

# Stability of food allergens to digestion in vitro

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**An integral part of the safety assessment of genetically modified plants is consideration of possible human health effects, especially food allergy. Prospective testing for allergenicity of proteins obtained from sources with no prior history of causing allergy has been difficult because of the absence of valid methods and models. Food allergens may share physicochemical properties that distinguish them from nonallergens, properties that may be used as a tool to predict the inherent allergenicity of proteins newly introduced into the food supply by genetic engineering. One candidate property is stability to digestion. We have systematically evaluated the stability of food allergens that are active via the gastrointestinal tract in a simple model of gastric digestion, emphasizing the major allergens of plant-derived foods such as legumes (peanuts and soybean). Important food allergens were stable to digestion in the gastric model (simulated gastric fluid). For example, soybean  $\beta$ -conglycinin was stable for 60 min. In contrast, nonallergenic food proteins, such as spinach ribulose bis-phosphate carboxylase/oxygenase, were digested in simulated gastric fluid within 15 sec. The data are consistent with the hypothesis that food allergens must exhibit sufficient gastric stability to reach the intestinal mucosa where absorption and sensitization (development of atopy) can occur. Thus, the stability to digestion is a significant and valid parameter that distinguishes food allergens from nonallergens.**

Keywords: allergens, food, safety, stability, digestion

Foods derived from genetically modified plants are entering the marketplace. Extensive assessment of potential human health and ecological effects are routinely performed to ensure the safety of these new products<sup>1,2</sup>. A significant challenge in food safety assessment is to devise a system and methodology to assess the impact that plant genetic engineering may have on food allergy. Preventing unwanted allergens from entering the food supply via transgenic plants depends on commonsense principles. Generally, the transfer of known allergens should be avoided, regardless of the technology used to introgress new genes into crops. Safety assessment necessarily focuses on the protein products of the gene(s) transferred into the food crop. In cases where genes are obtained from allergenic sources, such as peanuts, it should be assumed that the genes encode allergens unless clear scientific evidence establishes that the genes do not encode allergens. Genes from allergenic sources that obviously encode allergens can readily be identified by antigen-binding by IgEs of allergic patient serum, radioallergosorbent tests, and other clinical procedures. In cases where allergens or suspected allergens are transferred into plants, consumers must be informed of the existence of the allergen by appropriate labelling as suggested by the United States Food and Drug Administration (FDA)<sup>3</sup>. Indeed, Nordlee and coworkers<sup>4</sup> recently demonstrated the capability of these methods by demonstrating that a major Brazil nut allergen could easily be detected in soybean seeds engineered to contain the gene encoding the allergen. However, genes obtained from organisms not known to cause allergy, and genes that have no amino acid sequence similarity to known allergens, are more difficult to assess because there is no accepted method to prospectively test for inherent protein allergenicity<sup>5</sup>. For these proteins, a range of tests that compare their physicochemical properties to extant food allergens is appropriate.

Food allergens tend to be abundant in the food (e.g., ovalbumin represents 54% of egg white protein), resistant to food processing

(i.e., food allergens retain allergenicity after processing such as cooking), and stable to digestion by the gastrointestinal tract<sup>3-7</sup>. Therefore, proteins expressed in genetically modified plants are analyzed for these factors. Usually in vitro testing is performed to determine whether introduced proteins are stable to digestion under simulated human gastrointestinal conditions on the assumption that digestive stability is undesirable<sup>1,2,8,9</sup>. We propose that the ability of food allergens to reach the intestinal mucosa is a prerequisite to allergenicity. This ability necessarily implies survival of gastric digestion by pepsin secreted into the stomach. A protein that is stable to the proteolytic and acidic conditions of the digestive tract has an increased probability of reaching the intestinal mucosa where absorption can occur.

Soybeans and peanuts account for a large proportion of food allergies, representing a significant risk of fatal anaphylaxis<sup>10</sup>, and are the predominant sources of ingested allergens from plants in the United States<sup>6</sup>. Soybean allergy is of particular concern for children. Anaphylaxis to mustard spice also occurs, at times with fatal consequences<sup>11</sup>. The major allergens of soybean seeds appear to be the 11S globulins (mostly glycinin), the 7S globulins ( $\beta$ -conglycinin), and the oil-body associated Gly m BD 30K (Gly m 1)<sup>12-14</sup>. Soybean seeds also contain minor allergens that include a lectin and Kunitz trypsin inhibitor (SKTI)<sup>15</sup>. Characterized food allergens from peanuts include the 65-kD Ara h1 and the 17-kD Ara h2 (refs. 16, 17), while peanut agglutinin (lectin) is a minor allergen<sup>15</sup>. Both yellow and white mustard species contain allergenic 2S albumins: Sin a I from yellow mustard and Bra j IE from white mustard<sup>18,19</sup>. Other studies have suggested that the allergenicity of  $\beta$ -lactoglobulin (BLG), an important allergen in cow's milk allergy, may be due to its stability to digestion by the gastrointestinal tract<sup>20</sup>. Specifically, pepsin does not reduce the IgE reactivity of BLG<sup>21,22</sup>. Collectively, the importance of these food allergens and their high degree of characterization (including cloning, sequenc-

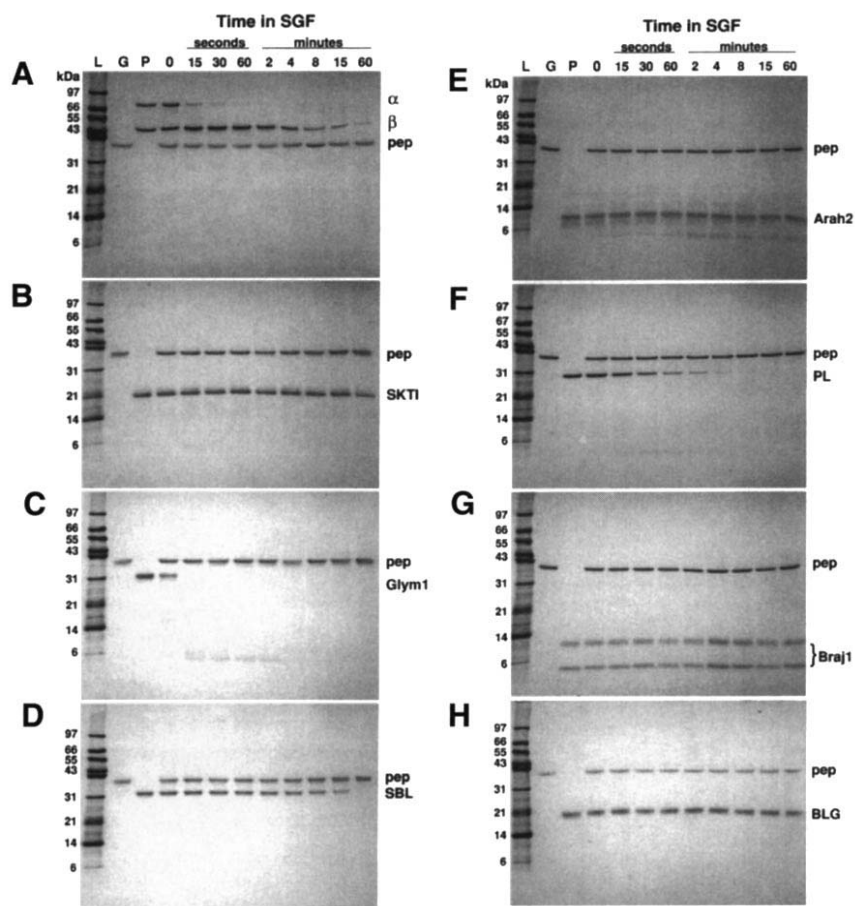


**Figure 1.** Analysis of food allergens in SGF. Tricine SDS-PAGE and Coomassie brilliant blue staining (panels A–H) were used to assess digestion of allergens. Molecular weight markers (lane L) are indicated on the left-hand side of the gels. SGF and allergen proteins were run alone (lane G and lane P, respectively). Allergens were incubated in SGF for the times indicated above the gels. The pepsin in SGF was visible and labelled “pep” for all gels. Individual allergens are labelled on the right-hand side as follows: (A) Soybean  $\beta$ -conglycinin subunits ( $\alpha$  and  $\beta$ ), (B) Soybean SKTI, (C) Soybean Gly m BD 30K (Gly m 1), (D) Soybean lectin (SBL), (E) Peanut Ara h2 (Ara h2), (F) Peanut lectin (PL), (G) Mustard Bra j 1E (Bra j 1), and (H) Milk  $\beta$ -lactoglobulin (BLG). Five microliters of digestion samples (corresponding to 500 ng of protein) were loaded for each lane.

ing, and identification of immunologic epitopes) made them ideal candidates for this comparative study.

We report the use of simulated gastric fluid<sup>23</sup> as a model of stomach digestion and systematically compare the stability of major food allergens to nonallergenic food proteins, especially those of plant origin such as soybean, peanut, and mustard. BLG was chosen as an initial control for stability in the model of gastric digestion. Other significant milk (casein, BSA) and egg (ovalbumin, ovomucoid, conalbumin, and phosvitin) allergens were also included in this assessment.

## Results

**Digestion of proteins in simulated gastric fluid.** Purified food allergens were evaluated individually for digestibility in simulated gastric fluid (SGF)<sup>23</sup>. Since SGF incubations for specific proteins were performed on different days, the proteolytic activity of each batch of SGF was estimated using a standard assay<sup>24</sup>. The proteolytic activity of SGF was 20,100 units  $\pm$  standard error (SE) = 449. For each protein, stability in SGF was defined as the last time point for which the protein or its proteolytic fragments could be detected by tricine SDS-PAGE and staining. The tricine SDS-PAGE system

was used to enable detection of proteolytic fragments of approximately 1000 daltons or larger relative molecular weight<sup>25</sup>.

Food allergens were not rapidly digested in SGF (Fig. 1). Three general patterns of stability were observed: (1) complete stability for 60 min with few or no detectable proteolytic fragments (e.g.,  $\beta$ -subunit of  $\beta$ -conglycinin, SKTI, Ara h2, Bra j 1E, and 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 (e.g.,  $\alpha$ -subunit of  $\beta$ -conglycinin, peanut lectin, and glycinin) and; (3) no stability for the whole protein with stable fragments observed for at least 8 min (e.g., Gly m 1).

Cow's milk allergen BLG was stable in SGF for the full 60 min, with no apparent fragments (Fig. 1). Similarly, all of the other egg and milk allergens (or their proteolytic fragments) were stable in SGF for at least 8 min and several were stable for 60 min, the last time point for which samples were analyzed (Table 1).

A variety of proteins obtained from typical plant food sources (spinach leaf ribulose-bisphosphate carboxylase/oxygenase and glycolate reductase, soybean seed lipoxygenase, wheat kernel sucrose synthetase, corn kernel PEP carboxylase, barley kernel  $\beta$ -amylase, and potato tuber acid phosphatase and phosphofructokinase) were evaluated for digestibility in SGF. In contrast to food allergens, these plant food proteins were completely digested by the first time point of 15 sec, with one (glycolate reductase) digested by the second time point of 30 sec (Fig. 2).

## Effect of food matrices on stability time in SGF.

To assess whether a food matrix affected the digestion rate, a crude soybean flour extract (CSE) was prepared and incubated in SGF. The digestion of  $\beta$ -conglycinin, a major protein component of soybean seeds, was monitored by immunoblots using anti- $\beta$ -conglycinin polyclonal antibody and compared to the digestion of purified  $\beta$ -conglycinin, also monitored by immunoblot. A similar amount of total protein was incubated in SGF for each case. For a valid comparison, the amount of purified  $\beta$ -conglycinin protein loaded on SDS-PAGE was adjusted to be comparable to the  $\beta$ -conglycinin present in CSE. The amount of  $\beta$ -conglycinin in CSE was assessed by comparison to known quantities of  $\beta$ -conglycinin on immunoblots. The proportion of  $\beta$ -conglycinin in the soy flour extract was estimated to be 18.5%, in good agreement with published reports<sup>26</sup>. The  $\alpha$ -subunit of  $\beta$ -conglycinin was stable for 120 sec independent of whether it was assayed as a purified protein or assayed as a component of CSE (Fig. 3). Likewise, the  $\beta$ -subunit was stable for 60 min in both cases. The CSE food matrix had no effect on digestion of protein by SGF.

**Characterization of SGF model: pepsin dilution effects.** To establish whether pepsin activity was limiting and whether pepsin activity could be saturated by the test protein, pepsin concentration in SGF was varied while holding all other SGF components constant. Rubisco and SKTI were selected to characterize the effect of pepsin concentration on SGF proteolytic activity because Rubisco and SKTI represented the extremes in protein stability, where Rubisco was extremely unstable and SKTI was extremely stable. Table 2 shows the effect of various concentrations of pepsin on digestion characteristics of SGF. By reducing the concentration

**Table 1. Summary of allergen and protein stability in SGF.**

| Protein                                   | Stability (min) |           |
|-------------------------------------------|-----------------|-----------|
|                                           | Whole Protein   | Fragments |
| <b>Egg allergens</b>                      |                 |           |
| Ovalbumin                                 | 60              | —         |
| Phosvitin                                 | 60              | —         |
| Ovomucoid                                 | 8               | —         |
| Conalbumin                                | 0               | 15        |
| <b>Milk allergens</b>                     |                 |           |
| $\beta$ -lactoglobulin                    | 60              | —         |
| Casein                                    | 2               | 15        |
| BSA                                       | 0.5             | 15        |
| <b>Soybean allergens</b>                  |                 |           |
| $\beta$ -conglycinin ( $\beta$ -subunit)  | 60              | —         |
| SKTI                                      | 60              | —         |
| Soy lectin                                | 15              | —         |
| $\beta$ -conglycinin ( $\alpha$ -subunit) | 2               | 60        |
| Gly m 1                                   | 0.5             | 8         |
| <b>Mustard allergens</b>                  |                 |           |
| Sin a 1                                   | 60              | —         |
| Bra j IE                                  | 60              | —         |
| <b>Peanut allergens</b>                   |                 |           |
| Ara h2                                    | 60              | —         |
| Peanut lectin                             | 8               | —         |
| <b>Common plant proteins</b>              |                 |           |
| Glycolate reductase (spinach leaf)        | 0.25 (15 sec)   | —         |
| Rubisco LSU (spinach leaf)                | 0 (<15 sec)     | —         |
| Rubisco SSU (spinach leaf)                | 0 (<15 sec)     | —         |
| Lipoxygenase (soybean seed)               | 0 (<15 sec)     | —         |
| PEP carboxylase (corn kernel)             | 0 (<15 sec)     | —         |
| Sucrose synthetase (wheat kernel)         | 0 (<15 sec)     | —         |
| $\beta$ -amylase (barley kernel)          | 0 (<15 sec)     | —         |
| Acid phosphatase (potato tuber)           | 0 (<15 sec)     | —         |
| Phosphofructokinase (potato tuber)        | 0 (<15 sec)     | —         |

**Table 2. Effect of pepsin concentration on SGF proteolytic activity.**

| Pepsin Concentration | Protein | Stability (min) |                  |
|----------------------|---------|-----------------|------------------|
|                      |         | Whole Protein   | Fragments        |
| 0.01X                | Rubisco | 60              | none detected    |
| 0.1X                 |         | 0               | 4 (3 fragments)  |
| 0.2X                 |         | 0               | 2 (2 fragments)  |
| 0.25X                |         | 0               | 1 (2 fragments)  |
| 0.5X                 |         | 0               | 0.5 (1 fragment) |
| 1X                   | SKTI    | 0               | 0                |
| 1X                   |         | 60              | none detected    |
| 5X                   |         | 60              | "                |
| 10X                  |         | 60              | "                |
| 50X                  |         | 60              | "                |
| 100X                 |         | 60              | "                |

of pepsin in SGF twofold, fragments of Rubisco were observed. Increasing the pepsin concentration by as much as 100-fold did not reduce the stability of SKTI. Pepsin concentration in 1X SGF was optimal for distinguishing the unstable plant protein Rubisco from stable food allergens like SKTI.

**Digestion in SGF assayed by immunoblots using monoclonal antibodies.** To further characterize the SGF system, the utility of using monoclonal antibodies to assess protein digestion was evaluated. Crude peanut extract (CPE) was incubated in SGF, and the digestion of Ara h1 was assayed by immunoblot using monoclonal antibody to Ara h1. The immunoblot detecting Ara h1 in CPE shows that Ara h1 lost immunoreactivity by 15 sec (Fig. 4). Stained SDS-PAGE gels of CPE incubated in SGF shows that a number of proteins (or fragments) were actually stable for 60 min (data not shown). It was suspected that the monoclonal antibody was not capable of identifying proteolytic fragments of Ara h1. To test whether monoclonal antibodies could be used to monitor stability in SGF, anti-Gly m 1 (monoclonal F5) was used to immunodetect Gly m 1 in crude soybean extract. Although SDS-PAGE showed that stable fragments of the purified Gly m 1 protein were gener-

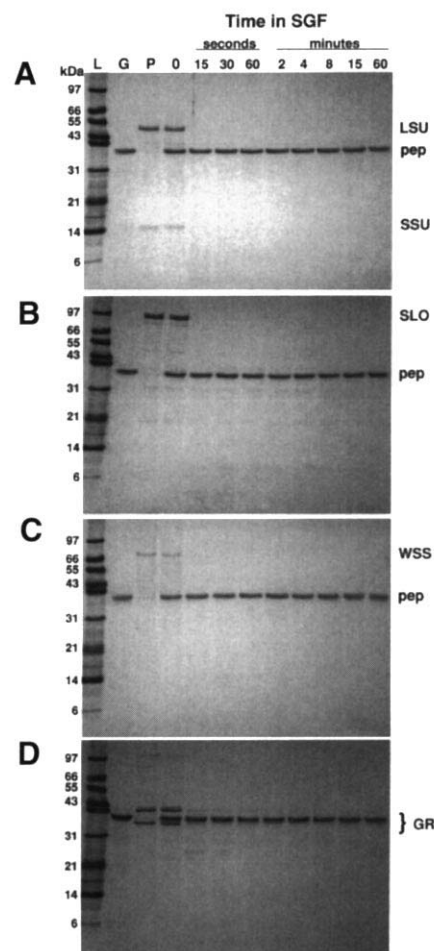


**Figure 2. Tricine SDS-PAGE and Coomassie brilliant blue staining of nonallergenic plant proteins in SGF.** Molecular weight markers (lane L) are indicated on the left-hand side of the gels. SGF and allergen proteins were run alone (lane G and lane P, respectively). Proteins were digested in SGF for the times indicated above the gels. The pepsin in SGF was visible and labelled "pep" for all gels. Individual proteins are labelled on the right-hand side of each panel as follows: (A) Spinach leaf Rubisco large subunit (LSU) and small subunit (SSU), (B) Soybean seed lipoxygenase (SLO), (C) Wheat seed sucrose synthetase (WSS), and (D) Spinach leaf glycolate reductase (GR). Five microliters of digestion samples (corresponding to 500 ng of protein) were loaded for each lane.

ated in SGF (Fig. 1), monoclonal F5 failed to detect these proteolytic fragments in immunoblots of CSE digested in SGF (Fig. 4). Monoclonal antibodies failed to detect fragments of Ara h1 and Gly m 1 produced by digestion in SGF.

## Discussion

Protein digestibility has been routinely evaluated as part of the safety assessment of proteins encoded by genes transferred into plants and subsequently used for food<sup>1,2,8,9</sup>. It has been assumed that proteins that are susceptible to gastrointestinal digestion are inherently safer than those that are stable, especially in relation to allergy. To validate this concept for allergens, we have evaluated the digestive stability of the major peanut, soybean, mustard, egg, and milk allergens relative to common nonallergen food proteins in the standard simulated gastric fluid described in *The United States Pharmacopeia*<sup>23</sup>.

In vitro models represent appropriate tools to evaluate digestibility of proteins, and have been widely employed. Legumes specifically have been examined using in vitro models. It has been suggested that the low nutritional value of legumes is due to the low digestibility of legume seeds<sup>27</sup>. For example, phaseolin, the 7S

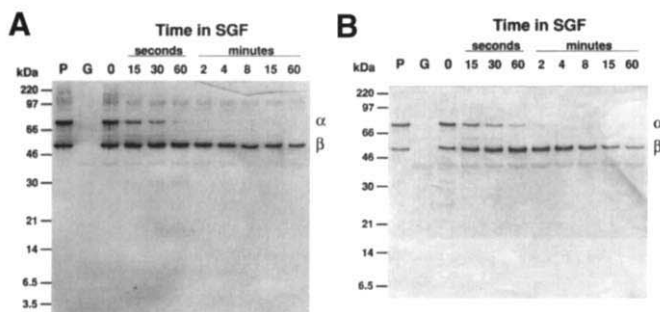


**Figure 3. Matrix effects on digestion of soybean  $\beta$ -conglycinin in SGF.** Proteins run in tricine SDS-PAGE gels were blotted onto nitrocellulose, detected with anti- $\beta$ -conglycinin, and visualized with goat anti-rabbit alkaline phosphatase-linked antibodies. Molecular weight markers are indicated on the left-hand side of the blots. SGF and allergens were run alone (lane G and lane P, respectively). Proteins were incubated in SGF for the times indicated above the blots. Individual proteins are labelled on the right-hand side as follows: (A) 95 ng of purified  $\beta$ -conglycinin subunits ( $\alpha$  and  $\beta$ ), and (B) Detection of  $\beta$ -conglycinin subunits ( $\alpha$  and  $\beta$ ) in 500 ng of crude soybean extract.

globulin storage protein of navy bean (*Phaseolus vulgaris*) and a homologue of soybean  $\beta$ -conglycinin, has been shown to be resistant to proteolysis by both pepsin and trypsin *in vitro*<sup>28</sup>. Similar models have been used to examine the stability of milk allergens<sup>21,22</sup>.

Under the conditions described for SGF in this study, all food allergens were considerably more resistant to pepsin hydrolysis than were common plant proteins. To our knowledge, this study is the first to systematically compare the digestive stability of food allergens to other food proteins in a highly controlled and reproducible system. SGF was further characterized by examining the effect of a food matrix on protein stability. Crude soybean extract, a typical food matrix, did not affect the observed stability of either subunit of  $\beta$ -conglycinin (a major component of soybean flour) in SGF. In addition, increasing the concentration of pepsin 100-fold in SGF did not increase the lability of SKTI, a highly stable protein in SGF. Decreasing the pepsin concentration of SGF by twofold or more leads to one or more observed fragments of labile proteins like Rubisco, and decreasing pepsin concentration by as much as 100-fold leads to complete stability of Rubisco. This experiment established that pepsin concentration was not limiting, and that SGF is a valid tool to measure the relative stability to gastric digestion, be they allergens or not. The pepsin concentration we used in SGF was optimal for discrimination of common labile food proteins and important food allergens.

Monoclonal antibodies might not be useful to assess digestibility in SGF. Digestion may eliminate important antibody-binding epitopes recognized by the monoclonal antibody and inaccurately indicate that the protein was rapidly and completely digested. This was demonstrated by the failure of anti-Gly m 1 monoclonal antibody to detect the fragments of Gly m 1 allergen generated by digestion in SGF. Thus, purified proteins and stained gels should be used for analysis of stability in SGF.

It is apparent from this study that the potent allergenicity of peanut, soybean, mustard, egg, and milk allergens may be due, at least in part, to resistance to proteolysis in the stomach. Stability to digestion may be a prerequisite to sensitization for these important food allergens. SGF represents a useful and simple tool to evaluate stability to gastric digestion, one factor contributing to the likelihood that a protein may be allergenic. Most importantly, these data support the use of digestive stability as a key criterion in the safety assessment of proteins encoded by genes obtained from organisms with no prior history of allergenicity. By validating stability to *in vitro* digestion as a factor correlating with food protein allergenic-

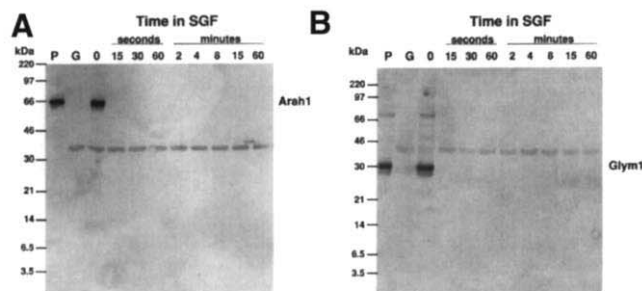


**Figure 4. Digestion in SGF assayed by immunoblots using monoclonal antibodies.** Proteins run in tricine SDS-PAGE gels were blotted onto nitrocellulose, detected with monoclonal antibodies, and visualized with goat anti-mouse alkaline phosphatase-linked antibodies. Molecular weight markers are indicated on the left-hand side of the blots. SGF and allergen proteins were run alone (lane G and lane P, respectively). Proteins were incubated in SGF for the times indicated above the blots. Individual proteins are labelled on the right-hand side as follows: (A) Detection of Ara h 1 in 500 ng of crude peanut extract using monoclonal antibody 5A6 and (B) Detection of Gly m 1 in 500 ng of crude soybean extract using monoclonal antibody F5.

ity, we have addressed the apparent lack of science-based methods for prospective assessments of inherent protein allergenicity. Thus, protein stability to gastric digestion should be used to determine allergenicity in conjunction with information about protein expression level in the resulting food, protein stability to cooking or processing, and sequence homology to known safe food proteins. These factors should be considered *in toto* when assessing the inherent allergenicity of foods derived from genetically modified plants.

A recent collaboration of the International Food Biotechnology Council and the Allergy and Immunology Institute of the International Life Sciences Institute recommended the digestibility of proteins as an important criterion in assessing the allergenicity of foods produced by genetic modification<sup>30</sup>. The results of this study validate SGF as an appropriate tool when assessing the allergenicity of proteins introduced into food crops as recommended by the FDA<sup>3</sup>. To date, all proteins introduced into plants intended for the marketplace have been demonstrated to be extremely labile in SGF<sup>31,32</sup>.

SGF may also be a useful tool for identifying previously uncharacterized allergens in food. SGF may be used in a selective allergen purification scheme where stable proteins are analyzed for allergenicity using IgE and *in vivo* criteria employed by allergists and the pharmaceutical industry. SGF-purified allergens may also have a role in the development of specific ELISAs and immunodiagnoses, critical components of food allergy prevention schemes<sup>32</sup>.

## Experimental protocol

**Crude soybean extract.** Soybean flour protein was prepared from commercial defatted soybean flakes (Cargill Protein Products, Cedar Rapids, IA). The soybean flakes were macerated 2 min with an Omni 2000 (Omni International, Waterbury, CT) hand-held ultrasonic grinder in phosphate buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 5.4 mM KCl, 0.5 M NaCl, pH 7.0). The mixture was agitated at 4°C for 6 h, centrifuged 15 min at 10,000 G, and the supernate was recovered and referred to as CSE.

**Crude peanut extract.** Commercial roasted peanuts were ground in a small scale flour mill. The resulting peanut flour was defatted by hexane extraction for 4 h. Defatted peanut flour was macerated 2 min with an Omni 2000 hand-held ultrasonic grinder in high salt phosphate buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 5.4 mM KCl, 1.0 M NaCl, pH 7.4). The mixture was centrifuged 30 min at 15,000 G, and the supernate was recovered. The supernate was dialyzed against phosphate buffered saline (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 137 mM NaCl, pH 7.4) overnight at 4°C using tubing with molecular weight cut off at 3500 daltons. Dialyzate was referred to as CPE.

**Purified allergens, proteins and antibodies.** Soybean  $\beta$ -conglycinin,

glycinin, SKTI, and lectin were obtained as purified freeze-dried samples from Martin Hessing, TNO Foods and Nutrition, The Netherlands. Rabbit anti-glycinin and anti- $\beta$ -conglycinin were obtained as protein-A purified IgG fractions. Soybean allergen Gly m Bd 30K (Gly m 1)<sup>14</sup> and monoclonal anti-Gly m 1 (F5)<sup>33</sup> were gifts of Tadashi Ogawa, Department of Nutrition, School of Medicine, University of Tokushima, Japan. Peanut (*Arachis hypogaea*) allergen Ara h2 (ref. 17), and anti-Ara h1 monoclonal antibody (5A6)<sup>16</sup> were gifts of A. Wesley Burks, Department of Pediatric Immunology and Allergy, Arkansas Childrens' Hospital, Little Rock, AR. Mustard (*Brassica juncea*) allergen Bra j IE (ref. 34) and mustard (*Sinapis alba*) allergen Sin a 1 (ref. 18) were gifts of Rosalía Rodríguez, Departamento de Bioquímica y Biología Molecular, Facultad de Química, Universidad Complutense, Madrid, Spain. All other purified proteins, allergens and antibodies were obtained from Sigma Chemical Co., St. Louis, MO.

**Protein assays.** The total protein content of all protein and allergen samples was estimated using the method of Bradford<sup>35</sup> and as adapted to the 96-well titer plate format of Bio-Rad Laboratories Ltd., (Richmond, CA). Known concentrations of bovine serum albumin were used to develop a standard curve.

**Incubation in SGF.** Simulated gastric fluid<sup>23</sup> contained 0.32% (w/v) pepsin (Sigma), 0.03M NaCl, at pH 1.2. Proteolytic activity of SGF preparations was estimated by measuring trichloroacetic acid precipitable hemoglobin after hydrolysis for 10 min<sup>24</sup>. Incubations in SGF were performed essentially as described<sup>8</sup> with the following modifications: 170 ng/ $\mu$ l of protein was digested in 200  $\mu$ l aliquots of SGF placed in 2-ml centrifuge tubes preheated to 37°C. Incubations were maintained at 37°C in a shaking waterbath and quenched by neutralization with 75  $\mu$ l of 160 mM Na<sub>2</sub>CO<sub>3</sub> 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<sup>35</sup> was added to the quenched samples followed by heating to 99°C.

**SDS-PAGE.** Digestion of proteins was evaluated by SDS-PAGE using precast 10% to 20% acrylamide minigels with tricine buffers (Integrated Separation Systems, Natick, MA)<sup>35</sup>. SDS-PAGE loading buffer contained 0.1% *m*-cresol purple<sup>36</sup>. Proteins were visualized by Coomassie brilliant blue colloidal staining<sup>37</sup>. Mid-range relative molecular weight protein markers were run with each gel (Promega, Madison, WI).

**Immunoblotting.** Gels were electroblotted onto nitrocellulose (Micron Separations, Westboro, MA) membranes<sup>35</sup>. Blotted membranes were placed in a blocking solution of 3% (w/v) gelatin in Tween Tris buffered saline (TTBS) for 30 min. TTBS was prepared by adding 0.05% tween-20 (v/v) to TBS (20 mM Tris, 500 mM NaCl, pH 7.5). For immunodetection, primary antibodies were diluted in TTBS containing 1% gelatin (w/v). Immunoblots were rinsed three times with TTBS prior to addition of secondary antibody. Antigen-antibody conjugates were visualized by reaction to alkaline phosphatase-linked goat anti-mouse IgG or goat anti-rabbit IgG secondary antibody (depending on the primary antibody used) diluted 1:7500 in TTBS containing 1% gelatin (w/v). Color reaction of alkaline phosphatase was according to the manufacturer's instructions (Bio-Rad Laboratories). Low-range or mid-range colored relative molecular weight protein markers were run with each gel (Amersham, Buckinghamshire, UK).

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