Appendix 17

Digestive Fate of Test Substance Arabidopsis Acetohydroxyacid Synthase (Lot #AtAHAS-0107) and AtAHAS produced in Imidazolinone Herbicide Tolerant Soybean BPS-CV127-9

BASF
The Chemical Company

Plant Science LLC

REPORT # BPS-012-07

DIGESTIVE FATE OF TEST SUBSTANCE ARABIDOPSIS ACETOHYDROXYACID SYNTHASE (LOT #ATAHAS-0107) AND ATAHAS PRODUCED IN IMIDAZOLINONE HERBICIDE TOLERANT SOYBEAN BPS-CV127-9

STUDY # BPS-CVSOY-07-001

EPA GUIDELINE #: N/A

AUTHOR:

[redacted]

STUDY COMPLETED ON NOVEMBER 5, 2007

TEST FACILITY/PERFORMING LABORATORY:

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

SEE PAGE 8 FOR ADDITIONAL PERFORMING FACILITIES

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: 5 Nov 2007

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

The study described in this volume was conducted in full compliance with Good Laboratory Practices as described in 40 CFR 160 with the following exception:

Phase B of the study, Digestibility of BPS-CV127-9 Soybean AtAHAS, was not conducted under GLP.

STUDY DIRECTOR:

[redacted] 5 Nov 07

Date

Senior Manager, Regulatory Science
BASF Plant Science, LLC

SPONSOR REPRESENTATIVE:

[redacted] 5 Nov 2007

Date

Regulatory Affairs Manager
BASF Plant Science, LLC

SUBMITTED BY:

[redacted] 5 Nov 2007

Date

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

QUALITY ASSURANCE UNIT STATEMENT

Study Number: BPS-CVSOY-07-001

Name/Number of Test Substance(s):

1. AtAHAS-0107
2. BPS-CV127-9 Soybean Leaf-expressed AtAHAS
3. BPS-CV127-9 Soybean Grain-expressed AtAHAS

Type of Study: Digestive Fate Study

THE QUALITY ASSURANCE UNIT OF THE TESTING FACILITY HAS INSPECTED THE STUDY AND/OR AUDITED THE FINAL REPORT AND REPORTED THE RESULTS OF THESE INSPECTIONS TO THE STUDY DIRECTOR AND TO MANAGEMENT.

Date of Inspection	Date reported to Study Director and to Management
May 29. 2007	**May 29, 2007**
May 30, 2007	**May 30, 2007**
October 23, 2007	**October 24, 2007**

[redacted]
Signature QAU

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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
HRP	horseradish peroxidase
I-con	simulated mammalian intestinal fluid without pancreatin
PVDF	polyvinylidene fluoride
S653N	serine at position 653 replaced by asparagine
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SGF	simulated mammalian gastric fluid containing pepsin
SIF	simulated mammalian intestinal fluid containing pancreatin

GENERAL INFORMATION

BASF Registration Document Number:	2007/7007586
BASF Plant Science Study Number:	BPS-CVSOY-07-001
Test Substance:	AHAS protein produced in an *Escherichia coli* over-expression system
Sample Lot No.:	AtAHAS-0107
Test Substance:	BPS-CV127-9 Soybean Leaf Extract
Test Substance:	BPS-CV127-9 Soybean Grain Extract
Sponsor and Testing Facility	BASF Plant Science, LLC P. O. Box 13528 26 Davis Drive Research Triangle Park, NC 27709
Additional Test Facility:	BASF Plant Science, GmbH BPH LI445 Limburgerhof D-67117 Germany
Study Director:	[redacted], Ph.D.

Digestive Fate of Test Substance Arabidopsis Acetohydroxyacid Synthase (Lot #AtAHAS-0107) and AtAHAS Produced in Imidazolinone Herbicide Tolerant Soybean BPS-CV127-9

Test Substance: AHAS protein produced in an *Escherichia coli* over-expression system

Sample Lot No.: AtAHAS-0107

SUMMARY

The purpose of this study was to characterize the digestive fate of a test substance, (Lot #AtAHAS-0107), and two plant tissue extracts, of imidazolinone herbicide tolerant soybean BPS-CV127-9 leaf and grain, all containing acetohydroxyacid synthase (AHAS) protein encoded by the imidazolinone-tolerant *ahasS653N* gene isolated from *Arabidopsis thaliana* and referred to as AtAHAS protein. The introduction of an imidazolinone-tolerant acetohydroxyacid synthase (*ahasS653N*) gene from *Arabidopsis thaliana* into the plant genome allows growth and hence selection of the transformed cells in the presence of imidazolinone herbicides. The *Arabidopsis* AHAS protein is a member of the class of AHAS (large subunit) proteins found ubiquitously in plants. The mutation in the *Arabidopsis ahas* gene responsible for imidazolinone herbicide tolerance is a single nucleotide change of guanine to adenine, which results in a codon change from AGT to AAT and a single amino acid substitution of serine to asparagine at position 653 of the AHAS protein. In addition, a second mutation was identified when this gene was introduced into soybean resulting in the transformation event designated BPS-CV127-9. The second mutation, R272K, does not impact the enzymatic function of the AHAS enzyme.

AtAHAS protein was purified from an *E. coli* over-expression system and designated sample lot number AtAHAS-0107. This AtAHAS protein preparation was characterized to determine identity, purity, functionality, concentration, and solubility and has been found to be substantially equivalent to that produced by BPS-CV127-9 plants. This material, together with extracts prepared from transgenic, imidazolinone tolerant soybean BPS-CV127-9 leaves and grain, was subjected to digestion in simulated mammalian gastric and intestinal fluids (SGF and SIF, respectively). The AHAS protein from all samples was found to be rapidly digested the same as conventional dietary protein. The pattern of digestion was similar with leaf, grain and *E. coli*-produced AHAS.

INTRODUCTION

The purpose of this study was to determine the susceptibility of 1) a test substance (Lot #AtAHAS-0107) containing AHAS protein encoded by the imidazolinone-tolerant acetohydroxyacid synthase *ahas* gene isolated from *Arabidopsis thaliana (*AtAHAS, containing R272K and S653N mutations) and 2) AHAS protein present in extracts of leaf

and grain from BPS-CV127-9 plants to digestion in both simulated mammalian gastric fluid (SGF) and simulated mammalian intestinal fluid (SIF).

The introduction of an imidazolinone-tolerant acetohydroxyacid synthase (*ahas*) gene from *Arabidopsis thaliana* into the plant genome allows growth and, hence, selection of the transformed cells in the presence of imidazolinone herbicides. The *Arabidopsis* AHAS (AtAHAS) protein is a member of the class of AHAS (large subunit) proteins found ubiquitously in plants. The mutation in the *Arabidopsis ahas* gene responsible for imidazolinone herbicide tolerance is a single nucleotide change of guanine to adenine, which results in a codon change from AGT to AAT and a single amino acid substitution of serine to asparagine at position 653 of the AHAS protein. In addition, a second mutation was identified after this gene was introduced into soybean resulting in the transformation event designated BPS-CV127-9. The second mutation, in which arginine at position 272 was replaced by lysine (R272K), does not impact the enzymatic function of the AHAS enzyme (Stevenson Paulik, 2006).

The AHAS enzyme catalyzes the first step in the biosynthesis of branched-chain amino acids in plants. In conventional plants, inhibition of the AHAS enzyme by imidazolinone herbicides leads to a deficiency in branched-chain amino acids and other compounds derived from this pathway that are needed for plant survival. The *ahas* gene from *Arabidopsis* confers tolerance to imidazolinone herbicides by encoding an AHAS enzyme (large subunit) with altered herbicide binding properties, but the enzyme has normal biosynthetic function in the transgenic plant.

The lot of test substance, AtAHAS-0107, was prepared from a recombinant *Escherichia coli* over-expression system and was intended for use in an acute oral mouse toxicity study, as well as other studies to confirm the food, feed and environmental safety of the protein. The AtAHAS protein preparation was characterized to determine identity, purity, functionality, concentration, and solubility (Privalle, 2007a) and has been found to be substantially equivalent to that produced by BPS-CV127-9 plants (Privalle, 2007b). The purpose of Phase A of this study was to confirm that the AtAHAS protein is rapidly digested, in a similar manner as typical dietary proteins. The purpose of also examining digestion of the AtAHAS protein as expressed in leaf and grain of BPS-CV127-9 (Phase B of this study) was to determine whether the plant matrix has an effect on the digestibility and to confirm that the plant-produced AtAHAS protein had a similar digestive fate as the *E. coli*-produced AtAHAS protein as well as the endogenous soybean AHAS protein. After incubation for various times in the appropriate simulated digestive fluids, the remaining protein was subjected to electrophoresis and visualized by either Coomassie blue staining or western blot analysis.

MATERIALS AND METHODS

PHASE A.

Preparation of test substance. The *ahas S653N* gene was cloned into the inducible, over-expression vector pTrcHis A® (Invitrogen; Carlsbad, CA) in *E. coli* strain

BL21DE3pLysS. The AHAS protein encoded by this vector lacks the 85 N-terminal amino acid leader sequence that targets the protein *in planta* to the chloroplast. This leader sequence has been replaced in this vector with 38 amino acids including a 6 x His tag to allow for ease in purification, a gene 10 leader sequence to enhance solubility, and an Xpress™ tag for detection. The remainder of the protein is identical in amino acid sequence to that produced by the native gene from *Arabidopsis thaliana* (Mazur, *et al*., 1987) except for the two aforementioned point mutations. The replacement of serine with asparagine at amino acid residue 653 of the AtAHAS protein results in decreased binding of imidazolinone herbicide to AtAHAS, and imidazolinone tolerance. The point mutation that results in replacement of arginine with lysine at amino acid residue 272 has no apparent impact on AHAS functionality.

AtAHAS protein was produced and purified by Invitrogen, Inc. (Madison, WI) and transferred as an ammonium sulfate pellet to the Regulatory Science Laboratory at BASF Plant Science, Research Triangle Park, NC where it was received on February 27, 2007. The AtAHAS protein was purified from 2,400 g of cell paste after lysis in 20 L buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM β-mercaptoethanol, 30 mM Imidazole) using an Emulsiflex cell disruptor (Avestin, Ontario, Canada). Insoluble material was removed by centrifugation. The His-tagged AtAHAS present in the supernatant was purified by chromatography using Talon® cobalt resin (Clontech, Inc., Mountain View, CA). Once eluted, the fractions containing AtAHAS were pooled and immediately precipitated by ammonium sulfate at 40% saturation. The ammonium sulfate pellet was resuspended in 100 mM Tris-HCl, pH 7.0 and dialyzed vs. 20 mM ammonium bicarbonate buffer, pH 7.9, containing 20 μM FAD. The dialyzed material was then lyophilized and designated AtAHAS-0107. This material has been characterized (Privalle, 2007a). The test substance in this study is also the test system. The test solution used in the digestion reactions below was prepared by resuspending 2.2 mg of AtAHAS-0107 in 1.1 ml of 8 mM sodium phosphate, 2 mM potassium phosphate, 0.14 M sodium chloride, 10 mM potassium chloride, pH 7.4, and used the day of preparation.

Control substances. An extract of nontransgenic soybean leaves was prepared by homogenizing 0.67 g of leaf material with two ml extraction buffer (8 mM sodium phosphate, 2 mM potassium phosphate, 0.14 M sodium chloride, 10 mM potassium chloride, pH 7.4). An extract of nontransgenic soybean grain was prepared similarly except that two grams of material was extracted with nine ml of buffer. Extracts were centrifuged 15 min at approx. 13,000 x g. The protein concentration was determined in the supernatants after centrifugation and the supernatants were used directly in the digestive reactions. Digestion reactions were conducted the same day that the extracts were prepared.

Protein quantification. Total protein in the leaf and grain extracts were quantified by the BCA™ procedure (bicinchoninic acid procedure; Pierce Biotechnology, Inc.; Rockford, IL) using bovine serum albumin as the standard. Dilutions of the extract (final volume 100 μl) were prepared such that the expected concentration of protein would be within the standard curve. 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.

PHASE B.

Preparation of test substances. Extracts of BPS-CV127-9 leaves were prepared by homogenizing two grams of leaf material with six ml extraction buffer (8 mM sodium phosphate, 2 mM potassium phosphate, 0.14 M sodium chloride, 10 mM potassium chloride, pH 7.4). Extracts were centrifuged 15 min at approx. 13,000 x g. An extract of BPS-CV127-9 grain was prepared similarly except that two grams of material was extracted with nine ml of buffer. The protein concentration was determined in the supernatants after centrifugation and the supernatants were used directly in the digestive reactions. Digestion reactions were conducted the same day extracts were prepared.

Protein quantification. Total protein in the leaf and grain extracts were quantified by the BCA™ procedure (bicinchoninic acid procedure; Pierce Biotechnology, Inc.; Rockford, IL) using bovine serum albumin as the standard. Dilutions of the extract were prepared such that the expected concentration of protein would be within the standard curve. Samples (25 µl) were loaded onto a multiwell plate in triplicate, reacted with 200 µl 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 550 nm was measured using a Multiskan Ascent V1.24 multiwell plate reader (Therma Labsystems, Helsinki, Finland) and concentrations were determined using a standard curve analyzed by linear regression.

PHASE A and B.

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 AtAHAS-0107 solution or BPS-CV127-9 leaf extract. For BPS-CV127-9 grain extract, a single tube containing sufficient reaction mix for all assay time points was prepared by mixing 665 µl 1 X SGF with 35 µl extract. 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 test protein. In addition, reactions that served as controls for pepsin auto-digestion and test protein stability were prepared containing SGF without test protein and SGF with test 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.

Simulated mammalian intestinal fluid reactions. Simulated mammalian intestinal fluid (SIF) containing phosphate (8.2 mg KH_2PO_4/ml), tap water and pancreatin (12 mg/ml), pH 7.5, was prepared as described in the United States Pharmacopoeia (2000) and was checked for proteolytic activity using azoalbumin as a substrate (SOP BPS 510.01). A single tube containing enough reaction mix for all time points was prepared by mixing 280 µl of SIF with 70 µl of AtAHAS-0107 solution or BPS-CV127-9 leaf or grain extract. The reaction mixtures were immediately placed in a 37°C water bath and 50 µl samples were removed at 0.5, 2, 5, 10, 30 and 60 min after initiation of the experiment. Each sample was quenched by addition of 50 µl of 2X Laemmli buffer (20% glycerol, 2% β-mercaptoethanol, 4% SDS, 0.13 M Tris, 0.02% bromophenol blue, pH 6.8). 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 was prepared by first heating SIF for 10 min at > 75°C and then adding the test protein and 50 µl 2X Laemmli buffer and reheating for 10 min at > 75°C. In addition, reactions that served as controls for pancreatin auto-digestion and test protein stability were prepared containing SIF without test protein and SIF with test protein but without pancreatin (I-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.

SDS-polyacrylamide gel electrophoresis. To monitor the integrity of AtAHAS (*ca*. 64,000 mol. wt.) in sample AtAHAS-0107 after incubation in the various digestion mixtures, aliquots of the quenched and heated mixes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, SOP BPS 510.02), using a 4 - 20% polyacrylamide gradient Tris-glycine gel (Invitrogen). Aliquots of the samples that contained *ca.* 2.5 µg of protein based on determinations made prior to digestion were loaded onto the gel. Mark 12™ molecular weight markers (Invitrogen) were used to establish approximate molecular weight of proteins. The protein bands were stained with 0.1% Coomassie Brilliant Blue R (Sigma Chemical; St. Louis, MO).

Western blot analysis. To monitor the integrity (intactness) of the AtAHAS protein in the leaf and grain extracts as well as in sample AtAHAS-0107 after incubation in the various digestion mixtures, western blot analysis was performed. Aliquots of the quenched and heated mixes 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. For the SGF samples, the amounts of protein loaded were 24 µg for leaves and grain, and 8 and 30 µg for BPS-CV127-9 and conventional soybean leaves, respectively, and for the SIF samples the amounts were 84 and 67 µg for BPS-CV127-9 and conventional soybean grain, respectively. For western blot analysis of the AtAHAS-0107 SIF reactions 0.2 µg of protein was loaded per lane. After electroblotting, the membranes were probed with rabbit anti-AHAS peptide 2 polyclonal antibody. Donkey 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 chemiluminescent ECL kit from Amersham (Buckinghamshire, UK) according to the manufacturer's instructions.

RESULTS

PHASE A.

Sensitivity of *E.coli*-produced AtAHAS (Lot # AtAHAS-0107) to degradation in SGF. The *E. coli*-produced AtAHAS protein in test material lot # AtAHAS-0107 was rapidly degraded in SGF (Figure 1A). AtAHAS (molecular weight *ca*. 64,000) is readily detected in the time 0 sample and in the G-con samples. However, no full-length protein is visible in the sample removed at 0.5 min after the initiation of the reaction. Some low molecular weight, Coomassie Blue staining, bands (<6000) were observed in the 0.5 min sample, but these were degraded after 2 min in the reaction. A low molecular weight band of approx. 6000 MW is apparent that is also present in the SGF only lane and hence must be derived from the pepsin enzyme preparation.

Sensitivity of *E.coli*-produced AtAHAS (Lot # AtAHAS-0107) to degradation in SIF. The *E. coli*-produced AtAHAS protein in test material lot #AtAHAS-0107 was also rapidly degraded in SIF (Figure 2A). Similar to the SGF results, AtAHAS is detected in the time 0 and I-con samples. However, no full-length protein is visible in the sample after 0.5 min of incubation. Some degradation of the AtAHAS is observed in the 60 min I-con sample indicating protein sensitivity to incubation at 60 min in simulated intestinal fluid in the absence of pancreatin. Pancreatin is comprised of multiple proteases and lipases and this accounts for the multiple staining bands in the SIF lanes. All of the lower molecular weight bands visible in the digestion time course correspond to those present in the SIF only sample(no AtAHAS-0107). The results of the AtAHAS digestion in SIF using western blot analysis (Figure 3A) confirm that no immunoreactive degradation products of AtAHAS were obscured by bands associated with the pancreatin, and confirm that the AtAHAS protein is rapidly degraded in SIF within 0.5 min incubation time.

PHASE B.

Sensitivity of BPS-CV127-9 soybean leaf and grain AtAHAS to degradation in SGF. The AHAS protein produced in BPS-CV127-9 soybean leaves and grain is a mixture of the endogenously encoded soybean AHAS protein and the AtAHAS transgenic protein encoded by the introduced imidazolinone-tolerant *ahasS653N* gene. Due to the high amino acid sequence similarity of these two proteins, they are immunologically indistinguishable. Both AHAS proteins are rapidly digested in SGF and, although the AHAS protein band is visible at time 0, no AHAS protein band is detectable within two minutes of incubation (Figure 1B). An additional immunoreactive band at approximately 50,000 molecular weight is observed in leaf extracts. It is stable in the absence of pepsin (G-con lanes) but is not visible during the digestion time course. A different immunoreactive band at approximately 36,000 molecular weight is observed in the grain extracts, and it is also rapidly digested by pepsin within 0.5 min (Figure 1C). These bands may represent proteins that cross-react with the AHAS-specific antibody or smaller fragments of the AHAS protein, but in either case, they are rapidly degraded.

Sensitivity of BPS-CV127-9 soybean leaf and grain AtAHAS to degradation in SIF. The full-length soybean leaf AHAS protein is rapidly digested and is no longer visible within 0.5 min in SIF (Figure 2B). Similar to the results observed with the *E. coli*-produced AtAHAS protein, the leaf AHAS protein is not stable in SIF without pancreatin (I-con). Also, a lower molecular weight protein of approximately 50,000 molecular weight appears to increase in intensity with time of digestion. Furthermore, an immunoreactive band of approximately 16,000 molecular weight appears to be associated with the pancreatin since the band was not visible in the absence of SIF (I-con lanes) or at the zero time point where the SIF was inactivated by heating prior to addition of the leaf extract. A smaller immunoreactive band of > 6000 molecular weight is also observed and since it is also present in the SIF only incubations, it appears to be associated with pancreatin. This band may be a degradation product of pancreatin. Full length AHAS protein is just barely detected by western blot analysis in the leaf extract of conventional nontransgenic soybean and the lower molecular weight protein at approximately 50,000 molecular weight (observed in the leaf extract of BPS-CV127-9 soybean) is apparently below the detection limit for this method as the 50,000 molecular weight band was not observed in the conventional nontransgenic leaf extract subjected to digestion in SIF (Fig. 3B). This result was not unexpected since the AHAS protein expression levels, as reported in da Silva (2007) were approximately 600 ng AHAS/g dry weight in young BPS-CV127-9 leaves as compared to approx. 75 ng AHAS/g dry wt in young leaves of conventional, nontransgenic soybean. The lower molecular weight bands at approximately 16,000 and >6000 molecular weights, previously observed in SIF digestion of leaf extracts of BPS-CV127-9, were again observed in SIF digestion of the leaf extract of conventional nontransgenic soybean (Fig. 3B), supporting the conclusion that these bands are associated with pancreatin and not with the AtAHAS transgenic protein encoded by the introduced imidazolinone-tolerant *ahas S653N* gene.

In the grain extracts of BPS-CV127-9, full-length AHAS is rapidly degraded by pancreatin and is not observed at 0.5 min (Fig. 2C). Full length AHAS protein was not detected in the SIF digest of the conventional nontransgenic soybean grain sample (Figure 3C). This result was not unexpected, since the expression levels, as reported in da Silva (2007) were below the level of quantification in the conventional nontransgenic soybean grain, and approximately 28 ng/g dry weight in BPS-CV127-9 grain. As observed with digestion of the leaf extract of BPS-CV127-9, where a lower molecular weight (approx. 50,000) protein band was observed, a lower molecular weight immuno-reactive band at approximately 36,000 molecular weight appears to accumulate with digestion time in SIF digestions of both BPS-CV127-9 and the conventional nontransgenic soybean grain (Figures 2C and 3C respectively). Also, the same immunoreactive bands associated with pancreatin (at approximately 16,000 and >6000 molecular weights), and described in the digest of the leaf extracts, are also observed in digestion of the grain extracts (Figs. 2C and 3C). The grain AtAHAS appears more stable in the SIF without pancreatin (I-con) and perhaps this is due to the presence of other components in the grain extract that stabilize AHAS (Fig. 2C).

In leaf and grain extracts, protein bands corresponding to approximately 50,000 and 36,000 molecular weights, respectively, appeared to increase in intensity with increasing time of SIF digestion. Because no lower molecular weight protein bands at

approximately 50,000 or 36,000 molecular weights were observed to accumulate in SIF digestion of AtAHAS-0107 (Fig. 3C), it is unlikely that these protein bands are a degradation product of the full-length AHAS protein produced in leaf or grain tissue, and are more likely immunoreactive peptides generated from digestion of a different protein present in the leaf or grain extracts.

CONCLUSIONS

E.coli-produced AtAHAS, and both forms of AHAS produced in BPS-CV127-9 leaves and grain (endogenous soybean AHAS and transgenic AtAHAS), are rapidly degraded in simulated mammalian gastric fluid. Full-length AHAS, regardless of source, is also rapidly degraded in simulated mammalian intestinal fluid. Degradation of the AHAS and AtAHAS proteins in plant extracts was slightly slower compared to the purified *E. coli*-produced protein in both SGF and SIF, showing that the plant matrix has only a slight effect on protein digestion. Therefore, AtAHAS expressed in BPS-CV127-9 soybean is digested the same as conventional dietary protein, the same as endogenous soybean AHAS has been digested in conventional plants to date.

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 predictive bioassays available to assess the allergenic potential of proteins in humans; however, physicochemical and human exposure profiles of the protein provide a basis for assessing potential allergenicity relative to known protein allergens. Results of the current study show that the AtAHAS protein expressed in BPS-CV127-9 soybean is highly digestible under the simulated digestion conditions, which is typical of most proteins exposed to the proteases of the mammalian digestive tract. Furthermore, the transgenic AtAHAS protein expressed in BPS-CV127-9 soybean was immunologically indistinguishable from the endogenous soybean AHAS protein in the current studies, and results suggested that the digestive fate of both proteins in SGF and SIF was identical. Therefore, the AtAHAS protein shares the same digestive fate properties with the endogenous soybean AHAS protein, which has a history of safe consumption in food and feed products.

GLP COMPLIANCE AND PROTOCOL CHANGES: This study was conducted in compliance with the relevant provisions of Good Laboratory Practice Standards (40 CFR 160, *Federal Register*, 1989) pursuant to the Federal Insecticide, Fungicide and Rodenticide Act and subsequent revisions with the exception of Phase B, the digestibility of AtAHAS produced in BPS-CV127-9 soybean . A protocol change was made to add digestion reactions with nontransgenic soybean grain and leaf preparations. This was judged to have no adverse impact on the study and allowed better interpretation of background protein bands.

RECORDS RETENTION: Raw data, the original copy of this report, and other relevant records are archived at BASF, 26 Davis Drive, Research Triangle Park, NC, USA 27709.

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

CRITICAL DATES:

Study initiation date: 29 May 07
Experimental start date: 30 May 07
Experimental end date: 3 October 07

REFERENCES

Standard Operating Procedures

SOP BPS 510.01 Simulated Mammalian *In vitro* Digestibility Method

SOP BPS 510.02 SDS-Polyacrylamide Gel Electrophoresis

SOP BPS 510.03 Western Blot Analysis

Literature References

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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. Susceptibility of AtAHAS to Digestion in Simulated Mammalian Gastric Fluid (SGF).

Incubation time at 37ºC is indicated in minutes. SGF is simulated mammalian gastric fluid containing pepsin, G-con is SGF without pepsin; SGF only indicates no AtAHAS sample was included. Panel A. Coomassie blue stained 4- 20% polyacrylamide gel containing digestion reactions carried out with test substance AtAHAS-0107. Panel B. Western blot analysis of digestion reactions carried out with leaf extract from BPS-CV127-9 soybean. Panel C. Western blot analysis of digestion reactions carried out with grain extract from BPS-CV127-9 soybean. AtAHAS is *ca.* 64,000 mol wt., pepsin is ca. 40,000 mol. wt. Molecular weight (x 10^{-3}) markers are indicated.

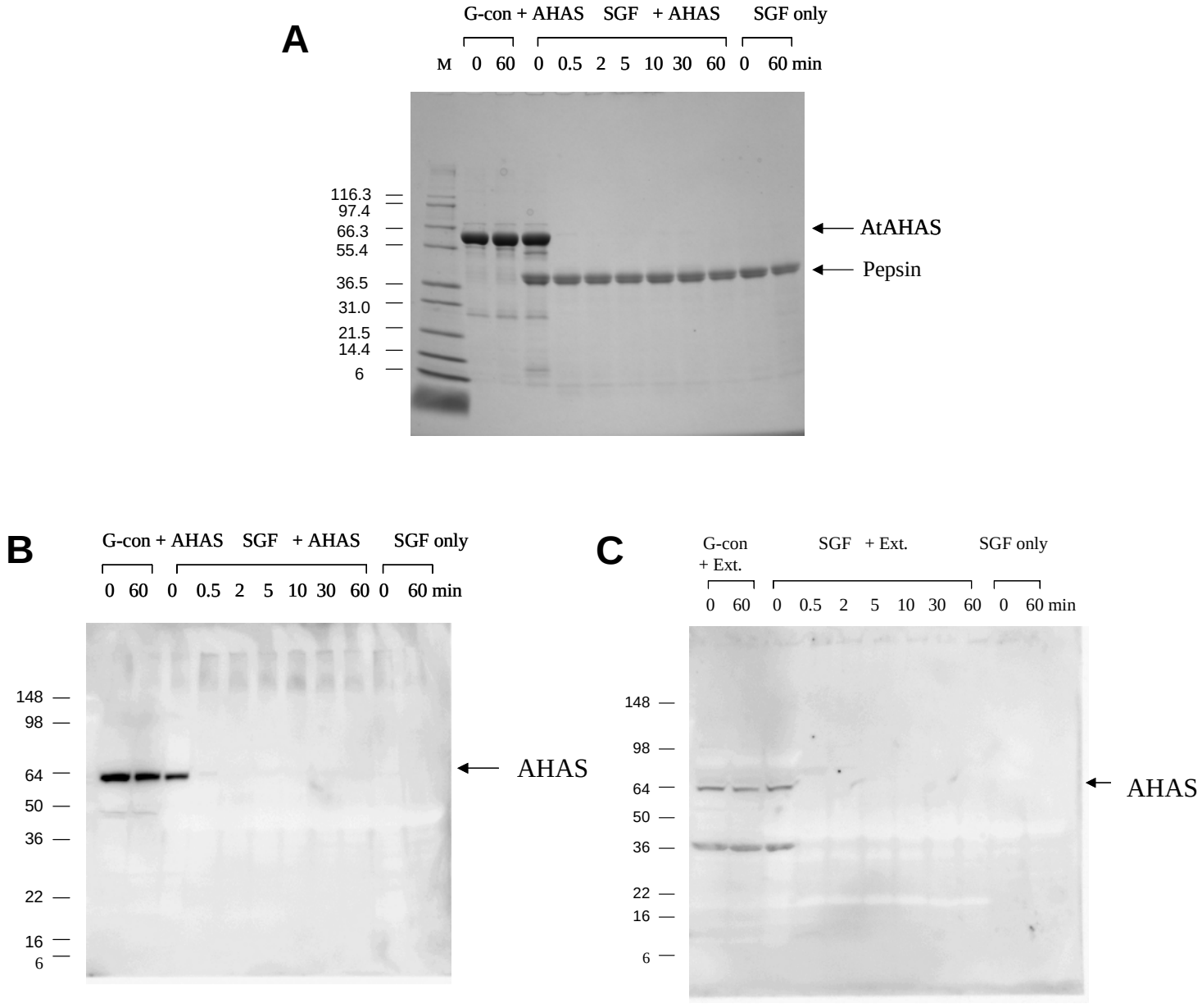

Figure 2. Susceptibility of AtAHAS to Digestion by Simulated Mammalian Intestinal Fluid (SIF).

Incubation time at 37ºC is indicated in minutes. SIF is simulated mammalian intestinal fluid containing pancreatin, I-con is SIF without pancreatin; SIF only indicates no AtAHAS sample was included. Panel A. Coomassie blue stained 4- 20% polyacrylamide gel containing digestion reactions carried out with the sample AtAHAS-0107. Panel B. Western blot analysis of digestion reactions carried out with leaf extract from BPS-CV127-9 soybean. Panel C. Western blot analysis of digestion reactions carried out with grain extract from BPS-CV127-9 soybean. AtAHAS is *ca.* 64,000 mol wt., and molecular weight (x 10^{-3}) markers are indicated.

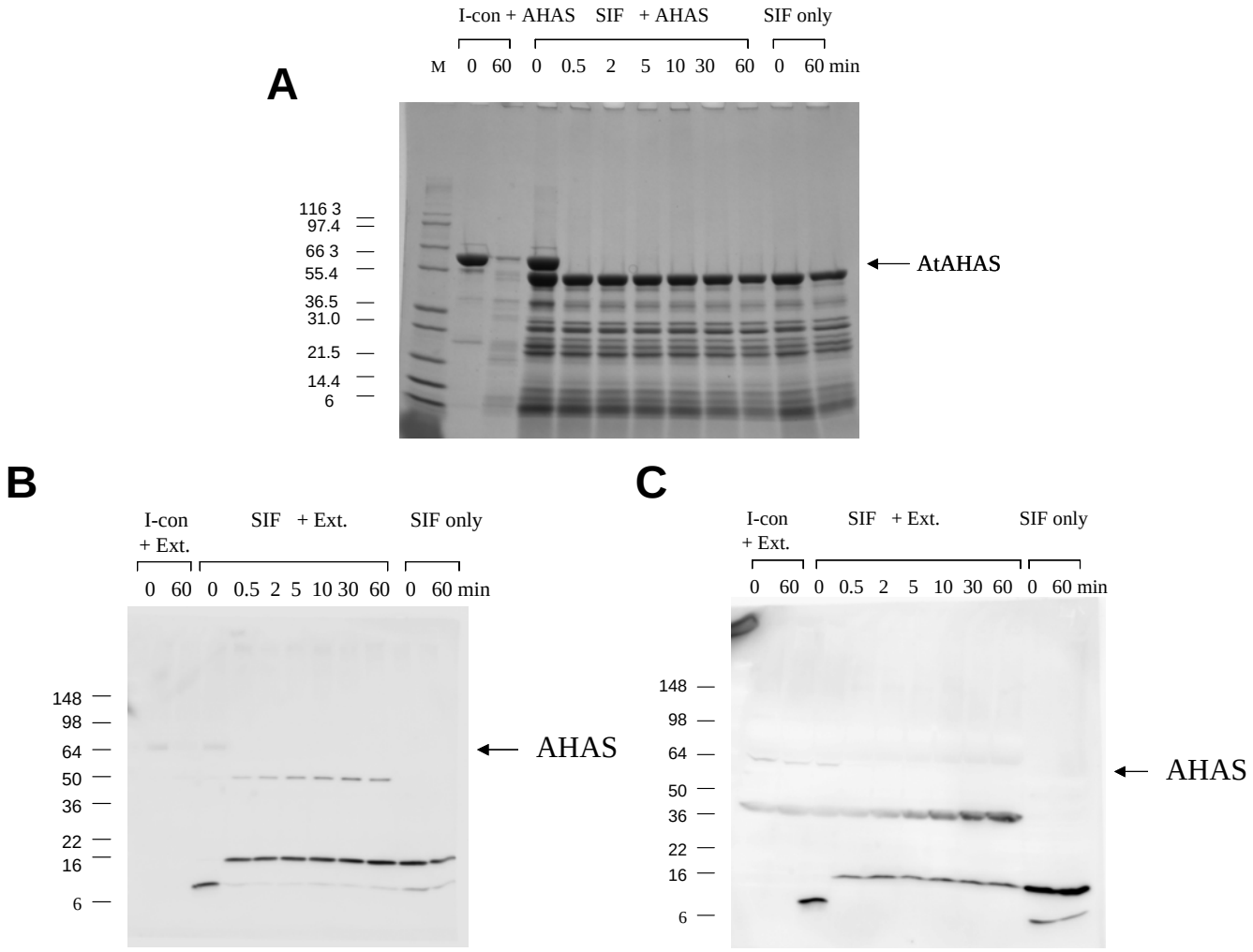

Figure 3. Western Blot Analysis of Simulated Mammalian Intestinal Fluid (SIF) Reactions for AtAHAS-0107, Conventional Soybean Leaf and Grain Protein Extracts.

Incubation time at 37ºC is indicated in minutes. SIF is simulated mammalian intestinal fluid containing pancreatin, I-con is SIF without pancreatin; SIF only indicates no AtAHAS sample was included. Panel A. Western blot analysis using a 4- 20% polyacrylamide gel containing the same digestion as in Figure 2A for AtAHAS-0107. Panel B. Western blot analysis of digestion reactions carried out with leaf extract from conventional, nontransgenic soybean leaves. Panel C. Western blot analysis of digestion reactions carried out with grain extract from conventional, nontransgenic soybeans. AtAHAS is *ca.* 64,000 mol wt., and molecular weight (x 10^{-3}) markers are indicated.