



Establishing objective detection limits for the pepsin digestion assay used in the assessment of genetically modified foods

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ABSTRACT

Rationale: Guidelines for assessing the potential allergenicity of genetically modified (GM) organisms recommend testing the digestibility of the introduced protein by pepsin. Previous studies detailed the digestion procedure but have not described a simple objective measurement of the extent of digestion nor evaluated the impact of variation in pepsin activity.

Methods: Samples of eight proteins were digested by pepsin at pH 1.2 and 2.0 using standard conditions (10,000 U of pepsin activity per mg test protein) as well as 5000 and 20,000 units per mg of test protein. An independent digestion assay of hemoglobin was used to verify pepsin activity for each assay. Digestion was stopped in timed samples between 0.5 and 60 min. Digestion samples and undigested protein (10% and 100%) were separated by SDS-PAGE. Residual stained protein bands were measured by image analysis.

Results: The differences in pH and pepsin concentration only had minor effects on digestion of intermediately stable proteins: concanavalin A, ovalbumin, and lysozyme, but not on rapidly digested or stable proteins.

Conclusions: Verification of pepsin activity and measurement of an objective endpoint of digestion (e.g. (90%)) should provide more comparable results for the safety assessment of novel food proteins.

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1. Introduction

International guidelines for the safety assessment of genetically modified (GM) organisms used for food products include evaluation for potential increases in food allergenicity compared to the similar non-GM crop variety (CODEX, 2003). This assessment focuses primarily on the newly expressed protein encoded by the transferred gene. The risk of allergic reactions to a specific food produced from a GM organism would be increased most directly through the introduction of a strongly allergenic protein from a different organism into a species that did not previously express that protein as individuals previously sensitized to the protein may experience severe unexpected reactions by consuming food from the new GM crop. That possibility is best assessed by testing for specific serum IgE binding if the gene is from a commonly allergenic source using sera from individuals who have proven clinical allergy to the source of the new gene (Metcalf et al., 1996; Goodman et al., 2005; Goodman, in press). There is a slightly lower risk

to allergic consumers by the transfer of protein that is sequentially and structurally similar to a protein that causes their allergic reactions as allergic cross-reactions may occur due to preexisting IgE. Potentially cross-reactive proteins are most efficiently identified by amino acid sequence comparison with known allergens (Goodman, 2006). If a significant identity is found, sera from individuals allergic to the source and having specific IgE to the matched protein would be used to test for IgE binding (Goodman et al., 2005; Goodman, in press). A less direct risk is posed by the introduction of an abundant protein that is also highly stable to digestion by pepsin (Bannon et al., 2003) as these factors increase the probability of *de novo* sensitization and elicitation (Goodman et al., 2005). To date, there are no documented cases of allergy to the introduced proteins in approved GM crops primarily because of the rigorous testing that GM products undergo before marketing (Lehrer and Bannon, 2005). Nordlee et al. (1996) evaluated a GM soybean undergoing development that had nutritionally superior amino acid composition due to the introduction of a gene from Brazil nuts that encodes a high methionine 2S albumin. That protein was found to bind IgE from sera of individuals allergic to Brazil nuts and is now known to be the major Brazil nut allergen, Ber e 1. The discovery was made using a testing strategy similar to that recommended by the CODEX Alimentarius Commission Guidelines (CODEX, 2003). As we learn more about allergy and allergens there

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are opportunities to improve the techniques used to assess the potential allergenicity of novel proteins in foods. The allergenicity assessment includes testing the stability of the introduced protein in a pepsin digestion assay to help evaluate the probability the protein may sensitize individuals and lead to food allergy as proposed in Metcalfe et al. (1996). Importantly, slow digestibility of food proteins by the gastric protease, pepsin, is a characteristic commonly found with food allergens. However, digestibility by pancreatic enzymes (trypsin and chymotrypsin) proposed as an additional test by Astwood et al. (1996), does not appear to be predictive for food allergy (Goodman and Hefle, 2005).

Since the first description of the protein digestion assay in pepsin at pH of 1.2 (Astwood et al., 1996), a number of suggested modifications and criticisms have been published. The FAO/WHO recommendations for GM safety assessment suggested using both pH 1.2 and 2.0 in the digestion assay as conditions in the stomach are often above the optimum pH 1.2 (FAO/WHO, 2001). Fu et al. (2002) demonstrated that some dietary proteins (e.g. lectins) which do not cause allergic disease are quite stable in pepsin. Others have demonstrated that a number of proteins identified as allergens in fresh fruits that cause oral allergy symptoms are highly digestible in pepsin (Yagami et al., 2002), although a recent report suggests that matrix components in pectin-rich fruits may reduce digestibility of the fruit proteins both in vivo and in vitro (Polovic et al., 2007). Yet the predictive value of this assay with pure proteins has been reported to be quite high (Bannon et al., 2003). The assay was found to be robust in a study by nine-laboratories using 10 common proteins, a fixed ratio of pepsin to protein and both pH 1.2 and 2.0 to evaluate interlaboratory variation (Thomas et al., 2004). The importance of choosing specific protein staining methods to ensure appropriate levels of sensitivity was described by Goodman and Hefle (2005).

A few potential improvements should be considered to improve the objectivity and reliability of the pepsin digestion (stability) assay across laboratories. The original publications simply reported the time of disappearance of the proteins under fixed conditions of protein to pepsin, pH, and relatively standard staining of protein residues in SDS–PAGE sample gels (Astwood et al., 1996; Thomas et al., 2004). However, different proteins have different staining ability and detection levels between stains vary considerably. Further, description of the time to visual disappearance (as reported in Astwood et al., 1996 and Thomas et al., 2004) can provide markedly different results between analysts. Herman et al. (2005) chose a time-kinetic analysis of digestion to evaluate rates of digestion with a selected concentration of each protein and a pepsin concentration based on weight to volume described by Astwood et al. (1996) with pepsin product P7000 from Sigma chemical, but with a more purified pepsin product (Sigma product # 6887) used by Thomas et al. (2004). While kinetics could offer an advantage of more accurate determination of the rate of digestion for a given protein by pepsin, e.g. half-life of the protein under specific conditions, the ratio of pepsin to protein must be varied and optimum assay conditions determined for each protein for a true kinetic analysis, thus complicating the assay. The amount of test protein used in the digestion assay described by Herman et al. (2007) was based on molarity, requiring different masses of each test protein (e.g. a sevenfold difference in protein mass for a protein of 10 kDa vs. one of 70 kDa). Since pepsin is an endopeptidase that cleaves the peptide bond between many amino acids in a given sequences based, on the abundance of the susceptible or resistant short sequences and not the number of proteins, use of a constant mass provides a more reliable comparison as used by others (Astwood et al., 1996; Thomas et al., 2004). We have retained the use of constant mass, but have added a quantitative measurement to allow estimation of 90% reduction in stained protein residue in the gel as an endpoint for comparing digestibility. In practical terms

this method is similar to the calculation of time to reach 50% inhibition, as described by Herman et al. (2007), however, 90% digestion times are likely more relevant to the propensity of proteins to induce intestinal allergic symptoms than 50% digestion times. Here we report the digestion of a number of common proteins under different ratios of pepsin to protein and at pH 1.2 and 2.0 to provide data to judge the robustness of the assay. Porcine pepsin is reported to be optimally active at pH 2.2, with less reactivity at pH 1.5 (~90%) and pH 3.0 (~25%) as described by Bohak (1969). However, in our experience different proteins are optimally digested at pH 1.2 or pH 2.0, which may be caused by differential unfolding or amino acid charge modification of the test protein. We therefore sought to test digestion efficiency of a number of test proteins at both pH 1.2 and 2.0 using three activity levels of pepsin, representing 50%, 100%, and 200% of the recommendation of Thomas et al. (2004).

In order to improve assay reliability, we have added additional controls compared to the methods described in a number of other studies. Some of these controls have been used by companies (e.g. Monsanto), to test GM products, but have not previously been reported. The first is to perform a preliminary SDS–PAGE stained gel with samples of serially diluted target protein to evaluate staining characteristics of each test protein and to ensure detection down to 10% of the target protein. The second is to verify the activity of pepsin in the digestion fluid every time an assay is performed using an independent digestion of hemoglobin with the extent of digestion measured by spectrophotometry using the method described by Sigma Company to determine pepsin activity (Sigma, 2007), modified from the method of Anson (1938). The third is to include the 10% diluted control sample of undigested protein in the assay gel to control for differences in staining between experiments.

2. Objectives

The primary aim of this study was to test an objective endpoint for this assay using samples representing 10% and 100% of the starting material as benchmarks for comparison of digestion times. The secondary aim of the study was to evaluate the potential impact on results if the activity of pepsin is between 50% and 200% of the intended activity. All digestions were performed at both pH 1.2 and 2.0 to evaluate the potential impact of minor differences in pH upon stability under all conditions.

3. Materials and methods

3.1. Materials

The following products were purchased from Sigma Chemical Co., St. Louis, MO: bovine serum albumin (BSA, # A0281), ovalbumin (OVA, # A5503, lot 025K7001), jack bean concanavalin A (Con A, # C2010), β -lactoglobulin (BLG, # L0130), hemoglobin (HB, # H2625), ribulose 1,5-diphosphate carboxylase (RUBISCO, # R8000), bovine lactoferrin (LFN, # L9507), hen's egg lysozyme (LYS, # L7651), purified porcine pepsin (# P6887) and Brilliant Blue G-250 colloidal stain (# B2025), monoclonal mouse anti-OVA (#A6075) and 6.1 N trichloroacetic acid (TCA, #T0699). Ovalbumin from Worthington Biochemicals (OVA # 3054) was also tested as the Sigma OVA included two molecular weight forms of OVA. Pre-cast 10–20% Tris–glycine (# EC61355) and Tris–tricine (# EC66255) gradient gels, PVDF (polyvinylidenedifluoride) membrane (# LC2005), transfer buffer (# LC3675), glycoprotein staining kit (# P21857) and Novex® Sharp Protein Standard (# LC5800) were purchased from Invitrogen. Polyclonal goat anti-mouse-IgG conjugated to HRP (# 31432) and Supersignal West Dura extended duration substrate (# 34075) were purchased from Pierce. Phos-

phate buffered saline (PBS # 1666 789) was obtained from Roche. Precision plus molecular weight marker proteins (# 161-0374), Tween 20 (# 161-0781) and Tris–glycine running buffer (# 161-0772) were purchased from BioRad. Fisherbrand® 0.45 µm PTFE syringe filters (# 09-719H) and BD 10 mL syringes (# 14-823-2A) were purchased from Fisher Scientific. Test proteins were dissolved fresh at 5 mg/mL each day of use (BLG, BSA, Con A, HB, LFN, LYS, and OVA in distilled water, RUBISCO in 1× PBS). Simulated gastric fluid (SGF) was prepared as a solution of 0.084 N HCl and 35 mM NaCl and the pH adjusted to 1.2 or 2.0 with dilute HCl and NaOH. Purified powdered pepsin (Sigma P6887) was added to SGF based on the units of activity per mg of solid on the certificate of analysis from Sigma. The target activity level used by Thomas et al. (2004) was 10,000 units per mg of test protein. The digests are performed by mixing 1.52 mL of SGF containing 4000 pepsin activity units (2632 units per mL) with 0.08 mL of stock test protein (5 mg/mL protein). The specific lot of pepsin used in this study was certified by Sigma as having 3280 U of pepsin activity per mg of solid. A 30 mL stock solution representing the 100% activity was prepared by the addition of 24.1 mg of pepsin powder. The 200% digestion fluid was prepared by adding 48.1 mg to 30 mL of SGF and the 50% digestion fluid was prepared by adding 12.0 mg of pepsin to 30 mL of SGF. The digestion fluids were tested after addition of pepsin using a calibrated pH meter to verify the intended acidity. Previous tests have demonstrated the activity of pepsin was stable in solution for >6 h at room temperature or >12 h at 4 °C. Acidified hemoglobin was prepared by dissolving 0.2 g of hemoglobin in 8 mL distilled water, then adding 2 mL of 300 mM HCl.

3.2. Verification of pepsin activity

The activity of digestion fluid (pepsin in SGF, both pH 1.2 and 2.0) was verified by digesting hemoglobin for each assay under fixed conditions at 37 °C using a modification of the method by Sigma Chemical Company (Sigma, 2007), with adjustment for a dilution of the digestion fluid based on reported activity per mg solid and filtration to remove undigested hemoglobin rather than centrifugation, as described in this paper. A 0.10 mL sample of digestion fluid (~2632 U of pepsin activity per mL SGF) was diluted to 2.68 mL (therefore the dilution factor is 26.8) with SGF and 0.25 mL samples were added to three replicate polypropylene tubes containing 1.25 mL of acidified hemoglobin as the pepsin digestion samples. Addition to replicates was temporally spaced for equal 10 min digests at 37 °C. After 10 min, 2.5 mL of 5% trichloroacetic acid (1:20 dilution of 6.1 N TCA) was added to each digestion tube. The contents were mixed briefly, then allowed to stand for an additional 10 min at room temperature. The fluid was filtered through 0.45 µm PTFE syringe filters to remove undigested hemoglobin. Identical samples of diluted pepsin digestion fluid was added to sample blank tubes containing 1.25 mL of acidified hemoglobin and 2.5 mL of 5% trichloroacetic acid (to prevent pepsin activity). Addition to blank tubes and incubation times at 37 °C were the same as sample tubes and the contents were filtered as above. The absorbance at 280 nm was measured from the filtrate of each sample and blank tube using a spectrophotometer. The mean absorbance of the three blank samples (e.g. 0.403) was subtracted from the mean of the pepsin digestion samples (e.g. 1.37). The difference (e.g. 0.967) is then multiplied by the dilution factor (e.g. 26.8) and then divided by a constant factor of 0.01 (Sigma's constant 0.001 times 10 min). The result is reported in units of pepsin activity per mL of the digestion fluid (e.g. 2591 activity units/mL). To calculate the activity per mg of solid, divide by the amount of pepsin in the digestion fluid (e.g. 0.803 mg/mL) and the result (e.g. 3227 units per mg solid) is compared to the Sigma certified value (e.g. 3280). Historically we have accepted the certified activity from Sigma ±1000 activity units per mg solid. In this

study statistical analysis of the activity of eight individual digestion fluid samples at pH 1.2 and eight at pH 2.0 that were performed over 16 months, using pepsin from three different vials of the same lot of pepsin purchased over 12 months were evaluated to determine an acceptable quality control limit (see Section 4). Statistical analysis of pepsin activity was performed using SAS 9.1 version 2003.

3.3. Analysis of target protein concentration by SDS–PAGE and densitometry

Test proteins of varying proteolytic stability were assayed in this pepsin digestion study. These proteins included known allergens; BSA, OVA, BLG, LYS (Herman et al., 2007), a rarely reported allergen, LFN (Natale et al., 2004), and putative non-allergens; Con A, RUBISCO and HB (Herman et al., 2007; Thomas et al., 2004). Verification of the ability to detect each test protein at 10% undigested residual mass was accomplished by diluting samples of the protein to concentrations representing <5–200% of the 100% target sample (1.47 µg/well) in Laemmli buffer. Diluted samples were separated by SDS–PAGE and stained with Coomassie colloidal blue. To ensure consistency of measurement, all the gels were stained for a minimum of 6 h, and then destained to a uniform, clear background with 25% methanol. Stained gels were scanned with a Kodak Gel Logic 440 imaging system. The pixel densities of the protein bands were determined using the Kodak 1D Image Analysis Software. Prior to commencing assays reported here, tests were performed to optimize the method of selecting the area to analyze by densitometry. A uniform densitometry scan area was defined by drawing a single rectangular box to enclose the test protein band of a single lane, then copying and pasting the box over the corresponding band in each lane of the gel. The quantity of protein in each band was estimated by automatically subtracting the background intensity (pixels) using the “minimum of perimeter” function. The data was analyzed by linear regression and the R^2 value (Pearson correlation coefficient) was determined using Microsoft Office Excel 2003 to evaluate linearity and samples representing all proteins were readily distinguished from background at ~2.5% (0.037 µg/well).

Since OVA from Sigma appeared to run as two nearly equal intensity bands around the MW reported for OVA, OVA was also obtained from Worthington. Protein samples were separated in SDS–PAGE Tris–tricine gels (10–20%) which were stained by Coomassie colloidal blue or by glycoprotein stain. The Worthington OVA matched the higher of the two Sigma bands in migration distance (~45 kDa). The lower band migrated at ~39 kDa. Both samples also appear to have an undetermined amount of ovomucoid that runs as a smear at ~35 kDa. Ovalbumin has previously been reported to be glycosylated (Sheares and Robbins, 1986). The OVA bands of both products also stained with glycoprotein stain as did the putative ovomucoid smeared band. Separated samples of OVA were also transferred to PVDF by electroblotting, blocked with 5% non-fat dry milk in 1X PBS with 0.05% Tween 20 detergent (PBST), then probed with diluted OVA-specific murine monoclonal antibody for 2 h. The membrane was washed repeatedly in PBST, and then incubated with diluted polyclonal goat anti-murine IgG–HRP, washed again and bound antibody was detected by chemiluminescence (ECL). Based on the results the Worthington OVA was selected for the dilution analysis and for digestion studies to allow evaluation of a single band.

3.4. Protein digestion

The method of Thomas et al. (2004) was followed for the determination of the digestibility of the selected proteins using pepsin at 10,000 units of activity per mg of test protein, with minor modi-

fications. Independent assays were performed using three concentrations of pepsin corresponding to 20,000, 10,000, and 5000 activity units per mg of test protein. In this study, similar to that of Thomas et al. (2004), the digestion of each test protein was measured using independent tests at pH 1.2 and 2.0. Simulated gastric fluid (SGF) was prepared as described in Section 3.1 and the appropriate amount of pepsin added as described, to provide the desired activity. An activity assay was used to verify the 100% pepsin concentration (10,000 units pepsin activity per mg test protein). Each reaction mixture was made up of 1.52 mL of the appropriate SGF plus pepsin digestion fluid, preheated to 37 °C and then 0.08 mL of test protein solution (at 5 mg/mL) was added and briefly vortexed before returning to the 37 °C waterbath digestion. Control samples of pepsin in SGF without test protein and test protein in SGF without pepsin were also prepared and treated at the same time to evaluate possible auto-digestion of pepsin and chemical hydrolysis of test protein (e.g. appearance of small molecular weight bands). At each time point (0.5, 2, 5, 10, 20, 30, 60 min) of digestion, 0.2 mL of the reaction mixture was withdrawn and added to a tube containing 70 μ L of NaHCO₃ buffer (pH 11) and 70 μ L of 5 $\times$ reducing Laemmli buffer (Laemmli, 1970) buffer, then mixed by vortexing and heated for 10 min at >80 °C to quench the activity of the pepsin. Time 0 samples were quenched before adding test protein. Samples of controls and all digestion samples were separated by SDS–PAGE the same day or stored at –20 °C for later analysis.

The extent of protein digestion of each test protein was measured by separating digested samples in SDS–PAGE, staining with Coomassie colloidal blue and measuring residual protein bands by image analysis as described above. Tris–glycine gels were used for all proteins except OVA for which Tris–tricine gels were used to allow better resolution of the OVA from pepsin. The stained image densities of residual intact protein bands were determined using the 1D Image Analysis software (Kodak). The time required to digest the specific test protein by 90% (down to 10% undigested level) was calculated by comparing pixel counts of digestion samples at each sample time point to the 100% and 10% control samples. Test proteins that digested so rapidly that 0.5 min sample was already below the 10% control band intensity were listed as requiring <0.5 min for 90% digestion. Test proteins that were digested slowly so that the protein band intensity at 60 min of digestion was still greater than the 10% control sample were listed as >60 min. For comparison purposes, two methods were used to calculate the time to 90% digestion for those reaching the 10% control level between 0.5 and 60 min (Con A, LYS, and OVA). The first was a linear interpolation to solve for “x” using a standard linear equation ($y = mx + b$), assuming “y” was equal to the pixel count of the 10% control, based on the two sample points having pixel counts that bracketed the 10% control sample value. The second method was by calculating the best fit line using the polynomial regression function of Microsoft Office Excel version 2003, and solving for “x”, setting “y” equal to the pixel count for the 10% control.

4. Results

4.1. Detection of test proteins by colloidal blue staining of samples separated by SDS–PAGE

All of the test proteins were detected below 10% of the initial sample concentration when stained with Coomassie colloidal blue dye (Fig. 1). The lowest R^2 values for the diluted proteins were for 0.79 (BLG) and HB (0.87), while all others ranged from 0.93 to 0.99 for concentrations representing from 2.5% to 189% or 200% of the target protein value (100% = 1.47 μ g/well).

The OVA from Sigma contained a mixture of two molecular weight proteins as the dominant stained proteins by SDS–PAGE

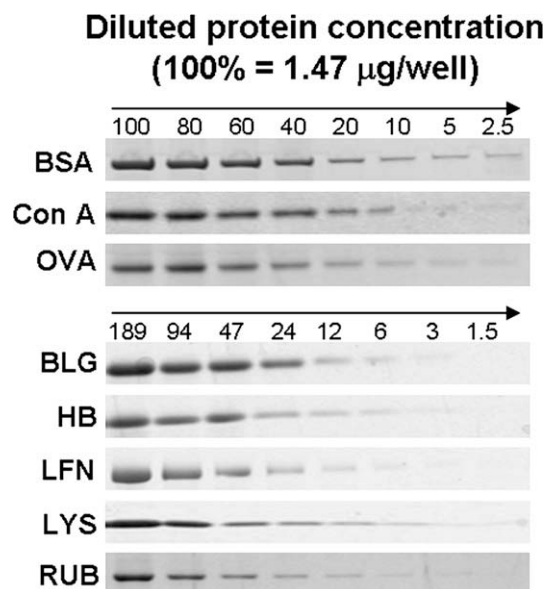


Fig. 1. Coomassie colloidal blue stained protein detection limits in SDS–PAGE. BSA, Con A, and OVA were diluted from 100% to 2.5% and LFN, RUB, LYS, HB, and BLG were diluted from 189% to 1.5%. Individual samples were separated in 10–20% Tris–glycine SDS–PAGE with reducing buffer, fixed briefly in acetic acid and stained for a minimum of 6 h in Coomassie colloidal blue. Gels were destained in 25% methanol, images were captured using a Kodak Gel Logic 440. Image analysis was performed using Kodak 1D software.

(Fig. 2). Since OVA is a glycoprotein it was assumed that the higher molecular weight band might be glycosylated and the lower one not contain glycan. The OVA from Worthington was primarily of a single band at the same apparent molecular weight as the more slowly migrating band from Sigma. The two OVA bands in the Sigma sample and the one band in the Worthington sample all stained well with glycoprotein stain as well as with Coomassie colloidal blue. An immunoblot with a monoclonal antibody to OVA demonstrated that the two stained bands from Sigma and the single stained band from Worthington apparently OVA (Fig. 2). Therefore the difference in the two bands in OVA from Sigma is not known.

4.2. Validation of pepsin activity

The pepsin activities of the SGF used for each of the digestion assays were within our previous historical range of the Sigma certified value (3280) $\pm$ 1000 units of the Sigma assayed value of 3280 units per mg solid (Table 1). However, we wanted to establish a statistically justified value for quality control purposes. The grand mean values were calculated from the eight tests at pH 1.2 and pH 2.0 (Table 1). These were found to be not statistically different from the labeled value of 3280 units per mg solid from Sigma ($p = 0.458$ for pH 1.2 and $p = 0.920$ for pH 2.0 using $\alpha = 0.05$). The variation of this assay was then estimated based on the percent represented by 3 times the standard error of the mean from this data set compared to the labeled value from Sigma. Table 1 shows the limit should be 21.9% based on data from pH 1.2 or 22.7% based on pH 2.0. Based on this data, digestion fluid (SGF plus pepsin) activity that falls outside of the range of the Sigma certified activity per mg, $\pm 23\%$ will be rejected in our laboratory and not used to evaluate test protein stability.

4.3. Digestibility of rapidly degraded proteins

Irrespective of the pH (pH 1.2–2.0) and pepsin-to-substrate ratio (activity from 5000 to 20,000 pepsin activity units per mg test protein) used; BSA, HB, LFN, and RUBISCO were all digested to less

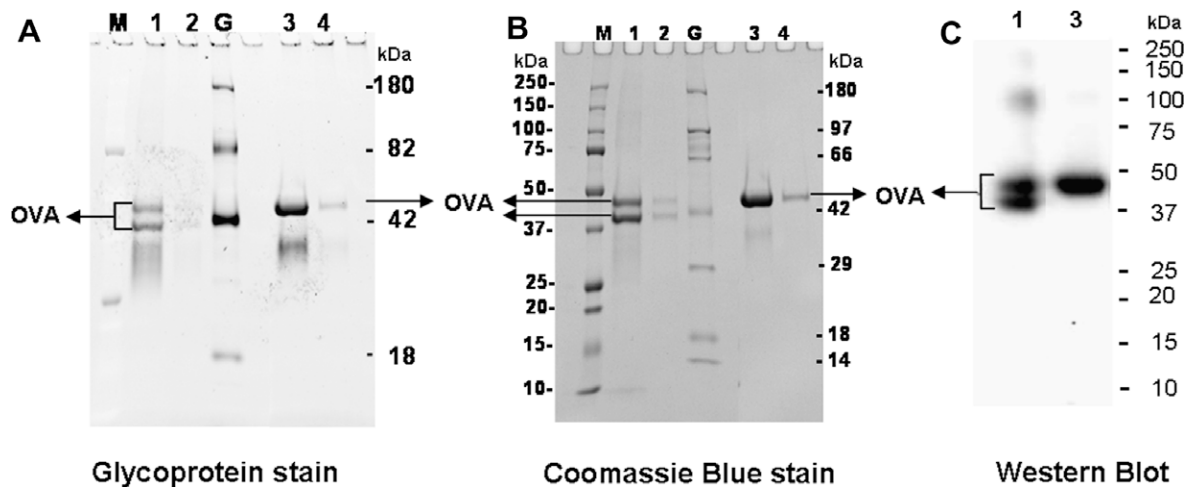


Fig. 2. Ovalbumin detection by glycoprotein stain, Coomassie blue and monoclonal anti-OVA antibody. Ovalbumin from Sigma Chemical Company (# A5503) and Worthington Biochemical Company (# 3054) were separated by SDS–PAGE with reducing Laemmli buffer. One gel was stained with glycoprotein stain from Sigma (A), one with Coomassie Colloidal blue stain (B) and one was blotted onto PVDF and the membrane was incubated with monoclonal anti-OVA, then with HRP-conjugated goat anti-murine IgG and detected by ECL. Samples included (lane M) Precision Plus molecular weight markers (BioRad); Sigma OVA at 1.47 µg (lane 1) and 0.147 µg (lane 2); Invitrogen CandyCane molecular weight standards (lane G); Worthington OVA at 1.47 µg (lane 3) or 0.147 µg (lane 4).

Table 1
Pepsin activity assay digestion of hemoglobin

Associated test protein assay	Mean-specific pepsin activity ± SD (units per mg solid)	
	pH 1.2	pH 2.0
BLG	3125 ± 27	3086 ± 49
BSA	2913 ± 160	2938 ± 62
Con A	2833 ± 32	2990 ± 210
HB	3450 ± 13	3215 ± 5
LFN	3925 ± 58	4025 ± 140
LYS	3990 ± 80	3921 ± 300
OVA	3427 ± 130	3002 ± 100
RUB	3525 ± 12	3175 ± 10
Grand mean ± SD	3399 ± 413	3296 ± 430
QC limit = to ± 3 × SEM	717	746
QC limit percent of Sigma label (3280 units per mg solid)	21.9%	22.7%

The activity of pepsin in SGF (at pH 1.2 and 2.0) was measured for each new digestion fluid. The test protein digests associated with the assayed SGF plus pepsin batch are shown. The labeled activity of Sigma Chemical Co. product P6887 used in these assays was 3280 activity units per mg of solid. Daily mean-specific activity values ($n = 3$) and the standard deviation are indicated for each protein assay and pH value. Grand mean ($n = 8$) and standard deviations are shown along with a calculated quality control limit equal to three times the standard error of the mean (SEM). Based on this data we have established a new quality control value as the labeled value $\pm 23\%$ (rounded up for simplicity).

than 10% residual full-length protein at the first sample point, 0.5 min (Fig. 3 and Table 2). However, digestion of both BSA (Fig. 3A) and LFN (Fig. 3C) produced small molecular weight ($<10,000$ Da) stable proteolytic fragments that were visible throughout the 60-min incubation period. The HB and RUBISCO digests (Figs. 3B and D, respectively) did not produce any significant level of detectable proteolytic fragments. Incubation of LFN at pH 1.2 in SGF without pepsin resulted in partial degradation of the protein with subsequent appearance of four prominent smaller molecular weight fragments and a number of minor bands. Subsequent investigations of stability of LFN in SGF without pepsin, at pH 1.2, 2.0, and 3.0 and different temperatures (25 and 37 °C) for up to 60 min revealed that LFN is auto-degraded most extensively at pH 1.2 and 37 °C by 60 min. Although some degradation was observed, with generation of minor amounts of similar smaller fragments at pH 2 and 3 at 37 °C, but not at 25 °C. This LFN product from Sigma was estimated to be more than 90% pure based on

stained gel densitometry (Sigma). Degradation may be due to a contaminant, or an inherent property of LFN. However, this phenomenon does not seem important in the digestion of LFN by pepsin as the primary protein is rapidly digested (<0.5 min) at both pH 1.2 and 2.0 in the presence of pepsin.

In order to more accurately estimate the size of the relatively stable fragments of BSA and LFN that were generated due to partial digestion by pepsin, samples of the digests at pH 1.2, with the target pepsin digestion fluid (10,000 units per mg solid) were separated in Tris–tricine SDS–PAGE (Fig. 4) along with Novex® Sharp protein standards that include a 3.5 kDa marker. Gels of both proteins show larger intermediately stable fragments and small fragments that are stable up to 60 min. The stable fragment in BSA is less than 4 kDa. Two stable fragments of LFN were detected (5.4 and 4.5 kDa). It is not possible to estimate the quantity of these peptides or the rate of digestion as the fragments are generated during digestion. The relative abundance of the intact proteins and fragments were estimated by densitometry (Fig. 4). The small stable fragments are potentially long enough to effectively cross-link IgE on the surface of mast cells, if they contain at least two IgE binding epitopes, based on synthetic allergen models as discussed in Bannon et al. (2003). However, they are also close to the size of the smallest known natural allergens and are thus less likely than larger proteins to act as allergenic targets. Melitin of honey bee venom is a weak allergen of approximately 2900 Da and has only one IgE binding epitope, but cross-links IgE on mast cells either through melitin–melitin interactions or by cell surface membrane and IgE interactions (Fehlner et al., 1991). The next smallest allergen reported is a short ragweed pollen allergen Amb a 5 and a western ragweed homologue, Amb p 5 (Ghosh et al., 1994), with mature peptide sizes of 5900 Da. Thus, the stable fragments of BSA and LFN appear to be near the theoretical limit in size to act as allergens.

4.4. Digestibility of relatively stable proteins

BLG was stable for 60 min at pH 1.2 and 2.0 in pepsin, even using 20,000 units of pepsin activity per mg of BLG (Fig. 5A and Table 2). Con A was digested within 50 min of incubation with pepsin although the resulting fragments were stable for more than 30 min (Fig. 5B). Although the calculated time to 90% digestion of Con A

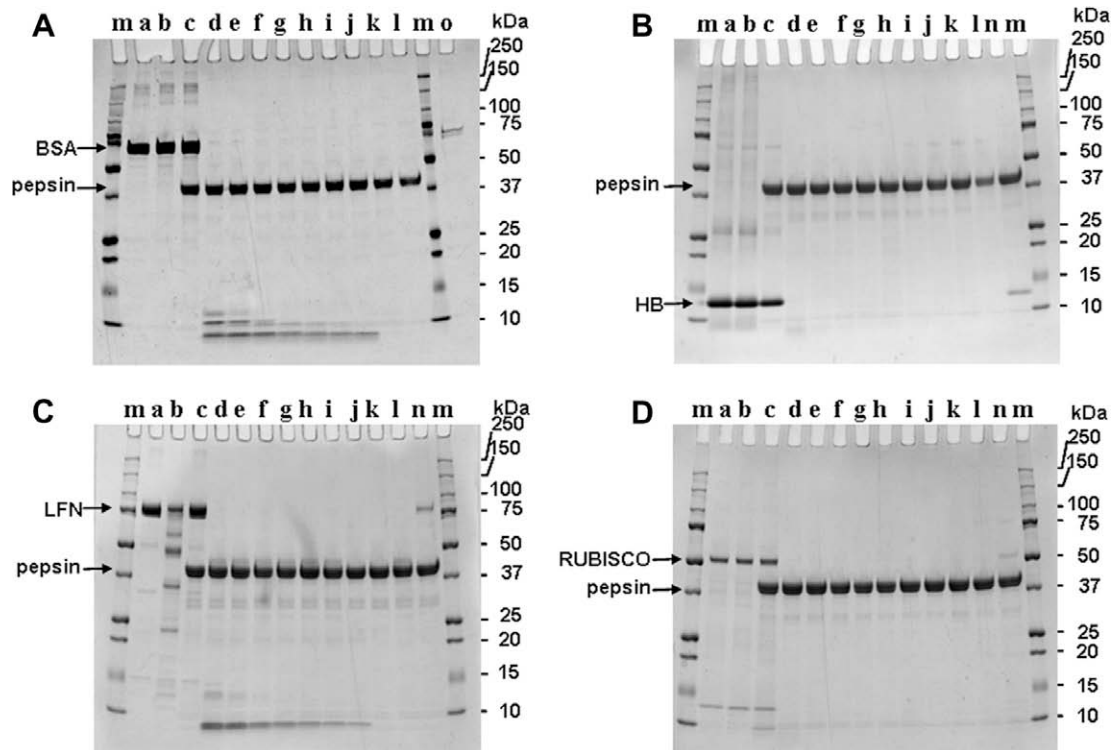


Fig. 3. Detection of pepsin-labile proteins. Samples of BSA (A), HB (B), LFN (C), and RUBISCO (D) were digested in pepsin at pH 1.2 with 10,000 pepsin activity units per mg test protein. Digestion products were separated in SDS-PAGE 10–20% gels under reducing conditions. Sample lanes: (m), Precision Plus molecular weight marker protein; 1.47 mg of undigested test protein without pepsin (a) at time 0; (b) at time 60 min; (c–j) test protein plus pepsin at times 0, 0.5, 2, 5, 10, 20, 30, and 60 min of digestion (respectively); (k) pepsin alone at time 0; (l) pepsin alone at time 60 min; (n) undigested test protein 0.147 μ g (10%) at time 0 with pepsin; (o) undigested test protein 0.147 μ g (10%) without pepsin.

varied from 19 to 50 min using different pH and pepsin to protein ratios, all times would be considered stable relative to the rapid digestion of BSA, HB, LFN, and RUBISCO. LYS was stable for 60 min in pepsin at pH 2.0 for all activity levels of pepsin tested although at pH 1.2 more than 90% of the protein was digested by 30 at the highest pepsin activity level, but not until approximately 50 min with either 10,000 or 5000 units of pepsin (Fig. 5C and Table 2). Digestion of OVA (Worthington) by 90% was achieved within 13–27 min of incubation with pepsin at pH 2.0 and between 8 and 26 min at pH 1.2 (Fig. 5D and Table 2).

4.5. Comparison of digestion times using endpoints of 50% and 10% residual protein

Digestibility assays performed by Astwood et al. (1996) and Thomas et al. (2004) have used of the loss of visually detected stained bands as an endpoint while Herman et al. (2005) used 50% loss as measured by densitometry. We have chosen 90% digestion (10% residual stained full-length protein) as an endpoint. Percent digestion of six test proteins were graphed at both pH 1.2 and 2.0, using the standard 10,000 pepsin activity units per mg of pro-

Table 2
Summary of test protein digestion times at pH 1.2 and 2.0

Test proteins	Digestion time in minutes ^{a,b} at pH 1.2 (pepsin activity: sample test protein)			Digestion time in minutes ^{a,b} at pH 2.0 (Pepsin activity: sample test protein)		
	20 kU:1 mg	10 kU:1 mg	5 kU:1 mg	20 kU:1 mg	10 kU:1 mg	5 kU:1 mg
BLG	>60	>60	>60	>60	>60	>60
BSA	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Con A	19 ^a (20 ^b)	23 ^a (23 ^b)	22 ^a (31 ^b)	30 ^a (23 ^b)	29 ^a (33 ^b)	47 ^a (46 ^b)
HB	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
LFN	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
LYS	28 ^a (29 ^b)	49 ^a (49 ^b)	51 ^a (52 ^b)	<60 ^a (65 ^b)	<60 ^a (62 ^b)	<60 ^a (64 ^b)
OVA	9 ^a (12 ^b)	19 ^a (21 ^b)	13 ^a (23 ^b)	13 ^a (16 ^b)	25 ^a (23 ^b)	27 ^a (25 ^b)
RUB	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

The digestion time (in minutes) is shown for each test protein at three pepsin activity levels (20,000; 10,000; and 5000 activity units per mg test protein) for pH 1.2 and for pH 2.0. For samples having a pixel count at 60 min markedly greater than the pixel count of the 10% undigested control, digestion was estimated to be >60 min (BLG and LYS at pH 2.0). For samples with the pixel count at time 0.5 being markedly less than the pixel count of the 10% undigested sample, the time for digestion is considered <0.5 min. The other sample digestion times were calculated using both a linear extrapolation method and polynomial regression.

^a Time of digest of the full-length protein to 10% residual mass was calculated using linear interpolation ($y = mx + b$), by solving for time (x) with $y = \#$ pixels of the band in the 10% control sample, b = the intercept when $X = 0$ and the slope, m is calculated from the pixel counts and times for the two sample points that bracketed the 10% control sample.

^b Time of digestion time of digestion of the full-length protein to 10% residual mass was calculated using the polynomial function of Microsoft Office Excel 2007 ($y = ax^2 + bx + c$), with $y = \#$ pixels of the band in the 10% control sample, and solving for x using the line calculated by Excel with all seven points from the individual digestion points.

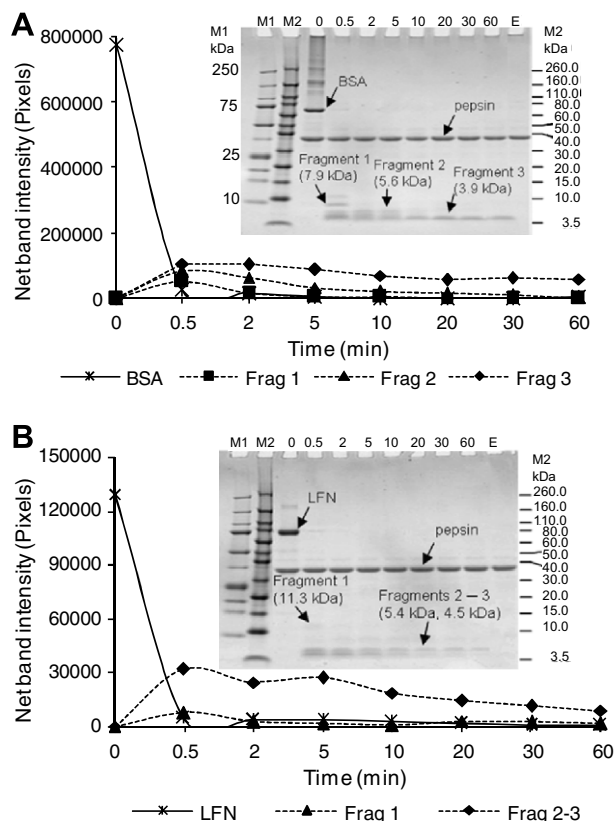


Fig. 4. Detection of pepsin-stable peptide fragments. Pepsin digestion samples for BSA (A) and LFN (B) were separated by SDS–PAGE in 10–20% Tris–tricine gels with reducing Laemmli buffer, then stained with Coomassie colloidal blue, destained and images were captured by Kodak Gel Logic 440. Pixel densities were analyzed by Kodak 1D software. Sample lanes: (M1), Precision Plus MW marker; (M2), Novex Sharp Protein Standard; (0), undigested protein with quenched pepsin, (0.5–60), pepsin digestion samples at times of 0.5 min to 60 min; (E), pepsin alone. Graphs represent the net pixel intensity (y-axis) over time in minutes of digestion (x-axis). Fragments are represented on the gel images and the graphs.

tein conditions to allow comparison of 50% and 90% digestion times (Fig. 6A and B, respectively). The BSA and RUBISCO samples are shown as representative rapidly digested proteins (LFN and HB are similar). The milk allergen, BLG was not reduced below 80% residual protein under any conditions and should clearly be considered a stable protein. Minor differences were observed for digestion times between pH 1.2 and 2.0 for Con A, LYS, and OVA. While it is clearly possible to differentiate between rapidly digested proteins (BSA and RUBISCO, which are weak or non-allergenic in foods) and highly stable proteins (e.g. BLG, that is an undisputed food allergen) by visual inspection, by measuring 50% digestion or 90% digestion times, the 90% digestion time seems to provide greater discrimination for the intermediately stable proteins OVA, Con A, and LYS (allergenic or immunomodulatory proteins).

5. Discussion

5.1. Detection of varying concentrations of proteins

Studies by Astwood et al. (1996) and Thomas et al. (2004) used visualization of undigested stained proteins in SDS–PAGE to estimate the time of disappearance of test proteins in the SGF with pepsin digestibility assay. However, specific proteins stain with different efficiencies using Coomassie blue, based upon the amino acids composition and it is therefore useful to include controls

within each assay. Further the studies did not present a relative concentration standard, besides the 100% undigested protein, within the assay gel, and did not report verification of the level of sensitivity for detecting residual protein. Yet the simple method is intuitively sound as it allows evaluation of residual intact protein as well as identification of fragments of the test protein that are highly stable in pepsin (Bannon et al., 2003). Herman et al. (2003) reported the use of densitometry to accurately quantify crystal proteins from *Bacillus thuringiensis* on SDS–PAGE, except when low protein concentrations were used, their results suggested probable over-estimation of residual protein. We sought to improve the reliability of the assay by verifying the detection sensitivity of the individual test proteins by using densitometry to estimate relative protein concentration of the undigested protein and verification of the sensitivity using a dilution series to verify the sensitivity is below 10% residual value. Our results show that the individual test proteins do stain slightly differently in the gels. Evaluation of linearity demonstrated staining was proportional to the concentration. In previous tests with collagen and ovomucoid (unpublished), we have found Coomassie stain did not effectively stain the protein and therefore this method of detection does not provide a reliable measure for this assay for those specific proteins. Thus, we strongly recommend including undigested sample of test protein at both 100% and 10% as controls for digestibility.

5.2. Activity of SGF with pepsin

In order to ensure reproducible results, the activity of the specific digestion fluid containing SGF and pepsin should be tested using an independent method. It would be possible to routinely digest an intermediately stable protein in parallel with the protein under test. However, we have chosen to use the hemoglobin digestion assay used by Sigma to evaluate pepsin activity as a fully independent measure. As shown by repeated assay at pH 1.2 and 2.0, the activity of pepsin from three bottles of one lot of pepsin was not statistically significantly different from the certified value over a 16 month test period, with three different analysts. Individual activity measurements by the method described in this paper demonstrate an expected variance of up to 23% from the Sigma certified label should be accepted as equivalent. Based on results in this paper (tested at 50% and 200% activity), an absolute difference of 25% in activity from the intended level is not expected to significantly impact the digestibility of test proteins.

5.3. Interpreting results of digestion of test proteins

This assay is used as one of a few simple tools for evaluating the potential risk that a new dietary protein might become an important food allergen when there is not a substantial, clear history of consumption and of allergenicity of the protein. The method first described by Astwood et al. (1996), and the verification ring-study by Thomas et al. (2004) used visual time disappearance as an endpoint, which is a very subjective measurement. Herman et al. (2003) estimated the half-life of the test protein, assuming pseudo-first-order kinetics. However, it is clear from the rapid digestion of a number of test proteins (BSA, HB, LFN, and RUBISCO) that an accurate estimate of half-life, or even a time to achieve 90% digestion is not possible with the simple conditions of protein and pepsin concentration used historically for this assay for such rapidly digested proteins. Those proteins should be considered highly digestible by pepsin and a more accurate estimate of rate of digestion is not necessary in the context of the safety assessment. It is likewise impossible to accurately estimate the half-life, or the 90% digestion time for a protein like BLG under the conditions of the assay as more than 50% of the starting material remained at

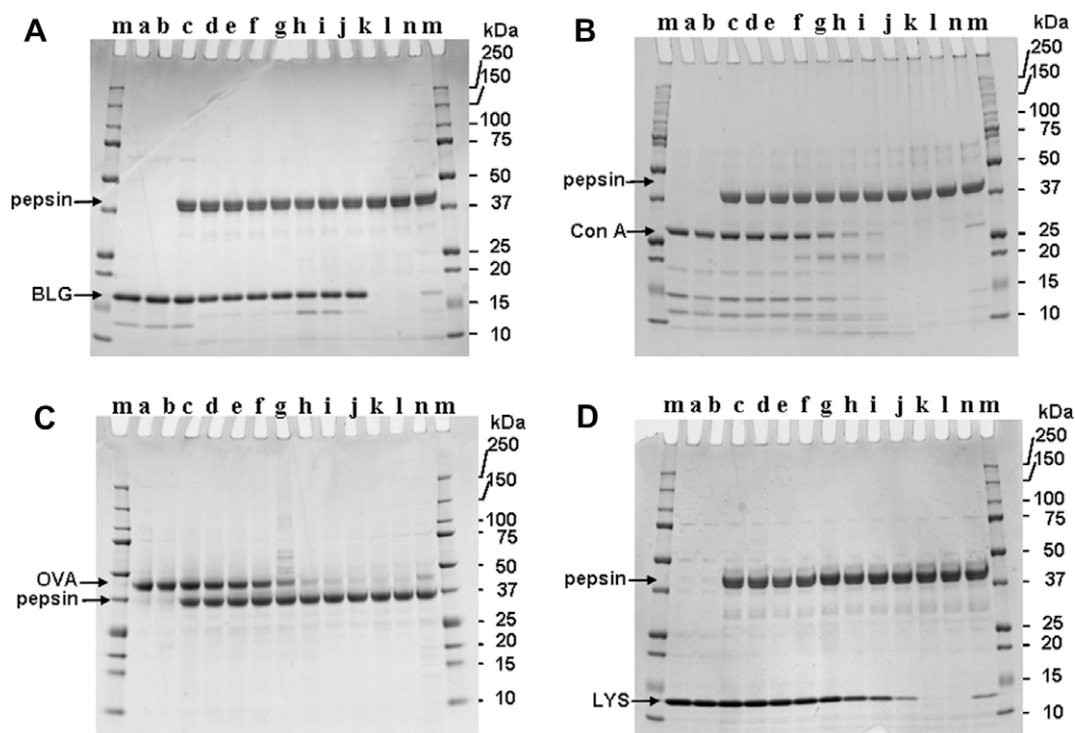


Fig. 5. Detection of pepsin-stable proteins. Samples of BLG (A), Con A (B), OVA (C), and LYS (D) were digested in pepsin at pH 1.2 with 10,000 pepsin activity units per mg test protein. Digestion products were separated in SDS–PAGE 10–20% gels under reducing conditions. Sample lanes: (m), Precision Plus molecular weight marker protein; 1.47 mg of undigested test protein without pepsin (a) at time 0; (b) at time 60 min; (c–j) test protein plus pepsin at times 0, 0.5, 2, 5, 10, 20, 30, and 60 min of digestion (respectively); (k) pepsin alone at time 0; (l) pepsin alone at time 60 min; (n) undigested test protein 0.147 μ g (10%) at time 0.

60 min. BLG is an abundant milk protein and a noted potent allergen. Other similarly stable food proteins should be suspected as being allergenic especially if highly abundant, unless there is a clear history of human dietary exposure without incidence of allergy. Similarly stable proteins can be assumed to carry an increased risk of sensitization or elicitation without a more accurate estimate of digestibility.

These results demonstrate that the conditions needed to accurately determine the kinetic characteristics of individual test proteins will not be met in each case. Clearly the rate of digestion of specific test protein substrates vary, meaning there are differences in rate constants and for some proteins first-order reaction rates cannot be assumed (Choisnard et al., 2002; EPA, 2005). One potential reason for differences is the combinations of amino acids, or modified amino acids that are encountered as pepsin processes the target proteins may alter the structure of pepsin at the active site (Sachdev and Fruton, 1975; Marciniszyn et al., 1976). The protein can in fact act as an inhibitor. The high degree of stability for BLG was also noted in other studies (Thomas et al., 2004; Takagi et al., 2003; Astwood et al., 1996; Guo et al., 1995). The BLG protein has five cysteines and four of these are reported as being involved in formation of intramolecular disulfide linkages to give BLG a highly folded and globular structure (Papiz et al., 1986). Reduction of the disulfide bonds of BLG renders it more susceptible to proteolysis by trypsin but not by pepsin (Walstra and Jenness, 1984). The chemical reduction of BLG at pH 8 is more effective compared to reduction at pH 2.0 (Walstra and Jenness, 1984; Guo et al., 1995) which is near the optimum pH for pepsin activity. Guo et al. (1995) reported an increase in digestibility of BLG after heating at 100 °C for 5–10 min or after addition of urea at a concentration greater than 3 M, conditions that should denature the protein, demonstrating, that the three-dimensional structure of BLG is important in preventing proteolysis by pepsin.

The resulting stability of such proteins is assumed to be a factor making such proteins more likely to act as food allergens. An accurate estimate of the kinetics of digestion is not necessary to evaluate the correlation of potential food allergy. We evaluated five proteins (BLG, BSA, Con A, OVA, and RUBISCO that were also tested by Thomas et al. (2004). The times we report for digestion are quite similar to those reported by Thomas et al. (2004) although they scored disappearance strictly based on the sample time, without extrapolating between times. Our results also verify the pattern of stability shown for LYS in the Thomas et al. (2004) paper, as it was identified as the visible contaminant in ovomucoid and was clearly stable, as well as results by Fu et al. (2002) and Yoshino et al. (2004). The use of 90% digestion as an endpoint measured by densitometry, and inclusion of 10% sample in the gel as well as verification of the pepsin activity should provide more objective testing across time and across laboratories as Thomas et al., demonstrated some marked variance in results that were probably due to methods of gel staining between laboratories, presumably due to differences in staining.

There is evidence that the relatively stable proteins LYS and OVA are food allergens (Walsh et al., 2005) and others have reported similar stability of these proteins (Astwood et al., 1996; Fu et al., 2002; Thomas et al., 2004; Yoshino et al., 2004). The other intermediately stable protein, Con A, while not known to be allergenic (Thomas et al., 2004) is a lectin with immunological activity and is known to resist digestion by pepsin (Thomas et al., 2004; Takagi et al., 2003; Haas et al., 1999). Based on our data using standard conditions (10,000 units pepsin activity per mg protein at either pH 1.2 or 2.0), proteins that are not digested to 10% or less residual mass by approximately 20 min could be considered to have an increased risk of causing food allergy.

It is also important to consider stable fragments of proteins that digest rapidly such as the dominant peanut allergen Ara h 2, which

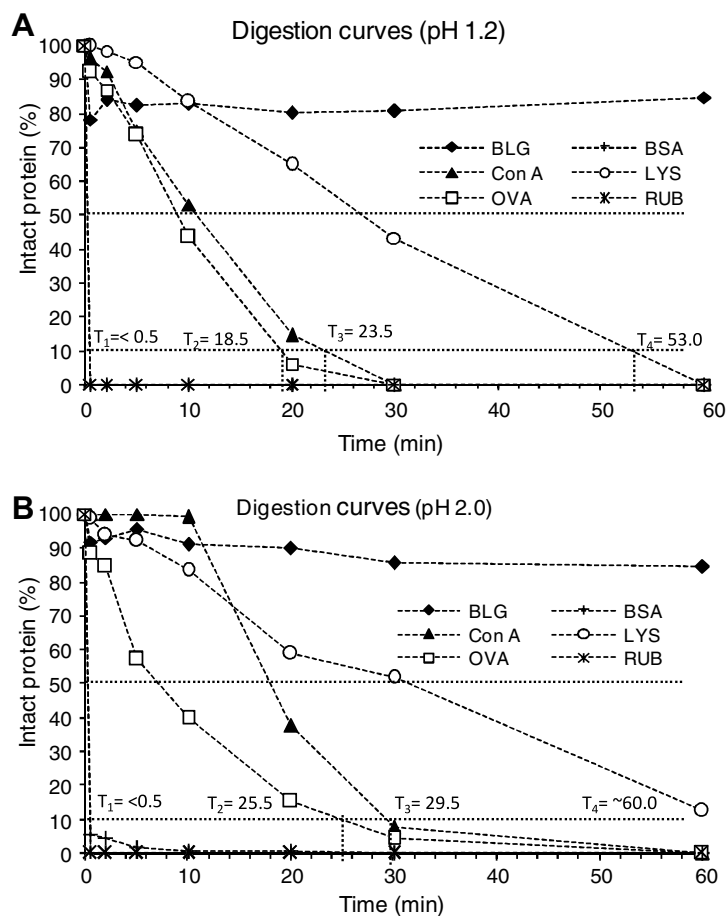


Fig. 6. Comparison of digestion results using 50% and 90% digestion endpoints. Degradation curves of the test proteins at pH 1.2 are shown in (A) and at pH 2.0 are shown in (B). Percent digestion is shown on the y-axis and time of digestion on the x-axis. Sample time points representing minutes of digestion are shown for time = 0 to time = 60 min. The line representing 10% of undigested protein is based on the actual pixel count of the 10% stained gel sample. The 50% line is estimated mathematically based on the 100% and 10% samples. Test proteins: BLG (solid diamond), Con A (solid triangle), OVA (open box), BSA (plus sign), LYS (open circle), RUBISCO (X). Ninety percent digestion times are marked at the indicated times for the different protein substrates: T_1 , BSA and RUB; T_2 , OVA, T_3 , Con A; T_4 , BLG.

produces a stable fragment of over 7 kDa that includes important IgE binding epitopes (Thomas et al., 2004; Sen et al., 2002). Since the stable fragments appear during digestion, it not possible to provide a quantitative estimate of digestion. We suggest that the appearance of stable fragments like fragment 3 from BSA (Fig. 4A) or the doublet labeled as fragment 2 and 3 in LFN (Fig. 4B) may indicate some risk of allergenicity, if the fragment size is greater than 5000 Da.

One of the criticisms of the pepsin assay for evaluating safety has been that pure proteins are tested, yet foods represent complex matrices that may influence protein stability (Guo et al., 1995). Also, Moreno (2007) inferred from the study by van Wijk et al. (2005) the possibility the food matrix may act as an adjuvant in sensitization. Experiments carried out by digesting pure proteins do not account for the effects of food matrices and therefore the allergenicity predictions regarding susceptibility of digestion correlating with the potential allergenicity of proteins in their native state may be misleading for proteins in certain matrices. Such an effect may be responsible for the less than perfect predictive value of pepsin digestion with allergenicity and deserves further study in order to improve the predictive value of the assay. Yet as discussed by Bannon et al. (2003), the current method already has a substantial correlative value, and is one of the studies that appears useful in the weight of evidence approach recommended by Codex and others (Goodman et al., 2005).

6. Conclusions

The use of image analysis (densitometry) of stained gels with a referenced endpoint of 90% loss of starting test protein using standard conditions is simple, convenient and provides reproducible results that are likely to improve laboratory to laboratory comparisons of digestibility. This assessment requires inclusion of a undigested samples representing 100% and 10% of the test protein sample run concomitantly with pepsin digests. Our results demonstrate differences of roughly 50% in reported digestion times for intermediately stable proteins when pepsin activity is 50% or 200% of the target level. Therefore we recommend verification of the activity of pepsin in the digestion fluid used to assess protein stability. Digestion results obtained at pH 1.2 are not substantially different from those at pH 2.0. Proteins that are not digested by 90% in 20 min carry an increased risk of causing food allergen. Proteins that produce pepsin-stable peptide fragments of more than 5 kDa may carry an increased risk of causing food allergy.

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