Appendix 9

Characterization of AtAHAS Protein Produced in Imidazolinone-tolerant Soybean BPS-CV127-9 and Comparison with AtAHAS Protein expressed in Recombinant *Escherichia coli*

BASF
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CHARACTERIZATION OF AtAHAS PROTEIN PRODUCED IN IMIDAZOLINONE-TOLERANT SOYBEAN BPS-CV127-9 AND COMPARISON WITH AtAHAS PROTEIN EXPRESSED IN RECOMBINANT *Escherichia coli*

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

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

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ABBREVIATIONS AND DEFINITIONS

AHAS	acetohydroxyacid synthase
AHASL	acetohydroxyacid synthase large subunit
AtAHAS	*Arabidopsis thaliana* acetohydroxyacid synthase
BCA	bicinchoninic acid
BSA	bovine serum albumin
CID	collision induced dissociation
CTP	chloroplast transit peptide
DIG	digoxigenin
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
FAD	flavin adenine dinucleotide
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
MS	mass spectroscopy
PBS	phosphate buffered saline
PVDF	polyvinylidene fluoride
R272K	arginine at position 272 replaced by lysine
S653N	serine at position 653 replaced by asparagine
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
TBST	tris buffered saline, tween
TPP	thiamine pyrophosphate

CHARACTERIZATION OF AtAHAS PROTEIN PRODUCED IN IMIDAZOLINONE-TOLERANT SOYBEAN BPS-CV127-9 AND COMPARISON WITH AtAHAS PROTEIN EXPRESSED IN RECOMBINANT *Escherichia coli*

Test Substance: AHAS protein produced in BPS-CV127-9 soybean

Control Substance: AHAS protein produced in the conventional soybean

Reference Substance: AHAS protein produced in an *Escherichia coli* over-expression system, lot # AtAHAS-0107

SUMMARY

The purpose of this study was to characterize the AtAHAS protein produced in the transgenic imidazolinone-tolerant soybean, referred to as BPS-CV127-9, to evaluate key biochemical and functional parameters and to demonstrate that it is substantially similar to the microbially-produced AtAHAS protein which has been used in various safety assessment studies. The introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene *csr1-2* 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 BPS-CV127-9. This mutation results in an arginine at position 272 of the protein being replaced by lysine, R272K, and this amino acid substitution does not impact the enzymatic function of the AHAS enzyme.

The microbially-produced AtAHAS test substance (Lot #AtAHAS-0107) contained acetohydroxyacid synthase (AHAS) protein encoded by the imidazolinone-tolerant *ahasl* gene *csr1-2* isolated from *Arabidopsis thaliana* and is referred to as AtAHAS protein. The gene encoding the microbially-produced AtAHAS protein also contained the second R272K mutation, resulting in arginine at position 272 of the AHAS protein being replaced by lysine. Both immuno-purified AHAS protein and ammonium sulfate treated extracts of BPS-CV127-9 leaves were used to evaluate the similarity of the plant-produced protein to the microbially-produced AtAHAS. The microbial test substance was previously characterized for purity, integrity, functionality and immunoreactivity (Privalle, 2007).

Western blot analyses using antibodies specific for AHAS showed the same molecular weight of approximately 64,000 and immunoreactivity for both the plant-produced and the microbially-produced proteins.

The functionality of the AtAHAS protein produced in BPS-CV127-9 and the microbially-produced protein was demonstrated by monitoring the enzymatic activity, its lack of sensitivity to inhibition by imidazolinone herbicide, and feedback inhibition by branched-chain amino acids. While both the plant-produced and microbially-produced proteins demonstrated the expected enzymatic activity with the substrate pyruvate, the specific activity of the AHAS protein in BPS-CV127-9 was found to be higher compared to the microbially-produced protein, most likely a result of enzyme activity losses incurred during extensive purification of the microbially-produced protein. Both BPS-CV127-9-produced and microbially-produced AHAS proteins showed tolerance to inhibition by the imidazolinone herbicide imazethapyr compared to the control plant-produced protein, and sensitivity to feedback inhibition by branched-chain amino acids was retained in the plant extracts but not in the microbially-produced material because of the lack of the small subunit of AHAS in the microbial AHAS preparation, which is responsible for mediating the feedback regulation.

As expected, no evidence of glycosylation associated with either the plant or microbially-produced AtAHAS was detected. Mass spectral analysis of the immuno-purified AtAHAS from young leaves of BPS-CV127-9 plants confirmed the expected amino acid sequence of the protein for 23% of the entire amino sequence of the protein.

This study confirmed that the AHAS protein produced in BPS-CV127-9 plants had the expected size, immunoreactivity, functionality and was substantially similar to that produced in a microbial over-expression system for use in safety assessment studies. Therefore, this study demonstrates the functional and chemical equivalence of the microbially-produced AtAHAS and the plant-produced AHAS proteins (AtAHAS and the endogenous soybean AHAS proteins) and therefore justifies the use of the microbial-produced protein for safety assessment studies of the AtAHAS protein produced in BPS-CV127-9 soybean.

INTRODUCTION

Soybean (*Glycine max* L.) plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants, referred to as BPS-CV127-9 in this report, were produced by introduction of the imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene *csr1-2* from *Arabidopsis thaliana* into the soybean plant genome. The *csr1-2* gene from *A. thaliana* encodes an acetohydroxyacid synthase protein (AtAHAS) that is tolerant to imidazolinone herbicides due to a point mutation that results in a single amino acid substitution in which the serine residue at position 653 of the protein is replaced by asparagine (S653N). This amino acid change in plant AHAS proteins is known to

prevent the binding of imidazolinone herbicides and thereby to result in tolerance to these herbicides with no effect on feedback regulation by branched chain amino acids or normal biosynthetic function (Newhouse *et al.*, 1992). In addition, a second mutation was discovered in the *csr1-2* gene that was introduced in the genome of BPS-CV127-9 soybean. This mutation results in an arginine at position 272 of the protein being replaced by lysine, and this amino acid substitution does not impact the enzymatic function of the AHAS enzyme or herbicide tolerance properties (Stevenson Paulik, 2007).

The *csr1-2* gene encodes a single polypeptide of 670 amino acids that includes the *A. thaliana* native chloroplast transit peptide CTP) on the N-terminus that is predicted to consist of 85 amino acids (Mazur *et al.*, 1987). During transport into the chloroplast, the chloroplast transit peptide is removed to produce the mature AtAHAS enzyme. Based on a sequence comparison of the deduced amino acid sequence of plant and microbial AHAS proteins, Mazur *et al.* (1987) predicted that the CTP of the AtAHAS is cleaved at the C-terminal side of residue 85 to produce a mature AHAS enzyme that is molecular weight approximately 64,000 and consists of 585 amino acids.

As part of the food, feed and environmental safety assessment of BPS-CV127-9 soybean, studies were conducted to confirm that the AtAHAS protein is equivalent to other AHAS proteins found ubiquitously among plant species and have a history of safe use in food and feed products. These safety assessments of the AtAHAS protein included a mouse acute gavage study and a digestive fate study conducted with purified AtAHAS protein. The AtAHAS protein is expressed at extremely low levels in tissues of BPS-CV127-9 soybean plants. For example, the AtAHAS protein is present in soybean grain at levels less than 1.5×10^{-6} % on a dry weight basis (Schwerz, 2007, 2008). Therefore, it was not technically feasible to extract sufficient quantities of the AtAHAS protein from soybean tissues for the safety assessments of the protein. The same AtAHAS coding sequence introduced in the BPS-CV127-9 soybean genome was introduced into *Escherichia coli* for over-expression of the AtAHAS protein. The AtAHAS protein, as encoded in this expression system, lacks the predicted 85 amino acid N-terminal CTP sequence that targets the protein *in planta* to the chloroplast. The DNA coding sequence for the CTP was replaced by a coding sequence for 38 amino acids including a six residue histidine tag to allow for ease in purification, a gene 10 leader sequence to enhance solubility, and an Xpress™ tag for detection. Furthermore, the microbially-produced AtAHAS protein contained the same amino acid substitutions corresponding to the S653N and R272K mutations in the *csr1-2* gene that are present in the plant-produced AtAHAS protein. Therefore, the coding sequences of the mature AtAHAS protein were the same in both the microbial production system and in BPS-CV127-9 soybean.

Microbial fermentation provided sufficient starting material for purification of gram quantities of the AtAHAS protein required for the safety assessment studies. The AtAHAS protein purified from the *Escherichia coli* over-expression system was characterized in a separate study (Privalle, 2007). The purpose of the current study was to characterize the AtAHAS protein produced in the transgenic imidazolinone-tolerant BPS-CV127-9 soybean to evaluate key biochemical and functional parameters and to

demonstrate that it is substantially similar to the microbially-produced AtAHAS protein which has been used in various safety assessment studies.

The AHAS characterized in this study was obtained from extracts of young leaves of field-grown BPS-CV127-9 plants. In some studies, immuno-purified AHAS was used, and in others, ammonium sulfate precipitated AHAS-enriched extract was utilized. These materials served as the test substances. As a control, the endogenous soybean AHAS from an ammonium sulfate precipitated AHAS-enriched extract of leaves from the isoline control soybean was included in some of the studies. The AtAHAS protein purified from the *Escherichia coli* over-expression system was used as a reference substance. This protein is identified as AtAHAS-0107 in this report. In this study, soybean-expressed AHAS protein was evaluated for its molecular weight, immunoreactivity, enzymatic activity, sensitivity to branched-chain amino acid feedback control and imidazolinone herbicide inhibition. In addition, the protein was evaluated for post-translational modifications and amino acid sequence confirmation.

MATERIALS AND METHODS

Preparation of test substance. AHAS expressed in BPS-CV127-9 used in these experiments was either from an ammonium sulfate precipitated AHAS-enriched fraction or it was immuno-purified from young leaves (collected from the F6 generation of CV127 line 127, grown in regulatory field trials in Santo Antônio de Goiás, GO, Brazil in 2007). Crude extracts were prepared by homogenizing frozen powdered leaves (1:3 w:v) in extraction buffer [50 mM potassium phosphate, pH 7.2, 100 mM sodium pyruvate, 5 mM $MgCl_2$, with HALT™ protease inhibitor cocktail (Pierce Biotechnology, Inc.; Rockford, IL)] using a Polytron™ (Brinkmann, Westbury, NY). Extracts were filtered through miracloth and subjected to centrifugation for 15 min at approx. 10,000 x g. The resulting supernatant was considered to be the crude extract. The ammonium sulfate precipitated AHAS-enriched fraction was obtained by adding an equal volume of saturated ammonium sulfate to the crude extract with stirring on ice for 30 min, centrifugation for 15 min at 10,000 x g, and resuspending the pellet with extraction buffer. Further purification was achieved by using goat anti-AtAHAS antibody (which had been purified using a Protein G affinity chromatography followed by chromatography on an AtAHAS affinity column).

Preparation of the control substance. Endogenous AHAS from the conventional soybean used in these experiments was from an ammonium sulfate precipitated AHAS-enriched fraction prepared from young leaves (collected from the isoline control soybean (F6 null) corresponding to the F6 generation of CV127 line 127, and grown in regulatory field trials in Santo Antônio de Goiás, GO, Brazil in 2007), as described above for the test substance.

Reference substance description.

Identification	Lot #	Gene Source	Expression System	Physical Description
Acetohydroxyacid Synthase R272K, S653N[1]	AtAHAS-0107	*Arabidopsis thaliana*	*Escherichia coli* strain BL21(DE3)pLysS	Light yellow proteinaceous powder

AtAHAS was purified by Invitrogen, Inc. and sent as an ammonium sulfate pellet to the Regulatory Science Laboratory at BASF Plant Science, Research Triangle Park, NC, where it was received on February 27, 2007. AtAHAS protein was purified from 2,400 g cell paste after lysis in 20 L buffer (50 mM Sodium phosphate, 300 mM NaCl, 10 mM β-mercaptoethanol, 30 mM Imidazole) using an Emulsiflex cell disruptor (Avestin, Ontario, Canada). Insoluble material was removed by centrifugation. The His-tagged AtAHAS present in the supernatant was purified by chromatography using Talon® cobalt resin (Clontech, Inc., Mountain View, CA). Once eluted, the fractions containing AtAHAS were pooled and immediately precipitated by ammonium sulfate at 40% saturation. The ammonium sulfate pellet was resuspended in 100 mM Tris-HCl, pH 7.0 and dialyzed vs. 20 mM ammonium bicarbonate buffer, pH 7.9, containing 20 µM FAD. The dialyzed material was then lyophilized and designated AtAHAS-0107. This reference substance has been characterized (Privalle, 2007).

Protein quantification. Protein in the AtAHAS reference substance and ammonium sulfate treated test and control AHAS-enriched fractions from plant tissues was quantified by the BCA™ procedure (bicinchoninic acid procedure; Pierce) in accordance with the manufacturer's instructions, using bovine serum albumin as the standard (SOP BPS 510.04). Samples were prepared such that the expected concentration of protein would be within the standard curve. Samples (25 µl) were loaded onto a multiwell plate in triplicate, reacted with 200 µl of a 1:50 (B:A) mixture of BCA reagent, incubated at 37°C for 30 min and allowed to cool at room temperature for 10 min. The absorbance at 550 nm was measured using a Multiskan Ascent V1.24 multiwell plate reader (Therma Labsystems, Helsinki, Finland). The results were analyzed using DeltaSoft PC software (Version 1.71.4, Biometallics, Inc.; Princeton, NJ) using the linear regression curve fit.

[1] The *ahas* gene was cloned into the inducible, over-expression pTrcHis A® vector (Invitrogen, Madison, WI) in *E. coli* strain BL21(DE3)pLysS. AHAS protein as encoded in this vector lacks the 85 N-terminal leader sequence that targets the protein *in planta* to the chloroplast. This leader has been replaced in this vector with 38 amino acids including a 6 x His tag to allow for ease in purification, a gene 10 leader sequence to enhance solubility and an Xpress™ tag for detection. The remainder of the protein is identical in amino acid sequence to that produced by the native gene from *Arabidopsis thaliana* (Mazur, *et al.*, 1987) except for the mutation at amino acid 653 that results in asparagine replacing serine and a mutation at amino acid 272 that results in lysine replacing arginine. The S653N mutation renders a decreased binding of the herbicide imparting the tolerance. The R272K mutation was found in the AtAHAS protein produced in BPS-CV127-9 soybean but has no apparent effect on activity or inhibitor sensitivity.

AHAS quantification. Samples 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. Nunc 96-well plates (VWR; West Chester, PA) were coated with rabbit anti-peptide 2 and incubated at 4°C overnight. 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 2 hours at 37°C. After washing twice, samples and standards were applied in triplicate. Plates were incubated at 4°C for 1.5 hr and 45 min at room temperature, 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 20 min at room temperature the absorbance at 620 nm was measured using a Multiskan Ascent V1.24 multiwell plate reader. The results were analyzed using DeltaSoft PC software (Version 1.71.4; Biometallics, Inc.; Princeton, NJ). The four-parameters algorithm was used to generate a curve. The AHAS component of the samples was quantified from the standard concentration curve generated from highly purified AtAHAS protein.

Molecular weight and immunoreactivity determination. To confirm that the AHAS produced in BPS-CV127-9 plants had the predicted molecular weight of AtAHAS (*ca.* 64,000), aliquots of the sample preparations 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) followed by electroblotting onto PVDF membrane (Invitrogen; SOP BPS 510.03). Samples of the ammonium sulfate AHAS-enriched preparations and AtAHAS-0107 preparations were loaded onto the gel. After electroblotting the membrane was probed with rabbit anti-AHAS peptide 2 polyclonal antibody. Donkey anti-rabbit IgG linked to horseradish peroxidase (Pierce), diluted 1:3,000 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 ECL Plus Western Blotting Detection Reagents (Amersham, GE Healthcare).

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 naphthol. One unit of AHAS activity is defined as 1 µmole acetoin produced per minute. The ammonium sulfate AHAS-enriched preparations of BPS-CV127-9 and the isoline control soybean, AtAHAS-0107, dissolved in 50 mM Tris-HCl, pH ~7.0, and diluted to desired concentration, were mixed with an equal volume of 2x assay buffer [100 mM potassium phosphate, pH 7.2, 10 mM $MgCl_2$, 200 mM sodium pyruvate, with 20 µM FAD and 2 mM thiamine pyrophosphate (TPP)] and incubated at 37°C for 90 minutes 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-naphthol (1.7% in 4 N NaOH, final concentration) for 15 min at 60°C and the absorbance was measured at 550 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.

Feedback inhibition of AHAS enzymatic activity by leucine and valine was confirmed by including leucine and valine in the assay mix. Leucine and valine solutions were prepared in 2x assay buffer at twice the desired final assay concentration (1 mM). Fifty µl of 20-fold diluted AtAHAS-0107, the ammonium sulfate AHAS-enriched preparations of BPS-CV127-9 and the isoline control soybean were incubated with 50 µl of 2x assay buffer or leucine and valine solution for 120 min at 37°C and AHAS enzymatic assays were performed in triplicate.

AHAS enzymatic activity sensitivity to inhibition by an imidazolinone herbicide was examined using a stock solution (50 mM) of imazethapyr, a commercial imidazolinone herbicide, 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. 500 µM. Fifty µl of the sample was incubated with 50 µl of 2x assay buffer or imazethapyr solution for 90 min at 37°C.

The absorbance was measured at 550 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 enzymatic activity obtained for each sample in the absence of inhibitor was assumed to be 100%.

Glycosylation analysis. The DIG Glycan Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) was used in accordance with the manufacturer's instructions (method B, described below) to monitor for glycosylation associated with BPS-CV127-9 expressed AHAS following the SDS-PAGE/electroblotting to membrane procedure. This method takes advantage of the adjacent hydroxyl residues in sugars of glyco-conjugates by oxidizing them to aldehyde groups by mild periodate treatment. The spaced linked steroid hapten digoxigenin (DIG) is then covalently attached to these aldehydes via a hydrazide group. Digoxigenin labeled glycoconjugates are then subsequently detected by western blot analysis using a digoxigenin specific antibody conjugated with alkaline phosphatase. Samples were run on the SDS-PAGE and transferred to PVDF membrane. After washing with phosphate buffered saline, PBS (50 mM potassium phosphate, pH 6.5, 150 mM NaCl), the membrane is treated with 10 mM sodium metaperiodate (in sodium acetate buffer, pH 5.5) for 20 minutes at room temperature (oxidation). The membrane is then washed three times with PBS and incubated with DIG-0-3-succinyl-ε-aminocaproic acid hydrazide for 1 hour at room temperature to label the glycoproteins. The membrane was washed three times with TBST (25 mM Tris, 3 mM KCl, 0.14 M NaCl, 0.05% Tween-20, pH 7.4) and blocked for at least 30 minutes, followed by incubation with anti-digoxigenin-AP for 1 hour. The chromagenic reaction with

nitroblue tetrazolium was used to monitor the alkaline phosphates bound to the membrane. Transferrin and creatinase were used as the positive and the negative controls, respectively. All incubations were conducted at room temperature with gentle agitation except for color development (room temperature, no agitation). The limit of detection for this method was determined to be 2 – 4 molecules of glucose equivalents per molecule of AtAHAS under these conditions.

Amino acid sequence analysis. To further confirm that the AtAHAS protein produced in BPS-CV127-9 was the protein expected, amino acid sequence was determined. The attempts to obtain N-terminal amino acid sequence using Edman degradation methods were unsuccessful. Therefore, a combination of SDS-PAGE, liquid chromatography coupled with tandem mass spectroscopy (LC/MS/MS) was used to obtain amino acid sequence data. Immunoaffinity purified AtAHAS from BPS-CV127-9 young leaves was subjected to SDS-PAGE and stained with Coomassie blue. The bands were excised and transferred into 0.5 ml Eppendorf cups. The gel pieces were washed consecutively with 100 µl of acetonitrile/ water (1:1, v:v), acetonitrile and acetonitrile/water (1:1). For each of the wash steps, the samples were shaken at 800 rpm for 5 min at 30°C, subjected to 1 min of sonication in an ultrasonic bath, centrifuged at 13,000 x g and the respective supernatants were discarded. Following addition of of 10 µl of 100 mM ammonium bicarbonate, the gel pieces were shaken for 5 minutes at 30°C. Then 50 µl of the trypsin solution [0.76 µg trypsin, (Roche Mannehiem), 0.12% $CaCl_2$, 8% acetonitrile, 100 mM ammonium bicarbonate] was added and incubated at 37°C for 16 hours with shaking (300 rpm). After stopping the reaction by addition of 90 µl stop solution (75% acetonitrile, 0.2% trifluoroacetic acid), shaking was continued for 1 hour followed by centrifugation for 1 min at 13,000 rpm. The supernatants were transferred to a fresh tube and 10 µl acetonitrile were added, shaken for 2 min and centrifuged as before. The wash was repeated a second time and the supernatants combined. The combined supernatants containing the peptides were concentrated in a Speed Vac until dry and used for analysis.

The peptide fragments were then subjected to nanoHPLC/ESI-MS/MS in the MS-laboratory of BASF Central Research, Ludwigshafen, Germany. MS-analysis of the trypsin-digested protein samples was carried out on a quadrupole ion-trap mass-spectrometer (LCQ, Fa. Thermofinnigan) with a gold-platinated spray capillary (Fa. New Objektive, Inc.). The spectrometer is directly linked to a µHPLC unit (Fa. SunChrom GmbH), allowing separation of the peptide mix prior to the MS-analysis.

The peptides were separated on the µHPLC unit using a reverse-phase column (Fa. LC-Packings) with 75 µm i.d. and a length of 15 cm. The column was packed with PepMap™ C18[2] material of 100 Å pore diameter and 3 µm particle size. A gradient of 5 – 50% solvent A and B (A: 95% H_2O, 5% ACN, 0.08% HCOOH; B: 95% ACN, 5% H_2O, 0.08% HCOOH) in 60 min was selected for separation of the trypsin-digested peptides. The 10 µl/min flow from an Eldex HPLC pump (Fa. SunChrome) was reduced using a split-system to achieve a column flow-rate of 180 nl/min.

[2] C18 material, i.e. covalently-bonded octadecylsilane, is used as bonded phase in the column.

The separated peptide mix was directly eluted into the mass spectrometer. MS-experiments were run in an alternating sequence of *"full MS-scan // MS/MS-scan"*. The full scan experiments provided an overview of the ions present at the respective elution time. In this study, these were mostly intact mono-, di-, and tri-protonated peptide ions. The most abundant ions of the full scan were then selected for MS/MS-analyses. In an MS/MS-experiment a specific ion is selected (by its m/z value) and subjected to collision with Helium atoms. This collision brings about a collision induced dissociation (CID) of the selected peptide ion into fragment ions. In the chosen experimental set-up, dissociation occurs mainly at the peptide bonds, with an ideally statistical distribution. Two series of ions are formed: b-ions, where the charge is retained on the N-terminal fragment, and y-ions, carrying the charge on the C-terminus of the fragmented peptide. The m/z-difference between two adjacent signals of one ion series corresponds to one amino-acid residue. In theory, the MS/MS-spectra feature the full amino-acid sequences of the analyzed proteolytic peptides. But usually not every expected signal is retrieved, so that instead of full-length sequences, sequence tags are obtained. A sequence tag is a data combination, consisting of the m/z-value of the entire peptide, and a partial, accurately localized amino-acid sequence of the peptide. Depending on the length/sequence coverage of the tag, a sequence tag of a proteolytic peptide can be highly specific for the originating protein. In summary, the MS-experiments provide the molecular masses of the proteolytic peptides and either full-length sequences or specific sequence tags.

In order to identify the originating protein of the peptide mix, the MS/MS-data were analyzed using the MASCOT MS/MS-ions search. In short, MASCOT compares the experimental MS/MS-data against a comprehensive primary sequence database. For this comparison, the entries of the sequence database are subjected to ion cleavage-rules, thus yielding calculated fragment-ion mass-values. The quality of the obtained matches is evaluated by an appropriate scoring algorithm. Depending on the contents of the database, the originating protein or a protein with close homology can be identified. The MASCOT search was carried out without specifying a digesting enzyme. All MASCOT results were checked manually against the experimental data to assure appropriate evaluation. In addition to the automated database search with MASCOT, amino-acid sequences were retrieved by hand from the MS/MS-spectra as far as possible.

RESULTS

Molecular weight and immunoreactivity determination. Western blot analysis, using rabbit anti-AHAS peptide 2 polyclonal antibodies, of extracts prepared from both BPS-CV127-9 and the isoline control soybean contained immuno-reactive bands at a molecular weight of approximately 64,000 (Figure 1). This was the same apparent molecular weight as the microbially-produced AtAHAS reference substance, AtAHAS-0107, indicating that the AHAS produced in the BPS-CV127-9 and control soybeans was of the same anticipated size and of similar immunoreactivity. Both plant extracts included a smaller immunoreactive band at approximately 47,000 molecular weight, which is most likely a degradation product of the mature AHAS protein. The molecular

weight of the microbially-produced AtAHAS and plant-produced AHAS proteins were also shown to be similar using SDS-PAGE and Coomassie blue staining. The approximate molecular weight of the immuno-purified AHAS protein from BPS-CV127-9 soybean plants and the AHAS in AtAHAS-0107 test substance were shown to be approximately 64,000 (Figure 5).

Based on a sequence comparison of the deduced amino acid sequence of AHAS proteins from several different plant and microbial sources, Mazur *et al.* (1987) predicted that the CTP of the AtAHAS is cleaved at the C-terminal side of residue 85 to produce a mature AtAHAS enzyme that consists of 585 amino acids. In the current study, the microbially-produced AtAHAS protein consisted of the predicted mature AtAHAS (585 amino acids) plus an N-terminal addition of 38 amino acid to facilitate protein purification, folding and solubility. Therefore, it was expected that the molecular weight of the microbially-produced protein would be slightly greater than that of the AHAS proteins produced in either BPS-CV127-9 soybean or the isoline control soybean. Because the apparent molecular weights of the plant-produced and microbially-produced AHAS proteins were equivalent, results of these studies suggest that the mature AHAS proteins produced in plant tissues contain more than the predicted 585 amino acid residues of the mature AtAHAS protein. These additional amino acids are most likely derived from the CTP component at the N-terminal end of the protein due to a different cleavage site in soybeans compared to Arabidopsis. This conclusion is supported by data presented in the later section of this report entitled "amino acid sequence determination".

Enzymatic activity. The microbially-produced AtAHAS and the plant produced AHAS proteins demonstrated the expected enzymatic activity with the substrate pyruvate, confirming the same functional activity of the AHAS proteins from both microbial and plant sources. However, the specific activity of ammonium sulfate AHAS-enriched plant extracts was considerably higher than the highly purified microbially-produced AHAS protein (Table 1). AHAS is a highly labile protein of low abundance in plants and has been extremely difficult to purify from plant preparations (Singh *et al.*, 1991; Durner and Boger, 1988; Muhitch *et al.*, 1987, Chang and Duggleby, 1997). In these leaf preparations, AHAS was present at very similar levels of 0.516 ng/ml and 0.384 ng/ml in the BPS-CV127-9 and the isoline control soybean extracts, respectively. This corresponds to only 0.06 ng/mg protein or 0.000006% of the protein as AHAS. The protein is stable in ammonium sulfate fractions but further purification results in greatly reduced enzymatic activity. The microbially-produced AtAHAS was purified extensively for the protein safety studies, whereas the plant AHAS used in these enzymatic studies was derived from an ammonium sulfate purification step. Hence, these differences in levels of purification accounted for the difference in specific activity between the microbially-produced material, AtAHAS-0107, and that in ammonium sulfate AHAS-enriched leaf extracts (Table 1). Activity was very similar between BPS-CV127-9 and the isoline control soybean, 0.33 and 0.30 Units AHAS activity/mg protein, respectively.

Both BPS-CV127-9-produced and microbially-produced AHAS proteins showed tolerance to inhibition by the imidazolinone herbicide imazethapyr compared to the control plant-produced protein (Figure 2). An intermediate level of inhibition of the

BPS-CV127-9-produced AHAS protein was observed, and this was as expected due to the mixture of sensitive (endogenous) and tolerant (transgenic) AHAS present in BPS-CV127-9. In general, the degree of herbicide inhibition observed for the AHAS proteins in the test and control soybeans was lower than anticipated but may reflect the tolerance that soybean naturally has to this particular imidazolinone herbicide, imazethapyr.

Feedback inhibition by branched-chain amino acids, leucine and valine, was very similar for both the BPS-CV127-9 AHAS and the nontransgenic conventional soybean isoline (Figure 3). No feedback inhibition was observed for the AtAHAS present in the microbially-produced reference substance because of the lack of the small subunit of AHAS responsible for mediating the feedback regulation, and which is present in the plant extracts but only the large subunit of AHAS is encoded in the over-expression microbial system (Singh *et al.*, 1991).

Glycosylation. No evidence of glycosylation was detected for either the BPS-CV127-9-produced or microbially-produced AHAS protein (Figure 4). As shown in Figure 5, there are only four N-glycosylation sites [NXS(T)X, where X can be any amino acid except for proline] in the mature AHAS polypeptide. At least one is most likely present in the chloroplast transit sequence and would not be present in the mature protein. There are, however, numerous O glycosylation sites (any serine or threonine). It was not anticipated that AHAS would be glycosylated as it does not enter the secretory pathway, a prerequisite of glycosylation, but rather it is destined to be localized in the chloroplast where proteins are not typically glycosylated. Likewise, the AtAHAS produced in the microbial expression system should not be glycosylated as glycosylation does not occur in prokaryotes. The results shown here demonstrate that no glycosylation was detected above the limit of detection (2 – 4 molecules of glucose equivalents/molecule of AtAHAS).

Amino acid sequence determination. Amino acid sequence data was obtained for approximately 23% of the protein produced in BPS-CV127-9, and approximately 73% coverage of the microbially-produced AtAHAS. The results (Figure 5) confirm that the major protein band at approximately 64,000 molecular weight in BPS-CV127-9 is AtAHAS since the amino acid sequences obtained from peptide fragments derived from it were identical to regions of the deduced amino acid sequence from the coding sequence of the *csr1-2* gene in the BPS-CV127-9 soybean genome. Similarly, sequences obtained from peptide fragments derived from the microbially-produced AtAHAS were identical to regions of the deduced amino acid sequence from the coding sequence of the *csr1-2* gene in the *Escherichia coli* over-expression system (data not presented). Also, where AHAS peptide fragments overlapped between the plant-produced and microbially-produced sources, the amino acid sequences were the same (data not presented). Since the same DNA coding sequence for the AtAHAS protein was introduced into the genome of BPS-CV127-9 soybean and in to the *Escherichia coli* over-expression system, these data confirm that the amino acid sequences of peptides produced from the predicted mature AtAHAS from the *Escherichia coli* over-expression system and from BPS-CV127-9 are the same and correspond to the predicted amino sequence of AtAHAS with the S653N and R272K amino acid substitutions.

Two of the four potential N-glycosylation sites were included in the amino acid sequence obtained, providing confirmatory support for the lack of glycosylation at these sites. If glycosylation had been present fragments of different masses would have been obtained.

The peptide fragment corresponding to residues 52 to 73 was not expected to be present in the mature AtAHAS protein produced in BPS-CV127-9. The *csr1-2* gene from *Arabidopsis thaliana* encodes a single polypeptide of 670 amino acids that includes a chloroplast transit peptide (CTP) on the N-terminus that has been hypothesized but not confirmed to consist of 85 amino acids (Mazur *et al.*, 1987, Chang and Duggleby, 1997). This hypothesized CTP was deduced by comparing the amino acid sequences of known AHAS proteins and looking for the first conserved residue (threonine 86). An identical CTP cleavage site was experimentally determined for the AHAS enzyme in maize (B. K. Singh, personal communication). During transport into the chloroplast, the chloroplast transit peptide is removed to produce the mature AtAHAS enzyme. For this reason, the peptide fragment corresponding to residues 52 to 73 was not expected to be present in BPS-CV127-9. The microbially-produced AHAS protein was designed to represent the mature form of the AHAS protein and lacks the 85 N-terminal amino acid sequence corresponding to the predicted chloroplast transit peptide sequence. The microbial protein does contain an additional 38 amino acids (see footnote on page 11) to aid in bacterial expression and purification. The molecular weights of the BPS-CV127-9-produced and microbially-produced proteins were shown to be similar and based on this information represent 623 amino acids in the *Escherichia coli*-produced AtAHAS and potentially 619 amino acids in BPS-CV127-9. Furthermore, the N-terminal amino acid for one of the soybean AHAS proteins (AHASL1) has been shown to be the serine 48, thus the chloroplast transit peptide for that protein is apparently only 47 amino acids in length (Rood, *et al.*, 2006).

CONCLUSIONS

Key biochemical and functional parameters were evaluated to demonstrate that the AtAHAS protein produced in BPS-CV127-9 is substantially similar to the microbially-produced AtAHAS protein which has been used in various safety assessment studies. The molecular weight and immunoreactivity of the AHAS produced in BPS-CV127-9 was shown to be similar to that of the endogenous AHAS from the isoline control soybean and the reference AHAS produced in a microbial over-expression system. The specific activity of the AHAS produced in BPS-CV127-9 was very similar to the endogenous soybean AHAS and was higher than the microbially-produced AtAHAS. This was attributed to AHAS being a very unstable protein that loses activity readily upon purification (Chang and Duggleby, 1997). Therefore, it is not unexpected that the AHAS specific activity in an impure state such as in the ammonium sulfate AHAS-enriched fraction is higher than that retained in the much more highly purified microbially-produced material. Both BPS-CV127-9-produced and microbially-produced AHAS proteins showed tolerance to inhibition by the imidazolinone herbicide imazethapyr compared to the control plant-produced protein. Sensitivity to feedback

inhibition by branched-chain amino acids was retained in the plant extracts but not in the microbially-produced material because of the lack of the small subunit of AHAS in the microbial AHAS preparation, which is responsible for mediating the feedback regulation. No evidence of glycosylation was found associated with the AtAHAS in BPS-CV127-9 leaves or the microbially-produced AtAHAS.

To compare the amino acid sequence of the AtAHAS protein produced in BPS-CV127-9 soybean with that produced in the *E. coli* expression system, the amino acid sequence of the AtAHAS protein of BPS-CV127-9 soybean was investigated. Attempts to obtain N-terminal amino acid sequence using Edman degradation methods were unsuccessful. This was most likely due to the low abundance of the AtAHAS protein in plant tissues, coupled with the repetitive amino acid content of the putative CTP. The high content of serine, threonine and proline within the first 85 amino acids of the full-length AtAHAS (the putative CTP segment of the protein) contribute to the difficulties in obtaining unambiguous results. Serines and threonines negatively impact yield of amino acid sequence and have high carryover into subsequent chromatograms, leading to ambiguous interpretation of results of analysis of the amino acid sequence. Proline is not efficiently cleaved (only 50 – 60%) by the Edman degradation chemistry. This also contributes to the high carryover into subsequent chromatograms. These three amino acids represent 60% (51/85) of the putative CTP. Therefore, the resulting data gave the appearance of N-terminal blockage of the AtAHAS protein.

Because the above approach was not successful in determining the N-terminus of the mature AtAHAS protein, a combination of SDS-PAGE, liquid chromatography coupled with tandem mass spectroscopy (LC/MS/MS) was performed. Peptides were derived from BPS-CV127-9 soybean and compared to the deduced amino acid sequence of the AtAHAS gene in the expression vector. The amino acid sequence obtained covered approximately 23% of the entire AtAHAS amino acid sequence. All amino acid sequence data obtained from the AtAHAS of BPS-CV127-9 soybeans was identical to the corresponding amino acid sequence of the *E. coli*-produced AtAHAS with the exception of a region near the N-terminus that is predicted to contain the chloroplast transit peptide. Based on a sequence comparison of the deduced amino acid sequence of plant and microbial AHAS proteins, Mazur *et al.* (1987) predicted that the chloroplast transit peptide of the AtAHAS is cleaved at the C-terminal side of residue 85. Amino acid sequence analysis of the AtAHAS protein produced in BPS-CV127-9 showed a peptide fragment corresponding to amino acid residues 52 to 73. Therefore, the CTP cleavage site of the AtAHAS protein produced in BPS-CV127-9 is most likely not at the predicted site at the C-terminal side of residue 85, but is located at least at the C-terminal side of residue 51. This conclusion was further confirmed because the apparent molecular weights of the BPS-CV127-9-produced AHAS protein and the microbially-produced AtAHAS protein were equivalent, yet the microbially-produced protein included an additional 38 amino acids attached to the predicted 585 amino acids of the mature AHAS protein.

However, even though the data in this study suggest that the mature AHAS protein produced in BPS-CV127-9 consists of the predicted mature protein (585 amino acids)

plus approximately 34 additional amino acids in the CTP predicted by Mazur *et al.* (1987), the microbially-produced AtAHAS protein is considered equivalent to the BPS-CV127-9 protein for the following reasons. The microbially-produced AtAHAS protein was functionally active and had the expected enzymatic activity with the substrate pyruvate. Furthermore, the microbially-produced protein lacked sensitivity to inhibition by the imidazolinone herbicide imazethapyr, similar to the lack of sensitivity of the BPS-CV127-9 protein to the same herbicide. In addition, the microbially-produced AtAHAS protein and BPS-CV127-9-produced AHAS proteins had similar immunoreactivity to antibodies specific to the AHAS protein based on results of both western blot and ELISA analyses. Both the microbially-produced AtAHAS protein and the BPS-CV127-9-produced protein showed no evidence of glycosylation. Finally, both proteins had the same amino acid sequence of the predicted mature protein of 585 amino acids, based on amino acid sequence analysis of overlapping peptide fragments.

Results of these studies justify the use of the microbially-produced AtAHAS protein as an appropriate substitute for the AtAHAS produced in BPS-CV127-9 in safety assessment studies of the AtAHAS protein.

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

STUDY PERSONNEL: Analytical work reported herein conducted by [redacted] M.S., BASF Plant Science, LLC., Research Triangle Park, NC 27709 a[redacted] Ph.D., [redacted] Ph.D., [redacted] nd [redacted] ASF AG, Ludwigshafen, Germany.

EXAMPLE CALCULATIONS:

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

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 510.09 Enzymatic Assay for Acetohydroxyacid Synthase (AHAS)

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Table 1. Specific activity of AHAS in BPS-CV127-9, the isoline control soybean, and the reference substance AtAHAS-0107

Source of AHAS	ng AHAS/ml extract	mg protein/ ml extract	AHAS activity nmole/min/ml extract	AHAS specific activity nmole/min/mg protein	AHAS specific activity Units/µg AHAS
BPS-CV127-9	0.516	8.16	2.697	0.33	5.227
Control	0.384	8.13	2.435	0.30	6.341
AtAHAS-0107	85411.000	0.32	436.003	1379.43	0.005

Figure 1. Western blot analysis of AHAS protein in BPS-CV127-9, the isoline control soybean and reference substance AtAHAS-0107.

The immunoreactivity and integrity of AHAS from BPS-CV127-9, isoline control soybean, and in sample AtAHAS-0107 was evaluated by western blot analysis. Lane 1, 0.4 ng AtAHAS from AtAHAS-0107; lane 2, 76 µg protein ammonium sulfate AHAS enriched fraction from BPS-CV127-9; and lane 3, 81 µg protein ammonium sulfate AHAS-enriched fraction from isoline control soybean. Blots were probed with rabbit anti-AHAS peptide 2 antibody. The molecular weight of intact AHAS corresponds to *ca.* 64,000 mol. wt. Molecular weight (x 10^{-3}) markers are indicated.

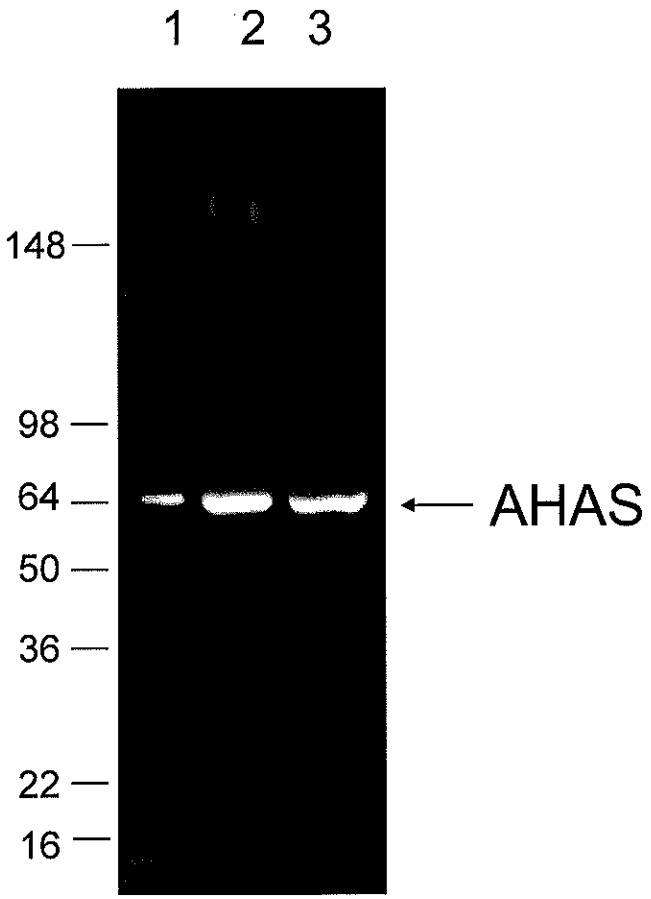


Figure 2. Sensitivity of the enzymatic activity of AHAS from BPS-CV127-9, isoline control soybean and reference substance AtAHAS-0107 to imazethapyr, an imidazolinone herbicide.

AHAS enzymatic activity was measured in the presence of increasing concentrations of the imidazolinone herbicide, imazethapyr, in AHAS extracts enriched by ammonium sulfate precipitation, prepared from BPS-CV127-9 leaves (-▲-), the isoline control soybean leaves (-■-), and in the reference AHAS microbially-produced test substance, AtAHAS-0107 (-▼-).

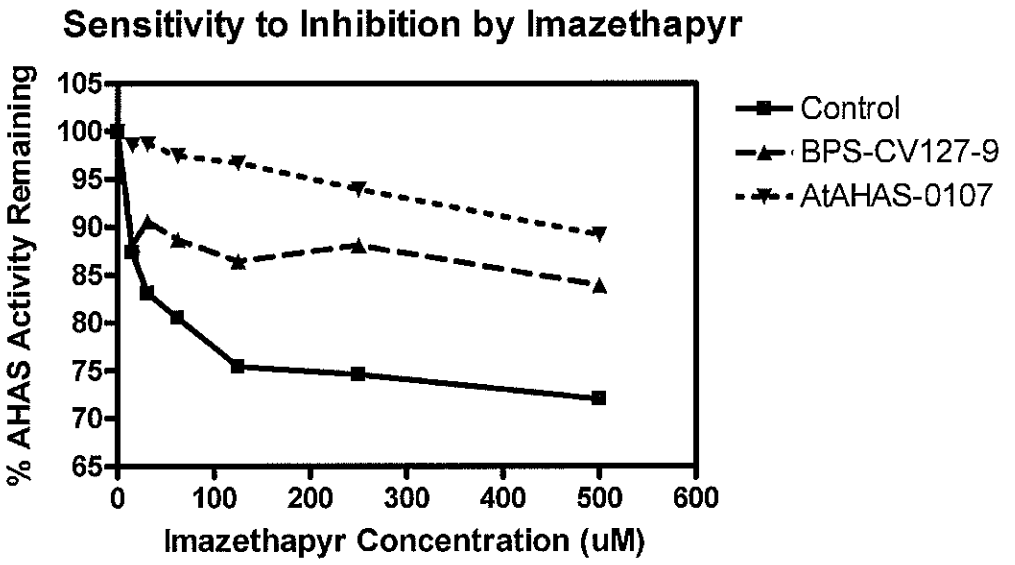


Figure 3. Sensitivity of the enzymatic activity of AHAS from BPS-CV127-9, isoline control soybean and reference substance AtAHAS-0107 to feedback inhibition by leucine and valine.

AHAS enzymatic activity was measured in the presence of increasing concentrations of the branched-chain amino acids, leucine and valine, in AHAS-enriched ammonium sulfate precipitation extracts prepared from BPS-CV127-9 leaves (-▲-), the isoline control soybean leaves, (-■-) and in the reference AHAS microbially-produced test substance, AtAHAS-0107 (-▼-).

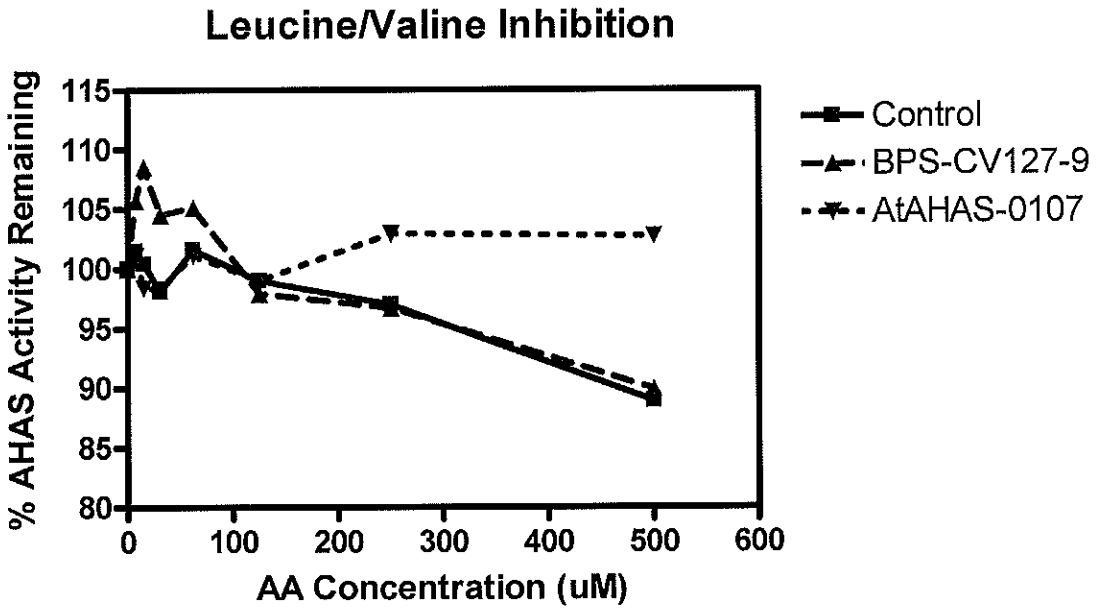


Figure 4. Examination of glycosylation of AHAS from BPS-CV127-9 and reference substance AtAHAS-0107.

Samples were subjected to SDS-PAGE on a single 8 – 16% polyacrylamide gel and electroblotted onto a PVDF membrane. The membrane was divided so that lane 1 and part of the marker lane (M) was developed by western blot analysis using goat anti-AtAHAS, Lanes 2 – 4 were developed in the absence of oxidation; and lanes 5 – 10 with oxidation. Lanes 1 and 5, 25 - 50 ng AHAS from the AHAS-enriched soybean leaf preparation, M, SeeBluePlus™ protein markers; lanes 2 and 10, 100 ng transferrin and lanes 8 and 9, 5 and 50 ng of transferrin (positive control), respectively; lanes 3 and 6, 2 ng AtAHAS-0107; lane 7, 100 ng creatinase (negative control). The AtAHAS position is indicated by arrows.

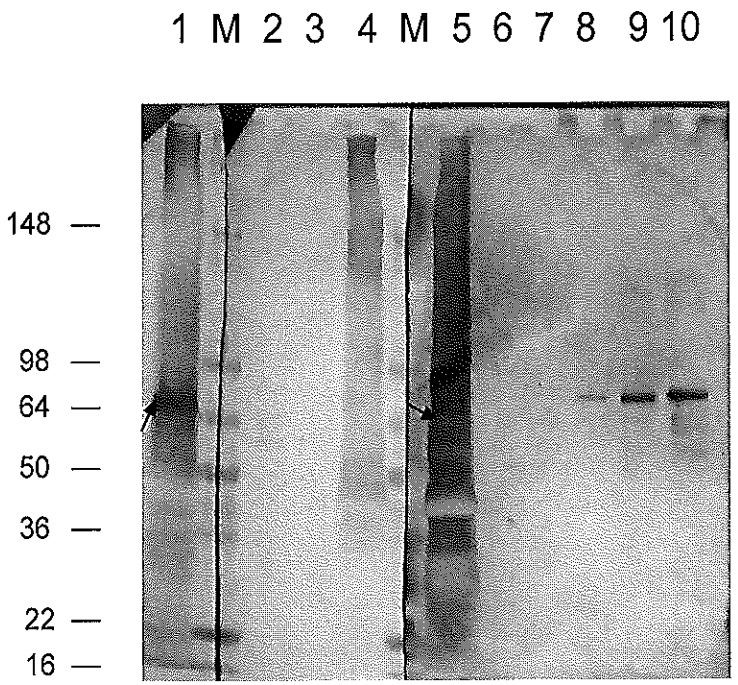
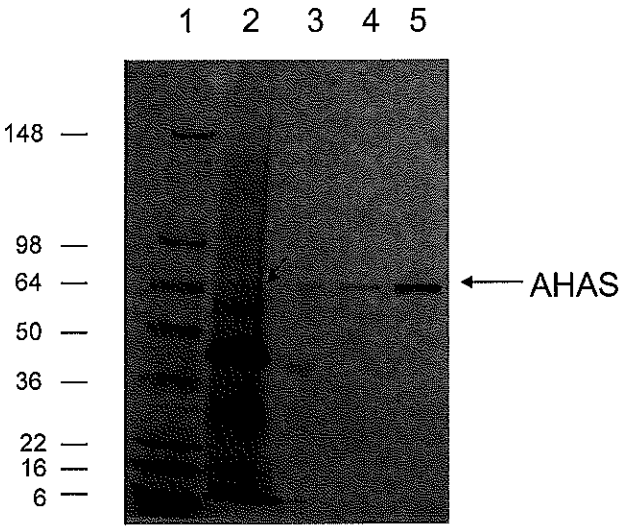


Figure 5. AtAHAS amino acid sequence. Panel A. SDS-PAGE with Coomassie blue staining of immuno-purified AtAHAS from BPS-CV127-9 (lane 2, arrow). Lanes 3 – 5, microbially-produced AtAHAS loaded at 25, 50, 250 ng AHAS. Molecular weight (x 10^{-3}) markers are indicated. Panel B. The predicted amino acid sequence of AtAHAS encoded by the gene transformed into soybean resulting in the event designated BPS-CV127-9. The amino acids underlined in Panel B were obtained by LC/MS/MS of the protein band indicated by the arrow in Panel A, lane 2. The four potential N-glycosylation sites [NX(S, T)X] are indicated by shading. The two amino acids that are different from wild type Arabidopsis AHAS are indicated in bold (K^{272}, N^{653})

A.



B.

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1   MAAATTTTTT SSSISFSTKP SPSSSKSPLP ISRFSLPFSL NPNKSSSSSR
51  RRGIKSSSPS SISAVLNTTI NVTTTPSPTK PTKPETFISR FAPDQPRKGA
101 DILVEALERQ GVETVFAYPG GASMEIHQAL TRSSSIRNVL PRHEQGGVFA
151 AEGYARSSGK PGICIATSGP GATNLVSGLA DALLDSVPLV AITGQVPRRM
201 IGTDAFQETP IVEVTRSITK HNYLVMDVED IPRIIEEAFF LATSGRPGPV
251 LVDVPKDIQQ QLAIPNWEQA MKLPGYMSRM PKPPEDSHLE QIVRLISESK
301 KPVLYVGGGC LNSSDELGKF VELTGIPVAS TLMGLGSYPC DDELSLHMLG
351 MHGTVYANYA VEHSDLLLAF GVRFDDRVTG KLEAFASRAK IVHIDIDSAE
401 IGKNKTPHVS VCGDVKLALQ GMNKVLENRA EELKLDFGVW RNELNVQKQK
451 FPLSFKTFGE AIPPQYAIKV LDELTDGKAI ISTGVGQHQM WAAQFYNYKK
501 PRQWLSSGGL GAMGFGLPAA IGASVANPDA IVVDIDGDGS FIMNVQELAT
551 IRVENLPVKV LLLNNQHLGM VMQWEDRFYK ANRAHTFLGD PAQEDEIFPN
601 MLLFAAACGI PAARVTKKAD LREAIQTMLD TPGPYLLDVI CPHQEHVLPM
651 IPNGGTFNDV ITEGDGRIKY
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