



# Rapid simulated gastric fluid digestion of in-seed/grain proteins expressed in genetically engineered crops

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## ARTICLE INFO

### Article history:

Received 27 April 2016

Received in revised form

2 August 2016

Accepted 3 August 2016

Available online 4 August 2016

### Keywords:

Genetically engineered

Genetically modified

Transgenic

Digestion

Simulated gastric fluid

Allergenicity

In-matrix

Purified proteins

Risk assessment

## ABSTRACT

The speed of simulated gastric digestion of proteins expressed in genetically engineered (GE) crops is commonly used to inform the allergenicity risk assessment. However, persistence of purified proteins in simulated gastric fluid (SGF) is poorly correlated with the allergenic status of proteins. It has been proposed that the plant or food matrix may affect the digestion of proteins and should be considered in interpreting digestion results. Here the SGF digestion of several GE proteins both as purified preparations and in soybean, corn, and cotton seed/grain extracts (in-matrix) are compared. Cry1F, Cry1Ac, phosphinothricin acetyltransferase (PAT), aryloxyalkanoate dioxygenase-1 (AAD-1), aryloxyalkanoate dioxygenase-12 (AAD-12), and double mutant 5-enol pyruvylshikimate-3-phosphate synthase (2mEPSPS) were all found to rapidly digest both as purified protein preparations and in seed/grain extracts from GE crops expressing these proteins. Based on these results, purified protein from microbial sources is a suitable surrogate for proteins in-matrix when conducting SGF digestion studies.

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## 1. Introduction

Digestion experiments with proteins introduced into crops via genetic engineering (GE) were first conducted to support the allergenicity risk assessment under the premise that greater digestion would reduce intestinal exposure and subsequent sensitization and allergy (Astwood et al., 1996). A simulated gastric fluid (SGF) method was adapted from the literature (United States Pharmacopeial Convention Inc., 2005) and was used to compare peptic digestion among allergens and non-allergens. While initial results appeared to show a good correlation between the allergenic status of proteins and their stability in SGF (Astwood et al., 1996), subsequent work revealed that the initial panel of proteins confounded the protein structural and functional families with the allergenic status of the proteins (Fu et al., 2002). Once this bias was corrected for in subsequent studies, the correlation was found to be poor or non-existent (Bøgh and Madsen, 2016; Fu et al., 2002; Herman et al., 2007; Schnell and Herman, 2009).

The belief that allergens should digest more slowly than non-

allergens prompted a search for modified digestion conditions that might support this correlation (e.g. more physiological conditions and/or incorporation of intestinal digestion) (Bøgh and Madsen, 2016; Devos et al., 2014). However, research thus far has not identified digestion conditions that better predict the allergenic status of a protein compared with the standard SGF assay conducted at pH 1.2 with 0.32% (w/v) pepsin (Bøgh and Madsen, 2016; Devos et al., 2014).

Another hypothesis often considered is that the plant matrix could be the driving factor for the assumed greater digestive persistence of allergens compared with non-allergens (Polovic et al., 2007). However, no such correlation has yet been shown (Bøgh and Madsen, 2016). The digestive stability of a subset of allergens has been used to justify the use of digestive stability in the risk assessment of GE proteins, but this logic is flawed because digestion-susceptible allergens and stable non-allergens are also known (Herman et al., 2007). A greater propensity for allergens to be stable, compared with non-allergens, is required to logically support the value of digestion results in the allergenicity risk assessment, and this relationship is poorly supported (Bøgh and Madsen, 2016).

SGF digestion experiments (as well as toxicological and ecotoxicological studies) are most often conducted with protein

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purified from GE microbial sources (Raybould et al., 2012). This is due to the often low expression of the GE protein in plants and the resultant difficulties in purifying sufficient quantities of the proteins from plants for use in various experiments (Bushey et al., 2014; Madduri et al., 2012). Over-expression of GE proteins in microbes is the standard for obtaining purified proteins for studies to support the safety assessment of proteins. Due to the potential to alter the structure or function of a GE protein during microbial expression or purification (from microbes or plants), the biochemical and functional equivalence of purified protein and those expressed in plants must be established prior to using this surrogate test material (Raybould et al., 2012).

The poor correlation between protein digestion and the allergenic status of proteins using purified protein has raised the question as to whether results from in-matrix digestion of GE proteins in plants would substantially change the interpretation of studies. Here we report and discuss the in-matrix (aqueous seed/grain extracts of soybean, corn, and cotton) SGF digestion of several GE proteins as compared with the digestion of GE proteins purified from microbial sources. Proteins include insecticidal proteins derived from *Bacillus thuringiensis* (Cry1F and Cry1Ac), and enzymes that confer herbicide tolerance (phosphinothricin acetyltransferase, PAT; aryloxyalkanoate dioxygenase-1, AAD-1; aryloxyalkanoate dioxygenase-12, AAD-12; and double mutant 5-enol pyruvylshikimate-3-phosphate synthase, 2mEPSPS) (Biosafety Unit, 2015).

## 2. Methods and materials

### 2.1. Proteins

Plant-derived matrix samples were lyophilized field grown GE soybean seed from events DAS-81419-2 and DAS-44406-6, corn grain of event DAS-40278-9, and delinted cottonseed of event DAS-81910-7 (Biosafety Unit, 2015). Non-GE soybean was also grown as a control. The seed/grain tissues were harvested, frozen, lyophilized, and held at  $-80^{\circ}\text{C}$  until use. The GE seed/grain extracts were analyzed using validated enzyme-linked immunosorbent assay (ELISA) methods to determine the concentration of the Cry1F (16.1 ng/mg), Cry1Ac (0.99 ng/mg), and PAT (0.75 ng/mg) proteins in DAS-81419-2 soybean; AAD-12 (21.8 ng/mg), 2mEPSPS (13.3 ng/mg), and PAT (1.44 ng/mg) proteins in DAS-44406-6 soybean; AAD-1 (2.79 ng/mg) protein in DAS-40278-9 corn; and AAD-12 (22.8 ng/mg) and PAT (3.53 ng/mg) proteins in DAS-81910-7 cotton. ELISA kits were used according to instructions from the various kit manufacturers. Cry1F and Cry1Ac ELISA kits were purchased from Romer Labs (Union, MO). PAT, AAD-1, and AAD-12 ELISA kits were purchased from Envirologix Inc. (Portland, ME). ELISA kits for detecting 2mEPSPS were produced at Dow AgroSciences (Indianapolis, IN).

The purified recombinant PAT, AAD-1, AAD-12, Cry1F, Cry1Ac, and 2mEPSPS proteins were expressed in, and purified from, *Pseudomonas fluorescens* (Pf) at Dow AgroSciences (Indianapolis, IN). The purity of each protein was determined to be >95% (protein of interest/total protein) by SDS-PAGE and the percentage of GE protein in each lyophilized powder was determined to be 34.0, 36.1, 35.3, 80.0, 14.0, and 66.5%, respectively, by amino-acid analysis (AAA). The lyophilized protein powders were stored at  $4^{\circ}\text{C}$  until solubilized for use in the experiments.

A rapidly digestible control protein, bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, Lots 118H0595, 110M7402V, 100K7420, 075K7572, >96% purity), and a persistent control protein,  $\beta$ -lactoglobulin A ( $\beta$ -lac; Sigma-Aldrich, St. Louis, MO, Lots SLBC4961V, 037K7017, 031K7052, 70K7049, >90% purity) were included in each digestion experiment (Herman et al., 2007).

### 2.2. Digestions

Equimolar (0.074-mM) solutions of the purified test and control proteins were prepared as follows: The PAT protein (21.7 mg) was dissolved in 5 mL of Tris buffer, pH 8.0. The Cry1Ac protein (36.0 mg) was dissolved in 2 mL of 25 mM sodium hydroxide, pH 11.0. The Cry1F protein (24.1 mg) was dissolved in 2 mL of 25 mM 3-cyclohexylamino-l-propane sulfonic acid (CAPS), pH 10.5. The proteins, AAD-1 (34.1 mg), AAD-12 (33.2 mg), and 2mEPSPS (26.2 mg) were dissolved in 5 mL phosphate buffered saline with Tween 20 (PBST), pH 7.4. Buffer selection was based on the solubility characteristics of each protein. For the seed/grain extractions, immediately prior to exposure to SGF, ground soybean seed and corn grain were suspended at 80 mg/mL in PBST, while the ground cottonseed was suspended in PBST at 60 mg/mL. The samples were then homogenized in a bead-mill (Geno-Grinder, Spex SamplePrep LLC, Metuchen, NJ) for 3 min at 1500 strokes/min. The insoluble particulates were removed by centrifugation at  $20,000 \times g$  for 10 min and the supernatant was decanted and held on wet ice until exposure to SGF. The control proteins, BSA (24.8 mg) and  $\beta$ -lac proteins (6.8 mg), were solubilized in 5 mL of Tris or PBST buffer depending on the experiment. The varying amounts of the test and control proteins reflect differences in purity and molecular weight.

Simulated gastric fluid (SGF, pH 1.2) containing a final concentration of 0.32% (w/v) pepsin (Sigma-Aldrich, St. Louis, MO; ranging from 73 to 97% w/w purity, and containing between 3370 and 3846 units of activity/mg protein) was prepared by weighing 0.187 g of pepsin into 50 mL of 34 mM NaCl, pH 1.2 as recommended by the United States Pharmacopeia (United States Pharmacopeial Convention Inc., 2005).

The digestions for all samples were performed in a water bath set to  $37^{\circ}\text{C}$ . The samples were digested as follows: Three 2.85-mL aliquots of SGF were placed in the  $37^{\circ}\text{C}$  water bath (GE sample, BSA, and  $\beta$ -lac). After 5 min, 150  $\mu\text{L}$  of the 0.074-mM solutions or tissue extracts were added to separate vials of SGF and a timer was set. After each specified incubation interval, 100  $\mu\text{L}$  of the reaction mixtures were removed and added to tubes containing stop solution (40  $\mu\text{L}$  of 200-mM sodium bicarbonate, pH 11.0). The stopped reactions were then placed on ice until all of the time points were sampled for all of the samples. The durations of the digestions varied and are indicated in figure legends.

An SGF control was prepared by substituting Tris buffer for the sample protein and incubating for the duration of the experiment at  $37^{\circ}\text{C}$ . The SGF control was prepared as follows: A 2.85-mL aliquot of SGF was heated in a  $37^{\circ}\text{C}$  water bath for 5 min, 150  $\mu\text{L}$  of Tris buffer was added, and a timer was set. A 100- $\mu\text{L}$  aliquot was immediately removed as the zero time point and placed into a tube containing the stop reaction (40  $\mu\text{L}$  of 200-mM sodium bicarbonate, pH 11.0). When all digestion reactions were complete, one final aliquot was taken at the last time point. For each of the samples mentioned above, a neutralized control was prepared as follows: First, a 2.85-mL aliquot of SGF was heated in a  $37^{\circ}\text{C}$  water bath for 5 min. The SGF was neutralized with 1.2 mL of 200-mM sodium bicarbonate and 150  $\mu\text{L}$  of the respective protein was added to the solution. In most cases, a 10-fold dilution (Tris or PBST) of the neutralized control was prepared allowing one to conclude at least 90% of the protein was digested when the proteins were not visible on the gels or blots (Ofori-Anti et al., 2008). This control was not included for the pure-protein digestions with Cry1Ac and Cry1F because these experiments were conducted prior to this convention being recommended.

### 2.3. Gels and blots

Aliquots of the SGF controls, neutralized samples, and digested

proteins/extracts were mixed with equal volumes of  $2 \times$  Laemmli sample buffer (Bio-Rad, Hercules, CA), containing 5% freshly added 2-mercaptoethanol ( $\beta$ -ME, Bio-Rad) and heated for 5 min at  $95^\circ\text{C}$ . Additionally, aliquots of all samples and the neutralized controls were diluted in Tris buffer to a concentration appropriate for western blot analysis, then mixed with equal volumes of the  $2 \times$  Laemmli sample buffer preparation and heated as described earlier.

Single 4–12% polyacrylamide gels (Bio-Rad) of BSA and  $\beta$ -lac, and duplicate gels of the recombinant proteins or transgenic extracts were prepared for each digestion experiment. For each recombinant protein (except purified Cry1F and Cry1Ac), a 10-fold dilution of the protein was prepared from the neutralized SGF and all samples were loaded as described in the figures. The samples were then separated on SDS-PAGE gels, and after separation, the proteins were stained with GelCode Blue stain (Thermo-Pierce). Recombinant proteins and the transgenic tissue extracts on the duplicate gel were electro-blotted to a nitrocellulose membrane (Bio-Rad) using Tris/Glycine buffer (Bio-Rad) containing 20% methanol. Following protein transfer, the membrane was blocked with PBST containing 5% non-fat dried milk (Sigma-Aldrich, St. Louis, MO). For immunodetection, the membrane was probed with a protein-specific polyclonal rabbit antibody (raised against highly purified, intact, active protein) and detected with a conjugate of goat anti-rabbit IgG and HRP (Thermo Fisher Scientific, Pierce Biotechnology, Rockford, IL). Chemiluminescent detection solution (Thermo Fisher Scientific, Pierce ECL, Rockford, IL) was used for visualization of the immunoreactive protein bands when the membrane was exposed to X-ray film and subsequently developed with a film developer. A non-transgenic soybean seed extract was

also probed with anti-PAT antibodies by western blot to identify any non-specific binding. To improve the visibility of the bands on the western blots of seed/grain extracts in Figs. 1–6, the image contrast was adjusted in Google Drive by setting the recolor preset to light 3, the brightness to  $-36$ , and the contrast to  $+90$ .

### 3. Results and discussion

Standard SGF digestion assays were conducted with both purified protein and seed/grain soybean, corn, or cotton extracts for several GE proteins that confer insect resistance or herbicide tolerance (Cry1F, Cry1Ac, PAT, AAD-1, AAD-12, and 2mEPSPS). The rapidly digesting and persistent controls, BSA and  $\beta$ -lac, respectively (Herman et al., 2007), responded as expected (data not shown). BSA was not detected at the 1 min time point when subjected to the SGF, and  $\beta$ -lac remained readily detectable for the duration of the digestions (up to 60 min).

For the GE proteins, both SDS-PAGE and western blot (Figs. 1–6) demonstrated that the neutralized samples and 10-fold dilutions of the neutralized samples were detectable (except for the purified Cry1F and Cry1Ac which did not include the 10% control, and the purified Cry1Ac which did not include the neutralized pepsin/Cry1Ac control). In addition to the band of the expected molecular weight, a higher molecular weight dimer band was seen on the western blot for the purified PAT protein (Fig. 1, Panel B). The formation and visualization of protein multimers on gels and blots is not uncommon for purified proteins (Herman et al., 2003). Lower molecular weight bands believed to be digestive fragments of the proteins of interest were also seen on gels and blots for the purified proteins and seed/grain extracts for the Cry1F and Cry1Ac proteins

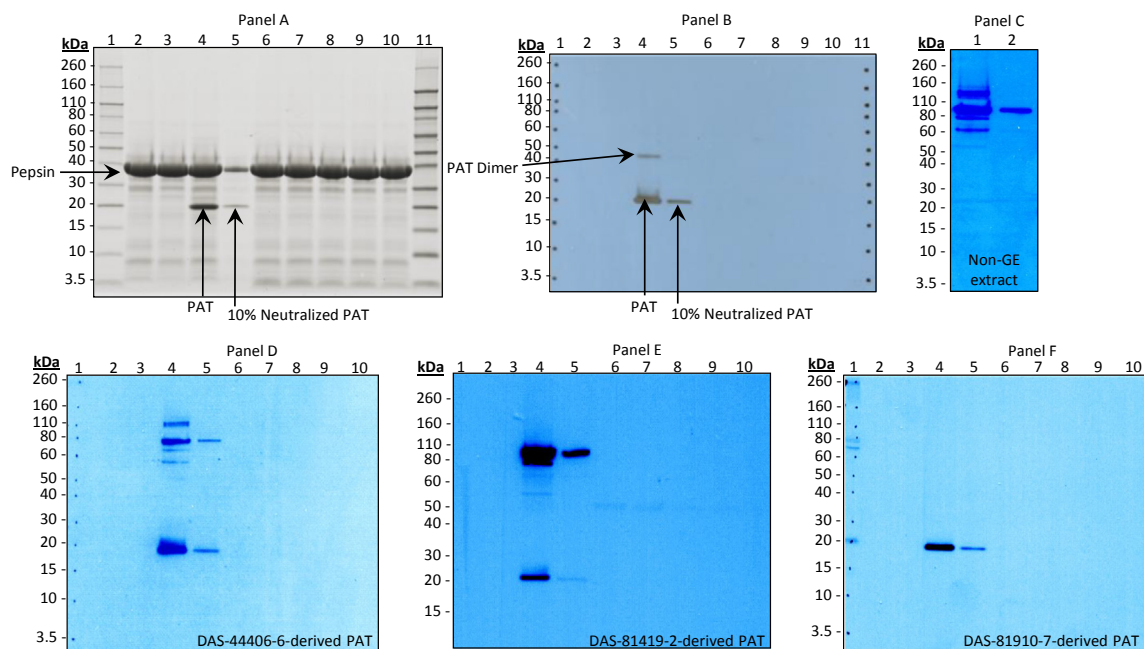


**Fig. 1. PAT gel and blots.** Panels A (SDS-PAGE gel) and B (western blot) of *Pf*-derived PAT protein: Lane 1 – MW markers; Lane 2 – SGF control, 0 min; Lane 3 – SGF control, 16 min, Lane 4 – Neutralized PAT, 0.53  $\mu\text{g}$  for gel and 0.013  $\mu\text{g}$  for blot; Lane 5 – 10% neutralized PAT, 0.053  $\mu\text{g}$  for gel and 0.0013  $\mu\text{g}$  for blot; Lanes 6 to 10 – 1, 2, 4, 8, and 16 min PAT digestion, respectively, 0.53  $\mu\text{g}$  for gel and 0.013  $\mu\text{g}$  for blot; Lane 11 – MW markers. Panel C: Western blot of non-GE soybean extract: Lane 1 – Neutralized non-GE soybean extract; Lane 2 – 10% Neutralized non-GE soybean extract. Panel D (DAS-44406-6 soybean), E (DAS-81419-2 soybean), and F (DAS-81910-7 cotton) – Western blots of GE seed extracts containing the PAT protein: Lane 1 – MW markers; Lane 2 – SGF control, 0 min; Lane 3 – SGF control, 16 min, Lane 4 – Neutralized extract, 0.041  $\mu\text{g}$ , 0.015  $\mu\text{g}$ , and 0.04  $\mu\text{g}$  for Panels D, E & F, respectively; Lane 5 – 10% neutralized extract, 0.0041  $\mu\text{g}$ , 0.0015  $\mu\text{g}$ , and 0.004  $\mu\text{g}$  for Panels D, E & F, respectively; Lanes 6 to 10 – 1, 2, 4, 8, and 16 min extract digestion, respectively, 0.041  $\mu\text{g}$ , 0.015  $\mu\text{g}$ , and 0.04  $\mu\text{g}$  for Panels D, E & F, respectively.

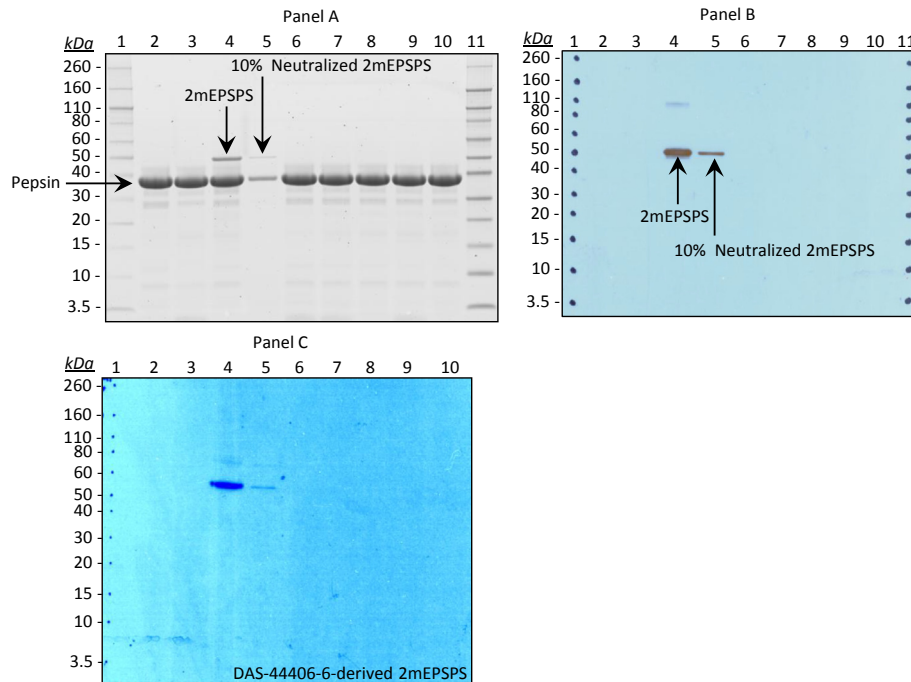


**Fig. 2. 2mEPSPS gel and blots:** Panel A (SDS-PAGE gel) and Panel B (western blot) of *Pf*-derived 2mEPSPS protein: Lane 1 – MW markers; Lane 2 – SGF control, 0 min; Lane 3 – SGF Control, 16 min; Lane 4 – Neutralized 2mEPSPS, 1.24  $\mu$ g for gel and 0.124  $\mu$ g for blot; Lane 5 – 10% Neutralized 2mEPSPS, 0.124  $\mu$ g for gel and 0.0124  $\mu$ g for blot; Lanes 6 to 10 – 1, 2, 4, 8, and 16 min 2mEPSPS digestion, respectively, 1.24  $\mu$ g for gel and 0.124  $\mu$ g for blot; Lane 11 – MW markers. Panel C: Western blot of GE soybean (DAS-44406-6) containing the 2mEPSPS protein: Lane 1 – MW markers; Lane 2 – SGF control, 0 min; Lane 3 – SGF control, 16 min; Lane 4 – Neutralized extract, 0.76 ng; Lane 5 – 10% neutralized extract, 0.076 ng; Lanes 6 to 10 – 1, 2, 4, 8, and 16 min extract digestion, respectively, 0.76 ng.

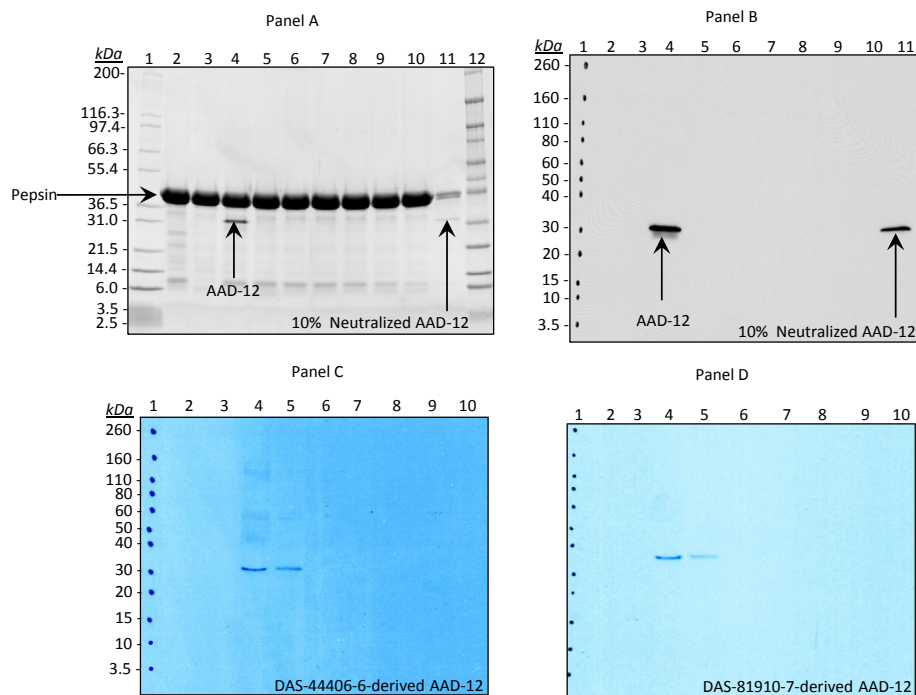


**Fig. 3. AAD-12 gel and blots:** Panel A (SDS-PAGE gel) and Panel B (western blot) of *Pf*-derived AAD-12 protein: Lane 1 – MW markers; Lane 2 – SGF control, 0 min; Lane 3 – SGF Control, 16 min; Lane 4 – Neutralized AAD-12, 1.67  $\mu$ g for gel and 0.17  $\mu$ g for blot; Lanes 5 to 10 – 0.5, 1, 2, 4, 8, and 16 min AAD-12 digestion, respectively, 1.67  $\mu$ g for gel and 0.17  $\mu$ g for blot; Lane 11 – 10% neutralized AAD-12, 0.167  $\mu$ g for gel and 0.017  $\mu$ g for blot; Lane 12 – (gel) MW markers. Panel C (DAS-44406-6 soybean) and Panel D (DAS-81910-7 cotton): Western blots of seed extracts containing the AAD-12 protein: Lane 1 – MW markers; Lane 2 – SGF control, 0 min; Lane 3 – SGF control, 16 min; Lane 4 – Neutralized extract, 1.2 ng and 0.98 ng, for Panels C and D, respectively; Lane 5 – 10% Neutralized extract, 0.12 ng and 0.098 ng, for Panels C and D, respectively; Lanes 6 to 10 – 1, 2, 4, 8, and 16 min extract digestion, respectively, 1.2 ng and 0.98 ng, for Panels C and D, respectively.

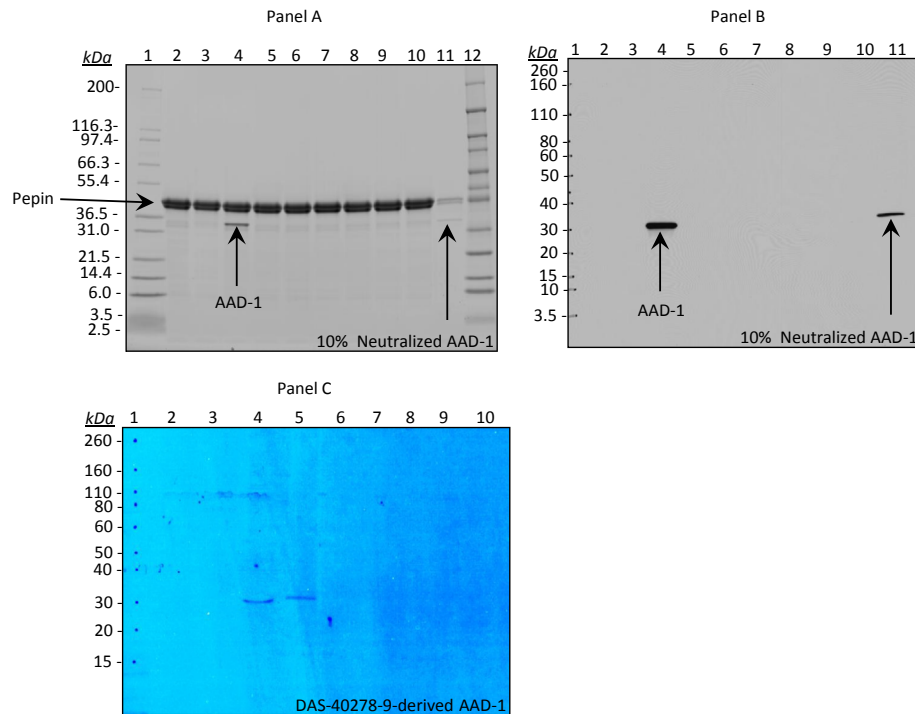


**Fig. 4. AAD-1 gel and blots:** Panel A (SDS-PAGE gel) and Panel B (western blot) of *Pf*-derived AAD-1 protein: Lane 1 – MW markers; Lane 2 – SGF control, 0 min; Lane 3 – SGF control, 16 min; Lane 4 – Neutralized AAD-1, 0.88  $\mu$ g for gel and 0.088  $\mu$ g for blot; Lanes 5 to 10 – 0.5, 1, 2, 4, 8, and 16 min AAD-1 digestion, respectively, 0.88  $\mu$ g for gel and 0.088  $\mu$ g for blot; Lane 11 – 10% neutralized AAD-1, 0.088  $\mu$ g for gel and 0.0088  $\mu$ g for blot; Lane 12 – (gel) MW markers. Panel C: Western blot DAS-40278-9 corn grain extract containing the AAD-1 protein: Lane 1 – MW markers; Lane 2 – SGF Control, 0 min; Lane 3 – SGF Control, 16 min; Lane 4 – Neutralized extract, 0.16 ng; Lane 5 – 10% Neutralized extract, 0.016 ng; Lanes 6 to 10 – 1, 2, 4, 8, and 16 min extract digestion, respectively, 0.16 ng.

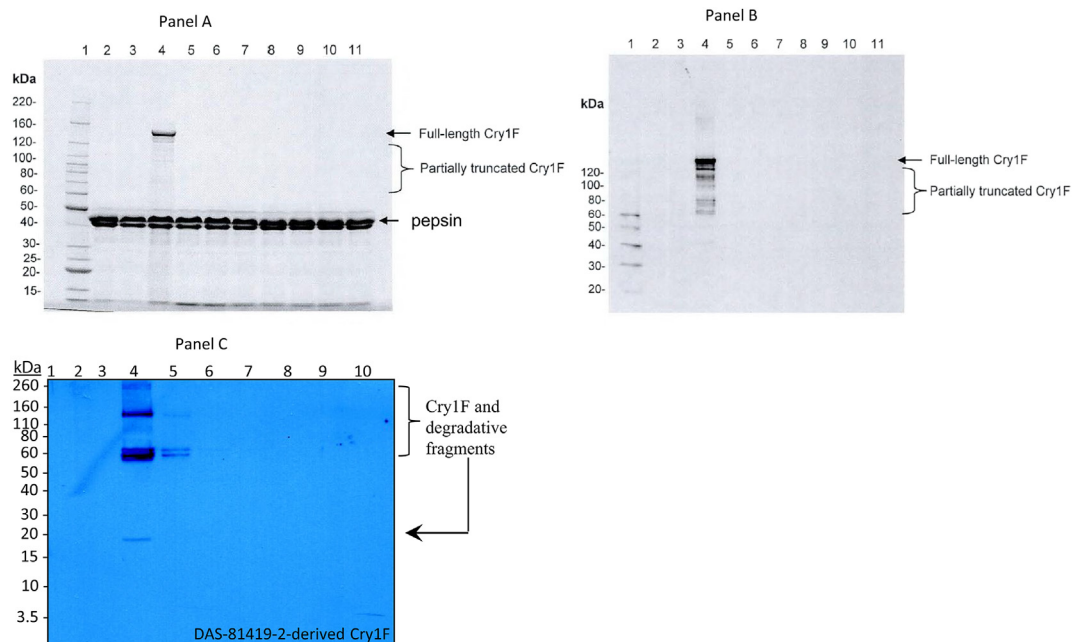


**Fig. 5. Cry1F gel and blots:** Panel A (SDS-PAGE gel) and Panel B (western blot) of *Pf*-derived Cry1F protein: Lane 1 – MW markers; Lane 2 – SGF control, 0 min; Lane 3 – SGF control, 60 min; Lane 4 – Neutralized Cry1F, 3.44  $\mu$ g for gel and 0.34  $\mu$ g for blot; Lanes 5 to 11 – 1, 3, 6, 10, 15, 30 and 60 min Cry1F digestion, respectively, 3.44  $\mu$ g for gel and 0.34  $\mu$ g for blot. Panel C: Western blot of DAS-81419-2 soybean containing the Cry1F protein: Lane 1 – MW markers; Lane 2 – SGF control, 0 min; Lane 3 – SGF control, 16 min; Lane 4 – Neutralized extract, 0.90 ng; Lane 5 – 10% neutralized extract, 0.090 ng; Lanes 6 to 10 – 1, 2, 4, 8, and 16 min extract digestion, respectively, 0.90 ng.

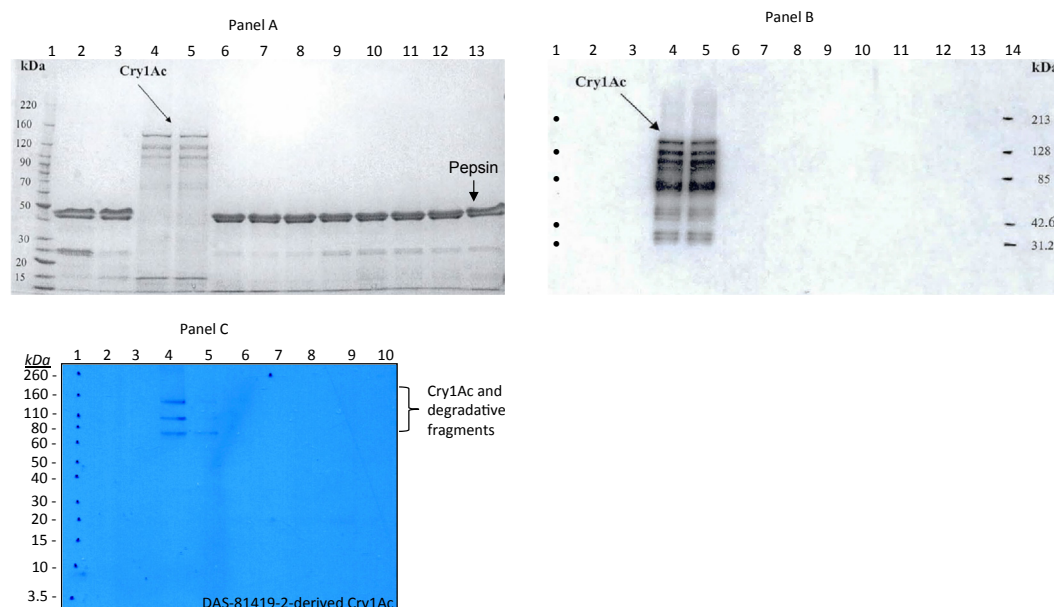


**Fig. 6. Cry1Ac gel and blots:** Panel A (SDS-PAGE gel) and Panel B (western blot) of *Pf*-derived Cry1Ac protein: Lane 1 – MW markers; Lane 2 – SGF control, 0 min; Lane 3 – SGF Control, 60 min; Lane 4 – Cry1Ac (0 min), 3.56  $\mu$ g for gel and 0.71  $\mu$ g for blot; Lane 5 – Cry1Ac (60 min), 3.56  $\mu$ g for gel and 0.71  $\mu$ g for blot; Lanes 6 to 13 – 1, 3, 6, 10, 15, 20, 30, and 60 min Cry1Ac digestion, respectively, 3.56  $\mu$ g for gel and 0.71  $\mu$ g for blot; Lane 14 – (blot) MW markers. Panel C: Western blot of DAS-81419-2 soybean containing the Cry1Ac protein: Lane 1 – MW markers; Lane 2 – SGF control, 0 min; Lane 3 – SGF control, 16 min; Lane 4 – Neutralized extract, 0.06 ng; Lane 5 – 10% neutralized extract, 0.006 ng; Lanes 6 to 10 – 1, 2, 4, 8, and 16 min extract digestion, respectively, 0.06 ng.

(Figs. 5 and 6). Furthermore, larger-than-expected molecular-weight bands were visible on the PAT western blot of the digested soybean seed extracts for both event DAS-44406-6 and DAS-81419-2 (Fig. 1, Panels D and E). These same immunoreactive bands were also seen for non-GE soybean seed extracts (Fig. 1, Panel C) and are therefore considered non-specific binding.

In all cases, the purified recombinant proteins and those in seed and grain (including multimers and fragments) were not detectable at the 1 min incubation point in SGF as demonstrated by either SDS-PAGE and/or western blot analyses (Figs. 1–6). Cry1F, Cry1Ac, PAT, AAD-1, AAD-12, and 2mEPS proteins were rapidly digested in SGF both as purified protein and in the seed/grain matrix.

#### 4. Conclusions

It has been hypothesized that the poor correlation between purified protein digestion and the allergenic status of proteins might be due to the exclusion of plant matrix components or alterations in protein structure during the purification process (Polovic et al., 2007; Schnell and Herman, 2009). Results reported here do not support this hypothesis. Here we show that SGF-digestion results with GE proteins in seed/grain extracts, and with purified proteins from microbes, were comparable. As seen for SGF digestion experiments where purified protein and pepsin concentrations have been varied near standard levels, the SGF assay is quite robust to moderate enzyme and substrate variation (Herman et al., 2005). Based on these results, purified protein from microbial sources is a suitable surrogate for proteins in-matrix when conducting SGF digestion studies. Rapidly digesting purified GE proteins also digested rapidly in the seed/grain matrix for the common GE crops soybean, corn, and cotton. These results also suggest that in-matrix digestion studies are a suitable alternative for GE proteins that cannot be readily purified in a functionally active form (Bushey et al., 2014; Madduri et al., 2012).

#### Acknowledgements

We thank Gaston Legris, Guomin Shan, Mark Krieger, Kathy Clayton, Eric Kraus, and Siva Kumpatla for reviewing a draft of this paper and we thank Benjamin S. Herman for improving the western blot image contrast in the figures.

#### Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.yrtph.2016.08.004>.

#### Conflict of interest

The authors are employed by Dow AgroSciences LLC which develops and markets GE crops.

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