

Digestion Stability as a Criterion for Protein Allergenicity Assessment

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ABSTRACT: Assessing the allergenic potential of transgenic proteins introduced into genetically engineered food remains a critical part of the overall safety assessment of these foods. Stability to digestion has been proposed as one of the steps in the decision tree approach to assess the allergenic potential of transgenic proteins. The validity of digestion stability as a criterion for protein allergenicity assessment, however, has encountered some criticism in recent years. This chapter gives an overview of the rationale behind the use of digestion stability as a criterion for protein allergenicity assessment and reviews the available data that may or may not support its use. The application of *in vitro* digestion assays for the assessment of allergenic potential of novel proteins and the factors that affect the assay results are also discussed. There is a need to establish standardized assay conditions so that direct comparison of results from different laboratories can be made. Consensus also needs to be reached on relating the measured digestibility to the allergenic potential of proteins.

KEYWORDS: digestion stability; food allergens; protein allergenicity assessment; *in vitro* digestion assays

INTRODUCTION

Avoidance of allergenic foods is the sole defense that allergic individuals have to prevent the occurrence of food allergy. The ability to identify foods containing allergens is therefore vital to these persons. Advances in biotechnology have resulted in an increased number of genetically engineered foods that contain proteins of nonfood origin. The allergenicity of these newly introduced proteins needs to be assessed to ensure the safety of these foods.

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Ann. N.Y. Acad. Sci. 964: 99–110 (2002). © 2002 New York Academy of Sciences.

For proteins that are derived from allergenic foods, protein allergenicity can be readily tested with serum from individuals allergic to those foods. Testing the allergenicity of novel proteins derived from nonfood sources, however, is challenging, because no human serum is available to test these proteins and there are no currently validated methods to predict protein allergenicity.

The strategy commonly adopted by the agricultural biotechnology industry to assess the allergenic potential of novel proteins is to evaluate whether these proteins share similar properties with those of known allergens. In 1996, the International Food Biotechnology Council (IFBC) and the International Life Sciences Institute (ILSI) jointly developed a decision-tree approach¹ and recommended that several factors be considered when assessing the allergenic potential of proteins derived from nonfood sources, including comparing the amino acid sequences and physicochemical properties of these proteins with those of known allergens. One of the physicochemical properties suggested was stability to digestion in the human gastrointestinal tract.

The 1996 IFBC/ILSI decision tree has been widely adopted by the industry and regulatory agencies around the world.² Digestion stability has been used as one of the criteria to assess the allergenic potential of many transgenic proteins introduced into foods^{3–8} and has been one of the major criteria that regulatory agencies consider when evaluating the safety of genetically engineered foods. Star Link™ corn, which recently received much media attention, was not approved for human consumption as a result of potential allergy concerns because the inserted gene product (the Cry9C protein) was found to be resistant to pepsin digestion.⁹

The validity of digestion stability as a criterion for protein allergenicity assessment, however, has encountered some criticism in recent years. This chapter gives an overview of the rationale behind the use of digestion stability as a criterion for protein allergenicity assessment and reviews the available data that may or may not support its use as a criterion. The application of digestion assays for the assessment of allergenic potential of genetically engineered foods and the factors that affect the assay results are also discussed.

DIGESTION STABILITY: A DEFINING CHARACTERISTIC OF FOOD ALLERGENS?

The rationale for the use of digestion stability as a criterion for protein allergenicity assessment stems from the observation that many food allergens exhibit proteolytic stability.^{10–13} Stability to digestion has thus been considered one of the properties shared by many food allergens.^{14,15} Nevertheless, in order to allow digestibility to be used as a predictive tool for the estimation or ranking of the allergenic potential of novel proteins, the comparative di-

gestibility of food allergens and nonallergenic proteins needs to be determined. Several questions need to be addressed:

- Are food allergens generally more resistant to digestion than nonallergenic proteins?
- Can digestibility be used as a parameter to distinguish food allergens from nonallergenic proteins?
- Does digestibility of food allergens correlate with their allergenic potential?

Answers to these questions require comparison of the relative digestibility among food allergens with varying degrees of allergenicity and between food allergens and nonallergenic proteins under well-defined conditions. *In vitro* models of mammalian digestion have been widely used to evaluate the digestibility of food proteins.^{16–18} Standard simulated gastric and intestinal digestive models as described in the U.S. Pharmacopeia¹⁹ provide simple and well-controlled systems for the estimation of the relative digestibility of proteins.

Digestibility: Food Allergens versus Nonallergenic Proteins

Astwood *et al.*²⁰ measured the digestive stability of a group of major food allergens and a group of nonallergenic enzymes in simulated gastric fluid (SGF). Although some food allergens were stable in SGF for the full 60-min reaction period, others were rapidly degraded within 30 sec (although peptide fragments were observed in these proteins for at least 8 min). All of the nonallergenic enzymes tested were degraded within 15 sec without forming any stable peptide fragments. It was concluded that allergens were more stable than nonallergenic proteins in SGF and that digestibility can be used as a parameter to distinguish allergens from nonallergenic proteins.

The notion that food allergens are stable to digestion, however, has been questioned by other researchers. Vieths *et al.*²¹ compared the digestibility of peanut and hazelnut allergens in SGF for 2 hr followed by a 45-min treatment in duodenal conditions and showed that while peanut proteins were relatively stable during digestion, the native hazelnut allergens were labile to digestion. Yagami *et al.*²² showed that most allergens extracted from five types of fruits degraded rapidly in SGF.

We compared the digestion stability of food allergens and nonallergenic proteins of similar cellular functions. Nineteen food allergens and 19 nonallergenic proteins belonging to each of the four protein groups (storage proteins, plant lectins, contractile proteins, and enzymes) were subjected to digestion in SGF as well as in simulated intestinal fluid (SIF). Our results showed that the digestive stability of the food allergens tested varied greatly, ranging from 0 to 120 min. A similar range of stability was observed among

the nonallergenic proteins tested. Food allergens were not necessarily more resistant to SGF and SIF digestion than nonallergenic proteins of similar cellular functions.²³

Several major food allergens, such as the milk allergen α -casein, the egg allergen ovomucoid, and shrimp tropomyosin, were quite labile in SGF (all were degraded within 30 sec); in contrast, a number of nonallergenic proteins (e.g., zein and the trypsin inhibitor from bovine pancreas) were very stable in SGF. Some major food allergens were rather rapidly degraded in SIF. For example, Ara h 2 was degraded within 30 sec; shrimp tropomyosin was degraded instantly, although degradation fragments stable for 30 sec were observed.

A similar observation was reported by Kenna and Evans,²⁴ who compared the digestibility of 17 food allergens with 24 proteins not associated with food allergy in SGF and found that, even though 13 out of the 17 food allergens were partially or completely stable for at least 60 min, 10 out of the 24 nonallergenic proteins also were completely stable or formed peptide fragments that were stable for at least 60 min.

The available data seem to indicate that resistance to digestion in SGF or SIF may not be a defining characteristic for food allergens. It may therefore be difficult to distinguish food allergens from nonallergenic proteins based on their stability in SGF and/or SIF.

Digestibility: Major Allergens versus Minor Allergens

The relative allergenicity of a food allergen is generally measured by the percentage of individuals having IgE for that specific allergen (referred to as percent allergenicity). Major allergens are proteins for which more than 50% of the allergic patients studied have specific IgE, and minor allergens are proteins for which fewer than 50% of the patients have IgE. Fuchs and Astwood¹⁵ ranked a selected group of egg, milk, and soybean allergens according to their percent allergenicity and compared their digestibility in SGF; they suggested that a correlation can be established between the digestibility and percent allergenicity.

When additional food allergens with varying percent allergenicity were compared, however, it was found that the relative resistance to digestion does not necessarily correspond to high allergenicity.²³ For example, ovalbumin, a major egg allergen to which up to 100% of patients have IgE, was degraded within 5 min in SGF; lysozyme, a minor egg allergen, was stable for at least 60 min. α -Casein, a major milk allergen, was degraded more rapidly than the minor milk allergen BSA.

A comparison of the SIF stability between the major and minor allergens also did not indicate any correlations between allergenicity and digestion stability.²³ Food allergens with a high percent allergenicity were not necessarily more resistant to SIF digestion than allergens with low percent allergenicity.

For example, Ara h 2, a major peanut allergen to which more than 95% of patients have IgE, was degraded within 30 sec in SIF; peanut lectin, a minor allergen, was stable for at least 120 min.

These results suggest that there is not a clear correlation between the digestibility of an allergen and its allergenic potential. It would therefore be difficult to rank the allergenic potential of proteins based on the SGF or SIF digestibility.

DIGESTION STABILITY: A PREREQUISITE FOR FOOD PROTEIN ALLERGENICITY?

The use of digestion stability as a criterion for protein allergenicity assessment also stems from the general belief that for a protein to elicit an allergic response, it must survive the acid and proteolytic environment of the human gastrointestinal system to reach and be absorbed through the intestinal mucosa.¹⁴ Digestive stability has therefore been considered a key prerequisite for food protein allergenicity.¹

The development of food allergy can be divided into two phases, the sensitization phase and the elicitation phase. When an allergen is first encountered by the immune system of an atopic individual, a series of responses occurs, including binding of the protein (or peptide fragments) to the B and T cells, formation of IgE antibodies, and binding of IgE antibodies to mast cells or basophils.²⁵ Details of why certain individuals develop sensitization to a particular protein are not clear. It is known, however, that sensitization does not necessarily need to be through the oral route. A person can be sensitized through skin contact or inhalation of the allergen. The second phase in the development of a food allergy is the elicitation phase. When an already-sensitized person encounters the allergen for the second time, the allergen cross-links two IgE antibody molecules bound on the surface of a mast cell and thus triggers the release of mediators, which in turn elicit the clinical symptoms of allergic diseases.

Few data are available in the literature suggesting that a protein needs to be in an intact form to sensitize an individual. However, there is some evidence indicating that peptide fragments could activate IgE responses. Haddad *et al.*²⁶ reported that a number of patients who were allergic to milk had only IgE antibodies to peptic digests or peptic or tryptic digest of β -lactoglobulin and did not have IgE antibodies to undigested β -lactoglobulin. It is not clear whether there is a minimal size requirement for a protein or a peptide to initiate the sensitization process.

As for the elicitation phase, a number of studies suggest that peptide fragments generated from the proteolytic digestion or hydrolysis of food allergens can elicit IgE responses in already-sensitized individuals. Maynard

*et al.*²⁷ showed that among the sera from patients with cow's milk allergy, 8 out of 19 possessed a specific IgE response to different tryptic peptides. Nils-son *et al.*²⁸ reported that allergic individuals showed responses to casein hydrolysates. Peptide fragments resulting from proteolysis of ovomucoid and ovalbumin were also reported to react with IgE of persons allergic to eggs.^{29,30}

There seems to be a size limitation on the ability of peptides to react with the IgE of already-sensitized individuals. van Beresteijn *et al.*³¹ tested the immunological properties of hydrolysates of whey protein concentrate and found that the minimal molecular mass to elicit immunogenicity and allergenicity appeared to be between 3000 and 5000 Da. Huby *et al.*³² suggested that the minimum size for a peptide to bridge two IgE molecules on the surface of mast cells to initiate degranulation was at least 3 kDa.

Although there are not enough data to validate (or invalidate) the idea that stability to digestion is a prerequisite for food protein allergenicity, consensus among the scientific community seems to exist that a protein that is resistant to proteolytic digestion in the digestive tract would retain sufficient structural integrity to have an increased probability of stimulating immune reactions. The *in vitro* digestion assay (SGF, SIF, or SGF and SIF in tandem) followed by SDS-PAGE analysis provides an estimate as to whether a protein would be degraded to fragments small enough that they are less likely to elicit an IgE reaction.

APPLICATIONS OF DIGESTION STABILITY AS A CRITERION FOR PROTEIN ALLERGENICITY ASSESSMENT

Digestibility Is Greatly Influenced by Assay Conditions Used

The applicability of digestion stability as a criterion for protein allergenicity assessment is widely accepted. The question now raised is, How stable does a protein need to be in order to be considered of high allergenic potential? The answer to this question is difficult, because available data do not support ranking of allergenicity based on SGF or SIF digestibility. One may not be able to estimate the allergenic potential of a protein by ranking the measured digestibility among those of food allergens with varying degrees of allergenicity as suggested.³³

The issue is further complicated in that the digestibility of a protein as measured by the *in vitro* SGF (or SIF) assay is greatly influenced by the assay conditions used. Changes in pH or the relative amount of enzymes used could affect the digestibility measured and thus the estimated allergenic potential. We have found that the digestibility of proteins as determined by the SGF assay could be greatly influenced by the relative amounts of pepsin and test pro-

TABLE 1. Enzyme/test protein ratios used for protein digestibility studies

Protein	Source	Enzyme used	Enzyme/test protein ratio	Reference
Albumin	Beans	Trypsin	1: 40	Marquez & Lajolo ³³
Globulins	(Phaseolus vulgaris)	Pepsin		
Glutelins		Pancreatin		
Phaseolin	Dry bean	Trypsin	1: 10 or 1 : 100	Nielsen <i>et al.</i> ¹⁸
Vicilin	Pea	Pepsin		
Glycinin	Soybean	Other enzymes		
β-Conglycinin	Soybean			
β-Conglycinin	Soybean	Trypsin	1 : 250	Kamata <i>et al.</i> ¹⁷
α-Lactalbumin	Milk	Pepsin	1 : 25	Asselin <i>et al.</i> ³⁵
β-Lactoglobulin		Chymotrypsin		
		Trypsin		
		Pancreatin		
α-Lactalbumin	Milk	Trypsin	1 : 100	Maynard <i>et al.</i> ³⁴
Globulins	Rice	Pepsin	1 : 100	Watanabe <i>et al.</i> ³⁶
		Actinase		
Neomycin phospho-transferase II (NPTII)	Recombinant <i>E. coli</i>	Pepsin	1600 : 1	Fuchs <i>et al.</i> ³
		Pancreatin	5000 : 1	
1-Aminopocyclopropane-1-carboxylic acid deaminase (ACCD)	Recombinant <i>E. coli</i>	Pepsin	800 : 1	Reed <i>et al.</i> ⁵
CRYIA(b)	Recombinant	Pepsin	100 : 1	Noteborn <i>et al.</i> ⁴
NPTII	<i>E. coli</i>	Chymotrypsin/ trypsin	25 : 1	

tein present in the assay.²³ For example, the stability of the major egg allergen ovalbumin changed from greater than 120 min to 0 min as the ratio of pepsin to test protein (by weight) increased from 0.1 to 100. Ovalbumin would therefore appear as highly stable or highly unstable depending on the pepsin/test protein ratio used. Changes in the pepsin/test protein ratio also affected the rate of degradation of nonallergenic proteins. Stable fragments were observed during the SGF digestion of lipoxidase and sucrose synthetase when the pepsin/test protein ratio decreased from 10 to 0.1, which would cause lipoxidase and sucrose synthetase to appear as stable proteins. That changes in enzyme concentration would affect the digestibility measured was also observed by Astwood *et al.*,²⁰ who showed that RUBISCO (D-ribulose 1,5-diphosphate carboxylase) would appear as a highly stable protein if the pepsin concentration used in the SGF assay were reduced by 100-fold.

It is not known what the relative amounts of pepsin and food proteins are during the human digestive process. However, variations seem to exist in the conditions employed by different laboratories to study protein digestibility *in vitro*. We examined a series of studies that assessed the digestibility of proteins from conventional and transgenic foods. As shown in TABLE 1, the ratio

TABLE 2. Interpretation of *in vitro* digestion assay results

Systems	Trait modified	SGF stability	SIF stability	Conclusion made	Reference
Soybean glycinin in transgenic rice	Improved nutritional value	10 min	30 min	Rapidly degraded	Momma <i>et al.</i> ⁸
Modified soybean glycinin in transgenic potato	Improved nutritional value	10 min	Not done	Rapidly degraded	Hashimoto <i>et al.</i> ⁷
Bacterial enzyme (ACCd) in transgenic tomato	Delay ripening	15 sec	>8 hours	Rapidly degraded	Reed <i>et al.</i> ⁵
CRYIA(b) from B. t. in transgenic tomato	Insect resistance	15-kDa fragments stable for 2 hr	—	Rapidly degraded	Noteborn <i>et al.</i> ⁴
Cry9C from B. t. in transgenic corn	Insect resistance	>2 hours	>2 hours	Stable	Noteborn <i>et al.</i> ³⁷

of enzyme to test protein (by weight) used in these studies varies greatly, from a 1:250 ratio of trypsin to soybean β -conglycinin used by Kamata *et al.*¹⁶ to a 5000:1 ratio of pancreatin to neomycin phosphotransferase II used by Fuchs *et al.*³ As discussed previously, these variations in relative amounts of enzyme and test protein can greatly affect the rate of enzymatic degradation of the proteins in question, and therefore have a significant impact on the estimated allergenic potential of those proteins.

Interpretation of Assay Results

Disparity also exists among different laboratories regarding interpretation of the *in vitro* digestion assay results. Fuchs and Astwood¹⁵ showed that nine different proteins expressed in genetically modified plants were rapidly degraded within 30 sec in SGF as opposed to the >2 min of stability shown by allergens. On the other hand, as shown in TABLE 2, different time frames were used by others to determine whether a protein is stable to digestion. For example, Noteborn *et al.*⁴ found that after 2 hr in the presence of pepsin, CRYIA(b) yielded a 15-kDa fragment that was successively degraded to smaller fragments after continued treatment with chymotrypsin and trypsin. The authors concluded that the CRYIA(b) protein was degraded rapidly under simulated gastrointestinal conditions. Hashimoto *et al.*⁷ considered the digestion of a modified soybean glycinin expressed in transgenic potato within 10 min as rapid. Momma *et al.*⁸ considered a soybean glycinin expressed in genetically engineered rice to be labile in SGF when the protein was digested within 10 min. With different experimental conditions and time criteria used to assess digestibility, it is difficult to establish a relative digestibility ranking (and thus the estimated allergenic potential) of the various proteins in these studies.

CONCLUDING REMARKS

Available data suggest that stability to digestion may not be a defining characteristic of food allergens. It would be difficult to differentiate food allergens from nonallergenic proteins based on digestive stability.

Nonetheless, it is widely accepted that a protein that is resistant to digestion would have an increased probability of stimulating allergic reactions; *in vitro* digestion assays provide an estimate of the relative integrity of a protein and thus the probability of eliciting the allergic reactions. Consensus needs to be reached, however, about how stable a protein needs to be, as shown by the *in vitro* digestion assays, in order for it to be considered potentially allergenic. There is also a need to establish standardized assay conditions (preferably ones that best relate a protein's integrity and its potential for stimulating im-

mune reactions) to be used among different laboratories so that direct comparisons of results can be made.

The use of digestion stability for protein allergenicity assessment, although widely accepted, has its limitations. It may screen out stable proteins that may not be allergenic, but, more seriously, it may fail to identify allergens that are labile to digestion.²² Furthermore, this criterion may not be relevant for allergens that stimulate allergic reactions through the respiratory route or by skin contact.

The approach currently employed for the assessment of the allergenic potential of novel proteins is based on comparison of the properties of proteins with those of known food allergens without taking into consideration the underlying mechanisms of food allergy. However, there seem to be no common physicochemical properties that truly distinguish food allergens from nonallergenic proteins. Caution needs to be taken when using this approach for protein allergenicity assessment. Further research is needed to develop an understanding of the underlying mechanisms of food allergy. Only then can accurate tests be developed for the assessment of protein allergenicity.

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