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

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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.

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 delineated 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 °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 °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, β-lactoglobulin A (β-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 × 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 β-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 °C. The samples were digested as follows: Three 2.85-mL aliquots of SGF were placed in the 37 °C water bath (GE sample, BSA, and β-lac). After 5 min, 150 μL 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 μL of the reaction mixtures were removed and added to tubes containing stop solution (40 μL 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 °C. The SGF control was prepared as follows: A 2.85-mL aliquot of SGF was heated in a 37 °C water bath for 5 min, 150 μL of Tris buffer was added, and a timer was set. A 100-μL aliquot was immediately removed as the zero time point and placed into a tube containing the stop reaction (40 μL 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 °C water bath for 5 min. The SGF was neutralized with 1.2 mL of 200-mM sodium bicarbonate and 150 μL 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