Appendix 16

AtAHAS-0107 Protein. Acute Oral Toxicity Study in CD®-1-mice

BASF
The Chemical Company

STUDY TITLE

Report

AtAHAS-0107 Protein

Acute Oral Toxicity Study in CD®-1-mice

AUTHOR(S)

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STUDY COMPLETED ON

January 22, 2008

PERFORMING LABORATORY

Experimental Toxicology and Ecology
BASF Aktiengesellschaft
67056 Ludwigshafen, FRG

LABORATORY PROJECT IDENTIFICATION

Project No.: 99C0295/07011

SPONSOR

BASF Plant Science L.L.C.
26 Davis Drive – Research Triangle Park
27709, North Carolina / USA

PART I OF III (REPORT SECTION AND SUMMARY TABLES)

The following statement is for US EPA submissions only:

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 sec.10(d)(1)(A), (B) or (C). This claim specifically supersedes any claim of implication of confidentiality contained in this document.

Company:

Company Agent: **Title:**

Signature: **Date:**

GLP COMPLIANCE STATEMENT

This study was conducted in accordance with the OECD Principles of Good Laboratory Practice and the GLP Principles of the German "Chemikaliengesetz" (Chemicals Act), which meet the United States Environmental Protection Agency Good Laboratory Practice Standards [40 CFR Part 160 (FIFRA) and Part 792 (TSCA)], with the exception that recognized differences exist between the GLP Principles/Standards of OECD and the Principles/Standards of FIFRA and TSCA.

Study Director [redacted] ..... 22 Jan 2008 .....
Date

To be filled for USA EPA submission only:

Sponsor ....
Date

Submitter ....
Date

The following statement is for US EPA submissions only:

FLAGGING CRITERIA

The flagging criteria of 40 CFR 158.34 are not applicable for this study type.

SIGNATURE PAGE

Study Director: [redacted]

Veterinarian Supervisor: [redacted]

Pathology: [redacted]

Management: [redacted]

CONTRIBUTORS TO THE STUDY / SUPERVISORY LABORATORY PERSONNEL

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Quality Assurance Unit (QAU):	[redacted]
Coordination QAU:	[redacted]
Mechanistic Laboratory:	[redacted]
Pathology:	[redacted]

STATEMENT OF THE QUALITY ASSURANCE UNIT

The Quality Assurance Unit (QAU) inspected the study and reported any inspection results to the Study Director and to Management.

The final report reflects the raw data.

Phase of study	Date of inspection (mm-dd-yyyy)	Reported to Study Director and to Management (mm-dd-yyyy)
Study Plan:	05-14-2007	05-14-2007
Conduct of study:	05-24-2007 06-01-2007	05-24-2007 06-01-2007
Report:	12-03-2007	12-03-2007

Ludwigshafen, Jan. 22, 2008 .....[redacted].....

GLP CERTIFICATE (FROM THE COMPETENT AUTHORITY)

RheinlandPfalz

Gute Laborpraxis / Good Laboratory Practice

GLP-Bescheinigung / Statement of GLP Compliance

(gem. /according to § 19 Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung und der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 88/320/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 88/320/EEC at::

Prüfeinrichtung / Test facility

BASF Aktiengesellschaft
Experimentelle Toxikologie und Ökologie
67056 Ludwigshafen

BASF Aktiengesellschaft
Experimental Toxicology and Ecology
67056 Ludwigshafen, Germany

Prüfung nach Kategorien / Areas of Expertise
(gem. / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1,2,3,4,5,8,9
Kat. 9 – Biochemische und pathologische Untersuchungen zu Wirkmechanismen /
Biochemical and pathological examinations concerning mode of action

Datum der Inspektion / Date of Inspection
(Tag.Monat Jahr / day.month year)
08.06.2005 und 25. bis 27.07.2005

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Unterschrift, Datum / Signature, Date

21/10/2005

Dr.-Ing. Karl-Heinz Rother - Präsident -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht
Kaiser-Friedrich-Straße 7
55116 Mainz

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Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht

CONTENTS OF PART I (REPORT SECTION AND SUMMARY TABLES)

Part A:
CLINICAL EXAMINATIONS
(MEAN VALUES AND SUMMARY TABLES)

Part B:
PATHOLOGY (MEAN VALUES AND SUMMARY TABLES)

CONTENTS OF PART II (TABLES SECTION; INDIVIDUAL VALUES)

CONTENTS OF PART III (SUPPLEMENT)

THIS REPORT CONSISTS OF PART I, II AND III.

1. SUMMARY

1.1. METHODS

AtAHAS-0107 was administered by gavage to groups of 5 male and 5 female CD®-1 mice at a dose of 5000 mg/kg body weight/day (*corresponding to 2620 mg full-length AtAHAS/kg bw).* The dose was given in two equal doses of 2500 mg/kg body weight in an interval of approximately one hour apart. Vehicle control animals received drinking water containing 0.5% carboxymethylcellulose by gavage. After the test substance administration, the animals were maintained over a post observation period of 14 days.

1.2. OBSERVATIONS

The animals were examined for any abnormal clinical signs before the first test substance administration and at regular intervals from 15 minutes to 6 hours thereafter. Subsequently, the animals were observed daily for any abnormal clinical signs.

Body weights were determined on the day of the test substance administration (day 0) and on study days 7 and 14.

The animals were sacrificed at the end of the study under anesthesia and assessed by gross pathology. Selected organ weights of all sacrificed animals were determined.

1.3. RESULTS

1.3.1. Analytics

The analytical determinations verified the concentration of the dosing solution.

1.3.2. Findings

As no animal died during the study period due to the test substance, a median lethal dose (LD_{50}) was not achieved. Thus, the LD_{50} can be considered as being higher than 5000 mg/kg bw (or higher than 2620 mg AtAHAS/kg bw) for male and female CD®-1 mice. In addition, no test substance-related findings were noted. As such, the no observed effect level (NOEL) was the limit dose of 5000 mg/kg bw (2620 mg AtAHAS/kg bw) for male and female CD-1 mice.

2. INTRODUCTION

2.1. OBJECTIVES

The aim of the study was to examine the potential toxic hazard after a single oral administration of AtAHAS-0107 protein to male and female CD®-1-mice.

2.2. SELECTION OF DOSES

By request of the sponsor a dose level of 5000 mg/kg bw/day was selected.

The oral route was selected since this route has been proven to be suitable for detecting toxicological hazards and corresponds to the anticipated route of exposure

2.3. TEST GUIDELINES

No test guideline exists for this type of study. As a reference while writing the study protocol, the following guidelines have been considered:

- U.S. EPA Health Effects Test Guidelines. OPPTS 870.1100; Acute Oral Toxicity (August 1998)
- OECD Guideline for Testing of Chemicals; Method No. 420: Acute Oral Toxicity-Fixed Dose Method; July 17, 1992

2.4. STUDY DATES

In the following table, the relevant intervals for certain study phases are given:

Date	Phase of study / examination	Study day
May 08, 2007	Experimental starting date: Arrival of the animals and start of acclimatization period	-9
May 16, 2007	Randomization	-1
May 17, 2007	Test substance administration[1)] (divided into two equal doses of 2,500 mg/kg bw and administered 1 hour apart)	0
May 31, 2007	Last weighing	14
June 01, 2007	Necropsy[2)]	15
November 21, 2007	Experimental completion date; Draft report to QAU	

[1)] = Fasting period (withdrawal of food) of about 16 hours before first dosing

[2)] = Fasting period (withdrawal of food) of about 16 to 20 hours before necropsy

2.5. RETENTION OF RECORDS

GLP-relevant records and materials are stored at BASF Aktiengesellschaft for at least the period of time specified in the GLP principles. Details concerning responsibilities or locations of archiving can be seen from the respective SOPs and from the raw data.

2.6. ANIMAL WELFARE

This study was performed in an AAALAC-approved laboratory in accordance with the German Animal Welfare Act and the European Council Directive 86/609/EEC.

3. MATERIAL AND METHODS

The analytical examinations of the test substance/test substance preparation were conducted by BASF Plant Science L.L.C. - Research Triangle Park, North Carolina, NC 27709 (USA).

3.1. TEST SUBSTANCE

Name of test substance:	AtAHAS-0107 Protein
Synonym:	AtAHAS-0107, Acetohydroxyacid synthase from Arabidopsis thaliana: R272K
Test substance No.:	07/0295-1
Batch-No.:	0107
CAS No.:	Not assigned
Content of active ingredient:	52.4%
Homogeneity:	Given
Stability under storage conditions:	The stability under transit and storage conditions was confirmed by reanalysis

ADDITIONAL TEST SUBSTANCE INFORMATION

Date of production:	March 12, 2007
Expiration date:	March 12, 2008
Aggregate state/ Appearance:	Light yellow, fluffy powder
Storage conditions:	Freezer, desiccated

3.2. TEST ANIMALS

3.2.1. Species and strain

Test species and strain:	Mice, Crl:CD1(ICR)
Supplier:	Charles River Laboratories, Research Models and Services, Germany GmbH
Sex:	Male and female
Age when supplied:	10 weeks
Age on study day 0:	11 weeks
Reason for the selection:	The mouse is a frequently used laboratory animal, and there is comprehensive experience with this animal species. Moreover, the mouse has been proposed as a suitable animal species in the literature for this type of study
Animal identification:	Ear tattoo (animal number)
Randomization:	The mice were randomized and allocated to the dose groups before administration (on the basis of their weight).

3.3. HOUSING AND DIET

The mice were housed singly in Polycarbonate cages, type M II with wire cover from Becker & Co., Castrop-Rauxel, Germany (floor area about 350 cm²). Bedding was type “Lignocel FS14 fibres” dust free bedding supplied by SSNIFF, Soest, Germany. The animals were housed in a fully air-conditioned room. Central air-conditioning guaranteed a range of 20 - 24°C for temperature and of 30 - 70% for relative humidity. The day/night rhythm was 12 hours (12 hours light from 06.00 a.m. - 06.00 p.m., 12 hours dark from 06.00 p.m. - 06.00 a.m.). Deviations from these ranges did not occur. The animal room was completely disinfected prior to the study using a disinfector ("AUTEX", fully automatic, formalin-ammonia-based terminal disinfector). The floor and the walls were cleaned once a week. The cleansing liquid used was water containing about 0.5% Incidin Extra N (supplied by Henkel, Düsseldorf, FRG).

The food used was ground Kliba maintenance diet mouse/rat “GLP”, meal, supplied by Provimi Kliba SA, Kaiseraugst, Switzerland. Food and drinking water (from water bottles) were available *ad libitum*.

3.4. TEST GROUPS AND DOSES

Dose group	Dose level (mg/kg body weight)	Number of animals	Animal No. males	Animal No. females
0	0	5	1 – 5	11 – 15
1	5000[1)]	5	6 –10	16 – 20

[1)] 5000 mg/kg AtAHAS-0107 (total protein approximately 58%; full length AtAHAS approximately 52.4%), corresponding to 2620 mg AtAHAS/kg.

3.5. TEST SUBSTANCE PREPARATIONS AND PREPARATION FREQUENCY

The test substance was applied as a suspension. The administration volume was 20 ml/kg body weight.
To prepare the suspension, the appropriate amount of test substance was weighed and brought up to the desired volume with the vehicle (drinking water containing 0.5% carboxymethyl-cellulose). Then the preparation was gently sonicated and homogenized using a high speed homogenizer and a magnetic stirrer.
During application the test substance preparation was kept homogeneous by using a magnetic stirrer.
The test substance preparation was prepared once before the first administration.
The test substance was administered into two equal oral doses (2500 mg/kg bw) approximately 1 hour apart.

3.6. ANALYSES

3.6.1. Analyses of the test substance preparation

On day 0, after administration, samples for concentration control analysis were drawn and stored frozen before being returned to the sponsor.

3.6.2. Food analyses

The supplier assayed the food used in the study for chemical and microbiological contaminants.

3.6.3. Drinking water analyses

The drinking water is regularly assayed for chemical contaminants by the municipal authorities of Frankenthal and the Technical Services of BASF Aktiengesellschaft as well as for the presence of microorganisms by a contract laboratory.

3.6.4. Bedding analyses

The bedding is regularly assayed for contaminants (chlorinated hydrocarbons and heavy metals).

3.7. EXPERIMENTAL PROCEDURE

On the day of arrival the animals were subjected to an acclimatization period during which they received ground diet and drinking water *ad libitum*. Prior to the day of the test substance administration, the animals were distributed according to weight among the individual test groups, separated by sex. The list of randomization instructions was compiled with a computer. The weight variation of the animals used did not exceed 20 percent of the mean weight of each sex.

The mice were maintained without food for about 16 hours before the first dosing.

The animals were maintained for a 14-day post-treatment period for observation. During this period, control and test animals received ground diet and drinking water *ad libitum*. All surviving animals were sacrificed under anesthesia.

3.7.1. Mortality

A check for moribund and dead animals was made twice daily on working days and once daily on Saturdays, Sundays and public holidays.

3.7.2. Clinical observations

3.7.3. Clinical signs

The animals were examined for any abnormal clinical signs before the test substance administration. Following the first test substance administration, the animals were examined after 15 and 30 minutes as well as after 1, 2, 4 and 6 hours.

Thereafter, the animals were observed daily for any abnormal clinical signs. For observation, the animals were removed from their cages and placed in a standard arena (50 x 37.5 x 25 cm). The following parameters were assessed:

1. Abnormal behavior in handling
2. Fur
3. Skin
4. Posture
5. Salivation
6. Respiration
7. Activity/arousal level
8. Tremors
9. Convulsions
10. Abnormal movements
11. Gait abnormalities
12. Lacrimation
13. Palpebral closure
14. Exophthalmus
15. Assessment of the feces discharged during the examination (appearance/ consistency)
16. Assessment of the urine discharged during the study
17. Pupil size

3.7.4. Food consumption

Food consumption was not determined.

3.7.5. Water consumption

Water consumption was observed daily by visual inspection of the water bottles for any overt changes in volume.

3.7.6. Body weight data

Body weights were determined on study day -1 in order to randomize the animals. During the study the body weights were determined on study days 0, 7 and 14.

3.7.7. Statistics of clinical examinations

Means and standard deviations of each test group were calculated for several parameters (see tables). Further statistical analyses were performed according to the following table:

Parameters	Statistical test	Markers in the tables	References
Body weight	A comparison of the dose group with the control group was performed using the Welch t-test (two sided) for the hypothesis of equal means	* for $p \leq 0.05$ ** for $p \leq 0.01$	Welch B.L. (1947): The generalization of Student's problem when several different population valiances are involved. Biometrika, 34, 28-35

3.8. PATHOLOGY

The animals were sacrificed by decapitation under anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology.

3.8.1. Organ weights

Weight assessment was carried out on all animals sacrificed at scheduled dates. The following weights were determined:

1. Anesthetized animals
2. Liver
3. Kidneys
4. Spleen
5. Brain
6. Heart

3.8.2. Organ/Tissue fixation

The following organs/tissues were fixed in 4% formaldehyde solution:

1. All gross lesions
2. Brain
3. Pituitary
4. Thyroids
5. Parathyroids
6. Oesophagus
7. Salivary glands (mandibular and sublingual glands)
8. Trachea
9. Lungs
10. Pharynx
11. Larynx
12. Nose (nasal cavity)
13. Thymus
14. Aorta
15. Heart
16. Liver
17. Gall bladder
18. Pancreas
19. Spleen
20. Kidneys
21. Adrenals
22. Testes
23. Ovaries
24. Oviducts, uterus and vagina

25. Epididymides, prostate and seminal vesicle
26. Stomach (forestomach and glandular stomach)
27. Duodenum, jejunum and ileum
28. Cecum, colon and rectum
29. Urinary bladder
30. Lymph nodes (mesenteric and axillary lymph nodes)
31. Sciatic nerve
32. Bone marrow (femur)
33. Spinal cord (cervical, thoracic and lumbar cords)
34. Eyes
35. Extraorbital lacrimal glands
36. Skin
37. Female mammary gland
38. Sternum with marrow
39. Femur with knee joint
40. Skeletal muscle

3.9. STATISTICS OF PATHOLOGY

Parameters	Statistical test	Markers in the tables	References
Weight parameters	A pair wise comparison of the dose group with the control group was performed using the WILCOXON test (two-sided) for the equal medians.	* for $p \leq 0.05$ ** for $p \leq 0.01$	SIEGEL, S. (1956): Non-parametric statistic for the behavioural sciences. McGraw-Hill New York.

4. RESULTS

Throughout the chapter "results and assessment of findings", the term "significant" implies that the inter-group differences have attained statistical significance ($p \leq 0.05$) when compared with the control group.

4.1. ANALYSES

4.1.1. Analyses of the test substance preparation

The analytical results confirm that the AtAHAS protein concentration was within the expected range. (details see Supplement)

4.1.2. Food analyses

On the basis of duration of use and the analytical findings with respect to chemical and microbiological contaminants the diet was found to be suitable. Fed. Reg. Vol. 44, No. 91 of May 9, 1979, p. 27354 (EPA), served as a guideline for maximum tolerable chemical contaminants. The number of microorganisms did not exceed $1*10^5$/g food. Individual results can be found in the archives of the Experimental Toxicology and Ecology of BASF Aktiengesellschaft.

4.1.3. Drinking water analyses

On the basis of the analytical findings the drinking water was found to be suitable. German Drinking Water Regulation (Trinkwasserverordnung) served as a guideline for maximum tolerable contaminants. Individual results can be found in the archives of the Experimental Toxicology and Ecology of BASF Aktiengesellschaft.

4.1.4. Bedding analyses

On the basis of the analytical findings the bedding was found to be suitable. Levels given in Lab. Animal, Nov. – Dec. 1979, pp. 24 - 33, served as a guideline for maximum tolerable contaminants. Individual results are to be found in the archives of the Experimental Toxicology and Ecology of BASF Aktiengesellschaft.

4.2. CLINICAL EXAMINATIONS

Summary tables of the results are given in the Appendix of PART I; individual values are given in Part A of PART II.

4.2.1. Mortality

As a consequence of a misgavage during the second administration, followed by reduced general state, one male animal (no. 7) was sacrificed under anesthesia on study day 8 in a moribund state.

4.2.2. Clinical examinations

(Tables IA-1 - IA-3)

With the exception of animal 7, which showed reduced general state as a consequence of being misgavaged, no other animals showed clinical signs of toxicity.

4.2.3. Water consumption

No overt changes in water consumption were observed.

4.2.4. Body weight data

(Figures 1 – 2; Tables IA-4 – IA-5)

No significant differences in body weight of animals treated with the test substance, when compared to the control group, were observed.

4.3. PATHOLOGY

Summary tables of the results are to be found in the Appendix of Volume I; individual tables are to be found in Part B of Volume II.

4.3.1. Weight parameters

4.3.1.1. Absolute weights

(Table IB-1 – 2)

There were no relevant differences in mean absolute weight parameters between treatment and control groups. The absolute weights are also considered to be within the normal biological range of test animals of that age.

4.3.1.2. Relative weights

(Table IB-3 – 4)

Compared to controls, treated male animals had an increase of 6% in relative liver weights. This finding is considered not to be treatment related, since absolute liver weights weren't changed compared to the control animals and no effect was seen in female animals.
For other organs, there were no relevant differences in mean relative weight parameters between treatment and control groups.
The relative weights are also regarded to be within the normal biological range of test animals of that age.

4.3.1.3. Gross lesions

(Table IB-5)

No substance related gross lesions were observed.

As a result of the misgavage of animal no. 7, (broncho)pneumonia was seen at gross pathology.

5. DISCUSSION

AtAHAS-0107 was administered by gavage to groups of 5 male and 5 female CD®-1 mice at a dose of 5000 mg/kg body weight/day.
After the test substance administration, the animals were maintained over a post observation period of 14 days.
There were no differences in clinical parameters and gross pathology that were related to the test substance administration.

6. CONCLUSION

As no animal died during the study period due to the test substance, a median lethal dose (LD_{50}) was not achieved. Thus, the LD_{50} can be considered as being higher than 5000 mg/kg bw (or higher than 2620 mg AtAHAS/kg bw) for male and female CD®-1 mice. In addition, no test substance-related findings were noted. As such, the no observed effect level (NOEL) was the limit dose of 5000 mg/kg bw (2620 mg AtAHAS/kg bw) for male and female CD-1 mice.

7. APPENDIX

7.1. LIST OF ABBREVIATIONS USED IN TABLES IA (CLINICAL EXAMINATIONS)

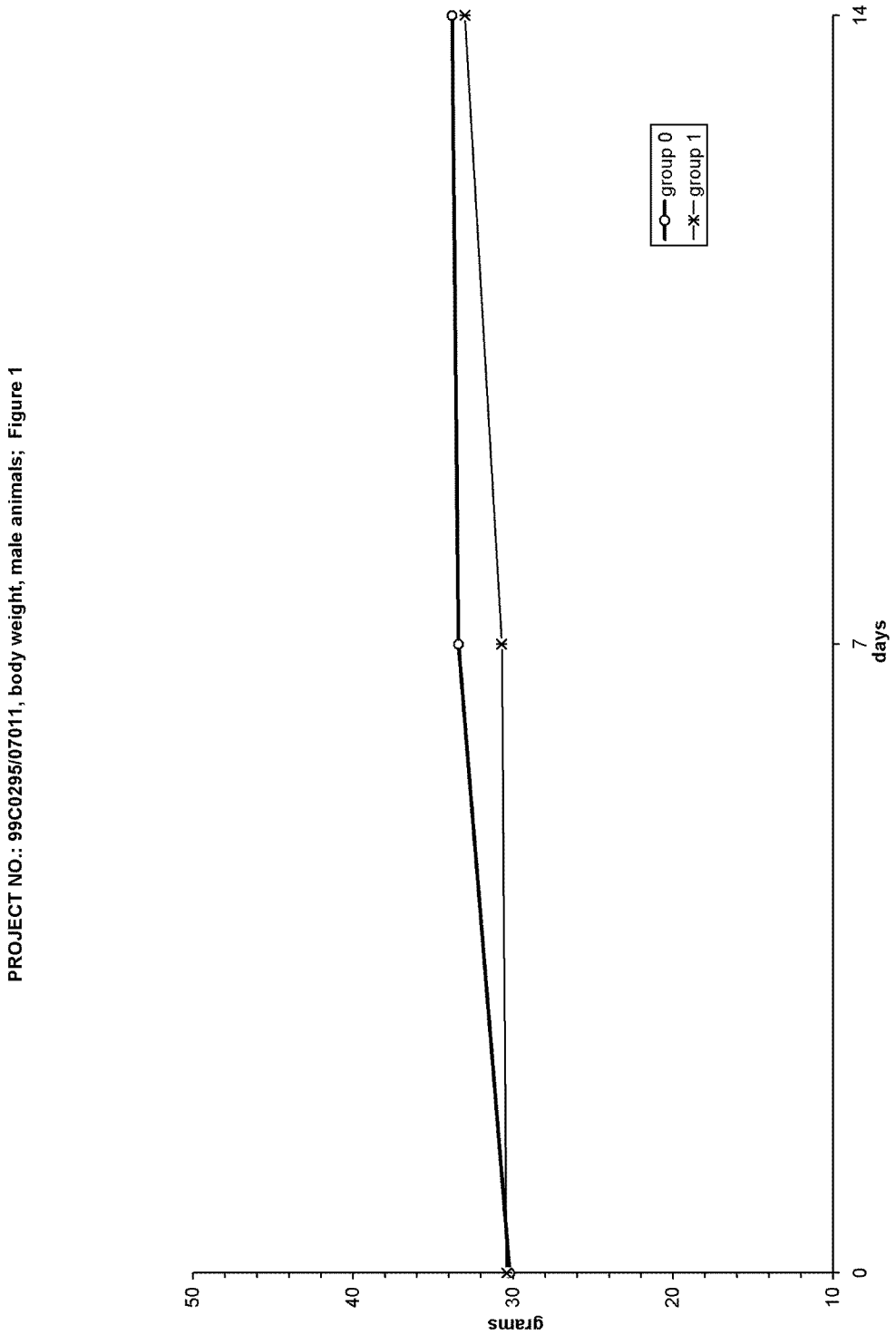
SD	=	standard deviation
N	=	number of animals for determining M and SD
%dev	=	per cent deviations
G, g	=	weight in gram
BW, bw	=	body weight

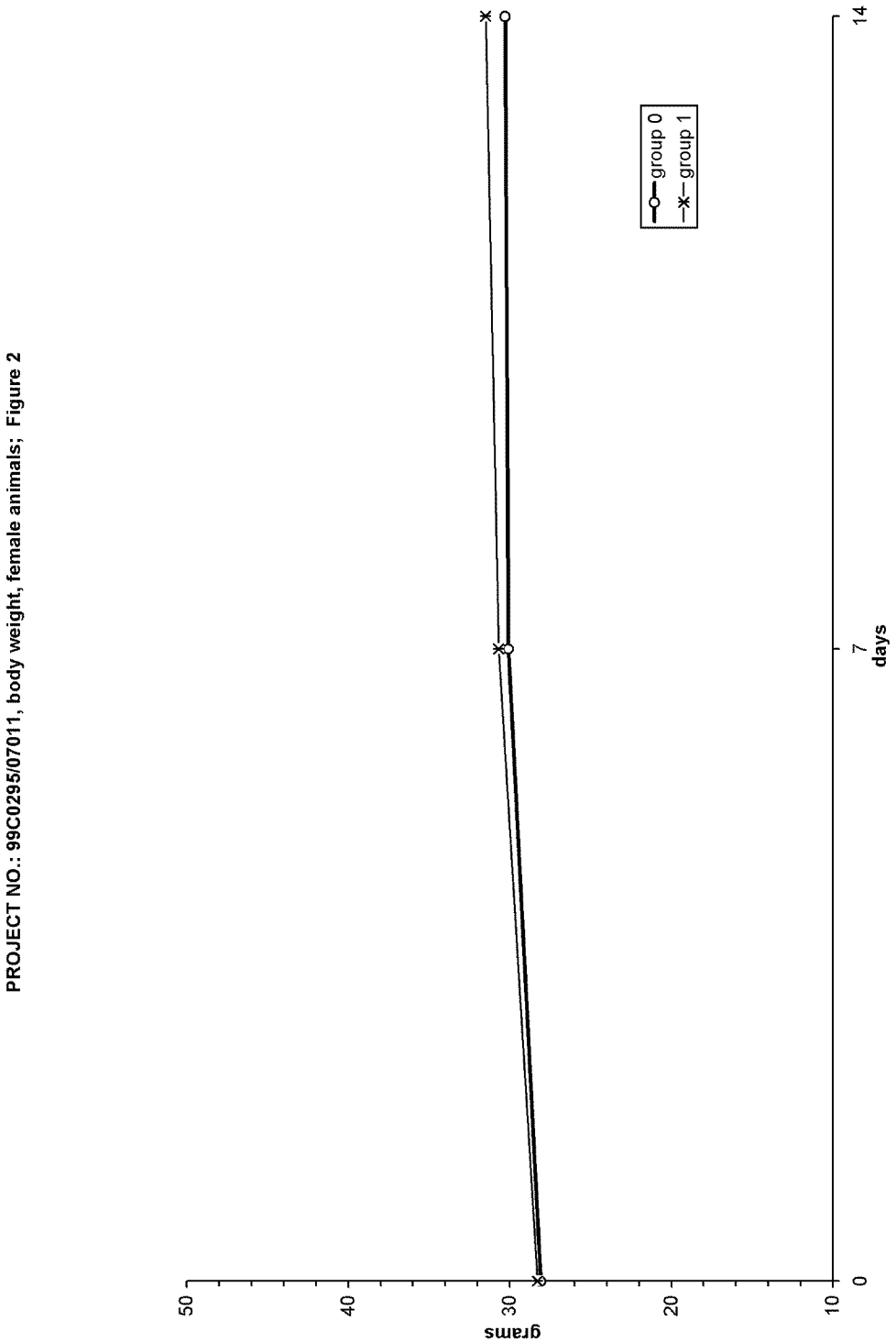
7.2. LIST OF ABBREVIATIONS USED IN TABLES IB (PATHOLOGY)

F	=	female animals
F1	=	final sacrifice groups (main groups)
g	=	weight determination in grams
M	=	male animals (under sex); mean value (on weight level)
mg	=	weight determination in milligrams
mg/kg BW	=	milligram per kilogram body weight under dose level
n	=	number of values measured for the determination of mean value and standard deviation
NAD	=	number of animals without gross lesions
SD	=	standard deviation
%	=	percentage related to the reference weight in relative organ weight calculations

Codes for the status at necropsy:

1	=	planned sacrifice
2	=	sacrificed in a moribund state
3	=	spontaneous death


PROJECT NO.: 99C0295/07011, body weight, male animals; Figure 1
group 0
group 1
grams
50
40
30
20
10
0
7
14
days



OBSERVATIONS REPORT - INCIDENCE

Study: 99C0295/07011
Sex: Male

Treatment Group	0	1
Dose	group 0	group 1
Animal Count	5	5
Gavage error		
Incidence		1 (20%)
Observed		1
Mean onset (Days)		0
TOTALS		
Incidence	0 (0%)	1 (20%)
Observed	0	1
Mean onset (Days)		0
General observation		
Nothing abnormal detected		
Incidence	5 (100%)	5 (100%)
Observed	110	91
Mean onset (Days)	0	0
TOTALS		
Incidence	5 (100%)	5 (100%)
Observed	110	91
Mean onset (Days)	0	0
Reduced general condition		
slight		
Incidence		1 (20%)
Observed		12
Mean onset (Days)		0
TOTALS		
Incidence	0 (0%)	1 (20%)
Observed	0	12
Mean onset (Days)		0
Sacrificed moribund		
Incidence		1 (20%)
Observed		1
Mean onset (Days)		8
TOTALS		
Incidence	0 (0%)	1 (20%)
Observed	0	1
Mean onset (Days)		8

BASF - DATATOX-F1 R14

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OBSERVATIONS REPORT - INCIDENCE

Study: 99C0295/07011
Sex: Male

Treatment Group	0	1
Dose	group 0	group 1
Animal Count	5	5
Sacrificed scheduled		
Incidence	5 (100%)	4 (80%)
Observed	5	4
Mean onset (Days)	15	15
TOTALS		
Incidence	5 (100%)	4 (80%)
Observed	5	4
Mean onset (Days)	15	15

OBSERVATIONS REPORT - INCIDENCE

Study: 99C0295/07011
Sex: Female

Treatment Group	0	1
Dose	group 0	group 1
Animal Count	5	5
General observation		
Nothing abnormal detected		
Incidence	5 (100%)	5 (100%)
Observed	110	110
Mean onset (Days)	0	0
TOTALS		
Incidence	5 (100%)	5 (100%)
Observed	110	110
Mean onset (Days)	0	0
Sacrificed scheduled		
Incidence	5 (100%)	5 (100%)
Observed	5	5
Mean onset (Days)	15	15
TOTALS		
Incidence	5 (100%)	5 (100%)
Observed	5	5
Mean onset (Days)	15	15

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BODY WEIGHT

Study: 99C0295/07011

	Body Weight g Day 0 t	Body Weight g Day 7 t	Body Weight g Day 14 t
Male, group 0			
Mean	30.2	33.4	33.8
SD	1.2	3.0	2.4
N	5	5	5
%dev			
Male, group 1			
Mean	30.4	30.7	33.0
SD	1.4	4.2	1.2
N	5	5	4
%dev	0.7	-8.2	-2.6

Key: t = Welch t-test, Two-sided, * p <= 0.050, ** p <= 0.010
Experimental Unit = Animal

BASF - DATATOX-F1 R14

BODY WEIGHT

Study: 99C0295/07011

	Body Weight g Day 0 t	Body Weight g Day 7 t	Body Weight g Day 14 t
Female, group 0			
Mean	28.1	30.1	30.3
SD	0.7	1.7	2.2
N	5	5	5
%dev			
Female, group 1			
Mean	28.3	30.7	31.5
SD	0.5	1.3	1.3
N	5	5	5
%dev	0.6	2.1	3.8

Key: t = Welch t-test, Two-sided, * p <= 0.050, ** p <= 0.010
Experimental Unit = Animal

BASF PATHOLOGY REPORT IB- 1/5
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

ABSOLUTE WEIGHTS - MEAN VALUES (MALE)

Sacrifice			F1	
Sex			M	
Group			0	1
Terminal body weight	g	M	29.4	28.05
		SD	2.527	1.303
		n	5	4
Brain	mg	M	494.8	518.5
		SD	22.038	21.703
		n	5	4
Heart	mg	M	147.8	146.5
		SD	10.986	10.408
		n	5	4
Kidneys	mg	M	468.8	436.5
		SD	50.351	40.146
		n	5	4
Liver	mg	M	1122.0	1139.5
		SD	78.16	34.799
		n	5	4
Spleen	mg	M	60.2	61.5
		SD	16.423	3.317
		n	5	4

*: P <= 0.05, **: P <= 0.01
Wilcoxon test, two sided

BASF PATHOLOGY REPORT IB- 2/5
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

ABSOLUTE WEIGHTS - MEAN VALUES (FEMALE)

Sacrifice			F1	
Sex			F	
Group			0	1
Terminal body weight	g	M	26.52	27.52
		SD	2.017	1.192
		n	5	5
Brain	mg	M	503.8	533.2
		SD	53.681	13.405
		n	5	5
Heart	mg	M	136.0	131.8
		SD	7.583	11.541
		n	5	5
Kidneys	mg	M	341.0	390.0
		SD	49.885	36.173
		n	5	5
Liver	mg	M	1093.8	1097.4
		SD	131.286	41.584
		n	5	5
Spleen	mg	M	88.0	82.8
		SD	15.232	16.724
		n	5	5

*: P <= 0.05, **: P <= 0.01
Wilcoxon test, two sided

BASF PATHOLOGY REPORT IB- 3/5
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

RELATIVE WEIGHTS - MEAN VALUES (MALE)

Sacrifice			F1	
Sex			M	
Group			0	1
Terminal body weight	%	M	100.0	100.0
		n	5	4
Brain	%	M	1.695	1.849
		SD	0.182	0.059
		n	5	4
Heart	%	M	0.506	0.522
		SD	0.059	0.015
		n	5	4
Kidneys	%	M	1.602	1.557
		SD	0.206	0.141
		n	5	4
Liver	%	M	3.821	4.065*
		SD	0.088	0.094
		n	5	4
Spleen	%	M	0.204	0.219
		SD	0.046	0.012
		n	5	4

*: P <= 0.05, **: P <= 0.01
Wilcoxon test, two sided

RELATIVE WEIGHTS - MEAN VALUES (FEMALE)

Sacrifice			F1	
Sex			F	
Group			0	1
Terminal body weight	%	M	100.0	100.0
		n	5	5
Brain	%	M	1.906	1.939
		SD	0.241	0.052
		n	5	5
Heart	%	M	0.514	0.479
		SD	0.035	0.036
		n	5	5
Kidneys	%	M	1.285	1.416
		SD	0.15	0.094
		n	5	5
Liver	%	M	4.116	3.991
		SD	0.226	0.154
		n	5	5
Spleen	%	M	0.331	0.301
		SD	0.037	0.063
		n	5	5

*: P <= 0.05, **: P <= 0.01
Wilcoxon test, two sided

BASF PATHOLOGY REPORT IB- 5/5
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

INCIDENCE OF GROSS LESIONS

Sacrifice	F1			
Sex	M		F	
Group	0	1	0	1
Animals in selected group	5	5	5	5
No abnormalities	3	3	4	4
Glandular stomach	.	.	.	.
Erosion/ulcer	1	1	1	.
Lungs	.	.	.	.
(Broncho)pneumonia	.	1	.	.
Ovaries	.	.	.	.
Paraovarian cyst(s)	.	.	.	1
Preputial glands	.	.	.	.
Cyst	1	.	.	.

STUDY TITLE

Report

AtAHAS-0107 Protein

Acute Oral Toxicity Study in CD®-1-mice

PERFORMING LABORATORY

Experimental Toxicology and Ecology
BASF Aktiengesellschaft
67056 Ludwigshafen/Rhein, FRG

LABORATORY PROJECT IDENTIFICATION

Project No.: 99C0295/07011

PART II OF III
(TABLES SECTION, INDIVIDUAL VALUES)

CONTENTS OF PART II (TABLES SECTION; INDIVIDUAL VALUES)

LIST OF ABBREVIATIONS USED IN TABLES IIA (CLINICAL EXAMINATIONS)

BW, bw	=	body weight
G, g	=	weight in gram
M	=	male
F	=	female
BT	=	before treatment

LIST OF ABBREVIATIONS USED IN TABLES IIB (PATHOLOGY)

F	=	female animals
F1	=	final sacrifice groups (main groups)
g	=	weight determination in grams
M	=	male animals (under sex); mean value (on weight level)
mg	=	weight determination in milligrams
mg/kg BW	=	milligram per kilogram body weight under dose level
n	=	number of values measured for the determination of mean value and standard deviation
NAD	=	number of animals without gross lesions
SD	=	standard deviation
%	=	percentage related to the reference weight in relative organ weight calculations

Codes for the status at necropsy:

1	=	planned sacrifice
2	=	sacrificed in a moribund state
3	=	spontaneous death

OBSERVATIONS REPORT - SUMMARY

Study: 99C0295/07011

Sex	Group	Animal	Observation	Days
M	0	1	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
M	0	2	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
M	0	3	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
M	0	4	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
M	0	5	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
M	1	6	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
M	1	7	Gavage error	0(1h)
			General observation, Nothing abnormal detected	0(BT)-0(30')
			Reduced general condition, slight	0(1h)-8
			Sacrificed moribund	8
M	1	8	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
M	1	9	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
M	1	10	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15

OBSERVATIONS REPORT - SUMMARY

Study: 99C0295/07011

Sex	Group	Animal	Observation	Days
F	0	11	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
F	0	12	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
F	0	13	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
F	0	14	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
F	0	15	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
F	1	16	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
F	1	17	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
F	1	18	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
F	1	19	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15
F	1	20	General observation, Nothing abnormal detected	0(BT)-15
			Sacrificed scheduled	15

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BASF - DATATOX-F1 R14

BODY WEIGHT

Study: 99C0295/07011

	Body Weight g Day 0	Body Weight g Day 7	Body Weight g Day 14
Male, group 0			
1	29.0	31.1	31.5
2	31.6	36.2	36.1
3	30.1	32.0	33.4
4	29.0	30.7	31.7
5	31.3	37.1	36.5
Male, group 1			
6	30.0	33.5	34.2
7	32.1	23.6	
8	30.5	32.0	32.2
9	28.3	30.5	31.6
10	31.1	33.8	33.8

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Table : IIA
Page : 4

BASF - DATATOX-F1 R14

BODY WEIGHT

Study: 99C0295/07011

	Body Weight g Day 0	Body Weight g Day 7	Body Weight g Day 14
Female, group 0			
11	28.4	32.0	33.4
12	27.0	28.4	28.5
13	28.7	28.5	27.8
14	27.7	30.0	31.2
15	28.7	31.6	30.7
Female, group 1			
16	28.2	30.9	30.9
17	27.5	28.5	29.7
18	28.2	31.5	33.2
19	28.6	30.9	32.0
20	28.9	31.8	31.6

BASF PATHOLOGY REPORT IIB- 1/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

ABSOLUTE WEIGHTS - INDIVIDUAL VALUES

Sacrifice F1
Sex M
Group 0

	Term. body weight g	Brain mg	Heart mg	Kidneys mg	Liver mg	Spleen mg
M	29.4	494.8	147.8	468.8	1122.0	60.2
SD	2.527	22.038	10.986	50.351	78.16	16.423
n	5	5	5	5	5	5
1	27.2	473.0	147.0	413.0	1065.0	50.0
2	31.6	500.0	158.0	501.0	1202.0	88.0
3	28.3	506.0	159.0	538.0	1063.0	61.0
4	27.3	523.0	142.0	455.0	1067.0	47.0
5	32.6	472.0	133.0	437.0	1213.0	55.0

BASF PATHOLOGY REPORT IIB- 2/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

ABSOLUTE WEIGHTS - INDIVIDUAL VALUES

Sacrifice F1
Sex M
Group 1

	Term. body weight g	Brain mg	Heart mg	Kidneys mg	Liver mg	Spleen mg
M	28.05	518.5	146.5	436.5	1139.5	61.5
SD	1.303	21.703	10.408	40.146	34.799	3.317
n	4	4	4	4	4	4
6	29.2	521.0	156.0	438.0	1169.0	59.0
8	27.3	526.0	137.0	380.0	1142.0	62.0
9	26.6	488.0	138.0	456.0	1090.0	59.0
10	29.1	539.0	155.0	472.0	1157.0	66.0

BASF PATHOLOGY REPORT IIB- 3/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

ABSOLUTE WEIGHTS - INDIVIDUAL VALUES

Sacrifice F1
Sex F
Group 0

	Term. body weight g	Brain mg	Heart mg	Kidneys mg	Liver mg	Spleen mg
M	26.52	503.8	136.0	341.0	1093.8	88.0
SD	2.017	53.681	7.583	49.885	131.286	15.232
n	5	5	5	5	5	5
11	29.2	527.0	142.0	406.0	1308.0	114.0
12	24.8	420.0	133.0	271.0	1040.0	81.0
13	24.3	555.0	130.0	349.0	956.0	79.0
14	27.6	483.0	129.0	320.0	1104.0	89.0
15	26.7	534.0	146.0	359.0	1061.0	77.0

BASF PATHOLOGY REPORT IIB- 4/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

ABSOLUTE WEIGHTS - INDIVIDUAL VALUES

Sacrifice F1
Sex F
Group 1

	Term. body weight g	Brain mg	Heart mg	Kidneys mg	Liver mg	Spleen mg
M	27.52	533.2	131.8	390.0	1097.4	82.8
SD	1.192	13.405	11.541	36.173	41.584	16.724
n	5	5	5	5	5	5
16	27.9	533.0	116.0	399.0	1135.0	77.0
17	25.9	523.0	128.0	328.0	1035.0	68.0
18	29.2	554.0	148.0	414.0	1094.0	70.0
19	27.3	536.0	133.0	417.0	1136.0	91.0
20	27.3	520.0	134.0	392.0	1087.0	108.0

BASF PATHOLOGY REPORT IIB- 5/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

RELATIVE WEIGHTS - INDIVIDUAL VALUES

Sacrifice F1
Sex M
Group 0

	Term. body weight	Brain	Heart	Kidneys	Liver	Spleen
	%	%	%	%	%	%
M	100.0	1.695	0.506	1.602	3.821	0.204
SD		0.182	0.059	0.206	0.088	0.046
n	5	5	5	5	5	5
1	100.0	1.739	0.54	1.518	3.915	0.184
2	100.0	1.582	0.5	1.585	3.804	0.278
3	100.0	1.788	0.562	1.901	3.756	0.216
4	100.0	1.916	0.52	1.667	3.908	0.172
5	100.0	1.448	0.408	1.34	3.721	0.169

BASF PATHOLOGY REPORT IIB- 6/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

RELATIVE WEIGHTS - INDIVIDUAL VALUES

Sacrifice F1
Sex M
Group 1

	Term. body weight	Brain	Heart	Kidneys	Liver	Spleen
	%	%	%	%	%	%
M	100.0	1.849	0.522	1.557	4.065	0.219
SD		0.059	0.015	0.141	0.094	0.012
n	4	4	4	4	4	4
6	100.0	1.784	0.534	1.5	4.003	0.202
8	100.0	1.927	0.502	1.392	4.183	0.227
9	100.0	1.835	0.519	1.714	4.098	0.222
10	100.0	1.852	0.533	1.622	3.976	0.227

BASF PATHOLOGY REPORT IIB- 7/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

RELATIVE WEIGHTS - INDIVIDUAL VALUES

Sacrifice F1
Sex F
Group 0

	Term. body weight	Brain	Heart	Kidneys	Liver	Spleen
	%	%	%	%	%	%
M	100.0	1.906	0.514	1.285	4.116	0.331
SD		0.241	0.035	0.15	0.226	0.037
n	5	5	5	5	5	5
11	100.0	1.805	0.486	1.39	4.479	0.39
12	100.0	1.694	0.536	1.093	4.194	0.327
13	100.0	2.284	0.535	1.436	3.934	0.325
14	100.0	1.75	0.467	1.159	4.0	0.322
15	100.0	2.0	0.547	1.345	3.974	0.288

BASF PATHOLOGY REPORT IIB- 8/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

RELATIVE WEIGHTS - INDIVIDUAL VALUES

Sacrifice F1
Sex F
Group 1

	Term. body weight	Brain	Heart	Kidneys	Liver	Spleen
	%	%	%	%	%	%
M	100.0	1.939	0.479	1.416	3.991	0.301
SD		0.052	0.036	0.094	0.154	0.063
n	5	5	5	5	5	5
16	100.0	1.91	0.416	1.43	4.068	0.276
17	100.0	2.019	0.494	1.266	3.996	0.263
18	100.0	1.897	0.507	1.418	3.747	0.24
19	100.0	1.963	0.487	1.527	4.161	0.333
20	100.0	1.905	0.491	1.436	3.982	0.396

```
BASF                                PATHOLOGY REPORT                IIB-        9/28
                                                                       99C0295/07011
Acute Oral Toxicity Study in CD-1-mice                              31.Jul.2007 SIGR


SINGLE ANIMAL SHEET
-------------------------------------------------------------------------------
                                                    Sacrifice                   F1
                                                    Sex                          M
                                                    Group                        0
                                                    Animal                       1
...............................................................................
General information
  Sex              : Male
  Group            : 0 (0 mg/kg)
  Sacrifice        : Final sacrifice group
  Necropsy status  : Planned sacrifice
  Date of death    : 01.Jun.2007
                     15 days after start of exposure
                     15 days after end of exposure

Macroscopic findings
  Glandular stomach
    Erosion/ulcer, few (2-5), to diameter 2.0 mm, dark red.
  All other organs without macroscopic findings.
```

```
BASF                                PATHOLOGY REPORT                IIB-       10/28
                                                                       99C0295/07011
Acute Oral Toxicity Study in CD-1-mice                              31.Jul.2007 SIGR


SINGLE ANIMAL SHEET
--------------------------------------------------------------------------------
                                                    Sacrifice                   F1
                                                    Sex                          M
                                                    Group                        0
                                                    Animal                       2
................................................................................
General information
  Sex              : Male
  Group            : 0 (0 mg/kg)
  Sacrifice        : Final sacrifice group
  Necropsy status  : Planned sacrifice
  Date of death    : 01.Jun.2007
                     15 days after start of exposure
                     15 days after end of exposure

Macroscopic findings
  Animal without particular findings.
```

```
BASF                                PATHOLOGY REPORT                IIB-       11/28
                                                                       99C0295/07011
Acute Oral Toxicity Study in CD-1-mice                              31.Jul.2007 SIGR


SINGLE ANIMAL SHEET
--------------------------------------------------------------------------------
                                                    Sacrifice                   F1
                                                    Sex                          M
                                                    Group                        0
                                                    Animal                       3
................................................................................
General information
  Sex              : Male
  Group            : 0 (0 mg/kg)
  Sacrifice        : Final sacrifice group
  Necropsy status  : Planned sacrifice
  Date of death    : 01.Jun.2007
                     15 days after start of exposure
                     15 days after end of exposure

Macroscopic findings
  Animal without particular findings.
```

```
BASF                                PATHOLOGY REPORT                IIB-       12/28
                                                                       99C0295/07011
Acute Oral Toxicity Study in CD-1-mice                              31.Jul.2007 SIGR


SINGLE ANIMAL SHEET
--------------------------------------------------------------------------------
                                                    Sacrifice                    F1
                                                    Sex                           M
                                                    Group                         0
                                                    Animal                        4
................................................................................
General information
  Sex              : Male
  Group            : 0 (0 mg/kg)
  Sacrifice        : Final sacrifice group
  Necropsy status  : Planned sacrifice
  Date of death    : 01.Jun.2007
                     15 days after start of exposure
                     15 days after end of exposure

Macroscopic findings
  Preputial glands
    Cyst, left side, diameter 2.0 mm.
  All other organs without macroscopic findings.
```

BASF PATHOLOGY REPORT IIB- 13/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

SINGLE ANIMAL SHEET

Sacrifice	F1
Sex	M
Group	0
Animal	5

General information
Sex : Male
Group : 0 (0 mg/kg)
Sacrifice : Final sacrifice group
Necropsy status : Planned sacrifice
Date of death : 01.Jun.2007
15 days after start of exposure
15 days after end of exposure

Macroscopic findings
Animal without particular findings.

BASF PATHOLOGY REPORT IIB- 14/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

SINGLE ANIMAL SHEET

Sacrifice	F1
Sex	M
Group	1
Animal	6

General information
Sex : Male
Group : 1 (5000 mg/kg)
Sacrifice : Final sacrifice group
Necropsy status : Planned sacrifice
Date of death : 01.Jun.2007
15 days after start of exposure
15 days after end of exposure

Macroscopic findings
Animal without particular findings.

BASF PATHOLOGY REPORT IIB- 15/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

SINGLE ANIMAL SHEET

Sacrifice	F1
Sex	M
Group	1
Animal	7

General information
Sex : Male
Group : 1 (5000 mg/kg)
Sacrifice : Final sacrifice group
Necropsy status : Sacrificed moribund
Date of death : 25.May.2007
8 days after start of exposure
8 days after end of exposure

Macroscopic findings
General Observations
* As a result of gavage error.
Lungs
(Broncho)pneumonia, yellow white, cloudy.
All other organs without macroscopic findings.

BASF PATHOLOGY REPORT IIB- 16/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

SINGLE ANIMAL SHEET

Sacrifice	F1
Sex	M
Group	1
Animal	8

General information
Sex : Male
Group : 1 (5000 mg/kg)
Sacrifice : Final sacrifice group
Necropsy status : Planned sacrifice
Date of death : 01.Jun.2007
15 days after start of exposure
15 days after end of exposure

Macroscopic findings
Animal without particular findings.

BASF PATHOLOGY REPORT IIB- 17/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

SINGLE ANIMAL SHEET

Sacrifice	F1
Sex	M
Group	1
Animal	9

General information
Sex : Male
Group : 1 (5000 mg/kg)
Sacrifice : Final sacrifice group
Necropsy status : Planned sacrifice
Date of death : 01.Jun.2007
15 days after start of exposure
15 days after end of exposure

Macroscopic findings
Animal without particular findings.

```
BASF                                PATHOLOGY REPORT                IIB-       18/28
                                                                       99C0295/07011
Acute Oral Toxicity Study in CD-1-mice                              31.Jul.2007 SIGR


SINGLE ANIMAL SHEET
--------------------------------------------------------------------------------
                                                    Sacrifice                   F1
                                                    Sex                          M
                                                    Group                        1
                                                    Animal                      10
................................................................................
General information
  Sex              : Male
  Group            : 1 (5000 mg/kg)
  Sacrifice        : Final sacrifice group
  Necropsy status  : Planned sacrifice
  Date of death    : 01.Jun.2007
                     15 days after start of exposure
                     15 days after end of exposure

Macroscopic findings
  Glandular stomach
    Erosion/ulcer, few (2-5), to diameter 2.0 mm, dark red.
  All other organs without macroscopic findings.
```

```
BASF                                PATHOLOGY REPORT                IIB-       19/28
                                                                       99C0295/07011
Acute Oral Toxicity Study in CD-1-mice                              31.Jul.2007 SIGR


SINGLE ANIMAL SHEET
--------------------------------------------------------------------------------
                                                    Sacrifice                    F1
                                                    Sex                           F
                                                    Group                         0
                                                    Animal                       11
................................................................................
General information
  Sex               : Female
  Group             : 0 (0 mg/kg)
  Sacrifice         : Final sacrifice group
  Necropsy status   : Planned sacrifice
  Date of death     : 01.Jun.2007
                      15 days after start of exposure
                      15 days after end of exposure

Macroscopic findings
  Animal without particular findings.
```

BASF PATHOLOGY REPORT IIB- 20/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

SINGLE ANIMAL SHEET

Sacrifice	F1
Sex	F
Group	0
Animal	12

General information
Sex : Female
Group : 0 (0 mg/kg)
Sacrifice : Final sacrifice group
Necropsy status : Planned sacrifice
Date of death : 01.Jun.2007
15 days after start of exposure
15 days after end of exposure

Macroscopic findings
Animal without particular findings.

BASF PATHOLOGY REPORT IIB- 21/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

SINGLE ANIMAL SHEET

Sacrifice	F1
Sex	F
Group	0
Animal	13

General information
Sex : Female
Group : 0 (0 mg/kg)
Sacrifice : Final sacrifice group
Necropsy status : Planned sacrifice
Date of death : 01.Jun.2007
15 days after start of exposure
15 days after end of exposure

Macroscopic findings
Animal without particular findings.

```
BASF                                PATHOLOGY REPORT                IIB-       22/28
                                                                       99C0295/07011
Acute Oral Toxicity Study in CD-1-mice                              31.Jul.2007 SIGR


SINGLE ANIMAL SHEET
-------------------------------------------------------------------------------
                                                    Sacrifice                   F1
                                                    Sex                          F
                                                    Group                        0
                                                    Animal                      14
...............................................................................
General information
  Sex              : Female
  Group            : 0 (0 mg/kg)
  Sacrifice        : Final sacrifice group
  Necropsy status  : Planned sacrifice
  Date of death    : 01.Jun.2007
                     15 days after start of exposure
                     15 days after end of exposure

Macroscopic findings
  Glandular stomach
    Erosion/ulcer, few (2-5), to diameter 2.0 mm, brown.
  All other organs without macroscopic findings.
```

```
BASF                                PATHOLOGY REPORT                IIB-       23/28
                                                                       99C0295/07011
Acute Oral Toxicity Study in CD-1-mice                              31.Jul.2007 SIGR


SINGLE ANIMAL SHEET
-------------------------------------------------------------------------------
                                                    Sacrifice                   F1
                                                    Sex                          F
                                                    Group                        0
                                                    Animal                      15
...............................................................................
General information
  Sex              : Female
  Group            : 0 (0 mg/kg)
  Sacrifice        : Final sacrifice group
  Necropsy status  : Planned sacrifice
  Date of death    : 01.Jun.2007
                     15 days after start of exposure
                     15 days after end of exposure

Macroscopic findings
  Animal without particular findings.
```

BASF PATHOLOGY REPORT IIB- 24/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

SINGLE ANIMAL SHEET

Sacrifice	F1
Sex	F
Group	1
Animal	16

General information
Sex : Female
Group : 1 (5000 mg/kg)
Sacrifice : Final sacrifice group
Necropsy status : Planned sacrifice
Date of death : 01.Jun.2007
15 days after start of exposure
15 days after end of exposure

Macroscopic findings
Animal without particular findings.

BASF PATHOLOGY REPORT IIB- 25/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

SINGLE ANIMAL SHEET

Sacrifice	F1
Sex	F
Group	1
Animal	17

General information
Sex : Female
Group : 1 (5000 mg/kg)
Sacrifice : Final sacrifice group
Necropsy status : Planned sacrifice
Date of death : 01.Jun.2007
15 days after start of exposure
15 days after end of exposure

Macroscopic findings
Animal without particular findings.

BASF PATHOLOGY REPORT IIB- 26/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

SINGLE ANIMAL SHEET

Sacrifice	F1
Sex	F
Group	1
Animal	18

General information
Sex : Female
Group : 1 (5000 mg/kg)
Sacrifice : Final sacrifice group
Necropsy status : Planned sacrifice
Date of death : 01.Jun.2007
15 days after start of exposure
15 days after end of exposure

Macroscopic findings
Ovaries
Paraovarian cyst(s), left side, diameter 3.0 mm, content bloody fluid.
All other organs without macroscopic findings.

BASF PATHOLOGY REPORT IIB- 27/28
99C0295/07011
Acute Oral Toxicity Study in CD-1-mice 31.Jul.2007 SIGR

SINGLE ANIMAL SHEET

Sacrifice	F1
Sex	F
Group	1
Animal	19

General information
Sex : Female
Group : 1 (5000 mg/kg)
Sacrifice : Final sacrifice group
Necropsy status : Planned sacrifice
Date of death : 01.Jun.2007
15 days after start of exposure
15 days after end of exposure

Macroscopic findings
Animal without particular findings.

```
BASF                                PATHOLOGY REPORT                IIB-       28/28
                                                                       99C0295/07011
Acute Oral Toxicity Study in CD-1-mice                              31.Jul.2007 SIGR


SINGLE ANIMAL SHEET
-------------------------------------------------------------------------------
                                                    Sacrifice                   F1
                                                    Sex                          F
                                                    Group                        1
                                                    Animal                      20
...............................................................................
General information
  Sex              : Female
  Group            : 1 (5000 mg/kg)
  Sacrifice        : Final sacrifice group
  Necropsy status  : Planned sacrifice
  Date of death    : 01.Jun.2007
                     15 days after start of exposure
                     15 days after end of exposure

Macroscopic findings
  Animal without particular findings.
```

STUDY TITLE

Report

AtAHAS-0107 Protein

Acute Oral Toxicity Study in CD®-1-mice

PERFORMING LABORATORY

Experimental Toxicology and Ecology
BASF Aktiengesellschaft
67056 Ludwigshafen/Rhein, FRG

LABORATORY PROJECT IDENTIFICATION

Project No.: 99C0295/07011

PART III OF III
(SUPPLEMENT)

CONTENTS OF PART III (SUPPLEMENT)

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:

LAURA PRIVALLE

STUDY COMPLETED ON: JULY 16, 2007

TEST FACILITY/PERFORMING LABORATORY:

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

SEE PAGE 8 FOR ADDITIONAL PERFORMING FACILITIES

BASF
The Chemical Company
Plant Science LLC

BASF Plant Science Study No. BPS-HTC-07-001

BASF Reg. Doc. No. 2007/7004168

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: James Ligon Date: 16 Jul 2007

Title: Regulatory Affairs Manager

Signature: James M. Ligon

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.

BASF
The Chemical Company
Plant Science LLC

BASF Plant Science Study No. BPS-HTC-07-001

BASF Reg. Doc. No. 2007/7004168

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:

16 Jul 07

Laura Privalle, Ph.D.
Senior Manager, Regulatory Science
BASF Plant Science, LLC

Date

SPONSOR REPRESENTATIVE:

16 Jul 2007

James Ligon
Regulatory Affairs Manager
BASF Plant Science, LLC

Date

SUBMITTED BY:

16 Jul 2007

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

Date

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

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:	Laura Privalle, Ph.D. 919-547-2823
Analysts:	Xiaoxu Jiang, 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 Xiaoxu Jiang, M.S., BASF Plant Science, LLC., Research Triangle Park, NC 27709 and Thomas Gaube, 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.

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.

Sathasivan, K., Haughn, G. W., and Murai, N. (1991) Molecular basis of imidazolinone herbicide resistance In *Arabidopsis thaliana* var. Columbia. Plant Physiol. 97:1044-1050.

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.

Tijssen, P. (1985) Processing of data and reporting of results of enzyme immunoassays. *In,* Practice and theory of enzyme immunoassays. Laboratory techniques in biochemistry and molecular biology, V. 15. Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 385-421.

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.

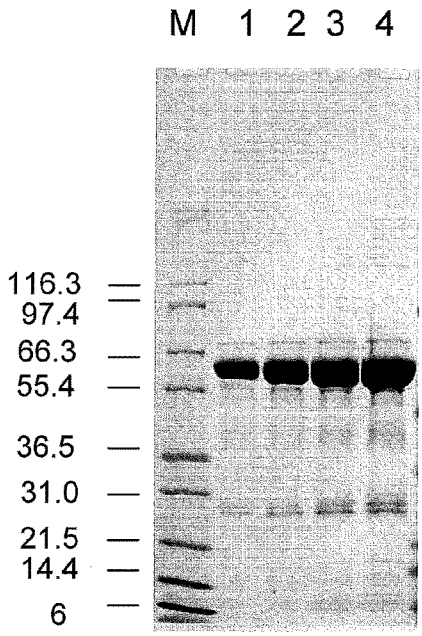

Figure 2. Western blot analysis of AtAHAS protein in sample AtAHAS-0107.

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.

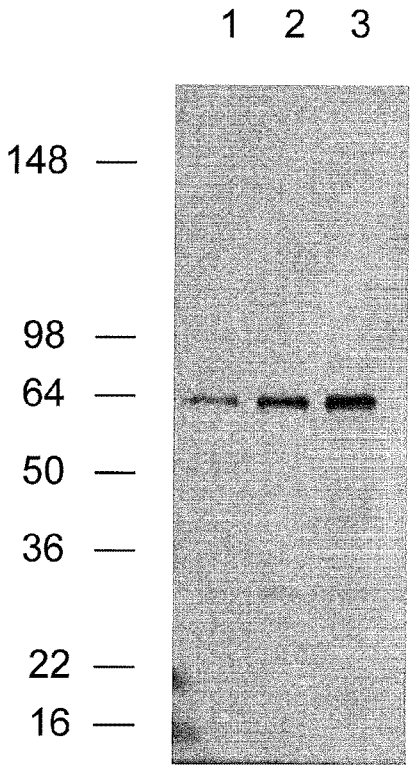

Figure 3. Sensitivity of AtAHAS protein in sample AtAHAS-0107 to imazethapyr, an imidazolinone herbicide. *E. coli* strain 256 expresses the wild type AtAHAS protein, and *E. coli* strain 346 expresses the herbicide tolerant AtAHAS R272K S653N protein.

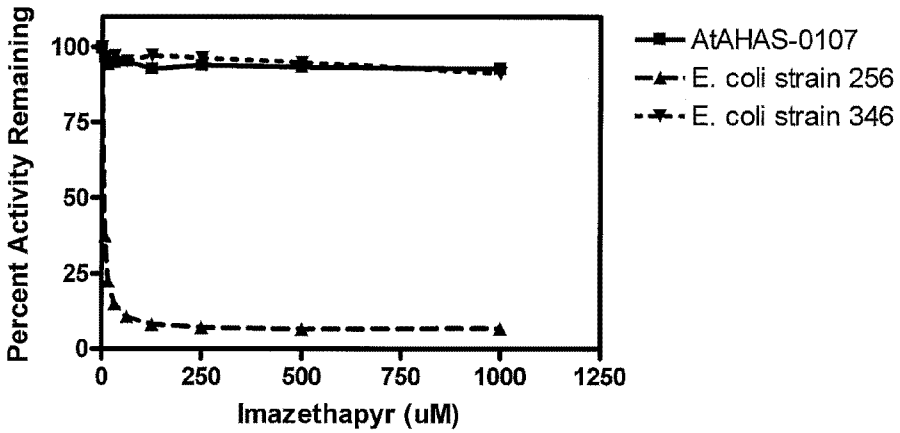

Figure 4. AtAHAS Amino Acid Sequence and N-terminal Amino Acid Sequence Results. The amino acids underlined were obtained by Edman degradation of tryptic peptide fragments derived from the 64,000 molecular weight band in AtAHAS-0107. The capital letters indicate the tag added by the expression vector,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

MGGSHHHHHHGMASMTGGQQMGRDLYDDDKDRWGSELtfisrfapdqprkgadilveal
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vicphqehvlpmipnggtfndvitegdgriky

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Plant Science LLC

BASF Plant Science Study No. BPS-HTC-07-001

BASF Reg. Doc. No. 2007/7004168

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

The Chemical Company

Plant Science LLC

REPORT # BPS-016-07

CONFIRMATION OF STABILITY OF TEST SUBSTANCE ARABIDOPSIS ACETOHYDROXYACID SYNTHASE (LOT #ATAHAS-0107) AFTER THE CONCLUSION OF THE IN-LIFE PHASE OF THE ACUTE ORAL MOUSE TOXICITY STUDY #99C0295/07011 AND ANALYSIS OF DOSING SOLUTION

STUDY # BPS-HTC-07-003

EPA GUIDELINE #: N/A

AUTHOR:

LAURA PRIVALLE

STUDY COMPLETED ON: SEPTEMBER 26, 2007

TEST FACILITY/PERFORMING LABORATORY:

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

2007/7009473

BASF
The Chemical Company
Plant Science LLC

BASF Plant Science Study No. BPS-HTC-07-003

BASF Reg. Doc. No. 2007/7009473

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: James Ligon Date: 25 Sep 2007

Title: Regulatory Affairs Manager

Signature: J. M. Ligon

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.

BASF
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Plant Science LLC

BASF Plant Science Study No. BPS-HTC-07-003

BASF Reg. Doc. No. 2007/7009473

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:

Laura Privalle, Ph.D.
Senior Manager, Regulatory Science
BASF Plant Science, LLC

26 Sep 07
Date

SPONSOR REPRESENTATIVE:

James Ligon
Regulatory Affairs Manager
BASF Plant Science, LLC

25 Sep 2007
Date

SUBMITTED BY:

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

25 Sep 2007
Date

BASF
The Chemical Company
Plant Science LLC

BASF Plant Science Study No. BPS-HTC-07-003

BASF Reg. Doc. No. 2007/7009473

QUALITY ASSURANCE UNIT STATEMENT

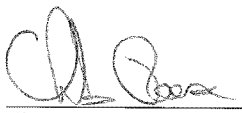
Study Number: BPS-HTC-07-003

Name/Number of Test Substance: AtAHAS-0107

Type of Study: Test Substance Re-characterization and Dosing Confirmation 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
7/26/07	7/26/07
8/7/07	8/8/07
9/20/07	9/21/07



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
CMC	Carboxymethyl cellulose
ELISA	enzyme-linked immunosorbent assay
FAD	flavin adenine dinucleotide
HRP	horseradish peroxidase
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:	2007/7009473
BASF Plant Science Study Number:	BPS-HTC-07-003
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:	Laura Privalle, Ph.D. 919-547-2823
Analysts:	Xiaoxu Jiang, M. S.

CONFIRMATION OF STABILITY OF TEST SUBSTANCE ARABIDOPSIS ACETOHYDROXYACID SYNTHASE (LOT #ATAHAS-0107) AFTER THE CONCLUSION OF THE IN-LIFE PHASE OF THE ACUTE ORAL MOUSE TOXICITY STUDY #99C0295/07011 AND ANALYSIS OF DOSING SOLUTION

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 confirm that the functional activity, concentration, purity and integrity of a test substance, (Lot #AtAHAS-0107), remained relatively unchanged during the in-life phase of a mouse acute oral toxicity study and to analyze the dosing solution for dose confirmation. The test substance contains AHAS protein encoded by the imidazolinone-tolerant acetohydroxyacid synthase *ahas* gene from *Arabidopsis thaliana.* The test substance containing the AtAHAS protein was produced and purified from an *E. coli* over-expression system, characterized in a separate study (Privalle, 2007), and was used in a study entitled :AtAHAS-0107 Protein Acute Oral Toxicity Study in CD®-1 Mice, Project Number 99C0295/07011". This 14-day, single acute dose study was carried out by the BASF Experimental Toxicology and Ecology Facility in Ludwigshafen, Germany. At the conclusion of the study, unused test substance AtAHAS-0107 together with an aliquot of the dosing solution was returned to the BASF Plant Science facility in Research Triangle Park, NC, U.S.A. Various biochemical parameters were evaluated to confirm the functional stability of the AtAHAS protein in the unused test substance and in the post-administration dosing solution. Parameters examined included enzymatic activity, as well as concentration, purity, and integrity.

The concentration, purity and integrity of the AtAHAS protein in the unused test substance and in the dosing solution preparations were assessed using ELISA with protein quantification, SDS-PAGE and western blot analysis, respectively. The unused test substance sample was shown to contain approximately 50.2% AtAHAS by weight as compared to the assessment of 52.4% in the original test substance characterization. This corresponded to 76.9% as the percent of total protein versus 90.6% shown originally. This difference was attributed to a higher overall percentage of protein (65.4%) determined to be present in the unused test substance compared to the lower value of 58% in the original characterization. The dosing solution was found to contain 47.9% protein by weight of which AtAHAS represented 89%, confirming that the dose of AtAHAS protein presented to the animals in the acute oral toxicity study was close to that estimated based on the original characterization of the test substance.

SDS-PAGE and western blot analyses of the sample revealed a major protein band at the predicted molecular weight of AtAHAS protein in both the unused AtAHAS-0107 test substance sample and the dosing solution. The molecular weight of the AtAHAS protein

was approximately 64,000 and the protein banding profiles were indistinguishable between the two samples.

The functionality of the AtAHAS protein in the unused AtAHAS-0107 test substance sample was monitored by measuring enzymatic activity. The sample was enzymatically active and had a specific activity of 0.549 ± 0.091 units/mg protein compared to the original characterization value of 0.790 ± 0.216 units/mg protein. The two values were not significantly different within the normal variability of the assay. The conditions used to solubilize the AtAHAS from the dosing solution were incompatible with AHAS assay conditions so it was not possible to confirm the functionality of AHAS in the dosing solution.

This study confirmed the purity, concentration, functionality and stability of the AtAHAS protein test substance preparation during the in-life phase of the mouse acute toxicity study as well as under shipping conditions between the analytical laboratory in Research Triangle Park, NC and the BASF Experimental Toxicology and Ecology Facility in Ludwigshafen, Germany. In addition, the analysis of the dosing solution confirmed the concentration, purity and integrity of AtAHAS used in the acute oral mouse toxicity study.

INTRODUCTION

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 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 purpose of this study was to confirm that the functional activity, concentration, purity and integrity of a test substance, (Lot #AtAHAS-0107), remained relatively

unchanged during the in-life phase of a mouse acute oral toxicity study and to analyze the dosing solution for confirmation of concentration, purity and integrity of the AtAHAS protein in the dose administered to the mice in the oral toxicity study. The test substance contains AHAS protein encoded by the imidazolinone-tolerant acetohydroxyacid synthase *ahas* gene isolated from *Arabidopsis thaliana* that was used in a study entitled "AtAHAS-0107 Protein Acute Oral Toxicity Study in CD®-1 Mice, Project Number 99C0295/07011". This 14-day, single acute dose study was carried out by the BASF Experimental Toxicology and Ecology Facility in Ludwigshafen, Germany. This test substance, lot # AtAHAS-0107 was originally characterized in April 2007 (Privalle, 2007) prior to the initiation of the acute oral mouse toxicity study, the experimental phase of which began on May 17, 2007. The test substance was formulated in to a dosing solution that was administered to the mice in the acute oral toxicity study, and unused test substance and residual dosing solution were stored at < –18°C and, at the conclusion of the in-life phase of the study, the unused test substance and dosing solution were returned to BASF Plant Science, Research Triangle Park, NC (termed the "analytical facility") for re-characterization. The unused test substance was analyzed for AtAHAS concentration, purity, integrity and functional activity, and the residual dosing solution was analyzed for AtAHAS concentration, purity and integrity. Because the conditions used to solubilize the AtAHAS from the dosing solution were incompatible with AHAS assay conditions, it was not possible to confirm the functionality of AHAS in the dosing solution.

MATERIALS AND METHODS

Test substance. The test substance, lot # AtAHAS-0107 was originally characterized in April of 2007 prior to the initiation of the acute oral mouse toxicity study, the experimental phase of which began on May 17, 2007. At the conclusion of the in-life phase of the acute oral mouse toxicity study, the remaining unused test substance that had been stored at < –18°C with desiccation during the in-life phase of the acute oral mouse toxicity study was returned to BASF Plant Science, Research Triangle Park, NC for re-characterization and was referred to as the "unused AtAHAS test substance". The original preparation and characterization of the test substance is described in BASF Plant Science Report No. BPS-011-07, Study No. BPS-HTC-07-001 (Privalle, 2007). In addition, the test substance was formulated in to a dosing solution (AtAHAS-0107 in 5% carboxymethyl cellulose [CMC] as the delivery vehicle) that was administered to the mice in the acute oral toxicity study, and an aliquot of the dosing solution was also analyzed for AtAHAS concentration, purity and integrity.

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

Sample preparation. The test substance, AtAHAS-0107, was shipped as a frozen dry powder to the BASF Experimental Toxicology and Ecology Facility in Ludwigshafen, Germany for the acute oral mouse toxicity study. The test substance was formulated in 5% CMC to prepare the dosing solution. Unused test substance was stored frozen as a dry powder, and residual dosing solution was also stored frozen. After the in-life phase

of the toxicity study, both the unused test substance and residual dosing solution were shipped frozen to BASF Plant Science, Research Triangle Park, NC for re-characterization. The unused test substance was resuspended in 100 mM CAPS, pH 11.0 (2 mg/ml) for concentration, purity and integrity analyses of the AtAHAS protein, Also a 1 mg/ml AtAHAS-0107 solution in 50 mM Tris-HCl, pH 7.0 was prepared for functional activity analysis of AtAHAS. The dosing solution was received as a 125 mg/ml 5% CMC suspension. An aliquot (20 µl) was dissolved in 100 mM CAPS, pH 11.0, and this solution was used for concentration, purity and integrity analyses.

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. The unused AtAHAS-0107 test substance sample and dosing solution were 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. 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 standard curve. The AtAHAS component of the unused AtAHAS-0107 test substance sample and dosing solution were quantified from the standard concentration curve generated from highly purified AtAHAS protein.

Molecular weight determination. To confirm that a major protein in the unused AtAHAS-0107 test substance sample and dosing solution had the predicted molecular weight of AtAHAS (*ca.* 64,000), aliquots of the sample and dosing solutions 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 the unused AtAHAS-0107 test substance sample and dosing solutions 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 loaded onto the gel such that the total protein in each lane was either *ca.* 2 and 6 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 the unused AtAHAS-0107 test substance sample and dosing solution, western blot analysis was performed on these samples. Aliquots of the returned AtAHAS-0107 sample and dosing solutions were subjected to SDS-PAGE on an 8 - 16% polyacrylamide gradient gel followed by electroblotting onto PVDF membrane (Invitrogen; SOP BPS 510.03). The samples were loaded onto the gel such that 20 and 50 ng AtAHAS protein was present in each lane. 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 in the unused AtAHAS-0107 test substance 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 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 the desired concentration 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 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.

Statistical analyses. Linear regression was used to fit absorbance values of standards for both protein quantification and AHAS enzymatic assays. The four-parameter algorithm was used to calculate the standard curve in the ELISA. Mean values of triplicate determinations are reported in all cases.

RESULTS

AHAS quantification. The unused AtAHAS-0107 test substance sample was determined by ELISA to contain *ca.* 0.502 g AHAS protein/g sample, or *ca.* 50.2% AHAS by weight. This was very similar to the determination of 0.524 g AHAS/g sample (52.4% AtAHAS by weight) in the original characterization of the test substance (Privalle, 2007). Analysis of AtAHAS-0107 using the BCA protein determination method determined that the sample contained 65.4% protein by weight. By calculation, the AtAHAS protein comprises 76.9% of the total protein in the sample which compares to 90.6% of the total protein determined originally. Therefore, the difference in percent AtAHAS protein of total protein in the unused sample compared to the original characterization was attributed to a higher overall percentage of protein (65.4%) determined to be present in the unused test substance compared to the lower value of 58% in the original characterization. The dosing solution was found to contain 47.7% protein of which 89% was found to be AtAHAS (Table 1).

Molecular weight determination. The major protein band in the unused AtAHAS-0107 test substance sample and dosing solution was determined to be *ca.* 64,000 molecular weight by SDS-PAGE analysis (Figure 1) corresponding to the predicted molecular weight of AtAHAS protein. The protein banding pattern of the two samples were indistinguishable and was similar to the pattern observed in the original characterization of the AtAHAS-0107 test substance. This confirms that the AtAHAS protein, as well as other proteins present in the AtAHAS-0107 test substance, remained unaltered during the course of the mouse toxicity study and when prepared as a dosing solution.

Immunoreactivity. Western blot analysis of the unused AtAHAS-0107 test substance sample and dosing solution 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. Again, the protein banding pattern of the two samples were indistinguishable and had a similar pattern to that observed in the original test substance characterization. This confirms that the AtAHAS protein in the AtAHAS-0107 test substance, retained its integrity and remained unaltered during the course of the mouse toxicity study and when prepared as a dosing solution.

Enzymatic activity. The returned AtAHAS-0107 sample was determined to have a specific activity of 0.549 ± 0.091 units/mg protein which was slightly lower than the original reported value of 0.790 ± 0.216 units/mg protein, confirming that the AtAHAS protein in sample AtAHAS-0107 is enzymatically active (Table 1). It was not possible to measure the enzymatic activity of the AtAHAS present in the dosing solution as the conditions used to solubilize the test substance in the suspension were incompatible with the AHAS enzymatic assay. This was confirmed by treating the returned test substance in a similar manner to the dosing solution, including suspending in 5% CMC and recovering substantially reduced activity.

CONCLUSIONS

The objectives of this study were two-fold:

a) Confirm that the AtAHAS protein concentration, purity, integrity and functional activity in the AtAHAS-0107 test substance preparation remained relatively unchanged during shipments between the laboratory where the acute oral mouse toxicity study was conducted (BASF Experimental Toxicology and Ecology Facility in Ludwigshafen, Germany) and the analytical laboratory where the test substance was originally characterized and re-characterized after the in-life phase of the toxicity study (BASF Plant Science facility in Research Triangle Park, NC, U.S.A).

b) Confirm that the concentration, purity, integrity and functional activity of the AtAHAS protein administered to the mice in the acute oral toxicity study were unchanged from the predicted values based on the original characterization of the test substance (Privalle, 2007).

The concentration of the unused test substance sample was shown to be approximately 50.2% AtAHAS by weight as compared to the assessment of 52.4% in the original test substance characterization. This corresponded to 76.9% as the percent of total protein versus 90.6% shown originally. This difference was attributed to a higher overall percentage of protein (65.4%) determined to be present in the unused test substance compared to the lower value of 58% in the original characterization, and this difference is within the normal variation of the assay. The dosing solution was found to contain 47.9% total protein by weight of which AtAHAS represented 89%, confirming that the dose of AtAHAS protein presented to the animals in the acute oral toxicity study is close to that estimated based on the original characterization of the test substance.

Results of SDS-PAGE and western blot analyses of the unused AtAHAS-0107 test substance and the AtAHAS dosing solution showed that the purity and integrity of the AtAHAS in these preparations was unchanged relative to the purity and integrity determinations in the original characterization of the AtAHAS test substance (Privalle, 2007).

Because the conditions used to solubilize the AtAHAS from the dosing solution were incompatible with AHAS assay conditions, it was not possible to confirm the functional or enzymatic activity of AHAS in the dosing solution. However, the AtAHAS protein in the unused AtAHAS-0107 test substance sample was shown to be enzymatically active and had a specific activity of 0.549 ± 0.091 units/mg protein compared to the original characterization value of 0.790 ± 0.216 units/mg protein. The two values were not significantly different within the normal variability of the assay. Therefore, based on this result, the AtAHAS in the dosing solution administered to the mice in the acute toxicity study was most probably functionally active at the time of administration.

Results of these studies confirm that the AtAHAS protein concentration, purity, integrity and functional activity in the AtAHAS-0107 test substance preparation remained relatively unchanged, within the normal variability of the assays, during shipments between the laboratory where the acute oral mouse toxicity study was conducted and the analytical laboratory where the test substance was originally characterized and re-characterized after the in-life phase of the toxicity study. These studies also confirmed that the concentration, purity, integrity and functional activity of the AtAHAS protein administered to the mice in the acute oral toxicity study were as predicted based on the original characterization of the test substance.

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, which was judged not to have adversely affected the study or it validity, was made. The original protocol did not specify how the dosing solution sample was going to be handled. A protocol change was written to document that this was to be determined empirically and recorded in the raw data.

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 Xiaoxu Jiang, M.S., BASF Plant Science, LLC., Research Triangle Park, NC 27709.

CRITICAL DATES:

Study initiation date:	26 July 07
Experimental start date:	7 August 07
Experimental end date:	20 August 07

EXAMPLE CALCULATIONS:

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

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

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 510.09	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.

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

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.

Tijssen, P. (1985) Processing of data and reporting of results of enzyme immunoassays. *In*, Practice and theory of enzyme immunoassays. Laboratory techniques in biochemistry and molecular biology, V. 15. Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 385-421.

Table 1. Concentration of the unused AtAHAS-0107 test substance sample and dosing solution, and enzymatic activity of the unused test substance compared to concentration and activity values determined in the original test substance characterization

	Protein	***AtAHAS* Concentration**			**Specific Activity**
AtAHAS preparation	% protein by weight	AtAHAS (g/g sample)	% AtAHAS by weight	AtAHAS as % total protein	Mean units/mg protein ± standard deviation
original characterization*	58.0	0.524	52.4	90.6	0.790 ± 0.216
unused test substance	65.4	0.502	50.2	76.9	0.549 ± 0.091
dosing solution	47.9			89.0	

*Reported originally in the BASF Plant Science Report No. BPS-011-07.

Figure 1. Molecular weight confirmation of AtAHAS protein in the unused AtAHAS-0107 test substance sample and the dosing solution.

Coomassie blue-stained 8 - 16% polyacrylamide SDS gel. AtAHAS is *ca.* 64,000 mol. wt. Lane M, molecular weight (x 10^{-3}) markers; lanes 2 – 4 contain 2 µg of protein, from the dosing solution, the unused test substance, AtAHAS-0107 resuspended in CAPS buffer, and the unused test substance resuspended in 5% CMC, respectively. Lanes 5 – 7 contain 6 µg of protein, from the unused test substance resuspended in 5% CMC, the unused test substance resuspended in CAPS buffer, and the dosing solution, respectively.

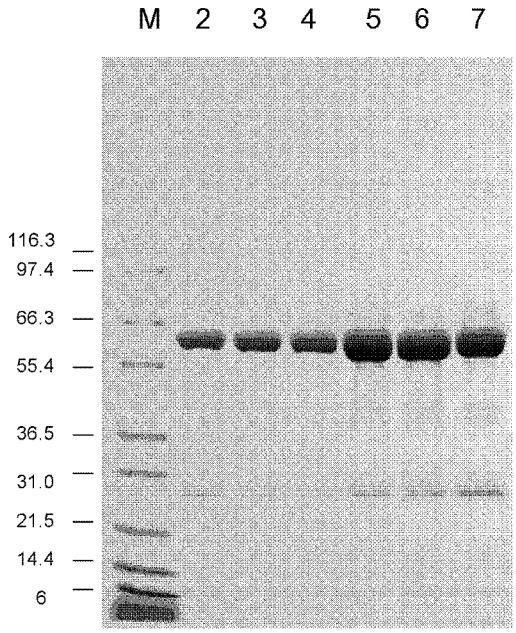

Figure 2. Western blot analysis of AtAHAS protein in unused AtAHAS-0107 test substance sample and the dosing solution.

The integrity of AtAHAS in sample AtAHAS-0107 was evaluated by western blot analysis. Lanes 1 – 3 contain 20 ng of protein, from the dosing solution, the unused test substance, AtAHAS-0107, resuspended in CAPS buffer, and the unused test substance resuspended in 5% CMC, respectively. Lanes 4 – 6 contain 50 ng of protein, from the unused test substance resuspended in 5% CMC, the unused test substance resuspended in CAPS buffer, and the dosing solution, respectively. The blot was 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.