Table 1. Stability of allergens derived from plant foods to digestion with pepsin^a.

Source	Allergen ^b	Size ^c (kDa)	Pepsin :Aller- gen mass ratio	Pepsin activity Unit/mg protein	pH	Diges- tion time (min)	Sta- bel	Sta- bility ^d (min)	Lar- gest frag- ments (kDa)	References
Brazil nut	Ber e 1 (2S albumin)	9+4	16.7	nd	1.2	60	no	15	nd	Murtagh et al., 2002
			16.7	nd	1.2	60	no	15	nd	Murtagh et al., 2003
			0.05	182	2.5	120	yes	-	-	Moreno et al., 2005c
			0.05 ^f	182	2.5	120	yes	-	-	Moreno et al., 2005c
			1.3	9,600	2.0	60	yes	-	-	Koppelman et al., 2005
Sesame seed	Ses i 1 (2S albumin)	9+4	0.05	182	2.5	120	yes	-	-	Moreno et al., 2005b
			0.05 ^f	182	2.5	120	yes	-	-	Moreno et al., 2005b
			0.05 ^h	182	2.5	120	yes	-	-	Moreno et al., 2005b
Sunflower seed	SFA-8 (2S albumin)	12.5	16.7	nd	1.2	60	no	5	nd	Murtagh et al., 2002
			16.7	nd	1.2	60	no	30	nd	Murtagh et al., 2003
Rice	RAP (Trypsin inhibitor)	~16	0.025	nd	1.2	120	yes	-	-	Yamada et al., 2006
Yellow Mustard	Sin a 1 (2S albumin)	9+4	19	nd	1.2	60	yes	-	-	Astwood et al., 1996
Oriental Mustard	Bra j 1 (2S albumin)	9+4	19	nd	1.2	60	yes	-	-	Astwood et al., 1996
Potato	Sola t 1 (Patatin)	43	12.8	nd	1.2	120	no	0	nd	Fu et al., 2002
Celery	Api g 1 (PR-10) ^g	15	0.43	nd	1.0	30	no	0	~1.2	Schimek et al., 2005
	Api g 2 (ns- LTP)	10	0.05	nd	2.0	120	yes	-	-	Gadermaier et al., 2011
Peach	Pru p 3 (ns- LTP)	10	20	nd	1.2	30	yes	-	-	Diaz-Perales et al., 2003
			6.4	nd	1.2	30	yes	-	-	Lopez-Torrejon et al., 2005
			0.017	nd	2.0	180	yes	-	-	Cavatorta et al., 2010
Grape	Vit v 1 (ns- LTP)	9	0.05	182	2.5	120	yes	-	-	Vassilopoulou et al., 2006
			0.05 ^f	182	2.5	120	yes	-	-	Vassilopoulou et al., 2006
Cherry	Pru av 1 (PR-10) ^g	9	nd ^e	≥60,000	2.5	120	no	30	nd	Scheurer et al., 2004
			nd ^e	≥60,000	2.5	120	yes	-	-	Scheurer et al., 2004
			nd ^e	≥60,000	2.5	120	no	1	nd	Scheurer et al., 2004
			nd ^e	≥60,000	2.5	120	no	<30	non visual	
Apple	Mal d 1 (PR-10) ^g	17.7	1.74	nd	2.0	30	no	0	nd	Jensen-Jarolim et al., 1999
			0.4	nd	1.0	30	no	0	~1.8	Schimek et al., 2005
	Mal d 2 (TLP)	31	13	42900	1.2	120	yes	-	-	Smole et al., 2008
Melon	Cuc m 2, (Profilin) ^g	14	6.4	nd	1.2	30	no	<30	non visual	Lopez-Torrejon et al., 2005
			nd ^e	≥60,000	2.5	120	yes	-	-	
Papaya	Papain	?	12.8	nd	1.2	120	no	0	nd	Fu et al., 2002
Pinapple	Papain	?	12.8	nd	1.2	120	no	0	nd	Fu et al., 2002
Kiwi	Act c 1 (Papain)	27.4	0.05	212	2.5	60	yes	-	-	Bublin et al., 2008
			12.8	nd	1.2	120	no	0	nd	Fu et al., 2002
Act c 2, (TLP)		24	16	nd	1.2	60	no	1	nd	Gavrovic-Jankulovic et al., 2002
			6.5	nd	1.2	60	no			Polovic et al., 2007

Avocado	Pers a 1 (chitinase) ^g	32	0.05 20	212 nd	2.5 1.2	120 30	yes no	- 0	- ~5-6	Bublin et al., 2008 Diaz-Perales et al., 2003
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Source	Allergen ^b	Size ^c (kDa)	Pepsin :Aller- gen mass ratio	Pepsin activity Unit/mg protein	pH	Diges- tion time (min)	Sta- bel	Sta- bility time ^d (min)	Lar- gest frag- ments (kDa)	References
Peanut	Ara h 1 (7S globulin)	64	12.8	nd	1.2	120	no	5	nd	Fu et al., 2002
			0.05	162	2.5	120	no	1	~5.6	Eiwegger et al., 2006
			3.04	10,000	1.2	60	no	0.5	nd	Koppelman et al., 2010
			0.30	1,000	1.2	60	no	0.5	nd	Koppelman et al., 2010
			0.03	100	1.2	60	no	0.5	nd	Koppelman et al., 2010
			nd ^e	170	2.5	120	no	4	4	Bøgh et al., 2012
	Ara h 2 (2S albumin)	17	19	nd	1.2	60	yes	-	-	Astwood et al., 1996
			0.4	nd	2.1	40	no	-	-	Sen et al., 2002
			12.8	nd	1.2	120	no	0.5	nd	Fu et al., 2002
			3	10,000	1.2	60	no	0-2	~10	Thomas et al., 2004
			3	10,000	2.0	60	no	0-30	~10	Thomas et al., 2004
			3.04	10,000	1.2	60	no	16	~10	Koppelman et al., 2010
	Ara h 3 (11S globulin)	60	0.002	nd	2.0	120	no	<10	<14	van Boxtel et al., 2008
			3.04	10,000	1.2	60	no	0.25	nd	Koppelman et al., 2010
			0.30	1,000	1.2	60	no	0.25	nd	Koppelman et al., 2010
			0.03	100	1.2	60	no	0.25	nd	Koppelman et al., 2010
	Ara h 6 (2S albumin)	15	nd ^e	6.49	3.0	120	yes	-	-	Suhr et al., 2004
			3.04	10,000	1.2	60	no	4	~10	Koppelman et al., 2010
			0.30	1,000	1.2	60	no	16	~10	Koppelman et al., 2010
			0.03	100	1.2	60	yes	-	-	Koppelman et al., 2010
Soy	Lectin	?	19	nd	1.2	60	no	8	non visual	Astwood et al., 1996
			12.8	nd	1.2	120	no	5	nd	Fu et al., 2002
	Gly m 1 (Hydropho- bic protein)	7	19	nd	1.2	60	no	0	non visual	Astwood et al., 1996
			12.8	nd	1.2	120	no	2	nd	Fu et al., 2002
	Gly m 5 (7S globulin) α - subunit	67	19	nd	1.2	60	no	2	non visual	Astwood et al., 1996
			12.8	nd	1.2	120	no	0	nd	Fu et al., 2002
			17.8	nd	1.2	60	yes	-	-	Fu et al., 2007
			19	nd	1.2	60	yes	-	-	Astwood et al., 1996
	Gly m 5 (7S globulin) β - subunit	48	12.8	nd	1.2	120	yes	-	-	Fu et al., 2002
			19	nd	1.2	120	yes	-	-	Astwood et al., 1996
	Gly m 6 (11S globulin)	60	0.002	nd	2.0	120	no	-	<25	van Boxtel et al., 2008
	STI (Trypsin- inhibitor)	19	19	nd	1.2	60	yes	-	-	Astwood et al., 1996
			0.94	nd	1.2	60	yes	-	-	Yagami et al., 2000
			12.8	nd	1.2	120	yes	-	-	Fu et al., 2002
			3.04	10,518	2.0	60	yes	-	-	Takagi et al., 2003
			3	10,000	1.2	60	yes	-	-	Thomas et al., 2004
			3	10,000	2.0	60	yes	-	-	Thomas et al., 2004
	Lectin	?	19	nd	1.2	60	no	15	non visual	Astwood et al., 1996
			12.8	nd	1.2	120	no	5	nd	Fu et al., 2002

Black gram	?	28	0.94	nd	1.2	60	no	15	~16	Kumari et al., 2012
Hazelnut	Cor a 1, (PR-10) ^g	17.5	0.4	nd	1.0	30	no	0	~1.6	Schimek et al., 2005

^aSummary of allergen stability to pepsin, simulating the digestion process that takes place in the stomach. Included in the Table is only digestibility results from pure plant food derived allergens, digested at 37 °C, for which it was possible to identify a pepsin to allergen ratio, either expressed by a mass ratio or an enzyme activity per mg allergen ratio, ^bAllergen name and biochemical name are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org. ^cSizes of allergens are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org, ^dStability time is based on either the one described by the given author or from the visual appearance in a presented SDS-PAGE, ^eImmobilised enzymes, ^fSurfactant, ^gNon-sensitising allergen, ^hPreheating, Abbreviations: nd, not described; ns-LTP, nonspecific-lipid transfer protein; PR-10, pathogenesis-related protein-10; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STI, soybean trypsin inhibitor; TLP, thaumatin-like protein.

Table 2. Stability of allergens derived from animal foods to digestion with pepsin^a.

Source	Allergen ^b	Size ^c (kDa)	Pepsin :Aller- gen mass ratio	Pepsin activity Unit/mg protein	pH	Diges- tion time (min)	Sta- bel	Sta- bility ^d (min)	Lar- gest frag- ments (kDa)	References			
Cow's milk	Bos d 4 (ALA)	14.2	0.02	nd	2.0	90	no	nd	~7.5	Schmidt et al., 1995			
			0.02	nd	3.0	90	no	nd	~7.5	Schmidt et al., 1995			
			0.02	nd	4.0	90	yes	-	-	Schmidt et al., 1995			
			12.8	nd	1.2	120	no	0	nd	Fu et al., 2002			
			0.05	182	2.5	120	no	5	< 6.5	Moreno et al., 2005a			
			0.05 ^f	182	2.5	120	no	30	< 6.5	Moreno et al., 2005a			
			13	42900	1.2	120	no	nd	< 6.1	Smole et al., 2008			
			0.05	182	2.5	120	no	2	< 6.1	Smole et al., 2008			
			Bos d 5 (BLG)	18.3	0.005	nd	2.0	120	yes	-	-		
			0.02	nd	2.0	90	yes	-	-	Schmidt et al., 1995			
Bos d 5 (BLG)			0.02	nd	3.0	90	yes	-	-	Schmidt et al., 1995			
			0.02	nd	4.0	90	yes	-	-	Schmidt et al., 1995			
			19	nd	1.2	60	yes	-	-	Astwood et al., 1996			
			0.94	nd	1.2	60	yes	-	-	Yagami et al., 2000			
			12.8	nd	1.2	120	yes	-	-	Fu et al., 2002			
			3.04	10,518	2.0	60	yes	-	-	Takagi et al., 2003			
			3	10,000	1.2	60	yes	-	-	Thomas et al., 2004			
			3	10,000	2.0	60	yes	-	-	Thomas et al., 2004			
			0.05	165	2.5	120	yes	-	-	Sanz et al., 2007			
			nd	10,000	1.5	60	yes	-	-	Lucas et al., 2008			
			3.05	10,000	1.2	60	yes	-	-	Ofori-Anti et al., 2008			
			0.05	182	2.5	60	yes	-	-	Macierzanka et al., 2009			
			0.05	182	2.5	60	yes	-	-	Mandalari et al., 2009b			
			16	55,356	1.2	60	yes	-	-	Misra et al., 2009			
			12.8	42,240	1.2	120	no	0	nd	Mandalari et al., 2009a			
			0.05	165	2.5	60	yes	-	-	Mandalari et al., 2009a			
			0.05 ^f	165	2.5	60	yes	-	-	Mandalari et al., 2009a			
			0.05 ^f	182	2.5	60	yes	-	-	Dupont et al., 2010			
			0.007 ^f	25	3.0	60	yes	-	-	Dupont et al., 2010			
Bos d 6 (BSA)			3.2	nd	1.2	60	yes	-	-	Zheng et al., 2010			
			nd ^e	180	2.5	120	yes	-	-	Bøgh et al., 2013			
			Bos d 6 (BSA)	37	0.02	nd	2.0	90	no	nd	~11		
			0.02	nd	3.0	90	no	nd	~11	Schmidt et al., 1995			
			0.02	nd	4.0	90	no	nd	~31	Schmidt et al., 1995			
			19	nd	1.2	60	no	0.5	non visual	Astwood et al., 1996			
			12.8	nd	1.2	120	no	0	nd	Fu et al., 2002			
			16.7	nd	1.2	60	no	5	nd	Murtagh et al., 2002			
			3.04	10,518	2.0	60	no	0.5	~3.9	Takagi et al., 2003			
			16.7	nd	1.2	60	no	0	nd	Murtagh et al., 2003			
			3	10,000	1.2	60	no	0-2	~6	Thomas et al., 2004			
			3	10,000	2.0	60	no	0-2	~6	Thomas et al., 2004			
			16	nd	1.2	60	no	0	nd	Pantoja-Uceda et al., 2004			
			0.025	nd	1.2	120	no	<10	non visual	Yamada et al., 2006			
			9.6	nd	1.2	60	no	<5	nd	Lin et al., 2006			
			13	nd	1.2	60	no	5	nd	Polovic et al., 2007			
			3.05	10,000	1.2	60	no	0.5	~3.9	Ofori-Anti et al., 2008			

		3.2	nd	1.2	60	no	0	nd	Zheng et al., 2010
Bos d 7 (IgG)	160	0.02	nd	2.0	90	no	nd	~30	Schmidt et al., 1995
		0.02	nd	3.0	90	yes	-	-	Schmidt et al., 1995
		0.02	nd	4.0	90	yes	-	-	Schmidt et al., 1995

Source	Allergen ^b	Size ^c (kDa)	Pepsin :Aller- gen mass ratio	Pepsin activity Unit/mg protein	pH	Diges- tion time (min)	Sta- bel	Sta- bility time ^d (min)	Lar- gest frag- ments (kDa)	References
Cow's milk	Bos d 8 (β - casein)	24	19	nd	1.2	60	no	2	non visual	Astwood et al., 1996
			0.05	182	2.5	60	no	20	nd	Macierzanka et al., 2009
			12.8	42,240	1.2	120	no	0.1	nd	Mandalari et al., 2009a
			0.05	165	2.5	60	no	10	≥ 3.5	Mandalari et al., 2009a
			0.05 ^f	165	2.5	60	no	10	≥ 3.5	Mandalari et al., 2009a
			0.007 ^f	25	3.0	60	no	10	7.4	Dupont et al., 2010
	Bos d 8 (α - casein)	?	12.8	nd	1.2	120	no	0	nd	Fu et al., 2002
	Lactoperoxi- dase?	?	12.8	nd	1.2	120	no	0	nd	Fu et al., 2002
	Lactoferrin	3.05	10,000	1.2	60	no	0.5	~ 5.4	Ofori-Anti et al., 2008	
Hen's Egg	Gal d 1 (OVM)	28	19	nd	1.2	60	no	8	non visual	Astwood et al., 1996
			0.05	nd	2.0	120	no	10	18	Kovacs-Nolan et al., 2000
			12.8	nd	1.2	120	no	0	nd	Fu et al., 2002
			3.04	10,518	2.0	60	no	0.5	<3	Takagi et al., 2003
			nd	10,000	2.0	60	no	0.5	4.5-6	Takagi et al., 2005
			nd	10,000	2.0	60	no	2	4.5-6	Takagi et al., 2005
			nd	10,000	2.0	60	no	30	4.5-6	Takagi et al., 2005
			0.05	nd	2.5	60	no	10	18	Jimenes-Saiz et al., 2011
			0.05 ^h	nd	2.5	60	no	10	18	Jimenes-Saiz et al., 2011
	Gal d 2 (OVA)	44	19	nd	1.2	60	yes	-	-	Astwood et al., 1996
			12.8	nd	1.2	120	no	5	nd	Fu et al., 2002
			8	nd	1.2	60	yes	-	-	Dearman et al., 2002
			2	nd	1.2	60	yes	-	-	Dearman et al., 2002
			12.8	nd	1.2	120	no	5	nd	Fu et al., 2002
			3.04	10,518	2.0	60	yes	-	-	Takagi et al., 2003
			3.04 ^h	10,518	2.0	60	no	0.5	non visual	Takagi et al., 2003
			3	10,000	1.2	60	yes	-	-	Thomas et al., 2004
			3	10,000	2.0	60	yes	-	-	Thomas et al., 2004
	Gal d 3 (OT)	78	3.05	10,000	1.2	60	yes	-	-	Ofori-Anti et al., 2008
			0.2	114	2.5	120	yes	-	-	Lopez-Exposito et al., 2008
			16	55,356	1.2	60	no	15	nd	Misra et al., 2009
			0.05	172	2.0	60	yes	-	-	Martos et al., 2010
			0.05 ^f	172	2.0	60	yes	-	-	Martos et al., 2010
			0.05	182	2.5	60	yes	-	-	Dupont et al., 2010
			0.007	25	3.0	60	yes	-	-	Dupont et al., 2010
			3.2	nd	1.2	60	no	0	nd	Zheng et al., 2010
			0.05	nd	2.5	60	yes	-	-	Jimenes-Saiz et al., 2011
			0.05 ^h	nd	2.5	60	no	10	~ 4	Jimenes-Saiz et al., 2011
			0.01 ^h	nd	1.8	60	yes	-	-	Golias et al., 2012

Gal d 4 (HEL)	14 3.05 16 3,2	12.8 10,000 55,356 nd	1.2 1.2 1.2 1.2	120 60 60 60	no yes no no	60 - 8 30	nd - nd nd	Fu et al., 2002 Ofori-Anti et al., 2008 Misra et al., 2009 Zheng et al., 2010	
Phosvitin, kinase?	?	19	nd	1.2	60	yes	-	-	Astwood et al., 1996

Source	Allergen ^b	Size ^c (kDa)	Pepsin :Aller- gen mass ratio	Pepsin activity Unit/mg protein	pH	Diges- tion time (min)	Sta- bel	Sta- bility ^d (min)	Lar- gest frag- ments (kDa)	References
Fish	Parvalbu- min, cod	12	0.03 ^h	nd	2.5	30	yes	-	-	de Jongh et al., 2011
	Lep w 1, whiff parvalbumin	12	0.05 ^h	212	2.5	120	yes/ no ⁱ	0	nd	Greismeier et al., 2010
Shrimp	Pen a 1, tropomyosin	36	12.8	nd	1.2	120	no	0	nd	Fu et al., 2002
	Lit v 1, tropomyosin	36	0.02	333	1.2	60	yes	-	-	Liu et al., 2011
Prawn	Pen m 1, tropomyosin	38	0.02	333	1.2	60	yes	-	-	Liu et al., 2011
Crab	Tropo- myosin	34	0.02	333	1.2	60	yes	-	-	Liu et al., 2010
			0.02	333	1.2	60	yes	-	-	Huang et al., 2010

^aSummary of allergen stability to pepsin, simulating the digestion process that takes place in the stomach. Included in the Table is only digestibility results from pure animal food derived allergens, digested at 37 °C, for which it was possible to identify a pepsin to allergen ratio, either expressed by a mass ratio or an enzyme activity per mg allergen ratio, ^bAllergen name and biochemical name are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org. ^cSizes of allergens are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org, ^dStability time is based on either the one described by the given author or from the visual appearance in a presented SDS-PAGE, ^eImmobilised enzymes, ^fSurfactant, ^gNon-sensitising allergen, ^hPreheating, ⁱThe protein was stable as a dimer but not as a monomer. Abbreviations: ALA, α -lactalbumin; BLG, β -

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lactoglobulin; BSA, bovine serum albumin; HEL, hen's egg lysozyme C; IgG, immunoglobulin G; nd, not described; OVA, ovalbumin; OT, ovotransferrin; OVM, ovomucoid; ns-LTP, nonspecific-lipid transfer protein; PR-10, pathogenesis-related protein-10; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 3. Stability of allergens from plant and animal foods to digestion with pepsin followed by trypsin and chymotrypsin^a.

Source	Allergen ^b	Size ^c (kDa)	Enzyme ^j :Aller-gen mass ratio	Enzyme activity Unit/mg protein	pH ^k	Diges-tion time (min)	Sta-bel	Sta-bility time ^d (min) ^l	Lar-gest frag-ments (kDa)	References
Peanut	Ara h 1 (7S globulin)	64	nd	162/ 34.5/ 0.44	2.5/ 6.5	120+ 15	no	4	2	Bøgh et al., 2009
			0.05/	162/	2.5/	120+	no	1	-	Eiwegger et al., 2006
			0.0025/	34.5/	6.5	16				
			0.01	0.44						
Brazil nut	Ber e 1 (2S albumin)	9+4	0.05/	182/	2.5/	120+	no	120+	1.1	Moreno et al., 2005c
			0.0025/	34.5/	6.5	120		nd		
			0.01	0.44						
			0.05/	182/	2.5/	120+	no	120+	1.1	Moreno et al., 2005c
Sesame seed	Ses i 1 (2S albumin)	9+4	0.05/	182/	2.5/	120+	no	120+	nd	Moreno et al., 2005b
			0.0025/	34.5/	6.5	120		nd		
			0.01	0.44						
			0.05/	182/	2.5/	120+	no	120+	nd	Moreno et al., 2005b
Grape	Vit v 1 (ns-LTP)	9	0.05/	182/	2.5/	120+	yes	-	-	Vassilopoulou et al., 2006
			0.0025/	34.5/	6.5	120				
			0.01	0.44						
			0.05/	182/	2.5/	120+	yes	-	-	Vassilopoulou et al., 2006
Peach	Pru p 3 (ns-LTP)	10	0.017/	nd	2.0/	180+	yes	-	-	Cavatorta et al., 2010
			0.017/0.		7.8	240				
Celery	Api g 2 (ns-LTP)	10	0.05/	nd	2.0/	120+	yes	-	-	Gadermeier et al., 2011
			0.0025/		7.8	120				
Kiwi	Act c 1 (papain)	27.4	0.05/	212/	2.5/	60+	yes	-	-	Bublin et al., 2008
			0.0025/	34.5/	6.5	30				
	Act c 2 (TLP)	24	0.05/	212/	2.5/	120+	yes	-	-	Bublin et al., 2008
			0.0025/	34.5/	6.5	120				
Apple	Mal d 1 (PR-10) ^g	17.7	1.74/	nd	2.0/	30+	no	0	nd	Jensen-Jarolim et al., 1999
			0.844/		7.7	30				
	Mal d 2 (TLP)	31	0.05/	182/	2.5/	120+	no	-	-	Smole et al., 2008
			0.0025/	34.5/	6.5	120				
			0.01	0.4						

Cow's milk	Bos d 4 (ALA)	14.2	0.05/ 0.0025/ 0.01	182/ 34.5/ 0.44	2.5/ 6.5	120+ 120	no	5	<<6.5	Moreno et al., 2005a
			0.05/ 0.0025/ 0.01 ^f	182/ 34.5/ 0.44	2.5/ 6.5	120+ 120	no	30	<<6.5	Moreno et al., 2005a
			0.05/ 0.0025/ 0.01	182/ 34.5/ 0.4	2.5/ 6.5	120+ 120	no	2	non visual	Smole et al., 2008

Source	Allergen ^b	Size ^c (kDa)	Enzyme ^j :Aller- gen mass ratio	Enzyme activity Unit/mg protein	pH ^k	Diges- tion time (min)	Sta- bel	Sta- bility ^d (min) ^l	Lar- gest frag- ments (kDa)	References
Bos d 5, (BLG)	Bos d 5, (BLG)	18.3	0.05/ 0.0025/ 0.01 ^f	182/ 34.5/ 0.4	2.5/ 6.5	60+ 30	yes	-	-	Duport et al., 2010
			0.05/ 0.0025/ 0.01 ^f	182/ 34.5/ 0.4	3.0/ 6.5	60+ 30	yes	-	-	Duport et al., 2010
			0.05/ 0.0025/ 0.01	165/ 34.5/ 0.5	2.5/ 6.5	60+ 30	yes	-	-	Mandalari et al., 2009a
	Bos d 5, (BLG)	18.3	0.05/ 0.0025/ 0.01 ^f	165/ 34.5/ 0.4	2.5/ 6.5	60+ 30	yes	-	-	Mandalari et al., 2009a
			0.05/ 0.0025/ 0.01 ^f	182/ 34.5/ 0.4	2.5/ 6.5	60+ 30	yes	-	-	Mandalari et al., 2009b
			0.05/ 0.0025/ 0.01	182/ 34.5/ 0.4	2.5/ 6.5	60+ 30	yes	-	-	Mandalari et al., 2009b
	Bos d 5, (BLG)	18.3	0.05/ 0.0025/ 0.01 ^f	182/ 35.5/ 0.4	2.5/ 6.5	60+ 30	yes	-	-	Mandalari et al., 2009b
			0.05/ 0.0025/ 0.01	182/ 34.5/ 0.4	2.5/ 6.5	60+ 30	no	60+ 15	nd	Macierzna et al., 2009
			nd	180/ 34.5/ 0.4	2.5/ 6.5	120+ 15	yes	-	-	Bøgh et al., 2013
Bos d 8 (β -casein)	Bos d 8 (β -casein)	24	0.05/ 0.0025/ 0.01 ^f	182/ 34.5 /0.4	2.5/ 6.5	60+ 30	no	10	~1.73	Duport et al., 2010
			0.05/ 0.0025/ 0.01 ^f	182/ 34.5/ 0.4	3.0/ 6.5	60+ 30	no	10	~3	Duport et al., 2010
			0.05/ 0.0025/ 0.01	165/ 34.5/ 0.5	2.5/ 6.5	60+ 30	no	10	non visual	Mandalari et al., 2009a
	Bos d 8 (β -casein)	24	0.05/ 0.0025/ 0.01 ^f	165/ 34.5 /0.4	2.5/ 6.5	60+ 30	no	10	non visual	Mandalari et al., 2009a
			0.05/ 0.0025/ 0.01	182/ 34.5/ 0.4	2.5/ 6.5	60+ 30	no	20	nd	Macierzna et al., 2009

Hen's egg	Gal d 1 (OVM)	28	0.005/ 0.002/0. 002	nd	2.0/ ~7.5	30+ 180	no	nd	>20	Kovacs-Nolan et al., 2000
Gal d 2 (OVA)		44	0.05/ 0.0025/ 0.01 ^f	182/ 34.5/ 0.4	2.5/ 6.5	60+ 30	yes	-	-	Duport et al., 2010
			0.05/ 0.0025/ 0.01 ^f	182/ 34.5/ 0.4	3.0/ 6.5	60+ 30	yes	-	-	Duport et al., 2010
			0.05/nd/ nd	172/ 40/ 0.5	2.0/ 7.0	60+ 60	yes	-	-	Martos et al., 2010

^aSummary of allergen stability to pepsin, simulating the digestion process that takes place in the stomach, followed by trypsin and chymotrypsin, simulating the digestion process that takes place in the duodenum. Included in the Table is only digestibility results from pure plant and animal food derived allergens, digested at 37 °C, for which it was possible to identify a pepsin to allergen ratio, either expressed by a mass ratio or an enzyme activity per mg allergen ratio,

^bAllergen name and biochemical name are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org. ^cSizes of allergens are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org, ^dStability time is based on either the one described by the given author or from the visual appearance in a presented SDS-PAGE, ^eImmobilised enzymes, ^fSurfactant, ^gNon-sensitising allergen,

^hPreheating, ^jenzymes presented in the order of; pepsin, trypsin and chymotrypsin, where pepsin is the enzyme used to simulate gastric digestion and trypsin and chymotrypsin to simulate duodenal digestion, ^kpH of gastric respectively duodenal digestion. Abbreviations: ALA, α -lacalbumin; BLG, β -lactoglobulin; nd, not described; ns-LTP, nonspecific-lipid transfer protein; OVA, ovalbumin; OVM, ovomucoid; PR-10, pathogenesis-related protein-10; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; thaumatin-like protein.