Appendix 18

Digestive Fate of Arabidopsis SEC61γ Subunit Protein

BASF
The Chemical Company

Plant Science LLC

REPORT # BPS-002-08

DIGESTIVE FATE OF ARABIDOPSIS SEC61γ SUBUNIT PROTEIN

EPA GUIDELINE #: N/A

AUTHOR:

[redacted]

STUDY COMPLETED ON AUGUST 28, 2008

TEST FACILITY/PERFORMING LABORATORY:

BASF PLANT SCIENCE, LLC
26 DAVIS DRIVE
RESEARCH TRIANGLE PARK, NC 27709

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d) (1) (A), (B), or (C).

Company: BASF Plant Science, LLC

Company Agent: [redacted] Date: 29 Aug 2008

Title: Regulatory Affairs Manager

Signature: [redacted]

These data are the property of BASF Plant Science, LLC and, as such, are considered to be confidential for all purposes other than compliance with FIFRA §10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under any other statute in any other country.

STATEMENT CONCERNING GOOD LABORATORY PRACTICES

This study was not conducted in compliance with the requirements of 40 CFR Part 160.

The data generated by BASF Plant Science in support of product safety comply with generally accepted scientific procedures. Record keeping is consistent with procedures used throughout the research community. This report accurately presents the raw data developed during the study.

PRINCIPAL INVESTIGATOR:

[redacted] 29 Aug 08
Date

Senior Manager, Regulatory Science
BASF Plant Science, LLC

SPONSOR REPRESENTATIVE:

[redacted] 29 Aug 2008
Date

Regulatory Affairs Manager
BASF Plant Science, LLC

SUBMITTED BY:

[redacted] 29 Aug 2008
Date

Regulatory Affairs Manager
BASF Plant Science, LLC
26 Davis Drive
Research Triangle Park, NC 27709
USA

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS AND DEFINITIONS

AHAS	acetohydroxyacid synthase
AHASL	acetohydroxyacid synthase large subunit
AtAHAS	*Arabidopsis thaliana* acetohydroxyacid synthase
BME	β-mercaptoethanol
G-con	simulated mammalian gastric fluid without pepsin
GST	glutathione-S transferase
HRP	horseradish peroxidase
PVDF	polyvinylidene fluoride
RT-PCR	reverse transcription-polymerase chain reaction
S653N	serine at position 653 replaced by asparagine
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SGF	simulated mammalian gastric fluid containing pepsin

DIGESTIVE FATE OF ARABIDOPSIS SEC61γ SUBUNIT PROTEIN

SUMMARY

Imidazolinone-tolerant soybean event BPS-CV127-9 was produced by the introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene from *Arabidopsis thaliana* into the soybean plant genome, resulting in soybean plants that are resistant to imidazolinone herbicides. The majority of the Arabidopsis *SEC61γ* subunit gene, including the entire Arabidopsis *SEC61γ* coding sequence, was also introduced into the soybean genome in BPS-CV127-9. This Arabidopsis *SEC61γ* subunit gene has been shown by reverse transcription-polymerase chain reaction (RT-PCR) experiments to be weakly transcribed in BPS-CV127-9 leaves (Shen, 2007). In this study, Sec61γ subunit protein was purified from an *E. coli* over-expression system as a gluthathione S-transferase (GST) fusion protein. The AtSec61γ subunit protein was subsequently cleaved proteolytically from GST and isolated. This AtSec61γ subunit protein preparation was subjected to digestion in simulated mammalian gastric fluid (SGF) and similar to conventional dietary proteins was found to be rapidly digested.

INTRODUCTION

Soybean (*Glycine max* L.) plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants, referred to as BPS-CV127-9, were produced by introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene from *Arabidopsis thaliana* into the soybean plant genome via biolistics using the PvuII fragment of transformation vector pAC321 (Figure 1). This fragment includes what, at the time of transformation, was defined as the Arabidopsis *AHASL* promoter, the herbicide-tolerant Arabidopsis *ahasl* coding sequence, and the Arabidopsis *AHASL* terminator. Also included in the region originally annotated as the *AHASL* promoter was the majority of the Arabidopsis *SEC61γ* subunit gene (Arabidopsis Genome Initiative locus code At3g48570), including the entire Arabidopsis *SEC61γ* coding sequence. This Arabidopsis *SEC61γ* subunit gene has since been shown by reverse transcription-polymerase chain reaction experiments to be weakly transcribed in BPS-CV127-9 leaves (Shen, 2007). Further examination of the *SEC61γ* subunit gene specific mRNA by 5' race experiments demonstrated that it is the expected size and that if it were translated it would encode the predicted *SEC61γ* polypeptide sequence (McKean, 2007). Sec61γ subunit, together with the α and β subunits, is part of the protein translocation complex associated with the endoplasmic reticulum and is ubiquitous and highly conserved in eukaryotes as well as being structurally related to analogous proteins in prokaryotes (Hartman, *et al*., 1994). There is a high degree of amino acid sequence homology (86%) between the soybean Sec61γ subunit and that from Arabidopsis (see Figure 2 for the alignment).

The purpose of this study was to demonstrate that the *E. coli*-produced AtSec61γ subunit protein has the same susceptibility to digestion in simulated mammalian gastric fluid (SGF) as other dietary proteins with a history of safe use in foods. After incubation for various times in the SGF, the remaining protein was subjected to electrophoresis and visualized by western blot analysis.

MATERIALS AND METHODS

Preparation of Sec61γ subunit protein. The At3g48570 gene, encoding the AtSec61γ subunit protein, was cloned into the expression cassette pGEX-6P (GE Healthcare Bio-Sciences Corp, Pistcaway, NJ) and transformed into *Escherchia coli* strain Origami B (DE3)pLysS (EMD Biosciences). This expression cassette encodes a glutathione S-transferase (GST)–AtSec61γ subunit fusion protein (mol. wt. *ca*. 35,000). The GST-AtSec61γ subunit fusion protein was purified from *E. coli* using an immobilized glutathione column according to the manufacturer's instructions (GE Healthcare). On-column digestion using PreScission Protease™ (GE Healthcare) was performed to cleave the (GST)–AtSec61γ subunit fusion protein at the site of the fusion to release the AtSec61γ subunit protein (mol. wt. *ca*. 7000). Protein in the preparation was quantified and used directly in the simulated gastric mammalian digestion reactions.

Protein quantification. Total protein in this preparation was quantified by the BCA™ procedure (bicinchoninic acid procedure; Pierce Biotechnology, Inc.; Rockford, IL) using bovine serum albumin as the standard. Samples were reacted with two ml of a 1:50 (B:A) mixture of BCA reagent, incubated at 37ºC for 30 min and allowed to cool at room temperature for 10 min. The absorbance at 562 nm was measured using a UV1600 Spectrophotometer (Shimadzu, Columbia, MD). The results were analyzed by the instrument's software using the linear regression curve fit.

Simulated mammalian gastric fluid reactions. Simulated mammalian gastric fluid (2X SGF; 0.016 N HCl, 0.75 mM NaCl, pH 1.2, and 3500 Units of pepsin (Sigma) {Thomas *et al.*, 2004} was prepared as described in the United States Pharmacopoeia (2000) and proteolytic activity was confirmed using azoalbumin as a substrate (SOP BPS 510.01). A single tube containing sufficient reaction mix for all assay time points was prepared by mixing 350 µl of SGF (2X) with 350 µl of AtSec61γ subunit protein preparation. The reaction mixtures were immediately placed in a 37°C water bath and 100 µl samples were removed at 0.5, 2, 5, 10, 30 and 60 min after initiation of the experiment. Each sample was quenched by the addition of 35 µl of 200 mM $NaHCO_3$, pH 11, and 35 µl 5X Laemmli buffer (40% glycerol, 5% β-mercaptoethanol, 10% SDS, 0.33 M Tris, 0.05% bromophenol blue, pH 6.8). Quenched samples were then heated to >75°C for 10 min and stored at *ca*. –20°C until subjected to electrophoresis as described below. The 0 time point for all reactions was prepared by first quenching SGF and then adding the Sec61γ subunit protein preparation. In addition, reactions that served as controls for pepsin auto-digestion and AtSec61γ subunit protein stability were prepared containing SGF without test protein and SGF with AtSec61γ subunit protein but without pepsin (G-con),

respectively. These control reactions were treated exactly as described above except samples were only taken at 0 and 60 minutes after the start of the 37°C incubation.

Western blot analysis. To monitor the integrity (intactness) of the AtSec61γ subunit protein after incubation in SGF, western blot analysis was performed. Aliquots of the quenched and heated samples were subjected to SDS-PAGE on a 4 - 20% polyacrylamide gradient gel followed by electroblotting onto PVDF membrane (Invitrogen; SOP BPS 510.03). The samples were loaded onto the gel to achieve maximum total protein based on determinations made prior to digestion. The amount of AtSec61γ subunit protein preparation loaded was 2.6 μg/lane. After electroblotting, the membranes were probed with either Protein A purified rabbit anti-AtSec61 peptides polyclonal antibody (blot shown in Figure 3A) or immunoaffinity purified rabbit anti-AtSec61 peptides polyclonal antibody (Figure 3B). The rabbit anti- AtSec61 γ peptides polyclonal antibody had been generated to two synthetic peptides that covered the entire 59 amino acid sequence of the mature Sec61 γ subunit-like protein. These antibodies were highly sensitive but not highly specific and therefore, the decision was made to immunoaffinity purify the antibodies over an immobilized peptide column prepared from the same set of peptides. This was designated as the immunoaffinity purified rabbit antiAtSec61 peptides antibody. The specificity of the antibody was increased, however, the sensitivity was concurrently decreased. Results with both antibody preparations are included in this report. Goat anti-rabbit IgG linked to horseradish peroxidase (Pierce), diluted 1:3000 in blocking buffer (3% nonfat dry milk in 0.1% Tween-20, 10 mM Tris-HCl, 150 mM NaCl, pH 7.5) was used to bind to the primary antibody and was visualized by development with the chromagenic substrate diaminobenzidine.

RESULTS

Sensitivity of *E.coli*-produced AtSec61 γ subunit protein to degradation in SGF. The *E. coli*-produced AtSec61 γ subunit protein was rapidly degraded in SGF (Figure 3A). AtSec 61 γ subunit protein (molecular weight *ca*. 7,000, the most intensely staining band in this size range) is readily detected in the time 0 sample and in the G-con samples. However, no full-length AtSec61 γ subunit protein is visible in the sample removed at 0.5 min after the initiation of the reaction. Some immunoreactive bands representing contaminating proteins of varying molecular weights (6000, 10,000, 25,000, 36,000,) were visible at all time points. Other immunoreactive protein bands at 20,000, 32,000, 63,000 and 98,000 molecular weights were also rapidly degraded similar to the AtSec61 γ subunit protein. The results shown in the blot pictured in Figure 3B in which the immunoaffinity-purified rabbit anti-Sec61 γ peptides was used, confirm the identity of the AtSec61 γ band and its rapid digestibility.

CONCLUSIONS

Although large quantities of a range of proteins are consumed in human diets each day, rarely do any of these tens of thousands of proteins elicit an allergenic response (Taylor, 1992). There are no definitive methods to assess potential allergenicity of proteins originating from sources not known to produce food allergy. However, there are some recognized procedures that can be used to evaluate a new protein to assess its allergenic potential. These procedures include evaluating the similarity of the new protein to known food allergens with respect to amino acid sequence and examining its biochemical characteristics such as molecular size, resistance to digestive degradation, and heat stability. These procedures together form the basis for evaluating whether a given protein is likely to be, or become, an allergen. Even known food allergens do not always share a group of characteristics and a weight of evidence approach must be used for the protein safety determination**.**

Digestive fate of the AtSec61γ subunit protein was evaluated as one of the predictive characteristics of a food allergen. Results of the current study show that the AtSec61γ subunit protein is highly digestible under the simulated digestion conditions, which is typical of most dietary proteins exposed to the proteases of the mammalian digestive tract. This result demonstrates that the Sec61γ subunit protein does not share one of the characteristics of some food allergens, resistance to digestive degradation. Furthermore, this result was as expected since the Sec61γ subunit protein is ubiquitous and highly conserved in eukaryotes, and is present in many plant species with a history of safe food use.

STUDY PERSONNEL: Analytical work reported herein was conducted by [redacted], Ph.D., BASF Plant Science, LLC, Research Triangle Park, NC 27709.

REFERENCES

Standard Operating Procedures

SOP BPS 510.01 Simulated Mammalian *In vitro* Digestibility Method

SOP BPS 510.03 Western Blot Analysis

Literature References

Hartmann, E., Sommer, T., Prehn, S., Görlich, D., Jentsch, S., Rapoport, T. A. (1994) Evolutionary conservation of components of the protein translocation complex. Nature 367:654-657

McKean, A. (2007) Determination of the 5' end of the *Arabidopsis thaliana SEC61γ* subunit transcript in Cultivance Soybean Event 127. BASF Plant Science Report No. BPS-002-06.

Shen, Y. (2007) Molecular characterization of Cultivance Soybean Event 127. BASF Plant Science Report No. BPS-001-06.

Taylor, S. L. (1992) Chemistry and detection of food allergens. Food Technol. 46:146-152.

Thomas, K., Aalbers, M., Bannon, G. A., Bartels, M.; Dearman, R. J., Esdaile, D. J., Fu, T. J., Glatt, C. M., Hadfield, N., Hatzos, C., Hefle, S. L., Heylings, J. R., Goodman, R. E., Henry, B., Herouet, C., Holsapple, M., Ladics, G. S., Landry, T. D., MacIntosh, S. C., Rice, E. A., Privalle, L. S., Steiner, H. Y., Teshima, R., van Ree, R., Woolhiser, M., and Zawodny, J. (2004) A multi-laboratory evaluation of a common *in vitro* pepsin digestion assay protocol used in assessing the safety of novel proteins. Regulatory Toxicology and Pharmacology. 39:87-98.

United States Pharmacopoeia, The National Formulary (2000) USP XXIV, NF XIX, U.S. Pharmacopoeial Convention, Inc. Mack Printing Co., Easton, PA, p. 2235.

Figure 1. Plasmid pAC321

The pAC321 PvuII fragment containing the *AHASL* 5' UTR, *csr1-2* coding sequence (AHASL), and *AHASL* 3' UTR was used for transformation to produce BPS-CV127-9. *AHASL* 5' UTR includes the coding sequence designated At3g48570, AtSec61γ subunit.

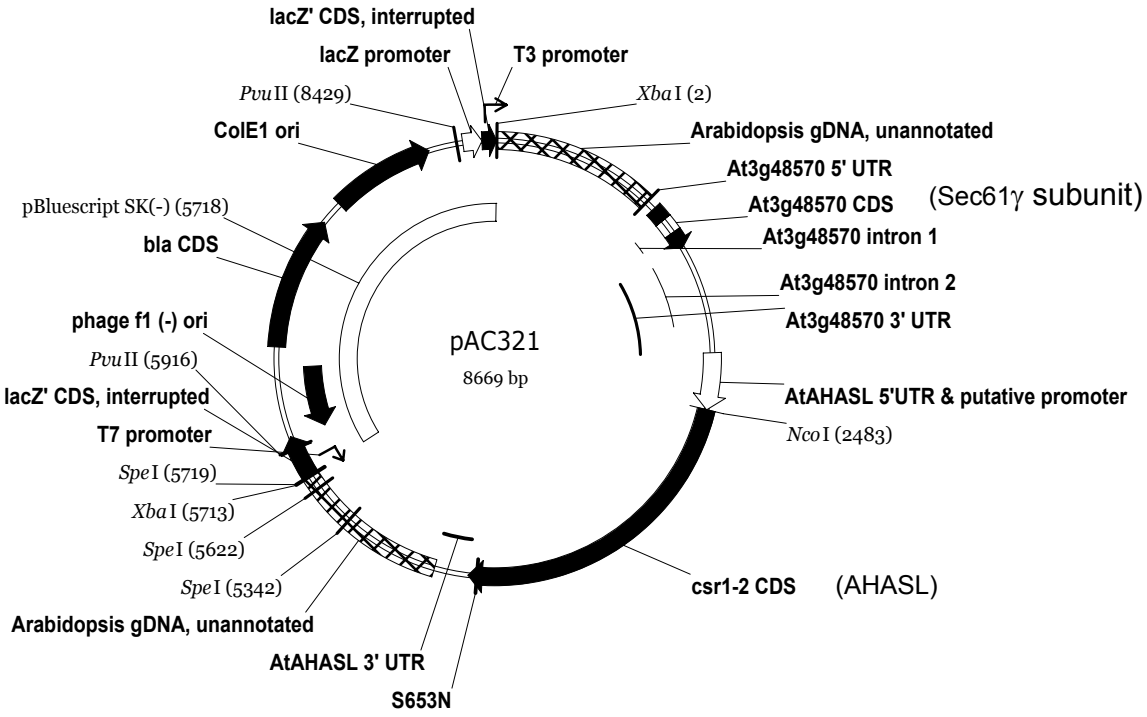

Figure 2. Amino acid sequence comparison of the Sec61γ subunit-like protein from Arabidopsis with Sec61 γ subunit-like proteins encoded by four genes from Soybean.

```
                  1                                                  50
At3g48570/Sec61   MEAIDSAIDP LRDFAKSSVR LVQRCHKPDR KEFTKVAVRT AIGFVVMGFV
   Soy_Sec61g_1   MDAIDSVFDP LREFAKDSVR LVKRCHKPDR KEFSKVAVRT AIGFVVMGFV
   Soy_Sec61g_2   MDAIDSVFDP LREFAKDSVR LVKRCHKPDR KEFSKVAVRT AIGFVVMGFV
   Soy_Sec61g_3   MDAIDSVFDP LREFAKDSVR LVKRCHKPDR KEFSKVAVRT AMGFVVMGFV
   Soy_Sec61g_4   MDAIDSVFDP LLEFAKDSVR LVKRCHKPDR KEFSKVAVRT AIGFVVMGFV

                  51                69
At3g48570/Sec61   GFFVKLVFIP INNIIVGSS
   Soy_Sec61g_1   GFFVKLIFIP INNIIVGSG
   Soy_Sec61g_2   GFFVKIIFIP INNIIVGSG
   Soy_Sec61g_3   GFFVKLIFIP INNIIVGSG
   Soy_Sec61g_4   GFFVKLIFIP INNIIVGSG
```

Percent Identitical Amino Acids

	At3g48570/Sec61	Soy Sec61γ 1	Soy Sec61γ 2	Soy Sec61γ 3	Soy Sec61γ 4
At3g48570/Sec61	100	87	86	86	86
Soy Sec61g 1		100	98	98	98
Soy Sec61g 2			100	97	97
Soy Sec61g 3				100	97
Soy Sec61g 4					100

Figure 3. Susceptibility of AtSec61γ subunit protein to digestion in Simulated Mammalian Gastric Fluid (SGF).

Western blot analysis of digestion reactions carried out with AtSec61γ subunit protein. Incubation time at 37ºC is indicated in minutes. SGF is simulated mammalian gastric fluid containing pepsin, G-con is SGF without pepsin; and the lanes labeled "SGF only" contained SGF without AtSec61γ subunit protein. AtSec61γ subunit protein is *ca.* 7000 mol wt., pepsin is ca. 40,000 mol. wt. Molecular weight (x 10^{-3}) markers are indicated. A. Protein A purified rabbit anti-Sec61 γ peptides was used as the detection antibody. B. Immunoaffinity purified rabbit anti-Sec61 γ peptides was used as the detection antibody.