Appendix 8

Characterization of Test Substance Arabidopsis Acetohydroxyacid Synthase (Lot #AtAHAS-0107)

BASF
The Chemical Company

Plant Science LLC

REPORT # BPS-011-07

CHARACTERIZATION OF TEST SUBSTANCE *ARABIDOPSIS* ACETOHYDROXYACID SYNTHASE (LOT #ATAHAS-0107)

STUDY # BPS-HTC-07-001

EPA GUIDELINE #: N/A

AUTHOR:

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STUDY COMPLETED ON: JULY 16, 2007

TEST FACILITY/PERFORMING LABORATORY:

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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: 16 Jul 2007

Title: Regulatory Affairs Manager

Signature: [redacted]

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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, N-terminal amino acid sequence identification, was not conducted under GLP

STUDY DIRECTOR:

[redacted] 16 Jul 07
Date

Senior Manager, Regulatory Science
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[redacted] 16 Jul 2007
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QUALITY ASSURANCE UNIT STATEMENT

Study Number: BPS-HTC-07-001

Name/Number of Test Substance: AtAHAS-0107

Type of Study: Test Substance Characterization 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
3/20/2007	**3/20/2007 Protocol audit**
3/27/2007	**3/27/2007 In-process audit, solubility and protein determination**
3/28/2007	**3/29/2007 In-process audit for lipopolysaccharide contamination**
6/192007	**6/19/2007 Final report and raw data audit**

[redacted]

Signature QAU

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ABBREVIATIONS AND DEFINITIONS

AHAS	acetohydroxyacid synthase
AHASL	acetohydroxyacid synthase large subunit
AtAHAS	*Arabidopsis thaliana* acetohydroxyacid synthase
BCA	bicinchoninic acid
BME	β-mercaptoethanol
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
ELISA	enzyme-linked immunosorbent assay
EU	endotoxin unit
FAD	flavin adenine dinucleotide
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
LPS	lipopolysaccharide (endotoxin)
S653N	serine at position 653 replaced by asparagine
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
TPP	thiamine pyrophosphate
WT	wild type

GENERAL INFORMATION

BASF Registration Document Number:	2007/7004168
BASF Plant Science Study Number:	BPS-HTC-07-001
Test Substance:	AHAS protein produced in an *Escherichia coli* over-expression system
Sample Lot No.:	AtAHAS-0107
Sponsor and Testing Facility	BASF Plant Science, LLC P. O. Box 13528 26 Davis Drive Research Triangle Park, NC 27709
Additional Test Facility:	BASF AG MicroProtein Analytical Laboratory GVC/C – A030 D-67056 Ludwigshafen, Germany
Study Director:	[redacted] Ph.D.
Analysts:	[redacted] M. S.

CHARACTERIZATION OF TEST SUBSTANCE *ARABIDOPSIS* ACETOHYDROXYACID SYNTHASE (LOT #ATAHAS-0107)

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 a test substance, (Lot #AtAHAS-0107), 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 dicot 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 Cultivance Soybean Event 127. 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 by affinity chromatography followed by precipitation by ammonium sulfate. Dialysis was used to remove salts. The dialyzed material was then lyophilized and the resulting test substance was designated sample lot number AtAHAS-0107. The AtAHAS protein preparation was characterized to determine identity, purity, functionality, concentration, and solubility. Stability of the protein was determined in a separate study.

Protein identity was demonstrated by amino acid sequence analysis of both the N-terminal and internal peptide fragments of the protein. In addition, western blot analysis using polyclonal antibodies specific for the AtAHAS protein confirmed immunoreactivity. The enzymatic activity of the protein as well as the enzymatic activity showing reduced sensitivity to inhibition by an imidazolinone herbicide confirmed the identity of the AtAHAS protein in test substance AtAHAS-0107.

The purity of the AtAHAS protein preparation was assessed using ELISA, and protein quantification. The preparation was shown to contain approximately 52.4% AtAHAS by weight, and corresponded to approximately 90.6% of the total protein in the preparation. SDS-PAGE and western blot analyses of the sample revealed a major protein band at the predicted molecular weight of AtAHAS. The molecular weight of the AtAHAS protein was approximately 64,000.

The functionality of the AtAHAS protein in sample AtAHAS-0107 was monitored by measuring enzymatic activity. The sample was enzymatically active and had a specific activity of 0.790 ± 0.216 units/mg protein and reduced sensitivity to the imidazolinone herbicide, imazethapyr.

The solubility of the AtAHAS-0107 test substance was approximately 10 mg/ml in 100 mM CAPs buffer at pH 11.

This study confirmed the identity, purity, concentration, functionality, and solubility of the AtAHAS protein test substance preparation.

INTRODUCTION

The purpose of this study was to characterize a test substance, (Lot #AtAHAS-0107), containing AtAHAS protein encoded by the imidazolinone-tolerant acetohydroxyacid synthase *ahas* gene isolated from *Arabidopsis thaliana.* The introduction of an imidazolinone-tolerant acetohydroxyacid synthase (*ahas*) gene from *Arabidopsis thaliana* into the dicot 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 when this gene was introduced into soybean resulting in the transformation event designated Cultivance Soybean Event 127. The second mutation, in which arginine at position 272 was replaced by lysine, 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.

This lot of test substance, AtAHAS-0107, was prepared from a recombinant *E. coli* over-expression system and is intended for use in product safety and characterization studies, including an acute oral mouse toxicity study. Various biochemical parameters were evaluated to confirm the identity of the AtAHAS protein test substance in sample lot number AtAHAS-0107, as well as to determine its concentration and integrity. The test substance was also evaluated for total protein concentration, enzymatic activity, solubility, and lipopolysaccharide content.

MATERIALS AND METHODS

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. AHAS protein, as encoded in this vector, lacks the 85 N-terminal amino acid leader sequence that targets the protein *in planta* to the chloroplast. This leader 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 two 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 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. AtAHAS protein was purified from 2,400 g 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. The test substance in this study is also the test system.

Protein quantification. Total protein in test substance AtAHAS-0107 was quantified by the BCA™ procedure (bicinchoninic acid procedure; Pierce Biotechnology, Inc.; Rockford, IL) in accordance with the manufacturer's instructions, using bovine serum albumin as the standard (SOP BPS 510.04). Samples of AtAHAS were prepared such that the expected concentration of protein would lie 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 37°C for 30 min and allowed to cool at room temperature for 10 min. The absorbance at 562 nm was measured using a Tecan Sunrise® multiwell plate reader. The results were analyzed using DeltaSoft PC software (Version 1.71.4, Biometallics, Inc.; Princeton, NJ) using the linear regression curve fit.

AHAS quantification. Sample AtAHAS-0107 was quantitatively analyzed for AHAS protein by a sandwich enzyme-linked immunosorbent assay (ELISA) [Tijssen, 1985] using immunoaffinity-purified polyclonal rabbit anti-AHAS peptide 2 antibody and

Protein G-purified goat antibodies specific for AHAS (SOP BPS 510.06 and BPS 510.16). Nunc 96-well plates (VWR; West Chester, PA) were coated with rabbit anti-peptide 2 and incubated at 37°C for 1 hr. The plate was washed two times with wash buffer and then blocked with 1% BSA in Tris buffered saline (25 mM Tris-HCl, 3 mM KCl, 0.14 M NaCl) with 0.05% Tween for 60 min. at 37°C. After washing twice, samples and standards were applied in triplicate. Plates were incubated overnight at 4°C, and then washed five times prior to the addition of the goat anti-AtAHAS followed by incubation for 1 hr at 37°C. Plates were then washed three times and donkey anti-goat-horseradish peroxidase (HRP) was added. After incubation at 37°C for 1 hr, the plates were washed three times and HRP substrate was added (1 Step Ultra TMB; Pierce). After 30 min at room temperature 1 M HCl was added to stop the reaction. The absorbance at 450 nm was measured using a Tecan Sunrise® multiwell plate reader. The results were analyzed using DeltaSoft PC software (Version1.71.4; Biometallics, Inc.; Princeton, NJ). The four-parameters algorithm was used to generate a curve. The AtAHAS component of AtAHAS-0107 was quantified from the standard concentration curve generated from highly purified AtAHAS protein.

Molecular weight determination. To confirm that a major protein in sample AtAHAS-0107 had the predicted molecular weight of AtAHAS (*ca.* 64,000), aliquots of the sample solution were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, SOP BPS 510.02), using an 8 - 16% polyacrylamide gradient Tris-glycine gel (Invitrogen). Aliquots of AtAHAS-0107 solution which had been mixed 1:1 with 2 X Laemmli buffer (20% glycerol, 2% β-mercaptoethanol (BME), 4% SDS, 0.13 M Tris, 0.02% bromophenol blue) were heated for 10 min at >75°C and were loaded onto the gel such that the total protein in each lane was either *ca.* 2, 4, 6, and 8 µg. Mark 12™ molecular weight markers (Invitrogen) were used to establish approximate molecular weight. The protein bands were stained with 0.1% Coomassie Brilliant Blue R (Sigma Chemical; St. Louis, MO).

Immunoreactivity. To assess the integrity (intactness) of the AtAHAS protein in AtAHAS-0107, western blot analysis was performed. Aliquots of the AtAHAS-0107 solution were subjected to SDS-PAGE on an 8 - 16% polyacrylamide gradient gel followed by electroblotting onto PVDF membrane (Invitrogen; SOP BPS 510.03). Sample AtAHAS-0107 was loaded onto the gel such that 5, 20 and 50 ng AtAHAS protein was present in the lanes. After electroblotting the membrane was 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 chromagenic substrate diaminobenzidine (Sigma Chemical).

Enzymatic activity. The enzymatic activity of AHAS was assayed according to Singh *et al.*, 1988 and SOP BPS 510.09. AHAS catalyzes the synthesis of acetolactate (an acetohydroxy acid) by the condensation of two molecules of pyruvate. The acetolactate produced by AtAHAS in this assay is converted to acetoin in the presence of acid and acetoin is detected colorimetrically ($A^{530\ nm}$) after interaction with creatine and napthol.

One unit of AHAS activity is defined as 1 μmole acetoin produced per minute. AtAHAS-0107 dissolved in 50 mM Tris-HCl, pH 7.0, diluted to desired concentration, and mixed with an equal volume of 2x assay buffer [100 mM Tris-HCl, 10 mM $MgCl_2$, 0.2 M sodium pyruvate, with 20 μM FAD and 2 mM thiamine pyrophosphate (TPP), pH 7.0] and incubated at 37°C for 1 h prior to the addition of 20 μl 5% H_2SO_4 and 15 min. incubation at 60°C. Background absorbance was determined by immediately quenching the reactions prior to incubation at 37°C. Acetoin color was developed by incubating the quenched reactions with creatine (0.17%, final concentration) and 1-napthol (1.7% in 4 N NaOH, final concentration) for 15 min at 60°C and the absorbance was measured at 530 nm. Absorbance values were compared to an acetoin standard curve and corrected for background absorbance. Several dilutions of a single dissolved sample of AtAHAS were prepared and each dilution was assayed in triplicate; the mean corrected value is reported.

A stock solution (50 mM) of imazethapyr, a commercial imidazolinone herbicide, was diluted in 2x AHAS assay buffer to two times the desired final assay concentration, where the final concentration of herbicide ranged from 0 to approx. 1000 μM. Fifty μl of the sample was incubated with 50 μl of 2x assay buffer or imazethapyr solution for 60 min at 37°C. For comparison when measuring imazethapyr inhibition, extracts (prepared as described below) of *E. coli* expressing either wild type AtAHAS (strain 256) or AtAHAS R272K S563N (strain 346) were assayed in parallel.

The absorbance was measured at 530 nm. Background samples were generated by pre-quenching with acid prior to assay incubation and the absorbances thus generated were subtracted from the test samples. All assays were conducted in triplicate and results are presented as mean values. The amount of activity obtained for each sample in the absence of inhibitor was assumed to be 100%.

Bacterial expression of recombinant AtAHAS polypeptides.

The open reading frame of wild type *AtAHASL* (WT), and *AtAHASL R272K S653N* were cloned into the bacterial expression vector, *pTrcHis* (Invitrogen). After sequence confirmation, the plasmids were transformed into AHAS minus *JMC1 E. coli* cells and designated strains 256 and 346 for WT and R272K S653N, respectively, and stored at –80° C as glycerol stocks.

Three ml liquid Luria Broth (LB) media plus carbenicillin (100 μg/ml, final concentration) was inoculated from the glycerol stock and placed on a rotary shaker at 220 rpm overnight at 37°C. One ml of the overnight culture was used to inoculate 50 ml LB plus carbenicillin in 250 ml flasks and cultures were shaken at 220 rpm at 37°C until the OD_{600} reached approx. 0.6 - 0.8. For induction of protein expression, isopropyl thiogalactoside (IPTG) was added at a final concentration of 500 μM and the cells were incubated at 37°C for about three hours with shaking at 220 rpm. and then centrifuged at *ca.* 6000 x g for 10 min. The pellets were immediately frozen at –20°C.

Preparation of bacterial extracts.

The frozen bacterial pellets were resuspended in 15 ml Tris buffered saline (TBS: 50 mM Tris-HCl, pH 7.0, 140 mM NaCl, 5 mM $MgCl_2$) supplemented with 1 mg/ml lysozyme,

vortexed, and then incubated on ice for 30 min. After incubation, the extract was sonicated (15 bursts, twice) and then centrifuged at ca. 23,000 x g for 15 min at 4°C. Protein was precipitated with ammonium sulfate by incubating the supernatant with an equal volume of saturated ammonium sulfate, which was added slowly drop-wise (final concentration 50% saturation of ammonium sulfate). Samples were incubated on ice for at least 30 min with constant stirring. Samples were centrifuged at *ca.* 23,000 x g for 15 min at 4°C and the supernatant was removed. The protein pellet was resuspended in 3 ml 50 mM Tris-HCl, pH 7.0, centrifuged and the supernatant was used in the inhibition assay.

Solubility determination. The solubility of AtAHAS-0107 in aqueous solutions, including specifically water; 100 mM CAPS, pH 11; and 50 mM Tris-HCl, pH 7.0, was determined by resuspending a known weight of test substance in a minimal volume of aqueous solution and diluting with this solution until the test substance is dissolved completely. The sample was rotated gently at room temperature for 15 min and evaluated visually for non-dissolved particulate matter (SOP BPS 510.07).

Lipopolysaccharide (Endotoxin) contamination. The Limulus Amoebocyte Lysate Pyrogen Plus Single Test kit (Cambrex BioScience; Walkersville, MD) was used to quantify the lipopolysaccharide (LPS) present in AtAHAS-0107 in accordance with the manufacturer's instructions. Lipopolysaccharide, specifically endotoxin, is often a contaminant of protein preparations produced in gram negative bacteria and results from contamination of the protein preparation by lipopolysaccharide outer membrane of the bacteria. AtAHAS-0107 was dissolved in HyPure Cell Culture water (HyClone, Logan, UT) and dilutions were prepared so that the lowest dilution tested did not form a gel upon incubation with the amoebocyte lysate. This dilution is compared with serially-diluted certified standard endotoxin (CSE, positive control), HyPure water (negative and buffer control), and AtAHAS-0107 solution containing 0.125 endotoxin units (EU)/ml CSE (to check for the presence of inhibitors in the test substance). All samples and controls were added to different vials of lysate and mixed by tilting and gentle swirling of the vial. After incubation at 37°C for 60 min, the vials were scored by carefully inverting each vial 180 degrees to check for gelling. Only those that were solid were scored as positive; all others were considered negative. An endotoxin unit is defined by the FDA as endotoxin activity of 0.2 ng of Reference Endotoxin Standard, EC-2 or 5 EU/ng.

N-terminal sequence analysis sample preparation. To further confirm the identity and integrity of the AtAHAS protein in test substance AtAHAS-0107, the N-terminal amino acid sequence of the AtAHAS-0107 protein preparation together with peptides generated from specific bands within the sample was performed at the BASF MicroProtein Analytical Laboratory, Ludwigshafen, Germany, as Phase B of this study. Approximately 100 µg of AtAHAS-0107 was dissolved in 100 µl 100 mM dithiothreitol, 2% SDS, 16% glycerol, 0.036% bromophenol blue, 80 mM Tris, pH 6.8 and subjected to SDS-PAGE divided into a total of 6 lanes. The main bands, corresponding to *ca.* 64,000 molecular weight, from four out of the six lanes were cut out of the gel and the gel pieces were diced and washed in an approximate ten-fold volume of water with shaking for 30 min. The supernatant was discarded and the wash step repeated, followed by sequential

washing with ten-fold volumes of methanol/water (1:1, v:v), acetonitrile, and finally acetonitrile/water 1:1. The gel pieces were then dried using a vacuum centrifuge. Freshly prepared trypsin digestion buffer (100 µl; 100 mM NH_4HCO_3, pH 8.5, 5% acetonitrile) was added to 10 µl of 1% acetic acid in which 25 µg trypsin (sequencing grade, Roche, Mannheim, GE) was dissolved. The gel pieces were soaked in the trypsin mixture overnight at 37°C. The mixture was centrifuged, the supernatant removed and retained and the gel pieces were washed with 5 x volume of 70% acetonitrile containing 1% trifluoroacetic acid. The supernatant was added to the retained supernatant and the gel pieces were washed with 5x volume of 100% acetonitrile containing 1% trifluoroacetic acid. The pooled supernatants were partially dried in a vacuum centrifuge. The resulting peptide fragments were separated by HPLC and the fractions were collected. Selected fractions were subjected to Edman degradation. Five fractions from the 64,000 mol. wt. band were subjected to Edman degradation using PROCISE 494 cLC and PROCISE 494 HT protein sequencers (Applied BioSystems; Foster City, CA). The resulting amino acid sequence data demonstrated that the five fractions contained peptide fragments derived from AtAHAS.

RESULTS

AHAS quantification. AtAHAS-0107 test substance was determined by ELISA to contain *ca.* 0.524 g AHAS protein/g sample, or *ca.* 52.4% AHAS by weight. Analysis of AtAHAS-0107 using the BCA protein determination method determined that the sample contained 58 % protein by weight. By calculation, the AtAHAS protein comprises 90.6 % of the total protein in the sample (Table 1).

Molecular weight determination. The major protein band in AtAHAS-0107 test substance was determined to be *ca.* 64,000 molecular weight by SDS-PAGE analysis (Figure 1) corresponding to the predicted molecular weight of AtAHAS protein.

Immunoreactivity. Western blot analysis of sample AtAHAS-0107 revealed that the major immunoreactive species corresponded to the predicted molecular weight of the AtAHAS protein (*ca.* 64,000 mol. wt.; Figure 2) using rabbit anti-AHAS peptide 2 polyclonal antibodies.

Enzymatic activity. Sample AtAHAS-0107 was determined to have a specific activity of 0.790 ± 0.216 units/mg protein, confirming that the AtAHAS protein in sample AtAHAS-0107 is enzymatically active (Table 1). The imidazolinone herbicide, imazethapyr was much less effective at inhibiting activity in comparison to *E. coli* expressed wild type (Figure 3). This AtAHAS contains an amino acid change (S653N) reducing its affinity for the herbicide and thus reducing inhibition (Sathasivan *et al.*, 1991).

Solubility determination. AtAHAS-0107 was determined to be soluble up to *ca.* 10 mg/ml in 100 mM CAPS, pH 11. It was less soluble in water or 50 mM Tris-HCl, pH 7.0.

Lipopolysaccharide determination. AtAHAS-0107 was determined to contain 588 EU/mg test substance.

N-terminal amino acid sequence determination. N-terminal amino acid sequence data was obtained after fractionation of AtAHAS-0107 by SDS-PAGE, elution from the gel, desalting over Prosorb-material and fixing on PVDF membrane. Other sequences were obtained by in-gel digestion of the SDS-PAGE bands with trypsin to generate peptide fragments that were separated by HPLC; selected fractions were subjected to Edman degradation. Peptides both within the AtAHAS protein and the expression tag were analyzed. The results (Figure 4) confirm that the major protein band at approx. 64,000 molecular weight is AtAHAS since the amino acid sequences obtained from peptide fragments derived from it were identical to regions of the deduced amino acid sequence of the AtAHAS gene in the expression vector.

CONCLUSIONS

The identity and concentration of the AHAS protein in the test substance was confirmed by various biochemical parameters. The test substance was also evaluated for total protein concentration, AHAS integrity, enzymatic activity, solubility, and lipopolysaccharide content.

Protein identity was demonstrated by amino acid sequence analysis of both the N-terminal and internal peptide fragments of the protein. These sequences were identical to the corresponding sequences encoded by the expression vector. The AtAHAS-0107 protein preparation was shown to contain approximately 52.4% AHAS by weight, and corresponded to approximately 90.6% of the total protein in the preparation. SDS-PAGE and western blot analyses of the sample revealed the major protein band at the predicted molecular weight of *ca.* 64,000 for AHAS. The AHAS protein preparation was shown to contain approximately 0.524 g AHAS protein / g of sample. The sample was enzymatically active and had a specific activity of 0.790 ± 0.216 units/mg protein and reduced sensitivity to the imidazolinone herbicide, imazethapyr. The composition of the lyophilized AtAHAS-0107 protein preparation was shown to contain approximately 58% protein, and the solubility of the AtAHAS-0107 protein preparation was up to *ca.* 10 mg/ml in 100 mM CAPs buffer at pH 11. Lipopolysaccharide content of AtAHAS-0107 was determined to be 588 EU/mg test substance.

GLP COMPLIANCE: 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 N-terminal amino acid sequence determination.

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 conducted by [redacted] M.S., BASF Plant Science, LLC., Research Triangle Park, NC 27709 and [redacted], BASF AG, Ludwigshafen, Germany.

CRITICAL DATES:

Study initiation date:	20 March 07
Experimental start date:	27 March 07
Experimental end date:	12 April 07

EXAMPLE CALCULATIONS:

$$\%\ \text{AtAHAS protein in AtAHAS-0107} = \frac{\text{mg AtAHAS/ml AtAHAS-0107}}{\text{mg total protein/ml AtAHAS-0107}} \times 100$$

$$\%\ \text{total protein in AtAHAS-0107} = \frac{\text{mg total protein}}{\text{mg AtAHAS-0107}} \times 100$$

$$\%\ \text{Inhibition by imazethapyr} = \frac{(\text{Final OD}^{350} - \text{Background OD with imazethapyr})}{(\text{Final OD}^{350} - \text{Background OD without imazethapyr})} \times 100$$

$$\text{Endotoxin Unit/mg test substance} = \frac{\text{Lowest positive standard conc. (in EU/ml)}}{\text{Lowest positive sample conc. (in mg/ml)}}$$

REFERENCES

Standard Operating Procedures

BPS 510.02	SDS-Polyacrylamide Gel Electrophoresis
BPS 510.03	Western Blot Analysis
BPS 510.04	Protein Determination Using the BCA Procedure
BPS 510.06	General Procedure for Enzyme Linked Immunosorbent Assay (ELISA)
BPS 510.07	Test Substance Solubility Determination
BPS 510.09	Enzymatic Assay for Acetohydroxyacide Synthase (AHAS)
BPS 510.16	*Arabidopsis* Acetohydroxyacid Synthase (*At*AHAS) ELISA

Literature References

Federal Register, Part IV, 40 CFR, Part 160, 17 August 1989 and subsequent revisions.

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Table 1. Characterization of Sample AtAHAS-0107

	AtAHAS Concentration			Specific Activity
Date of Analysis	AtAHAS (g/g sample)	% AtAHAS by weight	AtAHAS as % total protein	Mean units/mg protein ± standard deviation
April 12, 2007	0.524	52.4	90.6	0.790 ± 0.216

Figure 1. Molecular weight confirmation of AtAHAS protein in sample AtAHAS-0107.

Coomassie blue-stained 8 - 16% polyacrylamide SDS gel. AtAHAS is *ca.* 64,000 mol. wt. Lane 1, molecular weight (x 10^{-3}) markers; lanes 1 – 4 contain 2, 4, 6, and 8 μg of protein, respectively, from sample AtAHAS-0107.

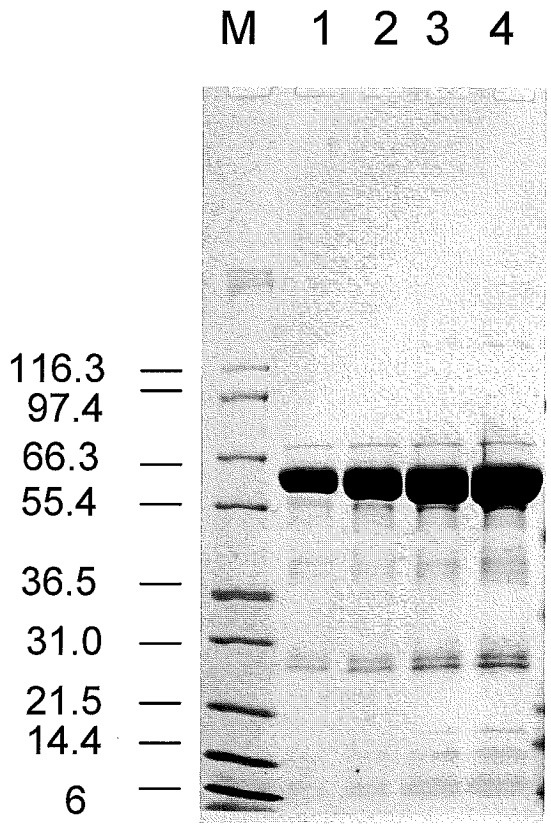

Figure 2. Western blot analysis of AtAHAS protein in sample AtAHAS-0107.

The integrity of AtAHAS in sample AtAHAS-0107 was evaluated by western blot analysis. Lanes 1 - 3 contain 5, 20, and 50 ng AtAHAS from AtAHAS-0107, respectively, and were probed with rabbit anti-AHAS peptide 2 antibody. The molecular weight of intact AtAHAS corresponds to *ca.* 64,000 mol. wt. Molecular weight (x 10^{-3}) markers are indicated.

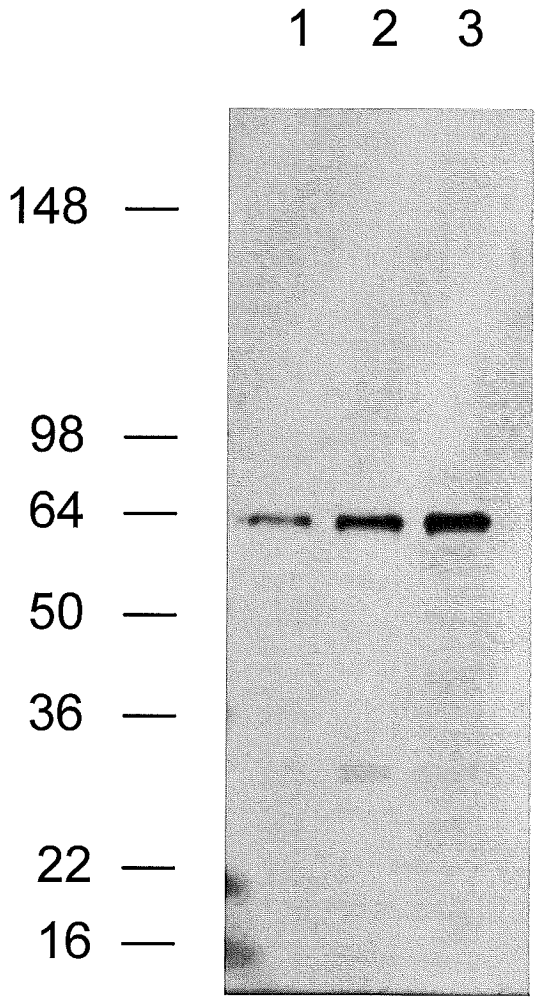

Figure 3. Sensitivity of AtAHAS protein in sample AtAHAS-0107 to imazethapyr, an imidazolinone herbicide. *E. coli* strain 256 expresses the wild type AtAHAS protein, and *E. coli* strain 346 expresses the herbicide tolerant AtAHAS R272K S653N protein.

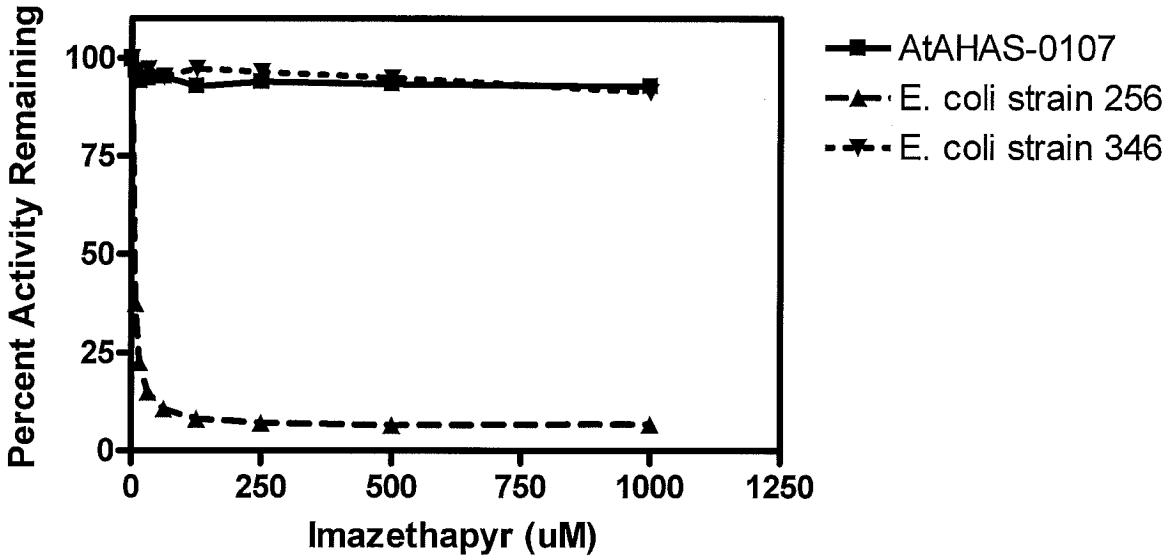

Figure 4. AtAHAS Amino Acid Sequence and N-terminal Amino Acid Sequence Results. The amino acids underlined were obtained by Edman degradation of tryptic peptide fragments derived from the 64,000 molecular weight band in AtAHAS-0107. The capital letters indicate the tag added by the expression vector, the lower-case letters correspond to the deduced amino acid sequence of AtAHAS encoded in the expression vector.

MGGSHHHHHHGMASMTGGQQMGRDLYDDDKDRWGSELtfisrfapdqprkgadilveal
erqgvetvfaypggasmeihqaltrsssirnvlprheqggvfaaegyarssgkpgicia
tsgpgatnlvsgladalldsvplvaitgqvprrmigtdafqetpivevtrsitkhnylv
mdvedipriieeafflatsgrpgpvlvdvpkdiqqqlaipnweqamrlpgymsrmpkpp
edshleqivrliseskkpvlyvgggclnssdelgkfveltgipvastlmglgsypcdde
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knktphvsvcgdvklalqgmnkvlenraeelkldfgvwrnelnvqkqkfplsfktfgea
ippqyaikvldeltdgkaiistgvgqhqmwaaqfynykkprqwlssgglgamgfglpaa
igasvanpdaivvdidgdgsfimnvqelatirvenlpvkvlllnnqhlgmvmqwedrfy
kanrahtflgdpaqedeifpnmllfaaacgipaarvtkkadlreaiqtmldtpgpylld
vicphqehvlpmipnggtfndvitegdgriky

Certificate of Analysis

Arabidopsis thaliana acetohydroxyacid synthase

Lot#: AtAHAS-0107

Substance Type: Protein

Description: Light yellow, fluffy powder

Date of Initial Analysis: April 12, 2007

Study: BPS-HTC-07-001

Sample Characterization

Description	AHAS Concentration: AtAHAS (g/g sample)	AHAS Concentration: % AtAHAS by weight	AHAS Concentration: AtAHAS as % total protein	Specific Activity: Mean units/mg protein ± standard deviation
APRIL 12, 2007				
AtAHAS-0107	0.524	52.4	90.6	0.790 ± 0.216

Storage Conditions: below 0°C, desiccated

Expiration Date: March 12, 2008

Study Director: Laura Privalle

Issued on: July 16, 2007