Appendix 14

Heat Stability of Arabidopsis Acetohydroxyacid Synthase Present in Test Substance AtAHAS-0107

BASF
The Chemical Company

Plant Science LLC

REPORT # BPS-018-07

Heat Stability of Arabidopsis Acetohydroxyacid Synthase Present in Test Substance AtAHAS-0107

STUDY # BPS-HTC-07-004

EPA GUIDELINE #: N/A

AUTHOR:

[redacted]

STUDY COMPLETED ON: MARCH 11, 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: 11 Mar 2008

Title: Regulatory Affairs Manager

Signature: [redacted]

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BASF Reg. Doc. No: 2007/701361

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.

STUDY DIRECTOR:

[redacted] 11 Mar 08
Date

Senior Manager, Regulatory Science
BASF Plant Science, LLC

SPONSOR REPRESENTATIVE:

[redacted] 11 Mar 2008
Date

Regulatory Affairs Manager
BASF Plant Science, LLC

SUBMITTED BY:

[redacted] 11 Mar 2008
Date

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

BASF
The Chemical Company
Plant Science LLC

BASF Reg. Doc. No: 2007/701361

QUALITY ASSURANCE UNIT STATEMENT

Study Number: BPS-HTC-07-004

Name/Number of Test Substance: AtAHAS-0107

Type of Study: Heat Stability of Test Substance AtAHAS-0107

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
9-17-07	**9-17-07**
9-19-07	**9-19-07**
3-10-08	**3-10-08**

[redacted]
Signature QAU

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ABBREVIATIONS AND DEFINITIONS

AHAS	acetohydroxyacid synthase
AtAHAS	*Arabidopsis thaliana* acetohydroxyacid synthase
FAD	flavin adenine dinucleotide
R272K	arginine at position 272 replaced by lysine
S653N	serine at position 653 replaced by asparagine
TPP	thiamine pyrophosphate

GENERAL INFORMATION

BASF Registration Document Number:	2007/701361
BASF Plant Science Study Number:	BPS-HTC-07-004
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:	none
Study Director:	[redacted] Ph.D.
Analyst:	[redacted] M. S.

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HEAT STABILITY OF ARABIDOPSIS ACETOHYDROXYACID SYNTHASE PRESENT IN TEST SUBSTANCE 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 monitor the heat stability of AtAHAS (test substance AtAHAS-0107) which is encoded by the imidazolinone-tolerant acetohydroxyacid synthase *ahas* gene isolated from *Arabidopsis thaliana (*AtAHAS, containing R272K and S653N mutation) in comparison to that of the wild-type *Arabidopsis thaliana* AHAS (reference substance AtAHAS-0207). The imidazolinone-tolerant acetohydroxyacid synthase (*ahas*) gene from *Arabidopsis thaliana* was transformed into soybean to produce an imidazolinone-tolerant soybean variety. The *Arabidopsis* AHAS protein is a member of the class of AHAS proteins found ubiquitously in plants. 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 with altered herbicide binding properties, but the enzyme has normal biosynthetic function in the soybean plant.

This lot of test substance, AtAHAS-0107, was prepared from a recombinant *Escherichia coli* over-expression system and has been used in an acute oral mouse toxicity study, as well as other studies to confirm the food, feed and environmental safety of the protein. The heat stability of AtAHAS gives an indication of the fate of the protein during processing of the soybean grain. The enzymatic activity of AHAS was monitored during incubation of a solution of the protein preparation at 4, 37, 60, 75 and 100°C over 60 minutes. AHAS activity was stable at both 4 and 37°C over the entire period. Activity at 75 and 100°C decreased to 0% of the original activity within two minutes. These results indicate that like most non-thermostable enzymes and the wild-type reference AHAS, imidazolinone tolerant AHAS activity is rapidly heat inactivated at temperatures above 60°C. Therefore activity of the AHAS protein shows the same sensitivity to heat treatment as other conventional dietary proteins with a history safe consumption in food products. Furthermore, this confirms the results found in previous studies with Clearfield crops containing a similar AHAS protein produced through mutation and breeding rather than via genetic engineering. In these studies, activity of the imidazolinone-tolerant AHAS protein produced in the Clearfield crop was inhibited by heat treatment the same as the control AHAS protein produced in the corresponding conventional crop.

INTRODUCTION

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 BPS-CV127-9. 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.

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 all these characteristics, however, and no one feature is predictive. Therefore, allergenicity assessments consider a group of characteristics and use a weight of evidence approach for the protein safety determination.

The purpose of this study was to monitor the heat stability of AtAHAS (test substance AtAHAS-0107) and compare it to the heat stability of the wild-type AHAS (reference substance AtAHAS-0207). This lot of test substance, AtAHAS-0107, was prepared from a recombinant *Escherichia coli* over-expression system and has been used in an acute oral mouse toxicity study, as well as other studies to confirm the food, feed and environmental safety of the protein. It was previously characterized with respect to purity, immunoreactivity, integrity and concentration, (Privalle, 2007). The heat stability of AtAHAS gives an indication of the fate of the protein during processing of the soybean grain. Specifically, the enzymatic activity of AHAS was monitored during incubation of a solution of the protein preparation at various temperatures over a period

of 60 minutes. Results of this study will be used in combination with results of companion safety studies in a weight of evidence approach to demonstrate that the AtAHAS protein lacks any characteristics of an allergenic protein and is as safe as other AHAS proteins present in conventional crops with a history of safe use in food and feed products.

MATERIALS AND METHODS

Test substance.

Identification	Lot #	Gene Source	Expression System	Physical Description
Acetohydroxyacid Synthase R272K, S653N[1]	AtAHAS-0107	*Arabidopsis thaliana*	*Escherichia coli* strain BL21DE3pLysS	Light yellow proteinaceous powder

AtAHAS was purified by Invitrogen, Inc. and sent as an ammonium sulfate pellet to the Regulatory Science Laboratory at BASF Plant Science LLC, 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. This material has been characterized for purity, integrity, functionality, solubility, and other biochemical parameters (Privalle, 2007).

The test substance was the test system in this study and there was no route of administration.

[1] The *ahas* gene was cloned into the inducible, over-expression pTrcHis A® vector (Invitrogen, Madison, WI) in *E. coli* strain BL21DE3pLysS. AHAS protein as encoded in this vector lacks the 85 N-terminal 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 the mutation at amino acid 653 that results in asparagine replacing serine and a mutation at amino acid 272 that results in lysine replacing arginine. The S653N mutation renders a decreased binding of the herbicide imparting the tolerance. The R272K mutation was found in the Cultivance Soybean Event 127 but has no apparent effect on activity or inhibitor sensitivity.

Reference substance.

Identification	Lot #	Gene Source	Expression System	Physical Description
Acetohydroxyacid Synthase	AtAHAS-0207	*Arabidopsis thaliana*	*Escherichia coli* strain BL21DE3pLysS	Light yellow proteinaceous powder

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 LLC, Research Triangle Park, NC where it was received on June 5, 2007. AtAHAS protein was purified from 3,100 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-0207. This material has been characterized for purity, integrity, functionality, solubility, and other biochemical parameters (Privalle, 2008).

Sample preparation. Stock solutions (1 mg/ml) of both AtAHAS-0107 and AtAHAS-0207 were prepared in 50 mM Tris-HCL, pH 7.0. Diluted samples of these stocks were incubated at 4, 37, 60, 75 and 100°C for 2, 10, 30 and 60 minutes. At sampling time the samples were placed on ice and all AHAS assays were initiated immediately after the 60 minute time point.

Enzymatic activity. The enzymatic activity of AHAS in each sample 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 naphthol. Each sample was assayed in at least triplicate. A 50 μl aliquot was mixed with an equal volume of 2x assay buffer [100 mM Tris-HCl, 10 mM $MgCl_2$, 200 mM sodium pyruvate, with 20 μM FAD and 2 mM thiamine pyrophosphate (TPP), pH 7.0] and incubated at 37°C for 1 hour prior to the addition of 20 μl 5% H_2SO_4, and 15 minute 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-naphthol (1.7% in 4 N NaOH, final concentration) for 15 minutes at 60°C. The absorbance was measured at 530 nm. Each aliquot was assayed in at least triplicate, with the mean corrected value reported as the percent activity remaining (with time 0 at 4°C being equal to 100%).

Statistical analyses. Mean values of triplicate determinations were determined, and compared to that of the time zero time point to calculate the percent activity remaining together with the coefficient of variation.

RESULTS AND DISCUSSION

AHAS activity in solutions of both the test substance AtAHAS-0107 and reference substance AtAHAS-0207 was found to be stable at both 4 and 37°C over one hour of incubation (Table 1, Figure 1). However at temperatures higher than 37°C, AHAS was found to be unstable. At 60°C, activity had decreased to zero by 30 minutes and within two minutes at both 75 and 100°C. These results indicate that like most non-thermostable enzymes, AHAS activity is rapidly inactivated at temperatures above 60°C. Therefore activity of the AHAS protein shows the same sensitivity to heat treatment as the wild-type AHAS as well as other conventional dietary proteins with a history safe consumption in food products. Furthermore, this confirms the results found in previous studies with Clearfield crops containing a similar AHAS protein produced through mutation and breeding rather than via genetic engineering. In these studies, activity of the imidazolinone-tolerant AHAS protein produced in the Clearfield crop was inhibited by heat treatment the same way as the control AHAS protein produced in the corresponding conventional crop.

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. The following protocol change was judged not to have adversely affected the results and conclusions of the study. In addition to evaluating test substance AtAHAS-0107 (containing the imidazolinone tolerant Arabidopsis AHAS R272K, S653N produced in *E. coli*) for its heat stability, the reference substance AtAHAS-0207 (containing the imidazolinone sensitive, wild type Arabidopsis AHAS produced in *E. coli*) was also examined for its heat stability, as a comparator to the test substance.

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.

CRITICAL DATES:

Study initiation date:	17 September 07
Experimental start date:	19 September 07
Experimental end date:	2 January 08

EXAMPLE CALCULATIONS:

$$\% \text{ Activity remaining} = \frac{(\text{Final OD}^{350} \text{ at each time point - background})}{(\text{Final OD}^{350} \text{ at time zero - background})} \times 100$$

REFERENCES

Standard Operating Procedures

BPS 510.09 Enzymatic Assay for Acetohydroxyacid Synthase (AHAS)

Literature References

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

Mazur, B. J., Chui, C.-F., and Smith, J. K. (1987) Isolation and characterization of plant genes coding for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiol. 85:1110-1117.

Privalle, L. (2007) Characterization of test substance Arabidopsis acetohydroxyacid synthase (Lot # AtAHAS-0107). BASF Plant Science Report No. BPS-011-07.

Privalle, L. (2008) Characterization of test substance Arabidopsis acetohydroxyacid synthase (Lot # AtAHAS-0107). BASF Plant Science Report No. BPS-020-07.

Singh, B. K., Stidham, M. A., and Shaner, D. L. (1988) Assay of acetohydroxyacid synthase. Analytical Biochemistry 171:173-179.

Stevenson Paulik, J. (2006) Comparison of inhibition of AtAHAS (S653N) and AtAHAS (S653N R272K) by imidazolinones and sulfonylurea. BASF Plant Science Report No. BPS-004-06.

Table 1. Percent AHAS Activity Remaining as a Function of Time at Different Incubation Temperatures

Results for both the test substance, AtAHAS-0107 (imidazolinone-tolerant AHAS, R272K, S653N) and the reference substance AtAHAS-0207 (wild-type AHAS)

Time (min)	4°C	37°C	60°C	75°C	100°C
	%Activity Remaining ± coefficient of variation				
AtAHAS-0107					
0	100.0 ± 4.7	100.0 ± 4.7	100.0 ± 4.7	100.0 ± 4.7	100.0 ± 1.3
2	95.0 ± 6.7	96.8 ± 1.1	94.4 ± 1.6	0.0 ± 1.7	0.1 ± 2.8
10	95.8 ± 3.2	96.3 ± 5.8	96.9 ± 2.9	0.1 ± 2.8	0.7 ± 9.9
30	99.7 ± 5.2	99.9 ± 1.8	0.0 ± 2.5	0.2 ± 3.8	0.1 ± 1.3
60	100.3 ± 0.9	101.1 ± 6.7	-0.1 ± 1.5	0.5 ± 6.6	0.3 ± 2.5
AtAHAS-0207					
0	100.0 ± 1.9	100.0 ± 1.9	100.0 ± 1.9	100.0 ± 1.9	100.0 ± 2.6
2	95.1 ± 1.7	96.5 ± 2.2	92.9 ± 2.9	0.0 ± 0.6	0.1 ± 0.0
10	96.6 ± 0.1	96.9 ± 2.3	94.0 ± 1.2	-0.6 ± 1.4	0.7 ± 1.6
30	95.7 ± 4.7	104.7 ± 3.1	0.1 ± 0.9	-0.3 ± 0.9	0.1 ± 0.6
60	98.7 ± 6.7	101.7 ± 2.8	0.3 ± 3.3	0.4 ± 4.6	0.3 ± 1.1

Figure 1. Percent AHAS Activity Remaining as a Function of Time at Different Incubation Temperatures. Results for both the test substance, AtAHAS-0107 (Panel A., imidazolinone-tolerant AHAS, R272K, S653N) and the reference substance AtAHAS-0207 (Panel B., wild-type AHAS) are shown.