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

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A B S T R A C T

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 isoline. 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, triazolopyrimidines, 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 (Metcalfe 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 cutoff = 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 (Metcalfe 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-

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