

## A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins

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Received 30 September 2003

### Abstract

**Rationale.** Evaluation of the potential allergenicity of proteins derived from genetically modified foods has involved a weight of evidence approach that incorporates an evaluation of protein digestibility in pepsin. Currently, there is no standardized protocol to assess the digestibility of proteins using simulated gastric fluid. Potential variations in assay parameters include: pH, pepsin purity, pepsin to target protein ratio, target protein purity, and method of detection. The objective was to assess the digestibility of a common set of proteins in nine independent laboratories to determine the reproducibility of the assay when performed using a common protocol.

**Methods.** A single lot of each test protein and pepsin was obtained and distributed to each laboratory. The test proteins consisted of Ara h 2 (a peanut conglutinin-like protein),  $\beta$ -lactoglobulin, bovine serum albumin, concanavalin A, horseradish peroxidase, ovalbumin, ovomucoid, phosphinothricin acetyltransferase, ribulose diphosphate carboxylase, and soybean trypsin inhibitor. A ratio of 10 U of pepsin activity/ $\mu$ g test protein was selected for all tests (3:1 pepsin to protein, w:w). Digestions were performed at pH 1.2 and 2.0, with sampling at 0.5, 2, 5, 10, 20, 30, and 60 min. Protein digestibility was assessed from stained gels following SDS-PAGE of digestion samples and controls.

**Results.** Results were relatively consistent across laboratories for the full-length proteins. The identification of proteolytic fragments was less consistent, being affected by different fixation and staining methods. Overall, assay pH did not influence the time to disappearance of the full-length protein or protein fragments, however, results across laboratories were more consistent at pH 1.2 (91% agreement) than pH 2.0 (77%).

**Conclusions.** These data demonstrate that this common protocol for evaluating the in vitro digestibility of proteins is reproducible and yields consistent results when performed using the same proteins at different laboratories.

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**Keywords:** Digestive stability; Protein allergenicity; Biotechnology; Genetically modified foods; Pepsinolysis

## 1. Introduction

Historically, the evaluation of the potential allergenicity of novel gene products (that is, proteins) derived from genetically modified (GM) foods has involved the use of a decision-tree strategy that incorporated an evaluation of protein digestibility using pepsin (Metcalf et al., 1996). The current assessment strategy, as outlined by the Ad Hoc International Task Force on Foods Derived from Biotechnology (*Codex Alimentarius* Commission, 2003), focuses on a weight-of-evidence approach that recognizes that no single endpoint can be used to predict human allergenic potential. In this context, the following factors are considered: the source of the gene, the similarity of the amino acid sequence of the protein of interest to that of known allergens, the stability of the protein in an in vitro pepsin digestibility assay, and, when necessary, in vitro human sera testing or in vivo clinical testing. The pepsin digestibility assay was first conceived as a means to determine the relative stability of a protein to the extremes of pH and pepsin protease encountered in the mammalian gastric environment. It was originally developed and utilized as a method to assess the nutritional value of protein sources by predicting amino acid bioavailability (Marquez and Lajolo, 1981; Nielson, 1988; Zikakis et al., 1977). For the purpose of safety evaluation, it seems reasonable that highly digestible proteins would be expected to have reduced potential for systemic exposure.

Before the use of recombinant technology became widely available, pepsin and other proteases were used by several investigators to identify the segments of proteins that bind IgE (Budd et al., 1983; Kawashima et al., 1992; Lorusso et al., 1986). In general, proteolytic digests of allergens retain the ability to bind IgE. The first systematic application of an in vitro pepsin digestion assay to evaluate food allergen digestibility was reported by Astwood et al. (1996). The relative resistance to pepsin digestion of some of the major allergenic proteins of peanut, soybean, egg and milk, and common non-allergenic plant proteins (for example, ribulose diphosphate carboxylase, RUBISCO), was determined in a pepsin digestion assay using simulated gastric fluid (SGF) prepared according to the US Pharmacopoeia (1995). Generally, the allergens and lectins examined in these experiments were resistant to pepsin digestion whereas the other proteins were more rapidly and completely digested. Since this initial report, there have been several studies examining the stability of a variety of proteins in pepsin digestion assays in which distinctions between allergens and non-allergenic proteins were less clear (Asero et al., 2001; Besler et al., 2001; Buchanan et al., 1997; del Val et al., 1999; Fu, 2002; Fu

et al., 2002; Kenna and Evans, 2000; Okunuki et al., 2002; Tanaka et al., 2002). Variability in the digestibility measured in these studies is likely to be due in part to the lack of a standardized protocol to assess the digestibility of proteins using SGF. Variations include differences in the pH of the assay, the purity of the pepsin, the pepsin to target protein ratio, target protein purity and structural conformation, and the method of detection. The objective of this study was to assess the reproducibility of a common digestion protocol in nine different laboratories with a standard set of proteins.

## 2. Materials and methods

### 2.1. Participating laboratories

The laboratories participating in this test represented industry: Bayer CropScience (Sophia Antipolis, France); The Dow Chemical (Midland, MI); DuPont, Haskell Laboratory (Newark, DE); Monsanto (St Louis, MO); Syngenta Biotechnology (Research Triangle Park, NC); and Syngenta Central Toxicology Laboratory (Alderley Park, UK); federal researchers: US Food and Drug Administration, National Center for Food Safety and Technology, (Summit-Argo, IL); National Institute of Health Sciences, Japan (Tokyo, Japan); and a private research foundation, Sanquin Research (Amsterdam, The Netherlands). All laboratories received the same lot of each test protein, used the same lot of pepsin, and performed the pepsin digestion assay using the same conditions.

### 2.2. Pepsin activity

A ratio of 10 U of pepsin activity/ $\mu$ g of test protein was used throughout the study. This pepsin to target protein ratio was selected based on an evaluation of the average activity of pepsin recommended in the US Pharmacopoeia (2000) and used in previous studies in some of the participating laboratories. Pepsin (Cat. #P-6887) was purchased from Sigma Chemical (St. Louis, MO) in a single lot having 3460 U/mg of protein as analyzed by Sigma, and was provided to each of the participating laboratories as a lyophilized powder. For this lot of pepsin, the pepsin/protein ratio in the digestion reaction was approximately 3:1 (w:w).

### 2.3. Test proteins

Ten milligrams of the following test proteins were supplied to each laboratory as lyophilized powders: conglutin-like peanut allergen Ara h 2 (IUIS Allergen

Nomenclature, 1994) that was purified from peanuts in the presence of the reducing agent dithiothreitol by Arkansas Children's Hospital Research Institute, (Little Rock, AR) for Monsanto, (St. Louis, MO), bovine  $\beta$ -lactoglobulin (BLG; Sigma, Cat. #L0130), bovine serum albumin (BSA; Sigma, Cat. #A0281), concanavalin A from Jack beans (ConA; Sigma, Cat. #C2010), horseradish peroxidase (HRP; Sigma, Cat. #P6782), chicken ovalbumin (Ova; Sigma, Cat. #A5503), chicken egg-white ovomucoid (Ovm; Sigma, Cat. #T2011), phosphinothricin acetyltransferase (PAT; provided by Syngenta Biotechnology, Research Triangle Park, NC), ribulose 1,5-diphosphate carboxylase from spinach leaves (RUBISCO; Sigma, Cat. #R8000), and Kunitz soybean trypsin inhibitor (STI; Sigma, Cat. #T9003). All proteins had been isolated from their native source (plant or animal) with the exception of PAT, that was produced as a recombinant protein in *Escherichia coli* by over-expression of the *bar* gene from *Streptomyces hygroscopicus* (Thompson et al., 1987). All proteins were dissolved at a concentration of 5 mg protein/ml in water or in 50 mM Tris-HCl, pH 9.5 (PAT and RUBISCO due to solubility constraints). All proteins were stable when dissolved at this concentration and stored at  $-20^{\circ}\text{C}$ . Each protein used in this study was at least 85% pure as determined by gel electrophoresis and densitometry.

#### 2.4. Pepsin digestion assay conditions

For each protein, a single tube containing 1.52 ml of simulated gastric fluid (SGF; 0.084 N HCl, 35 mM NaCl, pH 1.2 or 2.0, and 4000 U of pepsin) was pre-heated to approximately  $37^{\circ}\text{C}$  prior to the addition of 0.08 ml of test protein solution (5 mg/ml).

The tube contents were mixed by mild vortexing and the tube was immediately placed in a  $37^{\circ}\text{C}$  water bath. Samples of 200  $\mu\text{l}$  were removed at 0.5, 2, 5, 10, 20, 30, and 60 min after initiation of the incubation. Each 200  $\mu\text{l}$  sample was quenched by addition of 70  $\mu\text{l}$  of 200 mM  $\text{NaHCO}_3$ , pH 11, and 70  $\mu\text{l}$  5 $\times$  Laemmli (1970) buffer (40% glycerol, 5%  $\beta$ -mercaptoethanol, 10% SDS, 0.33 M Tris, 0.05% bromophenol blue, pH 6.8). Quenched samples were immediately heated to  $>75^{\circ}\text{C}$  for approximately 10 min and analyzed directly, or stored at  $-20^{\circ}\text{C}$ . The zero time point protein digestion samples were prepared by quenching the pepsin in the solution before adding the test protein. Control samples for pepsin auto-digestion (pepsin without test protein) and test protein stability (reaction buffer with test protein but without pepsin) were included. These control reactions were treated exactly as described above except samples were prepared only for the 0 and 60 min time points. Digestion samples were analyzed by visually inspecting stained protein bands following electrophoresis by one of the methods summarized in Table 1 and as described below.

#### 2.5. SDS-PAGE electrophoresis

Samples (15  $\mu\text{l}$ ) from each time point and control reactions were subjected to SDS-PAGE electrophoresis in reducing conditions, according to the method of Laemmli (1970), using either 15-well, 1 mm thick, 10–20% polyacrylamide Tricine gels (7 laboratories used gels from Invitrogen, Carlsbad, CA, one laboratory (#3) used gels from BioRad, Richmond, CA) or 10–20% polyacrylamide Tris-glycine gels (laboratory #2, BioRad Laboratories) [Table 1]. Mark12 standard markers (Invitrogen, Cat. # LC5677) containing 12 proteins

Table 1  
Laboratory, gel type, fixative, and staining type

| Laboratory # | Gel type Buffer                     | Gel Manuf. | Fix       | Stain                                       |
|--------------|-------------------------------------|------------|-----------|---------------------------------------------|
| 1            | Tricine<br>Tris/Tricine/SDS         | Invitrogen | No        | Colloidal Coomassie blue (G-250) Invitrogen |
| 2            | Tris-HCl 10–20%<br>Tris/glycine/SDS | Bio-Rad    | 2% HOAc   | Coomassie Blue (R-250) Bio-Rad              |
| 3            | Tricine<br>Tris/Tricine/SDS         | Bio-Rad    | No        | GelCode Blue (G-250) Pierce                 |
| 4            | Tricine<br>Tris/Tricine/SDS         | Invitrogen | MeOH/HOAc | Colloidal Coomassie blue (G-250) Sigma      |
| 5            | Tricine<br>Tris/Tricine/SDS         | Invitrogen | TCA       | Coomassie Blue (R-250)                      |
| 6            | Tricine<br>Tris/Tricine/SDS         | Invitrogen | TCA       | Coomassie Blue (R-250)                      |
| 7            | Tricine<br>Tris/Tricine/SDS         | Invitrogen | TCA       | Coomassie Blue (R-250)                      |
| 8            | Tricine<br>Tris/Tricine/SDS         | Invitrogen | No        | Colloidal Coomassie blue (G-250) Invitrogen |
| 9            | Tricine<br>Tris/Tricine/SDS         | Invitrogen | No        | Colloidal Coomassie blue (G-250) Invitrogen |

ranging in size from 2500 to 200,000 MW were included on all gels.

## 2.6. Gel staining

Investigators choose to use one of four different staining procedures in the course of this study (Table 1). Three laboratories (#5, 6, and 7) fixed the gels in 5% trichloroacetic acid (TCA) for less than 5 min and then washed them in 45.5% methanol/9% glacial acetic acid for 1 h. The gels were then stained with 0.1% Coomassie brilliant blue R-250 (Sigma #B-5133) for 10 min and destained in 25% methanol with 7.5% glacial acetic acid. One laboratory (# 2) stained the gels with Coomassie brilliant blue R-250 (Bio-Rad) following fixation in 2% acetic acid. Gels were destained in Bio-Rad destaining solution (Cat. #161-0438). One laboratory (# 4) stained the gels with colloidal Coomassie brilliant blue G-250 (Sigma Cat. # B-2025) for 2 h following 30 min of fixation in a solution of 40% methanol with 7% acetic acid. The gels were destained for 60 s in a solution of 25% methanol with 10% acetic acid, then overnight in 25% methanol. Three laboratories (# 1, 8, and 9) stained the gels using the colloidal Coomassie brilliant blue G-250 (Invitrogen, Cat. # LC6025) without pre-fixation. Gels were stained for 3–15 h in a mixture containing phosphoric acid, ammonium sulfate, methanol, and Coomassie blue G-250, prior to destaining with deionized water for 7 h to 3 days. One laboratory (# 3) stained with Gelcode Blue (Pierce, Rockford, IL, USA), a colloidal blue stain that includes phosphoric acid, methanol, and dimethyl sulfoxide with Coomassie blue G-250, without pre-fixation. Gels were washed three times with deionized water prior to staining for 2 h. Excess stain was removed by multiple washes in deionized water.

Images of stained gels were captured in participating laboratories using a variety of scanners or digital cameras and image capture programs. Whole image brightness and contrast adjustments were made during capture, but not altered thereafter. Gel images were adjusted to comparable size and merged into common Microsoft PowerPoint figures for each of the pH conditions (pH 1.2 and 2.0).

## 2.7. Data evaluation

Due to some inter-individual variation in the interpretation of gel images, a panel of 8 scientists representing 5 participating laboratories jointly viewed and scored the images from all 9 laboratories to provide a consensus view regarding the final time a full length protein or fragment bands were visible for each gel.

## 3. Results

Proteins used in this study were from plant, bacterial, or animal sources, and included allergens, non-allergens, and proteins with and without enzymatic activity (Table 2). Five proteins were isolated from plant sources: Ara h 2, ConA, HRP, RUBISCO, and STI, while four proteins were of animal origin: BLG, BSA, Ova, and Ovm. Phosphinothricin acetyltransferase (PAT) was produced as a recombinant protein in *E. coli*. Six of the proteins: Ara h 2 (Sen et al., 2002), BLG (Jarvinen et al., 2001), BSA (Tanabe et al., 2002), Ova (Honma et al., 1996), Ovm (Yamada et al., 2000), and STI (Gu et al., 2000), are known to be food allergens, while PAT, HRP, and RUBISCO have not been reported to be allergenic. One protein, ConA, while not considered to be an allergen, is

Table 2  
Biochemical properties of test proteins

| Protein | Source                    | Apparent size (MW) | Allergen/major or minor | Function             | Relative abundance |
|---------|---------------------------|--------------------|-------------------------|----------------------|--------------------|
| Ara h 2 | Plant-peanut              | 20,000             | Yes/major               | Seed storage protein | High <sup>a</sup>  |
|         |                           | 18,000             |                         |                      |                    |
|         |                           | 13,000             |                         |                      |                    |
| BLG     | Mammal-bovine             | 17,000             | Yes/major               | Milk protein         | High               |
| BSA     | Mammal-bovine             | 65,000             | Yes/minor               | Blood protein        | High               |
| ConA    | Plant-jack bean           | 30,000 (main)      | No                      | Seed protein         | High               |
|         |                           | 13,000             |                         |                      |                    |
|         |                           | 11,000             |                         |                      |                    |
| HRP     | Plant-horseradish         | 48,000             | No                      | Enzyme               | High               |
| Ova     | Avian-chicken egg         | 45,000             | Yes/major               | Storage protein      | High               |
| Ovm     | Avian-chicken egg         | 42,000             | Yes/major               | Storage protein      | High               |
| PAT     | Bacterium- <i>E. coli</i> | 23,000             | No                      | Enzyme               | Low <sup>b</sup>   |
| RUBISCO | Plant-spinach leaves      | 54,000             | No                      | Enzyme               | High               |
|         |                           | 20,000             |                         |                      |                    |
| STI     | Plant-soybeans            | 19,000             | Yes/minor               | Inhibitor trypsin    | Low                |

<sup>a</sup> Protein is present at greater than 1% of the protein content of source material.

<sup>b</sup> Protein is present at less than 1% of the protein content of source material.

a lectin and a T cell mitogen and is considered to be an anti-nutrient (Mendez et al., 1998). Three proteins: HRP, PAT (the selectable gene marker product in multiple transgenic seed products), and RUBISCO are enzymes. The proteins included in this study have diverse biochemical properties and all are consumed in foods commonly eaten in some cultures, albeit in widely different quantities.

Results from one of the test proteins, Ovm, were not included in the analytical summaries presented below, as there was no consensus among testing laboratories regarding detection. Ovm co-migrated with the pepsin enzyme as a protein-staining smear (Fig. 3C) due to its heavy glycosylation pattern making it difficult to definitively determine when the full-length protein was completely digested. In addition, the Ovm preparation was contaminated with a strongly staining protein band that was identified as lysozyme (sequence confirmed by Monsanto, data not shown). Lysozyme is stable to digestion by pepsin, making it difficult to determine when fragments of Ovm were last observed. This illustrates the importance of using well-characterized test proteins in this assay.

### 3.1. Test proteins exhibit different pepsin digestion characteristics

The susceptibility of the test proteins to the in vitro pepsin digestion assay utilized in this study varied significantly depending on the protein tested. Some proteins (for example, HRP) were rapidly and completely digested, with neither the full-length protein nor fragments of the protein detectable at the first time point (Fig. 1A). Other full-length proteins (for example, BSA) were undetectable 0.5 min after the start of digestion but fragments were observed up to 60 min (Fig. 1B). In another group of proteins (for example, ConA), both the full-length protein and fragments were detectable for at least 30 min after the start of the assay (Fig. 1C). Finally, there were proteins (for example, STI) that were resistant to digestion and relatively unchanged after 60 min of digestion (Fig. 1D). These results demonstrate that the proteins chosen for testing exhibited a wide range of susceptibility to pepsin digestion.

### 3.2. Comparison of the digestibility of the test proteins in pepsin as performed in the participating laboratories

In order to evaluate the results from all participants in a manner that would indicate whether substantive differences in protein digestibility were observed between laboratories, each participant's results were assessed for the final time point for which the full-length protein or fragment was detectable. The results from this analysis are depicted in Fig. 2. There was 91% agreement among

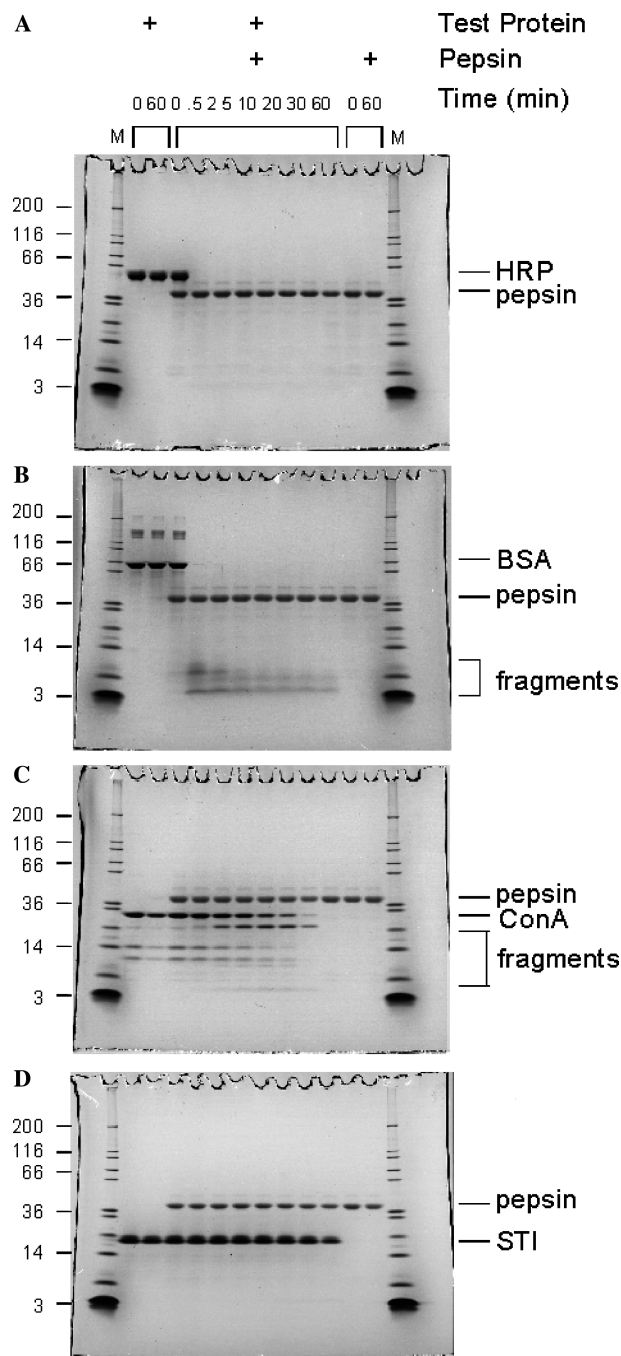


Fig. 1. Test proteins digested at different rates in a standardized in vitro pepsin digestion assay. Four patterns of digestion results were observed in this study. Some proteins were rapidly digested without visible fragments (A, HRP), some were rapidly digested, but produced resistant protein fragments (B, BSA), some were relatively resistant and also produced resistant fragments (C, Con A), and some were apparently fully resistant to digestion (D, STI). All gels in the study were loaded in the same order with the same amount of digestion reaction mix or markers. Mark12 markers, molecular weights ( $\times 10^{-3}$ ) are indicated for lanes labeled "M." All other lanes contained 15  $\mu$ l of quenched reaction mix sampled at the times indicated. The reactions contained either test protein alone in SGF (0, 60 min); test protein in SGF with pepsin (0, 0.5, 2, 5, 10, 20, 30, and 60 min); or SGF with pepsin alone (0, 60 min). These 10–20% polyacrylamide, Tricine gels are representative of reactions conducted at pH 1.2 from a single laboratory.

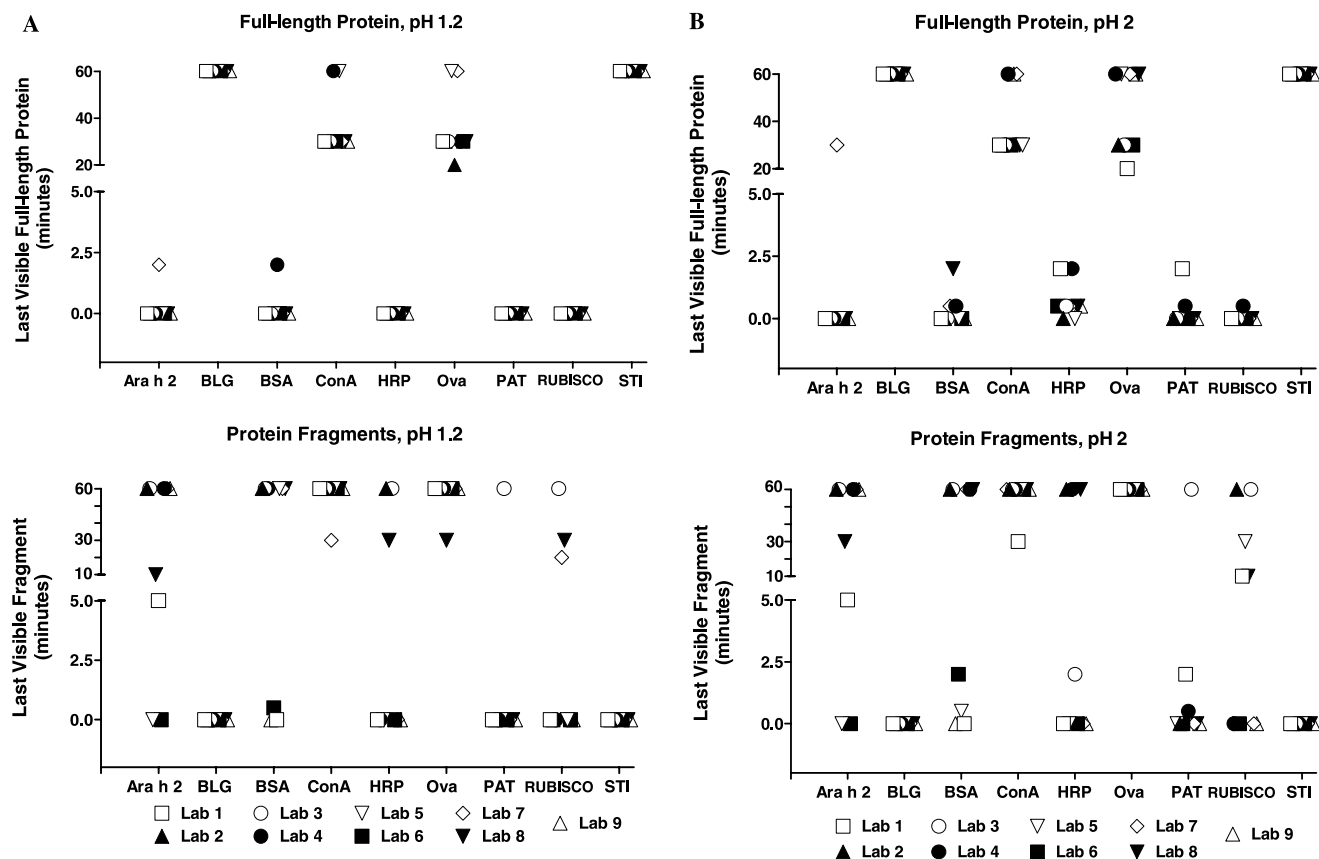


Fig. 2. Comparison of the digestibility of the test proteins across laboratories at pH 1.2 and 2.0. The last time-point each of the full-length test proteins or fragments were clearly visible in gels after staining in colloidal Coomassie blue G-250 or Coomassie brilliant blue R-250 from each of the nine laboratories are plotted. Protein samples digested in pH 1.2 pepsin buffer are shown in (A), samples digested in pH 2.0 buffer are shown in (B). Data from each laboratory are plotted.

laboratories regarding the digestibility of full-length proteins at pH 1.2 (Table 3, Fig. 2A) and 77% agreement on these proteins when digested at pH 2.0 (Table 4, Fig. 2B). For example, at pH 1.2 the PAT protein was undetectable after time zero in all laboratories. In contrast, at pH 2.0 the PAT protein was undetectable after time zero in 7/9 laboratories but in one laboratory this protein was last detected at the 0.5-min time point and in another laboratory it was last observed at the 2-min time point.

There was 80% agreement between laboratories regarding the detection of protein fragments at pH 1.2 (Table 3) and 75% agreement at pH 2.0 (Table 4). For example, at pH 1.2 the Ova protein fragments were detected at the 60-min time point in all labs. However, at pH 2.0 only 5/9 laboratories detected Ova fragments at the 60-min time point while 3/9 laboratories last detected them in the 30-min time point and 1/9 laboratories last detected them in the 20-min time point. The lower concordance between laboratory results for protein fragments compared to full-length proteins may be attributed to differences in staining procedures and gel electrophoresis methods as described below.

### 3.3. Effects of the gel electrophoresis system on detection of proteins and protein fragments

Laboratory # 2 used 10–20% polyacrylamide Tris–glycine gels while all other laboratories used 10–20% polyacrylamide Tricine gels. Overall, the two gel systems did not yield significantly different results. However, in some cases, the resolution of either the full-length protein or protein fragments was affected by the gel system used. For example, resolution of the full-length HRP and Ova proteins from the pepsin enzyme were substantially better in Tricine gels than that observed for these same proteins on Tris–glycine gels (Figs. 3A and B). In contrast, the full-length Ovm protein was more readily distinguished from the pepsin enzyme on Tris–glycine gels compared with Tricine gels (Fig. 3C).

### 3.4. Effects of the gel stain on detection of protein fragments

Several different gel fixing and staining procedures were used in the study (Table 1). Four laboratories used Coomassie brilliant blue R-250 staining procedures, while the other five laboratories used one of three

Table 3  
Concordance of digestibility between laboratories, pH 1.2

| Last time detected                            | 0                       | 0.5 | 2 | 5 | 10 | 20 | 30             | 60             |
|-----------------------------------------------|-------------------------|-----|---|---|----|----|----------------|----------------|
| <i>Full-length Protein<sup>a</sup> pH 1.2</i> |                         |     |   |   |    |    |                |                |
| Ara h 2                                       | 8 <sup>b</sup>          |     | 1 |   |    |    |                |                |
| BLG                                           |                         |     |   |   |    |    |                | 9 <sup>b</sup> |
| BSA                                           | 8 <sup>b</sup>          |     | 1 |   |    |    |                |                |
| ConA                                          |                         |     |   |   |    |    | 7 <sup>b</sup> | 2              |
| HRP                                           | 9 <sup>b</sup>          |     |   |   |    |    |                |                |
| Ova                                           |                         |     |   |   |    | 1  | 6 <sup>b</sup> | 2              |
| PAT                                           | 9 <sup>b</sup>          |     |   |   |    |    |                |                |
| RUBISCO                                       | 9 <sup>b</sup>          |     |   |   |    |    |                |                |
| STI                                           |                         |     |   |   |    |    |                | 9 <sup>b</sup> |
| Sum of labs in agreement <sup>b</sup>         | 74 (out of 81 possible) |     |   |   |    |    |                |                |
| Percent agreement                             | 91%                     |     |   |   |    |    |                |                |
| <i>Protein Fragment(s)<sup>a</sup> pH 1.2</i> |                         |     |   |   |    |    |                |                |
| Ara h 2                                       | 2                       |     |   | 1 | 1  |    |                | 5 <sup>b</sup> |
| BLG                                           | 9 <sup>b</sup>          |     |   |   |    |    |                |                |
| BSA                                           | 2                       | 1   |   |   |    |    |                | 6 <sup>b</sup> |
| ConA                                          |                         |     |   |   |    |    | 1              | 8 <sup>b</sup> |
| HRP                                           | 6 <sup>b</sup>          |     |   |   |    |    | 1              | 2              |
| Ova                                           |                         |     |   |   |    |    | 1              | 8 <sup>b</sup> |
| PAT                                           | 8 <sup>b</sup>          |     |   |   |    |    |                | 1              |
| RUBISCO                                       | 6 <sup>b</sup>          |     |   |   |    | 1  | 1              | 1              |
| STI                                           | 9 <sup>b</sup>          |     |   |   |    |    |                |                |
| Sum of labs in agreement <sup>b</sup>         | 65 (out of 81 possible) |     |   |   |    |    |                |                |
| Percent agreement                             | 80%                     |     |   |   |    |    |                |                |

Results of the number of laboratories reporting the indicated time (minutes) as the last observed time-point for each protein at pH 1.2.

<sup>a</sup> Ovomucoid (OVM) was not included in this evaluation due to the interference caused by the lysozyme contamination and the poor resolution of the full-length protein.

<sup>b</sup> The maximum number of laboratories in agreement regarding the last time each specific protein, or fragments of the specific protein were visible was summed in each experiment to calculate the percent agreement based on 81 (nine laboratories times 9 proteins).

versions of colloidal blue staining with Coomassie blue G-250. One difference noted among some laboratories that to some extent appears related to these staining procedures was the ability to detect protein fragments in digestion samples of Ara h 2, BSA, HRP, PAT, and RUBISCO (summarized in Fig. 2 and Tables 3 and 4). Even in examining data collected using a single staining method, some differences were found among laboratories, most notably for the detection of fragments of Ara h 2 (Fig. 4). In the stained gel images of seven laboratories, a proteolytic fragment of ~10,000 MW was visible through the first several digestion time points. However, two of the three laboratories that used Coomassie brilliant blue R-250 staining after fixing in TCA were unable to detect this band at any time point. This ~10,000 MW band has been identified as a fragment of Ara h 2 by N-terminal sequencing and MALDI-TOF (Monsanto, data not shown). These results suggest that the fixing and staining methods are likely to impact the ability to detect smaller molecular weight bands, and that factors that were not identified, such as time of fixing, staining or destaining may also be important. Detection of full-length proteins was markedly less variable as demonstrated by the closer agreement among laboratories on the final time of detection of the full-length proteins relative to the fragments (Tables 3 and 4).

#### 4. Discussion

There have been several studies reported in the literature that have examined pepsin digestibility of a variety of allergens and non-allergens (Asero et al., 2001; Astwood and Fuchs, 1996; Astwood et al., 1996; Besler et al., 2001; Buchanan et al., 1997; del Val et al., 1999; Fu, 2002; Fu et al., 2002; Kenna and Evans, 2000; Okunuki et al., 2002; Sen et al., 2002; Tanaka et al., 2002). These studies did not always come to the same conclusions regarding the susceptibility of proteins to pepsin digestion. Reported differences in digestibility may be attributable to variations in the pepsin digestion assay parameters including pH of the assay buffer, the purity of the pepsin, the ratio of pepsin to target protein, the target protein purity and structural conformation, and finally, the method of detection. Although the kinetics of protein degradation (that is, rate) can be assessed, the more simple “time to disappearance” was evaluated in this study addressing the reproducibility of the pepsin digestion assay in different laboratories using a common protocol and test proteins.

##### 4.1. Effect of pH on the *in vitro* pepsin digestion assay

The optimal pH range for porcine pepsin activity is 1.2–3.5, depending to some extent on the substrate

Table 4  
Concordance of digestibility between laboratories, pH 2

| Last time detected                          | 0                       | 0.5            | 2 | 5 | 10 | 20 | 30             | 60             |
|---------------------------------------------|-------------------------|----------------|---|---|----|----|----------------|----------------|
| <i>Full-length Protein<sup>a</sup> pH 2</i> |                         |                |   |   |    |    |                |                |
| Ara h 2                                     | 8 <sup>b</sup>          |                |   |   |    |    | 1              |                |
| BLG                                         |                         |                |   |   |    |    |                | 9 <sup>b</sup> |
| BSA                                         | 6 <sup>b</sup>          | 2              | 1 |   |    |    |                |                |
| ConA                                        |                         |                |   |   |    |    | 6 <sup>b</sup> | 3              |
| HRP                                         | 2                       | 5 <sup>b</sup> | 2 |   |    |    |                |                |
| Ova                                         |                         |                |   |   |    | 1  | 3              | 5 <sup>b</sup> |
| PAT                                         | 7 <sup>b</sup>          | 1              | 1 |   |    |    |                |                |
| RUBISCO                                     | 8 <sup>b</sup>          | 1              |   |   |    |    |                |                |
| STI                                         |                         |                |   |   |    |    |                | 9 <sup>b</sup> |
| Sum of labs in agreement <sup>b</sup>       | 63 (out of 81 possible) |                |   |   |    |    |                |                |
| Percent agreement                           | 77%                     |                |   |   |    |    |                |                |
| <i>Protein Fragment(s)<sup>a</sup> pH 2</i> |                         |                |   |   |    |    |                |                |
| Ara h 2                                     | 2                       |                |   | 1 |    |    | 1              | 5 <sup>b</sup> |
| BLG                                         | 9 <sup>b</sup>          |                |   |   |    |    |                |                |
| BSA                                         | 2                       | 1              | 1 |   |    |    |                | 5 <sup>b</sup> |
| ConA                                        |                         |                |   |   |    |    | 1              | 8 <sup>b</sup> |
| HRP                                         | 5 <sup>b</sup>          |                |   | 1 |    |    |                | 3              |
| Ova                                         |                         |                |   |   |    |    |                | 9 <sup>b</sup> |
| PAT                                         | 7 <sup>b</sup>          | 1              |   |   |    |    |                | 1              |
| RUBISCO                                     | 4 <sup>b</sup>          |                |   |   | 2  |    | 1              | 2              |
| STI                                         | 9 <sup>b</sup>          |                |   |   |    |    |                |                |
| Sum of labs in agreement <sup>b</sup>       | 61 (out of 81 possible) |                |   |   |    |    |                |                |
| Percent agreement                           | 75%                     |                |   |   |    |    |                |                |

Results of the number of laboratories reporting the indicated time (minutes) as the last observed time-point for each protein at pH 2.

<sup>a</sup> Ovomucoid (OVM) was not included in this evaluation due to the interference caused by the lysozyme contamination and the poor resolution of the full-length protein.

<sup>b</sup> The maximum number of laboratories in agreement regarding the last time each specific protein, or fragments of the specific protein were visible was summed in each experiment to calculate the percent agreement based on 81 (nine laboratories times 9 proteins).

(Collins and Fine, 1981; Crevieu-Gabriel et al., 1999). The pH in the lumen of the human stomach is typically between pH 1 and 2 under fasting conditions and increases above pH 5 during a meal, although there is considerable intra- and inter-individual variation (Evans et al., 1988; Lindahl et al., 1997; Russell et al., 1993). In an attempt to account for this pH range in the in vitro pepsin digestion assay, participants at a recent scientific advisory panel of the (FAO/WHO (2001)) recommended performing the pepsin digestion assay at pH 2.0. Results from the current study indicate that there was no appreciable difference in the time to disappearance of the full-length protein or protein fragments at pH 1.2 or 2.0. However, there was slightly greater consistency among laboratories at pH 1.2 than pH 2.0, perhaps because of differential buffering capacities of the test proteins, or solutions that might raise the reaction conditions beyond the optimal activity for pepsin. This result, together with the existence of an historical database of the in vitro digestive fate of proteins tested at pH 1.2 (Astwood et al., 1996; Fu et al., 2002; Kenna and Evans, 2000; Okunuki et al., 2002) as recommended by the (US Pharmacopoeia (1995, 2000)), suggests that pH 1.2 be utilized for analyses of protein digestibility by pepsin.

#### 4.2. Effects of the gel electrophoresis system on detection of proteins and protein fragments

Tris–glycine gels are based on the traditional Laemmli buffer system (Laemmli, 1970), while Tricine gels are considered more appropriate for the resolution of low molecular weight proteins and peptides (Schagger and von Jagow, 1987). Results from this study indicate that the interpretation of test protein digestibility by the in vitro pepsin digestion assay could be affected by the gel electrophoresis system chosen to analyze experimental results. Difficulties in interpretation were most often encountered when the migration of the test protein was in close proximity to pepsin. The selection of the gel system should take into account the size of the test protein and fragments being evaluated, and may have to be empirically derived to provide optimum resolution between pepsin and the test protein.

#### 4.3. Effects of the gel staining procedure on the pepsin digestion assay

Coomassie brilliant blue R-250 and G-250 stains can detect as little as 0.5 µg/cm<sup>2</sup> in a gel matrix (Srovy and

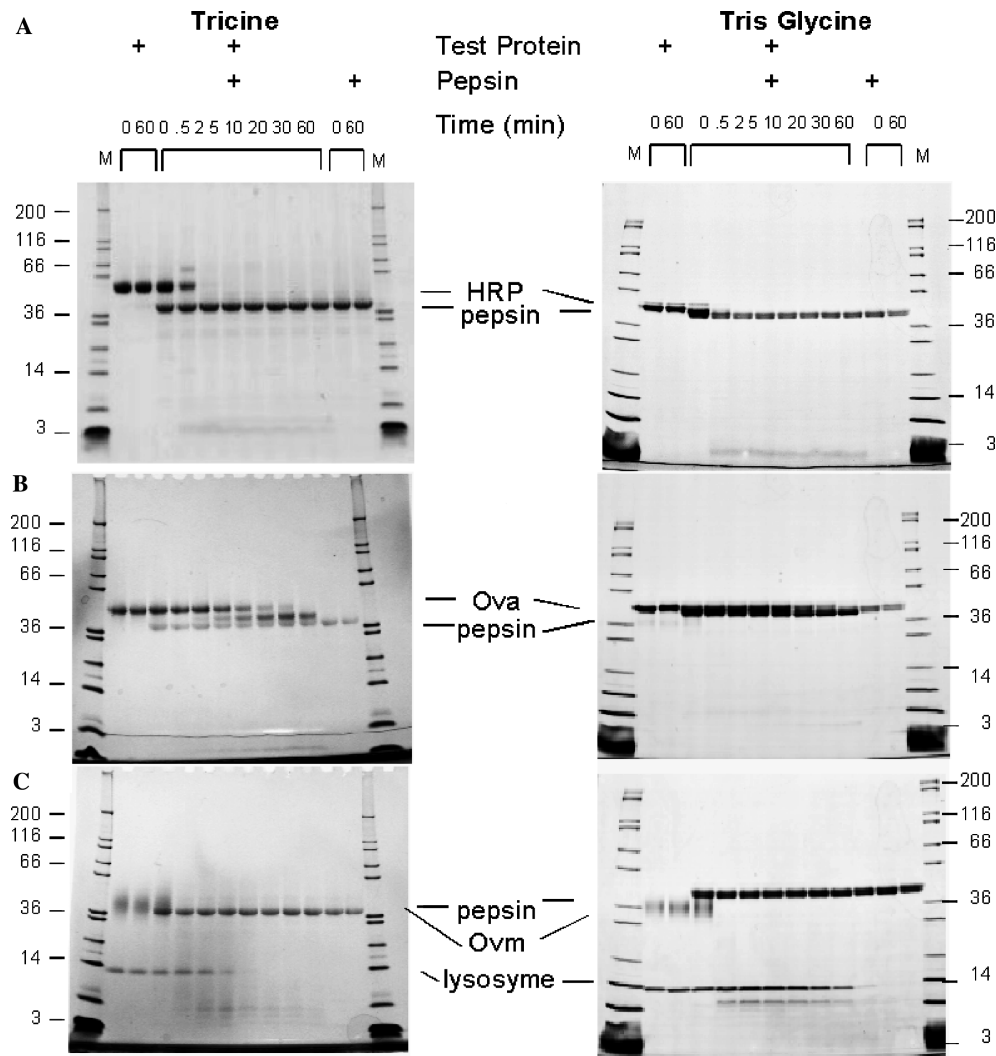


Fig. 3. Choice of gel type (Tricine vs Tris-glycine) can affect interpretation of in vitro pepsin digestion assay results. Digested samples were separated by electrophoresis SDS-polyacrylamide gels (10–20%) prior to fixing and staining. Eight laboratories used Tricine gels, one laboratory used Tris-glycine gels. Images of selected gels showing Ova (A), Ovm (B), and HRP (C) samples digested in pH 2.0 pepsin buffer.

Hodny, 1991). The dye binds primarily to basic and aromatic amino acid residues, especially arginine (Compton and Jones, 1985). Staining with colloidal Coomassie brilliant blue has been reported to detect a minimum of 0.1 ng of protein, which is slightly more sensitive than staining with other forms of Coomassie brilliant blue or methods employing other dyes (Syrov and Hodny, 1991). In this study there were differences noted in the detection of proteolytic fragments of Ara h 2, BSA, HRP, PAT, RUBISCO, and Ova, as well as minor differences in the detection of full-length ConA and Ova between laboratories. These variations in detection may be due, in part, to differences in staining procedures used by individual laboratories, the method of fixing the proteins prior to staining, the method and time of destaining, or image capture and processing. As demonstrated in this study, either staining system could be used effectively.

#### 4.4. Reproducibility of the assay, full-length proteins

In this study there was 91% agreement across all laboratories on the final time point at which the full-length protein was detected for all proteins tested at pH 1.2 and 77% agreement on all proteins tested at pH 2.0. Most of the differences noted were the result of laboratories reporting slightly different times at which the full-length protein was last detected. For example, at pH 1.2 seven laboratories reported that the ConA protein was last detected at the 30-min time point, while two labs reported it last detected at the 60-min time point. Similar small differences were noted when the assay was performed at pH 2.0. However, there was slightly more variation in the results when the assay was performed at this pH. It should be noted that none of the variation between participants was the result of widely disparate results of protein digestibility.

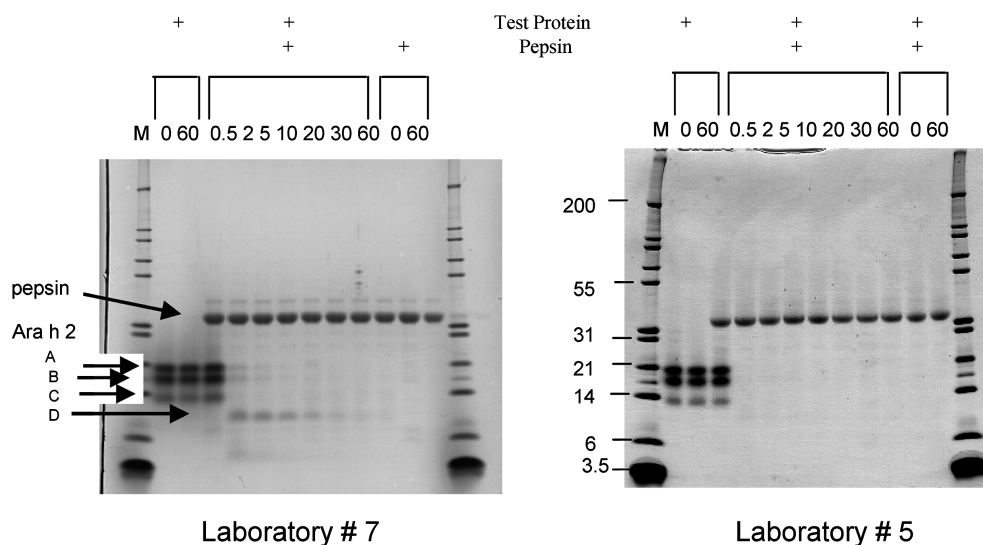


Fig. 4. Pepsin resistant fragments of Ara h 2. Seven of nine laboratories detected at least one fragment produced from proteolysis of Ara h 2 by pepsin. The left panel represents the gel from laboratory #7 for the digestion products of Ara h 2. Full-length Ara h 2 is represented by bands A and B. Band C is a contaminant. The dominant fragment (band D) was approximately 10 kDa, with the identity confirmed (data not shown). Laboratory #5 was one of the two laboratories that did not detect any fragment of Ara h 2 at any time point in the digestion assay. Both laboratory #5 and #7 fixed the gels in TCA prior to staining with Coomassie brilliant blue R-250.

One difference between this study and previous studies was noted for the peanut allergen Ara h 2. The rapid degradation of full-length Ara h 2 as observed in this study is similar to observations made by Fu et al. (2002) who showed that Ara h 2 was degraded within 0.5 min in a simulated gastric fluid containing pepsin. However, these results differ from those observed by both Sen et al. (2002) and Astwood et al. (1996) who showed that native Ara h 2 was resistant to digestion by pepsin for 20 and 60 min, respectively. One possible explanation for this discrepancy may be due to differences in the preparation of Ara h 2. The Ara h 2 for this study was prepared in the presence of dithiothreitol, a strong reducing agent, while the purification method used in the study by Astwood et al. (1996) was not reported. Sen et al. (2002) showed that the disulfide bonds of Ara h 2 contribute significantly to its overall structure and stability and if disrupted, as with a strong reducing agent, lead to its rapid degradation by GI enzymes including pepsin. These differences demonstrate the need to consider whether the test protein is present in the native conformation or a denatured form.

#### 4.5. Reproducibility of the assay-protein fragments

In this study there was 80% agreement on the last time point that protein fragments were detected across all laboratories for all proteins tested at pH 1.2 and 75% agreement on all proteins tested at pH 2.0. Consensus between laboratories on the final time point at which protein fragments were detected was not as high as that observed for the full-length protein. Possible explanations for differences in the detection of protein fragments

could be differences in gel staining methods and gel electrophoresis system as described above. In addition, variation was due in part to the detection in some laboratories of very small protein fragments. For example, 5/9 labs reported detecting protein fragments for RUBISCO. The protein fragments had migrated very close to the buffer front for those labs reporting their detection (<3000 MW) and could either be masked by the dye front, or have migrated out of the gel.

#### 4.6. Biological relevance of the assay

The rationale for using pepsin-resistance as part of a safety assessment of novel proteins has been challenged in the absence of significant modification(s) to the assay (Burnett et al., 2002; FAO/WHO, 2001; Houben et al., 1997). Reluctance to support pepsin-resistance in the safety assessment of proteins has been due to the lack of reproducible and consistent correlation between pepsin-resistance and allergenicity. While there are reports in the literature which demonstrate that many major food allergens are resistant to pepsin digestion (Asero et al., 2001; Astwood et al., 1996; Murtach et al., 2002; Tanaka et al., 2002), some recent studies report pepsin resistant proteins that are not allergenic (for example, ConA and Zein), as well as pepsin sensitive proteins that are allergenic [for example, shrimp tropomyosin and casein (Fu et al., 2002)]. The absence of a standardized digestibility protocol has contributed to inconsistencies in predicting allergenicity based on resistance of proteins to pepsin digestion. Therefore, the standardization of a digestibility protocol becomes essential to the utility of pepsin-resistance in the safety evaluation of proteins.

Urisu et al. (1999) and Yamada et al. (2000) demonstrated that pepsin-resistant fragments of ovomucoid bind IgE and that the binding correlates well with the pattern of persistent allergy to egg white proteins. Other studies have also tested the IgE binding capacity of the residual fragments following pepsin digestion of allergens and found that they bind IgE, or that they contain major IgE binding domains (Maleki et al., 2000; Sen et al., 2002; Tanaka et al., 2002), or epitopes. (Budd et al., 1983; Lorusso et al., 1986). Small protein fragments, between 1500 and 3500 MW, may not be able to elicit allergic symptoms as there are theoretical limits to the size of a peptide that can simultaneously bind to two IgE molecules attached to FcεRI on mast cells and induce mast cell degranulation (Bannon et al., 2002; Lack et al., 2002). Given the fact that pepsin-resistant proteins or pepsin-resistant fragments may have the potential to bind IgE, identifying this property is useful in the safety assessment process. It must be recognized, however, that this property is not absolutely predictive of allergenic potential.

The *Codex Alimentarius* Commission (2003) recommends a “weight of evidence” approach for the assessment of potential allergenicity of proteins introduced into crops through recombinant-DNA technology, and this approach includes an in vitro pepsin digestion assay such as the one described in this study. The results obtained in this study showed consistency between laboratories for individual proteins, lending support for the inclusion of this type of assay in the Codex approach. Further data regarding the predictive value of pepsin digestibility to human food allergy may be obtained using this protocol with additional allergenic and non-allergenic proteins.

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