



Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Safety assessment of a modified acetolactate synthase protein (GM-HRA) used as a selectable marker in genetically modified soybeans

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ARTICLE INFO

Article history:

Received 27 April 2009

Available online xxx

Keywords:

Acetolactate synthase

Bioinformatics

In vitro digestibility

Allergenicity

Toxicology

Genetically modified soybeans

ABSTRACT

Acetolactate synthase (ALS) enzymes have been isolated from numerous organisms including soybeans (*Glycine max*; GM-ALS) and catalyze the first common step in biosynthesis of branched chain amino acids. Expression of an ALS protein (GM-HRA) with two amino acid changes relative to native GM-ALS protein in genetically modified soybeans confers tolerance to herbicidal active ingredients and can be used as a selectable transformation marker. The safety assessment of the GM-HRA protein is discussed. Bioinformatics comparison of the amino acid sequence did not identify similarities to known allergenic or toxic proteins. *In vitro* studies demonstrated rapid degradation in simulated gastric fluid (<30 s) and intestinal fluid (<1 min). The enzymatic activity was completely inactivated at 50 °C for 15 min demonstrating heat lability. The protein expressed *in planta* is not glycosylated and genetically modified soybeans expressing the GM-HRA protein produced similar protein/allergen profiles as its non-transgenic parental isolate. No adverse effects were observed in mice following acute oral exposure at a dose of at least 436 mg/kg of body weight or in a 28-day repeated dose dietary toxicity study at doses up to 1247 mg/kg of body weight/day. The results demonstrate GM-HRA protein safety when used in agricultural biotechnology.

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1. Introduction

Acetolactate synthase (ALS) enzymes are widely distributed in nature. They have been identified and isolated from bacteria, fungi, algae, and plants including soybeans (Friden et al., 1985; Falco and Dumas, 1985; Mazur et al., 1987). In all plant species examined to date, at least one *als* gene is expressed in a constitutive manner, even though the level of expression may vary between tissues and developmental stages. The highest level of transcription of the *als* gene and activity of the ALS enzyme is found in the metabolically active meristematic tissues (Schmitt and Singh, 1990; Ouellet et al., 1992; Keeler et al., 1993).

ALS enzymes catalyze the first common step in biosynthesis of branched chain amino acids (Isoleucine [Ile], Leucine [Leu], Valine [Val]; LaRossa and Schloss, 1984; LaRossa and Falco, 1984; and Duggleby and Pang, 2000). Reactions catalyzed by ALS enzymes include condensation of two molecules of pyruvate to form acetolactate that leads to synthesis of Leu and Val and condensation of

pyruvate with 2-ketobutyrate to form 2-acetohydroxybutyrate in the Ile biosynthesis pathway. Studies in bacteria and tobacco have demonstrated that ALS is the primary site of action of several chemical classes of herbicides including sulfonylureas, imidazolinones, triazopyrimidines, pyrimidinylthio (or oxy)-benzoates, and sulfonylamino-carbonyltriazolinones (LaRossa and Schloss, 1984; Chaleff and Mauvais, 1984; Tan et al., 2006). The inhibiting effect of these herbicides on plant growth has been attributed to consequences of ALS enzyme inhibition resulting in amino acid content imbalance and inhibition of both DNA synthesis and cell division (Duggleby and Pang, 2000).

Bacteria, yeast, and plants have been identified that are tolerant to sulfonylurea and imidazolinone classes of herbicides (LaRossa and Schloss, 1984; Falco and Dumas, 1985; Chaleff and Mauvais, 1984). Isolation and sequencing of the *als* genes from these herbicide tolerant organisms identified 15 individual amino acid sequence changes in the corresponding proteins (relative to native ALS) that conferred the tolerant phenotype (Hartnett et al., 1990, 1991; Falco et al., 1989; Duggleby and Pang, 2000). Herbicide tolerance mutations have also been found in *als* genes that most frequently occur at four particular amino acid locations (Duggleby and Pang, 2000).

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The *gm-hra* gene which encodes the GM-HRA protein (*Glycine max* herbicide resistant acetolactate synthase) is a modified *als* gene that was produced by site-specific mutagenesis of two amino acids of the endogenous soybean *gm-als* gene. These particular mutations are analogous to those in herbicide tolerant tobacco (P196A and W573L) (Creason and Chaleff, 1988; Lee et al., 1988; Mazur and Falco, 1989; Hartnett et al., 1990). Expression of the GM-HRA protein in genetically modified soybeans confers tolerance to ALS-inhibiting herbicides. While soybeans expressing the GM-HRA protein are considered to be transgenic, it would actually be more accurate to consider them cisgenic since the *gm-hra* gene originated from soybeans.

Dietary proteins are requisite nutrients and typically not associated with adverse effects, however a small number of proteins are known to be allergenic or to cause acute toxicity (Metcalf et al., 1996; Sjoblad et al., 1992). Guidance documents have described procedures to assess the potential allergenicity and toxicity of transgenic proteins used in the context of agricultural biotechnology (CODEX, 2003; FAO/WHO, 2001; Delaney et al., 2008a). A weight-of-evidence approach has been recommended to assess the potential allergenicity of transgenic proteins used in genetically modified crops (CODEX, 2003; Taylor, 2006). Individual components of these assessments focus on comparison of the transgenic protein with the properties of known allergenic food proteins including information about the history of exposure, source of the gene, amino acid sequence similarity to allergenic proteins, *in vitro* resistance to digestive enzymes, and physicochemical properties including glycosylation (Astwood and Fuchs, 1996; Thomas et al., 2004; Ladics et al., 2006). The effect of processing (e.g., thermal lability) on heterologously produced transgenic proteins has also been assessed in some cases. Additionally, the impact of expression of transgenic proteins on the endogenous allergen profile has also been evaluated using serum obtained from humans with a documented clinical history of sensitivity to the particular crop in which the transgenic protein is expressed (Delaney et al., 2008b).

The potential toxicity of transgenic proteins used in genetically modified crops relies on a tiered approach. The first tier includes an assessment of the history of use of the organism from which the gene was obtained, bioinformatic analysis to assess similarity to known toxic proteins, mechanism of action of the protein, *in vitro* stability to digestive enzymes, and exposure assessment (Delaney et al., 2008a,b). When warranted, a second tier of analysis may be conducted. Elements within this tier include an assessment of the acute and repeated dose toxicity studies with purified transgenic proteins as have been requested by some regulatory authorities (European Commission, 2003) and hypothesis-based studies.

The current paper describes the safety assessment process conducted to evaluate the potential allergenicity and toxicity of the GM-HRA protein that has been used as a selectable marker in the context of agricultural biotechnology.

2. Materials and methods

2.1. Bioinformatic analysis of the amino acid sequence of the GM-HRA protein for similarity to allergens

The amino acid sequence of the GM-HRA protein was compared to the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 6.0 [January 2006] <http://www.allergenonline.com/about.asp>) using previously reported methods (Delaney et al., 2008a). Briefly, potential identities between the GM-HRA sequence and proteins in the allergen database were evaluated using the FASTA34 sequence alignment program (Pearson and Lipman, 1988) set to predetermined default parameters (word size = 2, scoring matrix = BLOSUM50, gap creation penalty = −10, gap extension penalty = −2, *E* score cut-

off = 10). Alignments were reviewed for identities >35% over 80 or more residues and for any 8 or more contiguous identical amino acid matches to known allergens (Metcalf et al., 1996; FAO/WHO, 2001; Ladics et al., 2007).

2.2. BLASTP bioinformatic search of the amino acid sequence of the GM-HRA protein for similarity to protein toxins

A sequence similarity search was conducted with the amino acid sequence of the GM-HRA protein using the BLASTP 2.2.13 algorithm against Release 153.0 (4/15/06) of the NCBI Protein dataset (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=protein>) using an *E* score cutoff of 1.0 using previously reported methods (Delaney et al., 2008a). Sequence annotations of proteins with similarity to the GM-HRA protein sequence were inspected manually to identify known toxic proteins. Generally, a statistically significant sequence similarity requires a match with an *E* score of <0.01; however a threshold *E* score of 1.0 provides greater assurance that proteins with even limited similarity will not be overlooked. Because of the large number of uncharacterized conceptual protein sequences derived from automated translations of genomic sequences, protein sequences from the RefSeq collection (<http://www.ncbi.nlm.nih.gov/RefSeq/>) were excluded from the search using the Entrez filtering statement “protein_all [filter] NOT srcdb_refseq [prop]” to delimit the results returned. The scoring matrix used was the default (BLOSUM62), low complexity filtering was turned off and the number of alignments returned was set to the maximum value of 2000 proteins.

2.3. Purification and characterization of bacterially-produced recombinant GM-HRA protein

Two separate lots of GM-HRA proteins were produced and characterized. The GM-HRA protein was expressed in *Escherichia coli* strain BL21 CodonPlus (DE3) RIPL (Stratagene, La Jolla, CA) as a fusion protein containing a His-T7 tag and was purified using immobilized metal affinity chromatography. Following purification, the His-T7 tag was cleaved from the affinity purified protein with thrombin. Then the fusion tag and thrombin were removed by dialysis (for the lot used in the acute toxicity study, digestion assays, thermal stability study and characterization figures) or diafiltration into 50 mM bicarbonate buffer (for the lot used in the repeated dose toxicity study). Thrombin cleavage resulted in one additional N-terminal amino acid (Glycine) on the microbially produced GM-HRA that is not found on the mature GM-HRA protein expressed *in planta*. Finally the protein was lyophilized, mixed and stored at −80 °C. Both lots of the GM-HRA proteins were independently characterized. Endotoxin and thrombin content were tested using aliquots of each lot prior to lyophilization. The purity (the percentage of the expected 65 kilodalton (kDa) GM-HRA protein on a total protein basis) was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by scanning densitometry. The concentration of total protein in each lot was determined using quantitative amino acid compositional analysis. The identity of both lots of the proteins was characterized using amino acid analysis, Western blot analysis with a proprietary GM-HRA specific antibody, electrospray mass spectroscopy, matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) of tryptic peptides, and N-terminal amino acid sequencing. Glycosylation of the GM-HRA protein was also assessed using a glycoprotein detection method.

2.4. Protein glycosylation analysis

A GelCode Glycoprotein staining kit (Pierce Biotechnology, Inc.) was used according to the manufacturer's instructions to deter-

mine whether the GM-HRA protein was glycosylated. The GM-HRA protein was isolated from genetically modified soybean leaf tissue by immunoaffinity chromatography. Additional characterization of this protein included SDS–PAGE, Western blot using a GM-HRA specific antibody, N-terminal amino acid sequencing, and MALDI-MS analyses. For the glycosylation analysis, the immunoaffinity purified GM-HRA protein was subjected to SDS–PAGE along with a positive control protein (horseradish peroxidase) and a negative control protein (soybean trypsin inhibitor). Following electrophoresis, the gel was fixed with 50% methanol for approximately 30 min and washed with 3% acetic acid. The gel was then incubated with oxidizing solution for approximately 15 min and washed three times with 3% acetic acid. The gel was incubated with Gel-Code glycoprotein staining reagent for 15 min and then treated with reducing reagent. The stained gel was then extensively washed with 3% acetic acid and deionized water. Glycoproteins were visualized as a magenta colored band on the gel. Following glycoprotein detection, the gel was scanned and the image was captured electronically. The same gel was then stained with Coomassie Blue to visualize all protein bands.

2.5. *In vitro* pepsin resistance of GM-HRA and control proteins in simulated gastric fluid (SGF)

In vitro pepsin resistance assays were conducted with heterologously produced GM-HRA protein using materials and methods as described previously (Delaney et al., 2008a). Bovine serum albumin (BSA) and β -Lactoglobulin proteins (Sigma–Aldrich, St. Louis, MO) were used as the positive and negative controls, respectively. Briefly, GM-HRA was incubated for 0, 0.5, 1, 2, 5, 10, 20, 30, and 60 min in SGF while BSA and β -Lactoglobulin were incubated for 0, 1, and approximately 60 min in SGF. The final concentrations of proteins in the SGF reaction mixture were 0.25 mg/ml GM-HRA protein and 0.74 mg/ml pepsin at pH 1.2, resulting in a molar ratio of enzyme (pepsin) to protein (GM-HRA) of 5.5:1. Samples were removed at the indicated time points and subjected to SDS–PAGE analysis. Resulting gels were either stained with RAPID-stain (G-Biosciences, St. Louis, MO) or used in subsequent Western blot analysis and evaluated for apparent disappearance of protein bands.

2.6. *In vitro* pancreatin resistance of GM-HRA and control proteins in simulated intestinal fluid (SIF)

Heterologously produced GM-HRA protein and commercially purchased positive and negative control proteins (BSA and β -Lactoglobulin, respectively) used in the pancreatin resistance assay were dissolved in water to a concentration of 5 mg/ml. *In vitro* pancreatin resistance assays were conducted using materials and methods as described previously (Delaney et al., 2008a). Briefly, GM-HRA was incubated for 0, 0.5, 1, 2, 5, 10, 20, 30, and 60 min in SIF while control substances (BSA and β -Lactoglobulin) were incubated for 0, 1, and approximately 60 min in SIF. The final nominal concentration of GM-HRA was 0.25 mg/ml in the SIF (50 mM KH_2PO_4 and 1% w/v pancreatin, pH 7.5) reaction mixture. Samples were removed at the indicated time points and subjected to SDS–PAGE. Resulting gels were stained with RAPIDstain (G-Biosciences) for at least 1 h and destained with deionized water. Western blot analysis of the GM-HRA SIF digestion reaction was conducted in order to clearly differentiate between the GM-HRA protein and the pancreatin protein bands. A digital image of the gel was generated.

2.7. Thermal treatment of GM-HRA protein

Thermal stability of microbially produced GM-HRA protein was determined by assessing the enzymatic activity of GM-HRA protein

following treatment with different thermal conditions. GM-HRA protein powder was dissolved in deionized water at 3 mg powder per ml and diluted with an equal volume of 2 $\times$ ALS buffer (4.0 mM magnesium chloride, 1.0 mM thiamine pyrophosphate, 40 μM flavin adenine dinucleotide, 20% glycerol, 200 mM phosphate buffer pH 7.5). The suspension was then centrifuged at 13,000 rpm for 5 min. The supernatant was removed and the soluble protein concentration was determined using Coomassie Plus Protein Assay Reagent (Pierce Biotechnology, Inc., Rockford, IL). The concentration of the solution was adjusted to a final concentration of 100 μg of protein per ml with 1 $\times$ ALS buffer. Fifty-microliters (μl) of the GM-HRA protein was dispensed into each well of a 96-well PCR plate. Individual wells were heated for 15 min at a designated temperature ranging from 36–60 $^{\circ}\text{C}$ with 2 $^{\circ}\text{C}$ increments in a Robocycler gradient thermocycler (Stratagene, La Jolla, CA). Samples were then centrifuged for 1 min (at 1000 rpm) and 20 μl of the supernatant was diluted to 100 μl with 1 $\times$ ALS buffer.

2.8. Enzymatic activity of GM-HRA protein

The spectrophotometric assay for assessing ALS activity was an indirect detection of the enzyme product, acetolactate. Following incubation of the enzyme with the substrate (pyruvate), the end product acetolactate is converted to acetoin by decarboxylation with sulfuric acid and high temperature. Acetoin is detected by formation of a creatine and α -naphthol complex which can be measured at 530 nm as described by Westerfeld (1945). In this study, GM-HRA samples were analyzed for activity by dispensing three 100- μl replicates of each non-heated and heat-treated GM-HRA protein sample into a 96-well plate. Five-microliters of 100 mM phosphate buffer was added to each well. Enzyme production of acetolactate from pyruvate was initiated by the addition of 10 μl of a 1.1 M pyruvate solution to each well, followed by incubation at 37 $^{\circ}\text{C}$ for 60 min (Chaleff and Mauvais, 1984). The acetolactate was then converted to acetoin by adding 5 μl of 2 N H_2SO_4 to each well, followed by incubation at 60 $^{\circ}\text{C}$ for 15 min. The plates were then allowed to cool at room temperature for 15 additional minutes. Indirect detection of the enzymatic reaction was performed by adding 50 μl of creatine/naphthol solution per well, followed by incubation at 60 $^{\circ}\text{C}$ for 15 min. Plates were allowed to cool at room temperature for an additional 15 min and read at 530 nm using SpectraMax spectrophotometer (Model 190, Molecular Devices Corporation, Sunnyvale, CA). The relative amount of product produced by the enzyme sample was interpolated from the acetoin standard curve.

2.9. SDS–PAGE analysis for GM-HRA protein characterization

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by first mixing GM-HRA protein samples with Laemmli sample buffer (Gradipore Limited, Frenchs Forest, NSW, Australia) containing 100 mM dithiothreitol and heating the solution at 100 $^{\circ}\text{C}$ for approximately 5 min. The prepared protein samples were loaded on a 10–20% gradient Ready Gel Tris–HCl gel (Bio-Rad Laboratories, Inc., Hercules, CA). PageRuler Unstained or Prestained Protein Ladder (#SM0661 or #SM0671; Fermentas, Inc., Hanover, MD) molecular weight markers were loaded on the gel to provide a visual estimate of molecular weight. Electrophoresis was conducted using the Ready Gel Cell system (Bio-Rad Laboratories, Inc.) with Tris–glycine running buffer (Gradipore Limited) and a constant 150 V for approximately 60 min or until the dye front neared the bottom of the gel. Upon completion of electrophoresis, the gels were removed from the gel cassette and were either prepared for Western blot analysis or were stained. Prior to staining, the gels were washed three times with deionized water for approximately 5 min each. The gels were then stained for approx-

imately 60 min with GelCode Coomassie Blue stain reagent (Pierce Biotechnology, Inc.), and destained with deionized water at least four times for approximately 10 min each.

2.10. Western blot analysis for GM-HRA protein characterization

Following electrophoresis, the resulting gel was soaked in Transfer Buffer (48 mM Tris-HCl, pH 8.6, 39 mM glycine, 0.0375% sodium dodecyl sulfate, and 20% methanol) for approximately 20 min. A polyvinylidene difluoride (PVDF, Bio-Rad Laboratories, Inc.) membrane was briefly placed in 100% methanol, followed by immersion in Transfer Buffer for 10–15 min. A Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell system (Bio-Rad Laboratories, Inc.) was used to transfer proteins from the gel to the membrane at 11 V for approximately 60 min. Following protein transfer, the membrane was washed four times for approximately 5 min each in Classic Buffer (50 mM Tris-HCl, pH 7.0, 500 mM sodium chloride, and 0.5% Tween-20), and then blocked by incubating in phosphate-buffered saline solution with Tween-20 (PBST: 8.1 mM phosphate buffer, pH 7.4, 137 mM sodium chloride, 2.7 mM potassium chloride, and 0.05% Tween-20) containing 5% non-fat dry milk for approximately 60 min. The blocked membrane was washed four times for approximately 5 min each in Classic Buffer, and then incubated for approximately 60 min with a GM-HRA specific antibody diluted 1:2500 in PBST containing 5% non-fat dry milk or a GM-ALS nonspecific antibody (recognizing both GM-ALS and GM-HRA) diluted 1:8000 in PBST containing 5% non-fat dry milk. The unbound antibody was rinsed from the membrane with four washes of Classic Buffer for approximately 5 min per wash. The membrane was then incubated for approximately 60 min with a horseradish peroxidase conjugated secondary antibody (Promega Corporation, Madison, WI) diluted 1:10,000 in Classic Buffer. The membrane was then washed four times with Classic Buffer for approximately 5 min each. The blot was then soaked in phosphate-buffered saline solution (PBS: 8.1 mM phosphate buffer, pH 7.4, 137 mM sodium chloride, and 2.7 mM potassium chloride) for approximately 5 min and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) according to the manufacturer's instructions. The molecular weight of the recognized protein band was estimated using PageRuler Prestained Protein Ladder (#SM0671; Fermentas, Inc.).

2.11. Acute toxicity of GM-HRA protein in mice

The acute toxicity study with purified GM-HRA protein obtained from a heterologous bacterial expression system was conducted at DuPont Haskell Global Centers for Health and Environmental Sciences (Newark, DE) in accordance with requirements for the US EPA FIFRA GLP (US EPA, 1989) and OECD 423 guidance (OECD, 2001). GM-HRA or control protein (BSA) suspended in deionized water were administered to CD-1 mice (5/sex) at a dose of 2000 mg/kg of body weight in accordance with OECD acute toxicity limit dose guidelines. Additional groups of mice (5/sex) were gavaged with deionized water. Individual body weights were determined at the time of dosing (Day 0) and at different time points (Days 1, 2, 4, 7, and 14) following administration of the test or control substances. Observation of clinical effects was conducted throughout the in-life phase of the study and a gross necropsy was conducted following sacrifice 14 days following exposure as described previously (Delaney et al., 2008a).

2.11.1. Repeated dose toxicity assessment of GM-HRA protein

The repeated dose dietary toxicity assessment of GM-HRA protein was conducted at Charles River Laboratories (Horsham, PA) in accordance with requirements for the US EPA FIFRA GLP (US EPA, 1989) and OECD 407 guidance (OECD, 1995). Healthy male and fe-

male Crl:CD-1(ICR) mice from Charles River Laboratories (Raleigh, North Carolina) were individually housed in stainless steel cages that conformed to standards set forth in the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996). On Study Day 1, the animals were approximately 10 weeks old. Males weighed 28–32 g and females weighed 22–26 g upon arrival. Animals consumed the respective diets for 28 consecutive days after which they were sacrificed. Filtered tap water was provided *ad libitum* throughout the study. Environmental controls were set to maintain temperatures of 19–25 °C with a relative humidity of 30–70%. A 12-h light/dark cycle was maintained in the animal room. The animal room was ventilated with at least 10 air changes per hour with 100% fresh air (no air recirculation). Twenty-five males and 25 females were randomly assigned to groups by a computer generated weight-ordered distribution such that group mean body weights did not exceed $\pm 20\%$ of the overall mean body weight for each sex.

2.11.2. Diet formulation and administration

Dietary formulations were prepared by Charles River Laboratories (Horsham, PA), stored under refrigeration and administered fresh daily based on estimated male body weights from facility historical control data. Calculations were based on a purity correction factor based on GM-HRA protein test substance (0.787 mg protein/mg lyophilized powder and 75% 65 kDa GM-HRA protein on a total protein basis) and on average estimated consumption of 6 g of diet per day per mouse. Control mice ($n = 5/\text{sex}$) consumed either PMI 5002 diets (meal form of Certified Rodent Diet® #5002, PMI® Nutrition International, St. Louis, MO) or PMI 5002 diets that were mixed with BSA at concentrations corresponding to the highest concentration of GM-HRA that was tested for a dose of approximately 1000 mg/kg of body weight of BSA per day. Mice in the test groups consumed one of three test diets consisting of PMI 5002 diets mixed with GM-HRA protein at concentrations to ensure daily exposure to doses of 100, 300, or 1000 mg/kg of body weight/day of GM-HRA protein. For mice consuming diets containing GM-HRA protein, a premix was prepared by blending the appropriate amount of GM-HRA protein with approximately 500 g of PMI 5002 diet in a Waring® 34BL97 homogenizer for approximately 10 s. The premix was then combined with the remaining PMI 5002 diet (approximately 1500 g) in a Hobart® mixer for approximately 15 min. The concentration, homogeneity, and stability of the GM-HRA protein in the diets were assessed using a GM-HRA specific antibody-based ELISA.

2.11.3. Clinical observations, body weights, feed consumption, ophthalmology, serum chemistry, organ weights, and histopathology

Clinical observations were recorded before the start of the study and weekly thereafter. Body weights were recorded on Study Days 4, 1, 8, 15, 22, and 29. Feed consumption was measured daily. Ophthalmic examinations were conducted for all animals pretest and on Study Day 26 (females) or 27 (males). A functional observational battery (FOB) was performed and motor activity quantified on all mice prior to exposure and again on Study Day 28. Blood samples obtained from fasted animals on Study Day 29 were evaluated for the following response variables: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), total bilirubin (BILI), blood urea nitrogen (BUN), creatinine (CREA), cholesterol (CHOL), triglycerides (TRIG), glucose (GLUC), total protein (TP), albumin (ALB), globulin (GLOB), albumin/globulin ratio (A/G), calcium (CALC), inorganic phosphorus (IPHS), sodium (NA), potassium (K), and chloride (CL). Following sacrifice, a gross necropsy (including all external surfaces and orifices, internal examination of tissues and organs, cranial, thoracic, and abdominal cavities with their associated organs and tissues) was conducted. Organ weights were recorded for the following organs prior to fixation: adrenal glands, brain, epididymides, heart,

kidneys, liver, ovaries, spleen, testes, thymus, and uterus with cervix. Paired organs were weighed together. Samples of these and additional tissues were collected, preserved and read for microscopic signs of pathology as described previously (Delaney et al., 2008a). Statistical analysis of data from the repeated dose toxicity study was conducted on body weight, feed intake, clinical chemistry, and organ weight data as described previously (Delaney et al., 2008a). In brief, response variables from the diet only control group were compared with each test group individually. When statistically significant differences ($p < 0.05$) were observed in particular response variables from this initial comparison, results from the individual test groups were compared with those of the BSA control group.

3. Results

3.1. Bioinformatics comparison of GM-HRA protein to allergenic proteins

No matches were returned when the GM-HRA protein sequence was used as a query in the search for continuous, identical stretches of amino acids 8 residues or greater in length with all known allergenic proteins (data not shown). FASTA34 analysis using the GM-HRA sequence as a query returned seven alignments to allergens in the dataset possessing E scores less than 10 (Table 1). The alignment with the highest degree of similarity ($E = 1.9$ corresponding to 28% over 50 amino acids) was between the GM-HRA protein sequence and a putative allergen Mal f 2 from the opportunistic skin yeast *Malassezia furfur* (Yasueda et al., 1998). This alignment between the GM-HRA and the allergen sequences is considerably lower than the 35% over 80 or greater amino acid threshold guideline for similarity to allergenic proteins. All the other remaining six alignments were of even lower percent identity and higher E score indicating that the GM-HRA protein does not possess sequence similarity to known allergenic proteins.

3.2. BLASTP comparison of GM-HRA protein to other proteins

A BLASTP similarity search was conducted using the amino acid sequence of GM-HRA to identify the closest 2000 matches. The highest E score returned was 0.85, confirming that sequences with limited similarity were not overlooked in the search. Ninety-five of the accessions returned by the searches displayed complete significance ($E = 0$) and represent very closely related ALS or acetohydroxyacid synthase (AHAS) proteins from various plant species. A total of 827 other sequences were identified as either AHAS or ALS proteins from various bacterial, archaeobacterial, and eukaryotic species. The remaining 1078 hits represented a variety of proteins that were all functionally related by the possession of one or more well characterized conserved thiamine pyrophosphate binding domains. None of the 2000 proteins returned by the BLASTP search identified similarities to proteins known to be toxic.

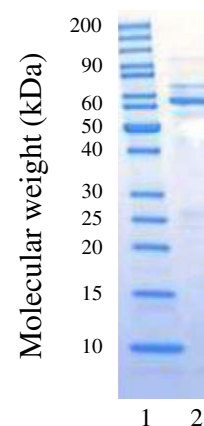


Fig. 1. Characterization of bacterially-produced GM-HRA protein. SDS-PAGE analysis of purified microbial GM-HRA protein. Lane 1 contains molecular weight markers (Fermentas Unstained Protein Molecular Marker #SM0661); Lane 2 contains GM-HRA protein (1 μ g).

3.3. Isolation and characterization of heterologously produced GM-HRA protein

The recombinant GM-HRA protein used in the studies described in subsequent sections of this paper was expressed in *E. coli* and purified as a soluble protein. The GM-HRA protein migrated as a major band with a molecular weight of approximately 65 kDa by SDS-PAGE (Fig. 1). The purity of the 65 kDa GM-HRA protein on a total protein basis was greater than 75% for both lots as determined by densitometry analysis following SDS-PAGE analysis (data not shown). By amino acid composition analysis, the protein concentration of the lyophilized powder was 29.1% for the lot used in the acute toxicity study and 78.7% for the lot used in the repeated dose toxicity study (data not shown). The GM-HRA protein demonstrated immunoreactivity in Western blot analysis using a GM-HRA specific antibody and recognized a predominant protein band migrating at approximately 65 kDa (Fig. 2A and B). The lot used in the acute toxicity study contained less than 200 units of endotoxin and 2.2 units of thrombin/mg of the lyophilized protein. The lot used in the repeated dose toxicity study contained less than 100 units of endotoxin and 8.4 units of thrombin/mg of lyophilized protein. MALDI-MS analysis showed 38% and 51.7% sequence coverage for the GM-HRA protein utilized in the acute and repeated dose toxicity studies, respectively. Further analytical studies included electrospray mass spectroscopy, N-terminal amino acid sequencing, glycosylation analysis and activity assay. These analyses demonstrated the purity and expected characteristics of the heterologously produced GM-HRA protein (data not shown).

3.4. Digestion of GM-HRA in SGF and SIF

In vitro resistance of GM-HRA protein to digestive enzymes was assessed by incubation in SGF (containing pepsin) and SIF (contain-

Table 1
Summary of FASTA34 allergen alignments to GM-HRA.

GI No.	Allergen description	Identity (%)	Length (aa)	E score
3445490	MF1 [<i>Malassezia furfur</i>]	28.0	50	1.9
130360	Putative peroxiredoxin-A (Thioredoxin reductase) (Peroxisomal membrane protein A) (PMP20) (Allergen C and b 2)	23.6	127	3.8
1168348	Alcohol dehydrogenase 1 (40 kDa allergen) (Allergen C and a 1) (Can a 1) (Can a 1)	28.0	100	3.8
58585250	Profilin [<i>Apis mellifera</i>]	44.4	27	6
398830	Phlp5 [<i>Phleum pratense</i>]	21.7	235	6.2
12005501	Vacuolar serine protease [<i>Penicillium citrinum</i>]	33.0	88	8.3
14423648	Calcium-binding allergen Ole e 8 (PCA18/PCA23)	24.1	108	9.6

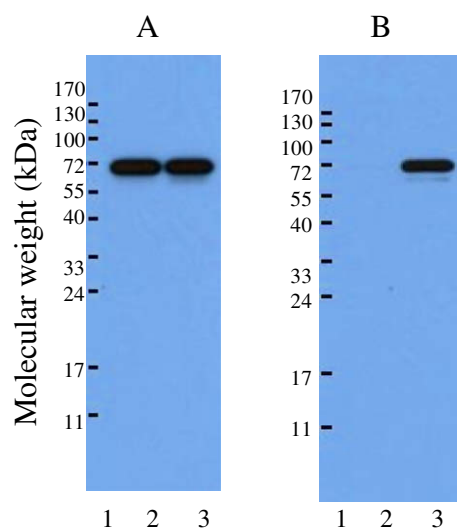


Fig. 2. Characterization of bacterially-produced GM-HRA protein. (A). Western blot analysis of microbial GM-HRA protein using a GM-ALS nonspecific antibody (recognizing both GM-HRA and GM-ALS). Lane 1 contains molecular weight markers (Fermentas Prestained Protein Molecular Marker (#SM0671)); Lane 2 contains microbial GM-ALS protein (20 ng); Lane 3 contains microbial GM-HRA protein (20 ng). The blot was probed with a GM-ALS nonspecific antibody. (B). Western blot analysis of microbial GM-HRA protein using a GM-HRA specific antibody. Lane 1 contains molecular weight markers (Fermentas Prestained Protein Molecular Marker (#SM0671)); Lane 2 contains microbial GM-ALS protein (20 ng); Lane 3 contains microbial GM-HRA protein (20 ng). The blot was probed with a GM-HRA specific antibody.

ing pancreatin). The 65 kDa GM-HRA protein was rapidly (<30 s) hydrolyzed in SGF containing pepsin by both SDS–PAGE and by Western blot analysis (Figs. 3 and 4). Similarly, no visible 65 kDa GM-HRA protein band was observed following SDS–PAGE or Western blot analysis at 30 s and 1 min, respectively, following incubation in SIF (Figs. 5 and 6). These results demonstrated that the GM-HRA protein is not resistant to degradation by digestive enzymes. Additional bands in the GM-HRA lanes appeared to be the aggregated or degraded forms of the GM-HRA protein as indicated by Western blot analysis (Figs. 4 and 6).

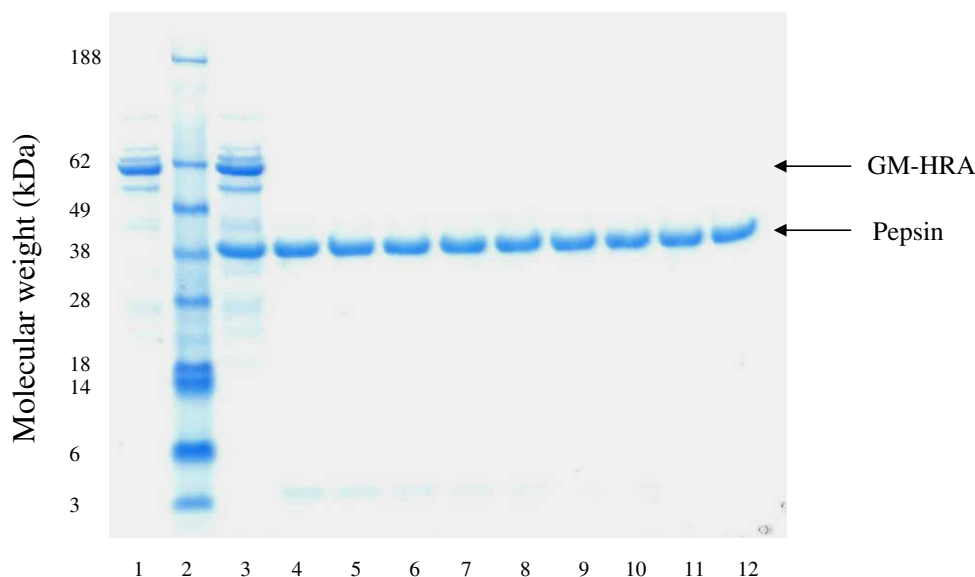


Fig. 3. Pepsin digestion of GM-HRA protein with SDS–PAGE. Purified heterologously produced GM-HRA protein was incubated in SGF containing pepsin for 0–60 min at 37 °C. Lane 1 contains GM-HRA protein (2.3 µg); Lane 2 contains molecular weight markers; Lane 3 contains GM-HRA at “Time 0”; Lanes 4–11 contain GM-HRA following SGF digestion of 30 s, 1, 2, 5, 10, 20, 30, and 60 min, respectively; Lane 12 contains SGF control ~60 min.

3.5. Thermal stability of GM-HRA

The thermal stability of GM-HRA was evaluated by comparison of residual enzyme activity following heat treatment relative to untreated GM-HRA protein. Enzymatic activity of GM-HRA was stable up to approximately 42 °C but decreased at higher temperatures and no enzymatic activity was observed at temperatures greater than 50 °C (Fig. 7).

3.6. Glycosylation analysis of the GM-HRA protein

The GM-HRA protein extracted from genetically modified soybean leaf tissue was not glycosylated as determined using a protein glycoprotein staining kit (Fig. 8). Characterization of the protein showed it was the GM-HRA form (data not shown).

3.7. Acute toxicity of GM-HRA protein

The acute toxicity of the GM-HRA protein was assessed in mice by oral administration of the purified heterologously expressed protein at 2000 mg/kg of body weight of the test substance (corresponding to approximately 436 mg/kg of body weight of GM-HRA protein) via oral gavage. Control groups were administered either vehicle (water) alone or BSA at 2000 mg/kg of body weight. All mice survived the duration of the study and no clinical signs of systemic toxicity were observed in any of the treatment groups (data not shown). Mice in all treatment groups gained weight relative to Day 0 of dosing (Table 2) and no gross lesions were present in any of the mice at necropsy indicating that the GM-HRA protein was not acutely toxic.

3.8. Repeated dose toxicity assessment of GM-HRA protein

Bacterially-produced GM-HRA was blended into diets at three different concentrations corresponding to daily doses of 100, 300, and 1000 mg/kg of body weight/day. The amount of protein incorporated into the diets was based on historical control data for male mice body weight and feed consumption. For comparison, mice were fed with either a Control diet (PMI 5002) or a diet containing control protein (BSA) at concentrations corresponding to those in the high GM-HRA dose group (i.e., 1000 mg/kg of body weight/

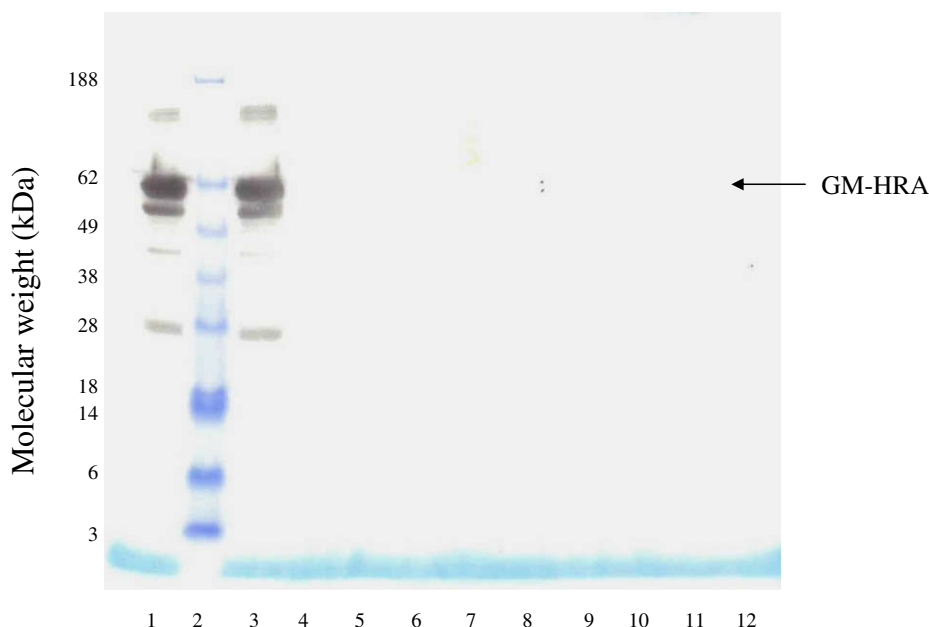


Fig. 4. Pepsin digestion of GM-HRA protein with Western blot analysis. Purified heterologously produced GM-HRA protein was incubated in SGF containing pepsin for 0–60 min at 37 °C. Lane 1 contains GM-HRA protein (2.3 µg); Lane 2 contains molecular weight markers; Lane 3 contains GM-HRA at “Time 0”; Lanes 4–11 contain GM-HRA following SGF digestion of 30 s, 1, 2, 5, 10, 20, 30, and 60 min, respectively; Lane 12 contains SGF control ~60 min. The blot was probed with a GM-HRA specific antibody.

day). Diets were fed to mice for a total of 28 consecutive days. Over the course of the in-life phase of the study, no statistically significant differences were observed in body weights (Figs. 9 and 10) or feed consumption between control groups and those consuming diets containing the GM-HRA protein (Figs. 11 and 12). Consumed dosages of GM-HRA protein averaged 107, 302, and 992 mg/kg of body weight/day in males and 123, 383, and 1247 mg/kg of body weight/day in females compared to 1066 and 1337 mg/kg of body weight/day, respectively in males and females from the BSA protein control groups.

No deaths occurred and there were no abnormal clinical or ophthalmological observations in any of the treatment groups (data

not shown). No treatment-related differences were observed in the FOB or motor activity evaluations (data not shown). No adverse effects were observed in the clinical chemistry response variables of any treatment group (Tables 3 and 4) and no treatment-related adverse effects were observed in organ weights from any of the treatment groups (Table 5). A statistically significant decrease in the relative spleen weight observed in male mice from the 300 and 1000 mg/kg of body weight/day GM-HRA protein dosage groups compared with the Control group was not considered adverse or related to the test substance because the differences were not dosage-dependent, not observed in female mice, and there were no histopathological observations observed in the spleen. A

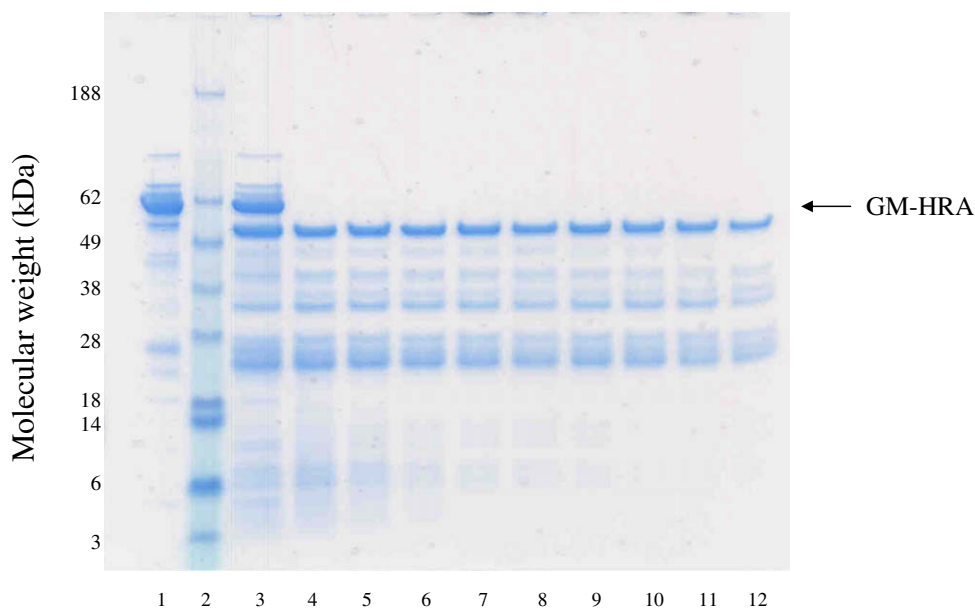


Fig. 5. SDS-PAGE of pancreatin digestion of GM-HRA protein. Purified heterologously produced GM-HRA protein was incubated in SIF containing pancreatin for 0–60 min at 37 °C. Lane 1 contains GM-HRA protein (3.3 µg); Lane 2 contains molecular weight markers; Lane 3 contains GM-HRA at “Time 0”; Lanes 4–11 contain GM-HRA following SIF digestion of 30 s, 1, 2, 5, 10, 20, 30, and 60 min, respectively; Lane 12 contains SIF control ~60 min.

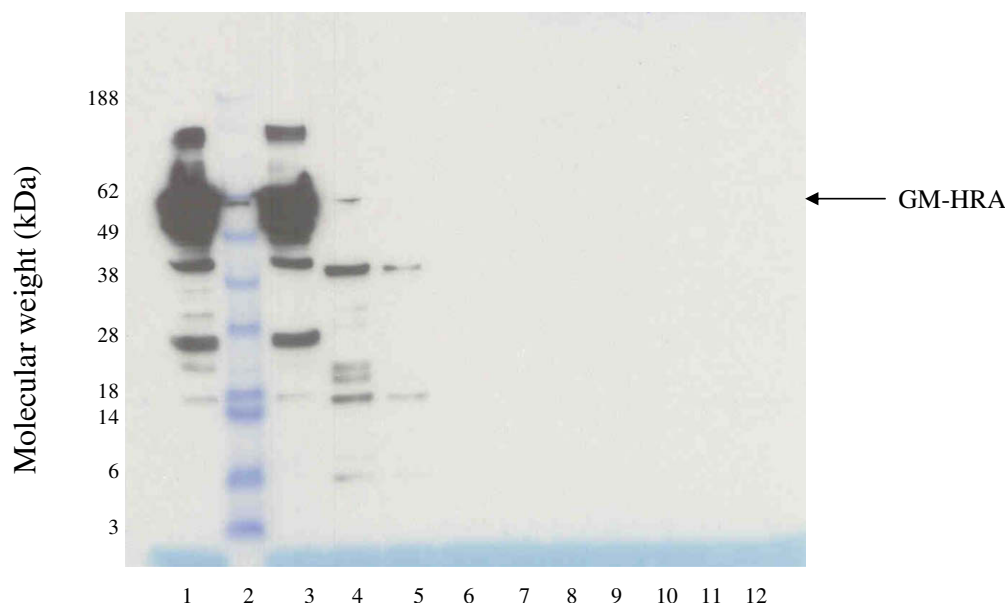


Fig. 6. Pancreatin digestion of GM-HRA protein with Western blot analysis. Purified heterologously produced GM-HRA protein was incubated in SIF containing pancreatin for 0–60 min at 37 °C. Lane 1 contains GM-HRA protein (3.3 µg); Lane 2 contains molecular weight markers; Lane 3 contains GM-HRA at “Time 0”; Lanes 4–11 contain GM-HRA following SGF digestion of 30 s, 1, 2, 5, 10, 20, 30, and 60 min, respectively; Lane 12 contains SIF control ~60 min. The blot was probed with a GM-HRA specific antibody.

statistically significant decrease in the mean relative paired adrenal weights was observed in male mice from the 300 mg/kg of body weight/day GM-HRA protein dosage group. This difference was not considered to be adverse or related to the test substance because it was not dosage-dependent, not observed in female mice, the value observed was comparable to the significant difference observed in the BSA exposure group, and there was no evidence of histopathological changes in the adrenals or kidneys or differences in clinical chemistry response variables indicating adverse effects in kidney function. No gross or microscopic lesions were identified in any tissues that were considered adverse or related to exposure to the GM-HRA protein in the experimental diets (data not shown).

4. Discussion

ALS enzymes catalyze the first common step in the biosynthesis of essential branched chain amino acids (LaRossa and Schloss, 1984; LaRossa and Falco, 1984; and Duggleby and Pang, 2000). Herbicide tolerant ALS enzymes have also been identified that are attributable to a small number of amino acid sequence changes relative to native ALS (LaRossa and Schloss, 1984; Falco and Dumas, 1985; Chaleff and Mauvais, 1984; Hartnett et al., 1990, 1991; Falco et al., 1989; Duggleby and Pang, 2000). The GM-HRA protein is the product of the *gm-hra* gene that was produced by site-specific mutagenesis of endogenous soybean *gm-als* gene with substitutions of two amino acids relative to the native soybean ALS

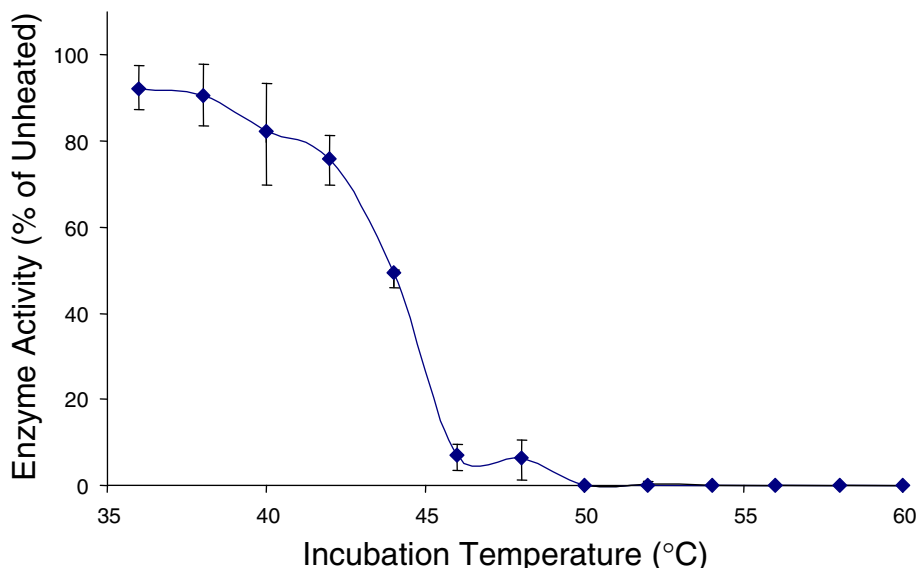


Fig. 7. Thermolability of GM-HRA protein. Heterologously produced GM-HRA was heated for 15 min over a temperature range of 36–60 °C after which the enzyme activity was measured.

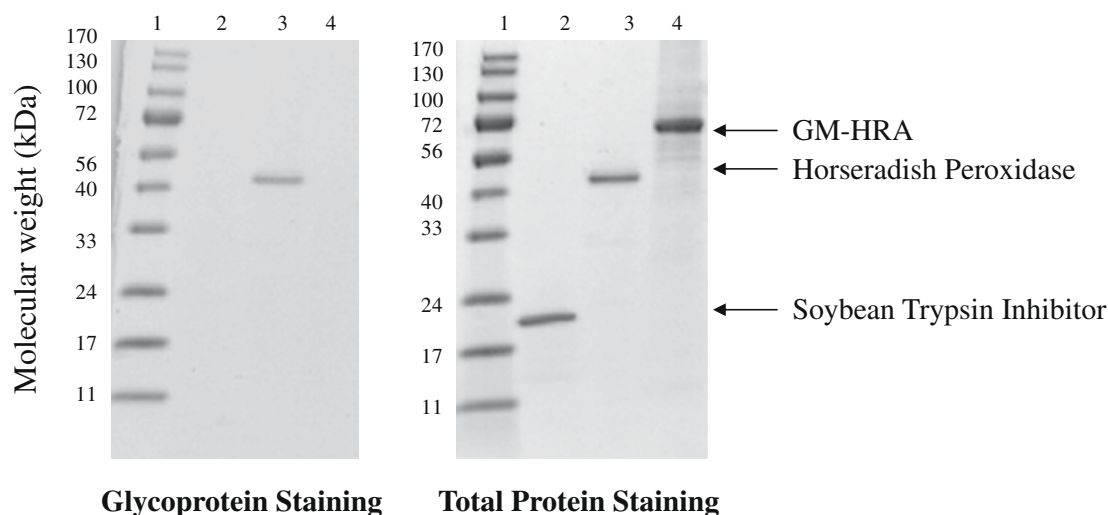


Fig. 8. Glycosylation analysis of GM-HRA protein expressed *in planta*. An SDS-PAGE gel was run with GM-HRA protein isolated from leaf tissue of genetically modified soybeans expressing the protein (lane 4), a glycoprotein-positive control (horseradish peroxidase; lane 3), a glycoprotein-negative control (soybean trypsin inhibitor; lane 2), or prestained molecular weight markers (Fermentas, lane 1). In the left figure, the gel was stained for glycoproteins using a glycoprotein staining kit. In the right figure, the same gel was stained with Coomassie Blue to visualize individual protein bands.

Table 2

Mean body weights (g $\pm$ SD) of mice from acute toxicity study with GM-HRA protein.

	Day					
	0	1	2	4	7	14
Males						
Vehicle	30.84 $\pm$ 1.09	31.84 $\pm$ 1.45	31.64 $\pm$ 1.49	32.84 $\pm$ 1.48	32.24 $\pm$ 1.43	32.68 $\pm$ 2.26
BSA	32.16 $\pm$ 0.92	33.80 $\pm$ 0.81	33.88 $\pm$ 0.91	34.98 $\pm$ 1.12	34.66 $\pm$ 1.12	36.24 $\pm$ 1.36
GM-HRA	31.54 $\pm$ 1.21	32.86 $\pm$ 1.26	32.66 $\pm$ 1.20	33.74 $\pm$ 1.62	33.48 $\pm$ 1.80	35.18 $\pm$ 1.75
Females						
Vehicle	23.10 $\pm$ 1.35	23.70 $\pm$ 1.68	23.54 $\pm$ 1.48	24.90 $\pm$ 1.67	24.16 $\pm$ 1.71	25.18 $\pm$ 1.71
BSA	23.04 $\pm$ 1.18	23.48 $\pm$ 1.42	23.44 $\pm$ 1.32	25.00 $\pm$ 1.37	24.84 $\pm$ 1.33	26.16 $\pm$ 1.24
GM-HRA	22.98 $\pm$ 1.61	23.72 $\pm$ 1.23	23.78 $\pm$ 1.12	24.98 $\pm$ 1.39	24.68 $\pm$ 1.47	26.28 $\pm$ 1.12

protein. These changes are analogous to those in herbicide tolerant tobacco (P196A and W573L) and equivalent to the mutations in naturally-occurring herbicide tolerant genotypes (Lee et al., 1988; Mazur and Falco, 1989; Hartnett et al., 1990; Duggleby and Pang, 2000). Expression of the GM-HRA protein in genetically modified soybeans confers tolerance to ALS-inhibiting herbicides and is used as a selectable biomarker in the context of agricultural biotechnology.

This paper describes the safety assessment that was conducted with the GM-HRA protein in a manner similar to that reported for

GAT4601, another transgenic protein used for herbicide tolerance in genetically modified crops (Delaney et al., 2008a). Differences between the methods used to assess the safety of transgenic proteins used in agricultural biotechnology and other types of food ingredients have been reviewed (Delaney, 2007). The assessments focus on evaluation of the potential for allergenicity and toxicity of transgenic proteins using weight-of-evidence and tiered approaches, respectively (CODEX, 2003; Delaney et al., 2008b).

The *gm-hra* gene was obtained from soybeans (*Glycine max*) which have a long history of safe use in the human food supply.

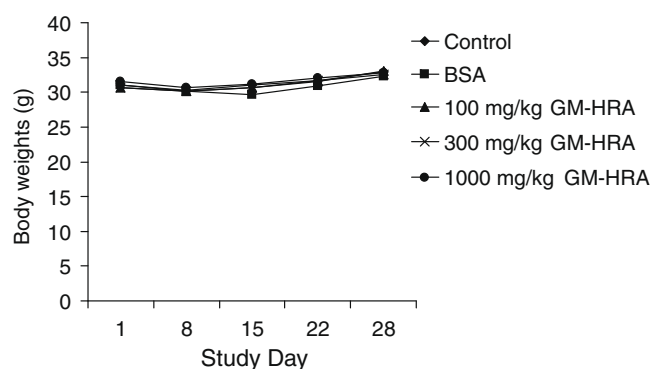


Fig. 9. Body weights of male mice following repeated dose dietary exposure to diets containing heterologously produced GM-HRA protein.

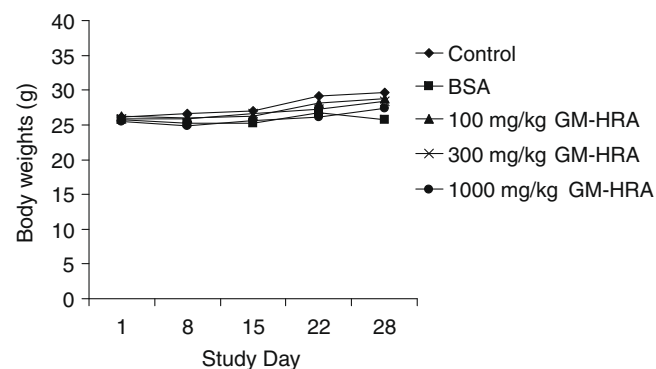


Fig. 10. Body weights of female mice following repeated dose dietary exposure to diets containing heterologously produced GM-HRA protein.

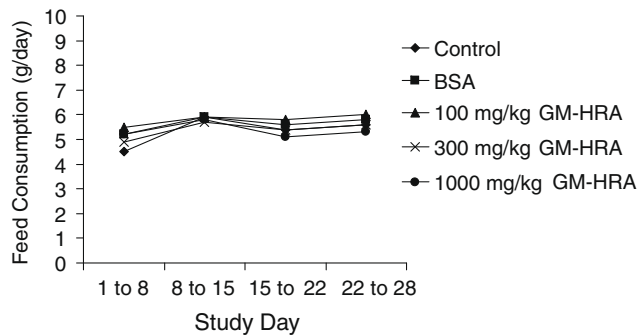


Fig. 11. Feed consumption of male mice following repeated dose dietary exposure to diets containing heterologously produced GM-HRA protein.

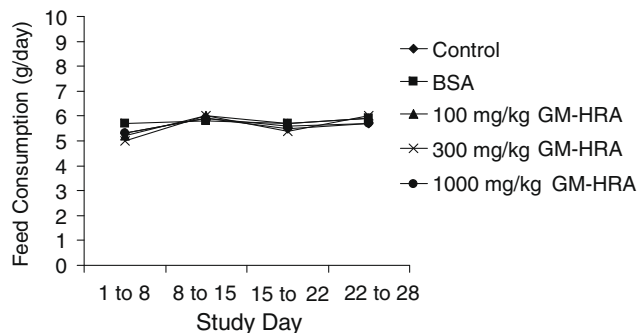


Fig. 12. Feed consumption of female mice following repeated dose dietary exposure to diets containing heterologously produced GM-HRA protein.

No amino acid sequence similarity was observed between the GM-HRA protein and known allergenic proteins according to published guidelines (CODEX, 2003; Taylor, 2006). Additionally, no amino acid sequence similarity was observed between GM-HRA and proteins known to be toxic following an extensive bioinformatics analysis. Bacterially-produced GM-HRA was not resistant to *in vitro* gastric and intestinal digestion models, and was heat labile. The heat lability of an enzyme (such as GM-HRA) as determined by

activity is required for the registration of genetically modified crops in some countries. In the context of the weight-of-evidence approach (CODEX, 2003; Taylor, 2006), these results demonstrated that the GM-HRA protein bears no similarity to allergenic proteins and therefore does not represent a risk for allergenicity.

Soybeans are considered one of the major foods known to cause allergic reactions and numerous allergenic proteins in soybeans have been identified (Metcalf et al., 1996; Ogawa et al. 1995, 2000; Cordle, 2004). Serum from soy allergic patients contains IgE antibodies that react with one or more allergenic soy proteins. In Delaney et al., 2008a, serum from clinically reactive soy allergic patients was used to demonstrate that the genetic modification used to produce DP-356043-5 soybeans did not alter the allergen content of the seed crop compared to non-transgenic soybeans (see Goodman and Leach, 2004). From these studies it was concluded that no new allergenic proteins were present in DP-356043-5 soybeans. In addition to the GAT4601 protein that was described in Delaney et al., 2008a to be in DP-356043-5 soybeans, the GM-HRA protein is also present, and therefore demonstrated to not produce new allergenic proteins.

Most dietary proteins are digested to constituent amino acids and small peptides in the mammalian gastrointestinal system and absorbed for nutritive purposes. However, because some proteins have been identified that are toxic to mammals and other species recommendations have been developed to assess the potential of transgenic proteins to cause toxicity using a two-tiered approach (Delaney et al., 2008b). Following components outlined in the first tier of evaluation, no evidence for potential toxicity was identified for the GM-HRA protein. It was obtained from soybeans; a common component of the human diet with a long history of safe use. Additionally, it does not have amino acid sequence similarity to proteins known to be toxic and was readily degraded in the presence of digestive enzymes.

Additional studies were conducted with the GM-HRA protein because the amino acid sequence is not identical to the form found naturally in soybeans and because they may also be requested in some cases by regulatory authorities (European Commission, 2003). No evidence of toxicity was observed in mice following acute or repeated oral exposure to heterologously produced GM-HRA protein. Accordingly, the No-Observed-Adverse-Effect-Level (NOAEL) for the GM-HRA protein from the current study was

Table 3

Mean clinical chemistry values of male mice ($\pm$ SD) following repeated dose dietary exposure to GM-HRA protein.

	Control	BSA (mg/kg)	GM-HRA (mg/kg)		
	n = 5	1000 n = 5	100 n = 5	300 n = 5	1000 n = 5
AST (U/L)	51.8 $\pm$ 4.5	68.6 $\pm$ 34.2	56.8 $\pm$ 18.0	69.6 $\pm$ 28.4	68.6 $\pm$ 25.4
ALT (U/L)	25.6 $\pm$ 3.4	34.8 $\pm$ 16.0	28.6 $\pm$ 14.5	32.6 $\pm$ 4.3	27.4 $\pm$ 2.1
ALKP (U/L)	55.6 $\pm$ 13.4	65.0 $\pm$ 22.4	55.4 $\pm$ 17.6	60.2 $\pm$ 11.6	48.4 $\pm$ 19.2
BILI (mg/dl)	0.20 $\pm$ 0.00	0.20 $\pm$ 0.00	0.26 $\pm$ 0.09	0.24 $\pm$ 0.05	0.20 $\pm$ 0.00 ^a
BUN (mg/dl)	18.8 $\pm$ 3.7	21.2 $\pm$ 1.8	18.2 $\pm$ 3.0	18.8 $\pm$ 8.1	27.4 $\pm$ 9.3
CREA (mg/dl)	0.16 $\pm$ 0.05	0.20 $\pm$ 0.00	0.18 $\pm$ 0.04	0.18 $\pm$ 0.04	0.18 $\pm$ 0.04
CHOL (mg/dl)	132.2 $\pm$ 38.2	147.6 $\pm$ 43.9	132.2 $\pm$ 30.0	130.4 $\pm$ 13.2	161.8 $\pm$ 31.7 ^a
TRIG (mg/dl)	115.6 $\pm$ 12.3	111.2 $\pm$ 33.4	133.6 $\pm$ 43.5	123.8 $\pm$ 26.6	133.0 $\pm$ 48.1
GLUC (mg/dl)	299.0 $\pm$ 94.8	350.6 $\pm$ 46.5	299.8 $\pm$ 10.9	345.6 $\pm$ 72.1	291.3 $\pm$ 12.8 ^a
TP (g/dl)	5.95 $\pm$ 0.13 ^a	6.03 $\pm$ 0.17 ^a	5.84 $\pm$ 0.11	6.02 $\pm$ 0.18	5.75 $\pm$ 0.25 ^a
ALB (g/dl)	3.08 $\pm$ 0.23	3.26 $\pm$ 0.05	3.14 $\pm$ 0.05	3.26 $\pm$ 0.11	3.04 $\pm$ 0.18
GLOB (g/dl)	2.78 $\pm$ 0.10 ^a	2.75 $\pm$ 0.13 ^a	2.70 $\pm$ 0.10	2.76 $\pm$ 0.09	2.73 $\pm$ 0.15 ^a
A/G ratio	1.13 $\pm$ 0.05 ^a	1.18 $\pm$ 0.05 ^a	1.14 $\pm$ 0.05	1.16 $\pm$ 0.05	1.10 $\pm$ 0.08 ^a
CALC (mg/dl)	10.20 $\pm$ 0.47 ^a	10.35 $\pm$ 0.47 ^a	10.22 $\pm$ 0.15	9.98 $\pm$ 0.08	10.38 $\pm$ 0.33 ^a
IPHS (mg/dl)	9.25 $\pm$ 0.88 ^a	8.64 $\pm$ 0.85	9.70 $\pm$ 1.20	8.62 $\pm$ 0.90	9.40 $\pm$ 1.58 ^a
NA (mmol/L)	151.6 $\pm$ 1.7	153.0 $\pm$ 2.6	151.6 $\pm$ 0.5	154.0 $\pm$ 3.3	154.0 $\pm$ 1.6
K (mmol/L)	12.04 $\pm$ 1.62	10.34 $\pm$ 0.32	12.30 $\pm$ 1.41	9.98 $\pm$ 1.67	10.06 $\pm$ 1.19
CL (mmol/L)	110.8 $\pm$ 1.3	110.2 $\pm$ 1.3	111.2 $\pm$ 1.6	111.6 $\pm$ 2.1	110.6 $\pm$ 1.1

No statistically significant or biologically important differences were observed.

^a n = 4.

Table 4Mean clinical chemistry values of female mice ($\pm$ SD) following repeated dose dietary exposure to GM-HRA protein.

	Control	BSA (mg/kg)	GM-HRA (mg/kg)		
	<i>n</i> = 5	1000 <i>n</i> = 5	100 <i>n</i> = 5	300 <i>n</i> = 5	1000 <i>n</i> = 5
AST (U/L)	57.8 $\pm$ 13.0	59.8 $\pm$ 10.6	67.6 $\pm$ 35.1	70.8 $\pm$ 22.3	85.2 $\pm$ 41.8
ALT (U/L)	25.8 $\pm$ 4.0	25.0 $\pm$ 7.2	24.0 $\pm$ 1.9	25.0 $\pm$ 4.1	28.2 $\pm$ 5.3
ALKP (U/L)	80.4 $\pm$ 19.5	90.2 $\pm$ 20.6	72.6 $\pm$ 11.0	95.2 $\pm$ 31.0	87.2 $\pm$ 23.1
BILI (mg/dl)	0.18 $\pm$ 0.04	0.20 $\pm$ 0.00	0.18 $\pm$ 0.05 ^a	0.20 $\pm$ 0.00 ^a	0.22 $\pm$ 0.04
BUN (mg/dl)	18.6 $\pm$ 5.2	19.4 $\pm$ 6.0	16.0 $\pm$ 2.6 ^a	16.2 $\pm$ 3.1	15.6 $\pm$ 2.9
CREA (mg/dl)	0.20 $\pm$ 0.00	0.20 $\pm$ 0.00	0.20 $\pm$ 0.00	0.18 $\pm$ 0.04	0.20 $\pm$ 0.00
CHOL (mg/dl)	91.0 $\pm$ 24.9 ^a	89.8 $\pm$ 5.2	112.0 $\pm$ 32.7 ^a	88.5 $\pm$ 16.7 ^a	100.6 $\pm$ 14.2
TRIG (mg/dl)	107.0 $\pm$ 26.9	103.0 $\pm$ 12.4	113.4 $\pm$ 31.1	107.0 $\pm$ 22.1	82.6 $\pm$ 6.7
GLUC (mg/dl)	218.0 $\pm$ 23.1 ^a	272.0 $\pm$ 41.8	260.0 $\pm$ 34.4 ^a	185.8 $\pm$ 7.4 ^a	220.4 $\pm$ 28.7
TP (g/dl)	5.58 $\pm$ 0.22 ^a	5.70 $\pm$ 0.16	5.93 $\pm$ 0.26 ^a	5.60 $\pm$ 0.29 ^a	5.90 $\pm$ 0.30
ALB (g/dl)	3.30 $\pm$ 0.22	3.36 $\pm$ 0.13	3.38 $\pm$ 0.18	3.26 $\pm$ 0.17	3.38 $\pm$ 0.19
GLOB (g/dl)	2.35 $\pm$ 0.06 ^a	2.34 $\pm$ 0.05	2.50 $\pm$ 0.14 ^a	2.35 $\pm$ 0.13 ^a	2.52 $\pm$ 0.13
A/G ratio	1.38 $\pm$ 0.05 ^a	1.44 $\pm$ 0.05	1.38 $\pm$ 0.10 ^a	1.40 $\pm$ 0.08 ^a	1.34 $\pm$ 0.05
CALC (mg/dl)	10.33 $\pm$ 0.32 ^a	10.34 $\pm$ 0.15	10.58 $\pm$ 0.29 ^a	10.03 $\pm$ 0.30 ^a	10.32 $\pm$ 0.13
IPHS (mg/dl)	10.10 $\pm$ 1.12 ^a	9.56 $\pm$ 0.63	10.28 $\pm$ 1.24 ^a	9.95 $\pm$ 1.18 ^a	9.56 $\pm$ 0.60
NA (mmol/L)	154.0 $\pm$ 1.4	152.2 $\pm$ 1.3	154.0 $\pm$ 1.6	152.6 $\pm$ 1.3	153.8 $\pm$ 2.8
K (mmol/L)	10.22 $\pm$ 0.97	10.34 $\pm$ 1.78	10.34 $\pm$ 0.96	10.14 $\pm$ 1.10	11.00 $\pm$ 2.04
CL (mmol/L)	111.4 $\pm$ 1.7	110.4 $\pm$ 2.5	111.0 $\pm$ 0.7	111.2 $\pm$ 1.3	111.8 $\pm$ 1.9

No statistically significant or biologically important differences were observed.

^a *n* = 4.

determined to be 992 and 1247 mg/kg of body weight/day for male and female mice, respectively.

Doses of the GM-HRA protein evaluated in the repeated dose toxicity study should be considered by comparison to the concentrations in soybean seeds obtained from genetically modified soybean plants that have been determined using GM-HRA specific ELISA (0.85 ng of GM-HRA protein per mg tissue on a dry weight basis). Humans with an average body weight of 60 kg would need to consume 88,023 kg/day of grain expressing the GM-HRA protein to approximate the same daily dose that was consumed in the high dose group from the 28-day repeated dose study. This is an extremely conservative estimate of human exposure to the GM-HRA protein because *in vitro* digestion studies demonstrated that it will

not be absorbed intact because it is likely to be degraded within the gut. Results from these studies further support the use of a tiered approach (Delaney et al., 2008b) by demonstrating that the Tier II studies (hazard characterization such as acute and repeated dose toxicology studies) did not provide additional information or results contradicting the results of the Tier I studies (Potential Hazard Identification – history of safe use, bioinformatics analysis, mode of action, *in vitro* digestibility and stability, and expression level and dietary intake) that did not identify evidence for potential toxicity of the GM-HRA protein.

This paper describes the assessment of GM-HRA for potential allergenicity and toxicity. It was concluded that the GM-HRA protein was not allergenic because it did not possess similarity to

Table 5Mean organ:body weight ratios of mice ($\pm$ SD) following repeated dose dietary exposure to GM-HRA protein.

	Control	BSA (mg/kg)	GM-HRA (mg/kg)		
	<i>n</i> = 5	1000 <i>n</i> = 5	100 <i>n</i> = 5	300 <i>n</i> = 5	1000 <i>n</i> = 5
Males					
Body wt (g, final)	30.9 $\pm$ 0.8	30.0 $\pm$ 1.6	30.6 $\pm$ 1.0	30.4 $\pm$ 2.2	29.9 $\pm$ 2.1
Adrenals	43.51 $\pm$ 10.71	23.20 $\pm$ 2.94 ^{**}	38.37 $\pm$ 18.91	24.34 $\pm$ 4.49 [*]	34.13 $\pm$ 6.35
Brain	1.82 $\pm$ 0.06	1.81 $\pm$ 0.11	1.88 $\pm$ 0.17	1.85 $\pm$ 0.17	1.83 $\pm$ 0.17
Epididymides	0.39 $\pm$ 0.07	0.39 $\pm$ 0.03	0.37 $\pm$ 0.03	0.33 $\pm$ 0.04	0.35 $\pm$ 0.04
Heart	0.60 $\pm$ 0.04	0.61 $\pm$ 0.04	0.59 $\pm$ 0.04	0.59 $\pm$ 0.04	0.61 $\pm$ 0.05
Kidneys	2.20 $\pm$ 0.18	2.16 $\pm$ 0.27	2.22 $\pm$ 0.24	2.15 $\pm$ 0.11	2.13 $\pm$ 0.10
Liver	5.50 $\pm$ 0.63	5.54 $\pm$ 0.37	5.49 $\pm$ 0.43	5.14 $\pm$ 0.49	5.26 $\pm$ 0.38
Spleen	0.37 $\pm$ 0.07	0.33 $\pm$ 0.03	0.34 $\pm$ 0.05	0.26 $\pm$ 0.04 ^{**}	0.30 $\pm$ 0.02 [*]
Testes	0.79 $\pm$ 0.14	0.88 $\pm$ 0.11	0.83 $\pm$ 0.05	0.78 $\pm$ 0.10	0.81 $\pm$ 0.13
Thymus ^a	159.90 $\pm$ 16.18	175.54 $\pm$ 53.46	180.26 $\pm$ 53.84	169.79 $\pm$ 39.57	179.85 $\pm$ 50.91
Females					
Body wt (g, final)	28.2 $\pm$ 1.6	26.3 $\pm$ 1.8	27.1 $\pm$ 1.2	26.7 $\pm$ 2.0	25.2 $\pm$ 1.0
Adrenals	52.36 $\pm$ 16.21	70.98 $\pm$ 15.04	63.45 $\pm$ 13.27	83.34 $\pm$ 31.30	83.31 $\pm$ 30.08
Brain	2.00 $\pm$ 0.10	2.21 $\pm$ 0.20	2.16 $\pm$ 0.20	2.14 $\pm$ 0.19	2.28 $\pm$ 0.18
Heart	0.59 $\pm$ 0.05	0.66 $\pm$ 0.03	0.59 $\pm$ 0.05	0.57 $\pm$ 0.10	0.61 $\pm$ 0.06
Kidneys	1.70 $\pm$ 0.21	1.95 $\pm$ 0.12	1.80 $\pm$ 0.21	1.88 $\pm$ 0.16	1.88 $\pm$ 0.15
Liver	5.30 $\pm$ 0.61	5.88 $\pm$ 0.56	5.49 $\pm$ 0.36	5.34 $\pm$ 0.51	5.43 $\pm$ 0.61
Ovaries ^a	111.35 $\pm$ 40.55	126.19 $\pm$ 47.83	129.61 $\pm$ 25.79	171.68 $\pm$ 33.58	193.07 $\pm$ 75.90
Spleen	0.44 $\pm$ 0.09	0.49 $\pm$ 0.12	0.43 $\pm$ 0.09	0.43 $\pm$ 0.04	0.44 $\pm$ 0.06
Thymus ^a	269.92 $\pm$ 71.34	265.59 $\pm$ 80.33	248.15 $\pm$ 61.80	355.51 $\pm$ 90.98	312.29 $\pm$ 108.21
Uterus w/Cervix	0.61 $\pm$ 0.24	0.87 $\pm$ 0.17	0.62 $\pm$ 0.23	0.57 $\pm$ 0.16	0.48 $\pm$ 0.18

^{*} *p* < 0.05 compared to control.^{**} *p* < 0.01 compared to control.^a Mean value multiplied by 1000.

known allergenic proteins using the step-wise, weight-of-evidence approach. Furthermore, serum screening studies with soy allergic patient serum indicated that the DP-356043-5 soybean, containing both the GM-HRA and GAT4601 proteins, and non-transgenic soybean flours are very similar in protein/allergen profiles without demonstrating significant changes in concentrations of total protein or individual protein/allergen profiles (Delaney et al., 2008a). The GM-HRA protein is not similar in sequence to known protein toxins and no evidence of adverse effects was observed in toxicology studies following single or repeated dose exposure. Finally, human exposure to the GM-HRA protein is likely to be extremely low because it is present at low concentrations in whole soybeans obtained from genetically modified soybean plants where it is expressed and because it is heat labile and has not been identified at measurable concentrations in soybean oil which is the primary component of soybeans that is consumed by humans. In conclusion, these data demonstrate the safety of the GM-HRA protein for use in food and feed applications and indicate that this protein presents no risk for adverse health effects when used in the context of agricultural biotechnology.

Acknowledgments

These studies were funded by Pioneer, A DuPont Company. Hugh Sampson and Luda Bardina from the Mount Sinai School of Medicine assisted in the preparation of the paper.

Special thanks to Tom Davis, Kent Brink, Natalie Weber, Ian Lamb, and Mary Locke (DuPont Agriculture and Nutrition) for guidance in the preparation of this paper.

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