

This article was downloaded by:[Advanced Institute of Sanita]
[Advanced Institute of Sanita]

On: 21 February 2007

Access Details: [subscription number 769486267]

Publisher: Informa Healthcare

Informa Ltd Registered in England and Wales Registered Number: 1072954

Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



International Journal of Food Sciences and Nutrition

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title-content=t713425816>

Stability of a set of allergens and non-allergens in simulated gastric fluid

To link to this article: DOI: 10.1080/09637480601149640

URL: <http://dx.doi.org/10.1080/09637480601149640>

Full terms and conditions of use: <http://www.informaworld.com/terms-and-conditions-of-access.pdf>

This article maybe used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

© Taylor and Francis 2007

Stability of a set of allergens and non-allergens in simulated gastric fluid

ROD A. HERMAN¹, MICHAEL M. WOOLHISER²,
GREGORY S. LADICS³, VALERIE A. KORJAGIN¹,
BARRY W. SCHAFER¹, NICHOLAS P. STORER¹,
SUSAN B. GREEN¹, & LYNN KAN²

¹Dow AgroSciences LLC, Indianapolis, IN, USA, ²The Dow Chemical Company, Midland, MI, USA, and ³DuPont Crop Genetics, Wilmington, DE, USA

Abstract

Stability in simulated gastric fluid has been suggested as a parameter for consideration in the allergenicity assessment of transgenic proteins. However, the relationship between the stability of proteins in simulated gastric fluid and allergenicity has been inconsistent among studies conducted with reference allergens and non-allergens. Differences in laboratory methods and data interpretation have been implicated as possible causes for conflicting study results. We attempted to mitigate some of the methodological inconsistencies among laboratory methods by applying a kinetic interpretation to results of digestion experiments conducted with a set of known allergens and putative non-allergens. We found that pepsinolysis in simulated gastric fluid generally followed an exponential (pseudo-first-order) pattern of decay, at least during the terminal (slower) phase of digestion, allowing the calculation of digestion half-lives. While digestibility estimates were reproducible and robust, results for the proteins evaluated in this study did not support a significant association between stability in simulated gastric fluid and allergenicity.

Keywords: *Digestion, food allergy, kinetics, model, simulated gastric fluid*

Introduction

Beneficial traits have been incorporated into crop plants through the introduction of genes that code for proteins that may subsequently be found in food derived from these crops. As part of the safety assessment for such proteins, the risk of allergenicity is evaluated. While the vast majority of proteins do not cause allergy, most allergens belong to subsets of structurally related proteins (Jenkins et al. 2005). However, no single property is known to differentiate between allergens and non-allergens. For this reason, a number of partially predictive characteristics are used in a weight-of-evidence evaluation of allergenic risk—and include the source of the gene, structural similarity of the protein to known allergens, prevalence of the protein in food, stability to food processing, and digestibility (FAO/WHO 2001; Metcalfe et al. 1996; Codex 2003).

Correspondence: Rod A. Herman, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268, USA. Tel: +1 317 337 3551. Fax: +1 317 337 3255. E-mail raherman@dow.com

ISSN 0963-7486 print/ISSN 1465-3478 online © 2007 Informa UK Ltd
DOI: 10.1080/09637480601149640

The source of the gene and its structural similarity to known allergens (based on bio-informatic analysis) can be used to formulate testable hypotheses concerning specific allergies. If a protein is isolated from a known allergenic source, susceptible individuals can be identified against which the transgenic protein can be evaluated *in vitro* or *in vivo* to identify whether the protein of interest is the allergenic agent. A similar approach can be used if a transgenic protein is found to be structurally similar to a known allergen.

Exposure is a prerequisite for allergenic reactivity. Exposure to the protein can be evaluated by considering the concentration of the transgenic protein in food, its stability during processing and food preparation, and its resistance to metabolism *in vivo*. The latter property has typically been investigated using a simulated gastric digestion assay first applied to allergenicity assessment by Astwood et al. (1996). While this initial study demonstrated a good correlation between stability in simulated gastric fluid (SGF) and the allergenicity of a reference set of proteins, subsequent studies have often been less definitive (Vieths et al. 1999; Yagami et al. 2000; Fu et al. 2002; Murtagh et al. 2002; Diaz-Perales et al. 2003; Lee et al. 2005). Differences in laboratory methods and data interpretation have been implicated as possible causes for conflicting study results (Bannon et al. 2002, 2003; Thomas et al. 2004; Metcalfe 2005). In this study, we attempted to mitigate some of the methodological inconsistencies between laboratories by applying a kinetic interpretation to results of digestion experiments conducted with a set of known allergens and putative non-allergens. To test whether this approach might improve the predictive ability of the SGF assay, the proteins used in this study were selected from those previously investigated (Astwood et al. 1996; Fu et al. 2002; Thomas et al. 2004; Herman et al. 2005).

Methods and materials

Substrate proteins and pepsin

The following substrate proteins were obtained from Sigma Chemical (St Louis, MO, USA): bovine serum albumin (BSA), A02081; collagen (type III) from calf skin, C3511; β -lactoglobulin (BLG) from cows milk, L0130; kidney bean lectin (KBL); phytohemagglutinin, L8629; lysozyme (LYS) from chicken egg white, L6876 d-ribulose 1,5-diphosphate carboxylase (RUBISCO) from spinach, R8000; lima bean trypsin inhibitor (LBTI), T9378; ovomucoid (OVM) trypsin inhibitor from chicken egg white, T2011; soybean trypsin inhibitor (STI), T9003; ovalbumin (OVA) from chicken egg white, A5503; horseradish peroxidase (HRP), P6782; concanavalin A (ConA) from jack bean, C2010; and pea lectin, L5380. Ara h 2 was purified from de-fatted peanut flour, and soy lipoxygenase (SLG) was isolated from de-fatted soy flour (TNO Nutrition and Food Research, Zeist, The Netherlands).

The purities of the test proteins as reported by the manufacturer were as follows: BSA, 99%; BLG, 90%; KBL, 85%; LYS, 95%; STI, 90%; OVA, 98%; HRP, highly purified; ConA, 96%; pea lectin, 100%; Ara h 2, 90+%; and SLG, 88%. The purity of the remaining proteins was estimated via sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and densitometry using BSA as a standard (not published). Purities were estimated as follows: collagen, 8.5%; RUBISCO, 8.9%; LBTI, 4.3%; and OVM, 35%. Pepsin (89% purity) was also purchased from Sigma Chemical (P6887, lot 062K7651).

Digestions

Digestion experiments were carried out at one of three laboratories (Dow AgroSciences LLC, Indianapolis, IN, USA; The Dow Chemical Company, Midland, MI, USA; Pioneer Hi-Bred International, Inc., Johnston, IA, USA). Gastric control solution (G-con) was prepared with 200 mg NaCl per 100 ml water and was adjusted to pH 1.2 using dilute HCl. SGF was prepared fresh on the day of use by adding pepsin to G-con at 0.32% w/v (United States Pharmacopeia 2000). Prior to addition to SGF, the OVA, ConA, HRP, STI, SLG and pea lectin were prepared in water at 0.2 or 0.04 mM. Ara h 2 was diluted in 25% G-con (diluted with water). OVM, BLG, KBL, LBTI and LYS were dissolved in G-con. RUBSCO and BSA were initially dissolved in Tris buffer (50 mM, pH 9.5) and water, respectively, prior to dilution in G-con.

For digestion experiments, SGF (1,900 μ l) was preheated for 2 min at 37 °C prior to addition of 100 μ l protein stock solutions. Due to poor solubility, collagen powder was added directly to SGF. The reaction was held at 37 °C and continuously mixed using a stir bar and a submersible stir plate. At designated time points (0.5, 1, 2, 5, 10, 15, 20, 30, 45 and 60 min), 120 μ l digestion mixture was removed and added to 42–48 μ l of 200 mM, pH 11, carbonate neutralizing buffer to stop the enzymatic reaction. Neutralized digestion mixtures were vortexed and then mixed with five times reducing buffer, which consisted of either Laemmli buffer (40% glycerol, 5% β -mercaptoethanol, 10% SDS, 0.33 M Tris, 0.05% bromophenol blue, pH 6.8) or ImmunoPure™ (50% glycerol, 100 mM dithiothreitol, 5% SDS, 0.3 M Tris-HCl, Lane Marker Tracking Dye, pH 6.8). For the initial time point (time 0), proteins were added directly to neutralized and reduced SGF. Neutralized mixtures were boiled for 5 min and either frozen or immediately analyzed via SDS-PAGE.

SDS-PAGE analysis

A variety of SDS-PAGE systems were used as deemed appropriate for discriminating protein bands for each substrate. Gels were run in triplicate for each digestion. Collagen, KBL, Ara h 2, ConA, STI, SLG, pea lectin, BLG, BSA, OVM and RUBISCO, each digested at 0.002 mM, were loaded at 40 μ l/well onto 4–20% Bio-Rad Criterion™ Tris-HCL gels (12+2 wells, 1.0-mm thickness; Bio-Rad Criterion; Hercules, CA, USA) and run for ~1 h between 170 and 200 V using Tris-glycine-SDS running buffer. BSA and RUBISCO, each digested at 0.01 mM, were run in the same manner except that 25 μ l each sample was loaded per well onto 10–20% Bio-Rad Criterion™ Tris-HCl gels.

OVA and HRP, each digested at 0.002 mM, and SLG and HRP, each digested at 0.01 mM, were loaded at 20 μ l/well onto NuPAGE 10% Novex Bis-Tris gels (12 wells, 1.0-mm thickness; NuPAGE, Invitrogen, Carlsbad, CA, USA) and run for 35 min at 200 V using [2-(N-morpholine)-ethane sulphonate acid]-SDS running buffer. OVA, STI, Ara h 2, and pea lectin, each digested at 0.01 mM, were run in the same manner except that 15-well, 1.5-mm-thick gels were used. ConA, digested at 0.01 mM, was run on 12-well, 1.0-mm-thick gels in the same manner using NuPAGE 4–12% Novex Bis-Tris gels (Invitrogen).

LYS and LBTI, each digested at 0.002 mM, and OVM, digested at 0.01 mM, were loaded at 25 μ l/well onto 10–20% Novex tricine gels (12 wells, 1.0-mm thickness) and run for ~2 h at 125 V using tricine-SDS running buffer. A repeat digestion of a different lot of OVM (Sigma lot 064K7007, with a LYS contaminant) digested at

0.01 mM was conducted with sample initially diluted in G-con or water. Samples were loaded at 40 μ l/well onto 4–20% Bio-Rad Criterion™ Tris–HCl gels (12 + 2 wells, 1.0-mm thickness) and run for ~1 h at 200 V using Tris–glycine–SDS running buffer.

Molecular-weight standards (Invitrogen SeeBlue Pre-Stained Standard, catalogue number LC5625 or Broad Range, catalogue number 1610318; Bio-Rad low range, catalogue number 161-0305; Pierce TriChromranger™, catalogue number 26691) were added to gels at 8–13 μ l/well. Each gel run also included SGF alone. Gels were stained with Pierce's GelCode Blue Stain Reagent (catalogue number 24562; Pierce's, Rockford, IL, USA). Gels were washed three times in water (5 min per wash) and then stained for at least 1 h in the GelCode stain. NuPAGE gels were fixed in 50% methanol, 7% acetic acid, for 15 min prior to being washed and stained as above. All gels were destained with several volumes of water over a period of at least 4 h.

Gels were scanned with a Bio-Rad Fluor-S MultiImager System using Quantity One software, a HP ScanJet 6200C (Hewlett Packard, Palo Alto, CA, USA), or a Molecular Dynamics Personal Densitometer SI (Sunnyvale, CA, USA) using ImagQuant software (Sunnyvale, CA, USA). All images were saved as 300 d.p.i. tif files. Densitometry measurements were made for all major bands on the SDS-PAGE gels using Kodak ID Image Analysis Software Version 3.6.6 (Kodak, Rochester, NY, USA).

Data analysis

The mean volumes of substrate–protein bands over time were fit to a three-parameter exponential model $[S] + B = [S_0]e^{-kt} + B$, where $[S]$ is an index of substrate concentration at time t , $[S_0]$ is an index of the initial concentration of substrate, k is the first-order rate constant, and B is the estimated background volume on the gel) using non-linear regression (SAS 1999). For Ara h 2, digestion of fragments was insufficient to allow an adequate estimation of background from the model. In this case, background densities were estimated from the lane containing only SGF. The conformation of the data to the regression line was examined visually to evaluate the goodness of fit and to identify outliers or systematic deviations from the model. Early time points that substantially deviated from the model were not included in the analysis, but are plotted in the figures for completeness. The data points for an initial fast phase of digestion were not modeled based on theoretical considerations (Herman et al. 2005, 2006a, 2006b). Digestion fragments that produced sufficiently dark bands to track quantitatively were also modeled using a three-parameter exponential model, but only data after the parent protein was largely digested were included, so that formation kinetics would not substantially affect the quantity of protein measured. Half-lives were calculated by dividing the natural logarithm of two (0.693) by each rate constant.

Results and discussion

A set of allergenic proteins (BSA, LYS, OVA, Ara h 2, STI, BLG and OVM) and putative non-allergens (RUBISCO, HRP, SLG, pea lectin, ConA, KBL, LBTI and collagen—hence forth referred to as non-allergens) were digested in SGF. Digestion was monitored on SDS-PAGE gels and quantified by densitometry. Digestion generally followed an exponential decline pattern (Figures 1–8), but for some

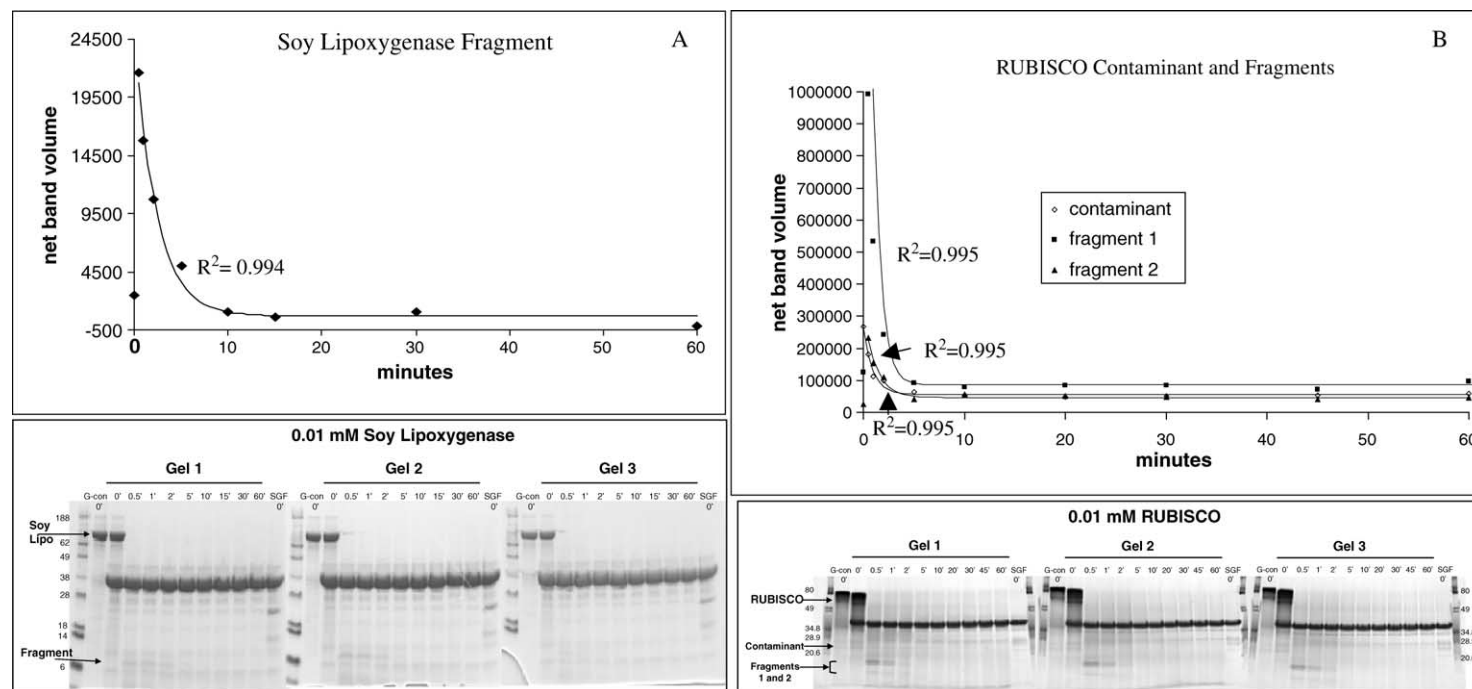


Figure 1. SGF digestion profiles of a SLG digestion fragment (a) and RUBISCO contaminant and digestion fragments (b). Solid lines illustrate the fit of a negative exponential (first-order) decline. Lower panels show SDS-PAGE gels of SGF digestion at indicated times (minutes of digestion).

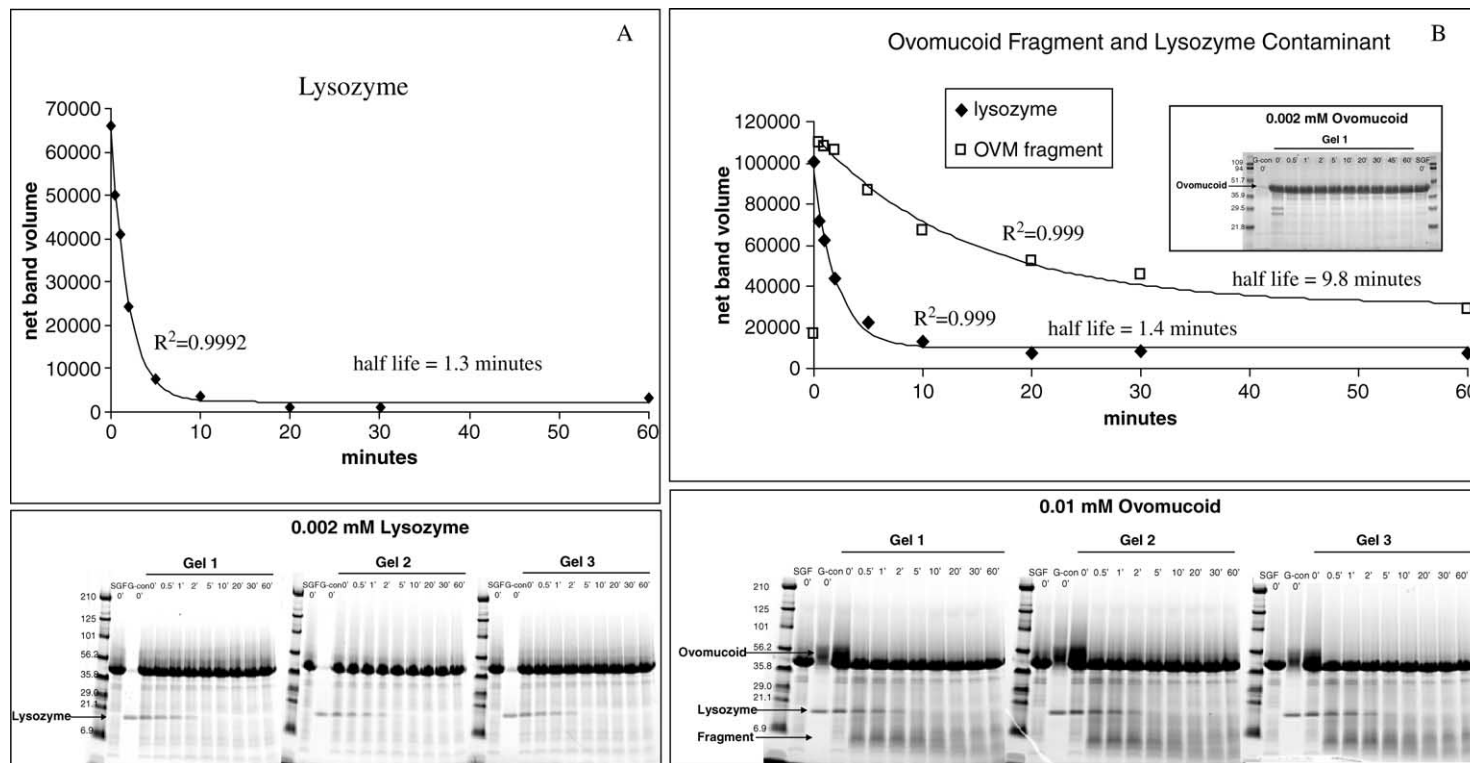


Figure 2. SGF digestion profiles of LYS (a) and an OVM digestion fragment and LYS contaminant of the OVM preparation (b). Solid lines illustrate the fit of a negative exponential (first-order) decline. Lower panels show SDS-PAGE gels of SGF digestion at indicated times (minutes of digestion).

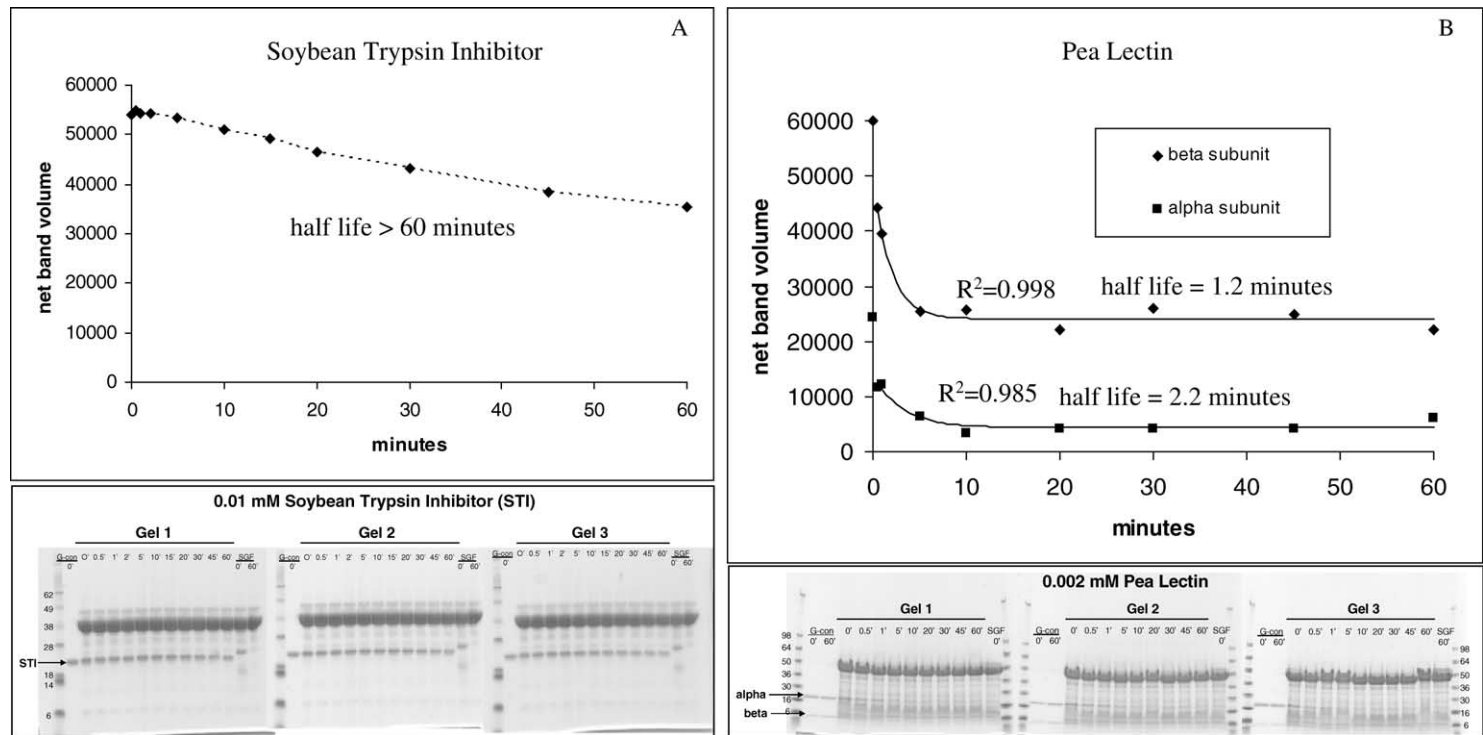


Figure 3. SGF digestion profiles of STI (a) and the alpha and beta subunits of pea lectin (b). Solid lines illustrate the fit of a negative exponential (first-order) decline. Lower panels show SDS-PAGE gels of SGF digestion at indicated times (minutes of digestion).

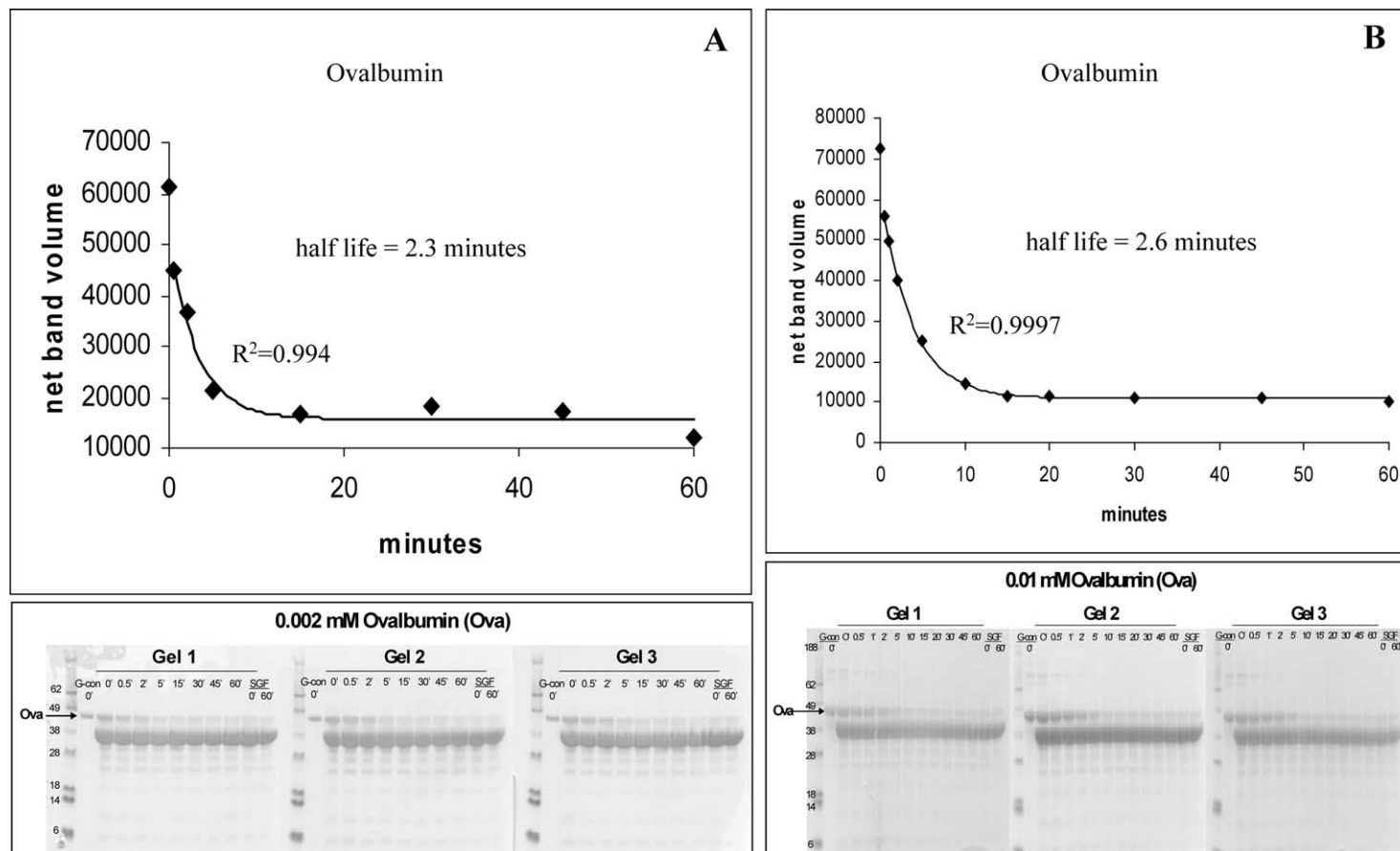


Figure 4. SGF digestion profiles of OVA at a concentration of 0.002 mM (a) and at 0.01 mM (b). Solid lines illustrate the fit of a negative exponential (first-order) decline. Lower panels show SDS-PAGE gels of SGF digestion at indicated times (minutes of digestion).

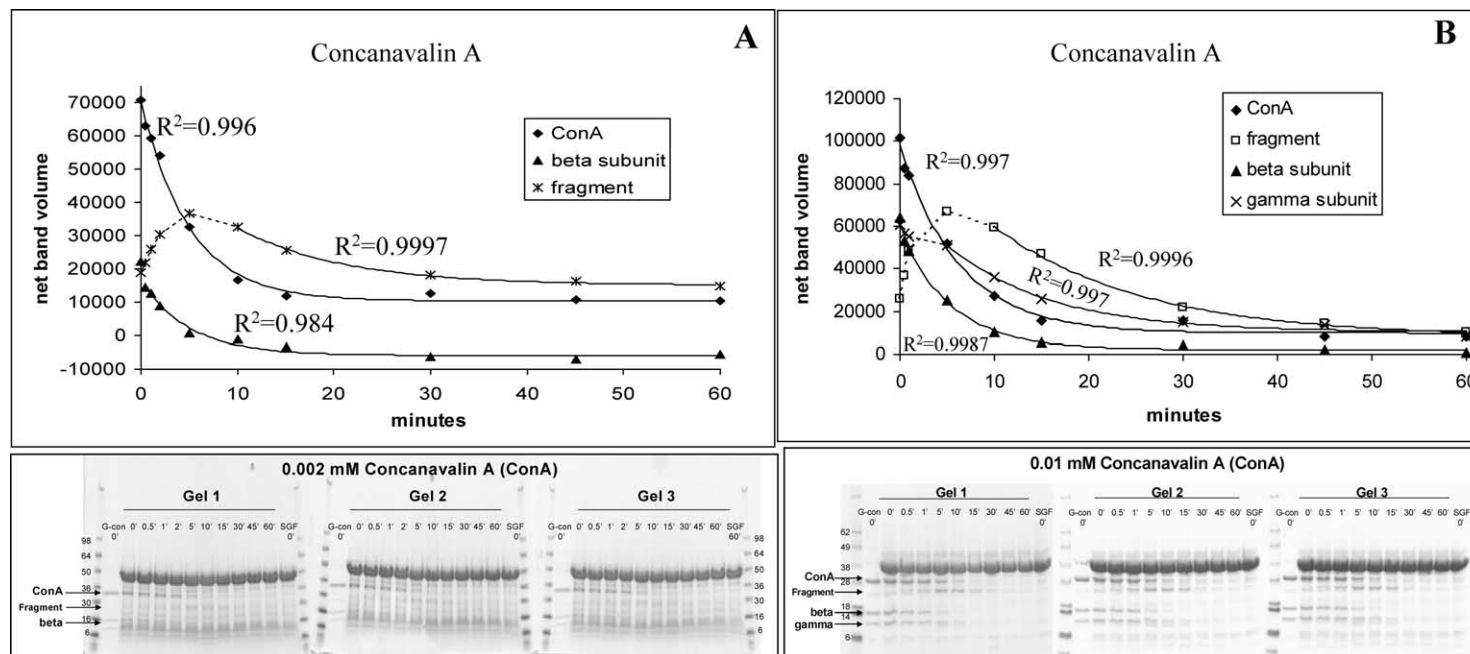


Figure 5. SGF digestion profiles of ConA at a concentration of 0.002 mM (a) and at 0.01 mM (b). Solid lines illustrate the fit of a negative exponential (first-order) decline. Lower panels show SDS-PAGE gels of SGF digestion at indicated times (minutes of digestion).

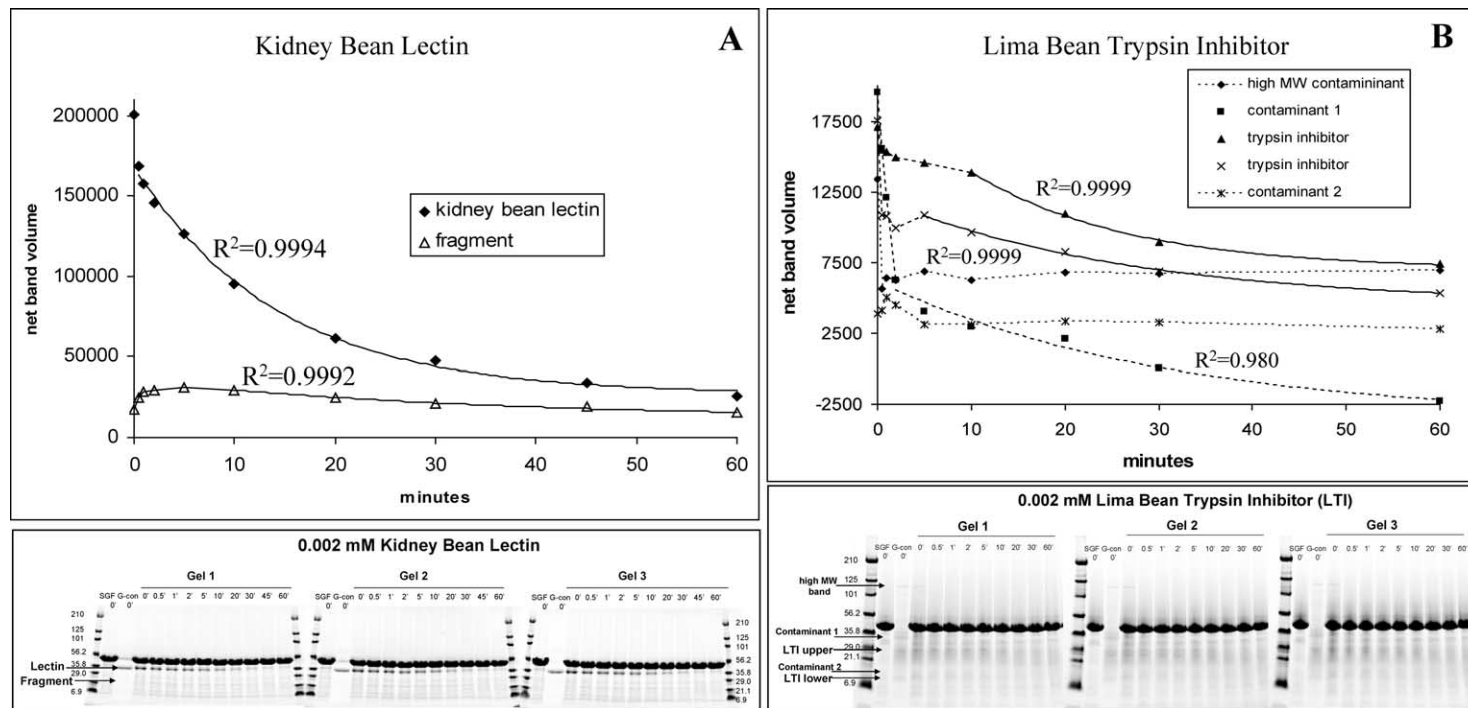


Figure 6. SGF digestion profiles of KBL and a digestion fragment (a), and LBTI and contaminating proteins present in the preparation (b). Solid lines illustrate the fit of a negative exponential (first-order) decline. Lower panels show SDS-PAGE gels of SGF digestion at indicated times (minutes of digestion).

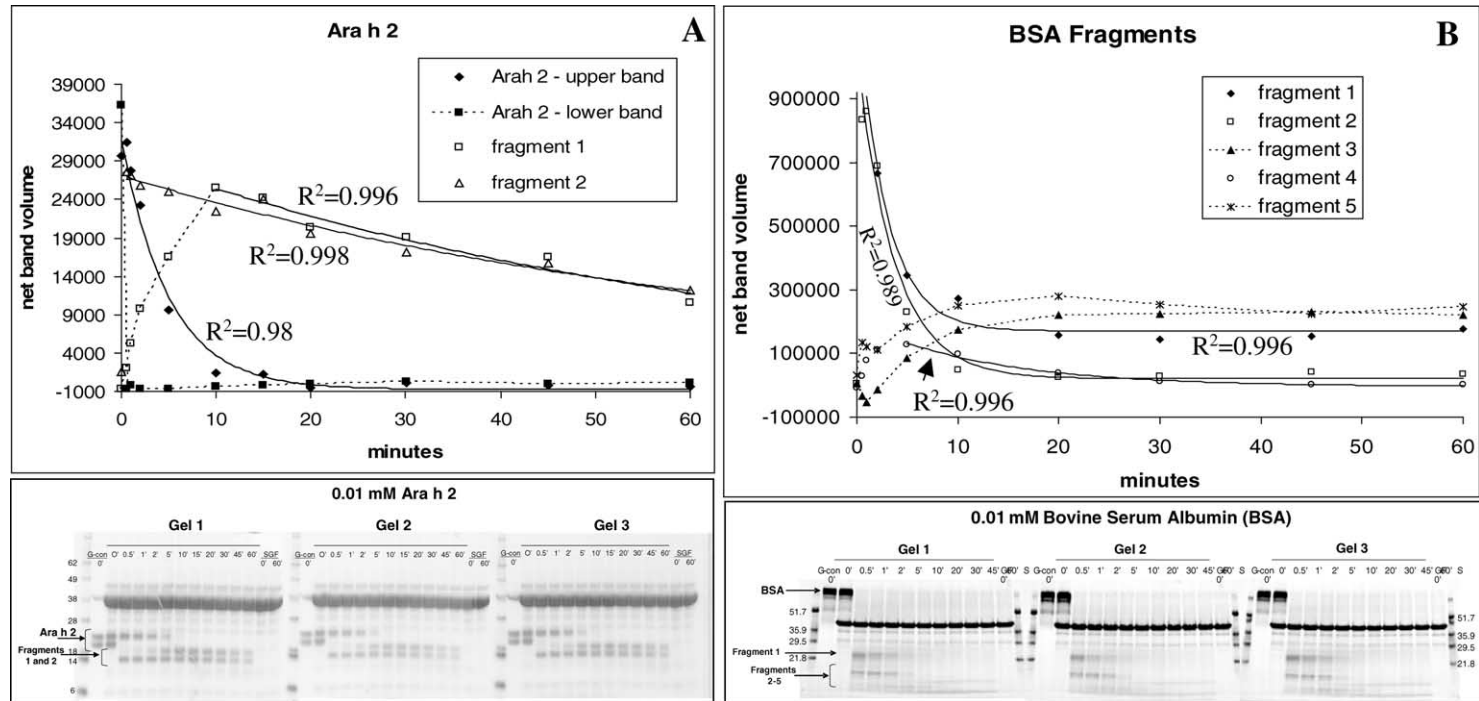


Figure 7. SGF digestion profiles of Ara h 2 and digestion fragments (a), and BSA digestion fragments (b). Solid lines illustrate the fit of a negative exponential (first-order) decline. Lower panels show SDS-PAGE gels of SGF digestion at indicated times (minutes of digestion).

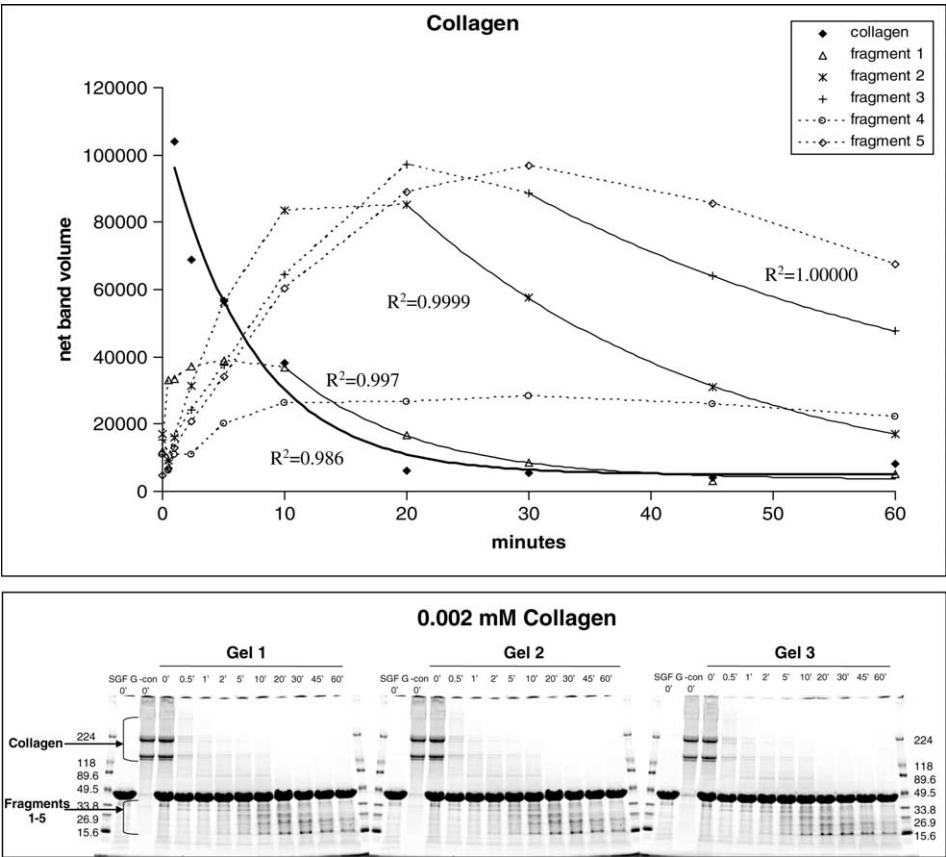


Figure 8. SGF digestion profiles of collagen and digestion fragments. Solid lines illustrate the fit of a negative exponential (first-order) decline. Lower panel shows SDS-PAGE gels of SGF digestion at indicated times (minutes of digestion).

proteins the exponential decline was preceded by a rapid initial digestive (burst) phase (pea lectin, Figure 3; OVA, Figure 4; ConA, Figure 5; KBL, Figure 6a; LBTI, Figure 6b; collagen; Figure 8). The stability of each protein (and digestion fragment) was estimated based on this most stable phase of digestion (terminal phase). Under the premise that stability favors allergenicity, focusing on the slowest digestive phase or most recalcitrant protein form seems logical for risk assessment purposes. The theoretical reasons for expecting exponential (pseudo-first-order) pepsinolysis of proteins in SGF has been discussed elsewhere (Herman et al. 2006b; Srividhya and Schnell 2006).

Specific protein results

Half-lives for each protein are summarized in Table I (ordered from the most labile to the most stable based on the most stable component). Several proteins digested very quickly in SGF. The non-allergen HRP, digested at either 0.002 or 0.01 mM, was not visible on the SDS-PAGE gels after 30 sec, and no digestion fragments were seen (data not shown). The non-allergen, parent RUBISCO protein was also highly digestible

Table I. Half-lives of allergens and non-allergens in SGF.

Protein	Allergenic status	Half-life in SGF	
		0.002 mM protein	0.01 mM protein
Horseradish peroxidase	Not reported as allergen	<30 sec	<30 sec
RUBISCO	Not reported as allergen	<30 sec	<30 sec
RUBISCO contaminant	Not reported as allergen	Not resolvable	39 sec
RUBISCO fragment 1	Not reported as allergen	Not resolvable	32 sec
RUBISCO fragment 2	Not reported as allergen	Not resolvable	49 sec
Lysozyme	Allergen	1.3 min	Not determined
Soy lipoxygenase	Not reported as allergen	<30 sec	<30 sec
SLG fragment	Not reported as allergen	Not resolvable	1.6 min
Pea lectin alpha subunit	Not reported as allergen	2.2 min	Not determined
Pea lectin beta subunit	Not reported as allergen	1.2 min	Not determined
Ovalbumin	Allergen	2.3 min	2.6 min
Concanavalin A	Not reported as allergen	3.5 min	4.3 min
ConA beta subunit	Not reported as allergen	3.5 min	3.8 min
ConA gamma subunit	Not reported as allergen	not resolvable	7.6 min
ConA digestion fragment	Not reported as allergen	7.6 min	11 min
Ovomucoid	Allergen	Not resolvable	Not resolvable
OVM fragment	Allergen	Not resolvable	9.8 min
OVM contaminant—lysozyme		Not resolvable	1.4 min
Kidney bean lectin	Not reported as allergen	9.8 min	Not determined
KBL fragment	Not reported as allergen	23 min	Not determined
Ara h 2 upper band	Allergen	3.3 min	3.5 min
Ara h 2 lower band	Allergen	<30 sec	<30 sec
Ara h 2 digestion fragment(s)	Allergen	50 min	47 min
Bovine serum albumin	Allergen	<30 sec	<30 sec
BSA fragment 1	Allergen	Not resolvable	2.0 min
BSA fragment 2	Allergen	Not resolvable	2.5 min
BSA fragment 4	Allergen	Not resolvable	9.1 min
BSA fragment 3 and 5	Allergen	Not resolvable	>60 min
Soybean trypsin inhibitor	Allergen	>60 min	>60 min
Bovine β -lactoglobulin	Allergen	>60 min	Not determined
Lima bean trypsin inhibitor, high molecular weight)	Not reported as allergen	<30 sec	Not determined
LBTI—contaminant 1	Not reported as allergen	21 min	Not determined
LBTI—upper band	Not reported as allergen	12 min	Not determined
LBTI—contaminant 2	Not reported as allergen	18 min	Not determined
LBTI—lower band	Not reported as allergen	>60 min	Not determined
Collagen	Hypo-allergenic	4.9 min	Not determined
Collagen fragment 1	Hypo-allergenic	7.3 min	Not determined
Collagen fragment 2	Hypo-allergenic	18 min	Not determined
Collagen fragment 3	Hypo-allergenic	27 min	Not determined
Collagen fragments 4 and 5	Hypo-allergenic	>60 min	Not determined

Bold text signifies the most stable component within a protein preparation.

(note: RUBISCO small subunit not visible at time 0 on the SDS-PAGE gels; Figure 1b). At 0.002 and 0.01 mM, RUBISCO was not visible after 30 sec. However, when digested at 0.01 mM, three digestion fragments of RUBISCO were observed that had half-lives ranging from 32 to 49 sec (Figure 1b).

The allergen LYS, digested at 0.002 mM, had a half-life of 1.3 min (Figure 2a). LYS was also present as a contaminant in the OVM sample (as seen by others; for

example, Thomas et al. 2004), and the half-life of LYS in this preparation was found to be 1.4 min (Figure 2b). The non-allergen, parent SLG protein, digested at 0.002 and 0.01 mM, was not visible on the SDS-PAGE gels after 30 sec. However at 0.01 mM, a digestion fragment of SLG with a half-life of 1.6 min was observed (Figure 1a). The non-allergen pea lectin was assayed only at 0.002 mM. The alpha subunit of pea lectin had a half-life of 2.2 min and the beta subunit had a half-life of 1.2 min (Figure 3b).

The allergen OVA, digested at 0.002 or 0.01 mM, had an estimated half-life of 2.3 or 2.6 min, respectively (Figure 4a,b). The non-allergen, parent ConA protein, digested at 0.002 or 0.01 mM, had an estimated half-life of 3.5 or 4.3 min, respectively, but produced digestion fragments with half-lives that ranged from 3.5 to 7.6 min or from 3.8 to 11 min, respectively (Figure 5a,b). The allergen, parent OVM protein, digested at either 0.002 and 0.01 mM, was difficult to resolve from the pepsin band on the SDS-PAGE gels, but appeared to digest very quickly (Figure 2b). At 0.01 mM, an OVM digestion fragment was visible with an estimated half-life of 9.8 min.

The non-allergen KBL was only assayed at 0.002 mM. The parent KBL protein had a half-life of 9.8 min, and a KBL digestion fragment had a half-life of 23 min (Figure 6a). The allergen, Ara h 2 parent, digested at 0.002 or 0.01 mM, had an estimated half-life of 3.3 or 3.5 min, respectively, but produced digestion fragments with half-lives of up to 47 or 50 min, respectively (Figure 7a, only the 0.01 mM concentration shown).

The allergen, parent BSA protein, digested at 0.002 or 0.01 mM, was not visible on SDS-PAGE gels after 30 sec of digestion (data not shown). However, several digestion fragments of BSA were visible at 0.01 mM with half-lives that ranged from 2 to >60 min (Figure 7b). The allergens STI and BLG, digested at 0.002 mM, had half-lives of over 60 min (data not shown). At 0.01 mM, STI also had a half-life in excess of 60 min (Figure 3a).

The non-allergen LBTI was assayed only at 0.002 mM. The parent LBTI protein was not visible on SDS-PAGE gels after 30 sec of digestion (Figure 6b). However, digestion fragments of LBTI were observed with half-lives that ranged from 12 to >60 min. The hypo-allergenic protein, collagen, digested at 0.002 mM, had a half-life of 4.9 min (Figure 8). However, digestion fragments of collagen had half-lives from 7.3 to >60 min.

Three known allergens digested relatively rapidly in this study (Figure 9). OVA had a half-life of 2.3–2.6 min, LYS had a half-life of 1.3–1.4 min, and OVM (fragment) had a half-life of 9.8 min. However, the latter two proteins were dissolved in G-con prior to digestion, potentially causing acid-induced unfolding that may have affected the speed of digestion (Herman et al. 2006b). For this reason, digestions were repeated comparing G-con solubilized proteins with water-solubilized proteins (new lot of OVM with LYS contaminant) to determine whether the initial solvent that was used to dilute these proteins played a role in the rapid digestion of these two allergens. An exponential decline model fit the data between 0.5 and 30 min (maximum exposure interval evaluated) very well for both proteins pre-diluted in either G-con or water (LYS and OVM digestion fragment; plots not shown). The half-lives for the new lot of LYS, initially diluted in G-con or water, were 1.1 or 1.8 min, respectively. Half-lives for the new lot of OVM, diluted in G-con or water, were 4.7 or 5.7 min for the digestion fragment, respectively. While initial dilution in G-con may have slightly increased the rate of digestion for each protein compared with initial dilution in water,

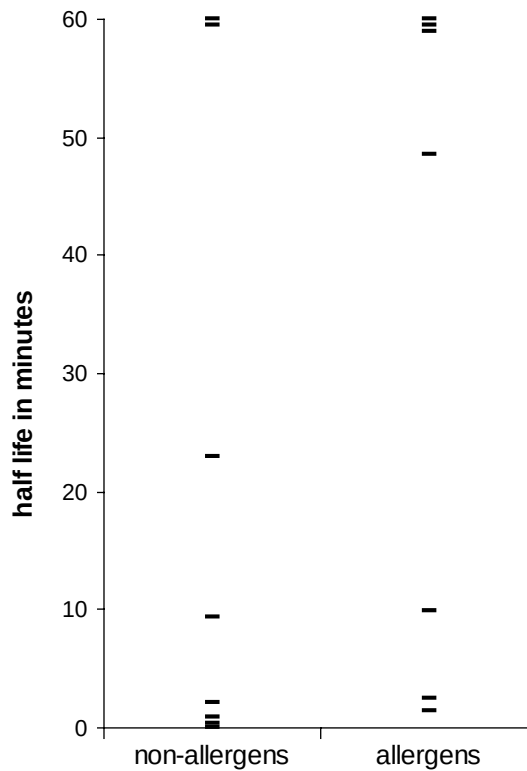


Figure 9. Half-lives for the most persistent species (parent protein or digestion fragment) of allergens and non-allergens in SGF. The position of half-life values for interfering data symbols are depicted in slightly altered positions to prevent some protein values from being obscured. The half-lives for the two non-allergens and the three allergens depicted near the top of the plot were estimated to be greater than 60 min. One non-allergen (HRP) depicted closest to the x axis had an estimated half-life of less than 30 sec.

these allergens remained relatively labile in SGF when initially diluted in water. Thus, the initial dilution methods did not appear to meaningfully skew results for these proteins.

Digestibility comparisons

The theory supporting the expectation of exponential pepsinolysis in SGF predicts relative insensitivity of the first-order rate constants (and thus half-lives) to relatively large shifts in the concentrations of the reactants (pepsin and protein substrates) as long as the pepsin concentration is relatively high, such as is specified for SGF (0.32%; United States Pharmacopeia 2000), and as long as the protein concentration is lower than the pepsin concentration (Herman et al. 2006b; Srividhya and Schnell 2006). This has been empirically tested and reported (Herman et al. 2005) and was confirmed for the subset of proteins evaluated here at two concentrations (Table I; Herman et al. 2006a).

A number of proteins produced digestion fragments that were more stable than the parent protein (Table I and Figures 1 and 2b and 5–8). As mentioned above, the premise that more stable proteins are at higher risk of being allergens makes it logical

to evaluate allergenic risk based on the most stable protein component (parent or fragment). For this reason, the most stable protein component was used to gauge the stability of a given protein. We therefore compared the half-lives for the most stable phase of digestion (terminal exponential phase) for the most recalcitrant protein form or fragment when evaluating the relationship between stability in SGF and allergenicity (Figure 9).

Figure 9 illustrates a poor correlation between SGF stability and allergenicity for this set of reference proteins. Application of a kinetic analysis to this particular enzymatic reaction resulted in reproducible estimates of persistence in SGF between protein concentrations of 0.002 and 0.01 mM, and previous work indicated similar reliability when different concentrations and different lots of pepsin were used (Herman et al. 2005). However, application of this kinetic approach did not overcome the previously reported poor capability of this assay for predicting allergenicity (Vieths et al. 1999; Yagami et al. 2000; Fu et al. 2002; Murtagh et al. 2002; Diaz-Perales et al. 2003; Lee et al. 2005). Potential reasons for this poor correlation have been reviewed previously, and include non-representative conformations of purified protein preparations, absence of whole-food matrix effects, no consideration of protein prevalence in food, poor simulation of *in-vivo* proteolysis conditions, and potential absorption of proteins in the mouth (Herman et al. 2006a).

Acknowledgements

The authors thank Kathryn Clayton, Penny Hunst, Laura Tagliani, Donald Stuart, John Cuffe and Janet Norton of Dow AgroSciences for reviewing a draft of this manuscript. They also thank Jean Schmidt of Pioneer Hi-Bred International for completing a number of high-quality digestion experiments reported here.

References

- Astwood JD, Leach JN, Fuchs RL. 1996. Stability of food allergens to digestion in vitro. *Nat Biotech* 14:1269–1273.
- Bannon GA, Goodman RE, Leach JN, Rice E, Fuchs RL, Astwood JD. 2002. Digestive stability in the context of assessing the potential allergenicity of food proteins. *Comments Toxicol* 8:271–285.
- Bannon GA, Fu T-J, Kimber I, Hinton DM. 2003. Protein digestion and relevance to allergenicity. *Environ Health Perspect* 111:1122–1124.
- Codex 2003. Codex Alimentarius Commission, Alinorm 03/34: Joint FAO/WHO Food Standard Programme. Codex Alimentarius Commission, Twenty-Fifth Session, Rome, Italy, 30 July 2003. Appendix III, Guideline for conduct of food safety assessments of foods derived from recombinant-DNA plants and Appendix IV, Annex on assessment of possible allergenicity. pp. 47–60.
- Diaz-Perales A, Blanco C, Sanchez-Monge R, Varela J, Carrillo T, Salcedo G. 2003. Analysis of avocado allergen (Prs a 1) IgE-binding peptides generated by simulated gastric fluid digestion. *J Allergy Clin Immunol* 112:1002–1007.
- FAO/WHO. 2001. Evaluation of allergenicity of genetically modified foods, report of a joint FAO/WHO expert consultation, food and agriculture organization of the United Nations, Rome.
- Fu T-J, Abbott UR, Hatzos C. 2002. Digestibility of allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid—a comparative study. *J Agric Food Chem* 50:7154–7160.
- Herman RA, Korjagin VA, Schafer BW. 2005. Quantitative measurement of protein digestion in simulated gastric fluid. *Reg Toxicol Pharmacol* 41:175–184.
- Herman RA, Gao Y, Storer NP. 2006a. Digestion assays in allergenicity assessment of GMO proteins. *Environ Health Perspect* 114:1154–1157.
- Herman RA, Gao Y, Storer NP. 2006b. Acid-induced unfolding kinetics in simulated gastric digestion of proteins. *Reg Toxicol Pharmacol* 46:93–99.

- Jenkins JA, Griffith-Jones S, Shewry PR, Breiteneder H, Mills ENC. 2005. Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an *in silico* analysis. *J Allergy Clin Immunol* 115:163–170.
- Lee S-K, Yoon S-H, Kim S-H, Choi J-H, Park H-S. 2005. Chestnut as a food allergen: identification of major allergens. *J Kor Med Sci* 20:573–578.
- Metcalf DD. 2005. Genetically modified crops and allergenicity. *Nat Immunol* 6:857–860.
- Metcalf DD, Astwood JD, Townsend R, Sampson HA, Taylor ST, Fuchs RL. 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit Rev Food Sci Nutr* 36:S165–S186.
- Murtagh GJ, Dumoulin M, Archer DB, Alcocer MJ. 2002. Stability of recombinant 2 S albumin allergens *in vitro*. *Biochem Soc Trans* 30:913–915.
- SAS 1999. SAS/STAT user's guide, version 8. Cary, NC: SAS Institute.
- Srividhya J, Schnell S. 2006. Why substrate depletion has apparent first-order kinetics in enzyme digestion. *Computat Biol Chem* 30:209–214.
- Thomas K, Aalbers M, Bannon GA, Bartels M, Dearman RJ, Esdaile DJ, Fu TJ, Glatt CM, Hadfield N, Hatzos C, et al. 2004. A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. *Reg Toxicol Pharmacol* 39:87–98.
- United States Pharmacopeia. 2000. Simulated gastric fluid, TS. In: Board of Trustees, Editors. The National Formulary 19. Rockville, MD: United States Pharmacopeial Convention, Inc. p. 2235.
- Vieths S, Reindl J, Muller U, Hoffmann A, Hausteiner D. 1999. Digestibility of peanut and hazelnut allergens investigated by a simple in vitro procedure. *Eur Food Res Technol* 209:379–388.
- Yagami T, Haishima Y, Nakamura A, Osuna H, Ikezawa Z. 2000. Digestibility of allergens extracted from natural rubber latex and vegetable foods. *J Allergy Clin Immunol* 106:752–762.