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Topic 5: Stability of Known Allergens (Digestive and Heat Stability)

Ricki M. Helm
University of Arkansas for Medical Sciences
Arkansas Children’s Hospital Research Institute
USA

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What makes an innocuous protein an allergen is unknown. There is no absolute set of characteristics that will define a protein as allergenic, i.e., the ability to sensitize with the production of IgE antibody and a subsequent re-exposure resulting in an allergic response. Each novel protein should be assessed on an individual basis and may require surveillance to determine its allergenicity. It is only by exposure and resulting allergic reactions in sensitized individuals that food allergens have been identified and characterized.

Sensitization and the subsequent allergic response to IgE-mediated food allergy is generally identified as a multifactorial process involving a genetic predisposition (the atopic syndrome) combined with environmental factors that results in the generation of allergenic determinants during food processing and/or intestinal digestion. Normally, proteins are readily digested and absorbed leading to oral tolerance; however, either through some intrinsic property of the protein and/or a breakdown in the intestinal epithelial barrier, there is an enhancement of protein-induced antigenicity and allergenicity. IgE-mediated food allergy consists of two phases: sensitization and triggering of the allergic response. The same food or allergen is not required for both steps of the allergic reactions. Symptoms are not specific to the gastrointestinal tract but can also involve the skin and respiratory tract. There is no definite evidence of a threshold, dose-dependent mechanism for the elicitation of food allergy (1). As identified by Wal (1), the questions raised are 1) is it possible to assess or predict an allergenic risk from any characteristic of the protein, e.g., primary, secondary, tertiary structure, function, origin, physico-chemical properties and as yet unidentified characteristics and 2) can a “new” protein produced by modern biotechnology, such as recombinant proteins expressed by genetically modified organisms be intrinsically more allergenic than a “natural” conventional protein.

In discussions of food allergy, the predominant hypothesis is that there is an adverse reaction to exogenous food proteins or food protein fragments, which escape lumen hydrolysis and are thus available for exposure to the gastrointestinal associated lymphoid tissues. The sensitizing food or food constituent is, to some extent, degraded by digestive enzymes in the GI tract, absorbed by the gut mucosa, processed by immunocompetent cells and then presented to the immune system. In a polarized Th2 environment, immunoglobulin class switching to allergen-specific IgE is generated. The mechanisms and what determines this class switch and ultimately an immediate hypersensitive food pathogenesis continues to be a major focus for research investigators. Coincidental to this research problem is a research focus into the characteristics of proteins that will lead to their predictive allergenic potential.

When characteristics of known food allergens are examined, the single most prominent trait attributed to food allergens is protein stability, i.e., heat stability and/or resistance to enzymatic degradation. Therefore, the current predictive methods or the evaluation of protein allergenicity of food allergens has included the detection of intact and/or residual antigenic fragments following artificial digestion procedures as determined by HPLC and SDS-PAGE/immunoblot analysis. Additional means have included immunogenic responses (production of IgE and IgG with subsequent release of allergic immune mediators (histamine, tryptase, ECP and TNF-alpha) from sensitized cells) following animal sensitizations which bypass the natural GI processing. The procedures represent artificial means for evaluating the potential allergenicity of novel proteins.

To assess food allergen stability, for which there is no standardized methodology to prospectively analyze the inherent ability of proteins to act as allergens, simulated gastric and intestinal fluid systems have been devised (SGF and SIF, respectively). These criteria are by no means definitive proof of a protein’s potential allergenicity; however, combined with other factors, they contribute to our best suggestion of allergenicity taking into consideration the plethora of proteins consumed. Only a few proteins (estimated at approximately 200) have been
shown to be true allergens, and the majority meets one or both of these criteria. Continued surveillance and documentation will undoubtedly identify additional proteins as allergens; however, the ability to predict allergenicity of novel proteins must rely on the characteristics of known food allergens.

It is therefore necessary to arrive at standardized methods and techniques that will determine the single most widely accepted trait of food allergens, protein stability. Unfortunately, there has been little advancement in this area from the time Drs. Steve Taylor and Sam Lehrer summarized their findings in 1996 (2). I quote from their article: “Although the assessment of the resistance to hydrolysis of proteins could offer valuable information regarding the potential allergenicity of specific proteins, a rigorous protocol for such experiments has not been established. Ideally, this protocol would mimic digestive proteolysis and include tests on the isolated protein and the protein in the appropriate food matrix. The experience with the evaluation of the immunogenicity of partial whey hydrolysis in animal models dictates that extreme caution be used in evaluation of results obtained from such animal models. The development of further data on the comparative stability of food allergens vs. other food proteins to digestion, proteolysis, and hydrolysis would be highly desirable to determine the ability of this tool for the assessment of the potential allergenicity of specific proteins.” What is relevant is that it is necessary to make the leap from simple artificial systems to confident statements on the enormously complex gastrointestinal tract digestibility of novel proteins, if such methods and assumptions are to be standardized and used. To do this, decision trees need to be validated that will be useful in predicting the allergic potential of novel proteins.

Protein Digestion:

A general consensus has been reached that proteins susceptible to gastrointestinal digestion are inherently safer than those that are stable with respect to allergenicity. In the analysis of digestibility of food allergens and non-food allergen proteins, several investigations will be highlighted. In the first, Astwood et al (3) demonstrated the validity of this concept for known allergens within peanuts, soybean, mustard, egg, and milk relative to common non-food proteins using a simulated gastric fluid as described in The United States Pharmacopoeia (4). Both purified and crude protein extracts were subjected to the following SGF conditions. Total protein was assessed using the method of Bradford adapted to 96-well tissue culture plate analysis (5) using bovine serum albumin to develop a standard curve. SGF contained 0.32% (w/v) pepsin (Sigma) in 0.03 M NaCl, at pH 1.2. Proteolytic activity was estimated by measuring TCA precipitable hemoglobin after hydrolysis for 10 min (6). The proteolytic activity was determined to be 20,100 units ± 449. SGF was performed with 170ng/µl of protein digested in 200µl aliquots placed in 2-ml centrifuge tubes preheated to 37°C. Serial dilutions of pepsin were used to determine the optimal concentration for discrimination of common labile food proteins and important food allergens. Incubations were maintained in a 37°C shaking water bath and quenched by neutralization with 75µl of 150mM Na2CO3 at the following times: 0, 15 and 30 sec; and 1, 2, 4, 8, 15, and 60 min. Seventy microliters of 5x Laemmli SDS-PAGE sample loading buffer was added to the quenched samples followed by heating to 90°C. Digestion of proteins was evaluated by SDS-PAGE using 10-20% precast acrylamide gradient gels with tricine buffers. Five microliters corresponding to 500 ng of protein were loaded for each lane. Proteins were visualized by staining with Coomassie brilliant blue or colloidal gold. Digestion was also evaluated by using immunoblot analysis with monoclonal antibodies to the respective allergen. Gels were electroblotted onto nitrocellulose membranes, blocked in a solution of 3% gelatin in Tween Tris buffered saline (TTBS) for 30 min. For immunodetection, primary antibodies were diluted in TTBS buffer containing 1% gelatin (w/v). Immunoblots were rinsed three times with TTBS and secondary antibody was added. Antigen-antibody conjugates were visualized by the
addition of alkaline-phosphatase-linked goat anti-mouse or goat anti-rabbit IgG secondary antibody (depending on the primary antibody used) diluted 1:7500 in TTBS containing 1% gelatin. Color reaction was determined according to manufacturer’s instructions.

Three general patterns of stability were observed:

1. complete stability for 60 minutes with few or no detectable proteolytic fragments (beta subunit of beta-conglycinin, SKTI, Ara h 2, Bra j IE, Sin a I);
2. intermediate stability (less than 60 min but more than 30 sec) for the whole protein with observed fragments that were stable for additional periods of time (alpha subunit of beta-conglycinin, peanut lectin, and glycamin) and;
3. no stability for the whole protein with stable fragments observed for at least 8 min (Gly m 1 (Gly m Bd 30 kD).

Cow’s milk beta-lactoglobulin was stable for the full 60 min with no apparent fragments. Egg proteins (ovalbumin, ovomucoid, conalbumin, and phosvitin) and other milk proteins (casein, bovine serum albumin) were stable for at least 8 min and up to 60 min. Non-allergen food proteins, including spinach leaf ribulose-bis-phosphate carboxylase/oxygenase, wheat kernel sucrose synthetase, soybean seed lipoxigenase, wheat kernel sucrose synthetase, corn kernel PEP carboxylase, barley kernel beta-amylase, and potato tuber acid phosphatase and phosphofructokinase were all completely digested by 15 sec. In crude extracts to determine the effects of matrix on digestibility, it was estimated that beta-conglycinin represented 18.5% of the soy flour extract. The crude soybean extract did not have any effect on digestion of beta-conglycinin by SGF. It was noted that monoclonal antibodies to the proteins might not have been useful to assess digestibility in SGF. The conditions of digestion may have produced fragments that were not detectable using the monoclonal antibodies.

However, notable exceptions of characterized food allergens to SGF stability include many oral allergy syndrome pollen/fruit/vegetable cross-reactive allergens. These include Bet v 1-related proteins and profilins (7-9) that are heat labile and susceptible to enzyme digestion.

In the second investigation, to address the efficacy of digestibility for cross-reactive food allergens, Yagami et al (10) extracted proteins from natural rubber latex and vegetable foods and dissolved them in both SGF and SIF. In this investigation, sera from allergic patients were used to assess IgE-binding proteins and fragments following digestion procedures. Protein extraction and enrichment of latex proteins and food proteins from avocado, kiwi, banana, potato, melon, and peach were used in the analysis. The BCA Protein Assay Reagent kit (Pierce) determined protein concentration. The SGF procedure for the most part was that used by Astwood in the above citation. Briefly, protein sample (680µg of crude proteins and 34-680µg of separated latex allergens) were dissolved in 200µl of prewarmed SGF containing 0.32 wt/v percentage of pepsin A (Sigma). Digestion was maintained at 37°C with constant rocking and 20ul aliquots were removed at 0.5, 1, 2, 4, 8, 16, and 60 mins. Each aliquot was mixed with a 26µl sample buffer containing 2% 2-ME and 4% SDS together with 6µl Na2CO3 (200mM) for SDS-PAGE analysis. Mixtures were boiled for 5 minutes and stored at -30°C until further analysis. Reducing agents were removed when hevein was the analyte. For the 0 minute sampling, 68µg of crude protein was first dissolved in 26µl sample buffer, mixed with 26µl of SGF that had been inactivated by boiling for 5 minutes after neutralization with Na2CO3. Control experiments included protein samples dissolved in SGF that did not contain pepsin A. Milk lactoglobulin and soybean trypsin inhibitor was used to assess the food allergen activity of SGF and soybean lipoxygenase and potato acid phosphatases were selected as representative non-allergen protein sources.
For digestibility by SIF, crude vegetable food proteins (340µg) were dissolved in 130µl of US Pharmacopoeia solutions containing 1.0 wt/vol percentage of pancreatin and incubated at 37°C with constant rocking. Twenty-six microliter aliquots were removed at 0.25, 1, 4, and 16 hours, quickly mixed with an equal volume of sample buffer and boiled for 5 mins. Controls were treated in the same fashion as described for SGF.

The digestibility of proteins (7.65µl samples) was analyzed by Coomassie blue stained 12.5% acrylamide SDS-PAGE gels and immunoblotting using sera from either individuals with latex allergy or individuals diagnosed with OAS. Both representative nonallergenic food allergens were digested within 30 seconds in SGF whereas the two food allergens were not completely digested within 60 minutes coinciding with findings by Astwood. Although most latex proteins were digested by SGF within 4 minutes, a 28kD allergen and hevein (Hev b 6.02, 4.7kD) were stable and not completely digested within 1 hour when analyzed in crude extracts. Hevein, irrespective of its purity, was shown to be digestible when SDS-PAGE was carried out under reducing conditions. Most cross-reactive allergens from fruits were digested within 4 minutes. In contrast, potato proteins were stable in SGF with several bands (10, 20, 24kD) clearly detectable even after 1-hour of SGF digestion. Astwood (personal communication) has confirmed this data; however, the potato allergens are not regarded to be clinically relevant. In SIF, the cross-reactive allergens were gradually degraded with some persisting for greater than 16 hours.

The authors suggest that criteria established by Aalberse (11) be considered when determining food allergens. Food proteins having both the ability to sensitize and the ability to induce symptoms should be termed complete food allergens. While food proteins that can perorally induce allergic symptoms, even though they are digestible and cannot perorally sensitize individuals, should be termed incomplete food allergens. Irrespective of this criterion, the authors note that their results implied that SGF was not an effective criterion for determining predicting allergenicity of food proteins. As pointed out by the authors, caution must be considered in that indigestibility is not a prerequisite for food protein to induce allergic symptoms and caution must be taken in these determinations. Evidence substantiating these claims include those individuals presensitized by inhaling birch pollen allergens, such as Bet v 1 and birch profilin, often experience OAS to various vegetable foods containing Bet v 1-related proteins or profilin, which have been shown to be labile to both heat and enzymatic digestion. Care must be taken in considering threshold sensitization and elicitation processes of different food proteins. One must also consider new evidence that many OAS allergens are now being identified that contribute to systemic allergic reactions. The disease and allergen source material may need to be reclassified.

In considering protein digestion, it may be relevant to establish a criterion that is more physiological in nature than the USP designed protocols. Remarks made to Dr. Barbara Henry by Dr. Jerry D. Gardner, regarding the digestibility of Cry9c may be significant. Dr. Gardner exhaustively cites Hasler (12). The following information was provided using this textbook:

1) Gastric emptying is not usually discussed in terms of transit time, but instead, in terms of the time for 50% of the ingested food or liquid to pass from the stomach.

2) Gastric emptying is usually measured after ingesting isotopically labeled food or liquid and measuring disappearance of the isotope from the stomach with an external detection device or breath test.

3) These measurements indicate that in normally healthy subjects, the T$_{1/2}$ for solid emptying is about 2 hours and for liquid emptying is 1-1.5 hours.
4) Most individuals with disordered gastric emptying have delayed gastric emptying.

5) It is also important to remember that most digestion of ingested protein occurs in the upper small intestine as a result of enzymes secreted from the pancreas.

6) The digestion of protein that occurs in the stomach is often referred to as the initiation of digestion.

7) Thus, digestion by pepsin will usually be accompanied by more extensive digestion by pancreatic enzymes.

Dr. Gardner further commented that measured gastric pH using pH-sensitive electrodes in 24 healthy subjects, the gastric pH at 3:00 A.M. was $0.77 \pm 0.04$ and at 8:00 A.M. $1.33 \pm 0.10$. These results were considered representative of similar results obtained from over 100 healthy subjects measuring gastric pH on aspirated gastric fluid or with a pH-sensitive electrode.

In an animal/mathematical model of digestion of protein (13), simulated digestion results were consistent with observed data. A pig model was used to integrate current knowledge on the transit of digesta along the small intestine, endogenous secretions, digestion of proteins, and absorption of amino acids into a mechanistic representation of digestion. Sensitivity analysis showed that parameters of protein hydrolysis largely determined protein digestibility. It further showed that amino acid absorption could be limiting to protein digestibility when large amounts of protein are eaten in a single daily meal. This may relate to exposure levels of digestible as well as non-digestible proteins. It was suggested that the model could be useful in evaluating protein digestion of different feedstuffs and feeding strategies; however, validation would be required for determining these simulation results.

A gastrointestinal model (TIM) has been developed that simulates to a high degree the physiology of the stomach and small intestine of monogastric animals and man (14). The model could be useful to study the digestibility of ingested components that very closely resembles that of the stomach and small intestine. Simulation of the following successive kinetic physiological events takes place: temperature, pH, saliva, gastric and intestinal secretions (electrolytes, enzymes, co-factors, bile, and pancreatic juice); gastric and intestinal mixing. The model was developed as an alternative for human and animal experiments and validated successfully in comparison to in vivo experiments with human volunteers and fistulated pigs and calves for the digestion of proteins.

Research efforts reported by Dr. T-J Fu at the recent symposium on “Assessment of the Potential Allergenicity of Genetically Modified Food” by the National Center for Food Safety and Technology were undertaken to compare the digestion stability of food allergens and non-allergenic proteins of similar cellular functions in SGF and SIF. The study was conducted primarily with plant proteins including storage proteins (7S and trypsin inhibitors), plant lectins, contractile proteins (tropomyosin) and enzymes. The test proteins (0.17 mg/ml) were reacted with 3.2 mg/ml pepsin in 0.03 M NaCl, pH 1.2 at 37°C for varying periods (pepsin/test protein – 640µg/34µg). At each time interval, 75µl of 0.6 M Na₂CO₃ was added to neutralize the reaction prior to mixing with 70µl of 5x Laemmli sample buffer. The samples were boiled and 5µl samples electrophoresed in 10-20% acrylamide minigels in a tricine buffer and visualized by Coomassie brilliant blue staining. The results of both SGF and SIF suggested a wide range of stabilities were exhibited among both food allergens and non-allergenic proteins. Many proteins with unproven allergenicity exhibited high stability; however, proteins belonging to the same protein family appeared to exhibit similar stability to digestion in SGF, SIF and acids irrespective of their allergenicity.
Discussions during food allergenicity symposium and ISLI/HESI meetings led to the following conclusions:

1- Research is needed to validate the criterion that stability to digestion in simulated human gastrointestinal systems is prerequisite for food protein allergenicity.

2- A consensus needs to be reached on standardized protocols for SGF and SIF and what emphasis should be placed on the results.

3- Additional research is needed to characterize food allergens to determine specific characteristics that can be used to assess potential allergenicity of novel proteins.

The working committee on the “Characteristics of Protein Food Allergens” held by ISLI/HESI following the symposium established that the following criteria be taken into consideration.

1- Protein Stability

- Disulfide bonds and their contribution to 3-D structure
- Glycosylation: contribution to 3-D structure
- Heat stability: temperature and duration need to be standardized. Heating at 90°C for 5 min was recommended.
- Enzyme resistance:
  - SGF: complete pepsin digestion at pH 1.0 – 2.0 for minimal of 5 minutes.
    (Intact protein and fragments should be analyzed by SDS-PAGE and immunological endpoints (polyclonal and monoclonal inhibition assays)
  - SIF: No discussion

  Consideration should be given to protein families (e.g., different albumins) when stability is considered.

2- Abundance: A consideration should be given to dry weight or protein of the major allergen in the food source. Exposure levels were considered to be relevant to the exposed populations and should be uniformly expressed in some unitage.

3- Delivery: Consideration should be given to how the material will be introduced into the diet. Assessment of allergenicity should be based on the matrix/matrices that the novel protein would be introduced into the diet.

For protein digestibility studies, the following considerations were identified:

1- The pepsin source and ratio of pepsin/protein needs to be standardized

2- The SDS-PAGE gel system should be standardized for analysis of digestion with respect to conditions and limits of detection relative to molecular size.

3- The staining methods should be standardized: Coomassie brilliant blue, silver stain, or colloidal gold.

4- Interpretation: molecular size estimates of fragments that would be acceptable in the diet.

5- Purified proteins from both recombinant and plant source material should be assessed.

6- Should the plant protein matrix be assessed? Using what methods?
7- Should the diet and physiological issues of eating a meal be considered?

Other topics at the meetings deemed important for consideration included exposure, allergen characterization, dose, sequence and problems associated with the GI tract that may relate to allergenicity. A brief discussion of each follows.

**Allergen Characterization**

Allergens must contain B-cell epitopes to which immunoglobulin E can bind, and T-cell epitopes capable of inducing type 2 T-lymphocyte responses. Although important to clinical responses, T- and B-cell epitopes alone are insufficient to endow a protein with allergenic potential. Careful consideration should be given to resistance to proteolysis, post-translational glycosylation, and function, e.g., enzymatic activity, PR-proteins and contractile proteins (protein families). Relative stability in SGF and SIF often correlate with allergenic activity. Post-translational modification appears to enhance allergenicity by increasing uptake and detection by the immune system. Functional activity, in particular enzyme activity, may also enhance allergenicity by non-specific activation of cells participating in the immunologic response, e.g., inflammation. A similar circumstance may be attributed to lectins, superantigens, and antigen-antibody complexes. By better defining the limits within which these factors operate, we may be in a better position to identify and characterize the hazards and risks of allergic disease associated with novel protein (15).

**Exposure and Dose**

Prevalence, exposure and dose of the suspected food source were regarded to be important factors that should be considered when attempting to assign threshold levels for sensitivity and reactivity for labeling of potential food sources. For example, Martens (16) focused on the degradation half-life of the protein and the ILSI decision tree stating the following: “When a protein is quickly digested (e.g. degradation half-time of less than 10s) and is completely deactivated by processing temperatures then the GM food containing the protein should not be labeled as allergenic. However, no limits or declarations of what the prevalence, dose or exposure was recommended. Some informative data exists in the literature that may be useful in these determinations with respect to an allergic response. Very little information is available for threshold levels involving food allergen sensitization.

A milk-allergic 3-year-old boy experienced symptoms of an allergic response including vomiting after ingestion of 4-6 oz (ca. 113.4-170.1g) of lemon sorbet. In analyzing the sorbet, trace amounts of milk allergens were identified. The authors concluded that <200 micrograms of whey proteins could elicit systemic reactions in milk-allergic individuals (17).

In open challenges with lyophilized ovalbumin, 10 mg was required to elicit symptoms in children (18). For fish, 6 mg induced DBPCFC (19). Peanuts have received considerable interest because of the frequency of anaphylactic episodes associated with their ingestion. Hourihane et al established levels of peanut allergen from 0.1 to 1 mg that caused significant symptoms during DBPCFC’s (20). In oral provocation studies, 25% of peanut allergic cases responded to 100mg of peanut seeds, a threshold considerably lower than that for egg challenges (21). In peanut challenges, highly sensitive individuals are not usually challenged because of risk. Hong et al (22) submitted raw peanut extract to pepsin digestion and demonstrated that IgE binding by immunoblots analysis was eliminated. The digest did induce T-cell proliferation suggesting that fragments maintained their immunogenicity. Immunogenicity may not be relative to allergenicity. All allergens are antigens but not all antigens are allergens, so caution must be taken when considering when proteins or protein fragments are considered to be immunogenic.
In a review of the stability and allergenicity of allergens from animal sources, Besler et al. (23) concluded that cow’s milk, hen’s egg, fish and crustaceae allergens are stable to heat treatment and enzymatic hydrolysis with meat allergens being partially stable to heat treatment and susceptible to enzymatic hydrolysis. In this review, positive challenges with the milk elucidating symptoms in DBPCFC ranged from 5g – 250g; egg, 5mg – 5 g egg protein; fish, 6mg – 6.7g; and crustaceae, 4 – 6 g. Overall, threshold levels eliciting symptoms form DBPCFC ranged from 1mg to 250 grams. Threshold levels were reported to be strongly dependent on the patient’s individual susceptibility and the allergenic potency of the particular food. Although several allergens of animal origin have been identified and characterized, it is important to note that not all allergens have been carefully characterized, nor has their stability and allergenicity been determined in processed foods under standardized conditions.

The oral dose of beta-lactoglobulin causing sensitization has been estimated to be between 1 ng and several milligrams (24).

Protein Families:

The sequence identity of lipocalin is often less than 20%, but they contain between one and three structurally conserved regions and their 3-D structures are similar. Lipocalins share common biological functions, predominantly related to the transport of small hydrophobic molecules, such as vitamins and pheromones.

Summary:

In reviewing the literature and attending several meetings regarding the prediction of novel proteins as potential allergens, there is abundant confusion on what protein characteristics contribute to make a novel protein an allergen. What makes a protein an allergen is not known. Our knowledge about food allergens is based solely on the characteristics of known food allergens that have been identified, isolated and characterized following an allergic response.

Ruling out all other common characteristics between normal dietary proteins and food allergens, the characteristic that best describes known food allergens can be limited primarily to protein stability, i.e., heat stability and resistance to enzymatic degradation. Other factors, both known and unknown may contribute to an individual’s recognition of a protein as an allergen. However, at present, protein stability is the only reliable characteristic that is being used to characterize known food allergens from normal dietary proteins. As established by the review, this is not a guarantee that a novel protein will present as an allergen. There exist several food proteins that are both heat labile and easily digested by enzymatic treatment that cause allergic reactions. Each novel protein will have to be individually assessed using the best available criteria available.

Recommendations:

In order to use protein stability as a reliable criterion in predicting a novel protein as a potential allergen, standardized methods should be followed that can be repeated in any laboratory. To assist in this standardization, the following recommendations should be taken into consideration. These are by no means the only criteria in the decision tree that will be useful in predicting the potential of a novel protein of being an allergen, but the methodology and interpretation should be succinct.

Heat Stability: The definition of heat stability should be standardized using the following criteria.

1-Heat treatment of the novel protein, native and recombinant, should be for 5 minutes at 90°C.
2-Assessment of stability by a combination of molecular sieving using HPLC and standardized SDS-PAGE analysis (both native and denaturing/reducing gels). See SDS-PAGE protocol below.

**Enzymatic Digestion:** The definition of enzymatic digestion should be standardized using the following criteria. The SGF protocol should be used in preference to SIF; however, recommendations will be given for both protocols. In both assays, the following limitations are recognized. Most proteins will pass through the stomach undigested. Foods act as natural antacids altering the pH of the stomach acidity. Acidity of the stomach may alter food characteristics, e.g., curdling of milk. Enzyme sources have inherent variability. Denaturing conditions will change the pH optimum for digestion. Digestion starts in the oral cavity with amylase activity, followed by initiation in the stomach and complete digestion in the small intestine. Many proteins and fragments of digested proteins can pass through the mucosal epithelium.

**Simulated gastric fluid (SGF)**

1-Standardized source materials and pH ranges.

   a. Pepsin should be from a reliable source and enzymatic activity should be expressed in arbitrary units prior to assessment of novel protein degradation. For this, the method used by Ryle (6) could be applied, i.e., enzymatic activity based upon measuring TCA precipitable hemoglobin after hydrolysis for 10 min.

   b. A standardized enzyme/protein ratio should be established.

   c. Bovine serum albumin should be used as a digestible protein.

   d. Peanut allergens (and/or a stable protein readily available in pure form) should be used as a non-digestible protein.

   e. The novel protein should be assessed in enriched or pure form, both recombinant and natural sources. If the matrix is to be assessed, assessment should be from both the natural and transgenic form.

   f. The effects of pH determinations should be made at 1.0, 1.5, 2.0, 4.0 and 6.0 due to the pH variation in the stomach following a meal.

   g. Sampling of digestion should be taken at the following time points, 0, 15, and 30 seconds; and 1, 2, 4, 8, 15 and 60 minutes.

   h. A scale in arbitrary units should be established using the digestible and non-digestible proteins to characterize the novel protein.

   i. Reasonable criteria of digestibility for acceptance should be determined. (This could be based upon the data being collected by members testing the protocol recommended by the ILSI/HESI working subgroup).

   j. All analyses should be made at 37°C.

2-Standardized analytical methods for determining degree of degradation.

   a. Column chromatography (e.g., HPLC) should be used to assess the degree of degradation.

   b. SDS-PAGE analysis, both denaturing and non-denaturing conditions, should be standardized according to the following criteria.

      i. A common gel system should be used, e.g., Novex system.

      ii. 10-20% acrylamide gradient gels.
iii. A sensitive staining method should be used (Silver stain or colloidal gold)

c. Immunoblot analysis.

i. A standardized blotting system should be used, e.g., Novex system.

ii. Both polyclonal and monoclonal antibody assessments should used to determine degree of degradation.

d. Data should be provided in a publishable format.

Simulated intestinal fluid (SGF) This assay should only be used if there are considerable amounts of undegraded or protein fragments identified in the SGF. A gastroenterologist should be consulted for best physiologic conditions. Pancreatin sources are too variable, therefore a standardized mixture of enzymes should be used.

1-A minimal composition to that of physiological state, i.e., pancreatic drainage fluid of animal to enzyme mixtures in test sample, should be used.

   a. Homogenous sources of trypsin/amylase/lipase/elastase/chymotrypsin are recommended from reliable sources (Worthington). (This will be difficult to manage, as sources may be limited and purity questionable).

2- Standards and conditions for SGF should be applied.

References:


