SUMMARY

(In accordance with 40 CFR part 152, this summary is available for public release after registration)

STUDY TITLE

Characterization of the Aryloxyalkanoate Dioxygenase-12 (AAD-12) and double mutant 5-Enol Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) proteins derived from transgenic soybean event DAS-444Ø6-6

DATA REQUIREMENTS

Not Applicable

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STUDY COMPLETED ON

21 – July – 2011

PERFORMING LABORATORY

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LABORATORY STUDY ID

101707

Characterization of the Aryloxyalkanoate Dioxygenase-12 (AAD-12) and double mutant 5-Enol Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) proteins derived from transgenic soybean event DAS-444Ø6-6

SUMMARY

The purpose of this study was to characterize the biochemical properties of the recombinant AAD-12 and 2mEPSPS proteins derived from the transgenic soybean event DAS-444Ø6-6 (event 44Ø6) and compare them with the previously characterized *Pseudomonas fluorescens*-derived proteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, stained with Coomassie blue and glycoprotein detection methods), western blot, lateral flow strip assay (LFS), and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses were used to characterize the biochemical properties of both proteins. In addition, enzymatic activity and glyphosate tolerance of the 2mEPSPS was verified. Using these methods, the AAD-12 and 2mEPSPS proteins from *P. fluorescens* and transgenic soybean (event 44Ø6) were shown to be biochemically equivalent. These data support the use of the microbial-derived proteins for use in studies supporting the registration of transgenic soybeans expressing the AAD-12 and 2mEPSPS proteins.

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Characterization of the Aryloxyalkanoate Dioxygenase-12 (AAD-12) and double mutant 5-Enol Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) proteins derived from transgenic soybean event DAS-444Ø6-6

DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

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STUDY COMPLETED ON

21 – July – 2011

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: AAD-12 and 2mEPSPS

Title: Characterization of the Aryloxyalkanoate Dioxygenase-12 (AAD-12) and double mutant 5-Enol Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) proteins derived from transgenic soybean event DAS-444Ø6-6

- STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS:

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Company: Dow AgroSciences LLC

Company Agent: M. S. Krieger

Title: Regulatory Manager

Signature: 

Date: 28 June 2014

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Characterization of the Aryloxyalkanoate Dioxygenase-12 (AAD-12) and double mutant 5-Enol Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) proteins derived from transgenic soybean event DAS-444Ø6-6

Study Initiation Date: 16-Jun-2010

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ENV/MC/CHEM(98)17, Paris January 26, 1998

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160 with the following exceptions. The protein standards from Invitrogen, Applied Biosystems, and Thermo-Pierce were non-GLP as the chain of custody was not monitored. The mass spectrometry analysis of the *P. fluorescens*-derived AAD-12 and 2mEPSPS proteins was performed in a non-GLP laboratory.

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28 June 2011
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21-July-2011
Study Completion Date

Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Study ID: 101707

Title: Characterization of the Aryloxyalkanoate Dioxygenase-12 (AAD-12) and double mutant 5-Enol Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) proteins derived from transgenic soybean event DAS-44406-6

Study Initiation Date: 16-Jun-2010 **Study Completion Date:** 21-Jul-2011

GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
14-Jun-2010	14-Jun-2010	Protocol Review
23-Jun-2010	23-Jun-2010	Planting
13-Jul-2010	14-Jul-2010	Repeat Planting
30-Nov, 1-Dec-2010	1-Dec-2010	Repeat Planting with new source ID
18-Jan-2011	19-Jan-2011	Western Blot
9-Feb-2011	18-Mar-2011	Western Blot (Repeat)
23-Mar-2011	23-Mar-2011	Western Blot (Repeat 2)
11, 12, 13, 15, 18, & 19-Jul-2011	20-Jul-2011	Report and Raw Data Review; Test Substance Container Verification

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.

Tracey Froggatt
Dow AgroSciences, Quality Assurance

21-Jul-2011
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ABSTRACT

The purpose of this study was to characterize the biochemical properties of the recombinant AAD-12 and 2mEPSPS proteins derived from the transgenic soybean event DAS-444Ø6-6 (event 44Ø6) and compare them with the previously characterized *Pseudomonas fluorescens*-derived proteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, stained with Coomassie blue and glycoprotein detection methods), western blot, lateral flow strip assay (LFS), and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses were used to characterize the biochemical properties of both proteins. In addition, enzymatic activity and glyphosate tolerance of the 2mEPSPS was verified. Using these methods, the AAD-12 and 2mEPSPS proteins from *P. fluorescens* and transgenic soybean (event 44Ø6) were shown to be biochemically equivalent. These data support the use of the microbial-derived proteins for use in studies supporting the registration of transgenic soybeans expressing the AAD-12 and 2mEPSPS proteins.

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ABBREVIATIONS

AAD-12	aryloxyalkanoate dioxygenase-12 protein
ACTH	adrenocorticotropic hormone
C-terminus	carboxyl-terminus
ESI-LC/MS	electrospray ionization-liquid chromatography mass spectrometry
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kDa	kiloDalton
L	liter
LFS	lateral flow strip assay
LSB	Laemmli sample buffer
MALDI-TOF MS	matrix assisted laser desorption/ionization time-of-flight mass spectrometry
µg	microgram
mA	milliAmps
µL	microliter
min	minutes
ng	nanogram
N-terminus	amino-terminus
PBS	phosphate buffered saline, pH 7.4
PBST	phosphate buffered saline + 0.05% Tween 20, pH 7.4
PEP	phosphoenolpyruvate
PMF	peptide mass fingerprinting
PMSF	phenylmethylsulphonyl fluoride
PVPP	polyvinylpyrrolidone
S3P	shikimate-3-phosphate
sec	seconds
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TSN	test substance number

INTRODUCTION

The purpose of this study was to characterize the biochemical properties of the recombinant AAD-12 and 2mEPSPS proteins derived from the transgenic soybean event DAS-444Ø6-6 (Event 4406) and compare them with the previously characterized microbe-derived AAD-12 (Karnoup and Kuppannan 2008; Embrey 2011) and 2mEPSPS proteins (Karnoup and Kuppannan 2010; Schafer 2010). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, stained with Coomassie blue and glycoprotein detection methods), western blot, lateral flow test strips, enzymatic assay, and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) peptide analysis were used to characterize the biochemical properties of the protein.

The biochemical and immunological methods employed in this study are among those that have been well established for protein analysis. SDS-PAGE separates proteins based on the apparent molecular weight (mass). Western blotting of proteins to a membrane support following SDS-PAGE, and immunodetection with a protein specific antibody are widely used to identify the authenticity and relative quantity of a molecule in a crude preparation (in addition to LFS). In addition, staining for carbohydrate moieties linked to polypeptides (following electrophoresis) is a standard test to detect post-translational glycosylation of proteins. Peptide mass fingerprinting and sequence analysis (by MALDI-TOF-MS and MALDI-TOF-MS/MS following proteolysis) are among the most powerful tools for comparing the amino acid sequence of related proteins.

MATERIALS AND METHODS

Test Substance/Test System

The seeds (T4 generation) from event DAS-444Ø6-6 were used to produce the tissue containing the AAD-12 and 2mEPSPS transgenes [(Source ID: YX09KX590371.018 and YX09KX950434) — planted on June 23rd and July 7th, 2010 and harvested on September 9, 2010]. Leaf punches from each individual plant were harvested fresh at the Dow AgroSciences greenhouse in

Indianapolis, IN and were analyzed by a lateral flow strip assay (American Bionostica Inc.) to confirm the presence of the AAD-12 protein (through inference, the plants were considered to contain 2mEPSPS as it is part of the molecular stack). After confirmation of the presence of the AAD-12 protein, all above ground- and root-tissues were harvested separately and transported to the laboratory and processed as follows. The tissues were placed in bags, frozen at -80 °C, and placed in a lyophilizer to remove moisture. After the tissues were completely dry, they were ground to a fine powder with dry ice and stored at -80 °C until needed. As needed throughout the study, additional plantings were required to produce fresh tissue for protein isolation. All plantings and harvests were documented in the study file.

Control Substance

1. The control substance used in this study was a non-transgenic soybean plant extract (*Glycine max* cv Maverick). Seeds of the Maverick soybean line (Source ID: YX09KX540002) were planted, grown, harvested, and processed under the same conditions as the transgenic plants described above. Soybean leaf punches were harvested fresh at the Dow AgroSciences greenhouse in Indianapolis, IN and each individual plant was tested by a LFS assay (American Bionostica Inc.) to confirm the absence of the AAD-12 protein (2mEPSPS was also considered to be absent).

2. Recombinant AAD-12 microbial protein, (Lot #: 466-028A), molecular weight: 31.7 kDa, 35.3% active ingredient (a.i.) AAD-12 to powder mass (Embrey 2011). The microbial preparation was produced and purified from *P. fluorescens* (Lin 2006). The lyophilized powder was sent to the Test Substance Coordinator and designated TSN030732-0001.

3. Recombinant 2mEPSPS microbial protein (Lot #: DMMG_033110), molecular weight: 43.7 kDa, ~66.5% active ingredient (a.i.) 2mEPSPS to powder mass (Schafer 2010). The microbial preparation was produced and purified from *P. fluorescens* (Lin 2010). The lyophilized powder was sent to the Test Substance Coordinator and designated TSN033171-0001.

Table 1: Commercially available reference substances used (non-GLP):

Reference Substance	Product Name	Assay	Supplier
Soybean Trypsin Inhibitor (STI)	A component of the GelCode glycoprotein staining kit	Glycosylation assay	Thermo-Pierce
Horseradish Peroxidase (HRP)	A component of the GelCode glycoprotein staining kit	Glycosylation assay	Thermo-Pierce
Bovine Serum Albumin Fraction V (BSA)	Pre-diluted BSA protein assay standard set	SDS-PAGE, western blot, & glycosylation assay	Thermo-Pierce
Protein Standard Markers	Novex Sharp unstained protein standards	SDS-PAGE, glycosylation assay & western blot analysis	Invitrogen: molecular weight markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10 and 3.5 kDa
Prestained Protein Standard Markers	Novex Sharp prestained protein standards		
des-Arg1-Bradykinin 904.4681 Da Angiotensin I 1,296.6853 Da Glu1-Fibrinopeptide 1,570.6774 Da ACTH (clip 1–17) 2,093.0867 Da ACTH (clip 18–39) 2,465.1989 Da ACTH (clip 7–38) 3,657.9294 Da	Applied Biosystems 4700 Proteomic Analyser Mass Standards Kit	MALDI MS & MALDI MS-MS	Applied Biosystems

Note: ACTH = adrenocorticotropic hormone

Purification of the AAD-12 protein

The AAD-12 protein was extracted from the lyophilized root tissue with a Tris-based buffer (see Table 2 for buffer components) by blending approximately 12.0 grams of lyophilized root in a Waring blender cup containing 120 mL of extraction buffer.

Table 2: Soybean extraction buffer

Ingredient	Supplier	Final Concentration
1M Tris-HCl	Sigma	50 mM
Sodium Ascorbate	Sigma	20 mM
Sodium Metabisulfite	Sigma	10 mM
Protease Inhibitor Cocktail	Sigma	0.5%
NaCl	N/A	250 mM

Notes:

a. The buffer pH was adjusted to 8.0 before bringing buffer up to final volume.
b. The buffer was made fresh the day of use.

The blended material was strained through 4 layers of cheesecloth and the tissue was re-extracted with an additional 120 mL of buffer as described earlier. The cheesecloth filtrates were combined and centrifuged at 10,000 ×g for 20 minutes and the resulting supernatant (160 mL) was filtered through diatomaceous earth. Ammonium sulfate was slowly added to the extract to a concentration of 1 M and the extract was stirred for 1 hour at 4 °C and centrifuged at 10,000 ×g for 20 minutes. The supernatant was collected and filtered through a 0.2 µ nylon filter and the extract was loaded onto a 5 mL Phenyl HP Hi-Trap column (GE Healthcare) equilibrated with 1 M ammonium sulfate, 50 mM Tris, pH 8.0. The column was washed with 2 CV (column volume) of the equilibration buffer and the bound proteins were eluted with a 20 CV elution gradient to 100% 5 mM Tris, pH 8.0. After the gradient, the column was eluted with 20 CV of elution buffer. Five-milliliter fractions were collected and assayed for AAD-12 content by ELISA (Acadia BioScience, LLC) and by western blot analysis. Fractions containing the AAD-12 protein were pooled and PVPP (Sigma) was added to a final concentration of 3% at 4 °C. The sample was centrifuged 10,000 ×g for 10 min and the resulting supernatant was applied onto an immunoaffinity column that consisted of covalently cross-linked anti-AAD-12 monoclonal antibody (mAb 539B304, lot #: 609.04-2-4) to a Protein A/G resin (Thermo-Pierce). The affinity resin was equilibrated with 25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol; pH 7.4 (equilibration buffer) and the PVPP treated sample was incubated with the resin on an orbital shaker for 1 hour at room temperature. The resin was pelleted by centrifugation at 1000 ×g for 1 min and was transferred to a gravity flow column and washed with 15 mL of equilibration buffer followed by 2.5 mL of conditioning buffer (Thermo-Pierce). The bound

AAD-12 protein was eluted with 6.5 mL of elution buffer (Thermo-Pierce) into tubes containing 1 M Tris HCl (Sigma). Six fractions were collected and analyzed by SDS-PAGE, western blot, and ELISA. The flow-through from the column contained AAD-12 protein and was reapplied to the column and purified as previously described. Fractions containing the AAD-12 protein were pooled (5 fractions total) and buffer exchanged into 5 mM ammonium bicarbonate using a PD-10 column (GE Healthcare). Seven 1-mL aliquots were collected, lyophilized and stored at - 80 °C until used for subsequent analyses.

Purification of 2mEPSPS protein

The 2mEPSPS protein was extracted from lyophilized leaf tissue with a HEPES-based buffer (see Table 3 for buffer components) by weighing ~2.5 g of lyophilized tissue into a Waring blender cup and adding ~50 mL of buffer. The tissue was blended for 45 seconds followed by stirring on a magnetic stir-plate at 4 °C for an additional five minutes. The extract was strained through four layers of cheesecloth and the filtrate was collected and centrifuged at 10,000 ×g for 20 minutes. Ammonium sulfate was added to the clarified extract slowly to a final concentration of 2.0 M and stirred on a magnetic stir plate at 4 °C for one hour. The solution was then centrifuged at 10,000 ×g for 10 minutes and approximately 30 mL of supernatant was recovered. The supernatant was then filtered through a 0.2 µm nylon syringe filter and loaded onto a 1 mL Phenyl HP Hi-trap column (GE Healthcare) equilibrated with 50 mM HEPES, 2.0 M ammonium sulfate, pH 7.0. After loading, the column was washed with 2 CV of phenyl equilibration buffer and the bound proteins were eluted with a 20 CV gradient to 100% phenyl elution buffer (50 mM HEPES, pH 7.0). The eluted proteins were collected in 1-mL fractions. The fractions were assayed for 2mEPSPS content by western blot using a mouse monoclonal antibody raised against the 2mEPSPS protein (DAS mAb lot 609.48A-2-4) and detected with a goat, anti-mouse IgG horseradish peroxidase conjugated secondary antibody (Thermo-Pierce). The membrane was developed using ECL substrate according to the manufacturer's protocol (GE Healthcare) and exposed to X-Ray film (Thermo-Pierce). Fractions containing 2mEPSPS were pooled and two 2.5-mL aliquots were sequentially desalted into 50 mM HEPES, pH 7.0 buffer using a PD-10 desalting column (GE Healthcare) according to manufacturer's protocol. The Phenyl HP Pool

(~7.5 mL) was then loaded onto a 1-mL Q Sepharose FF Hi-Trap Column (GE Healthcare) equilibrated with 50 mM HEPES, pH 7.0. After loading, the column was washed with 2 CV of the same buffer and eluted with a gradient over 20 column volumes to 100% Q elution buffer (50 mM HEPES, 500 mM NaCl, pH 7.0). The eluted proteins were collected in 1-mL fractions. Fractions were assayed for 2mEPSPS content by western blot as described above. Fractions containing 2mEPSPS were pooled and desalted into 50 mM HEPES, pH 7.0 buffer using a PD-10 desalting column. The desalted Q-pool (~7.0 mL) was then loaded onto a 5 mL Blue Sepharose HP Hi-Trap column (GE Healthcare) equilibrated with 50 mM HEPES, pH 7.0 buffer. The column was washed with 2 CV of the same buffer and the bound protein was eluted with a gradient over 20 column volumes to 100% Blue elution buffer (50 mM HEPES, 500 mM NaCl, pH 7.0). Five-mL fractions were collected and assayed for 2mEPSPS content by Coomassie staining a SDS-PAGE gel. The fraction (1.5 mL) containing the highest level of 2mEPSPS by visual inspection was concentrated to ~130 µL using a 10,000 kDa MWCO centrifugal filter device (Amicon) according to the manufacture's protocol. The final purified sample was used for subsequent analyses.

Table 3: Composition of Extraction Buffer for Soybean-Derived 2mEPSPS

Ingredient	Supplier	Final Concentration
HEPES	Fisher	50 mM
Sodium Ascorbate	Sigma	20 mM
Sodium Metabisulfite	Sigma	10 mM
Polyvinylpyrrolidone	Sigma	1.0%
Protease Inhibitor Cocktail	Sigma	0.5%

Notes:

a. The buffer pH was adjusted to 7.0 before bringing buffer up to final volume.
b. The buffer was made fresh the day of use.

SDS-PAGE and polyclonal antibody western blot analysis of AAD-12 and 2mEPSPS proteins

The soybean leaf tissues of the transgenic and nontransgenic events were harvested fresh from the greenhouse on the day of testing. Extracts were prepared by Geno-Grinding (Spex) ~30 mg of tissue with steel ball bearings in a Tris based buffer (Table 4) containing PVP for 3 minutes in

a chilled Teflon microfuge tube holder. For the SDS-PAGE gel, the supernatants were clarified by centrifuging the samples at 4 °C for 5 minutes at 20,000 ×g and 15 µL of each extract was mixed with 15 µL of 2x Laemmli sample buffer (Bio-Rad, containing freshly added β - mercaptoethanol) and heated for 5 minutes at ~95 °C. The western blot analysis of the recombinant proteins required the extracts to be further diluted (data not shown). After a brief centrifugation (2 min @ 20,000 ×g), the resulting supernatants were loaded directly on the gel. The reference standards, microbe-derived AAD-12 (TSN030732-0001), 2mEPSPS (TSN033171-0001), and control standard, BSA (Thermo-Pierce), were prepared and diluted to a concentration that was similar to the expression level observed in DAS-444Ø6-6 and mixed with Bio-Rad 2x Laemmli sample buffer and processed as described earlier. SDS-PAGE and western blot analysis of the recombinant AAD-12, 2mEPSPS, BSA, nontransgenic Maverick, and DAS-444Ø6-6 soybean extracts were performed with Bio-Rad Criterion gels (Bio-Rad) fitted in a Criterion Cell gel module with XT-MES running buffer (Bio-Rad). Two gels were prepared and the electrophoresis was conducted at a constant voltage of 150 V for ~60 minutes. After separation, one gel was stained with Thermo Scientific GelCode Blue protein stain and scanned with a densitometer (GE Healthcare) to obtain a permanent record of the image. The remaining gel was electro-blotted to a nitrocellulose membrane (Bio-Rad) with a Criterion trans-blot electrophoretic transfer cell for 60 minutes under a constant voltage of 100 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad. After transfer, the membrane was cut in half and one half was probed with an AAD-12-specific rabbit polyclonal antibody (Lot #: DAS F1197-167-2, 1.0 mg/mL) and the remaining half was probed with a 2mEPSPS-specific rabbit polyclonal antibody (Lot #: G2874, 3.3 mg/mL). A conjugate of goat anti-rabbit IgG (H+L) antibody and horseradish peroxidase (Thermo-Pierce) was used as the secondary detection antibody. GE Healthcare chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membranes were exposed to CL-XPosure detection film (Thermo Scientific) for various time points and subsequently developed with an All-Pro 100 film developer.

Table 4: Western blot extraction buffer

Ingredient	Supplier	Final Concentration
1M Tris-HCl	Sigma	50 mM
Sodium Ascorbate	Sigma	20 mM
Sodium Metabisulfite	Sigma	10 mM
PVP	Sigma	0.7%
2-mercaptoethanol	Bio-Rad	0.2%
Protease Inhibitor Cocktail	Sigma	3.3%
NaCl	N/A	250 mM

Notes:

a. The buffer pH was adjusted to 8.0 before bringing buffer up to final volume.
b. The buffer was made fresh the day of use.

SDS-PAGE and monoclonal antibody western blot analysis of the 2mEPSPS protein

The soybean leaf tissues of the transgenic and nontransgenic events were harvested fresh from the greenhouse and stored at -80 °C until used for testing. On the day of analysis, soybean leaf material was ground in liquid nitrogen using a pre- chilled mortar and pestle and approximately 30 mg of tissue was transferred to a micro-centrifuge tube and the soluble proteins were extracted by Geno-Grinding as described earlier. For the SDS-PAGE gel, the supernatants were clarified by centrifuging the samples at 4 °C for 5 minutes at 20,000 ×g and 50 µL of each extract was mixed with 50 µL of 2x Laemmli sample buffer (Bio-Rad, containing freshly added β -mercaptoethanol) and heated for 5 minutes at ~95 °C. After a brief centrifugation (2 min @ 20,000 ×g), the resulting supernatants were loaded directly on the gel. The reference standard, 2mEPSPS (TSN033171-0001), and control standard, BSA (Thermo-Pierce), were prepared and diluted to a concentration that was similar to the expression level observed in DAS-444Ø6-6 and mixed with Bio-Rad 2x Laemmli sample buffer and processed as described earlier. SDS-PAGE and western blot analysis of the recombinant 2mEPSPS, BSA, nontransgenic Maverick, and DAS-444Ø6-6 soybean extracts were performed with Bio-Rad Criterion gels (Bio-Rad) fitted in a Criterion Cell gel module with XT-MES running buffer (Bio-Rad). One gel was prepared and the electrophoresis was conducted at a constant voltage of 150 V for ~60 minutes. After

separation the gel was cut in half and one half of the gel was stained with Thermo Scientific GelCode Blue protein stain and scanned with a densitometer (GE Healthcare) to obtain a permanent record of the image. The remaining gel half was electro-blotted to a nitrocellulose membrane (Bio-Rad) with a Criterion trans-blot electrophoretic transfer cell for ~60 minutes under a constant voltage of 100 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad. After transfer, the membrane was probed with a 2mEPSPS-specific mouse monoclonal antibody (Lot #: 609.48A-2-4, 2.1 mg.mL). A conjugate of goat anti-mouse IgG (H+L) antibody and horseradish peroxidase (Thermo-Pierce) was used as the secondary detection antibody. GE Healthcare chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membranes were exposed to CL-XPosure detection film (Thermo Scientific) for various time points and subsequently developed with an All-Pro 100 film developer.

Detection of post-translational glycosylation (AAD-12)

The immunoaffinity chromatography-purified, soybean-derived AAD-12 protein (lyophilized, desalted pool) was resuspended with 1x Laemmli sample buffer (LSB). The microbe-derived AAD-12, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase were diluted with 2x Bio-Rad LSB to the approximate concentration of the concentrated plant-derived AAD-12 protein. After mixing the proteins with Laemmli sample buffer, the proteins were heated at ~95 °C for 5 minutes and centrifuged at 20000 ×g to obtain a clarified supernatant. The resulting supernatants were applied directly to a Bio-Rad Criterion Gel and electrophoresed for 50 minutes at 125 V. After electrophoresis, the gel was first stained with a GelCode Glycoprotein Staining Kit (Thermo-Pierce) according to the manufacturers' protocol to visualize the glycoproteins. The glycoproteins were visualized as magenta bands on a light pink background. After the glycoprotein staining was complete, the gel was scanned with a GE Healthcare densitometer to obtain a permanent visual record of the gel. Once the gel and gel scans were verified by two independent scientists, the gel was stained for total protein with GelCode Blue stain according to the manufacturers' protocol. After the second staining was complete, the gel was scanned with a densitometer to obtain a permanent visual record of the gel.

Detection of post-translational glycosylation (2mEPSPS)

The immunoaffinity chromatography-purified, soybean-derived 2mEPSPS protein (Blue Fraction #19) was mixed 1:1 with 2x LSB. The microbe-derived 2mEPSPS, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase were diluted with 2x Bio-Rad LSB to the approximate concentration of the concentrated plant-derived 2mEPSPS protein. After mixing the proteins with Laemmli sample buffer, the proteins were heated at ~95 °C for 5 minutes and centrifuged at 20,000 ×g for 2 minutes to obtain a clarified supernatant. The resulting supernatants were applied directly to a Bio-Rad Criterion Gel and electrophoresed for 61 minutes at 150 V. After electrophoresis, the gel was cut in half and one half was stained with GelCode Blue stain for total protein according to the manufacturers' protocol. After the staining was complete, the gel was scanned with a densitometer to obtain a permanent visual record of the gel. The remaining half of the gel was stained with a GelCode Glycoprotein Staining Kit (Thermo-Pierce) according to the manufacturers' protocol to visualize the glycoproteins. The glycoproteins were visualized as magenta bands on a light pink background. After the glycoprotein staining was complete, the gel was scanned to obtain a permanent visual record of the gel.

Mass spectrometry peptide mass fingerprinting and sequence analysis of soybean- and *P. fluorescens*-derived AAD-12

Matrix Assisted Laser Desorption Ionization – Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) and Electrospray Ionization-Liquid Chromatography Mass Spectrometry (ESI-LC/MS) analysis of the microbe-derived AAD-12 protein (TSN030732) was conducted at the Analytical Sciences Laboratory of The Dow Chemical Company (Midland, MI). MALDI-TOF MS/MS of the soybean-derived AAD-12 protein (lyophilized, desalted pool purified from DAS-444Ø6-6) was conducted at Dow AgroSciences Biotechnology Regulatory Sciences Group (Indianapolis, IN). The analytical summaries, which contain the methods and results, can be found in Appendix 2 and Appendix 3.

Mass spectrometry peptide mass fingerprinting and sequence analysis of soybean- and *P. fluorescens*-derived 2mEPSPS

MALDI-TOF MS and ESI-LC/MS analysis of the microbe-derived 2mEPSPS protein (TSN033171) was conducted at the Analytical Sciences Laboratory of The Dow Chemical Company (Midland, MI) and Dow AgroSciences (Indianapolis, IN). MALDI-TOF MS/MS analysis of the soybean-derived 2mEPSPS (Blue Fraction #19) was conducted at the Dow AgroSciences Biotechnology Regulatory Sciences Group (Indianapolis, IN). The analytical summaries, which contain the methods and results, can be found in Appendix 4 and Appendix 5.

Enzymatic assay of soybean-derived 2mEPSPS protein

The enzyme activity of the soybean-derived 2mEPSPS protein was measured by inorganic phosphate (P_i) production in a modified procedure described by Lanzetta (Lanzetta, Alvarez et al. 1979). Assays were performed in 96-well plate format in a total of 50 µL on a Spectra-Max M2e plate reader (Molecular Devices). Typical assays contained 50 mM HEPES, 100 mM KCl, 1 mM dithiothreitol, pH 7.5, and 1 mM S3P (shikimate-3-phosphate). PEP (phosphoenolpyruvate) and glyphosate concentrations were varied as indicated in the study file. Glyphosate was obtained from Sigma as the free acid and was resuspended in Milli-Q H_2O. Glyphosate was solubilized by addition of NaOH until the mixture was at a neutral pH. Assays were initiated by addition of 2.5 µL (~0.075 µM) of the soybean-derived 2mEPSPS enzyme. Reactions were terminated by the addition of 235 µL of a 3:1 mixture of malachite green:ammonium molybdate solution. After complete color development (2 min for K_m and 4 min for glyphosate inhibition measurements), the absorbance change at 660 nm was recorded and the amount of P_i formed was calculated from a standard curve. Control reactions lacking enzyme were used to correct for background absorbance. The data were fitted to the Michaelis-Menten equation which allowed for the determination of K_m and V_{max} (Equation 1). Data was analyzed using GraFit version 5 software (Erithacus Software Limited).

Equation 1

$$v = \frac{V_{max}.[S]}{K_m + [S]}$$

Data Analysis and Calculations

SOFTmax PRO software was used with the Molecular Devices plate reader. SOFTmax PRO allows the creation of computer generated data files containing all of the parameters required for acquiring and analyzing data from any MAXline instrument. The calibration curve for the AAD-12 ELISA kit was constructed using a quadratic curve regression of the known concentration of the standard calibration solutions and their subsequent absorbance (optical density).

The equation fits the best parabola to the standard curve based on Equation 2:

Equation 2

$$y = A + Bx + Cx^2$$

Where:

y = mean absorbance value (OD)
x = protein concentration

The SOFTmax PRO software was used to determine the presence or absence of the AAD-12 proteins in each sample. The absorbance value and calculated concentration, as well as individual well results, mean sample result, standard deviation and the percent coefficient of variation are reported on the SOFTmax PRO data report but were not used in the final report.

Statistical Treatment of Data

No statistical treatments were used during this study.

RESULTS AND DISCUSSION

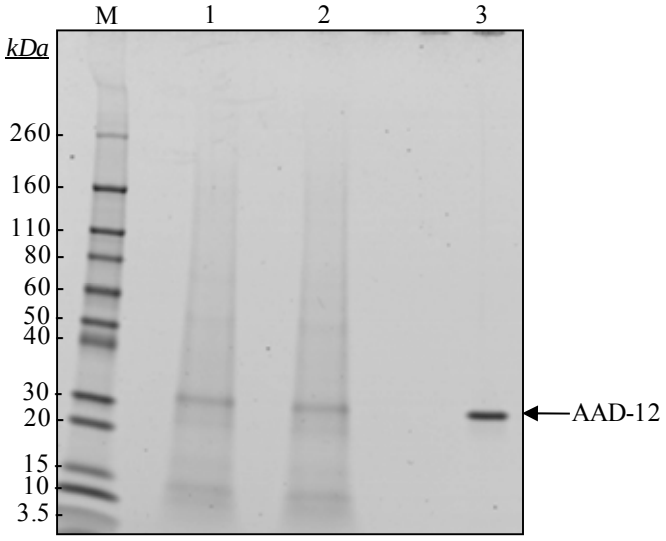
Lateral Flow Test Strip Assay

The presence of the AAD-12 protein (and by inference the 2mEPSPS protein) in the fresh leaf tissue (T4 generation) of event DAS-444Ø6-6 was confirmed using commercially prepared lateral flow test strips from American Bionostica Inc. The strips, capable of detecting between 1-10 ppb of AAD-12, easily discriminated between transgenic and non-transgenic tissue. All of the transgenic plants were positive for AAD-12 protein and none of the non-transgenic extracts of Maverick contained detectable amounts of immunoreactive protein. This result was also confirmed by the western blot analysis using polyclonal antibodies specific to the AAD-12 and 2mEPSPS proteins.

Purification of the AAD-12 protein from transgenic event DAS-444Ø6-6 root extracts

Hydrophobic interaction chromatography followed by monoclonal antibody based immunoaffinity chromatography was conducted on an aqueous extract of ~12 grams of lyophilized DAS-444Ø6-6 transgenic root (grown from T4 seed). The protein that bound to the respective columns was examined by SDS-PAGE, ELISA, and western blot (ELISA and western blot data not shown) which demonstrated that the final concentrated fractions contained the AAD-12 protein at an approximate molecular weight of 32 kDa (Figure 1). Once isolated, the soybean-derived AAD-12 was then compared with the microbe-derived protein as described below.

Figure 1: SDS-PAGE analysis of DAS-444Ø6-6 derived AAD-12 protein

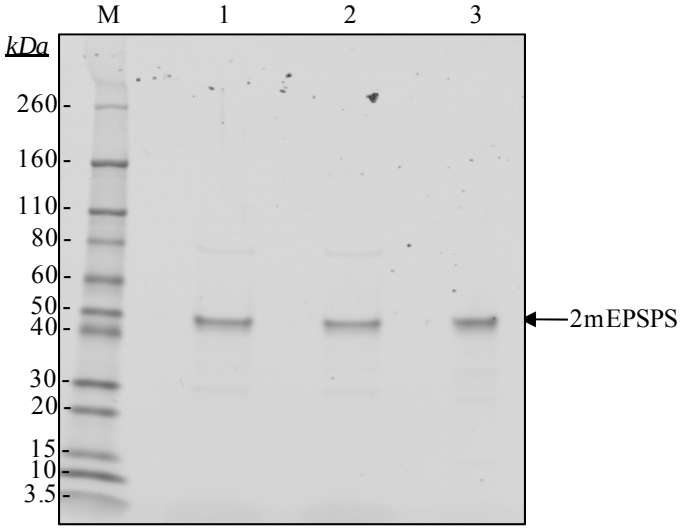


Lane	***Sample***	***Amount***
M	Novex Sharp Protein Standard	10 µL
1	DAS-44406-6 derived-AAD-12	~200 ng
2	DAS-44406-6 derived-AAD-12	~200 ng
3	*P. fluorescens* derived AAD-12	780 ng

Purification of the 2mEPSPS protein from transgenic event DAS-444Ø6-6 leaf extracts

Hydrophobic interaction chromatography, anion-exchange, and Blue Sepharose chromatography was conducted on an aqueous extract of ~2.5 grams of lyophilized DAS-444Ø6-6 transgenic leaf (grown from T4 seed). The protein that bound to the respective columns was examined by SDS-PAGE and western blot (data not shown) which demonstrated the final concentrated fractions contained the 2mEPSPS protein at an approximate molecular weight of 47 kDa (Figure 2). Once isolated, the soybean-derived 2mEPSPS protein was compared with the microbe-derived protein as described below.

Figure 2: SDS-PAGE analysis of DAS-44406-6 derived 2mEPSPS protein



Lane	Sample	Amount
M	Novex Sharp Protein Standard	10 µL
1	DAS-44406-6 derived-2mEPSPS	~500 ng
2	DAS-44406-6 derived-2mEPSPS	~500 ng
3	*P. fluorescens* derived-2mEPSPS	520 ng

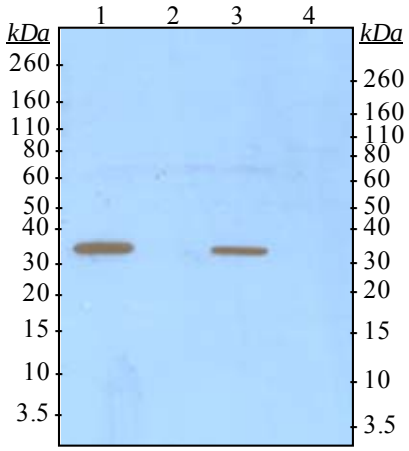
SDS-PAGE analysis of soybean-derived AAD-12 and 2mEPSPS proteins

In the toxicology-lot preparations of *P. fluorescens*-produced AAD-12 (TSN030732) and 2mEPSPS (TSN033171) the major protein bands, as visualized on Coomassie stained SDS-PAGE gels, were approximately 32 and 47 kDa, respectively. As expected, the corresponding soybean-derived proteins were identical in size to the microbe-expressed proteins (Figure 1 and Figure 2) suggesting no major post-translational modifications (e.g. glycosylation) occurred *in planta*. Predictably, the plant purified fractions contained a minor amount of impurities in addition to the recombinant proteins. The co-eluted proteins were likely retained by the columns by weak interactions with the column matrix or antibody leaching from the immunoaffinity column under the harsh elution conditions. Other researchers have also reported the non-specific adsorption of peptides and amino acids on Sepharose column matrices (Holroyde, Chesher et al. 1976; Kennedy and Barnes 1983; Williams, Norcross et al. 2006) as well as antibody leaching from the column (Goldberg, Knudsen et al. 1991).

Western blot analysis of soybean-derived AAD-12 proteins

The microbe-derived AAD-12 protein showed a positive signal of the expected size by polyclonal western blot analysis (32 kDa). This was also observed in the DAS-444Ø6-6 transgenic soybean leaf extract (Figure 3, lane 3).

Figure 3: Polyclonal antibody western blot of *P. fluorescens*- and DAS-444Ø6-6 derived AAD-12 protein



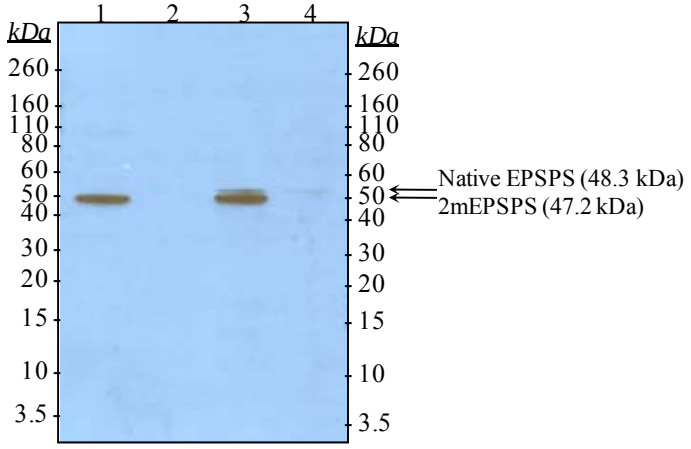
Lane	*Sample*	*Amount Loaded*
kDa	Novex Pre-stained MW Marker	
1	Microbe derived AAD-12 (TSN030732-0001)	~1 ng
2	Bovine Serum Albumin (BSA)	~1 ng
3	Event DAS-444Ø6-6 Leaf Extract	10 µL
4	Non-transgenic (Maverick) Leaf Extract	10 µL
kDa	Novex Pre-stained MW Marker	

In the AAD-12 western blot analysis, no immunoreactive proteins were observed in the control Maverick extract and no alternate size proteins (aggregates or degradation products) were seen in the transgenic samples. These results add to the evidence that the protein expressed in soybean was not glycosylated which would add to the overall protein molecular weight.

Western blot analysis of soybean-derived 2mEPSPS proteins

The microbe-derived 2mEPSPS protein showed a positive signal of the expected size by polyclonal western blot analysis (47.2 kDa). This was also observed in the DAS-444Ø6-6 transgenic soybean leaf extract (Figure 4, lane 3).

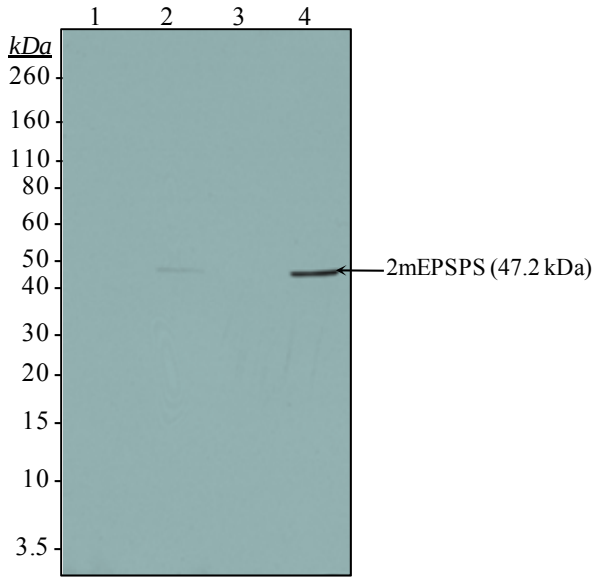
Figure 4: Polyclonal antibody western blot of *P. fluorescens*- and DAS-444Ø6-6 derived 2mEPSPS protein



Lane	Sample	Amount Loaded
kDa	Novex Pre-stained MW Marker	
1	Microbe-derived 2mEPSPS (TSN033171)	~1 ng
2	Bovine Serum Albumin (BSA)	~1 ng
3	Event DAS-444Ø6-6 Leaf Extract	10 µL
4	Non-transgenic (Maverick) Leaf Extract	10 µL
kDa	Novex Pre-stained MW Marker	

However, in the 2mEPSPS western blot analysis, the native soybean EPSPS protein was also observed (48.3 kDa) in the control Maverick extract as well as the transgenic event DAS-444Ø6-6 extract. This result was expected as the native soybean endogenous EPSPS has >75% homology to the 2mEPSPS protein and likely cross-reacted with the polyclonal antibody. To prove this hypothesis, a monoclonal antibody (lot #: 609.48A-2-4) was used to probe the soybean leaf extracts and only the 2mEPSPS protein was detected (Figure 5, lane 4). This result added evidence that the polyclonal antibody was reacting to the native EPSPS protein at the expected molecular weight (48.3 kDa).

Figure 5: Monoclonal antibody western blot of *P. fluorescens*- and DAS-444Ø6-6 derived 2mEPSPS protein



Lane	Sample	Amount Loaded
kDa	Novex Pre-stained MW Marker	
1	Bovine Serum Albumin (BSA)	~1 ng
2	Microbe-derived 2mEPSPS (TSN033171)	~1 ng
3	Non-transgenic (Maverick) Leaf Extract	10 µL
4	Event DAS-444Ø6-6 Leaf Extract	10 µL

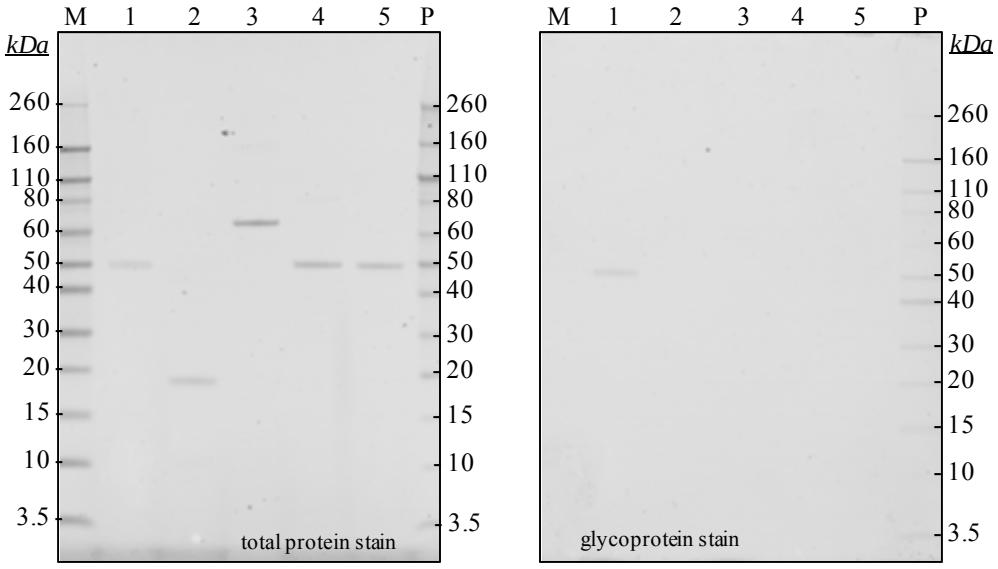
In both the polyclonal and monoclonal analyses, no alternate size proteins (aggregates or degradation products) were seen in the transgenic samples. These results add to the evidence that the protein expressed in soybean is not glycosylated or post-translationally modified which would add to the overall protein molecular weight.

Detection of possible glycosylation of soybean-derived AAD-12 and 2mEPSPS proteins

Detection of carbohydrates, possibly covalently linked to soybean-derived AAD-12 and 2mEPSPS proteins, was assessed by the GelCode Glycoprotein Staining Kit from Thermo-Pierce. The purified soybean-derived AAD-12 and 2mEPSPS proteins and were electrophoresed simultaneously with a set of control and reference protein standards. A glycoprotein, horseradish peroxidase, was loaded as a positive indicator for glycosylation, and non-glycoproteins; microbe-

derived AAD-12, microbe-derived 2mEPSPS, soybean trypsin inhibitor, and bovine serum albumin, were employed as negative reference controls. The results showed that both the soybean- and microbe-derived 2mEPSPS (Figure 6) and AAD-12 (Figure 7) proteins had no detectable covalently linked carbohydrates.

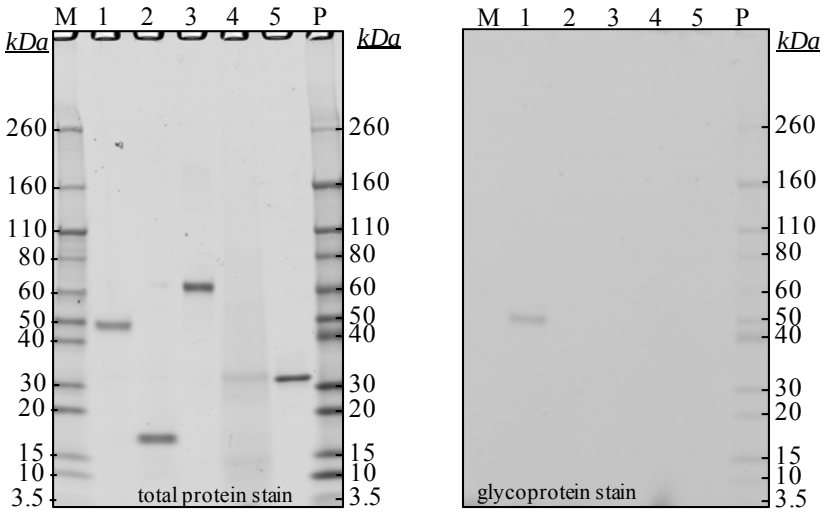
Figure 6: SDS-PAGE gel containing 2mEPSPS protein stained with GelCode Blue total protein and glycoprotein stains



Note: The Novex Sharp protein standard does not appear on the glycoprotein stained gels as they do not contain glycoproteins.

Lane	***Sample***	***Amount Loaded***
M	Novex Sharp Protein Standard	10 µL
1	Horseradish peroxidase (+ control)	~500 ng
2	Soybean trypsin inhibitor (- control)	~500 ng
3	Bovine serum albumin (- control)	~500 ng
4	DAS-44406-6 derived 2mEPSPS	~500 ng
5	*P. fluorescens* derived 2mEPSPS	~500 ng
P	Novex Pre-stained MW Marker	10 µL

Figure 7: SDS-PAGE gel containing AAD-12 protein stained with GelCode Blue total protein and glycoprotein stains



Note: The Novex Sharp protein standard does not appear on the glycoprotein stained gels as they do not contain glycoproteins.

Lane	*Sample*	*Amount Loaded*
M	Novex Sharp Protein Standard	10 µL
1	Horseradish peroxidase (+ control)	~500 ng
2	Soybean trypsin inhibitor (- control)	~500 ng
3	Bovine serum albumin (- control)	~500 ng
4	DAS-444Ø6-6 derived AAD-12	~200 ng
5	*P. fluorescens* derived AAD-12	~500 ng
P	Novex Pre-stained MW Marker	10 µL

Mass spectrometry analysis of the soybean- and microbe-derived AAD-12 proteins

The AAD-12 protein derived from transgenic soybean tissue (event DAS-444Ø6-6) was separated by SDS-PAGE (Figure 1) and the respective bands were excised and subjected to in-gel digestion by trypsin and chymotrypsin. The resulting peptide mixture was analyzed by MALDI-TOF MS/MS to determine the peptide sequences (Appendix 3). The masses of the detected peptides were compared to those deduced based on potential trypsin or chymotrypsin cleavage sites in the sequence of the soybean-derived AAD-12 protein. Figure 8 illustrates the theoretical cleavage of the AAD-12 protein when subjected to endoprotease digestion *in silico*

using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC. The theoretical and observed amino acid digest (and molecular weights) of the soybean-derived AAD-12 protein is also described in Tables I and II of the Mass Spectrometry Report for the AAD-12 protein (Appendix 3). The AAD-12 protein, once denatured, is readily digested by endoproteases and will generate numerous peptide peaks.

Figure 8: Theoretical cleavage of the AAD-12 protein [with trypsin (top panel) and chymotrypsin (bottom panel)] generated in silico using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC.
Alternating blocks of upper (black) and lower (red) case letters within the amino acid sequence are used to differentiate the potential peptides after protease digestion. The numbers on the left and right sides indicate the amino acid residue numbers.

Trypsin cleavage of AAD-12

1	M	A	Q	T	T	L	Q	I	T	P	T	G	A	T	L	G	A	T	V	T	G	V	H	L	A	T	L	D	D	A	30
31	G	F	A	A	L	H	A	A	W	L	Q	H	A	L	L	I	F	P	G	Q	H	L	S	N	D	Q	Q	I	T	F	60
61	A	K	r	F	G	A	I	E	R	i	g	g	g	d	i	v	a	i	s	n	v	k	A	D	G	T	V	R	q	h	90
91	s	p	a	e	w	d	d	m	m	k	V	I	V	G	N	M	A	W	H	A	D	S	T	Y	M	P	V	M	A	Q	120
121	G	A	V	F	S	A	E	V	V	P	A	V	G	G	R	t	c	f	a	d	m	r	A	A	Y	D	A	L	D	E	150
151	A	T	R	a	l	v	h	q	r	S	A	R	h	s	l	v	y	s	q	s	k	L	G	H	V	Q	Q	A	G	S	180
181	A	Y	I	G	Y	G	M	D	T	T	A	T	P	L	R	P	L	V	K	v	h	p	e	t	g	r	p	s	l	l	210
211	i	g	r	H	A	H	A	I	P	G	M	D	A	A	E	S	E	R	f	l	e	g	l	v	d	w	a	c	q	a	240
241	p	r	V	H	A	H	Q	W	A	A	G	D	V	V	V	W	D	N	R	c	l	l	h	r	A	E	P	W	D	F	270
271	K	l	p	r	V	M	W	H	S	R	l	a	g	r	p	e	t	e	g	a	a	l	v								293

Chymotrypsin cleavage of AAD-12

1	M	A	Q	T	T	L	q	i	t	p	t	g	a	t	I	G	A	T	V	T	G	V	H	L	a	t	I	D	D	A	30
31	G	F	a	a	I	H	A	A	W	I	Q	H	A	L	I	I	F	P	G	Q	H	L	s	n	d	q	q	i	t	f	60
61	A	K	R	F	g	a	i	e	r	i	g	g	g	d	i	v	a	i	s	n	v	k	a	d	g	t	v	r	q	h	90
91	s	p	a	e	w	D	D	M	M	K	V	I	V	G	N	M	A	W	h	a	d	s	t	y	M	P	V	M	A	Q	120
121	G	A	V	F	s	a	e	v	v	p	a	v	g	g	r	t	c	f	A	D	M	R	A	A	Y	d	a	I	D	E	150
151	A	T	R	A	L	v	h	q	r	s	a	r	h	s	I	V	Y	s	q	s	k	I	G	H	V	Q	Q	A	G	S	180
181	A	Y	i	g	y	G	M	D	T	T	A	T	P	L	r	p	I	V	K	V	H	P	E	T	G	R	P	S	L	I	210
211	I	G	R	H	A	H	A	I	P	G	M	D	A	A	E	S	E	R	F	I	E	G	L	v	d	w	A	C	Q	A	240
241	P	R	V	H	A	H	Q	W	a	a	g	d	v	v	v	w	D	N	R	C	L	I	H	R	A	E	P	W	d	f	270
271	K	L	P	R	V	M	W	h	s	r	I	A	G	R	P	E	T	E	G	A	A	L	v								293

In the endoproteinase digest of the transgenic-soybean-derived AAD-12 protein, the peptide sequence coverage was excellent (84.3%) and 76.1% of the peptide primary sequence was confirmed by MS/MS analysis. The detected peptide fragments covered nearly the entire protein sequence lacking only 4 peptide fragments (Figure 9), one near the N-terminus (L^{40} to L^{45}), two in the middle of the protein (Q^{89} to Y^{114} and S^{125} to F^{138}) and one near the C-terminus (I^{211} to R^{213}). The protein sequence that was missed did not contain sequence motifs that are typically required for glycosylation (Asn-Xxx-Ser/Thr, (Hamby and Hirst 2008).

Figure 9: Overall sequence coverage of trypsin and chymotrypsin digests for AAD-12 (DAS-444Ø6-6) by MALDI-TOF MS and MALDI MS/MS

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
1	M	A	Q	T	T	L	Q	I	T	P	T	G	A	T	L	G	A	T	V	T	G	V	H	L	A	T	L	D	D	A	30	30	27
31	G	F	A	A	L	H	A	A	W	L	Q	H	A	L	L	I	F	P	G	Q	H	L	S	N	D	Q	Q	I	T	F	60	24	20
61	A	K	R	F	G	A	I	E	R	I	G	G	G	D	I	V	A	I	S	N	V	K	A	D	G	T	V	R	Q	H	90	28	22
91	S	P	A	E	W	D	D	M	M	K	V	I	V	G	N	M	A	W	H	A	D	S	T	Y	M	P	V	M	A	Q	120	6	0
121	G	A	V	F	S	A	E	V	V	P	A	V	G	G	R	T	C	F	A	D	M	R	A	A	Y	D	A	L	D	E	150	16	16
151	A	T	R	A	L	V	H	Q	R	S	A	R	H	S	L	V	Y	S	Q	S	K	L	G	H	V	Q	Q	A	G	S	180	30	30
181	A	Y	I	G	Y	G	M	D	T	T	A	T	P	L	R	P	L	V	K	V	H	P	E	T	G	R	P	S	L	L	210	30	30
211	I	G	R	H	A	H	A	I	P	G	M	D	A	A	E	S	E	R	F	L	E	G	L	V	D	W	A	C	Q	A	240	30	27
241	P	R	V	H	A	H	Q	W	A	A	G	D	V	V	V	W	D	N	R	C	L	L	H	R	A	E	P	W	D	F	270	30	28
271	K	L	P	R	V	M	W	H	S	R	L	A	G	R	P	E	T	E	G	A	A	L	V								293	23	23
																															Tot. a.a. covered =	*247*	*223*

A = Tryptic MS coverage
A = Tryptic MS-MS data
A = Chymotryptic MS coverage
A = Chymotryptic MS-MS data

MALDI PMF sequence coverage = 84.3 %
MALDI MS-MS sequence coverage = 76.1 %

This analysis confirmed the soybean-derived protein amino acid sequence matched that of the microbe-derived AAD-12 [Appendix 2, (Karnoup and Kuppannan 2008)] protein at both the N- and C-terminus as well as a major portion of the internal sequence. In the MS chromatograms, there were several unidentified peptides detected in the enzyme digest preparations (data not shown). Many factors contribute to the formation of these unidentified peptides, such as over digestion (which results in non-specific cleavage), self-digestion products of trypsin and chymotrypsin, as well as random breakage of peptides during ionization. Unidentified peptides do not indicate the protein deviates from the predicted amino acid sequence. Results of these analyses indicate that the amino acid sequence of the soybean-derived AAD-12 protein was equivalent to the *P. fluorescens*-expressed protein characterized earlier (Karnoup and Kuppannan 2008) and (Embrey 2011).

N- and C-terminal sequence of the AAD-12 proteins

The amino acid residues at the N-and C-termini of the soybean-derived AAD-12 protein (immunoaffinity purified from soybean event DAS-444Ø6-6) were determined and compared with the sequence of the microbe-derived protein (Karnoup and Kuppannan 2008). The soybean-derived AAD-12 protein sequences were determined by MALDI-TOF MS/MS (Appendix 3). The chymotrypsin and trypsin digestions were performed on the soybean-derived AAD-12 protein followed by mass spectrometry analysis and two forms of the N-terminus were determined (Table 5, Tables III and IV in Appendix 3).

Table 5: Summary of N-terminal sequence data of AAD-12 soybean- and microbe-derived proteins

Source	Expected N-terminal Sequence[1]
P. fluorescens	M^{1} A Q T T L Q I T P T G A T L G A T V T G V H L A T L^{27}
Soybean Event DAS-444Ø6-6	M^{1} A Q T T L Q I T P T G A T L G A T V T G V H L A T L^{27}

Source	Detected N-terminal Sequence[2, 3, 4]
P. fluorescens	A^{2} Q T T L Q I T P T G A T L G A T V T G V H L A T^{27}
Soybean Event DAS-444Ø6-6	M^{1} A Q T T L Q I T P T G A T L G A T V T G V H L A T^{27}
Soybean Event DAS-444Ø6-6[4]	$^{N\text{-}Ac}A^{2}$ Q T T L Q I T P T G A T L G A T V T G V H L A T^{27}

Notes:

[1]Expected N-terminal sequence of the first 27 amino acid residues of *P. fluorescens*- and soybean-derived AAD-12.

[2]Detected N-terminal sequences of *P. fluorescens*- and soybean-derived AAD-12 (Appendix 2 and 3).

[3]Numbers in superscript (R^{X}) indicate the amino acid residue number in the sequence. The N-terminal amino acid sequence was confirmed by peptide mass fingerprinting and MS/MS sequencing.

[4]The MALDI-TOF MS/MS data for the N-terminal peptide revealed that the soybean-derived AAD-12 protein had a portion of the peptide that was acetylated (*N-Acetyl*-AQTTL).

Amino acid residue abbreviations:

A:	alanine	G:	glycine	H:	histidine
I:	isoleucine	L:	leucine	M:	methionine
P:	proline	Q:	glutamine	S:	serine
T:	threonine	V:	valine		

These results demonstrate that the N-terminus of the AAD-12 protein was intact and as predicted (Table 5 and Figure 8). In addition, a portion of the protein was missing the N-terminal methionine and the second amino acid, alanine, was acetylated (Table 5). This result is encountered frequently with eukaryotic (plant) expressed proteins as approximately 80-90% of the N-terminal residues are modified in such a way (Wellner, Panneerselvam et al. 1990) and (Polevoda and Sherman 2003). This result determined that during or after translation in soybean and *P. fluorescens*, the N-terminal methionine was cleaved by a methionine aminopeptidase (MAP). MAPs cleave methionyl residues rapidly when the second residue on the protein is small, such as Gly, Ala, Ser, Cys, Thr, Pro, and Val (Walsh 2005). Also, it has been shown that proteins with serine and alanine at the N-termini are the most frequently acetylated (Polevoda and Sherman 2002). The two co-translational processes, cleavage of N-terminal methionine residue and N-terminal acetylation, are by far the most common modifications and occur on the vast majority (~85%) of eukaryotic proteins (Polevoda and Sherman 2002). However, examples demonstrating biological significance associated with N-terminal acetylation are rare (Polevoda and Sherman 2000).

The C-terminal sequence of the soybean- and microbe-derived AAD-12 proteins were determined essentially as described above and compared with the expected amino acid sequences. The results indicated the measured sequences (Appendix 2, Table V) were identical to the expected sequences, and both the soybean- and microbe-derived AAD-12 proteins were indistinguishable and unaltered at the C-terminus (Table 6 and Figure 8).

Table 6: Summary of C-terminal sequence data of AAD-12 soybean- and *P. fluorescens*-derived proteins

Source	**Expected C-terminal Sequence[1]**
P. fluorescens	^{281}L A G R P E T E G A A L V^{293}
Soybean Event DAS-444Ø6-6	^{281}L A G R P E T E G A A L V^{293}

Source	**Detected C-terminal Sequence[2]**
P. fluorescens	^{281}L A G R P E T E G A A L V^{293}
Soybean Event DAS-444Ø6-6	^{281}L A G R P E T E G A A L V^{293}

Notes:

[1]Expected C-terminal sequence of the last 13 amino acid residues of *P. fluorescens*- and soybean-derived AAD-12.

[2]Detected C-terminal sequences of *P. fluorescens*- and soybean-derived AAD-12.

Numbers in superscript (R^x) indicate amino acid residue numbers in the sequence.

Amino acid residue abbreviations:

A:	alanine	E:	glutamate	G:	glycine
L:	leucine	P:	proline	R:	arginine
T:	threonine	V:	valine		

Enzymatic assay of soybean-derived AAD-12

The biological equivalence of soybean- and microbe-derived AAD-12 proteins has been demonstrated previously for a soybean event containing the identical *aad-12* gene and expressing the equivalent AAD-12 protein (Kuppannan and Karnoup 2009; Schafer 2009; Cicchillo and Barnett 2011). In the previous characterization, the protein sequence was verified and found to be identical to that expressed in DAS-444Ø6-6 and the enzyme kinetics were found to be equivalent to the *P. fluorescens*-derived protein (Table 7).

Table 7: AAD-12 derived from *P. fluorescens* and transgenic soybean root reaction rates with various substrates.

	Reaction Rates (µM/min)						
	***R*-dichlorprop**	***S*-dichlorprop**	**2,4-D**	***R,S*-fenoxaprop**	**MCPA**	***R,S*-Quizalofop**	***R,S*-haloxyfop**
AAD-12 *Pf*	4.01	21.73	31.55	7.80	26.81	4.26	4.48
AAD-12 Soy Root	4.51	9.89	7.43	2.68	8.23	1.48	1.64

In addition, the excellent tolerance exhibited by DAS-444Ø6-6 to 2,4-D demonstrates its proper function in plants (Simpson 2011). Together these studies indicate biological equivalence between the AAD-12 protein in the *P. fluorescens*-derived preparation and the AAD-12 protein expressed in event DAS-444Ø6-6.

Mass spectrometry analysis of the soybean- and microbe-derived 2mEPSPS proteins

The 2mEPSPS protein derived from transgenic soybean tissue (event DAS-444Ø6-6) was separated by SDS-PAGE (Figure 2) and the respective bands were excised and subjected to in-gel and in-solution digestion by trypsin and chymotrypsin. The resulting peptide mixture was analyzed by MALDI-TOF MS/MS to determine the peptide sequences (Appendix 5). The masses of the detected peptides were compared to those deduced based on potential trypsin or chymotrypsin cleavage sites in the sequence of the soybean-derived 2mEPSPS protein. Figure 10 illustrates the theoretical cleavage of the 2mEPSPS protein when subjected to endoprotease digestion *in silico* using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC.

Figure 10: Theoretical cleavage of the 2mEPSPS protein (with trypsin [top panel] and chymotrypsin [bottom panel]) generated in silico using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC.
Alternating blocks of upper (black) and lower (red) case letters within the amino acid sequence are used to differentiate the potential peptides after protease digestion. The numbers on the left and right sides indicate the amino acid residue numbers.

Trypsin cleavage of 2mEPSPS

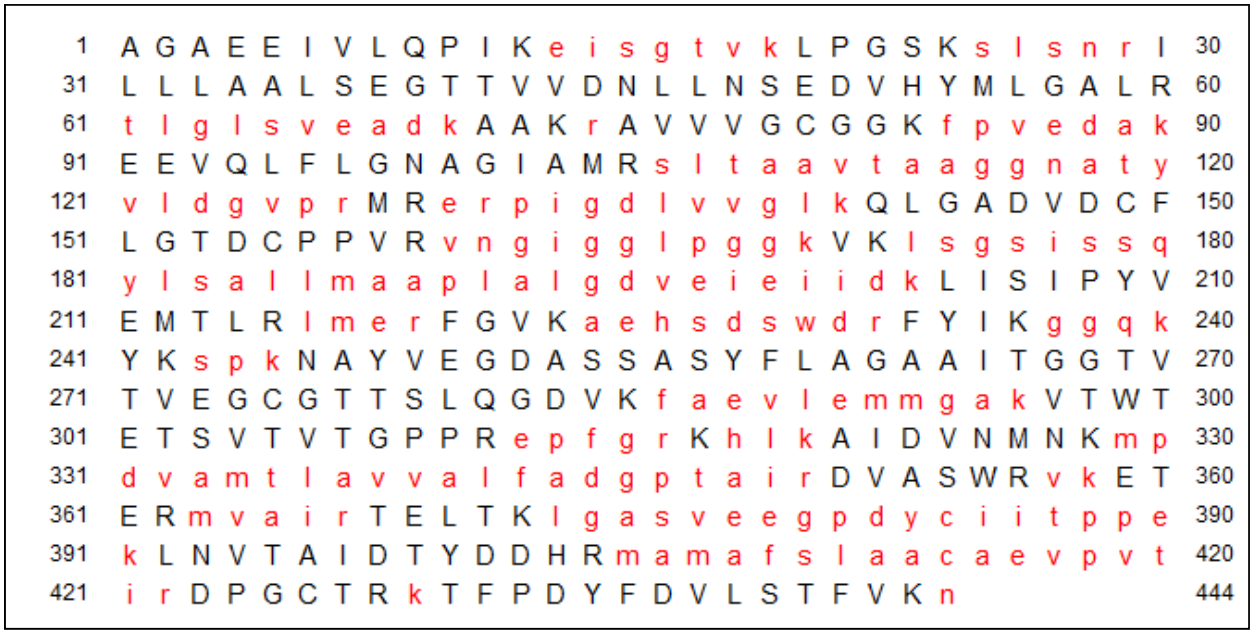


Figure 10: (continued)

Chymotrypsin cleavage of 2mEPSPS

1	A	G	A	E	E	I	V	L	q	p	i	k	e	i	s	g	t	v	k	l	p	g	s	k	s	l	S	N	R	I	30
31	L	l	L	a	a	l	S	E	G	T	T	V	V	D	N	L	l	N	S	E	D	V	H	Y	m	l	G	A	L	r	60
61	t	l	G	L	s	v	e	a	d	k	a	a	k	r	a	v	v	v	g	c	g	g	k	f	p	v	e	d	a	k	90
91	e	e	v	q	l	F	l	G	N	A	G	I	A	M	R	S	L	t	a	a	v	t	a	a	g	g	n	a	t	y	120
121	V	L	d	g	v	p	r	m	r	e	r	p	i	g	d	l	V	V	G	L	k	q	l	G	A	D	V	D	C	F	150
151	l	G	T	D	C	P	P	V	R	V	N	G	I	G	G	L	P	G	G	K	V	K	L	s	g	s	i	s	s	q	180
181	y	L	s	a	l	L	m	a	a	p	l	A	L	g	d	v	e	i	e	i	i	d	k	l	I	S	I	P	Y	v	210
211	e	m	t	l	R	L	m	e	r	f	G	V	K	A	E	H	S	D	S	W	d	r	f	Y	i	k	g	g	q	k	240
241	y	K	S	P	K	N	A	Y	v	e	g	d	a	s	s	a	s	y	F	l	A	G	A	A	I	T	G	G	T	V	270
271	T	V	E	G	C	G	T	T	S	L	q	g	d	v	k	f	A	E	V	L	e	m	m	g	a	k	v	t	w	T	300
301	E	T	S	V	T	V	T	G	P	P	R	E	P	F	g	r	k	h	l	K	A	I	D	V	N	M	N	K	M	P	330
331	D	V	A	M	T	L	a	v	v	a	l	F	a	d	g	p	t	a	i	r	d	v	a	s	w	R	V	K	E	T	360
361	E	R	M	V	A	I	R	T	E	L	t	k	l	G	A	S	V	E	E	G	P	D	Y	c	i	i	t	p	p	e	390
391	k	l	N	V	T	A	I	D	T	Y	d	d	h	r	m	a	m	a	f	S	L	a	a	c	a	e	v	p	v	t	420
421	i	r	d	p	g	c	t	r	k	t	f	p	d	y	F	d	v	l	S	T	F	v	k	n							444

The theoretical and observed amino acid digest (and molecular weights) of the soybean-derived 2mEPSPS protein are also described in Tables I and II of the Mass Spectrometry Report for the 2mEPSPS protein (Appendix 5). The 2mEPSPS protein, once denatured, is readily digested by endoproteases and will generate numerous peptide peaks.

In the endoproteinase digest of the transgenic-soybean-derived 2mEPSPS protein, the peptide sequence coverage was excellent (86.3%) and 70.0% of the peptide primary sequence was confirmed by MS/MS analysis. The detected peptide fragments covered nearly the entire protein sequence lacking only six peptide fragments (Figure 11), two near the N-terminus (S^{65} to K^{70} and A^{75} to K^{83}), three in the middle of the protein (V^{249} to Y^{258}, A^{287} to K^{296}, and A^{321} to K^{328}) , and one near the C-terminus (M^{405} to R^{422}). The protein sequence that was missed did not contain sequence motifs that are typically required for glycosylation (Asn-Xxx-Ser/Thr, (Hamby and Hirst 2008).

Figure 11: Overall sequence coverage of trypsin and chymotrypsin digests for 2mEPSPS (DAS-444Ø6-6) by MALDI-TOF MS and MALDI TOF-TOF.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
1	A	G	A	E	E	I	V	L	O	P	I	K	E	I	S	G	T	V	K	L	P	G	S	K	S	L	S	N	R	I	30	30	30
31	L	L	L	A	A	L	S	E	G	T	T	V	V	D	N	L	L	N	S	E	D	V	H	Y	M	L	G	A	L	R	60	30	29
61	T	L	G	L	S	V	E	A	D	K	A	A	K	R	A	V	V	V	G	C	G	G	K	F	P	V	E	D	A	K	90	15	15
91	E	E	V	Q	L	F	L	G	N	A	G	I	A	M	R	S	L	T	A	A	V	T	A	A	G	G	N	A	T	Y	120	30	30
121	V	L	D	G	V	P	R	M	R	E	R	P	I	G	D	L	V	V	G	L	K	Q	L	G	A	D	V	D	C	F	150	30	30
151	L	G	T	D	C	P	P	V	R	V	N	G	I	G	G	L	P	G	G	K	V	K	L	S	G	S	I	S	S	Q	180	30	27
181	Y	L	S	A	L	L	M	A	A	P	L	A	L	G	D	V	E	I	E	I	I	D	K	L	I	S	I	P	Y	V	210	30	30
211	E	M	T	L	R	L	M	E	R	F	G	V	K	A	E	H	S	D	S	W	D	R	F	Y	I	K	G	G	Q	K	240	30	30
241	Y	K	S	P	K	N	A	Y	V	E	G	D	A	S	S	A	S	Y	F	L	A	G	A	A	I	T	G	G	T	V	270	20	8
271	T	V	E	G	C	G	T	T	S	L	Q	G	D	V	K	F	A	E	V	L	E	M	M	G	A	K	V	T	W	T	300	20	7
301	E	T	S	V	T	V	T	G	P	P	R	E	P	F	G	R	K	H	L	K	A	I	D	V	N	M	N	K	M	P	330	22	20
331	D	V	A	M	T	L	A	V	V	A	L	F	A	D	G	P	T	A	I	R	D	V	A	S	W	R	V	K	E	T	360	30	14
361	E	R	M	V	A	I	R	T	E	L	T	K	L	G	A	S	V	E	E	G	P	D	Y	C	I	I	T	P	P	E	390	30	23
391	K	L	N	V	T	A	I	D	T	Y	D	D	H	R	M	A	M	A	F	S	L	A	A	C	A	E	V	P	V	T	420	14	2
421	I	R	D	P	G	C	T	R	K	T	F	P	D	Y	F	D	V	L	S	T	F	V	K	N							444	22	16
																															Tot. a.a. covered =	*383*	*311*

A = Tryptic MS coverage

A = Tryptic MS-MS data

A = Chymotryptic MS coverage

A = Chymotryptic MS-MS data

MALDI PMF sequence coverage = 86.3 %

MALDI MS-MS sequence coverage = 70.0 %

This analysis confirmed the soybean-derived protein amino acid sequence matched that of the microbe-derived 2mEPSPS protein (Karnoup and Kuppannan 2010) at both the N- and C-terminus as well as a major portion of the internal sequence. In the MS chromatograms, there were several unidentified peptides detected in the enzyme digest preparations (as discussed above). Results of these analyses indicate that the amino acid sequence of the soybean-derived 2mEPSPS protein was equivalent to the *P. fluorescens*-expressed protein characterized earlier (Karnoup and Kuppannan 2010) and (Schafer 2010).

N- and C-terminal sequence of the 2mEPSPS proteins

The amino acid residues at the N-and C-termini of the soybean-derived 2mEPSPS protein (purified from soybean event DAS-444Ø6-6) were determined (Appendix 5) and compared with the sequence of the previously characterized microbe-derived protein [Appendix 4, (Karnoup and Kuppannan 2010)]. The soybean-derived 2mEPSPS protein sequences were determined by MALDI-TOF MS/MS. The chymotrypsin and trypsin digestions were performed on the soybean-derived 2mEPSPS protein followed by mass spectrometry analysis and the N-terminus

was determined to be identical to the expected sequences, and both the soybean- and microbe-derived 2mEPSPS proteins were indistinguishable and unaltered (Table 8 and Figure 10).

Table 8: Summary of N-terminal sequence data of 2mEPSPS soybean- and microbe-derived proteins

Source	Expected N-terminal Sequence[1]
P. fluorescens	A^{1} G A E E I V L Q P I K E I S G T V K L P G S K S L S^{27}
Soybean Event DAS-444Ø6-6	A^{1} G A E E I V L Q P I K E I S G T V K L P G S K S L S^{27}
Source	**Detected N-terminal Sequence[2]**
P. fluorescens	A^{1} G A E E I V L Q P I K E I S G T V K L P G S K S L S^{27}
Soybean Event DAS-444Ø6-6	A^{1} G A E E I V L Q P I K E I S G T V K L P G S K S L S^{27}

Notes:

[1]Expected N-terminal sequence of the first 13 amino acid residues of *P. fluorescens*- and soybean-derived 2mEPSPS.

[2]Detected N-terminal sequences of *P. fluorescens*- and soybean-derived 2mEPSPS.

Numbers in superscript (R^{x}) indicate amino acid residue numbers in the sequence.

Amino acid residue abbreviations:

A:	alanine	E:	glutamic acid	G:	glycine
I:	isoleucine	K:	lysine	L:	leucine
P:	proline	Q:	glutamine	S:	serine
T:	threonine	V:	valine		

The C-terminal sequence of the soybean- and microbe-derived 2mEPSPS proteins was determined essentially as described above and compared with the expected amino acid sequences. The results indicated the measured sequences were identical to the expected sequences, and both the soybean- and microbe-derived 2mEPSPS proteins were indistinguishable and unaltered at the C-terminus (Table 9 and Figure 10).

Table 9: Summary of C-terminal sequence data of 2mEPSPS soybean- and microbe-derived proteins

Source	Expected C-terminal Sequence[1]
P. fluorescens	K^{429} T F P D Y F D V L S T F V K N^{444}
Soybean Event DAS-444Ø6-6	K^{429} T F P D Y F D V L S T F V K N^{444}

Source	Detected C-terminal Sequence[2]
P. fluorescens	K^{429} T F P D Y F D V L S T F V K N^{444}
Soybean Event DAS-444Ø6-6	K^{429} T F P D Y F D V L S T F V K N^{444}

Notes:

[1]Expected C-terminal sequence of the last 16 amino acid residues of *P. fluorescens*- and soybean-derived 2mEPSPS.

[2]Detected C-terminal sequences of *P. fluorescens*- and soybean-derived 2mEPSPS.

Numbers in superscript (R^x) indicate amino acid residue numbers in the sequence.

Amino acid residue abbreviations:

D:	aspartic acid	F:	phenylalanine	K:	lysine
L:	leucine	N:	asparagine	P:	proline
S:	serine	T:	threonine	V:	valine
Y:	tyrosine				

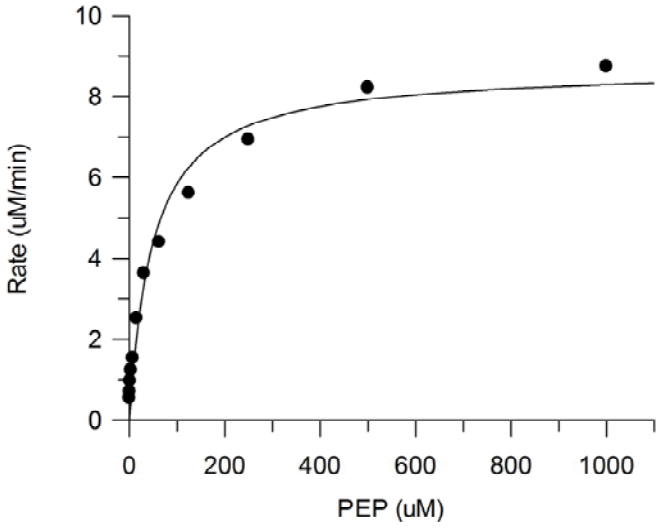
Enzymatic assay of soybean-derived 2mEPSPS

The enzyme activity of the soybean-derived 2mEPSPS protein was measured by inorganic phosphate (P_i) production in a modified procedure described by Lanzetta (Lanzetta, Alvarez et al. 1979). The soybean-derived 2mEPSPS enzyme (isolated from event DAS-444Ø6-6) was catalytically active and displayed typical Michaelis-Menten saturation kinetics. The 2mEPSPS protein displayed expected K_m values for PEP (~49 µM) and maintained activity (~70%) when incubated with glyphosate up to ~4 mM and 1 mM PEP (data not shown). These values are consistent with those observed for the *P. fluorescens*-derived protein (Lin 2010) and other glyphosate tolerant enzymes expressed in *E. coli* (Cicchillo, Lira et al. 2010). The activity assay provided additional evidence that the 2mEPSPS protein expressed in event DAS-444Ø6-6 was substantially equivalent to that expressed and isolated from *P. fluorescens*.

Table 10: Kinetic measurements for soybean-derived 2mEPSPS enzyme.

V_{max} (µM/min)	[Enzyme] (µM)	K_{cat} for PEP (1/sec)	K_m for PEP (µM)	K_{cat}/K_m ($M^{-1}s^{-1}$)
8.70	0.075	1.93	48.70	3.97×10^4

Figure 12: K_m and V_{max} determination of 2mEPSPS enzyme



Parameter	Value	Std. Error
Vmax	8.6964	0.4029
Km	48.6967	8.5494

CONCLUSIONS

It was demonstrated that the biochemical identity of *P. fluorescens*-produced AAD-12 and 2mEPSPS proteins were equivalent to the protein purified from tissues of soybean event DAS-444Ø6-6 (except for the addition of a 42-Dalton acetyl group to a portion of the N-terminus of the soybean-derived AAD-12 protein). Both the soybean- and microbe-derived proteins had the correct apparent molecular weights (32 and 47 kDa, respectively) and were immunoreactive to protein-specific antibodies in lateral flow strip and western blot assays. The amino acid sequences of both proteins were confirmed by enzymatic peptide mass fingerprinting and sequence analysis by MALDI-TOF MS/MS. In addition, the lack of glycosylation of the soybean-derived proteins provided additional evidence that the AAD-12 and 2mEPSPS proteins produced by *P. fluorescens* and transgenic soybean were substantially equivalent molecules.

ARCHIVING

The original copy of the final report, protocol and all raw data records are filed in the Dow AgroSciences LLC archives at 9330 Zionsville Road in Indianapolis, IN 46268. The raw data for the mass spectrometry characterization of the microbe-derived AAD-12 and 2mEPSPS proteins is located in The Dow Chemical Company Analytical Sciences archives in the 1897 building, Midland, MI 48667.

APPENDIX 1

Amendments

1. The original protocol did not specify multiple planting dates would be performed. An additional planting date was required to account for the poor germination of the first two plantings.
2. The original protocol specified that the tissue used in the study would be harvested, frozen, and lyophilized prior to use. However, fresh tissue was used for the western blot detection of the AAD-12 and 2mEPSPS proteins.
3. The isolation of 2mEPSPS required additional tissue for purification probe work. Therefore additional seeds were planted on a bi-weekly basis so that tissue was available as needed.
4. The original protocol did not specify that enzymatic activity from plant-purified 2mEPSPS would be characterized. The 2mEPSPS activity was characterized by measuring the release of inorganic phosphate by the malachite green dye assay. The initial rate of reaction with respect to the substrate, phosphoenolpyruvate, as well as glyphosate inhibition of the enzyme's activity was studied.

Deviations

1. The original protocol did not specify multiple planting dates would be performed. An additional planting date was required to account for the poor germination of the first planting.
2. Amendment #1 specified the source ID of the seeds to be planted on 30-Nov-2010 was YX09KX590371-018, the same source ID of the seeds planted in the original protocol. However, the seeds from that lot had a poor germination rate and were replaced with new seeds from source ID: YX09KX950434. Both seed lots were of the T4 generation.

3. New equipment (iBlot and Snap-i.d. detection system) was used to perform western blot analysis of proteins derived from event DAS-444Ø6-6. The use of this equipment was not covered under SOP ECL-27 which was referenced in protocol 101707. The experimental procedures using this new equipment deviated from the issued protocol and SOP.
4. Wild type EPSPS (wtEPSPS) was listed in the protocol as a control substance. This substance was not used in the study.

APPENDIX 2

Mass Spectrometry Analysis of *P. fluorescens*-derived AAD-12 Protein

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Technology Report The Dow Chemical Company		CRI Number Laboratory Report Code ML-AL MD-2008-003833
Department	Geographic Location	Date Issued
Analytical Sciences	Midland	10/21/2008
Page Count	Protocol Study Number	Report Status
33		Final

Title

Characterization of AAD-12: Batch TSN030732-002

Author(s): Last Name and Initials (Master Numbers)	Author(s) Signature / Date
Karnoup, Anton (AS) (u369292) Kuppannan, Krishna (K) (u386368)	AKarnoup 10/21/2008 Rkuppannan 10.21.2008
Reviewer Name(s)	**Reviewer and/or Supervisor Signature(s)/Date**
Young, Scott (SA) (u289561)	10/22/2008

Patent Status

Disclosure Submitted	Case Filed	No Action Required
[ ]	[ ]	[x]

A batch of purified recombinant aryloxyalkanoate dioxygenase (AAD-12) (batch TSN030732-002) was submitted by Barry Schafer of Dow AgroSciences for characterization. In conjunction with Dow AgroSciences characterization, Analytical Sciences Laboratory was requested to provide analytical data on the intact molecular weight, peptide mass fingerprinting, and N-terminal and C-terminal sequencing.
Intact molecular weight analyses were accomplished by electrospray ionization/liquid chromatography/mass spectrometry (ESI/LC/MS). The mass spectrum revealed the presence of a principal mass component at 31,599.2 (*des*-Met[1]). An earlier eluting peak, which accounts for ~14 % by peak area, primarily contained the protein with Met residue(s) oxidized: the base peak corresponds to *des*-Met[1] AAD-12 with two oxidized Met (or double oxidation of one Met) with a mass 31,631.2. The experimentally observed mass of *des*-Met[1] AAD-12 (31,599.2 Da) is within 0.004% of the calculated average mass of AAD-12 lacking a methionine, based on the expected amino acid sequence. Peptide mass fingerprinting was accomplished by in-solution trypsin, chymotrypsin, Arg-C, Asp-N, and Glu-C digests followed by ESI-LC/MS analysis. The peptide mass fingerprinting resulted in 100% overall mass coverage for the AAD-12 recombinant protein sample (batch TSN030732-002) (taking into account post-translational removal of Met[1]). The N-terminal and C-terminal sequences for AAD-12 (batch TSN030732-002) were determined by a combination of in-solution digestion with endoproteinases trypsin, Arg-C, and chymotrypsin, followed by tandem MS. The tandem MS data for both the N-terminal and C-terminal peptides confirmed the following sequences, ^{2}AQTTLQITPTGATLGATVTGVHLATLDDAGFAALHAAWLQHALLIFPGQHLSNDQQITFAK^{62} and ^{281}LAGRPETEGAALV^{293}, respectively.

DISTRIBUTION LIST

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INTRODUCTION

A sample of purified recombinant aryloxyalkanoate dioxygenase (AAD-12) (Batch TSN030732-002) was submitted by Barry Schafer of Dow AgroSciences for characterization. In conjunction with Dow AgroSciences characterization, Analytical Sciences Laboratory was requested to provide analytical data on peptide mass fingerprinting and N-terminal and C-terminal sequencing. Original experimental data are stored in the raw data packet ML-AL MD-2008-003833 [1].

EXPERIMENTAL

Sample Preparation:

A sample of microbial recombinant purified AAD-12 (Batch TSN030732-002) (dark grey lyophilized material; several mg), was submitted for analysis by Barry Schafer (Dow AgroSciences, Indianapolis, IN). Prior to analyses by mass-spectrometry, the sample was prepared as follows:

AAD-12 (Batch TSN030732-002) material (1.16 mg) was resuspended in 1.16 mL of 25 mM ammonium bicarbonate/ 0.1M Gu:HCl, pH8, buffer to a final concentration of 1 mg/mL. An aliquot of the protein solution was stored at +4 ^{o}C prior to ESI/LC/MS analysis, and the rest of the solution was frozen at -20 ^{o}C. Preparation of enzymatic digests for peptide mass fingerprinting and sequencing is described separately below.

ESI/LC-MS for Intact Protein:

Reagents and Standards:

1. Acetonitrile (HPLC grade, 99.9%, Fisher Scientific), Lot no. 082100
2. Trifluoroacetic Acid (Aldrich, 99+%), Lot no. 00339JD
3. Deionized water, 18.2 MΩcm, MilliQ gradient A10, Millipore, freshly drawn
4. Poly-DL-Alanine, Sigma, Catalog no. P9003, Lot no. 97H5912
5. Ribonuclease A (RNAse A), Sigma, Catalog no. R5000, Lot no. 122K1319
6. Bovine serum albumin (BSA), Sigma, Catalog no. A1900, Lot No. 036K7575
7. Lysozyme (from chicken egg white), Sigma, Catalog no. L7651, Lot no. 072K7062
8. Myoglobin (from horse heart), Sigma, Catalog no. M1882

Analytical Procedure:

ESI/LC/MS: The sample preparations were analyzed directly by mass spectrometry. All mass spectra were acquired on a Waters Q-Tof Micro MS system (S/N YA137). The mass spectrometer was calibrated prior to use in the mass range 500-1950 amu using 0.1 mg/mL Poly-DL-Alanine solution (in acetonitrile). A mixture of proteins with known molecular masses (RNAse A, BSA, Lysozyme, Myoglobin; solutions in deionized water at 10 mg/mL were used) was run as a test standard. The following mass spectrometer settings were used.

LC : Acquity UPLC system
Mobile Phase A : 0.01 % trifluoroacetic acid (TFA) in water
Mobile Phase B : 0.01 % trifluoroacetic acid (TFA) in acetonitrile (ACN)
Column : 2.1x150 mm Symmetry 300 C18 3.5 µm 300 Å; S/N: 01283608610502 Part No: 186000188
Flow rate : 100 µL/min
Column temperature : 50 °C
Injection volume : 10 µL
Injection loop : 20 µL
UV detection : 214 nm, 40 pts/sec

Gradient table:

Time, min	Flow rate, mL/min	%A	%B
Initial	0.1	90	10
3	0.1	76	24
19	0.1	44	56
21	0.3	10	90
25	0.3	10	90
26	0.3	90	10
32	0.3	90	10
33	0.1	90	10
35	0.1	90	10

Q-Tof Micro with Micromass lock-spray interface: MS Parameters:

Capillary : 2800 V
Desolvation Gas : 550 L/hr
Desolvation Temperature: 345 °C
Source Temperature : 90 °C
Sample Cone : 15 V
Extraction Cone : 0.9 V
Collision Energy : 10.0 V
MCP : 2350 V
Mode : ESI-TOF-MS +
Scan Range : 500 – 1950 amu
Scan Rate : 0.98 sec/scan

The Micromass-supplied electrospray maximum entropy algorithm (MAXENT 1) was used to transform the spectra to a mass axis and to resolution enhance the transformed spectra. The maximum entropy algorithm was set to optimize the spectra with a resolution of 1 Da/channel. The resulting resolution-enhanced spectral peaks were centered and integrated to display the accurate mass for intact molecular mass analysis.

Table: Molecular weight of intact standard proteins determined by ESI/LC/MS:

Protein	Theoretical Mass, Da	Observed Mass, Da	Delta Mass, Da
RNase A	13681.3	13682.4	+ 1.1
Lysozyme	14303.9	14304.5	+ 0.6
BSA	66433.2	66432.3	- 1.2
Myoglobin	16951.5	16951.7	+ 0.2

In-solution Protein Processing and enzymatic digests:

Equipment:

a) Mettler AE168 analytical balance serial no. F00518
b) Eppendorf Centrifuge, Model 5415D, serial no. 5425 17645
c) Eppendorf, Thermomixer R, serial no. 5355 20846
d) Centrifugal evaporator (Centrivap), Labconco, cat. no. 7812013, S/N 051146935 A
e) Eppendorf adjustable pipettes: 2.5µL serial no. 296447, 2-20µL serial no. 286820, 10-100µL serial no. 289560, and 1000µL serial no. 33165
f) Fisher Vortex Genie 2, serial no. 2-156856
g) Siliconized microcentrifuge tubes, 1.5mL, Fisher, cat no. 02-681-320
h) Parafilm
i) Eppendorf pipette tips (epTips) 10µL
j) Fisher brand Reditip General Purpose, 200µL and 1000µL

Reagents and Standards:

1. Fisher, acetonitrile, cat no. A998-1
2. Sigma, ammonium bicarbonate, cat no. A-6141
3. Pierce, Dithiothreitol (DTT), cat no. 20290

4. Sigma, Iodoacetamide (IAA), Sigma, cat no. I-1149
5. Roche, Trypsin, cat no. 11-418-025-001 (Lot no. 13556621)
6. Roche, Chymotrypsin, cat no. 11-418-467-001 (Lot no. 13998020)
7. Roche, Asp-N, cat no. 11-054-589-001 (Lot no. 13883820)
8. Roche, Arg-C, cat no. 11-370-529-001 (Lot no. 11377132(13))
9. Roche, Glu-C, cat no. 11-047-817-001 (Lot no. 13390420)
10. Fluka, 98% Formic Acid (FA), Lot no. 1255194
11. Fisher, Trifluoroacetic acid (TFA), cat no. 04902-100
12. Milli-Q deionized water

Reagent Solution Preparation:

a. 25 mM Ammonium Bicarbonate buffer: dissolved 98.83 mg NH_4HCO_3 in 50 mL of Milli-Q water; filtered through 0.22 μm sterile syringe filter.
b. 100 mM Tris buffer: dissolved 121.1 mg Tris in 10 mL of Milli-Q water; adjusted pH to 8.11 with HCl; filtered through 0.22 μm sterile syringe filter.
c. Protein dissolution buffer (6M guanidine hydrochloride (Gu:HCl)/ 400 mM ammonium bicarbonate, pH 7.8): to 316 mg of ammonium bicarbonate, 7.5 mL of 8M Gu:HCl solution and 2.5 mL of water were added. pH was adjusted to 7.8 with NaOH. Buffer was filtered through 0.22 µm sterile syringe filter.
d. DTT solution (100 mM; prepared fresh): dissolved 15.4 mg DTT in 1 mL of water.
e. Alkylating reagent (IAA) (200 mM; prepared fresh): dissolved 37 mg IAA in 1 mL of water.
f. Trypsin solution. Step 1: Dissolved 25 µg of dried trypsin in 320 µL of 100 mM Tris buffer immediately prior to digestion procedure. Step 2: Dissolved 50 µg of dried trypsin in 320 µL of 100 mM Tris buffer immediately prior to digestion procedure.
g. Chymotrypsin solution. Step 1: Dissolved 25 µg of dried chymotrypsin in 66 µL of 1 mM HCl immediately prior to digestion procedure. Step 2: Dissolved 50 µg of dried chymotrypsin in 160 µL of 1 mM HCl immediately prior to digestion procedure.
h. Asp-N solution: Dissolved 2 µg of dried Asp-N in 50 µL of Milli-Q water immediately prior to digestion procedure.
i. Glu-C solution. Step 1: Dissolved 50 µg of dried Glu-C in 65 µL of Milli-Q water immediately prior to digestion procedure. Step 2: Dissolved 50 µg of dried Glu-C in 160 µL of Milli-Q water immediately prior to digestion procedure.
j. Arg-C solution. Step 1: Immediately prior to digestion procedure, dissolved 5 µg of dried Arg-C in 30 µL of Milli-Q water, and combine with 50 µL of activation solution (reconstituted in 100 µL of Milli-Q water, as per manufacturer's procedure). Step 2: Immediately prior to digestion procedure,

dissolved 10 µg of dried Arg-C in 30 µL of Milli-Q water, and combine with 50 µL of activation solution (reconstituted in 100 µL of Milli-Q water, as per manufacturer's procedure).

In-solution Protein Processing (Reduction/ alkylation/ digestion):

a. Five 180-µL aliquots of 1 mg/mL AAD-12 (Batch TSN030732-002) protein solution were dried in the centrifugal evaporator to completeness.
b. Reduction and carboxyamidomethylation (alkylation) of protein: approximately 180-µL of protein dissolution buffer, 6M Gu:HCl/ 0.4M ammonium bicarbonate, pH 7.8, was added to the dry AAD-12 [Batch TSN030732-002] samples, and samples were mixed by pipette action. Twenty microliters of 100 mM DTT (reducing reagent) solution was added to each tube. Tubes were sealed, vortexed, and incubated at 65 ºC for 40 min in a thermomixer at 1100 rpm. Tubes were then cooled to room temperature, centrifuged for 30 sec. and 40 µL of 200 mM IAA (alkylating reagent) solution was added to each tube. Tubes were incubated in the dark at room temperature for 1 hour. Eighty microliters of DTT solution was added to consume unreacted IAA and the tubes were allowed to stand for 20 min at room temperature. The total reaction volume was approximately 320 µL in each tube.
c. Desalting of the reduced/alkylated protein samples was performed using NAP-5 gravity cartridges (Sephadex G-25) as per the manufacturer's procedure. NAP-5 cartridges were pre-equilibrated with the corresponding digestion buffer (100 mM Tris buffer, pH 8.11, for tryptic and Arg-C digests; 25 mM ammonium bicarbonate, pH 7.8, for chymotryptic, Asp-N, and Glu-C digests), and protein elution was performed with the same buffer (final volume 1-mL for each sample).
d. Tryptic digestion of reduced/alkylated protein: Step 1: 100-µL of trypsin solution (25 µg in 320 µL of 100 mM Tris buffer, pH8.11) was added to the 1-mL of reduced/alkylated protein AAD-12 [Batch TSN030732-002] sample in 100 mM Tris buffer, pH8.11. The digest was incubated for 2 hours at 37 ºC in a thermomixer at 900 rpm. Step 2: 100-µL of trypsin solution (50 µg in 320 µL of 100 mM Tris buffer) was added to the digestion reaction. The digest was incubated for 16 hours at 37 ºC in a thermomixer at 900 rpm. Sample was frozen at -20 ^{o}C until ready for analysis by mass-spectrometry.
e. Arg-C digestion of reduced/alkylated protein: Step 1: 25-µL of Arg-C solution (5 µg in 30 µL of Milli-Q deionized water, combined with 50 µL of activation solution) was added to the 1-mL of reduced/alkylated protein AAD-12 [Batch TSN030732-002] sample in 100 mM Tris buffer, pH8.11. The digest was incubated for 2 hours at 37 ºC in a thermomixer at 900 rpm. Step 2: 25-µL of Arg-C solution (10 µg in 30 µL of Milli-Q deionized water, combined with 50 µL of activation solution) was added to the digestion reaction. The

digest was incubated for 16 hours at 37 ºC in a thermomixer at 900 rpm. Sample was frozen at -20 °C until ready for analysis by mass-spectrometry.

f. Chymotryptic digestion of reduced/alkylated protein: Step 1: 20-µL of chymotrypsin solution (25 µg in 66 µL of 1 mM HCl) was added to the 1-mL of reduced/alkylated protein AAD-12 [Batch TSN030732-002] sample in 25 mM ammonium bicarbonate buffer, pH7.8. The digest was incubated for 2 hours at 22 ºC in a shaker. Step 2: 50-µL of chymotrypsin solution (50 µg in 160 µL of 1 mM HCl) was added to the digestion reaction. The digest was incubated for 16 hours at 22 ºC in a shaker. Sample was frozen at -20 °C until ready for analysis by mass-spectrometry.

g. Asp-N digestion of reduced/alkylated protein: 50-µL of Asp-N solution (2 µg in 50 µL of Milli-Q deionized water) was added to the 1-mL of reduced/alkylated protein AAD-12 [Batch TSN030732-002] sample in 25 mM ammonium bicarbonate buffer, pH7.8. The digest was incubated for 16 hours at 37 ºC in a thermomixer at 900 rpm. Sample was frozen at -20 °C until ready for analysis by mass-spectrometry.

h. Glu-C digestion of reduced/alkylated protein: Step 1: 20-µL of Arg-C solution (50 µg in 65 µL of Milli-Q deionized water) was added to the 1-mL of reduced/alkylated protein AAD-12 [Batch TSN030732-002] sample in 25 mM ammonium bicarbonate buffer, pH7.8. The digest was incubated for 2 hours at 22 ºC in a shaker. Step 2: 50-µL of Glu-C solution (50 µg in 160 µL of Milli-Q deionized water) was added to the digestion reaction. The digest was incubated for 16 hours at 22 ºC in a shaker. Sample was frozen at -20 °C until ready for analysis by mass-spectrometry.

ESI-LC/MS and MS/MS of proteolytic digests

Reagents and Materials:

1. Acetonitrile (Baker analyzed HPLC solvent, JT Baker), Lot no. C10827
2. Milli-Q water
3. 98% Formic Acid (Fluka), Lot no. 1255194
4. Poly-DL-Alanine, Sigma, cat. no. P9003, Lot no. 97H5912
5. Leucine Enkephalin acetate salt, Sigma, cat. no. L-9133, Lot no. 095K5109
6. Waters total recovery HPLC vials, P/N 186000384c, lot no. 0384661180

Analytical Procedure:

ESI-LC/MS: The samples (digests) were dried to completeness in a centrifugal evaporator, resuspended in deionized water (180 μL; to a final concentration of approximately 1 mg/mL) and analyzed by LC/MS. All mass spectra were acquired on a Waters Q-Tof Micro MS system (S/N YA137). The mass spectrometer was calibrated prior to use in the mass range 350 – 1900 amu (MS for peptide mass finger printing) or 80 – 1900 amu (tandem MS) using 0.1 mg/mL Poly-DL-Alanine solution in acetonitrile. The following liquid chromatography and mass spectrometer settings were used:

LC :	Acquity UPLC system
Mobile Phase A :	0.1 % formic acid (FA) in water
Mobile Phase B :	0.1 % formic acid (FA) in acetonitrile
Column :	2.1x150 mm Acquity BEH C18 1.7 µm 135 Å; S/N: 01245523640B05 Part No: 186002353
Flow rate :	100 µL/min
Column temperature :	50 °C
Injection volume :	10 µL
Injection loop :	20 µL
UV detection :	214 nm, 40 pts/sec

Gradient table:

Time, min	Flow rate, mL/min	%A	%B
Initial	0.1	95	5
5	0.1	95	5
63	0.1	60	40
63.5	0.3	60	40
69	0.3	10	90
70	0.3	10	90
71	0.3	95	5
79	0.3	95	5
80	0.1	95	5
85	0.1	95	5

MS :	QTOF-micro mass spectrometer (S/N YA137)
ESI :	Micromass lock-spray electrospray interface
Mode :	+TOFMS

MS Parameters (peptide mass fingerprinting):

Capillary	:	2850 V
Desolvation Gas	:	650 L/hr
Desolvation Temperature	:	300 °C
Source Temperature	:	110 °C
Sample Cone	:	15 V
Extraction Cone	:	0.9 V
Collision Energy	:	10.0 V
MCP	:	2350 V
Mode	:	ESI-TOF-MS +

Scan Range	:	350 – 1900 amu (PMF) or 80 – 1900 amu (tandem MS)
Scan Cycle Time	:	0.98 sec/scan
MS/MS Parameters:		
Capillary	:	2850 V
Desolvation Gas	:	650 L/hr
Desolvation Temperature	:	300 °C
Source Temperature	:	110 °C
Sample Cone	:	15 V
Extraction Cone	:	0.9 V
MCP	:	2350 V
Mode	:	ESI-TOF-MS +
Scan Range	:	80 – 1900 amu
Survey Scan		
Collision Energy	:	10.0 V
Scan Cycle Time	:	0.98 sec/scan
Precursor Selection	:	Included Masses only
Include Window	:	+/- 300 mDa
Include Retention Time	:	240 sec
Peak Detection Window	:	1 Da
MS/MS Scan		
MS to MSMS Switch Criteria	:	Intensity
MS to MSMS Switch Threshold	:	10 counts/sec
MSMS to MS Switch Criteria	:	Intensity falling below threshold
Switchback Threshold	:	3 counts/sec
MSMS Switch After Time	:	12 sec
Scan Cycle Time	:	1.98 sec/scan

Methods:

The samples were injected using a partial loop configuration. After sample injection, the column was held at 5 % MPB for 5 minutes. The gradient from 5 % MPB to 40 % MPB was then employed. At the end of the gradient, the MPB concentration was increased to 90% to allow removal of any hydrophobic components. The column was then re-equilibrated to the initial conditions.

The Time of Flight (ToF) analyzer was calibrated daily using a 0.1 mg/mL (100 ppm) solution (in acetonitrile) of Poly-Alanine at 20 µL/min flow rate. The same instrument parameter file (with the calibration parameters) was used for MS data acquisitions. Data acquisition was performed with cycle times of 1 sec/scan (scan acquisition time: 0.88 sec; interscan delay: 0.1sec) and 2 sec/scan (scan acquisition time: 1.88 sec; interscan delay: 0.1sec) in the MS mode and MSMS mode, respectively. The lock mass data was acquired using 2.5 µM Leucine-Enkephalin peptide solution (0.1 % formic acid in 50 % acetonitrile was used as the solvent) flowing at 3 – 5 µL/min. The lock mass channel was sampled every 7 sec during MS analysis and 10 sec during MS/MS analysis. The reference ion used was the singly charged Leucine-Enkephalin ion at m/z 556.2771. The tandem MS experimental parameters used

in the analyses of N- and C- terminal peptides from both tryptic and chymotryptic digests of AAD-12 (TSN030732-002) are listed above.

Peptide mass fingerprinting of the UPLC-MS data was performed manually. The spectrum of each chromatographic peak was summed, smoothed (SG, 2x3 channels), centroided (4 channels, top 80 %, by height) and m/z error corrected (lock mass channel: 10 scans, m/z 556.2271 ± 0.5 Da). In-source fragmentation observed was used to further confirm the identity of the peptides. For some peptides eluting later in the gradient MaxEnt1 was used. The Micromass-supplied electrospray maximum entropy algorithm (MAXENT 1) was used to transform the spectra to a mass axis and to resolution-enhance the transformed spectra. The maximum entropy algorithm was set to optimize the spectra with a resolution of 1 Da/channel. The resulting resolution-enhanced spectral peaks were centered and integrated to display the accurate mass.

The spectra from tandem MS experiments were also summed, smoothed (SG, 2x3 channels), centroided (4 channels, top 80 %, by height) and m/z error corrected (lock mass channel: 10 scans, m/z 556.2271 ± 0.5 Da). The fragments were assigned using a theoretical fragmentation ion table generated using either a local copy of Protein Prospector (v 3.2.1) or using Micromass BioLynx software.

RESULTS AND DISCUSSION

In this study, the numbering of the amino acid residues is in accordance with the theoretical sequence of the recombinant AAD-12 protein starting with Met[1] and containing a total of 293 residues (**Figure 1**).

ESI Intact Mass Spectral Characterization:

The purified AAD-12 (Batch TSN030732-002) was processed by directly solubilizing the dry protein material in PBS buffer supplemented with 0.1M Gu:HCl (to ease solubilization and to prevent immediate protein precipitation).

The solubilized proteins were then analyzed by ESI-LC/MS using a Symmetry C18 column for separation. The chromatography for AAD-12 (Batch TSN030732-002) revealed the presence of one major peak at retention time of 12.35 min, and two small satellite peaks at 11.86 min (~14% by LC-UV peak area) and 13.61 min (~4% by LC-UV peak area) (**Figure 2**). Unique features of the corresponding mass spectra were the broad charge distribution and partial resolution of the peaks. Ions related to the monomer form of AAD-12 were observed. The transformed and integrated maximum entropy spectra revealed the presence of a principal mass component at m/z 31,599.2 (LC peak II, 12.35 min). This mass is consistent with the calculated molecular weight for *des*-Met[1] AAD-12 (theor. average mass: 31,598 Da, see **Table I**).

Ions related to the monomer form of AAD-12 (*des*-Met[1]) were observed under the non-reducing conditions of the described ESI-LC/MS experiment. Thus the observed mass of the major sample component is within 0.004% of the theoretical molecular weight of AAD-12 (*des*-Met[1]) (**Table I**). Peak I, which accounts for approximately 14% by peak area, primarily contains oxidized Met residue(s) (peak I, 11.86 min; **Figure 2**). The majority of the oxidized AAD-12 protein contained two oxidation sites (either on two separate Met, or a single doubly-oxidized Met; **Table I**).

Peptide Mass Fingerprinting:

ESI-LC/MS analysis was used to generate peptide coverage maps, N-terminal and C-terminal sequences, and to determine post-translational processing sites. For that purpose, in-solution trypsin, chymotrypsin, Arg-C, Asp-N, and Glu-C digests of reduced and alkylated AAD-12 were generated and analyzed by ESI-LC/MS. **Figures 3** through **7** show the corresponding LC chromatograms of the digested AAD-12 (Batch TSN030732-002). The corresponding mass spectral data with assignments from ESI-LC/MS analyses are presented in **Tables II** through **VI** for the tryptic, Arg-C, chymotryptic, Asp-N, and Glu-C digests, respectively (in that order). The combined sequence coverage is full (100%), given the expected post-translational removal of N-terminal Met[1] (**Figure 1**). Most peptides observed in the proteolytic digests of AAD-12 (Batch TSN030732-002) exhibited in-source fragmentation patterns consistent with their expected theoretical amino acid sequences (**Tables II** through **VI**). The N- and C-terminal peptides were further analyzed by LC tandem MS to confirm their amino acid sequences.

LC Tandem MS:

N- and C-terminal peptides observed by LC-MS analyses were further analyzed by tandem MS to confirm their amino acid sequences. The results are presented in **Tables VII** and **VIII**. Sequence tags were generated from the tryptic fragments with m/z 642.36 (C-terminal peptide, $[M+2H]^{2+}$) and m/z 1063.22 (N-terminal peptide, $[M+6H]^{6+}$), and chymotryptic fragments with m/z 585.82 (C-terminal peptide, $[M+2H]^{2+}$) and m/z 533.29 (N-terminal peptide, $[M+H]^{1+}$). LC tandem MS ion spectra were acquired for each individual peptide at specific retention time obtained in the preceding peptide mass fingerprinting study. Tandem MS experiments were performed with multiple collision energies for each peptide. The tandem MS fragments observed for N-terminal peptides from both tryptic and chymotryptic digests were consistent with the N-terminal peptide sequence, AQTTLQITPTGATLGATVTGVHLATLDDAGFAALHAAWLQHALLIFPGQHLSNDQQITFAK (**Table VIII**). The tandem MS fragments observed for C-terminal peptides from both tryptic and chymotryptic digests were consistent with the C-terminal peptide sequence, LAGRPETEGAALV (**Table VII**).

REFERENCES

1. Raw data packet ML-AL MD-2008-003833

Table I: Molecular weight of intact AAD-12 (Batch TSN030732-002) determined by ESI-LC/MS

Sample Lot # / Peak #	Residues	Theoretical Average Mass	Observed	Comment
TSN030732-002/ I	2-293 (des-Met[1])	31598.0	31594.6	
	2-293 (des-Met[1])		31612.3	One oxidation site
	2-293 (des-Met[1])		31631.2*	Two oxidation sites
	2-293 (des-Met[1])		31645.7	Three oxidation sites
	2-293 (des-Met[1])		31661.7	Four oxidation sites
TSN030732-002/ II	2-293 (des-Met[1])	31598.0	**31599.2***	
	2-293 (des-Met[1])		31615.1	One oxidation site
	4-293 (des-Met[1])	31398.8	31399.1	
			31579.8	unidentified
TSN030732-002/ III	2-293 (des-Met[1])	31598.0	31596.1	
	2-293 (des-Met[1])		31612.1	One oxidation site
			31714.3	unidentified
			31720.9	unidentified
			31733.4	unidentified

* Primary mass component within the LC peak

Table II: Tryptic digest peptide mass fingerprinting of AAD-12 (Batch TSN030732-002).

Peptide	# of missed cleavages	a.a. ##	Sequence	m/z theor.							m/z obs. (ESI-LC/MS)	UPLC elution time, min	In-source fragments observed (matching amino-acid sequence)	Comment
				$(M+H)^{1+}$	$(M+2H)^{2+}$	$(M+3H)^{3+}$	$(M+4H)^{4+}$	$(M+5H)^{5+}$	$(M+6H)^{6+}$	$(M+7H)^{7+}$				
T1	0	2-62	(-) AQTTLQITPTGATLGATVTGVHLATLDDAGFAALHAAWLQHALLIFPGQHLSNDQQITFAK (R)	6366.22	3183.62	2122.75	1592.31	1274.05	1061.88	910.32	1592.09 (4+), 1273.90 (5+), 1061.84 (6+), 910.28 (7+)	63.42		processed N-terminus; no oxidation observed; average masses
				6365.22 [0 charge, ave.]										
T3	0	64-69	(R) FGAIER (I)	692.37	346.69	231.46	173.85	139.28			692.37 (1+)	21.69	545.31 (1+, y5), 488.28 (1+, y4), 417.25 (1+, y3)	
T4	0	70-82	(R) IGGGDIVAISNVK (A)	1242.71	621.86	414.91	311.43	249.35			1242.70 (1+), 621.86 (2+)	34.84	1129.64 (1+, y12), 1072.59 (1+, y11), 843.55 (1+, y8), 796.45 (1+, b9), 631.38 (1+, y6), 612.33 (1+, b7), 560.33 (1+, y5), 513.27 (1+, b6), 447.26 (1+, b4), 400.19 (1+, b5), 360.23 (1+, y3)	
T5	0	83-88	(K) ADGTVR (Q)	618.32	309.66	206.78	155.34	124.47			618.32 (1+)	4.70		
T6	0	89-100	(R) QHSPAEWDDMMK (V)	1474.61	737.81	492.21	369.41	295.73			737.81 (2+), 492.21 (3+)	31.31	954.37 (1+, y7), 825.33 (1+, y6), 650.29 (1+, b6), 639.25 (1+, y5), 524.23 (1+, y4), 521.24 (1+, b5), 504.24 (2+, y8-H2O)	
T6-Met-ox1	0	89-100	(R) QHSPAEWDDMMK (V)	1490.61	745.81	497.54	373.40	298.92			ND	ND		
T6-Met-ox2	0	89-100	(R) QHSPAEWDDMMK (V)	1506.61	753.81	502.87	377.40	302.12			ND	ND		
T7	0	101-135	(K) VIVGNMAWHADSTYMPVMAQGAVFSAEVVPAVGGR (T)	3617.77	1809.39	1206.60	905.20	724.36			1206.60 (3+), 905.22 (4+)	53.51	838.87 (2+, b15), 655.39 (1+, y7), 556.32 (1+, y6)	
T7-Met-ox1	0	101-135	(K) VIVGNMAWHADSTYMPVMAQGAVFSAEVVPAVGGR (T)	3633.77	1817.39	1211.92	909.19	727.55			ND	ND		
T7-Met-ox2	0	101-135	(K) VIVGNMAWHADSTYMPVMAQGAVFSAEVVPAVGGR (T)	3649.77	1825.39	1217.26	913.19	730.75			ND	ND		
T8	0	136-142	(R) TCFADMR (A)	900.37	450.69	300.80	225.85	180.88			900.37 (1+), 450.69 (2+)	24.42	639.29 (1+, y5), 595.22 (1+, b5), 492.22 (1+, y4), 421.19 (1+, y3)	
T8-Met-ox1	0	136-142	(R) TCFADMR (A)	916.37	458.69	306.12	229.84	184.07			ND	ND		
T9	0	143-153	(R) AAYDALDEATR (A)	1195.56	598.28	399.19	299.65	239.92			1195.67 (1+), 598.29 (2+)	26.67	1053.49 (1+, y9), 1021.45 (1+, b10), 920.44 (1+, b9), 890.44 (1+, y8), 849.33 (1+, b8), 775.40 (1+, y7), 720.33 (1+, b7), 704.34 (1+, y6), 591.27 (1+, y5), 527.25 (2+, y9), 492.22 (1+, b5), 476.24 (1+, y4), 421.18 (1+, b4)	
T10	0	154-159	(R) ALVHQR (S)	723.43	362.22	241.81	181.61	145.49			723.43 (1+)	7.82	440.24 (1+, y3), 539.30 (1+, y4)	
T12	0	163-171	(R) HSLVYSQSK (L)	1048.54	524.78	350.19	262.89	210.51			1048.54 (1+), 524.78 (2+)	15.99		
T13	0	172-199	(K) LGHVQQAGSAYIGYGMDTTATPLRPLVK (V)	2944.54	1472.77	982.18	736.89	589.71			1472.64 (2+), 982.19 (3+), 736.90 (4+)	40.74	919.99 (2+, y17), 860.46 (2+, y16), 831.96 (2+, y15), 750.41 (2+, y14), 723.39 (2+, b14), 641.82 (2+, b13), 613.33 (2+, b12), 556.77 (2+, b11), 475.25 (2+, b10)	
T13-Met-ox1	0	172-199	(K) LGHVQQAGSAYIGYGMDTTATPLRPLVK (V)	2960.54	1480.77	987.51	740.89	592.91			ND	ND		
T14	0	200-213	(K) VHPETGRPSLLIGR (H)	1531.87	766.44	511.30	383.72	307.18			766.45 (2+), 511.30 (3+)	29.31	777.41 (1+, b7), 648.37 (2+, y12), 594.34 (2+, b11), 537.80 (2+, b10), 481.26 (2+, b9), 458.32 (1+, y4)	
T15	0	214-228	(R) HAHAIPGMDAAESER (F)	1591.73	796.37	531.25	398.69	319.15			796.38 (2+), 531.26 (3+)	21.95	1062.50 (1+, y10), 965.41 (1+, y9), 908.38 (1+, y8), 777.35 (1+, y7), 684.37 (1+, b7), 662.32 (1+, y6), 591.28 (1+, y5), 530.28 (1+, b5), 520.24 (1+, y4), 465.72 (2+, b9), 417.20 (1+, b4), 391.20 (1+, y3)	
T15-Met-ox1	0	214-228	(R) HAHAIPGMDAAESER (F)	1607.73	804.37	536.58	402.68	322.35			ND	ND		
T16	0	229-242	(R) FLEGLVDWACQAPR (V)	1661.81	831.41	554.61	416.21	333.17			831.41 (2+), 554.61 (3+)	49.66	774.41 (1+, b7), 702.34 (1+, y6), 695.83 (2+, b12), 659.39 (1+, b6), 631.30 (1+, y5), 560.30 (1+, b5), 502.23 (2+, y8), 471.27 (1+, y4), 390.21 (1+, b3)	
T17	0	243-259	(R) VHAHQWAAGDVVVWDNR (C)	1959.96	980.48	653.99	490.75	392.80			980.49 (2+), 654.00 (3+)	34.84	1172.54 (1+, b11), 1073.49 (1+, b10), 788.41 (1+, y6), 788.89 (2+, b14), 689.34 (1+, y5), 685.85 (2+, b13), 636.33 (2+, b12), 590.27 (1+, y4), 586.78 (2+, b11), 572.80 (2+, a11), 537.26 (2+, b10), 404.19 (1+, y3), 394.72 (2+, y6)	
T18	0	260-264	(R) CLLHR (A)	698.38	349.69	233.46	175.35	140.48			698.37 (1+)	17.57	425.27 (1+, y3)	
T19	0	265-271	(R) AEPWDFK (L)	892.42	446.71	298.15	223.86	179.29			892.42 (1+), 446.72 (2+)	34.41	692.34 (1+, y5), 595.30 (1+, y4), 571.26 (1+, a5), 409.21 (1+, y3)	
T20	0	272-274	(K) LPR (V)	385.26	193.13	129.09	97.07	77.86			385.26 (1+)	8.57		
T21	0	275-280	(R) VMWHSR (L)	815.40	408.20	272.47	204.61	163.89			815.39 (1+), 408.21 (2+)	18.07	716.32 (1+, y5), 585.29 (1+, y4), 399.22 (1+, y3)	
T21-Met-ox1	0	275-280	(R) VMWHSR (L)	831.40	416.20	277.80	208.60	167.08			ND	ND		
T22	0	281-293	(R) LAGRPETEGAALV (-)	1283.70	642.35	428.57	321.68	257.55			1283.71 (1+), 642.36 (2+)	30.86	1053.54 (1+, b11), 982.50 (1+, b10), 886.46 (1+, y9), 854.43 (1+, b8), 583.82 (2+, b12), 527.27 (2+, b11), 491.77 (2+, b10), 398.25 (1+, b4)	**C-terminus**
T1-2	1	2-63	(-) AQTTLQITPTGATLGATVTGVHLATLDDAGFAALHAAWLQHALLIFPGQHLSNDQQITFAKR (F)	6522.41	3261.71	2174.81	1631.36	1305.29	1087.91	932.64	1304.92 (5+), 1087.71 (6+), 932.46 (7+)	62.27		Processed N-term.; missed cleavage
T2-3	1	63-69	(K) RFGAIER (I)	848.47	424.74	283.50	212.87	170.50			424.74 (2+)	19.93		missed cleavage
non-spec1		106-135	(N) MAWHADSTYMPVMAQGAVFSAEVVPAVGGR (T)	3135.49	1568.25	1045.83	784.63	627.90			1568.19 (2+), 1045.85 (3+), 784.68 (4+)	51.04	1290.64 (2+, b24), 860.72 (3+, b24), 761.34 (2+, b13), 754.44 (1+, y8), 655.38 (1+, y7), 597.74 (2+, b10), 556.32 (1+, y6),	non-specific cleavage
non-spec1-Met-ox1		106-135	(N) MAWHADSTYMPVMAQGAVFSAEVVPAVGGR (T)	3151.49	1576.25	1051.16	788.62	631.10			ND	ND		
non-spec1-Met-ox2		106-135	(N) MAWHADSTYMPVMAQGAVFSAEVVPAVGGR (T)	3167.49	1584.25	1056.50	792.62	634.30			ND	ND		
non-spec1-Met-ox3		106-135	(N) MAWHADSTYMPVMAQGAVFSAEVVPAVGGR (T)	3183.49	1592.25	1061.83	796.62	637.50			ND	ND		

"Met-ox#" =number of possible oxidations of Met residue(s) in a peptide

Cys residues are reduced and carboxyamidomethylated

Table III: Arg-C digest peptide mass fingerprinting of AAD-12 (Batch TSN030732-002).

Peptide	# of missed cleavages	a.a. ##	Sequence	m/z theor.							m/z obs. (ESI-LC/MS)	UPLC-MS elution time (min)	In-source fragments observed (matching amino-acid sequence)	Comment
				$(M+H)^{1+}$	$(M+2H)^{2+}$	$(M+3H)^{3+}$	$(M+4H)^{4+}$	$(M+5H)^{5+}$	$(M+6H)^{6+}$	$(M+7H)^{7+}$				
				6522.41	3261.71	2174.71	1631.36	1305.29	1087.91	932.64				
1	0	2-63	(-) AQTTLQITPTGATLGATVTGVHLATLDDAGFAALHAAWLQHALLIFPGQHLSNDQQITFAKR (F)	6521.40 [0 charge]							6523.47 (± 0.03) (z=0)	61.96		average mass, transformed
2	0	64- 69	(R) FGAIER (I)	692.37	346.69	231.46	173.85	139.28			692.37 (1+)	21.60	545.30 (1+, y5), 488.28 (1+, y4), 417.25 (1+, y3)	
3	0	70- 88	(R) IGGGDIVAISNVKADGTVR (T)	1842.01	921.51	614.67	461.26	369.21			921.51 (2+), 614.68 (3+)	33.83	836.45 (2+, y17), 807.93 (2+, y16), 779.45 (2+, y15), 721.91 (2+, y14), 683.37 (1+, b8), 665.38 (2+, y13), 580.32 (2+, y11), 523.76 (2+, y10), 513.26 (1+, b6), 432.26 (1+, y4), 400.18 (1+, b5)	
5	0	136-142	(R) TCFADMR (A)	900.37	450.69	300.79	225.85	180.88			900.37 (1+), 450.70 (2+)	24.41	639.29 (1+, y5), 595.22 (1+, b5), 492.23 (1+, y4), 421.18 (1+, y3), 409.16 (1+, b3), 400.17 (2+, y6)	
6	0	143-153	(R) AAYDALDEATR (A)	1195.56	598.28	399.19	299.65	239.92			1195.56 (1+), 598.28 (2+)	26.68	1053.47 (1+, y9), 1021.48 (1+, b10), 920.35 (1+, b9), 890.42 (1+, y8), 849.35 (1+, b8), 775.39 (1+, y7), 720.33 (1+, b7), 704.36 (1+, y6), 591.27 (1+, y5), 527.25 (2+, y9), 492.21 (1+, b5), 476.25 (1+, y4), 421.17 (1+, b4)	
7	0	154-159	(R) ALVHQR (H)	723.43	362.22	241.81	181.61	145.49			723.42 (1+), 362.22 (2+)	6.71	539.30 (1+, y4), 440.24 (1+, y3),	
10	0	214-228	(R) HAHAIPGMDAAESER (F)	1591.73	796.37	531.25	398.69	319.15			796.37 (2+), 531.25 (3+)	21.93	1062.44 (1+, y10), 965.40 (1+, y9), 777.34 (1+, y7), 684.35 (1+, b7), 662.32 (1+, y6), 601.27 (2+, b12), 591.26 (1+, y5), 530.28 (1+, b5), 520.24 (1+, y4), 501.24 (2+, b10), 465.72 (2+, b9), 417.20 (1+, b4), 408.20 (2+, b8), 391.20 (1+, y3)	
11	0	229-242	(R) FLEGLVDWACQAPR (V)	1661.81	831.41	554.61	416.21	333.17			1661.80 (1+), 831.41 (2+), 554.61 (3+)	49.58	1390.68 (1+, b12), 1319.57 (1+, b11), 1191.54 (1+, b10), 1031.52 (1+, b9), 1003.42 (1+, y8), 960.51 (1+, b8), 774.40 (1+, b7), 702.34 (1+, y6), 701.34 (2+, y12), 895.82 (2+, b12), 659.37 (1+, b6), 631.30 (1+, y5), 596.27 (2+, b10), 560.30 (1+, b5), 551.76 (2+, y9), 516.27 (2+, b9), 502.22 (2+, y8), 471.26 (1+, y4), 444.71 (2+, y7), 390.21 (1+, b3)	
12	0	243-259	(R) VHAHQWAAGDVVVWDNR (C)	1959.96	980.48	653.99	490.74	392.80			980.49 (2+), 653.99 (3+)	34.78	1172.57 (1+, b11), 1073.44 (1+, b10), 901.45 (1+, b8), 887.50 (1+, y7), 830.41 (1+, b7), 788.40 (1+, y6), 778.89 (2+, b14), 689.34 (1+, y5), 685.86 (2+, b13), 636.32 (2+, b12), 590.27 (1+, y4), 586.79 (2+, b11), 572.78 (2+, a11), 537.25 (2+, b10), 451.23 (2+, b8), 424.55 (3+, b12), 417.71 (2+, b7), 404.19 (1+, y3), 394.71 (2+, y6)	
13	0	260-264	(R) CLLHR (A)	698.38	349.69	233.46	175.35	140.48			698.37 (1+), 349.69 (2+)	17.33	425.26 (1+, y3)	
14	0	265-274	(R) AEPWDFKLPR (V)	1258.66	629.83	420.22	315.42	252.54			629.83 (2+), 420.22 (3+)	40.60	961.54 (1+, y7), 775.43 (1+, y6), 660.41 (1+, y5), 529.79 (2+, y8), 513.34 (1+, y4), 481.26 (2+, y7), 385.26 (1+, y3), 399.16 (1+, PWD int fr)	
15	0	275-280	(R) VMWHSR (L)	815.40	408.20	272.47	204.60	163.89			815.40 (1+), 408.21 (2+)	17.92	716.34 (1+, y5), 585.29 (1+, y4), 399.21 (1+, b3-H2O), 358.58 (2+, y5)	
16	0	281-293	(R) LAGRPETEGAALV (-)	1283.70	642.35	428.57	321.68	257.54			1283.69 (1+), 642.35 (2+)	30.75	1166.62 (1+, b12), 1053.52 (1+, b11), 982.47 (1+, b10), 911.44 (1+, b9), 886.44 (1+, y9), 854.42 (1+, b8), 624.35 (1+, b6), 583.81 (2+, b12), 569.80 (2+, a12), 574.80 (2+, b12-H2O), 527.27 (2+, b11), 513.27 (2+, a11), 518.26 (2+, b11-H2O), 491.75 (2+, b10), 398.25 (1+, b4)	
8-16	8	160-293	(R) SARHSLVYSQSKLGHVQQAGSAYIGYGMDTTATPLRPLVKVHPETGRPSLLIGRHAHAIPGMDAAESERFLEGLVDWACQAPRVHAHQWAAGDVVVWDNRCLLHRAEPWDFKLPRVMWHSRLAGRPETEGAALV (-)	14946.03 [0 charge]							**14946.03** (± 0.02) (z=0)	52.73		Incomplete C-term. cleavage; average masses, transformed; ave. 17+ charge; traces of Na- & K-salts are observed
non-spec1		196-206	(R) PLVKVHPETGR (P)	1232.71	616.86	411.58	308.93	247.35			616.86 (2+), 411.58 (3+)	19.11	795.44 (1+, y7), 696.32 (1+, y6), 674.43 (1+, b6), 568.34 (2+, y10), 559.28 (1+, y5), 511.79 (2+, y9), 462.25 (2+, y8)	unusual R-P cleavages
non-spec2		163-171	(R) HSLVYSQSK (L)	1048.54	524.78	350.19	262.89	210.51			1048.53 (1+), 524.77 (2+)	15.75	612.29 (1+, y5), 449.23 (1+, y4)	tryptic-like cleavage
non-spec3		172-195	(K) LGHVQQAGSAYIGYGMDTTATPLR (P)	2507.24	1254.12	836.42	627.56	502.25			1254.11 (2+), 836.42 (3+)	36.51	1282.63 (1+, y12), 1225.64 (1+, y11), 1167.05 (2+, b23), 1112.50 (1+, b11), 1061.99 (2+, b21), 1011.48 (2+, b20), 976.00 (2+, b19), 925.42 (2+, b18), 817.43 (2+, b16), 759.43 (1+, y7), 751.90 (2+, b15), 723.37 (2+, b14), 658.37 (1+, y6), 641.81 (2+, b13), 613.31 (2+, b12), 556.79 (2+, b11), 486.30 (1+, y4), 385.26 (1+, y3)	tryptic-like & R-P cleavage; trace of K-salt also observed

Cys residues are reduced and carboxyamidomethylated

Table IV: Chymotrypsin digest peptide mass fingerprinting of AAD-12 (Batch TSN030732-002).

Peptide	# of missed cleavages	a.a. ##	Sequence	m/z theor.					m/z obs. (ESI-LC/MS)	UPLC-MS elution time (min)	In-source fragments observed (matching amino-acid sequence)	Comment
				$(M+H)^{1+}$	$(M+2H)^{2+}$	$(M+3H)^{3+}$	$(M+4H)^{4+}$	$(M+5H)^{5+}$				
Y1	0	2-6	(-) AQTTL (Q)	533.29	267.15	178.44	134.08	107.46	533.29 (1+)	18.93	515.29 (1+, pep - H2O), 402.20 (1+, b4), 384.19 (1+, b4-H2O)	
Y2	0	7-15	(L) QITPTGATL (G)	901.50	451.25	301.17	226.13	181.11	901.50 (1+)	31.96	770.39 (1+, b8), 752.38 (1+, b8-H2O), 669.36 (1+, b7), 651.35 (1+, b7-H2O), 598.30 (1+, b6), 559.31 (1+, y6), 385.70 (2+, b8), 376.70 (2+, b8-H2O)	
Y3	0	16- 24	(L) GATVTGVHL (A)	854.47	427.74	285.50	214.37	171.70	854.48 (1+), 427.74 (2+)	31.73	726.41 (1+, y7), 625.37 (1+, y6), 586.32 (1+, b7), 526.30 (1+, y5), 390.20 (2+, y8-H2O), 363.72 (2+, y7), 354.71 (2+, y7-H2O)	
Y5	0	28- 32	(L) DDAGF (A)	524.20	262.60	175.40	131.81	105.65	524.20 (1+)	19.39	506.19 (1+, pep - H2O), 359.13 (1+, b4)	
Y7	0	36- 39	(L) HAAW (L)	484.23	242.62	162.08	121.81	97.65	484.23 (1+)	18.67	466.22 (1+, pep - H2O)	
Y11	0	46- 52	(L) IFPGQHL (S)	811.45	406.23	271.15	203.62	163.10	811.44 (1+), 406.23 (2+)	31.29	698.37 (1+, y6), 551.30 (1+, y5), 533.28 (1+, y5-H2O), 454.24 (1+, y4), 397.22 (1+, y3), 380.19 (1+, y3-17)	
Y12	0	53- 60	(L) SNDQQITF (A)	952.44	476.72	318.15	238.86	191.29	952.45 (1+)	31.96	787.37 (1+, b7), 769.34 (1+, b7-H2O), 686.31 (1+, b6), 573.22 (1+, b5), 433.25 (2+, y7), 394.18 (2+, b7), 380.22 (2+, a7)	
Y13	0	61- 64	(F) AKRF (G)	521.32	261.16	174.44	131.09	105.07	521.32 (1+)	6.00		
Y24	0	168-172	(Y) SQSKL (G)	562.32	281.66	188.11	141.34	113.27	562.32 (1+)	9.15		
Y27	0	186-194	(Y) GMDTTATPL (R)	906.42	453.72	302.81	227.36	182.09	906.43 (1+)	30.58	718.37 (1+, y7), 700.34 (1+, y7-H2O), 678.28 (1+, b7), 660.27 (1+, b7-H2O), 577.23 (1+, b6), 559.21 (1+, b6-H2O), 506.20 (1+, b5), 416.15 (2+, y8-H2O), 405.15 (1+, b4)	
Y27-Met-ox1	0	186-194	(Y) GMDTTATPL (R)	922.42	461.71	308.14	231.36	185.28	ND	ND		
Y28	0	195-197	(L) RPL (V)	385.26	193.13	129.09	97.07	77.86	385.26 (1+)	11.42		
Y29	0	198-209	(L) VKVHPETGRPSL (L)	1319.74	660.38	440.59	330.69	264.75	660.34 (2+), 440.57 (3+)	20.35		
Y36	0	249-256	(W) AAGDVVVW (D)	816.43	408.72	272.81	204.86	164.09	816.43 (1+)	41.12	698.37 (1+, x6), 657.36 (1+, y6-17), 612.34 (1+, b7), 594.33 (1+, b7-H2O), 584.33 (1+, a7), 513.27 (1+, b6), 495.26 (1+, b6-H2O), 485.27 (1+, a6), 414.21 (1+, b5), 403.25 (1+, y3), 396.20 (1+, b5-H2O), 386.20 (1+, a5)	
Y39	0	263-268	(L) HRAEPW (D)	795.39	398.20	265.80	199.60	159.88	795.40 (1+), 398.20 (2+)	20.76	658.32 (1+, y5), 591.30 (1+, b5), 494.24 (1+, b4), 485.09 (1+, y4-17), 466.26 (1+, a4)	
Y41	0	271-277	(F) KLPRVMW (H)	929.54	465.27	310.52	233.14	186.71	929.53 (1+), 465.27 (2+)	37.65	688.36 (1+, y5)	
Y41-Met-ox1	0	271-277	(F) KLPRVMW (H)	945.54	473.27	315.85	237.14	189.91	ND	ND		
Y42	0	278-281	(W) HSRL (A)	512.29	256.65	171.44	128.83	103.26	512.29 (1+)	5.50		
Y43	0	282-292	(L) AGRPETEGAAL (V)	1071.54	536.28	357.85	268.64	215.11	1071.53 (1+), 536.28 (2+)	21.89	940.44 (1+, b10), 869.42 (1+, b9), 811.38 (1+, x8), 470.73 (2+, b10), 435.22 (2+, b9)	
Y23-24	1	166-172	(L) VYSQSKL (G)	824.45	412.73	275.49	206.87	165.70	824.46 (1+), 412.73 (2+)	26.92		
Y29-30	1	198-210	(L) VKVHPETGRPSLL (I)	1432.83	716.92	478.28	358.96	287.37	1432.80 (1+), 716.93 (2+), 478.28 (3+)	28.13	969.53 (1+, y9), 872.45 (1+, y8), 810.46 (1+, c7), 667.39 (2+, y12), 651.38 (2+, b12), 642.38 (2+, b12-H2O), 603.34 (2+, y11), 553.82 (2+, y10), 485.28 (2+, y9), 464.30 (1+, b4), 434.59 (3+, b12), 425.25 (3+, a12)	
Y37-38	1	257-262	(W) DNRCLL (H)	790.39	395.70	264.13	198.35	158.88	790.39 (1+), 395.71 (2+)	28.80	659.30 (1+, b5), 561.32 (1+, y4), 546.20 (1+, b4)	
Y38-39	1	262-268	(L) LHRAEPW (D)	908.47	454.74	303.50	227.87	182.50	908.48 (1+), 454.75 (2+)	23.51	658.34 (1+, y5), 607.32 (1+, b5), 352.70 (2+, b6)	
Y43-44	1	282-293	(L) AGRPETEGAALV (-)	1170.61	585.81	390.88	293.41	234.93	1170.60 (1+), 585.82 (2+)	27.64	1053.54 (1+, b11), 940.45 (1+, b10), 886.46 (1+, y9), 869.41 (1+, b9), 741.37 (1+, b7), 527.27 (2+, b11), 518.27 (2+, b11-H2O), 513.28 (2+, a11), 470.73 (2+, b10), 435.21 (2+, b9)	
Y12-14	2	53- 95	(L) SNDQQITFAKRFGAIERIGGGDIVAISNVKADGTVRQHSPAEW (D)	4639.38	2320.19	1547.13	1160.60	928.68	1547.31 (3+), 1160.54 (4+), 928.64 (5+)	40.87		
Y32-34	2	230-236	(F) LEGLVDW (A)	831.42	416.22	277.81	208.61	167.09	831.43 (1+)	47.16	672.33 (1+, EGLVDW-28 int.fr.), 627.33 (1+, b6), 512.35 (1+, b5), 484.31 (1+, a5), 419.19 (1+, y3), 413.24 (1+, b4), 385.25 (1+, a4)	
non-spec1		125-135	(F) SAEVVPAVGGR (T)	1041.57	521.29	347.86	261.15	209.12	1041.56 (1+), 521.29 (2+)	25.28	833.51 (1+, y9), 754.45 (1+, y8), 655.39 (1+, y7), 556.32 (1+, y6), 486.26 (1+, b5), 458.26 (1+, a5), 442.25 (2+, y9), 387.19 (1+, b4), 359.20 (1+, a4)	partially-tryptic cleavage
non-spec2		125-136	(F) SAEVVPAVGGRT (C)	1142.62	571.81	381.54	286.41	229.33	1142.57 (1+), 571.82 (2+)	25.98	855.49 (1+, y9), 756.44 (1+, y8), 657.37 (1+, y7), 486.25 (1+, b5), 390.21 (1+, y4), 387.20 (1+, b4)	
non-spec3		65-80	(F) GAIERIGGGDIVAISN (V)	1541.83	771.42	514.61	386.21	309.17	1541.78 (1+), 771.43 (2+)	36.01	705.40 (2+, b15), 661.88 (2+, b14), 605.34 (2+, b13), 404.22 (1+, y4)	
non-spec4		65-78	(F) GAIERIGGGDIVAI (S)	1340.75	670.88	447.59	335.94	268.96	1340.80 (1+), 670.89 (2+)	42.20	1138.64 (1+, b12), 1039.58 (1+, b11), 926.53 (1+, b10), 640.38 (1+, b6), 605.34 (2+, b13), 569.82 (2+, b12), 555.82 (2+, a12), 520.28 (2+, b11)	
non-spec5		269-275	(W) DFKLPRV (M)	874.52	437.76	292.18	219.38	175.71	874.51 (1+)	51.35	583.38 (1+, b5-H2O), 484.31 (1+, y4), 466.30 (1+, y4-H2O), 391.20 (1+, b3), 371.23 (1+, y3), 353.22 (1+, y3-H2O)	

Cys residues are reduced and carboxyamidomethylated

Table V: Asp-N digest peptide mass fingerprinting of AAD-12 (Batch TSN030732-002).

Peptide	# of missed cleavages	a.a. ##	Sequence	m/z theor.					m/z obs. (ESI-LC/MS)	UPLC elution time, min	In-source fragments observed (matching amino-acid sequence)
				$(M+H)^{1+}$	$(M+2H)^{2+}$	$(M+3H)^{3+}$	$(M+4H)^{4+}$	$(M+5H)^{5+}$			
D1	0	2-27	(-) AQTTLQITPTGATLGATVTGVHLATL (D)	2536.40	1268.70	846.14	634.85	508.09	1268.72 (2+), 846.15 (3+)	51.58	1152.63 (2+, b24), 802.47 (3+, b25),
D9	0	111-139	(A) DSTYMPVMAQGAVFSAEVVPAVGGRTCFA (D)	3018.42	1509.71	1006.81	755.36	604.49	1006.84 (3+)	52.53	598.24 (1+, b5), 518.28 (2+, y10)
D10	0	140-145	(A) DMRAAY (D)	726.32	363.67	242.78	182.34	146.07	726.34 (1+)	19.17	545.25 (1+, b5), 480.26 (1+, y4)
D17	0	257-268	(W) DNRCLLHRAEPW (D)	1566.76	783.88	522.92	392.45	314.16	783.88 (2+), 522.93 (3+)	30.73	633.32 (2+, b10)
D18	0	269-293	(W) DFKLPRVMWHSRLAGRPETEGAALV (-)	2836.50	1418.76	946.17	709.88	568.11	946.17 (3+), 709.89 (4+)	40.57	907.13 (3+, b24), 869.47 (3+, b23), 845.77 (3+, b22), 680.87 (4+, b24), 652.35 (4+, b23), 634.61 (4+, b22), 413.25 (1+, y5-17)
D4-5	1	55- 83	(N) DQQITFAKRFGAIERIGGGDIVAISNVKA (D)	3074.67	1537.84	1025.56	769.42	615.74	1025.57 (3+), 769.45 (4+)	44.31	
D14-15	1	222-251	(M) DAAESERFLEGLVDWACQAPRVHAHQWAAG (D)	3377.59	1689.30	1126.53	845.15	676.32	845.19 (4+), 676.56 (5+)	54.79	
non-spec1		23-100	(V) HLATLDDAGFAALHAAWLQHALLIFPGQHLSNDQQITFAKRFGAIERIGGGDIVAISNVK ADGTVRQHSPAEWDDMMK (V)	8492.61 [0 charge]					8492.65 [0 charge; transformed, average mass]	60.95	

Cys residues are reduced and carboxyamidomethylated

Table VI: Glu-C digest peptide mass fingerprinting of AAD-12 (Batch TSN030732-002).

Peptide	# of missed cleavages	a.a. ##	Sequence	m/z theor.					m/z obs. (ESI-LC/MS)	UPLC elution time, min	In-source fragments observed (matching amino-acid sequence)
				$(M+H)^{1+}$	$(M+2H)^{2+}$	$(M+3H)^{3+}$	$(M+4H)^{4+}$	$(M+5H)^{5+}$			
A11	0	112-127	(D) STYMPVMAQGAVFSAE (V)	1688.77	844.89	563.59	422.95	338.56	844.89 (2+)	44.29	1206.57 (1+, y12), 810.35 (1+, b7), 679.34 (1+, b6), 483.20 (1+, b4)
A12	0	128-140	(E) VVPAVGGRTCFAD (M)	1348.67	674.84	450.23	337.92	270.54	674.84 (2+)	29.45	575.78 (2+, y11)
A13-14	1	141-150	(D) MRAAYDALDE (A)	1154.52	577.76	385.51	289.38	231.71	577.76 (2+)	28.09	892.43 (1+, b8)
A17-18	1	204-225	(E) TGRPSLLIGRHAHAIPGMDAAE (S)	2270.18	1135.60	757.40	568.30	454.84	1135.63 (2+), 747.40 (3+), 568.30 (4+)	31.43	790.97 (2+, b15), 734.42 (2+, b14), 708.39 (3+, b21), 698.93 (2+, b13), 684.71 (3+, b20), 675.37 (3+, a20), 661.03 (3+, b19), 630.34 (2+, b12), 622.69 (3+, b18), 579.01 (3+, b17), 531.55 (4+, b21), 513.78 (4+, b20), 496.02 (4+, b19), 489.95 (3+, b14), 466.27 (3+, b13)
A1-4	3	2-68	(-) AQTTLQITPTGATLGATVTGVHLATLDDAGFAALHAAWLQHALLIFPGQHLSNDQQITFA KRFGAIE (R)	7039.99	3520.50	2347.33	1760.75	1408.80	1408.67 (5+), 1174.11 (6+), 1006.52 (7+), 880.81 (8+)	62.58	
				7038.99 [0 charge]					7040.57 [0 charge; transformed]		
A12-14	3	128-150	(E) VVPAVGGRTCFADMRAAYDALDE (A)	2484.17	1242.59	828.73	621.80	497.64	1245.55 (2+), 828.74 (3+), 621.82 (4+)	40.82	1169.07 (2+, b22), 1143.52 (2+, y21), 1111.55 (2+, b21), 1055.00 (2+, b20), 1019.50 (2+, b19), 1009.98 (2+, y18), 961.98 (2+, b18), 880.42 (2+, b17), 779.71 (3+, b22), 762.69 (3+, y21), 741.37 (3+, b21), 725.30 (1+, y6), 703.68 (3+, b20), 562.24 (1+, y5), 466.29 (1+, b5), 447.21 (1+, y4), 376.17 (1+, y3)

Cys residues are reduced and carboxyamidomethylated

Table VII:

(A) Amino acid sequence obtained by in-source fragmentation for the C-terminal peptide ^{281}LAGRPETEGAALV^{293} (m/z 642.35) from Arg-C digest of sample AAD-12 (Batch TSN030732-002) eluting at 30.7 min.

Sequence: LAGRPETEGAALV
Fragment ion masses: monoisotopic
Peptide mass $[M+2H]^{2+}$: 642.35

Ion		*L*	*A*	*G*	*R*	*P*	*E*	*T*	*E*	*G*	*A*	*A*	*L*	*V*
y	theoretical	-	1170.61	1099.57	1042.55	886.45	789.40	660.36	559.31	430.27	373.25	-	-	-
	experimental					**886.41**				**430.26**				
y^{2+}	theoretical		585.81^{+2}	550.29^{+2}	521.78^{+2}	443.73^{+2}	395.20^{+2}	-	-	-	-	-	-	-
	experimental													
b	theoretical	-	-	-	398.25	495.30	624.35	725.39	854.44	911.46	982.50	1053.53	1166.62	-
	experimental				**398.25**		**624.35**		**854.40**	**911.44**	**982.47**	**1053.52**	**1166.61**	
b^{2+}	theoretical	-	-	-	-	-	-	363.20^{+2}	427.72^{+2}	456.23^{+2}	491.75^{+2}	527.27^{+2}	583.81^{+2}	-
	experimental										**491.75**	**527.27**	**583.81**	
a	theoretical	-	-	-	370.26	467.31	596.35	697.40	826.44	883.46	954.50	1025.54	1138.62	-
	experimental					**467.22**								
a^{2+}	theoretical	-	-	-	-	-	-	-	413.73^{+2}	442.24^{+2}	477.75^{+2}	513.27^{+2}	569.81^{+2}	
	experimental											**513.27**	**569.80**	

** only ions with m/z between 350 and 1900 were recorded in this experiment (LC/MS).*

(B) Amino acid sequence obtained by tandem MS for the C-terminal peptide ^{281}LAGRPETEGAALV^{293} (m/z 642.36) from tryptic digest of sample AAD-12 (Batch TSN030732-002) eluting at 29.65 min.

Sequence: LAGRPETEGAALV
Fragment ion masses: monoisotopic
Theoretical monoisotopic peptide mass $[M+2H]^{2+}$: 642.35

Ion		*L*	*A*	*G*	*R*	*P*	*E*	*T*	*E*	*G*	*A*	*A*	*L*	*V*
y	theoretical	-	1170.61	1099.57	1042.55	886.45	789.40	660.36	559.31	430.27	373.25	302.21	231.17	118.09
	experimental												**231.18**	**118.09**
b	theoretical	114.09	185.13	242.15	398.25	495.30	624.35	725.39	854.44	911.46	982.50	1053.53	1166.62	-
	experimental		**185.14**	**242.17**	**398.23**		**624.37**	**725.41**	**854.43**	**911.43**	**982.51**	**1053.55**	**1166.66**	
b^{2+}	theoretical	-	93.07^{+2}	121.58^{+2}	199.63^{+2}	248.16^{+2}	312.68^{+2}	363.20^{+2}	427.72^{+2}	456.23^{+2}	491.75^{+2}	527.27^{+2}	583.81^{+2}	-
	experimental									**456.25**	**491.77**	**527.29**		
a	theoretical	86.10	157.13	214.16	370.26	467.31	596.35	697.40	826.44	883.46	954.50	1025.54	1138.62	-
	experimental	**86.10**	**157.14**	**214.15**	**370.27**		**596.38**		**826.46**		**954.54**	**1025.55**		
a^{2+}	theoretical	-	-	107.58^{+2}	185.63^{+2}	234.16^{+2}	298.68^{+2}	349.20^{+2}	413.73^{+2}	442.24^{+2}	477.75^{+2}	513.27^{+2}	569.81^{+2}	
	experimental										**477.76**			

** only ions with m/z between 80 and 1900 were recorded in this experiment (MS/MS).*

Table VII: (continued)

(C) Amino acid sequence obtained by tandem MS for the C-terminal peptide ^{282}AGRPETEGAALV^{293} (m/z 585.82) from chymotryptic digest of sample AAD-12 (Batch TSN030732-002) eluting at 26.6 min.

Sequence: AGRPETEGAALV
Fragment ion masses: monoisotopic
Theoretical monoisotopic peptide mass $[M+2H]^{2+}$: 585.81

Ion		*A*	*G*	*R*	*P*	*E*	*T*	*E*	*G*	*A*	*A*	*L*	*V*
y	theoretical	-	1099.57	1042.55	886.45	789.40	660.36	559.31	430.27	373.25	302.21	231.17	118.09
	experimental											**231.18**	**118.09**
b	theoretical	-	129.07	285.17	382.22	511.26	612.31	741.35	798.37	869.41	940.45	1053.53	-
	experimental			**285.18**	**382.18**	**511.26**	**612.30**	**741.37**	**798.38**	**869.42**	**940.46**	**1053.56**	
b^{2+}	theoretical	-	-	143.09^{+2}	191.61^{+2}	256.14^{+2}	306.66^{+2}	371.18^{+2}	399.69^{+2}	435.21^{+2}	470.73^{+2}	527.27^{+2}	-
	experimental								**399.68**	**435.20**	**470.72**		
a	theoretical	-	101.07	257.17	354.23	483.27	584.32	713.36	770.38	841.42	912.45	1025.54	-
	experimental		**101.07**	**257.18**		**483.25**		**713.40**	**770.39**	**841.42**	**912.46**	**1025.54**	
a^{2+}	theoretical	-	-	129.09^{+2}	177.62^{+2}	242.14^{+2}	292.66^{+2}	357.18^{+2}	385.69^{+2}	421.21^{+2}	456.73^{+2}	513.27^{+2}	
	experimental									**421.20**			

** only ions with m/z between 80 and 1900 were recorded in this experiment (MS/MS).*

Table VIII:

(A) Amino acid sequence obtained by tandem MS for the N-terminal peptide ^{2}AQTTLQITPTGATLGATVTGVHLATLDDAGFAALHAAWLQHALLIFPGQHLSNDQQITFAK^{62} (m/z 1063.22) from tryptic digest of sample AAD-12 (Batch TSN030732-002) eluting at 59.93 min.

Sequence: AQTTLQITPTGATLGATVTGVHLATLDDAGFAALHAAWLQHALLIFPGQHLSNDQQITFAK
Fragment ion masses: monoisotopic
Theoretical peptide average mass $[M+6H]^{6+}$: 1061.88

Ion		A	Q	T	T	L	Q	I	T	P	T	G	A	T	L	G	A	T	V	T	G	V
y^{5+}	theoretical	-	1259.06	1233.45	1213.24	1193.03	1170.41	1144.80	1122.19	1101.98	1082.56	1062.35	1050.95	1036.74	1016.53	993.92	982.51	968.30	948.09	928.28	908.07	896.67
	experimental									**1102.02**												
y^{4+}	theoretical	-	1573.58	1546.57	1516.30	1491.04	1462.77	1430.76	1402.49	1377.22	1352.96	1327.70	1313.44	1295.68	1270.42	1242.15	1227.90	1210.14	1184.87	1160.11	1134.84	1120.59
	experimental															**1242.18**						
b	theoretical	-	200.10	301.15	402.20	515.28	643.34	756.43	857.47	954.53	1055.57	1112.60	1183.63	1284.68	1397.76	1454.79	1525.82	1626.87	1725.94	1826.99	1884.01	1938.08
	experimental		**200.12**	**301.17**		**515.30**		**756.51**														
b^{2+}	theoretical	-	100.56	151.08	201.60	258.15	322.17	378.72	429.24	477.77	528.29	556.80	592.32	642.84	699.39	727.90	763.42	813.94	863.47	914.00	942.51	992.04
	experimental																					
a	theoretical	-	172.11	273.16	374.20	487.29	615.35	728.43	829.48	926.53	1027.58	1084.60	1155.64	1256.69	1369.77	1426.79	1497.83	1598.88	1697.94	1798.99	1856.01	1955.08
	experimental																					

Ion		H	L	A	T	L	D	D	A	G	F	A	A	L	H	A	A	W	L	Q	H
y^{2+}	theoretical	2190.64	2122.11	2065.57	2030.05	1979.52	1922.98	1865.47	1807.96	1772.44	1743.93	1670.39	1634.87	1599.35	1542.81	1474.28	1438.76	1403.25	1310.21	1253.66	1189.63
	experimental																				
b^{2+}	theoretical	1060.57	1117.11	1152.63	1203.16	1259.70	1317.21	1374.72	1410.24	1438.75	1512.29	1547.81	1583.33	1639.87	1708.40	1743.92	1779.43	1872.47	1929.02	1993.04	2061.57
	experimental																				

Ion		A	L	L	I	F	P	G	Q	H	L	S	N	D	Q	Q	I	T	F	A	K
y	theoretical	2241.20	2170.17	2057.08	1944.00	1830.91	1683.85	1586.79	1529.77	1401.71	1264.65	1151.57	1064.54	950.49	835.47	707.41	579.35	466.27	365.22	218.15	147.11
	experimental																				
y^{2+}	theoretical	1121.11	1085.59	1029.04	972.50	915.96	842.43	793.90	765.39	701.36	632.83	576.29	532.77	475.75	418.24	354.21	290.18	233.64	183.11	109.58	-
	experimental																				

** only ions with m/z between 80 and 1900 were recorded in this experiment (MS/MS).*

Table VIII: (continued)

(B) Amino acid sequence obtained by tandem MS for the N-terminal peptide ^{2}AQTTL^{6} (m/z 533.29) from chymotryptic digest of sample AAD-12 (Batch TSN030732-002) eluting at 18.52 min.

Sequence:	AQTTL
Fragment ion masses:	monoisotopic
Theoretical peptide average mass $[M+H]^{1+}$:	533.29

Ion		*A*	*Q*	*T*	*T*	*L*
y	theoretical	-	462.26	334.20	233.15	132.10
	experimental				**233.16**	**132.11**
y°	theoretical	-	444.25	316.19	215.14	114.09
	experimental				**215.13**	
b	theoretical	-	200.10	301.15	402.20	-
	experimental		**200.11**	**301.15**		
b°	theoretical	-	182.09	283.14	384.19	-
	experimental			**283.13**	**384.16**	
b*	theoretical	-	183.08	284.12	385.17	-
	experimental		**183.08**	**284.11**		
a	theoretical	-	172.11	273.16	374.20	-
	experimental					

** only ions with m/z between 80 and 1900 were recorded in this experiment (MS/MS).*

Figure 1: Theoretical amino acid sequence of AAD-12 with sequence coverage for AAD-12 (Batch TSN030732-002) by ESI-LC/MS. Cys residues were alkylated with Iodoacetamide. Overall sequence coverage was 100%. Complete removal of N-terminal Met[1] was observed (shown with an arrow). No Asn deamidation was observed.
Theoretical average mass of processed (*des*-Met[1]) AAD-12 is 31769.17 Da (Cys reduced and carboxyamidomethylated) [31598.02 Da for unmodified reduced Cys].

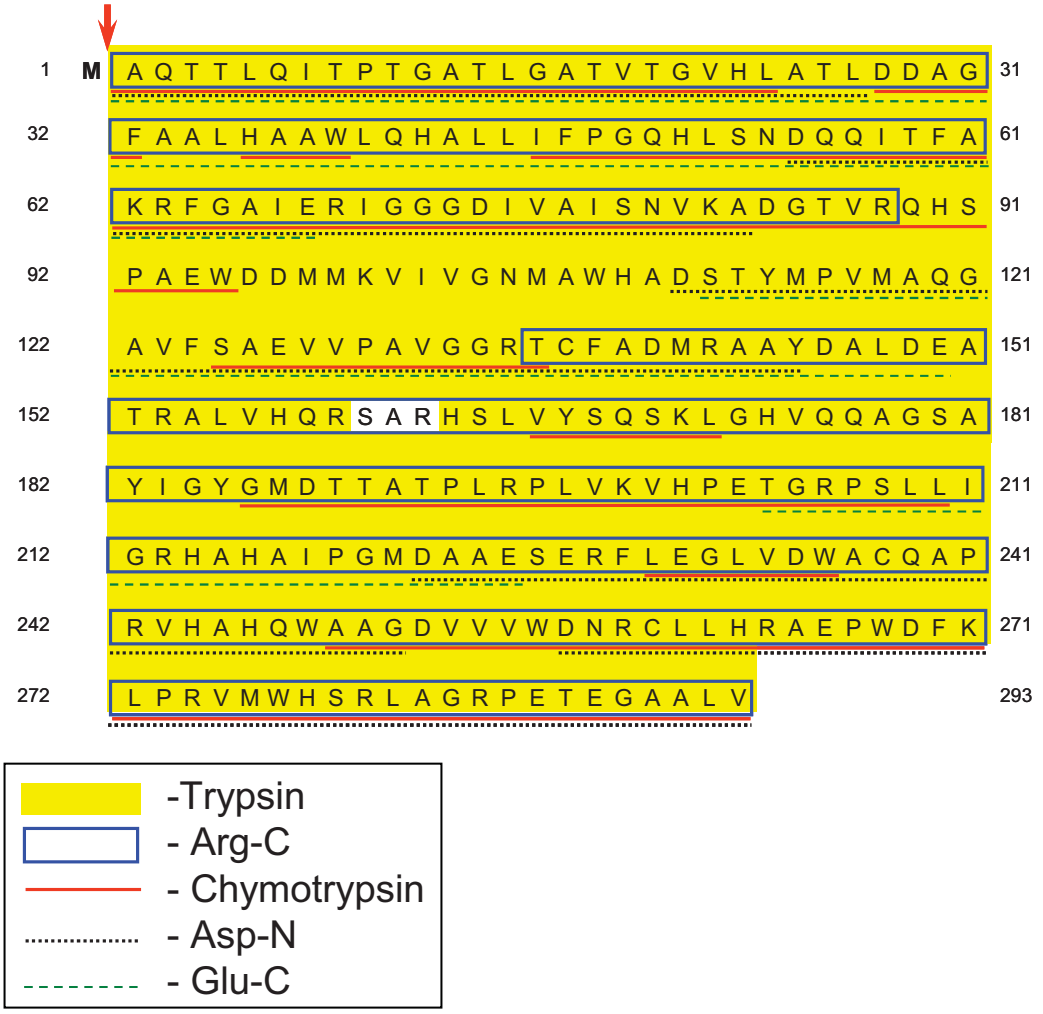


Figure 2: AAD-12 sample (Batch TSN030732-002) prepared in PBS/0.1M Gu:HCl buffer, analyzed by UPLC-MS. (**a**) Chromatograph (UV 214 nm trace); (**b**) multiple charge envelope mass spectrum (top; m/z axis) of component in Peak I, and the corresponding deconvoluted and centered mass spectrum (bottom; true mass axis); (**c**) multiple charge envelope mass spectrum (top; m/z axis) of component in Peak II, and the corresponding deconvoluted and centered mass spectrum (bottom; true mass axis); (**d**) multiple charge envelope mass spectrum (top; m/z axis) of component in Peak III, and the corresponding deconvoluted and centered mass spectrum (bottom; true mass axis). See **Table I** for peak assignments.

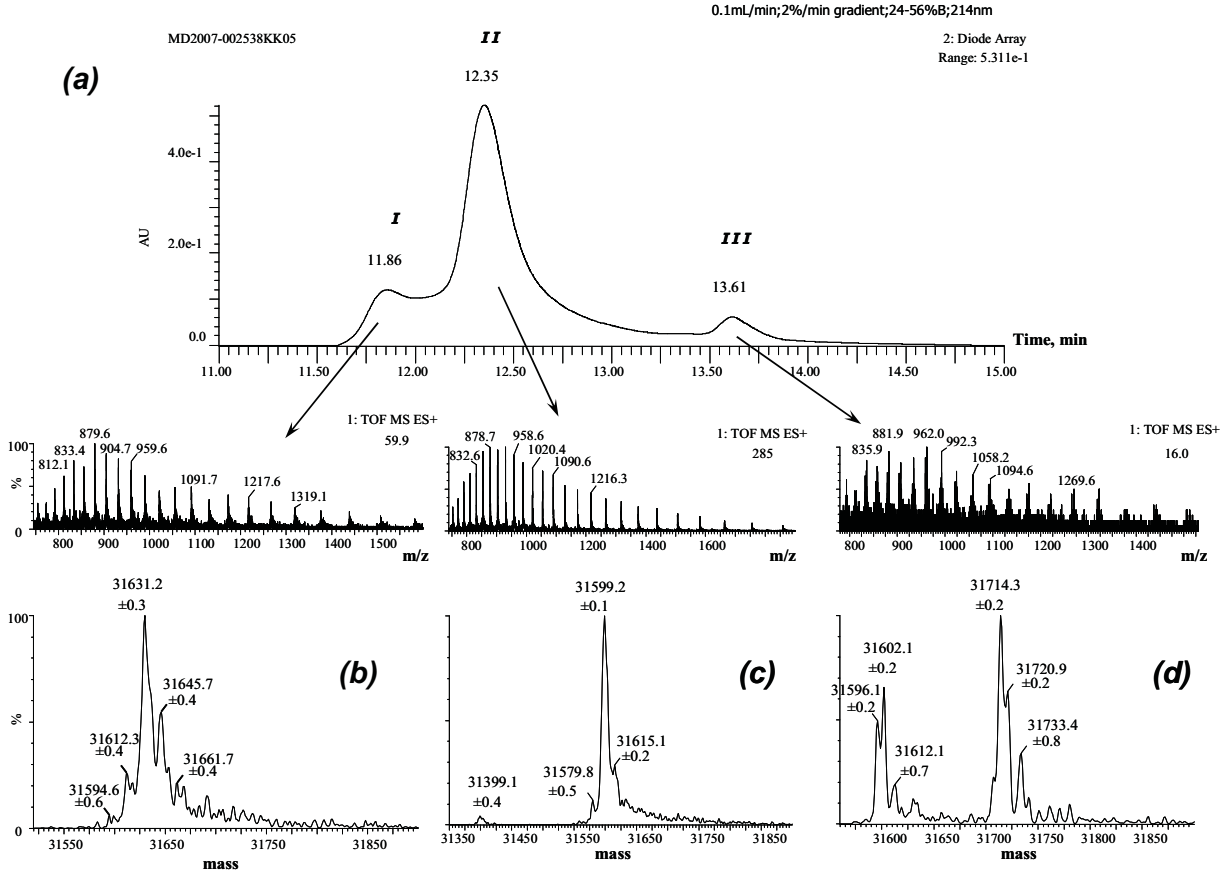


Figure 3: ESI-LC/MS chromatogram (MS TIC) for AAD-12 (Batch TSN030732-002) tryptic digest.

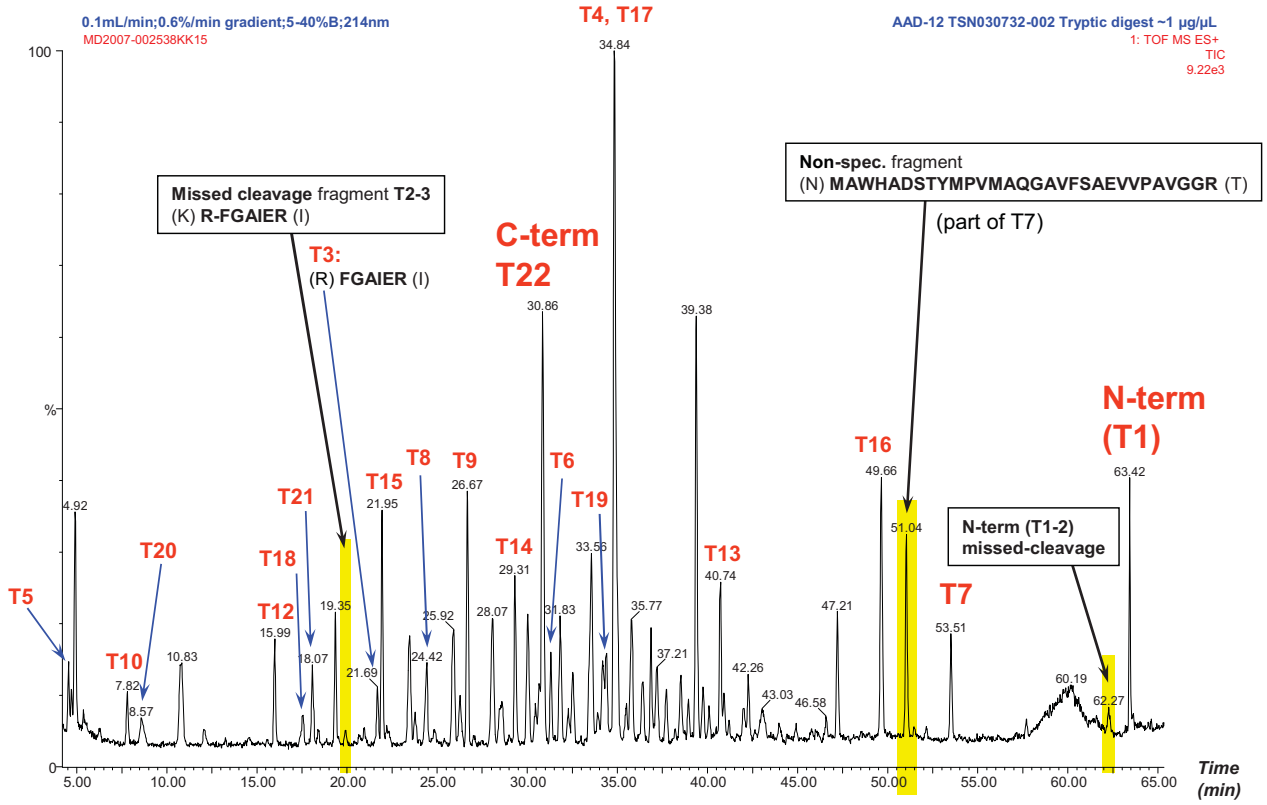


Figure 4: ESI-LC/MS chromatogram (MS TIC) for AAD-12 (Batch TSN030732-002) Arg-C digest.

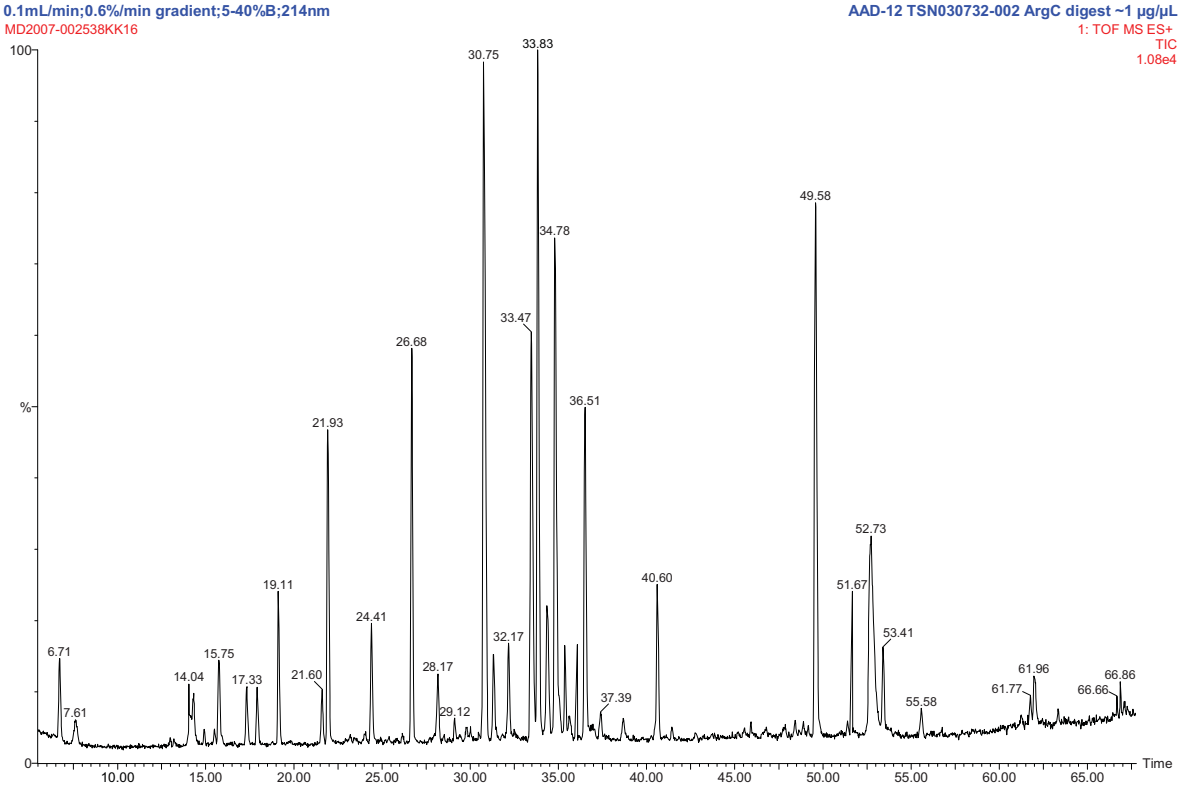


Figure 5: ESI-LC/MS chromatogram (MS TIC) for AAD-12 (Batch TSN030732-002) chymotryptic digest.

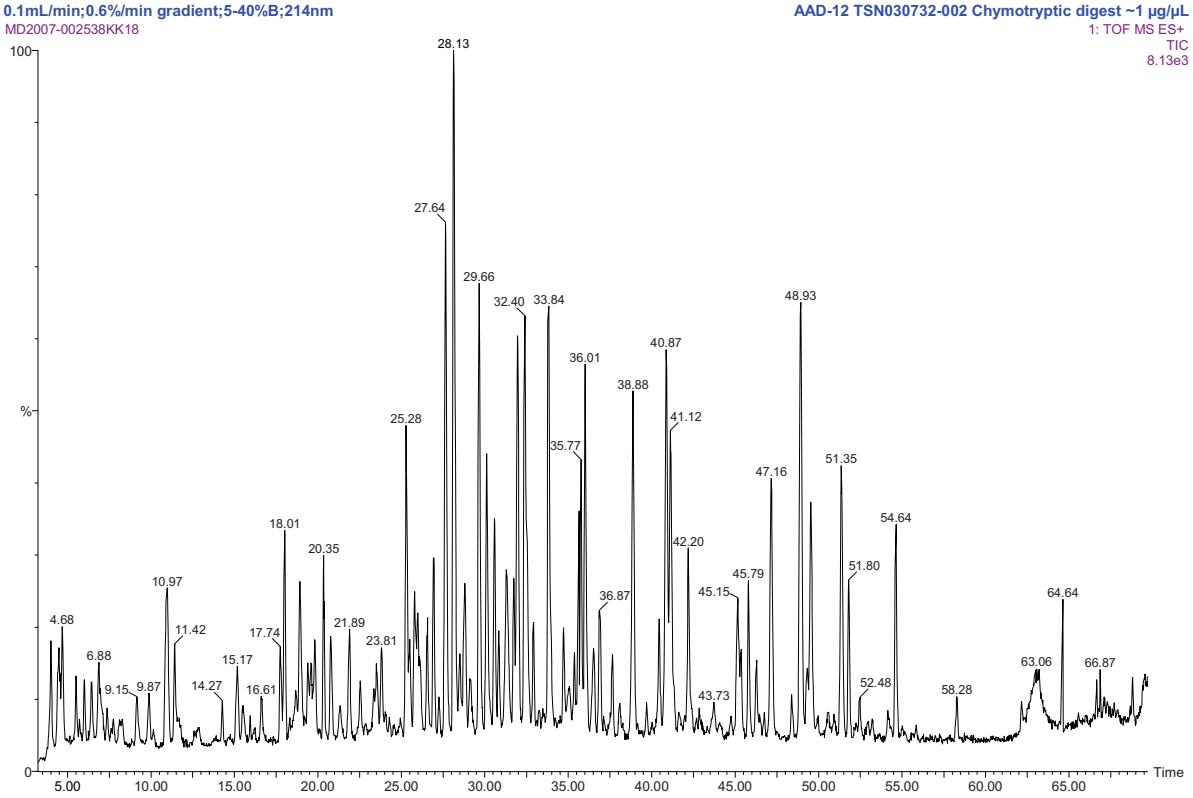


Figure 6: ESI-LC/MS chromatogram (MS TIC) for AAD-12 (Batch TSN030732-002) Asp-N digest.

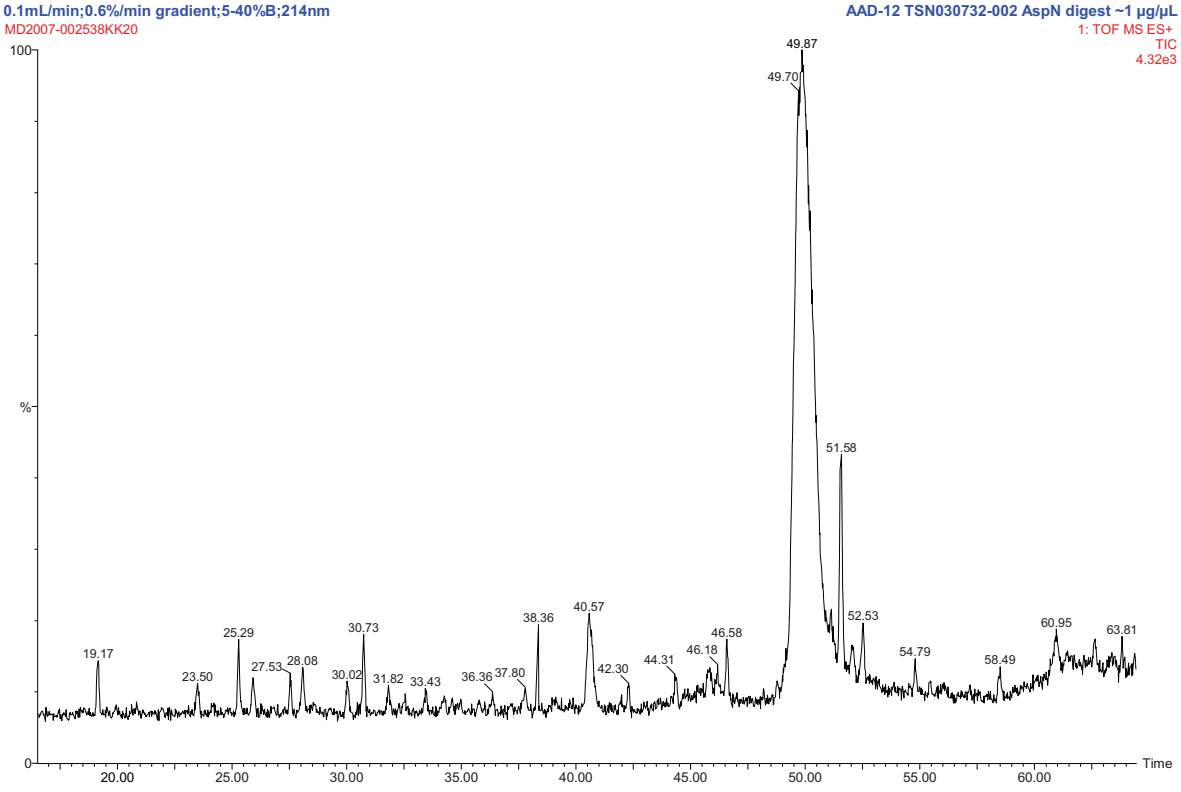


Figure 7: ESI-LC/MS chromatogram (MS TIC) for AAD-12 (Batch TSN030732-002) Glu-C digest.

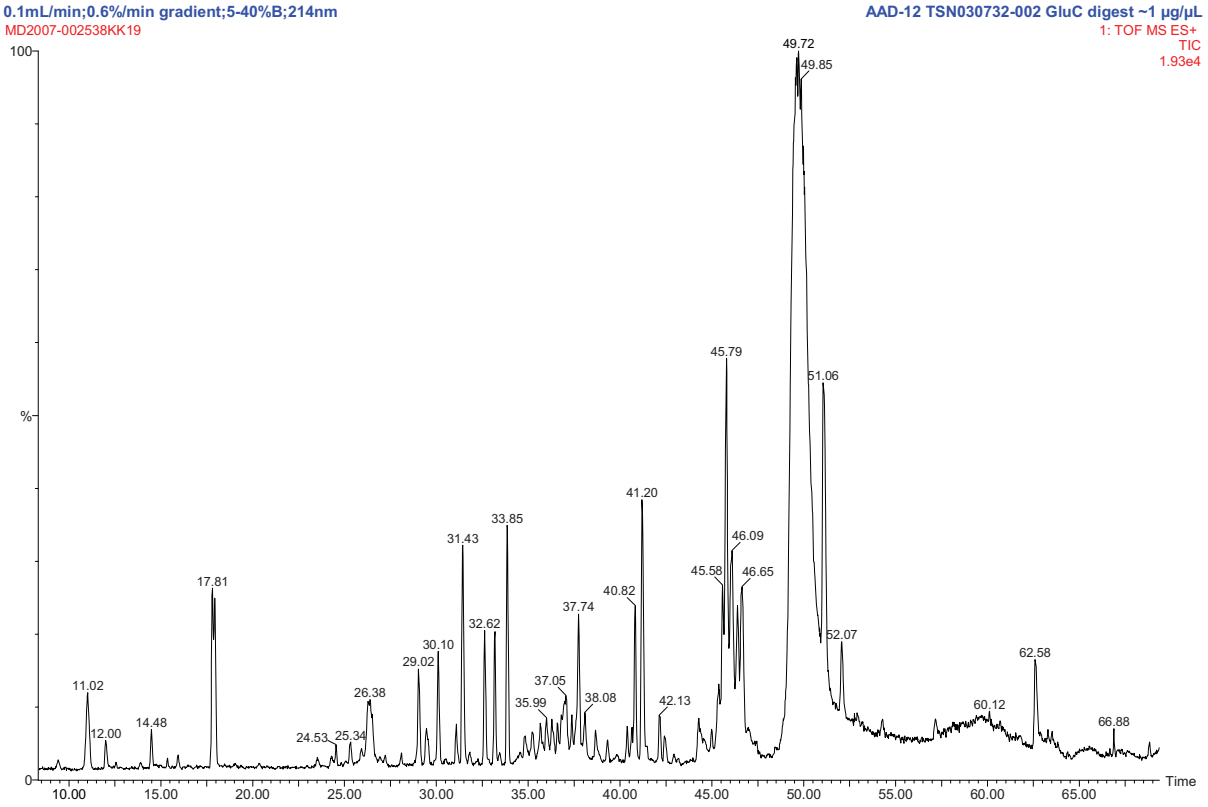
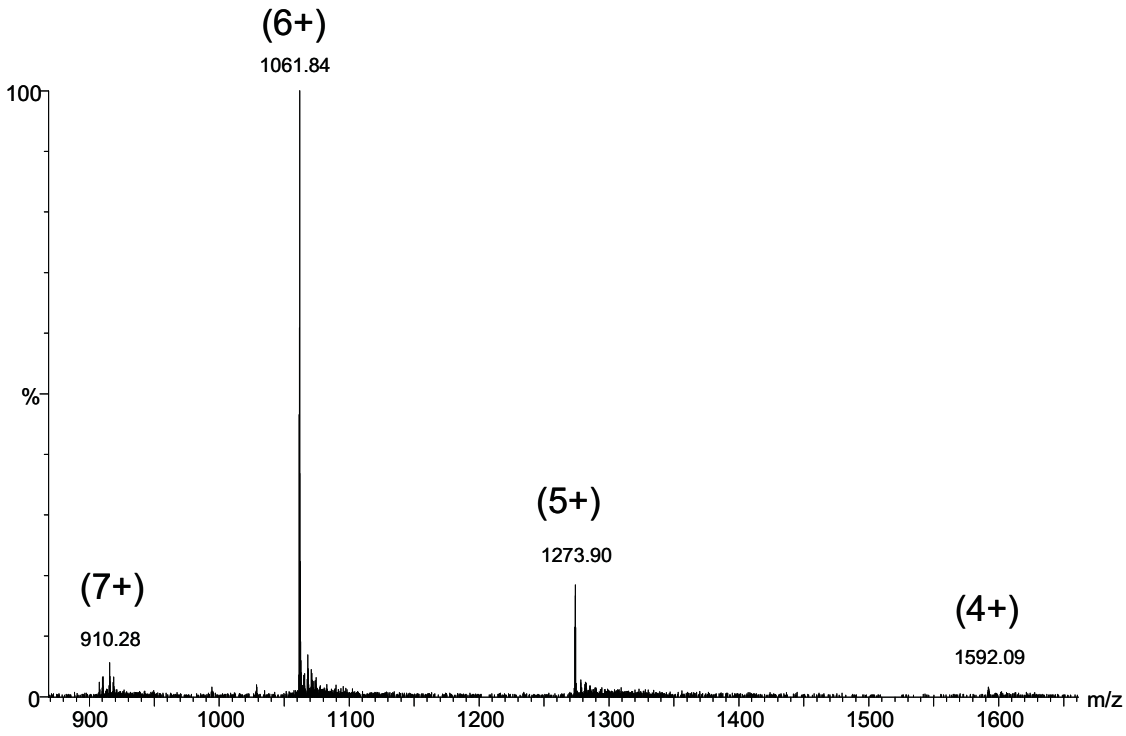


Figure 8: A representative ESI-MS spectrum of N-terminal tryptic fragment T1 (*des*-Met[1]): [2]AQTTLQITPTGATLGATVTGVHLATLDDAGFAALHAAWLQHALLIFPGQHLSNDQQITFAK[62]. Charged states and m/z values are indicated above peaks (see **Figure 3**, and **Tables II** and **VIII** for details). Sample: AAD-12 (Batch TSN030732-002) tryptic digest. Similar spectra of N-terminal fragments were also observed in Arg-C and Glu-C digests (not shown; see **Tables III** and **VI**).



APPENDIX 3

Mass Spectrometry Analysis of Soybean-Derived AAD-12 Protein (Event DAS-444Ø6-6)

Mass Spectrometry Report

Dow AgroSciences LLC

Department	Geographic Location	
Biotechnology Regulatory Sciences	Indianapolis	
Page Count	Protocol Study Number	Report Status
18	101707	Final

Title

Characterization of AAD12 partially purified from soybean event DAS-44406-6

Author(s): Last Name and Initials (Master Numbers)	Author(s) Signature / Date
Harpham, Nicholas (NJ) (U407575)	20-JULY-2011
Reviewer(s): Last Name and Initials (Master Numbers)	**Reviewer Signature(s)/Date**
Singletary, Linda (L) (U411575)	Linda Singletary 20-Jul-11
Karnoup, Anton (AS) (U369292)	06/24/2011
Schafer, Barry (BW) (U097380)	20-JUL-2011

Patent Status		
Disclosure Submitted	Case Filed	No Action Required
[ ]	[ ]	[x]

Descriptive Summary and Conclusions

A partially purified AAD12 herbicide tolerance protein from soybean event DAS-44406-6 was submitted as a band on a Coomassie stained gel by Brian Barnett, on behalf of Barry Schafer of Dow AgroSciences Biotechnology Regulatory Sciences Group for characterization. Comprehensive peptide mapping, analysis of N- and C-terminal sequences and characterization of possible post-translational modifications by MALDI MS, and MALDI MS-MS were requested. The quantity of SDS-PAGE separated AAD12 protein submitted was approximately 100 ng per band.

The sequence coverage obtained from a tryptic digest of the AAD12 (DAS-44406-6) protein analyzed by MALDI Peptide Mass Fingerprint (PMF) MS measurements was 44.7% and coverage by MALDI MS-MS measurements was 41.0 %. The sequence coverage obtained from a chymotryptic digest of the AAD12 protein analyzed by MALDI PMF MS measurements was 59.4% and coverage by MALDI MS-MS measurements was 56.0%. The combined MALDI PMF MS sequence coverage obtained from all methods of measurement was 84.3% with MS-MS data covering 76.1% of the peptides in the protein sequence. The methods used included MALDI-TOF MS and MALDI-TOF MS-MS of in-gel tryptic and chymotryptic digests of the AAD12 protein.

The N-terminal peptide ^{1}MAQTTL^{6} (from the chymotryptic digest) was determined to be present in both a full length form and in an acetylated form from which Met^{1} had been removed, Ac-^{2}AQTTL^{6}. The majority of the peptides observed in the proteolytic digests exhibited collision induced dissociation fragmentation patterns (in MALDI TOF-TOF MS-MS) that were consistent with their expected theoretical amino acid sequences.

The sequence of the C-terminal fragment ^{281}LAGRPETEGAALV^{293} (m/z 1283.6954) from the tryptic digest was confirmed by MALDI TOF-TOF MS-MS following collision induced dissociation.

No other post-translational or in-process modifications were detected for the AAD12 protein.

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INTRODUCTION

A sample of partially purified and SDS-PAGE resolved AAD12 herbicide tolerance protein (DAS-44406-6) was submitted by Brian Barnett, on behalf of Barry Schafer, of Dow AgroSciences for characterization. Analytical data on peptide mass fingerprinting and N- and C-terminal sequencing was requested.

EXPERIMENTAL

In-gel protein processing and enzymatic digests:

Equipment:

a) AB Sciex 4800 MALDI ToF-ToF
b) Mettler AT200 analytical balance
c) Eppendorf, Thermomixer R
d) Centrifugal evaporator (Jouan Speed Vac)
e) Gilson and Eppendorf adjustable pipettes
f) Siliconized microcentrifuge tubes, 0.5 and 1.5mL, (Fisher)

Reagents and Standards:

1. Acetonitrile, Fluka (LC-MS grade)
2. Ammonium bicarbonate, (Sigma)
3. Dithiothreitol (DTT), GE Healthcare
4. Iodoacetamide (IAA), GE Healthcare
5. Trypsin, (Roche)
6. Chymotrypsin, (Roche)
7. Formic Acid (FA), Fluka
8. Trifluoroacetic Acid (TFA), Pierce
9. Milli-Q deionized water (freshly drawn)

Reagent Solution Preparation:

a. 25 mM Ammonium Bicarbonate buffer: dissolved 98.83 mg NH_4HCO_3 in 50 mL of Milli-Q water; filtered through 0.22 μm sterile syringe filter.
b. DTT solution (100 mM; prepared fresh): dissolved 13.9 mg DTT in 0.903 mL of water.
c. Alkylating reagent (IAA) (200 mM; prepared fresh): dissolved 35.7 mg IAA in 1 mL of water.
d. Trypsin solution: Dissolved 25 µg of dried trypsin in 400 µL of 25 mM ammonium bicarbonate buffer immediately prior to digestion procedure.
e. Chymotrypsin solution: Dissolved 25 µg of dried chymotrypsin in 400 µL of 1 mM HCl immediately prior to digestion procedure.

In-gel Protein Processing Procedure:

a. For each enzyme digestion SDS-PAGE bands at ~ 30 kDa corresponding to AAD12 protein (approximately 100 ng) were excised with a sterile scalpel from the gel, and processed as follows (a blank section of the gel at approximately the same height in the gel was processed alongside with the protein sample, in a separate tube, using identical procedure):
b. Gel pieces were crushed with a sterile micro-pestle in a siliconized microcentrifuge tube, and destained as follows: 0.4 mL of 50% ACN and 0.4 mL of ammonium bicarbonate buffer were added, the tube was sealed and shaken at room temperature for 30 min in a Thermomixer R at 1100 rpm; the tube was centrifuged to settle the gel pieces, and the supernatant was removed with a pipette tip and discarded; the destain procedure was repeated 3 times.
c. The protein was reduced and alkylated in-gel as follows: (1) 0.2 mL of DTT solution was added to gel pieces, and the tube was incubated at room temperature for 30 min in a Thermomixer R; (2) the tube was centrifuged, and the DTT solution was removed by a pipette tip; (3) the gel pieces were washed with 0.5 mL of 25 mM ammonium bicarbonate buffer, the tube was centrifuged, and the buffer was removed; (4) 0.2 mL of IAA solution was added to the gel pieces, and the tube was incubated in darkness at ambient temperature for 1 hour; (5) the gel pieces were washed twice with 0.5 mL of 25 mM ammonium bicarbonate buffer (the tube was centrifuged, and the buffer was removed after each wash).
d. After the destaining/ reduction/ alkylation procedures, the gel pieces were dried in a centrifugal evaporator for 30 min.
e. In-gel digestion with Trypsin: the dried gel pieces were re-hydrated with trypsin solution (25 μg in 400 μL of 25 mM ammonium bicarbonate buffer, pH 7.8; prepared fresh) and incubated in a Thermomixer R at 900 rpm and 37 ºC for 16 hours (overnight).

f. In-gel digestion with Chymotrypsin: the dried gel pieces were re-hydrated with chymotrypsin solution (25 μg in 400 μL of 1mM HCl; prepared fresh) and incubated in a Thermomixer R at 900 rpm and 25 ºC for 16 hours (overnight).
g. Extraction of peptides from gel: peptides were extracted sequentially with 0.4 mL of 50% ACN/ 0.1% TFA, then 0.4 mL of 50% ACN/ 5% FA, and then 0.4 mL of 70% ACN/ 5% FA (30 min per extraction in a Thermomixer R at room temperature, shaking at 1100 rpm). The extracts for each sample were combined and dried in a centrifugal evaporator.
h. Dried peptides were reconstituted in 15 μL of 0.1% TFA and were purified for MALDI MS analysis using C18 zip-tips, as described below.

MALDI-TOF MS analysis of AAD12 proteolytic digests:

Reagents and Standards:

1. Trifluoroacetic Acid (TFA), Pierce
2. Deionized water, 18.2 MΩ/cm, MilliQ, Millipore, freshly drawn
3. Acetonitrile, Fluka (LC-MS grade)
4. 4700 Proteomics Analyzer Mass Standards Kit, (ABSciex)
5. CHCA (α-Cyano-4-hydroxycinnamic acid), Sigma

Analytical Procedure:

Sample preparation and deposition: Tryptic (in-gel digest), and Chymotryptic (in-gel digest) peptides were purified using Millipore C18 zip-tips as shown on the relevant run sheet. Purified peptides were eluted directly onto MALDI plate sequentially, with aqueous 10%, 25%, 50%, and 70% ACN (supplemented with 0.1% TFA). The C18 zip-tip fractions were mixed on-plate with 1 μL of CHCA matrix and air-dried.

MALDI-TOF MS: The sample preparations were analyzed directly by MALDI-TOF mass spectrometry. All mass spectra were acquired on an AB Sciex 4800 MALDI-TOF-TOF mass spectrometer (S/N AK011030605H). Mass calibration was performed with peptide standards utilizing Applied Biosystems 4700 Proteomics Analyzer Mass Standards Kit, consisting of the calibration mixture (monoisotopic $(M+H)^+$ m/z values used): des-Arg^1-Bradykinin, m/z 904.4681; Angiotensin I, m/z 1296.6853; Glu^1-Fibrinopeptide B, m/z 1570.6774; ACTH (fragment 1-17), m/z 2093.8532; ACTH (fragment 18-39), m/z 2465.1989; ACTH (fragment 7-38) m/z 3657.9294. The plate wide calibration model was used for MS calibration.

Peak matching for plate wide calibration were as follows;

Minimum signal to noise set to 10
Mass tolerance ± 0.3 m/z
Minimum peaks to match set to 5
Max outlier error set to 10 ppm
Only monoisotopic peaks were used
Weighted fit was set to S/N

The following mass spectrometer settings were used for MS spectral acquisitions:

Mode of operation: reflector
Extraction mode: delayed
Polarity: positive
Acquisition control: manual
Accelerating voltage: 20000 V
Grid 1 voltage 16000 V
Source Lens voltage 10000 V
Lens 1 voltage 4000
Grid to Source 1 voltage ratio: 0.8000
Mirror 2 to Mirrior 1 voltage ratio: 1.4639
Mirror 2 to Source 1 voltage ratio: 1.1200
Extraction delay time: 390 nsec
Acquisition mass range: 300 - 6000 Da (in segments 300 – 1000, 600 – 4000 Da, and 1900 – 6000 Da)
Number of laser shots: 400/spectrum
Laser intensity: 2900
Calibration matrix: α-cyano-4-hydroxycinnamic acid (CHCA)
Timed ion selector: enabled
Bin size: 0.5 nsec
Vertical scale: 0.50 mV
Vertical offset: 0.00%
Input bandwidth: 500 MHz

A total of 3 to 6 spectra were summed per MS analysis and the following settings used to process the spectra;

Minimum signal to noise filter of 10
Local noise window width set to 250
Min peak width at full width half max (bins) set to 2.9

MALDI-TOF-TOF MS-MS: The sample preparations were analyzed directly by MALDI-TOF-TOF tandem mass spectrometry. All mass spectra were acquired on an AB Sciex 4800 MALDI-TOF-TOF mass spectrometer (S/N AK011030605H). Mass calibration was performed with peptide standards utilizing Applied Biosystems 4700 Proteomics Analyzer Mass Standards Kit, consisting of the calibration mixture (monoisotopic $(M+H)^+$ m/z values used): des-Arg^1-Bradykinin, m/z 904.4681; Angiotensin I, m/z 1296.6853; Glu^1-Fibrinopeptide B, m/z 1570.6774; ACTH (fragment 1-17), m/z 2093.087; ACTH (fragment 18-39), m/z 2465.1989; ACTH (fragment 7-38) m/z 3657.9294. For MS-MS calibration b- and y-ions derived from the Angiotensin I peptide were used to produce the calibration values for MS-MS data. The following is a list of the monoisitopic (M+H)+ m/z values used in MS-MS calibration; His immonium ion, m/z 110.072; y2, m/z 269.161; b2, m/z 272.136; y3, m/z 416.230; y4, m/z 513.283; b4-17, m/z 517.241; y5, m/z 650.341; b6-17 m/z 767.384; b6, m/z 784.411; b8+H_2O, m/z 1046.542; b9, m/z 1165.591; y9, m/z 1181.658; y9+H_2O, 1183.601. Peak matching for MS-MS calibration were as follows;

Minimum signal to noise set to 7
Mass tolerance ± 0.3 m/z
Minimum peaks to match set to 5
Max outlier error set to 0.05 m/z
Only monoisotopic peaks were used

Weighted fit was set to S/N

Masses obtained from the MS experiment were used to manually set the parent mass to acquire MS-MS data. The following mass spectrometer settings were used for MS-MS spectral acquisitions:

Mode of operation: reflector
Extraction mode: delayed
Polarity: positive
Acquisition control: manual
Accelerating voltage: 8000 V
Grid 1 voltage 7300 V
Source 1 focus voltage 4200 V
Source 1 Lens voltage 3200 V
Lens 1 voltage 2700 V
Deceleration stack voltage 6300 V
Collision cell offset -40 V
Source 2 voltage 15000 V
Lens 3 voltage 4000 V
Mirror 2 voltage 18000 V
Reflector detector voltage 2190 V
Combined precursor 1650 V
Grid to Source 1 voltage ratio: 0.9189
Mirror 2 to Mirror 1 voltage ratio: 1.7192
Mirror 2 to Source 2 voltage ratio: 1.2000
Number of laser shots: 400/spectrum

A total of 3 to 6 spectra were summed per MS analysis and the following settings used to process the spectra;

Minimum signal to noise filter of 20
Local noise window width set to 250
Min peak width at full width half max (bins) set to 2.9

The mass-spectra of proteolytic fragments were analyzed using Applied Biosystems Data Explorer v 4.9 (Build 115) and GPMAW v. 9.02 (Lighthouse Data) software.

RESULTS AND DISCUSSION

In this study, the numbering of the amino acid residues is in accordance with the theoretical sequence of the recombinant AAD12 protein starting with Met[1] and containing a total of 293 residues (**Figure 1**).

Band Excision:

The purified AAD12 (DAS-44406-6) was analyzed by SDS-PAGE in order to confirm sample integrity, and to estimate protein quantity (needed for subsequent digest preparations). The annotated SDS-PAGE gel image is presented in **Figure 2** and the post excision gel is shown in **Figure 3**.

Peptide Mass Fingerprinting and MS-MS analysis:

In order to generate a peptide (sequence) coverage map, N-terminal and C-terminal sequences, and examine possible presence of post-translational modifications, in-gel trypsin digest and in-gel chymotrypsin digest of reduced and alkylated (carboxyamidomethylated) AAD12 (DAS-44406-6) were generated and analyzed by MALDI-TOF MS and MALDI TOF-TOF MS-MS. A summary of the mass spectral data of in-gel digested AAD12 (DAS-44406-6) with assignments from MALDI-TOF MS and MALDI TOF-TOF MS-MS analyses are presented in **Table I** and **Table II** for the tryptic and chymotryptic digests, respectively. The peptide mass fingerprint (PMF) sequence coverage for the tryptic digest was 44.7% (**Figure 4**) and the PMF coverage for the chymotryptic digest was 59.4% (**Figure 5**). The combined PMF sequence coverage from both analyses is extensive at 84.3% (**Figure 6**). Most peptides observed in the proteolytic digests of AAD12 (DAS-44406-6) exhibited collision induced fragmentation patterns (MALDI TOF-TOF, MS-MS) consistent with their expected theoretical amino acid sequences. Tandem MS analysis of the PMF ions showed MS-MS sequence coverage for the tryptic digest was 41.0% (**Figure 4**) and the MS-MS coverage for the chymotryptic digest was 56.0% (**Figure 5**). The combined MS-MS sequence coverage from both analyses was extensive at 76.1% (**Figure 6**) The N-terminal amino acid was shown to be present as both an intact N-terminal Met[1] variant and as an acetylated (Ac) variant with the N-terminal Met[1] removed (*des*-Met[1]/ *Ac*-Ala[2] form). The sequences of the both N-terminal fragments, ^{1}MAQTTL^{9} (m/z 664.3334) and Ac-^{2}AQTTL^{9} (m/z 575.3035) of AAD12 (DAS-44406-6) from the chymotryptic digest, were confirmed by MALDI TOF-TOF tandem MS (**Table III** and **Table IV)**. The sequence of the C-terminal fragment, ^{281}LAGRPETEGAALV^{293} (m/z 1283.6954) of AAD12 (DAS-44406-6) from the tryptic digest, was confirmed by MALDI TOF-TOF MS-MS (**Table V)**.

Table I Tryptic digest coverage analysis of AAD12 (DAS-44406-6): Proteolytic fragments observed by MALDI-TOF MS and MALDI TOF-TOF MS-MS from in-gel tryptic digests.

Fragment #	# of missed cleavages	Start	End	Sequence	Charged State	Theor. mono. m/z	% ACN (elution from C18-zt; MALDI)	Observed m/z MALDI-MS	Comment	Mass range identifier	Base peak intensty	Mass difference (ppm)	MALDI MS-MS data (all fragments are in a singularly charged)
T1	0	1	62	(-) MAQTTLQITPTGATLGATVTGVH	1	6493.3765	ND	ND					
T1-des-Met	0	2	62	(-) AQTTLQITPTGATLGATVTGVHLA	1	6362.3360	ND	ND					
T1-des-Met-N-Ac	0	2	62	**Ac**-AQTTLQITPTGATLGATVTGVHL	1	6404.3466	ND	ND					
T3	0	64	69	FGAIER	1	692.3726	10%	692.3998		001	2096	-39	b2=205.12, b3=276.16, b4=389.26, b5=518.36, y1=175.13, y2=304.19, y3=417.29, y4=488.32, y5=545.34
T4	0	70	82	IGGGDIVAISNVK	1	1242.7052	10%	1242.6914		001	89	11	b2=171.11, b5=400.22, b7=613.34, b8=683.44, y4=447.28, y6=631.44, y7=730.49, y10=1015.63
T5	0	83	88	ADGTVR	1	618.3206	10%	618.3471		001	139	-43	y1=175.13, y3=375.22
T9	0	143	153	AAYDALDEATR	1	1195.5590	10%	1195.6277		001	962	-57	b4=421.21, b5=492.26, b6=605.38, y1=175.12, y2=276.17, y3=347.22, y4=476.29, y6=704.41, y7=775.48
T10	0	154	159	ALVHQR	1	723.4260	10%	723.4578		001	1501	-44	b2=185.16, b4=421.31, b5=549.36, y1=175.13, y2=303.21
T12	0	163	171	HSLVYSQSK	1	1048.5422	10%	1048.5966		001	277	-52	b2=225.12, b3=338.21, b4=437.32, b5=600.40, b6=687.45, b7=815.47, b8=902.54, y1=147.07, y3=362.23, y4=449.29, y5=612.38, y6=711.44, y7=824.55
T15	0	214	228	HAHAIPGMDAAESER	1	1591.7282	10%	1591.8308		001	328	-64	b1=138.10, b2=209.11, b3=346.20, b4=417.21, b5=530.31, b6=627.34, y2=304.16, y3=391.20, y5=591.31, y6=662.36, y7=777.44, y12=1246.67
T16	0	229	242	FLEGLVDWACQAPR	1	1661.8104	**50%**, 70%	1661.8397		001	76	-18	b2=261.19, b4=447.26, b5=560.39, b6=659.46, b7=774.53, y1=175.12, y2=272.19, y3=343.23, y4=471.29, y6=702.42
T17	0	243	259	VHAHQWAAGDVVVWDNR	1	1959.9573	20%	1960.0011		001	103	-22	b2=237.13, b3=308.18, b4=445.26, b5=573.33, b6=759.45, b7=830.50, b8=901.52, b9=958.54, y1=175.12, y2=289.15, y4=590.28, y5=689.39, y6=788.52
T18	0	260	264	CLLHR	1	698.3766	10%	698.4036		001	375	-39	y1=175.12, y3=425.26
T19	0	265	271	AEPWDFK	1	892.4199	10%	892.4665		001	534	-52	b2=201.13, b5=599.32, y2=294.22, y3=409.27, y5=692.44, y6=821.47
T20	0	272	274	LPR	1	385.2558	10%	385.2610		002	342	-13	b1=114.09, b2=211.17, y3=175.15, y2=272.22
T21	0	275	280	VMWHSR	1	815.3981	10%	815.4349		001	212	-45	b1=100.09, y1=175.12, y2=262.17
T22	0	281	293	LAGRPETEGAALV	1	1283.6954	10%	1283.7648		001	457	-54	b2=185.13, b3=242.16, b4=398.28, b6=624.45, b11=1053.62, b12=1166.71
T2-T3	1	63	69	RFGAIER	1	848.4737	10%	848.5129		001	803	-46	b3=361.23, b5=545.39, b6=674.44, y1=175.13, y6=545.39, y6=692.44
T10-T11	1	154	162	ALVHQRSAR	1	1037.5963	10%	1037.5427		001	1118	52	b1=72.08, y1=175.11, y6=754.50

Cys residues were alkylated with Iodoacetamide. Background and auto-proteolytic peaks were removed.

Table 11 Chymotryptic digest coverage analysis of AAD12 (DAS-44406-6): Proteolytic fragments observed by MALDI-TOF MS and MALDI TOF-TOF MS-MS from in-gel chymotryptic digests.

Fragment #	# of missed cleavages	Start	End	Sequence	Charged State	Theor. mono. m/z	% ACN (elution from C18-zt; MALDI)	Observed m/z MALDI-MS	Comment	Mass range identifier	Mass difference (ppm)	MALDI MS-MS data (all fragments are in a singularly charged)
Y1	0	1	6	(-) MAQTTL	1	664.3334	10%	664.3997		002	-100	b4=432.29, y3=334.17, y4=462.17
Y1-des-Met-N-Ac	0	2	6	**Ac**-AQTTL	1	575.3035	10%	575.3415		002	-66	b3=343.19, b4=444.22, y1=132.08, y3=334.19,
Y3	0	16	24	GATVTGVHL	1	854.4730	10%	854.4699		002	4	b2=129.11, y2=269.18, y3=368.15
Y5	0	28	32	DDAGF	1	524.1987	10%, **50%**	524.1815		002	33	b1=116.07, b2=231.10, y3=294.11, y4=409.22
Y11	0	46	52	IFPGQHL	1	811.4461	**10%**, 20%	811.4938		001	-59	b3=358.19, y2=269.16, y4=454.24, y5=551.23, y6=698.35
Y12	0	53	60	SNDQQITF	1	952.4370	10%	952.5063		001	-73	b2=202.09, b4=445.27, b5=573.30, b6=686.37, y6=751.44
Y13	0	61	64	AKRF	1	521.3194	10%	521.3550		002	-68	b1=72.09, b2=200.16, b3=450.29, y1=166.11, y2=322.21, y3=450.29
Y17	0	115	124	MPVMAQGAVF	1	1050.5111	10%	1050.6185	No MS-MS signals	001	-102	
Y19	0	139	145	ADMRAAY	1	797.3610	**10%**, 20%	797.4417		001	-101	b1=72.09, y5=611.33
Y25	0	173	182	GHVQQAGSAY	1	1017.4748	10%	1017.5503		001	-74	b2=195.09, b3=294.18, b4=442.25, b5=550.34, b6=621.31, b7=724.41, y3=240.18, y5=468.27, y6=596.33, y7=724.41
Y26	0	183	185	IGY	1	352.1867	20%	352.1663		002	58	a2=143.12, b2=171.13, y2=239.15
Y27	0	186	194	GMDTTATPL	1	906.4237	50%, **70%**	906.5071	weak MS-MS signals	001	-92	b6=577.36
Y28	0	195	197	RPL	1	385.2558	**10%**, 20%	385.2479		002	21	b1=157.12, b2=254.19
Y29	0	198	209	VKVHPETGRPSL	1	1319.7430	10%	1319.7747		001	-24	b1=100.09, b11=1188.64, y3=316.15
Y39	0	263	268	HRAEPW	1	795.3896	**10%**, 20%, 50%	795.4434		001	-68	b1=138.01, b2=294.18, b3=356.24, b4=494.28, y2=302.18, y4=502.23, y5=658.36
Y42	0	278	281	HSRL	1	512.2940	20%	512.2999		002	-12	b2=225.14, b3=381.26, y2=288.17, y3=375.25
Y1-Y2-des-Met-N-Ac	1	2	15	**Ac**-AQTTLQITPTGATL	1	1457.7846	10%	1457.8035	weak MS-MS signals	001	-13	b1=114.10, b4=444.30, y6=559.37
Y4-Y5	1	25	32	ATLDDAGF	1	809.3676	10%	809.4346	weak MS-MS signals	001	-83	b1=72.09, y3=294.15
Y5-Y6	1	28	35	DDAGFAAL	1	779.3570	50%	779.4248		001	-87	b1=116.06, b3=302.19, b6=577.40
Y6-Y7	1	33	39	AALHAAW	1	739.3886	**10%**, 20%	739.3867	weak MS-MS signals	002	3	b3=302.18, b7=648.32
Y12-Y13	1	53	64	SNDQQITFAKRF	1	1454.7386	10%	1454.7699	weak MS-MS signals	001	-22	b2=202.09, b5=573.38
Y20-Y21	1	146	155	DALDEATRAL	1	1074.5426	10%	1074.6075		001	-60	b5=544.39, b8=872.47, y6=660.37
Y22-T23	1	156	167	VHQRSARHSLVY	1	1452.7819	20%	1452.8715	weak MS-MS signals	001	-62	b4=521.34, b6=697.45
Y23-Y24	1	166	172	VYSQSKL	1	824.4512	**10%**, 20%, 50%	824.5040		001	-64	b1=100.09, b5=565.33, y2=260.16, y3=347.28
Y26-Y27	1	183	194	IGYGMDTTATPL	1	1239.5926	10%, 20%, **50%**	1239.6486		001	-45	b10=1011.79, b11=1108.59, y10=1069.91, y11=1126.74
Y29-Y30	1	198	210	VKVHPETGRPSLL	1	1432.8271	10%, 20%, **70%**	1432.8699		001	-30	b3=327.27, b4=464.30, b9=1004.56, b12=1301.73, y5=585.43, y6=642.52, y7=743.54, y9=969.68, y10=1106.73
Y32-Y33	1	230	233	LEGL	1	431.2500	50%	431.2188		002	72	b1=114.11, y1=132.11, y2=189.14, y3=318.27
Y34-Y35	1	234	248	VDWACQAPRVHAHQW	1	1860.8711	10%	1860.8606	very weak MS-MS signals	001	6	y5=678.41
Y38-Y39	1	262	268	LHRAEPW	1	908.4737	**10%**, 20%	908.5378		001	-71	b2=251.16, b3=407.29, b5=607.41, y1=205.14, y2=302.19, y6=795.51
Y41-Y42	1	271	281	KLPRVMWHSRL	1	1422.8151	20%	1422.8635		001	-34	b1=129.11, b2=242.14, b5=594.45, b7=911.53, b8=1084.74, y2=288.15, y8=1084.74
Y43-Y44	1	282	293	AGRPETEGAALV	1	1170.6113	**10%**, 20%	1170.6936		001	-70	b1=72.08, b2-129.09, b3=285.17, b5=511.32, b7=741.43, b8=798.50, b9=869.52, y9=886.75

Cys residues were alkylated with Iodoacetamide. Background and auto-proteolytic peaks were removed.

Table III Amino acid sequence obtained for N-terminal peptide identified from the chymotryptic digest of AAD12 (DAS-44406-6) (1-6; m/z 664.3997). Fragment ions positively identified from the MS-MS spectrum are shown in bold.

N-terminal peptide from chymotryptic digest 1-6 (theoretical m/z = 664.3334)
Sequence: MAQTTL
Parent peptide mass $[M+H]^{1+}$(monoisotopic): 664.3997

	M	***A***	***Q***	***T***	***T***	***L***
a	104.05	175.09	303.15	404.20	505.24	
	104.08	**175.11**	**303.16**	**404.22**		
b	132.05	203.09	331.14	432.19	533.24	
				432.29	**533.24**	
c	151.09	222.13	350.19	451.23	552.28	
x		559.27	488.24	360.18	259.13	158.08
						158.11
y		533.29	462.26	334.20	233.15	132.10
			462.17	**334.17**		
z		517.27	446.24	318.18	217.13	116.08
		517.27	**446.26**			

Table IV Amino acid sequence obtained for N-terminal peptide identified from chymotryptic digest of AAD12 (DAS-44406-6) with clipped methionine and acetylation (2-6; m/z 575.3415). Fragment ions positively identified from the MS-MS spectrum are shown in bold.

N-terminal peptide from chymotryptic digest 2-6 (theoretical m/z = 575.3035)
Acetylation of N-terminal amino acid
Sequence: Ac-AQTTL
Parent peptide mass $[M+H]^{1+}$(monoisotopic): 575.3415

	Ac-A	***Q***	***T***	***T***	***L***
a	86.06	214.12	315.17	416.21	
	86.10	**214.14**	**315.20**		
b	114.06	242.11	343.16	444.21	
	114.07		**343.19**	**444.22**	
c	133.10	261.16	362.20	463.25	
		261.14		**463.22**	
x		488.24	360.18	259.13	158.08
		488.24	**360.22**		
y		462.26	334.20	233.15	132.10
z		446.24	318.18	217.13	116.08
					116.07

Table V Amino acid sequence obtained for C-terminal peptide from tryptic digest (281-293; m/z 1283.7646) of AAD12 (DAS-44406-6). Fragment ions positively identified are shown in bold.

C-terminal peptide from tryptic digest 281-293 (theoretical m/z = 1283.6954)
Sequence: LAGRPETEGAALV
Parent peptide mass $[M+H]^{1+}$(monoisotopic): 1283.7646

	L	***A***	***G***	***R***	***P***	***E***	***T***	***E***	***G***	***A***	***A***	***L***	***V***
a	86.10	157.13	214.16	370.26	467.31	596.35	697.40	826.44	883.46	954.50	1025.54	1138.62	
	86.10	**157.14**	**214.19**	**370.29**	**467.36**			**826.53**				**1183.69**	
b	114.09	185.13	242.15	398.25	495.30	624.35	725.39	854.44	911.46	982.50	1053.53	1166.62	
		185.13	**242.16**	**398.28**		**624.45**				**982.57**	**1053.62**	**1166.71**	
c	133.13	204.17	261.19	417.29	514.35	643.39	744.44	873.48	930.50	1001.54	1072.57	1185.66	
	133.08	**204.12**	**261.18**								**1072.78**		
x		1196.59	1125.55	1068.53	912.43	815.38	686.34	585.29	456.25	399.22	328.19	257.15	144.07
		1196.79						**585.31**			**328.17**	**257.16**	**144.08**
y		1170.61	1099.57	1042.55	886.45	789.40	660.36	559.31	430.27	373.24	302.21	231.17	118.09
		1170.67	**1099.76**				**660.43**					**231.15**	
z		1154.59	1083.56	1026.53	870.43	773.38	644.34	543.29	414.25	357.23	286.19	215.15	102.07
				1026.61						**357.21**		**215.19**	**102.08**

Figure 1 Theoretical amino acid sequence of AAD12.

[1-293] mass = 31708.959

Small polar:	D(17)	E(11)	N(4)	Q(14)			
Large polar:	K(6)	R(18)	H(16)				
Small non-polar:	S(13)	T(18)	A(44)	G(25)			
Large non-polar:	L(24)	I(11)	V(24)	M(10)	F(8)	Y(5)	W(8)
Special:	C(3)	P(14)					

1	M	A	Q	T	T	L	Q	I	T	P	T	G	A	T	L	G	A	T	V	T	G	V	H	L	A	T	L	D	D	A	30
31	G	F	A	A	L	H	A	A	W	L	Q	H	A	L	L	I	F	P	G	Q	H	L	S	N	D	Q	Q	I	T	F	60
61	A	K	R	F	G	A	I	E	R	I	G	G	G	D	I	V	A	I	S	N	V	K	A	D	G	T	V	R	Q	H	90
91	S	P	A	E	W	D	D	M	M	K	V	I	V	G	N	M	A	W	H	A	D	S	T	Y	M	P	V	M	A	Q	120
121	G	A	V	F	S	A	E	V	V	P	A	V	G	G	R	T	C	F	A	D	M	R	A	A	Y	D	A	L	D	E	150
151	A	T	R	A	L	V	H	Q	R	S	A	R	H	S	L	V	Y	S	Q	S	K	L	G	H	V	Q	Q	A	G	S	180
181	A	Y	I	G	Y	G	M	D	T	T	A	T	P	L	R	P	L	V	K	V	H	P	E	T	G	R	P	S	L	L	210
211	I	G	R	H	A	H	A	I	P	G	M	D	A	A	E	S	E	R	F	L	E	G	L	V	D	W	A	C	Q	A	240
241	P	R	V	H	A	H	Q	W	A	A	G	D	V	V	V	W	D	N	R	C	L	L	H	R	A	E	P	W	D	F	270
271	K	L	P	R	V	M	W	H	S	R	L	A	G	R	P	E	T	E	G	A	A	L	V								293

Figure 2 SDS-PAGE analysis of AAD12 (DAS-44406-6). SDS-PAGE image (supplied by Brian Barnett) of affinity enriched fraction for AAD12 loaded in lanes 3 and 5. To aid with band excision and estimate the amount of protein per band, a bacterially expressed standard AAD12 was loaded in lane 8 at 780ng.

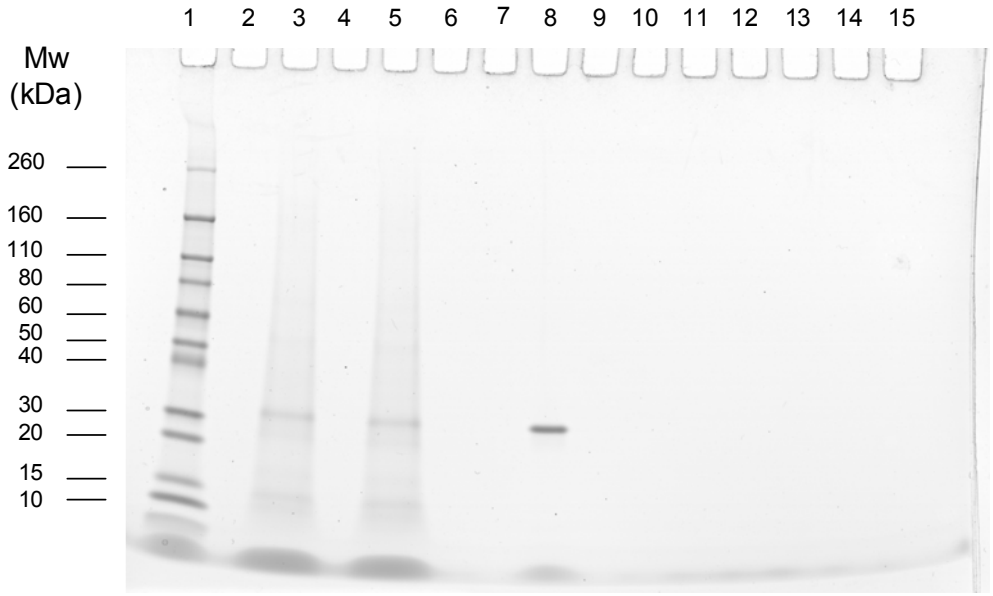
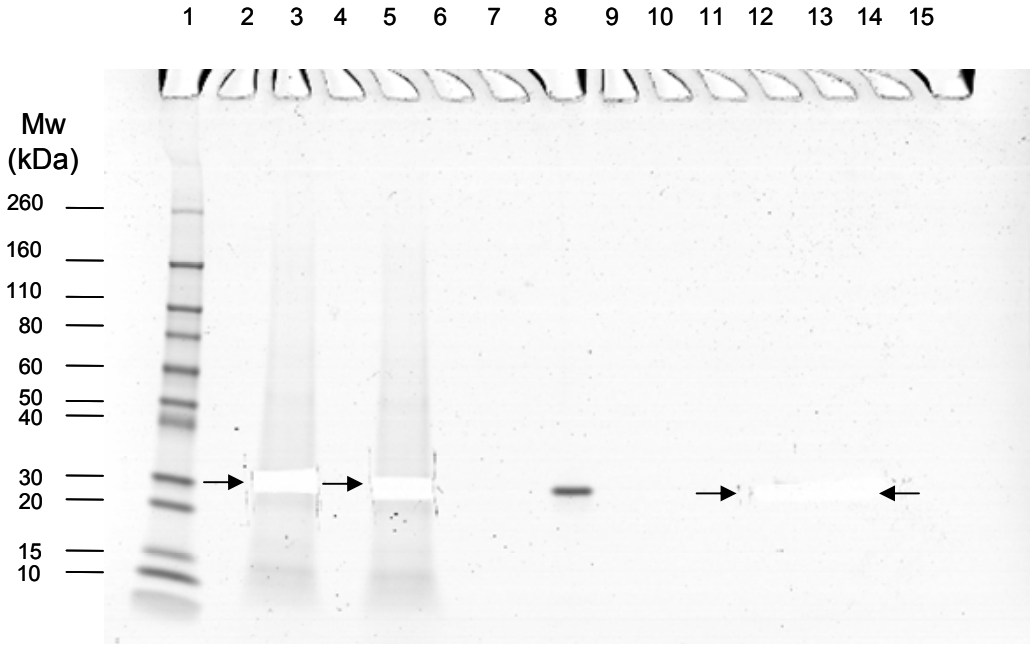


Figure 3 SDS-PAGE analysis of AAD12 (DAS-44406-6) after excision of the putative AAD12 bands and the blank control bands. (arrowed)



* AAD12 bands (~ 32 kDa) were excised from gel, digested with trypsin or chymotrypsin and analyzed by MALDI-TOF MS and MALDI TOF-TOF MS-MS. Bands were removed from the blank part of the gel at a similar height to provide control samples for processing.

Figure 4 Tryptic digest sequence coverage map for AAD12 (DAS-44406-6) by MALDI-TOF MS and MALDI TOF-TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 44.7% with PMF data and 41.0% by tandem MS. The C-terminal peptide was observed in this digestion.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
1	M	A	Q	T	T	L	Q	I	T	P	T	G	A	T	L	G	A	T	V	T	G	V	H	L	A	T	L	D	D	A	**30**	0	0
31	G	F	A	A	L	H	A	A	W	L	Q	H	A	L	L	I	F	P	G	Q	H	L	S	N	D	Q	Q	I	T	F	**60**	0	0
61	A	K	R	**F**	**G**	**A**	**I**	**E**	**R**	**I**	**G**	**G**	**G**	**D**	**I**	**V**	**A**	**I**	**S**	**N**	**V**	**K**	**A**	**D**	**G**	**T**	**V**	**R**	Q	H	**90**	25	19
91	S	P	A	E	W	D	D	M	M	K	V	I	V	G	N	M	A	W	H	A	D	S	T	Y	M	P	V	M	A	Q	**120**	0	0
121	G	A	V	F	S	A	E	V	V	P	A	V	G	G	R	T	C	F	A	D	M	R	**A**	**A**	**Y**	**D**	**A**	**L**	**D**	**E**	**150**	8	8
151	**A**	**T**	**R**	**A**	**L**	**V**	**H**	**Q**	**R**	S	A	R	**H**	**S**	**L**	**V**	**Y**	**S**	**Q**	**S**	**K**	L	G	H	V	Q	Q	A	G	S	**180**	18	18
181	A	Y	I	G	Y	G	M	D	T	T	A	T	P	L	R	P	L	V	K	V	H	P	E	T	G	R	P	S	L	L	**210**	0	0
211	I	G	R	**H**	**A**	**H**	**A**	**I**	**P**	**G**	**M**	**D**	**A**	**A**	**E**	**S**	**E**	**R**	**F**	**L**	**E**	**G**	**L**	**V**	**D**	**W**	**A**	**C**	**Q**	**A**	**240**	27	27
241	**P**	**R**	**V**	**H**	**A**	**H**	**Q**	**W**	**A**	**A**	**G**	**D**	**V**	**V**	**V**	**W**	**D**	**N**	**R**	**C**	**L**	**L**	**H**	**R**	**A**	**E**	**P**	**W**	**D**	**F**	**270**	30	25
271	**K**	**L**	**P**	**R**	**V**	**M**	**W**	**H**	**S**	**R**	**L**	**A**	**G**	**R**	**P**	**E**	**T**	**E**	**G**	**A**	**A**	**L**	**V**								**293**	23	23
																															Tot. a.a. covered =	***131***	***120***

A = Tryptic MS coverage

A = Tryptic MS-MS data

MALDI PMF sequence coverage = 44.7 %

MALDI MS-MS sequence coverage = 41.0 %

Figure 5 Chymotryptic digest sequence coverage map for AAD12 (DAS-44406-6) by MALDI-TOF MS and MALDI TOF-TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 59.4% with PMF data and 56.0% by tandem MS. Removal of N-terminal Met[1] was observed and acetylation was detected on the N-terminal peptide from which the Met[1] had been removed.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
1	**M***	**A**	**Q**	**T**	**T**	**L**	**Q**	**I**	**T**	**P**	**T**	**G**	**A**	**T**	**L**	**G**	**A**	**T**	**V**	**T**	**G**	**V**	**H**	**L**	**A**	**T**	**L**	**D**	**D**	**A**	30	30	30
31	**G**	**F**	**A**	**A**	**L**	**H**	**A**	**A**	**W**	L	Q	H	A	L	L	**I**	**F**	**P**	**G**	**Q**	**H**	**L**	**S**	**N**	**D**	**Q**	**Q**	**I**	**T**	**F**	60	24	24
61	**A**	**K**	**R**	**F**	G	A	I	E	R	I	G	G	G	D	I	V	A	I	S	N	V	K	A	D	G	T	V	R	Q	H	90	4	4
91	S	P	A	E	W	D	D	M	M	K	V	I	V	G	N	M	A	W	H	A	D	S	T	Y	**M**	**P**	**V**	**M**	**A**	**Q**	120	6	6
121	**G**	**A**	**V**	**F**	S	A	E	V	V	P	A	V	G	G	R	T	C	F	**A**	**D**	**M**	**R**	**A**	**A**	**Y**	**D**	**A**	**L**	**D**	**E**	150	16	16
151	**A**	**T**	**R**	**A**	**L**	**V**	**H**	**Q**	**R**	**S**	**A**	**R**	**H**	**S**	**L**	**V**	**Y**	**S**	**Q**	**S**	**K**	**L**	**G**	**H**	**V**	**Q**	**Q**	**A**	**G**	**S**	180	30	20
181	**A**	**Y**	**I**	**G**	**Y**	**G**	**M**	**D**	**T**	**T**	**A**	**T**	**P**	**L**	**R**	**P**	**L**	**V**	**K**	**V**	**H**	**P**	**E**	**T**	**G**	**R**	**P**	**S**	**L**	**L**	210	30	30
211	I	G	R	H	A	H	A	I	P	G	M	D	A	A	E	S	E	R	F	**L**	**E**	**G**	**L**	V	D	W	A	C	Q	A	240	4	4
241	P	R	V	H	A	H	Q	W	A	A	G	D	V	V	V	W	D	N	R	C	L	**L**	**H**	**R**	**A**	**E**	**P**	**W**	D	F	270	7	7
271	**K**	**L**	**P**	**R**	**V**	**M**	**W**	**H**	**S**	**R**	**L**	**A**	**G**	**R**	**P**	**E**	**T**	**E**	**G**	**A**	**A**	**L**	**V**								293	23	23
																															Tot. a.a. covered =	***174***	***164***

A = Chymotryptic MS coverage

A = Chymotryptic MS-MS data

MALDI PMF sequence coverage = 59.4 %

MALDI MS-MS sequence coverage = 56.0 %

*The N-terminal peptide ^{1}MAQTTL^{6} was determined to be present in both a full length form and in a form from which Met[1] had been removed and the alanine was acetylated, Ac-^{2}AQTTL^{6}

Figure 6 Overall sequence coverage of trypsin and chymotrypsin digests for AAD12 (DAS-44406-6) by MALDI-TOF MS and MALDI TOF-TOF. Cys residues were alkylated with iodoacetamide. Overall sequence coverage was 84.3% with PMF data and 76.1% by tandem MS.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
1	M*	A	Q	T	T	L	Q	I	T	P	T	G	A	T	L	G	A	T	V	T	G	V	H	L	A	T	L	D	D	A	30	30	27
31	G	F	A	A	L	H	A	A	W	L	Q	H	A	L	L	I	F	P	G	Q	H	L	S	N	D	Q	Q	I	T	F	60	24	20
61	A	K	R	F	G	A	I	E	R	I	G	G	G	D	I	V	A	I	S	N	V	K	A	D	G	T	V	R	Q	H	90	28	22
91	S	P	A	E	W	D	D	M	M	K	V	I	V	G	N	M	A	W	H	A	D	S	T	Y	M	P	V	M	A	Q	120	6	0
121	G	A	V	F	S	A	E	V	V	P	A	V	G	G	R	T	C	F	A	D	M	R	A	A	Y	D	A	L	D	E	150	16	16
151	A	T	R	A	L	V	H	Q	R	S	A	R	H	S	L	V	Y	S	Q	S	K	L	G	H	V	Q	Q	A	G	S	180	30	30
181	A	Y	I	G	Y	G	M	D	T	T	A	T	P	L	R	P	L	V	K	V	H	P	E	T	G	R	P	S	L	L	210	30	30
211	I	G	R	H	A	H	A	I	P	G	M	D	A	A	E	S	E	R	F	L	E	G	L	V	D	W	A	C	Q	A	240	30	27
241	P	R	V	H	A	H	Q	W	A	A	G	D	V	V	V	W	D	N	R	C	L	L	H	R	A	E	P	W	D	F	270	30	28
271	K	L	P	R	V	M	W	H	S	R	L	A	G	R	P	E	T	E	G	A	A	L	V								293	23	23
																															Tot. a.a. covered =	*247*	*223*

A = Tryptic MS coverage

A = Tryptic MS-MS data

A = Chymotryptic MS coverage

A = Chymotryptic MS-MS data

MALDI PMF sequence coverage = 84.3 %

MALDI MS-MS sequence coverage = 76.1 %

*The N-terminal peptide ^{1}MAQTTL^{6} was determined to be present in both a full length form and in a form from which Met^{1} had been removed and the alanine was acetylated, Ac-^{2}AQTTL^{6}

APPENDICES (AS CONTAINED IN THE STUDY FILE)

Appendix A: SDS-PAGE data, scan of gels, scan of post excision gel and sequences data
Appendix B: Experimental run-sheets
Appendix C: Sample and calibration logs
Appendix D: MS calibration and settings
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Appendix F: MS data record sheets and coverage maps for tryptic and chymotryptic digests
Appendix G: Tryptic digest MS spectral print outs
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Appendix I: Tryptic digest MS-MS spectral print outs
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Appendix K: N- and C-terminal peptide coverage maps and MS-MS spectral print outs
Appendix L: Data not used

APPENDIX 4

Mass Spectrometry Analysis of *P. fluorescens*-Derived 2mEPSPS Protein

ACCESSION #: 2010003338

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Technology Report **The Dow Chemical Company**		Laboratory Report Code ML AL-2010-007963
Department	Geographic Location	Date Issued
Analytical	Midland	08/24/2010
Page Count	Protocol Study Number	Report Status
40	Databook 201001156A	Final

Title

Analytical characterization of 2m-EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) containing TIPS mutation.

Author(s): Last Name and Initials (Master Numbers)	Author(s) Signature / Date
Karnoup, Anton (AS) (U369292)	08/24/2010
Kuppannan, Krishna (K) (U386368)	8/24/2010
Reviewer Name(s)	**Reviewer and/or Supervisor Signature(s)/Date**
Young, Scott (SA) (U289561)	8/24/2010
Deshmukh, Balasaheb (BK) (U099523)	8/24/2010

Patent Status		
Disclosure Submitted	Case Filed	No Action Required
[ ]	[ ]	[x]

Descriptive Summary and Conclusions

2m-EPSPS is a recombinant herbicide tolerance enzyme, similar to the wild-type EPSPS, except that amino acids 102 and 106 have been modified from a T (Thr) to I (Ile), and P (Pro) to S (Ser), respectively (TIPS mutation). A bacterially-expressed 2m-EPSPS sample from DAS Regulatory Labs was examined by peptide mass fingerprinting (using both MALDI-TOF MS and ESI LC/MS), with the purpose of detailed analysis of the protein's primary structure.

The sample of 2m-EPSPS was examined for purity and integrity using SDS-PAGE, and for intact MW by ESI LC/MS. The sample of 2m-EPSPS was subsequently reduced and alkylated, and digested by 4 proteolytic enzymes (as 4 separate digests: Trypsin, Asp-N, Arg-C, and Glu-C at pH 8.0). The resulting proteolytic digests were examined by MALDI-TOF and ESI LC/MS mass-spectrometry techniques in order to obtain confirmation of the protein's amino-acid sequence, with the focus on terminal sequences and the sites of TIPS mutations.

SDS-PAGE resulted in an apparent molecular weight for 2m-EPSPS of approximately 45 ±2 kDa. LC/MS analysis of the intact 2m-EPSPS sample resulted in an accurate MW = 47285.8 ±0.5 Da for the principal component of the 2m-EPSPS sample. This result is within 0.0024% of the theoretical average MW for the 2m-EPSPS protein (47284.68 Da). No N-terminal Met^1 was present, as expected (the protein was expressed without Met^1 in the coding sequence). The presence of the TIPS-mutation, and the absence of the non-mutated variants were also confirmed.

Peptide mass fingerprinting of 2m-EPSPS, based on four proteolytic digests, resulted in 100% sequence coverage for its 444- amino-acid-long sequence. The accurate mass of the main sample component measured by LC/MS also confirmed the identity of 2m-EPSPS.
To further confirm the details of the primary structure of 2m-EPSPS sample, N- and C-terminal proteolytic fragments, as well as proteolytic fragments containing the TIPS mutation were subjected to MS/MS fragmentation analysis. The following N-terminal sequence was confirmed by MS/MS analysis: ^{1}AGAEEIVLQPIK^{12}. The following C-terminal sequences were confirmed by MS/MS analysis: ^{436}DVLSTFVKN^{444}, and ^{429}KTFPDYFDVLSTFVKN^{444}. No other variants of the N- and C-terminal sequences were detected. Amino acid sequences of the following fragments containing TIPS mutation were confirmed: ^{106}**S**LTAAVTAAGGNATYVLDGVPR^{127}, and ^{91}EEVQLFLGNAGIAMR^{105}. No fragments representing the absence of the TIPS mutation or partial TIPS mutation were detected.

ACCESSION #: 2010003338

DISTRIBUTION LIST

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INTRODUCTION

A sample of purified recombinant 2m-EPSPS (also known as Double Maize Mutant Gene (DMMG) protein) herbicide tolerance protein (Batch TSN033171-0001) was submitted by Barry Schafer of Dow AgroSciences for analytical characterization. In conjunction with Dow AgroSciences characterization, Analytical Sciences Laboratory was requested to provide analytical data on full protein sequence coverage, N- and C-terminal sequencing, and the TIPS mutation sites. TIPS mutation is a mutation, in which, relative to the wild-type protein, Thr residue is replaced with Ile residue, and Pro residue is replaced with Ser residue. In 2m-EPSPS, Thr^{102} was replaced with Ile^{102}, and Pro^{106} was replaced with Ser^{106}.

Original experimental data are stored in the raw data archive MD-2010-007963 [1].

EXPERIMENTAL

Samples

Sample of 2m-EPSPS protein, lot no. TSN033171-0001, was received in the form of dry powder from Barry Schafer of Dow AgroSciences, LLC. A stock solution at 1 mg/mL (by dry weight) in PBS buffer, pH7.4, was prepared and stored at 4 °C.

SDS-PAGE analysis

Equipment:

a) Centrifugal evaporator (Centrivap), Labconco, cat. no. 7812013, S/N 051146935 A
b) Bio-Rad Criterion Cell, cat. no. 165-6001
c) Bio-Rad PowerPac 1000, cat. no. 165-5054
d) Traceable Digital Thermometer, model NEW 15-078J
e) Fisher brand Heating Block
f) Eppendorf Centrifuge, model 5415D, serial no. 5425 17645
g) Eppendorf adjustable pipettes: 2-20 µL, and 10-100 µL
h) Aros 160 Orbital Shaker
i) Fisher Vortex Genie 2, serial no. 2-156856
j) Eppendorf safe-lock microfuge tubes 1.5 mL, cat. no. 22 36 332-8
k) Bio-Rad gel loading tips, cat. no. 223-9917
l) Parafilm
m) Graduated cylinder, 1000 mL
n) Fluor-S MultiImager, Bio-Rad, cat. no. 170-7700; Quantity One Version 4.2 software

Reagents and Standards:

1. Laemmli Sample Buffer, Bio-Rad, cat. no. 161-0737, lot no. 310008426

2. β-mercaptoethanol, Fisher, Certified lot no. 004508
3. 4-20% Tris-HCl Criterion Precast Gel, Bio-Rad, lot no. C072410A1
4. Tris/Glycine/SDS Running Buffer, Bio-Rad, cat no. 161-0732, lot no. 68199A
5. Coomassie Stain Solution, Bio-Rad, lot no. 68198A
6. Destaining solution I: 45% methanol/ 45% Milli-Q water/ 10% acetic acid
7. Destaining solution II: 5% methanol/ 88% Milli-Q water/ 7% acetic acid
8. Certified Precision Plus Unstained Protein Standards, Bio-Rad, cat. no. 161-0363, lot no. 300000271
9. Bovine serum albumin (BSA), Sigma, Cat. no. A1900, Lot no. 036K7575 (prepared at 1.0 mg/mL in PBS buffer, pH7.4, and stored at +4 ^{o}C)
10. Wt-EPSPS, lot no. TSN032933-003: provided by B. Schafer (DAS)

Analytical Procedure:

The apparent molecular weight of 2m-EPSPS was determined by high-resolution SDS-PAGE analysis. Preparation of reagents, samples, and standards are shown below:

a. Aliquots of the sample (1, 2, 4, 5 μL of sample solution, approximately 1mg/mL based on dry weight) were dried to completeness in a centrifugal evaporator.
b. Final Laemmli sample buffer was prepared by adding 50 µL of β-mercaptoethanol to ~950 µL of Bio-Rad Laemmli buffer. The sample buffer was thoroughly mixed by a vortex.
c. The dried 2m-EPSPS samples were dissolved in a final Laemmli sample buffer (10 µL). After briefly mixing 2m-EPSPS samples in the Laemmli buffer, the microfuge tubes were sealed with Parafilm, and placed in a pre-heated heat block set at 95 °C for ~1.5 minutes. The microfuge tubes were removed from the heating block and briefly centrifuged. The entire 2m-EPSPS sample was loaded on an SDS-PAGE gel in one lane.
d. Known amounts of wt-EPSPS reference material (batch TSN032933-0003[2]) were also prepared alongside 2m-EPSPS samples, using identical procedures.
e. Known amounts of BSA protein standard were also prepared (from a 1 mg/mL stock solution). Aliquots of 0.5, 1, 2, 3 μL (0.5, 1, 2, 3 μg) of BSA were loaded onto separate SDS-PAGE gel lanes.

Instrument Conditions:

A 4 - 20 % Tris-HCl Criterion pre-cast gel was removed from the storage container, the comb removed from the gel, the wells thoroughly rinsed with deionized water, and the tape removed from the bottom of

the cassette. The Criterion gel cassette was inserted into one of the slots in the Criterion electrophoresis tank. The upper buffer chamber of the Criterion gel was filled with approximately 1X Tris/Glycine/SDS (100 mL of 10X Tris/Glycine/SDS mixed with 900 mL of deionized water) premixed running buffer. The remaining running buffer was added to the lower buffer chamber. Approximately 12 µL of certified unstained precision protein standards, Bio-Rad, was loaded into far right and far left wells with a pipette using gel-loading tips. After applying the samples and reference materials to the gels, the lid was placed on the tank, the electrical leads were plugged into the power supply, and the power was turned on. Constant current of 50 mA was applied to the Criterion cell for ~1.5 hours, until the dye reached the bottom of the gel. After the electrophoresis was complete, the power supply was turned off and the electrical leads were disconnected. The gel was removed from the Criterion gel cassette, transferred to Coomassie Stain Solution (Bio-Rad) in the gel cassette tray to cover the gel (~40-mL), and placed on an orbital shaker at 35 rpm for approximately 30 minutes. The Coomassie Stain Solution was discarded and replaced with ~40-mL of Destaining Solution I (45% deionized water, 45% methanol, 10% acetic acid), and placed on an orbital shaker at 35 rpm for ~30 minutes. The Destaining Solution I was replaced with Destaining Solution II (88% deionized water, 5% methanol, 7% acetic acid) and the gel was destained for approximately 16 hours.

Methods for determining protein's apparent molecular weight: After destaining of the SDS-PAGE gel was complete, a gel image was acquired using the Bio-Rad Fluor-S MultiImager, as specified by the manufacturer. The captured image was then analyzed using Quantity One version 4.2 software utilizing tools for determining molecular weight. The molecular weight value was determined relative to the certified Bio-Rad protein standards defined for the gel, and the band's position in the lane. To estimate the quantity of the 2m-EPSPS material on the SDS-PAGE gel, a calibration curve was obtained from the measured band densities and the known µg amounts of BSA reference material on the same gel. The calibration curve was applied to the lanes containing 2m-EPSPS material to estimate the amount of 2m-EPSPS on the gel.

In-solution protein processing and enzymatic digests

Equipment:

a) Mettler AE168 analytical balance serial no. F00518
b) Eppendorf Centrifuge, Model 5415D, serial no. 5425 17645
c) Eppendorf, Thermomixer R, serial no. 5355 20846
d) Centrifugal evaporator (Centrivap), Labconco, cat. no. 7812013, S/N 051146935 A
e) Eppendorf adjustable pipettes: 2.5µL serial no. 296447, 2-20µL serial no. 286820, 10-100µL serial no. 289560, and 1000µL serial no. 33165

f) Fisher Vortex Genie 2, serial no. 2-156856
g) Siliconized microcentrifuge tubes, 1.5mL, Fisher, cat no. 02-681-320
h) NAP-5 SEC gravity cartridge, GE Healthcare, Cat. no. 17-0853-02, Lot no. 354677
i) Parafilm
j) Eppendorf pipette tips (epTips) 10µL
k) Fisher brand Reditip General Purpose, 200µL and 1000µL

Reagents and Standards:

1. Acetonitrile, Fisher Scientific, Cat. no. A998-1, Lot no. 094014
2. Ammonium bicarbonate, Sigma, cat no. A-6141
3. Dithiothreitol (DTT), Pierce, cat no. 20290
4. Iodoacetamide (IAA), Sigma, cat no. I-1149
5. Guanidinium Hydrochloride (Gu:HCl), Pierce, Cat. no. 24110, Lot no. DH54867
6. Trypsin, Roche, cat no. 11-418-025-001, Lot no. 11366139
7. Asp-N, Roche, cat no. 11-054-589-001, Lot no. 11210921
8. Glu-C, Roche, cat no. 11-047-817-001, Lot no. 14530520
9. Arg-C, Roche, cat no. 11-370-529-001, Lot no. 10960720
10. Formic Acid (FA) (98%), Fluka, Lot no. 1255194
11. Trifluoroacetic Acid (99+%) (TFA), Aldrich, Lot no. 00339JD
12. Milli-Q deionized water

Reagent Solution Preparation:

a. 25 mM Ammonium Bicarbonate buffer: dissolved 98.83 mg NH_4HCO_3 in 50 mL of Milli-Q water; filtered through 0.22 µm sterile syringe filter.
b. 100 mM Tris buffer: dissolved 121.1 mg Tris in 10 mL of Milli-Q water; adjusted pH to 8.04 with HCl; filtered through 0.22 µm sterile syringe filter.
c. Protein dissolution buffer (6M guanidine hydrochloride (Gu:HCl)/ 400 mM ammonium bicarbonate, pH 7.8): to 316 mg of ammonium bicarbonate, 7.5 mL of 8M Gu:HCl solution and 2.5 mL of water were added. pH was adjusted to 7.8 with NaOH. Buffer was filtered through 0.22 µm sterile syringe filter.
d. DTT solution (100 mM; prepared fresh): dissolved 15.4 mg DTT in 1 mL of water.
e. Alkylating reagent (IAA) (200 mM; prepared fresh): dissolved 37 mg IAA in 1 mL of water.

f. Trypsin solution: Dissolved 50 µg of dried trypsin (contents of 2 vials) in 200 µL of 100 mM Tris buffer immediately prior to digestion procedure.
g. Arg-C solution: Dissolved 15 µg of dried Arg-C (contents of 3 vials) in 150 µL of Milli-Q deionized water immediately prior to digestion procedure. Reconstituted activation solution concentrate in 100 µL of Milli-Q deionized water.
h. Asp-N solution: Dissolved 8 µg of dried Asp-N (contents of 4 vials) in 200 µL of Milli-Q deionized water immediately prior to digestion procedure.
i. Glu-C solution: Dissolved 150 µg of dried Glu-C in 150 µL of Milli-Q deionized water immediately prior to digestion procedure.

In-solution Protein Processing Procedure (Reduction/ alkylation/ digestion):

a. Two 560-µL aliquots of 2m-EPSPS protein stock solution were dispensed in 1.5-mL siliconized microcentrifuge tubes and dried in a centrifugal evaporator to completeness.
b. Reduction and carboxyamidomethylation (alkylation) of protein: approximately 180-µL of protein dissolution buffer, 6M Gu:HCl/ 0.4M ammonium bicarbonate, pH 7.8, was added to each dry 2m-EPSPS [Batch TSN033171-0001] aliquot, and the samples were mixed by pipette action. Twenty microliters of 100 mM DTT (reducing reagent) solution was added to each microfuge tube. The microfuge tubes were sealed, vortexed, and incubated at 65 ºC for 30 min in a thermomixer at 1100 rpm. The microfuge tubes were cooled to room temperature, centrifuged for 30 sec, and 40 µL of 200 mM IAA (alkylating reagent) solution was added to each tube. The microfuge tubes were incubated in the dark at room temperature for 1 hour. Eighty microliters of DTT solution was added to each tube to consume unreacted IAA, and the tubes were allowed to incubate for 20 min at room temperature.
c. Desalting of the reduced/alkylated protein sample was performed using a NAP-5 gravity cartridge (Sephadex G-25) as per the manufacturer's procedure. NAP-5 cartridges were pre-equilibrated with 100 mM Tris buffer, pH 8.04, and protein elution was performed with the same buffer (final volume: 1-mL). The reduced and alkylated protein sample from each tube was split into 2 equal parts (500 µL each in a separate tube; final total number of tubes = 4) prior to digestion with various enzymes.
d. In-solution Tryptic digestion of reduced/alkylated protein: 20 µL of trypsin solution was added to the 500-µL of reduced/alkylated 2m-EPSPS protein sample in 100 mM Tris buffer, pH8.04. The digest was incubated for 16 hours (overnight) at 37 ºC in a Thermomixer, shaking at 800 rpm.

e. Arg-C digestion of reduced/alkylated protein: 75 µL of Arg-C solution was added to the 500-µL of reduced/alkylated 2m-EPSPS protein sample, followed by addition of 25 µL of Arg-C activation solution. The digest was incubated for 16 hours (overnight) at 37 ºC in a Thermomixer, shaking at 800 rpm.
f. Glu-C (pH8) digestion of reduced/alkylated protein: 75 µL of Glu-C solution was added to the 500-µL of reduced/alkylated 2m-EPSPS protein sample. The digest was incubated for 16 hours (overnight) at room temperature (~23 ºC), shaking at ~800 rpm.
g. Asp-N digestion of reduced/alkylated protein: 100 µL of Asp-N solution was added to the 500-µL of reduced/alkylated 2m-EPSPS protein sample. The digest was incubated for 16 hours (overnight) at 37 ºC in a Thermomixer, shaking at 800 rpm.
h. Prior to mass-spectrometry analyses, digested samples were concentrated in a centrifugal evaporator: dried to completeness and reconstituted in 100 µL of 0.1% aqueous TFA. The samples were centrifuged, and 80 µL of each sample were taken for LC/MS and LC/MS/MS analyses. The remaining 20 µL of each sample were purified for MALDI-TOF MS analysis using C18 zip-tips (procedure is described separately under "*MALDI-TOF MS analysis of 2m-EPSPS proteolytic digests*").

MALDI-TOF MS of 2m-EPSPS proteolytic digests:

Reagents and Materials:

1. Acetonitrile, Fisher, cat no. A998-1, Lot no. 080757
2. Milli-Q deionized water (18 $M\Omega cm^{-1}$, TOC 30-20 ppb)
3. Siliconized 0.6-ml microcentrifuge tubes, Fisher, cat no. 02-681-330
4. Pipette tips 0.2-10 µL, Eppendorf
5. Zip Tips C18, Millipore, cat no. ZTC18S096
6. α-cyano-4-hydroxycinnamic acid (CHCA), Fluka, cat no. 28480
7. Trifluoroacetic acid (TFA), Fisher, cat no. 04902-100
8. ProteoMass MALDI-MS calibration kit, Sigma, cat. no. MS-CAL2

Analytical Procedure:

MALDI-TOF MS: The in-solution proteolytic digests were purified using C18 ZipTips, according to the manufacturer's procedure. For each sample, 4-µL fractions eluting off C18 ZipTips in 10 %, 25 %, 50 % and 70 % acetonitrile/ 0.1 % TFA were deposited onto a MALDI plate, mixed with 1 µL of CHCA matrix solution (prepared as saturated solution in 50% ACN/ 0.1% TFA, and centrifuged), air-dried, and

analyzed by MALDI-TOF MS. All mass spectra were acquired on a Voyager DE STR MALDI-TOF mass spectrometer (S/N 4260). The following mass spectrometer settings were used:

Mode of operation: reflector
Extraction mode: delayed
Polarity: positive
Acquisition control: manual
Accelerating voltage: 20000 V
Grid voltage: 62%
Mirror voltage ratio: 1.12
Extraction delay time: 200 nsec
Acquisition mass range: segments 300-1000 Da, 600-4000 Da, and 1900-6000 Da
Number of laser shots: 500/spectrum
Laser intensity: 1600 – 2000 (varied)
Low mass gate: 500-1900 Da (varied)
Timed ion selector: off

External mass calibration was performed with peptide standards utilizing a Sigma mass calibration kit (cat. no. MS-CAL2), consisting of the calibration mixture (monoisotopic $[M+H]^{1+}$ m/z values used): Bradykinin (fragment 1-7), m/z 757.3997; Angiotensin II, m/z 1046.5423; P14R synthetic peptide, m/z 1533.8582; ACTH (fragment 18-39), m/z 2465.1989; and Insulin oxidized B chain (bovine), m/z 3494.6513.

The proteolytic fragments for all digests were assigned using theoretical ion tables generated using Micromass BioLynx and GPMAW (v 7.01a) software packages.

ESI-LC/MS of intact 2m-EPSPS protein

Reagents and Standards:

1. Acetonitrile (HPLC grade, 99.9%, J.T. Baker), Lot no. 10827
2. Isopropanol (HPLC grade, EMD), Lot n. LH44299
3. Formic acid (Sigma), Lot no. 12026KD
4. 0.1 % formic acid in water (HPLC grade, J.T. Baker), Lot no. GI6503
5. Deionized water, 18.2 $M\Omega cm^{-1}$, MilliQ gradient A10, Millipore, freshly drawn
6. Poly-DL-Alanine, Sigma, Catalog no. P9003, Lot no. 97H5912
7. Ribonuclease A (RNAse A), Sigma, Catalog no. R5000, Lot no. 122K1319
8. Bovine serum albumin (BSA), Sigma, Catalog no. A3059, Lot No. 034K0598
9. β-Galactosidase (from *E.coli*), Sigma, Catalog no. G8511, Lot no. 105K6020

Analytical Procedure:

ESI-LC/MS: The sample preparations were analyzed directly by mass spectrometry. All mass spectra were acquired on a Waters Q-ToF Micro MS system (S/N YA137). The mass spectrometer was calibrated prior to use in the mass range 700-2300 amu using 0.1 mg/mL Poly-DL-Alanine solution (in acetonitrile). A mixture of proteins with known molecular masses (RNAse A, BSA, β-Galactosidase; solutions in deionized water at 20 mg/mL were used) was run as a test standard. The following mass spectrometer settings were used.

LC : Acquity UPLC system
Mobile Phase A : 0.1 % TFA in water
Mobile Phase B : 0.1 % TFA in isopropanol (IPA)
Post-LC/ Pre-MS : addition of 7% glycerol solution at 40 μL/min
Column : 2.1x100 mm BEH C4 1.7μ 300 Å, P/N 186004496, Lot no. 0105191051
Flow rate : 300 μL/min
Column temperature : room temperature (~23 ^{o}C)
Injection volume : 10 μL
Injection loop : 20 μL
UV detection : 215 and 280 nm, 2 points/sec

Gradient table:

Time, min	Flow rate, mL/min	%A	%B
Initial	0.3	95	5
22.5	0.3	50	50
25	0.3	50	50
25.1	0.3	95	5

Waters Q-ToF Micro MS system: MS Parameters:

Capillary : 2850 V
Desolvation Gas : 600 L/hr
Desolvation Temperature: 410 °C
Source Temperature : 100 °C
Sample Cone : 35 V
MCP : 2350 V
Mode : ESI-TOF-MS +
Scan Range : 700 – 2300 amu
Scan Cycle Time : 0.98 sec/scan

The Micromass-supplied electrospray maximum entropy algorithm (MAXENT 1) was used to transform the spectra to a mass axis and to resolution enhance the transformed spectra. The maximum entropy algorithm was set to optimize the spectra with a resolution of 2 Da/channel. The resulting resolution-enhanced spectral peaks were centered and integrated to display the accurate mass for intact molecular mass analysis.

ESI-LC/MS and MS/MS of 2m-EPSPS proteolytic digests

Reagents and Materials:

1. Acetonitrile, HPLC grade, Fisher Scientific, Lot no. 094014
2. Milli-Q deionized water
3. 98% Formic Acid (Fluka), Lot no. 1255194
4. Poly-DL-Alanine, Sigma, cat. no. P9003, Lot no. 97H5912
5. Leucine Enkephalin acetate salt, Sigma, cat. no. L-9133, Lot no. 095K5109
6. Waters polypropylene plastic HPLC vials, P/N 186002640, lot no. 2640500710

Analytical Procedure:

ESI-LC/MS: All mass spectra were acquired on a Waters Q-ToF Micro MS system (S/N YA137). The mass spectrometer was calibrated prior to use in the mass range 80 – 1900 amu using 0.1 mg/mL Poly-D,L-Alanine solution in acetonitrile. The following liquid chromatography and mass spectrometer settings were used:

LC :	Acquity UPLC system
Mobile Phase A :	0.1 % formic acid (FA) in water
Mobile Phase B :	0.1 % formic acid (FA) in acetonitrile
Column :	2.1x100 mm Acquity BEH C18 1.7 µm 130 Å; P/N 186002578, Lot no.115A1430810
Flow rate :	100 µL/min
Column temperature :	50 °C
Injection volume :	4 µL
Injection loop :	20 µL
UV detection :	214 nm, 10 pts/sec

Gradient table:

Time, min	Flow rate, mL/min	%A	%B
Initial	0.1	95	5
5	0.1	95	5
35.4	0.1	60	40
37	0.3	10	90
40	0.3	10	90
40.1	0.3	95	5
46	0.3	95	5

46.1	0.1	95	5
49	0.1	95	5
51	0	95	5

MS : QTOF-micro mass spectrometer (S/N YA137)

ESI : Micromass lock-spray electrospray interface

Mode : +TOFMS

MS Parameters (peptide mass fingerprinting):

Capillary	:	2850 V
Desolvation Gas	:	250 L/hr
Desolvation Temperature	:	250 °C
Source Temperature	:	100 °C
Sample Cone	:	35 V
Extraction Cone	:	1.5 V
Collision Energy	:	10.0 V
MCP	:	2350 V
Mode	:	ESI-TOF-MS +
Scan Range	:	80 – 1900 amu
Scan Cycle Time	:	0.90 sec/scan

MS/MS Parameters:

Capillary	:	3153 V
Desolvation Gas	:	250 L/hr
Desolvation Temperature	:	120 °C
Source Temperature	:	80 °C
Sample Cone	:	51 V
Extraction Cone	:	1.0 V
MCP	:	2753 V
Mode	:	ESI-TOF-MS +
Scan Range	:	80 – 1900 amu

Survey Scan

Collision Energy	:	10.0 V
Scan Cycle Time	:	0.98 sec/scan
Precursor Selection	:	Included Masses only
Include Window	:	+/- 500 mDa
Include Retention Time	:	60 sec
Peak Detection Window	:	3 Da

MS/MS Scan

MS to MSMS Switch Criteria	:	Intensity
MS to MSMS Switch Threshold	:	1 counts/sec
MSMS to MS Switch Criteria	:	Intensity falling below threshold
Switchback Threshold	:	3 counts/sec
MSMS Switch After Time	:	20 sec
Scan Cycle Time	:	1 sec/scan

Methods:

The samples were injected using partial loop configuration. After sample injection, the column was held at 5 % MPB for 5 minutes. The gradient from 5 % MPB to 40 % MPB was then employed. At the end of

the gradient, the MPB concentration was increased to 90% to allow removal of any hydrophobic components. The column was then re-equilibrated to the initial conditions.

The Time of Flight (ToF) analyzer was calibrated daily using a 0.1 mg/mL solution (in acetonitrile) of Poly-D,L-Alanine at 20 µL/min flow rate. The same instrument parameter file (with the calibration parameters) was used for MS data acquisitions. Data acquisition was performed with cycle times of 1 sec/scan (scan acquisition time: 0.88 sec; interscan delay: 0.1sec). The lock mass data was acquired using 2.5 µM Leucine-Enkephalin peptide solution (0.1 % formic acid in 50 % acetonitrile was used as the solvent) flowing at 3 – 5 µL/min. The lock mass channel was sampled every 7 sec during MS analysis and 10 sec during MS/MS analysis. The reference ion used was the singly charged Leucine-Enkephalin ion at m/z 556.2771.

Peptide mass fingerprinting of the UPLC-MS data was performed manually. The spectrum of each chromatographic peak was summed, smoothed (SG, 2x3 channels), centroided (4 channels, top 80 %, by height) and m/z error corrected (lock mass channel: 10 scans, m/z 556.2271 ± 0.5 Da). In-source fragmentation observed was used to further confirm the identity of the peptides. The mass-spectra of proteolytic fragments, as well as in-source fragments, were analyzed using MassLynx (Micromass) and GPMAW v. 7.01a (Lighthouse Data) software.

The spectra from tandem MS experiments (for N- and C-terminal fragments, and fragments containing TIPS mutation) were also summed, smoothed (SG, 2x3 channels), centroided (4 channels, top 80 %, by height) and m/z error corrected (lock mass channel: 10 scans, m/z 556.2271 ± 0.5 Da). The fragments were assigned using a theoretical fragmentation ion table generated using Micromass BioLynx and GPMAW v. 7.01a (Lighthouse Data) software packages.

The raw data are archived in a raw data archive MD-2010-007963 [1].

RESULTS

The sample of 2m-EPSPS was examined for purity and integrity by SDS-PAGE (**Figure 1**). A wild-type reference (wt-EPSPS) and a generic standard protein (BSA) were also loaded on the same SDS-PAGE gel to facilitate analysis.

SDS-PAGE analysis resulted in an apparent molecular weight of 2m-EPSPS of approximately 45 ±2 kDa (**Figure 1**). Based on known quantities of a BSA standard loaded alongside 2m-EPSPS on the same SDS-PAGE gel, concentration in the original "1 mg/mL" stock solution was determined to be 0.62 mg/mL. The absence of other protein bands on the SDS-PAGE gel indicated high homogeneity and integrity of the 2m-EPSPS sample.

LC/MS analysis of the intact 2m-EPSPS sample (**Figure 2**) resulted in an accurate MW = 47285.8 ±0.5 Da for the principal component of the 2m-EPSPS sample. This result is within 0.0024% of the theoretical average MW for the 2m-EPSPS protein (47284.68 Da).

The sample of 2m-EPSPS was subsequently reduced, alkylated, and digested by four proteolytic enzymes (as 4 separate digests: Trypsin, Asp-N, Arg-C, and Glu-C at pH8). The resulting proteolytic digests were examined by MALDI-TOF and ESI LC/MS mass-spectrometry techniques in order to obtain confirmation of the protein's amino-acid sequence and sequence details. Peptide mass fingerprinting of 2m-EPSPS based on the four proteolytic digests resulted in 100% sequence coverage (**Figure 3** and **Tables 1** through **4**). The LC/MS chromatograms of the 4 digests are shown in **Figures 4** through **7**.

To further confirm the details of the primary structure of 2m-EPSPS sample, N- and C-terminal proteolytic fragments, as well as proteolytic fragments containing the TIPS mutation were subjected to MS/MS fragmentation analysis. **Tables 5** through **11** list the results of these experiments. The following N-terminal sequence was confirmed by MS/MS analysis: ^{1}AGAEEIVLQPIK^{12} (T1 fragment from tryptic digest, **Table 5**; i.c.1 fragment from Asp-N digest, **Table 6**). The following C-terminal sequences were confirmed by MS/MS analysis: ^{436}DVLSTFVKN^{444} (D27 fragment from Asp-N digest, **Table 7**), ^{429}KTFPDYFDVLSTFVKN^{444} (R20 fragment from Arg-C digest, **Table 8**). No other variants of the N- and C-terminal sequences were detected.
Amino acid sequences of the following fragments containing TIPS mutation were confirmed: ^{106}**S**LTAAVTAAGGNATYVLDGVPR^{127} (R5 and T12 fragments from Arg-C and tryptic digests; **Tables 9** and **10**, respectively), ^{91}EEVQLFLGNAG**I**AMR^{105} (T11 fragment from tryptic digest, **Table 11**). No fragments representing the absence of the TIPS mutation or partial TIPS mutation were detected.

REFERENCES

1. Raw data archive MD-2010-007963
2. MD-2010-007962 (AL report “Confirmation of primary structure of wt-EPSPS protein by MALDI-TOF MS peptide mass fingerprinting”; A. Karnoup)

Table 1. Observed tryptic fragments of 2m-EPSPS. Cys residues were reduced and carboxyamidomethylated.

Fragment #	# of missed cleavages	Start - End	Sequence	Theor. mono. m/z (MH^+)	Retention Time, min	Observed m/z, LC/MS *	Observed in-source peptide fragments	% ACN (elution from C18-zt; MALDI)	Observed m/z MALDI-MS	Modification	Comment
T1	0	1-12	(-) AGAEEIVLQPIK	1267.73	20.83	1267.74 (1+), 634.36 (2+)	in-source fragments match sequence	10, 25, 50, 70%	1267.58	no Met[1]	major LC peak; sequence confirmed by MS/MS fragmentation
T1 + Met	0	(-1)-12	(-) MAGAEEIVLQPIK	1398.77	ND	ND		ND	ND		
T2	0	13- 19	EISGTVK	733.41	6.45	733.41 (1+)	in-source fragments match sequence	ND	ND		
T3	0	20- 24	LPGSK	501.30	3.98	501.30 (1+)	some in-source fragments match	ND	ND		coelutes with T39
T4	0	25- 29	SLSNR	576.31	3.21	576.31 (1+)		ND	ND		coelutes with T32. T13, T43
T5	0	30- 60	ILLLAALSEGTTVVDNLLNSEDVHYMLGALR	3340.78	36.33	1670.87 (2+), 1114.26 (3+), 835.94 (4+)	in-source fragments match sequence	50, 70%	3340.47		colutes with T18
incorrect cleavage 1	n/a	33-60	LAALSEGTTVVDNLLNSEDVHYMLGALR	3001.52	31.59	1501.27 (2+), 1001.17 (3+), 751.14 (4+)	some in-source fragments match	ND	ND		incorrect (tryptic/chymotryptic) cleavage
incorrect cleavage 2	n/a	34-60	AALSEGTTVVDNLLNSEDVHYMLGALR	2888.44	30.10	1444.72 (2+), 963.48 (3+), 722.84 (4+)	some in-source fragments match	ND	ND		incorrect (tryptic/chymotryptic) cleavage
T6	0	61- 70	TLGLSVEADK	1032.56	17.42	1032.56 (1+), 516.78 (2+)	in-source fragments match sequence	10, 25%	1032.29		coelutes with T41
T9	0	75- 83	AVVVGCGGK	846.45	9.78	846.46 (1+)	in-source fragments match sequence	10%	846.10		coelutes with T21
T10	0	84- 90	FPVEDAK	805.41	13.18	805.41 (1+), 403.21 (2+)	in-source fragments match sequence	10, 25%	805.20		
T11	0	91-105	EEVQLFLGNAGİAMR	1647.85	25.48	1647.87 (1+), 824.43 (2+)	in-source fragments match sequence	10, 25, 50, 70%	1647.68		sequence confirmed by MS/MS fragmentation
T11 no mut	0	91-127	EEVQLFLGNAGTAMRPLTAAVTAAGGNATYVLDGVPR	3730.92	ND	ND		ND	ND		
T11 no PS-mut	0	91-127	EEVQLFLGNAGİAMRPLTAAVTAAGGNATYVLDGVPR	3742.96	ND	ND		ND	ND		
T11 no TI-mut	0	91-105	EEVQLFLGNAGTAMR	1635.82	ND	ND		ND	ND		
T12	0	106-127	**S**LTAAVTAAGGNATYVLDGVPR	2104.10	23.87	1052.54 (2+), 702.03 (3+)	in-source fragments match sequence	10, 25, 50, 70%	2103.88		sequence confirmed by MS/MS fragmentation
T13	0	128-129	MR	306.16	3.21	306.16 (1+)		ND	ND		coelutes with T32. T32, T4
T14	0	130-141	ERPIGDLVVGLK	1295.77	20.67	1295.78 (1+), 648.38 (2+)	in-source fragments match sequence	10, 25, 50, 70%	1295.63		
T15	0	142-159	QLGADVDCFLGTDCPPVR	2019.93	23.18	1010.46 (2+), 673.98 (3+)	in-source fragments match sequence	25, 50, 70%	2019.71		
T16	0	160-170	V**N**GIGGLPGGK	968.55	16.10, 16.71	969.54 (1+), 485.26 (2+)	in-source fragments match sequence	ND	ND	deamidated Asn161	deamidation
T18	0	173-203	LSGSISSQYLSALLMAAPLALGDVEIEIIDK	3217.73	36.33	1609.38 (2+), 1073.26 (3+)	some in-source fragments match	ND	ND		coelutes with T5
incorrect cleavage 3	n/a	182-203	LSALLMAAPLALGDVEIEIIDK	2295.28	34.76	1148.15 (2+), 765.76 (3+)	some in-source fragments match	ND	ND		incorrect (tryptic/chymotryptic) cleavage; minor LC peak
incorrect cleavage 4	n/a	187-203	MAAPLALGDVEIEIIDK	1797.96	28.65	1797.93 (1+), 899.49 (2+)	some in-source fragments match	ND	ND		incorrect (tryptic/chymotryptic) cleavage; minor LC peak
T19	0	204-215	LISIPYVEMTLR	1434.80	27.55	1434.82 (1+), 717.89 (2+)	in-source fragments match sequence	10, 25, 50, 70%	1434.66		
T20	0	216-219	LMER	548.29	4.60	548.29 (1+)	in-source fragments match sequence	ND	ND		
T21	0	220-223	FGVK	450.27	9.78	450.27 (1+)	some in-source fragments match	ND	ND		coelutes with T9
T22	0	224-232	AEHSDSWDR	1102.46	4.85	1102.46 (1+), 551.73 (2+)	in-source fragments match sequence	10, 25, 50%	1102.32		
T23	0	233-236	FYIK	570.33	14.95	570.33 (1+)	some in-source fragments match	10, 25%	570.17		
T27	0	246-285	NAYVEGDASSASYFLAGAAITGGTVTVEGCGTTSLQGDVK	3924.84	ND	ND		50, 70%	3924.38		
T28	0	286-296	FAEVLEMMGAK	1225.60	24.03	1225.60 (1+), 613.29 (2+)	in-source fragments match sequence	25, 50, 70%	1225.46		
T29	0	297-311	VTWTETSVTVTGPPR	1630.84	19.55	1630.92 (1+), 815.92 (2+)	in-source fragments match sequence	10, 25, 50, 70%	1630.68		
T30	0	312-316	EPFGR	605.30	10.33	605.31 (1+)	in-source fragments match sequence	10, 25%	605.15		
T32	0	318-320	HLK	397.26	3.21	397.26 (1+)		ND	ND		coelutes with T43. T13, T4
T33	0	321-328	AIDVNMNK	904.46	13.81	904.46 (1+), 452.72 (2+)	in-source fragments match sequence	ND	ND		

Table 1 (continued). Observed tryptic fragments of 2m-EPSPS. Cys residues were reduced and carboxyamidomethylated.

Fragment #	# of missed cleavages	Start - End	Sequence	Theor. mono. m/z (MH^+)	Retention Time, min	Observed m/z, LC/MS *	Observed in-source peptide fragments	% ACN (elution from C18-zt; MALDI)	Observed m/z MALDI-MS	Modification	Comment
incorrect cleavage 5	n/a	329-341	MPDVAMTLAVVAL	1330.70	33.28	1330.71 (1+), 665.85 (2+)	in-source fragments match sequence	**ND**	**ND**		incorrect (tryptic/chymotryptic) cleavage
T34	0	329-350	MPDVAMTLAVVALFADGPTAIR	2259.19	35.88	1130.10 (2+), 753.73 (3+)	in-source fragments match sequence	50, 70%	2258.96		
T35	0	351-356	DVASWR	733.36	13.88	733.37 (1+)	in-source fragments match sequence	**ND**	**ND**		
T38	0	363-367	MVAIR	589.35	13.01	589.35 (1+)	in-source fragments match sequence	10, 25%	589.20		
T39	0	368-372	TELTK	591.34	3.98	591.33 (1+)	in-source fragments match sequence	**ND**	**ND**		coelutes with T3
T40	0	373-391	LGASVEEGPDYCIITPPEK	2075.00	21.01	1038.00 (2+), 692.33 (3+)	many in-source fragments match sequence	25, 50, 70%	2074.78		
T41	0	392-404	LNVTAIDTYDDHR	1532.73	17.42	1532.74 (1+), 766.87 (2+)	in-source fragments match sequence	10, 25, 50, 70%	1532.57		coelutes with T6
T42	0	405-422	MAMAFSLAACAEVPVTIR	1937.97	**ND**	**ND**		50, 70%	1937.77		
incorrect cleavage 6	n/a	405-409	MAMAF	570.23	22.20	570.24 (1+)	in-source fragments match sequence	**ND**	**ND**		incorrect (tryptic/chymotryptic) cleavage
incorrect cleavage 7	n/a	410-422	SLAACAEVPVTIR	1386.73	20.25	1386.76 (1+), 693.87 (2+)	in-source fragments match sequence	**ND**	**ND**		incorrect (tryptic/chymotryptic) cleavage
T43	0	423-428	DPGCTR	705.30	3.21	705.30 (1+)		**ND**	**ND**		coelutes with T32. T13, T4
T45	0	430-443	TFPDYFDVLSTFVK	1678.84	32.53	1678.86 (1+), 839.90 (2+)	in-source fragments match sequence	10, 25, 50, 70%	1678.64		
T10-11	1	84-105	FPVEDAKEEVQLFLGNAGIAMR	2434.24	28.51	1217.63 (2+), 812.08 (3+)	some in-source fragments match	25, 50, 70%	2433.99		
T38-39	1	363-372	MVAIRTELTK	1161.67	**ND**	**ND**		25, 50, 70%	1161.66		
T44-45	1	429-443	KTFPDYFDVLSTFVK	1806.93	29.77	903.96 (2+)	some in-source fragments match	50, 70%	1806.73		
T8-10	2	74- 90	RAVVVGCGGKFPVEDAK	1788.94	**ND**	**ND**		50%	1788.59		
T9-11	2	75-105	AVVVGCGGKFPVEDAKEEVQLFLGNAGIAMR	3261.68	**ND**	**ND**		50%	3261.15		
T36-39	3	357-372	VKETERMVAIRTELTK	1904.06	**ND**	**ND**		25, 50, 70%	1903.68		

ND = not detected

Table 2. Observed Arg-C fragments of 2m-EPSPS. Cys residues were reduced and carboxyamidomethylated.

Fragment #	# of missed cleavages	Start - End	Sequence	Theor. mono. m/z (MH^+)	Retention Time, min	Observed m/z, LC/MS *	Observed in-source peptide fragments	% ACN (elution from C18-zt; MALDI)	Observed m/z MALDI MS	Modification	Comment
R1	0	1 - 29	(-) AGAEEIVLQPIKEISGTVKLPGSKSLSNR	3021.69	ND	ND		ND	ND		N-term
R1 + Met	0	(-1) - 29	(-) MAGAEEIVLQPIKEISGTVKLPGSKSLSNR	3152.73	ND	ND		ND	ND		N-term
i.c. 1	n/a	1 - 19	(-) AGAEEIVLQPIKEISGTVK	1982.11	24.92	991.55 (2+), 661.37 (3+)	in-source fragments match sequence	ND	ND		N-term., tryptic-like cleavage; major LC peak
i.c. 2	n/a	1 - 24	(-) AGAEEIVLQPIKEISGTVKLPGSK	2464.39	26.08	1232.77 (2+), 822.13 (3+), 616.85 (4+)	in-source fragments match sequence	ND	ND		N-term., tryptic-like cleavage; trace LC peak
i.c. 3	n/a	20 - 24	LPGSK	501.30	4.02	501.30 (1+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
R2	0	30- 60	ILLLAALSEGTTVVDNLLNSEDVHYMLGALR	3340.78	36.34	1670.90 (2+), 1114.26 (3+), 835.94 (4+)	in-source fragments match sequence	25, 50, 70%	3341.02		
R3	0	61- 74	TLGLSVEADKAAKR	1458.83	14.52	729.92 (2+), 486.94 (3+)	in-source fragments match sequence	10, 25%	1458.80		
i.c. 4	n/a	75 - 83	AVVVGCGGK	846.44	9.89	846.45 (1+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
i.c. 5	n/a	84-105	FPVEDAKEEVQLFLGNAG**I**AMR	2434.24	28.46	1217.63 (2+), 812.08 (3+), 609.31 (4+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
R4	0	75-105	AVVVGCGGKFPVEDAKEEVQLFLGNAG**I**AMR	3261.68	28.04	1631.35 (2+), 1087.89 (3+), 816.16 (4+)	in-source fragments match sequence	10, 25, 50, 70%	3261.61		major LC peak
R4 no TI mut	0	75-105	AVVVGCGGKFPVEDAKEEVQLFLGNAG**T**AMR	3249.64	ND	ND		ND	ND		
R5	0	106-127	**S**LTAAVTAAGGNATYVLDGVPR	2104.10	23.85	1052.56 (2+), 702.03 (3+)	in-source fragments match sequence	10, 25, 50, 70%	2104.08		
R4-5 no mut	0	75-127	AVVVGCGGKFPVEDAKEEVQLFLGNAG**T**AMR**P**LTAAVTAAGG NATYVLDGVPR	5344.75	ND	ND		ND	ND		
R4-5 no PS mut	0	75-127	AVVVGCGGKFPVEDAKEEVQLFLGNAG**I**AMR**P**LTAAVTAAGG NATYVLDGVPR	5356.78	ND	ND		ND	ND		
i.c. 6	n/a	130-141	ERPIGDLVVGLK	1295.76	20.75	1295.75 (1+), 648.38 (2+), 432.59 (3+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
i.c. 7	n/a	132-141	PIGDLVVGLK	1010.62	22.89	1010.63 (1+), 505.81 (2+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
i.c. 8	n/a	142-159	QLGADVDCFLGTDCPPVR	2019.92	23.18	1010.47 (2+), 673.97 (3+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
i.c. 9	n/a	132-159	PIGDLVVGLKQLGADVDCFLGTDCPPVR	3011.53	32.74	1506.25 (2+), 1004.51 (3+), 753.62 (4+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
i.c. 10	n/a	204-215	LISIPYVEMTLR	1434.80	27.54	1434.82 (1+), 717.90 (2+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
R9	0	216-219	LMER	548.29	4.65	548.29 (1+)	in-source fragments match sequence	10, 25%	548.27		
R10	0	220-232	FGVKAEHSDSWDR	1533.71	13.22	1533.69 (1+), 767.36 (2+), 511.90 (3+)	in-source fragments match sequence	10, 25, 50, 70%	1533.69		
i.c. 11	n/a	233-236	FYIK	570.32	14.94	570.33 (1+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
i.c. 12	n/a	233-240	FYIKGGQK	940.52	11.14	940.51 (1+), 470.76 (2+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
i.c. 13	n/a	286-296	FAEVLEMMGAK	1225.59	24.07	1225.60 (1+), 613.30 (2+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
i.c. 14	n/a	297-311	VTWTETSVTVTGPPR	1630.84	19.59	1630.83 (1+), 815.93 (2+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
R12	0	312-316	EPFGR	605.30	10.36	605.31 (1+)	in-source fragments match sequence	10, 25%	605.29		
i.c. 15	n/a	317-328	KHLKAIDVNMNK	1410.78	11.64	1410.73 (1+), 705.89 (2+), 470.93 (3+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
i.c. 16	n/a	329-350	MPDVAMTLAVVALFADGPTAIR	2259.18	35.87	1130.10 (2+), 753.73 (3+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
R14	0	351-356	DVASWR	733.36	13.93	733.37 (1+)	in-source fragments match sequence	10, 25%	733.35		
R15	0	357-362	VKETER	761.42	3.21	761.42 (1+)	some in-source fragments match	10, 25%	761.40		coelutes with R19
R16	0	363-367	MVAIR	589.35	13.01	589.35 (1+)	in-source fragments match sequence	10, 25%	589.33		
i.c. 17	n/a	368-372	TELTK	591.33	4.02	591.33 (1+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
i.c. 18	n/a	373-391	LGASVEEGPDYCIITPPEK	2074.99	21.04	1038.01 (2+), 692.33 (3+)	most in-source fragments match	ND	ND		tryptic-like cleavage
i.c. 19	n/a	392-404	LNVTAIDTYDDHR	1532.73	17.43	1532.72 (1+), 766.87 (2+), 511.58 (3+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
i.c. 20	n/a	373-404	LGASVEEGPDYCIITPPEKLNVTAIDTYDDHR	3588.71	23.46	1794.81 (2+), 1196.91 (3+), 897.93 (4+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
R18	0	405-422	MAMAFSLAACAEVPVTIR	1937.96	27.07	969.49 (2+), 646.67 (3+)	in-source fragments match sequence	10, 25, 50, 70%	1937.93		
R19	0	423-428	DPGCTR	705.30	3.21	705.30 (1+)	some in-source fragments match	ND	ND		coelutes with R15
i.c. 21	n/a	429-443	KTFPDYFDVLSTFVK	1806.92	29.70	1806.91 (1+), 903.97 (2+), 602.97 (3+)	in-source fragments match sequence	ND	ND		tryptic-like cleavage
R20	0	429-444	KTFPDYFDVLSTFVKN (-)	1920.97	29.18	960.99 (2+), 640.99 (3+)	in-source fragments match sequence	10, 25, 50, 70%	1920.95		C-term; major LC peak

ND = not detected **i.c.** = "incorrect" cleavage

Table 3. Observed Asp-N fragments of 2m-EPSPS. Cys residues were reduced and carboxyamidomethylated.

Fragment #	# of missed cleavages	Start - End	Sequence	Theor. mono. m/z (MH^+)	Retention Time, min	Observed m/z, LC/MS *	Observed in-source peptide fragments	% ACN (elution from C18-zt; MALDI)	Observed m/z MALDI-MS	Modification	Comment
i.c. 1	2	1-12	(-) AGAEEIVLQPIK	1267.72	20.93	1267.73 (1+), 634.36 (2+)	in-source fragments match sequence	ND	ND		N-terminal
i.c. 2	3	1-37	(-) AGAEEIVLQPIKEISGTVKLPGSKSLSNRILLLAALS	3816.21	30.18	1272.73 (3+), 954.80 (4+), 764.03 (5+)	some in-source fragments match	ND	ND		N-terminal
i.c. 3	0	13-37	EISGTVKLPGSKSLSNRILLLAALS	2567.51	26.36	1284.24 (2+), 856.50 (3+), 642.62 (4+)	in-source fragments match sequence	ND	ND		
i.c. 4	1	13-43	EISGTVKLPGSKSLSNRILLLAALSEGTTVV	3153.80	27.81	1577.39 (2+), 1051.94 (3+), 789.19 (4+)	in-source fragments match sequence	ND	ND		
D1	0	1-43	(-) AGAEEIVLQPIKEISGTVKLPGSKSLSNRILLLAALSEGTTVV	4402.52	30.78	1468.12 (3+), 1101.38 (4+), 881.28 (5+)		25, 50, 70%	4402.53	no Met[1]	N-terminal
D1 + Met	0	(-1)-43	(-) MAGAEEIVLQPIKEISGTVKLPGSKSLSNRILLLAALSEGTTVV	4533.56	ND	ND		ND	ND		N-terminal
D2	0	44- 50	DNLLNSE	804.37	16.28	804.36 (1+)	in-source fragments match sequence	ND	ND		
D3	0	51- 68	DVHYMLGALRTLGLSVEA	1945.02	29.36	973.00 (2+), 649.01 (3+)	in-source fragments match sequence	10, 25, 50, 70%	1945.05		
D4	0	69- 87	DKAAKRAVVVGCGGKFPVE	1988.08	15.33	994.54 (2+), 663.36 (3+), 497.76 (4+)	in-source fragments match sequence	10, 25, 50, 70%	1988.08		
D5	0	88-122	DAKEEVQLFLGNAGIAMRSLTAAVTAAGGNATYVL	3522.83	36.18	1174.92 (3+)		50, 70%	3522.90		trace LC peak
D5 deamidated	0	88-122	DAKEEVQLFLGNAGIAMRSLTAAVTAAGGNATYVL	3523.83	34.96	1175.24 (3+)	some in-source fragments match	ND	ND		Asn deamidation; trace LC peak
D5 no mut	0	88-122	DAKEEVQLFLGNAGTAMRPLTAAVTAAGGNATYVL	3520.81	ND	ND		ND	ND		
D5 no PS-mut	0	88-122	DAKEEVQLFLGNAGIAMRPLTAAVTAAGGNATYVL	3532.85	ND	ND		ND	ND		
D5 no TI-mut	0	88-122	DAKEEVQLFLGNAGTAMRSLTAAVTAAGGNATYVL	3510.79	ND	ND		ND	ND		
D6	0	123-134	DGVPRMRERPIG	1382.73	13.38	1382.71 (1+), 691.86 (2+), 461.57 (3+), 346.44 (4+)	in-source fragments match sequence	10, 25, 50, 70%	1382.75		coelutes with D14
i.c. 5	0	130-134	ERPIG	571.31	4.88	571.32 (1+)	some in-source fragments match	ND	ND		
D7	0	135-145	DLVVGLKQLGA	1112.67	23.73	1112.66 (1+), 556.83 (2+)	in-source fragments match sequence	50, 70%	1112.67		
D9	0	148-153	DCFLGT	712.30	18.90	712.29 (1+)	in-source fragments match sequence	ND	ND		
D10	0	154-194	DCPPVRVNGIGGLPGGKVKLSGSISSQYLSALLMAAPLALG	4064.20	32.21	1355.73 (3+), 1017.02 (4+)	in-source fragments match sequence	25, 50, 70%	4064.24		Asn deamidation likely
D11	0	195-201	DVEIEII	830.45	26.57	830.44 (1+)	in-source fragments match sequence	10, 25%	830.35		
i.c. 6	0	202-217	DKLISIPYVEMTLRLM	1922.04	31.26	961.52 (2+), 641.34 (3+)	in-source fragments match sequence	ND	ND		
D12	0	202-227	DKLISIPYVEMTLRLMERFGVKAEHS	3062.62	ND	ND		25, 50, 70%	3062.61		
i.c. 7	0	218-224	ERFGVKA	806.44	11.39	806.44 (1+), 403.73 (2+)	some in-source fragments match	ND	ND		trace LC peak
i.c. 8	0	218-227	ERFGVKAEHS	1159.58	6.70	1159.58 (1+), 580.29 (2+), 387.20 (3+)	in-source fragments match sequence	ND	ND		
D14	0	231-251	DRFYIKGGQKYKSPKNAYVEG	2448.27	13.38	1224.63 (2+), 816.75 (3+), 612.81 (4+), 490.45 (5+)	some in-source fragments match	10, 25, 50, 70%	2448.29		coelutes with D6
i.c.9	1	291-311	EMMGAKVTWTETSVTVTGPPR	2278.11	20.64	1139.57 (2+), 760.04 (3+)	in-source fragments match sequence	ND	ND		
i.c. 10	2	291-322	EMMGAKVTWTETSVTVTGPPREPFGRKHLKAI	3554.85	19.28	1777.86 (2+), 1185.62 (3+), 889.46 (4+), 711.77 (5+)	in-source fragments match sequence	ND	ND		

Table 3 (continued). Observed Asp-N fragments of 2m-EPSPS. Cys residues were reduced and carboxyamidomethylated.

Fragment #	# of missed cleavages	Start - End	Sequence	Theor. mono. m/z (MH^+)	Retention Time, min	Observed m/z, LC/MS *	Observed in-source peptide fragments	% ACN (elution from C18-zt; MALDI)	Observed m/z MALDI-MS	Modification	Comment
D17	0	323-330	DVNMNKMP	948.43	15.56	948.43 (1+), 474.72 (2+)	some in-source fragments match	ND	ND		coelutes with i.c.17 fragment
D18	0	331-343	DVAMTLAVVALFA	1320.72	36.32	1320.73 (1+), 660.87 (2+)	in-source fragments match sequence	ND	ND		
D19	0	344-350	DGPTAIR	729.39	10.18	729.39 (1+)	in-source fragments match sequence	10, 25%	729.32		
i.c. 11	0	351-358	DVASWRVK	960.52	14.71	960.52 (1+), 480.76 (2+)	in-source fragments match sequence	ND	ND		
D20	0	351-381	DVASWRVKETERMVAIRTELTKLGASVEEGP	3457.81	24.18	1729.40 (2+), 1153.26 (3+), 865.20 (4+), 692.36 (5+), 577.12 (6+)	in-source fragments match sequence	25, 50, 70%	3457.84		
i.c. 12	2	351-368	DVASWRVKETERMVAIRT	2147.13	18.45	1074.08 (2+), 716.38 (3+), 537.53 (4+), 430.22 (5+)	in-source fragments match sequence	ND	ND		
i.c. 13	1	359-368	ETERMVAIRT	1205.62	14.52	1205.63 (1+), 603.31 (2+)	in-source fragments match sequence	ND	ND		
i.c. 14	4	359-381	ETERMVAIRTELTKLGASVEEGP	2516.30	22.81	1258.65 (2+), 839.43 (3+), 629.81 (4+)	some in-source fragments match	ND	ND		trace LC peak
i.c. 15	0	369-381	ELTKLGASVEEGP	1329.68	17.43	1329.71 (1+), 665.34 (2+)	in-source fragments match sequence	ND	ND		
D21	0	382-397	DYCIITPPEKLNVTAI	1846.96	25.77	1846.98 (1+), 923.98 (2+)	in-source fragments match sequence	10, 25, 50, 70%	1846.97		
i.c. 16	1	401-415	DDHRMAMAFSLAACA	1666.71	23.04	1666.73 (1+), 833.86 (2+)	in-source fragments match sequence	ND	ND		trace LC peak
i.c. 17	0	416-422	EVPVTIR	813.48	15.56	813.48 (1+), 407.24 (2+)	in-source fragments match sequence	ND	ND		coelutes with D17
D25	0	423-432	DPGCTRKTFP	1178.56	12.38	1178.56 (1+), 589.78 (2+), 393.52 (3+)	in-source fragments match sequence	10, 25, 50%	1178.58		
D27	0	436-444	DVLSTFVKN (-)	1022.55	21.41	1022.55 (1+), 511.77 (2+)	in-source fragments match sequence	10, 25%	1022.46		C-terminal; sequence confirmed by MS/MS fragmentation
D6-7	1	123-145	DGVPRMRERPIGDLVVGLKQLGA	2476.38	ND	ND		10, 25, 70%	2476.29		
D23-24	1	401-422	DDHRMAMAFSLAACAEVPVTIR	2461.18	ND	ND		10, 25%	2461.08		

ND = not detected; **i.c.** = "incorrect" cleavage (for Asp-N, cleavage may occur N-terminal to E, in addition to D)

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Table 4. Observed Glu-C (pH8) fragments of 2m-EPSPS. Cys residues were reduced and carboxyamidomethylated.

Fragment #	# of missed cleavages	Start - End	Sequence	Theor. mono. m/z (MH^+)	Retention Time, min	Observed m/z, LC/MS *	Observed in-source peptide fragments	% ACN (elution from C18-zt; MALDI)	Observed m/z MALDI-MS	Modification	Comment
A1	0	1-5	(-) AGAEE	476.20	ND	ND		ND	ND	no Met[1]	N-terminal
A1 + Met	0	(-1) - 5	(-) MAGAEE	607.24	ND	ND		ND	ND		N-terminal
A2	0	6-13	IVLQPIKE	939.59	18.39	939.59 (1+), 470.29 (2+)	in-source fragments match sequence	10, 25, 70%	939.61		Coelutes with A33
A3	0	14- 38	ISGTVKLPGSKSLSNRILLLAALSE	2567.51	25.89	1284.25 (2+), 856.51 (3+), 642.62 (4+)	in-source fragments match sequence	10, 25, 50, 70%	2567.62		
A5	0	45- 50	NLLNSE	689.35	16.32	689.35 (1+)	some in-source fragments match	ND	ND		coelutes with A50
A7	0	52- 67	VHYMLGALRTLGLSVE	1758.96	ND	ND		10, 25, 50, 70%	1759.04		
i.c. 1	n/a	58-67	ALRTLGLSVE	1058.61	20.56	1058.63 (1+), 529.81 (2+)	in-source fragments match sequence	ND	ND		
A12	0	93-123	VQLFLGNAG**I**AMR**S**LTAAVTAAGGNATYVLD	3065.61	ND	ND		25, 50, 70%	3065.67		
A12 no TI mut.	0	93-123	VQLFLGNAGTAMR**S**LTAAVTAAGGNATYVLD	3053.57	ND	ND		ND	ND		
A12 no PS mut.	0	93-123	VQLFLGNAG**I**AMRPLTAAVTAAGGNATYVLD	3075.63	ND	ND		ND	ND		
A12 no mutations	0	93-123	VQLFLGNAGTAMRPLTAAVTAAGGNATYVLD	3063.59	ND	ND		ND	ND		
A13	0	124-130	GVPRMRE	844.45	9.11	844.44 (1+), 422.72 (2+)	in-source fragments match sequence	10, 25%	844.45		
A23	0	212-218	MTLRLME	893.46	21.05	893.46 (1+), 447.23 (2+)	in-source fragments match sequence	25, 50, 70%	893.49		
A24	0	219-225	RFGVKAE	806.45	9.35	806.45 (1+), 403.73 (2+), 269.49 (3+)	in-source fragments match sequence	10, 25%	806.47		
A27	0	232-250	RFYIKGGQKYKSPKNAYVE	2276.22	ND	ND		10, 25, 50, 70%	2276.31		
A33	0	292-301	MMGAKVTWTE	1153.54	18.39	1153.54 (1+), 577.27 (2+)	in-source fragments match sequence	70%	1153.60		coelutes with A2
A34	0	302-323	TSVTVTGPPREPFGRKHLKAID	2406.33	ND	ND		10, 25, 70%	2406.41		
i.c. 2	n/a	302-315	TSVTVTGPPREPFG	1444.74	19.08	1444.76 (1+), 722.87 (2+)	in-source fragments match sequence	ND	ND		
A37	0	345-351	GPTAIRD	729.39	ND	ND		50, 70%	729.46		
A38	0	352-359	VASWRVKE	974.54	12.61	974.55 (1+), 487.77 (2+), 325.52 (3+)	in-source fragments match sequence	10, 25, 50, 70%	974.58		
A40	0	362-369	RMVAIRTE	975.54	12.24	975.54 (1+), 488.27 (2+), 325.84 (3+)	in-source fragments match sequence	ND	ND		
A41	0	370-379	LTKLGASVEE	1046.57	14.75	1046.58 (1+), 523.79 (2+)	in-source fragments match sequence	25%	1046.62		
A43	0	383-390	YCIITPPE	992.48	20.92	992.48 (1+)	in-source fragments match sequence	ND	ND		
A47	0	403-416	HRMAMAFSLAACAE	1565.70	ND	ND		50, 70%	1565.79		
A48	0	417-433	VPVTIRDPGCTRKTFPD	1959.01	15.94	980.01 (2+), 653.67 (3+), 490.50 (4+)	in-source fragments match sequence	10, 25, 50, 70%	1959.10		
A50	0	437-444	VLSTFVKN (-)	907.53	16.32	907.53 (1+), 454.26 (2+)	in-source fragments match sequence	ND	ND		C-terminal; coelutes with A5

Table 4 (continued). Observed Glu-C (pH8) fragments of 2m-EPSPS. Cys residues were reduced and carboxyamidomethylated.

Fragment #	# of missed cleavages	Start - End	Sequence	Theor. mono. m/z (MH^+)	Retention Time, min	Observed m/z, LC/MS *	Observed in-source peptide fragments	% ACN (elution from C18-zt; MALDI)	Observed m/z MALDI-MS	Modification	Comment
A1-2 + Met	1	(-1) - 13	(-) MAGAEEIVLQPIKE	1527.81	**ND**	**ND**		**ND**	**ND**		N-terminal
A1-2	1	1- 13	(-) AGAEEIVLQPIKE	1396.77	**ND**	**ND**		10, 25%	1396.82		N-terminal
A4-5	1	39- 50	GTTVVDNLLNSE	1261.63	23.07	1261.64 (1+), 631.31 (2+)	in-source fragments match sequence	**ND**	**ND**		
A6-7	1	51- 67	DVHYMLGALRTLGLSVE	1873.98	29.30	937.50 (2+)	in-source fragments match sequence	10, 25, 50, 70%	1874.07		
A8-9	1	68- 87	ADKAAKRAVVVGCGGKFPVE	2059.11	**ND**	**ND**		10, 25, 70%	2059.18		
A13-14	1	124-135	GVPRMRERPIGD	1382.73	**ND**	**ND**		10, 25%	1382.81		
A21-22	1	200-211	IIDKLISIPYVE	1402.82	26.27	1402.84 (1+), 701.91 (2+)	in-source fragments match sequence	50, 70%	1402.86		
A25-26	1	226-231	HSDSWD	746.27	7.26, 7.67	746.27 (1+)	some in-source fragments match	**ND**	**ND**		
A30-31	1	274-288	GCGTTSLQGDVKFAE	1569.72	18.18	1569.70 (1+), 785.36 (2+)	some in-source fragments match	10, 25, 50, 70%	1569.79		
A34-35	1	302-331	TSVTVTGPPREPFGRKHLKAIDVNMNKMPD	3335.74	17.51	1668.36 (2+), 1112.58 (3+), 834.69 (4+), 667.95 (5+)	in-source fragments match sequence	10, 25, 50, 70%	3335.85		
A36-37	1	332-351	VAMTLAVVALFADGPTAIRD	2031.09	33.09	1016.05 (2+), 677.69 (3+)	in-source fragments match sequence	25, 50, 70%	2031.18		
A39-40	1	360-369	TERMVAIRTE	1205.63	**ND**	**ND**		10, 70%	1205.53		
A48-49	1	417-436	VPVTIRDPGCTRKTFPDYFD	2384.17	20.33	1192.61 (2+), 795.39 (3+), 596.79 (4+)	in-source fragments match sequence	25, 50, 70%	2384.26		
A49-50	1	434-444	YFDVLSTFVKN (-)	1332.68	**ND**	**ND**		50, 70%	1332.75		C-terminal
A8-10	2	68- 88	ADKAAKRAVVVGCGGKFPVED	2174.14	15.07	1087.56 (2+), 725.37 (3+)	some in-source fragments match	10, 25, 70%	2174.24		
A9-11	2	70- 92	KAAKRAVVVGCGGKFPVEDAKEE	2445.29	13.48	1223.18 (2+), 815.76 (3+), 612.08 (4+)	some in-source fragments match	**ND**	**ND**		
A17-19	2	149-197	CFLGTDCPPVRVNGIGGLPGGKVKLSGSISSQYLSALLMAAP LALGDVE	4985.59	**ND**	**ND**		50, 70%	4986.72		Asn161 could be deamidated
A25-27	2	226-250	HSDSWDRFYIKGGQKYKSPKNAYVE	3003.48	**ND**	**ND**		10, 25, 50, 70%	3003.59		
A31-33	2	284-301	VKFAEVLEMMGAKVTWTE	2069.05	**ND**	**ND**		70%	2069.14		
S23-24	1	380-416	GPDYCIITPPEKLNVTAIDTYDDHRMAMAFSLAACAE	4156.91	**ND**	**ND**		70%	4156.96		
A48-50	2	417-444	VPVTIRDPGCTRKTFPDYFDVLSTFVKN (-)	3272.68	**ND**	**ND**		10, 25, 50, 70%	3272.80		C-terminal
A4-7	3	39- 67	GTTVVDNLLNSEDVHYMLGALRTLGLSVE	3116.59	**ND**	**ND**		50, 70%	3116.70		
A8-11	3	68- 92	ADKAAKRAVVVGCGGKFPVEDAKEE	2631.36	14.02	1316.17 (2+), 877.79 (3+), 658.59 (4+)	in-source fragments match sequence	10, 25, 50, 70%	2631.46		
A39-42	3	360-382	TERMVAIRTELTKLGASVEEGPD	2502.29	**ND**	**ND**		10, 25%	2502.42		
A44-47	3	391-416	KLNVTAIDTYDDHRMAMAFSLAACAE	2914.35	24.02	1457.74 (2+), 972.13 (3+), 729.35 (4+)	in-source fragments match sequence	25, 50, 70%	2914.46		

ND = not detected **i.c.** = "incorrect" cleavage

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Table 5. Amino acid sequence obtained for N-terminal T1 peptide of 2m-EPSPS from tryptic digest (a.a. 1-12; m/z 634.41; retention time 20.95 min).

Sequence: AGAEEIVLQPIK
Fragment ion masses: monoisotopic
Peptide mass $[M+H]^{2+}$(monoisotopic): 634.37

	A	G	A	E	E	I	V	L	Q	P	I	K
a(+1)	44.05	101.07	172.11	301.15	430.19	543.28	642.35	755.43	883.49	980.54	1093.63	
		101.07	**172.11**	**301.15**	**430.20**	**543.27**	**642.33**					
b(+1)	72.04	129.07	200.10	329.15	458.19	571.27	670.34	783.43	911.48	1008.54	1121.62	
		129.07	**200.10**	**329.14**	**458.19**	**571.27**	**670.33**	**783.40**	**911.52**		**1121.54**	
y(+1)		1196.69	1139.67	1068.63	939.59	810.55	697.46	598.39	485.31	357.25	260.20	147.11
		1196.72		**1068.64**	**939.57**	**810.54**	**697.46**	**598.39**	**485.31**	**357.25**	**260.20**	**147.11**

Upper rows = theoretical ions.
Lower rows (in **bold**) = observed fragment ions.

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Table 6. Amino acid sequence obtained for N-terminal "i.c.1" peptide (see Table 3) of 2m-EPSPS from endoproteinase Asp-N digest (a.a. 1-12; m/z 634.37; retention time 20.99 min)

Sequence: AGAEEIVLQPIK
Fragment ion masses: monoisotopic
Peptide mass $[M+H]^{2+}$(monoisotopic): 634.37

	A	G	A	E	E	I	V	L	Q	P	I	K
a(+1)	44.05	101.07	172.11	301.15	430.19	543.28	642.35	755.43	883.49	980.54	1093.63	
		101.07	**172.11**	**301.15**	**430.19**	**543.26**	**642.34**					
b(+1)	72.04	129.07	200.10	329.15	458.19	571.27	670.34	783.43	911.48	1008.54	1121.62	
		129.07	**200.10**	**329.15**	**458.19**	**571.27**	**670.34**	**783.42**				
y(+1)		1196.69	1139.67	1068.63	939.59	810.55	697.46	598.39	485.31	357.25	260.20	147.11
				1068.57	**939.56**	**810.53**	**697.46**	**598.39**	**485.30**	**357.25**	**260.20**	**147.11**

Upper rows = theoretical ions.
Lower rows (in **bold**) = observed fragment ions.

Table 7. Amino acid sequence obtained for C-terminal D27 peptide of 2m-EPSPS from endoproteinase Asp-N digest (a.a. 436-444; m/z 1022.59; retention time 21.55 min)

Sequence: DVLSTFVKN
Fragment ion masses: monoisotopic
Peptide mass $[M+H]^{2+}$(monoisotopic): 1022.5522

	D	V	L	S	T	F	V	K	N
a(+1)	88.04	187.11	300.19	387.22	488.27	635.34	734.41	862.50	
		187.13	**300.18**			**635.31**	**734.39**		
b(+1)	116.03	215.10	328.19	415.22	516.27	663.34	762.40	890.50	
		215.11	**328.19**	**415.20**	**516.25**	**663.32**	**762.40**	**890.46**	
y(+1)		907.53	808.46	695.37	608.34	507.29	360.22	261.16	133.06
		907.49	**808.46**	**695.35**	**608.33**	**507.31**	**360.23**	**261.16**	

Upper rows = theoretical ions.
Lower rows (in **bold**) = observed fragment ions.

Table 8. Amino acid sequence obtained for C-terminal R20 peptide of 2m-EPSPS from Arg-C digest (a.a. 429-444; m/z 961.54; retention time 29.22 min).

Sequence: KTFPDYFDVLSTFVKN
Fragment ion masses: monoisotopic
Peptide mass $[M+H]^{2+}$(monoisotopic): 960.99

	K	T	F	P	D	Y	F	D	V	L	S	T	F	V	K	N
a(+1)	101.11	202.16	349.22	446.28	561.30	724.37	871.44	986.46	1085.53	1198.61	1285.65	1386.69	1533.76	1632.83	1760.93	
	101.11		**349.21**						**1085.56**	**1198.50**						
b(+1)	129.10	230.15	377.22	474.27	589.30	752.36	899.43	1014.46	1113.53	1226.61	1313.64	1414.69	1561.76	1660.83	1788.92	
	129.10	**230.15**	**377.19**		**589.30**	**752.34**		**1014.46**	**1113.49**	**1226.61**		**1414.67**				
y(+1)		1792.88	1691.83	1544.76	1447.71	1332.68	1169.62	1022.55	907.53	808.46	695.37	608.34	507.29	360.22	261.16	133.06
				1544.78		**1332.75**	**1169.61**	**1022.56**		**808.43**	**695.37**	**608.31**		**360.19**	**261.16**	

Upper rows = theoretical ions.
Lower rows (in **bold**) = observed fragment ions.

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Table 9. Amino acid sequence obtained for internal R5 peptide of 2m-EPSPS from Arg-C digest (a.a. 106-127; m/z 1053.13; retention time 23.93 min) containing P to S mutation.

Sequence: **S**LTAAVTAAGGNATYVLDGVPR
Fragment ion masses: monoisotopic
Peptide mass $[M+H]^{2+}$(monoisotopic): 1052.56

	S	*L*	*T*	*A*	*A*	*V*	*T*	*A*	*A*	*G*	*G*	*N*	*A*	*T*	*Y*	*V*	*L*	*D*	*G*	*V*	*P*	*R*
a(+1)	60.04	173.13	274.18	345.21	416.25	515.32	616.37	687.40	758.44	815.46	872.48	986.53	1057.56	1158.61	1321.68	1420.74	1533.83	1648.85	1705.88	1804.94	1902.00	
		173.11			**416.24**	**515.29**																
b(+1)	88.04	201.12	302.17	373.21	444.25	543.31	644.36	715.40	786.44	843.46	900.48	1014.52	1085.56	1186.61	1349.67	1448.74	1561.82	1676.85	1733.87	1832.94	1929.99	
		201.13	**302.17**	**373.20**	**444.24**	**543.29**	**644.36**	**715.39**	**786.41**	**843.37**	**900.47**			**1187.57**								
y(+1)		2017.07	1903.99	1802.94	1731.90	1660.87	1561.80	1460.75	1389.71	1318.68	1261.65	1204.63	1090.59	1019.55	918.50	755.44	656.37	543.29	428.26	371.24	272.17	175.12
						1660.81	**1561.78**	**1460.65**	**1389.75**	**1318.68**	**1261.70**	**1204.59**	**1090.51**	**1019.55**	**918.48**	**755.43**	**656.36**	**543.29**	**428.26**	**371.20**	**272.17**	**175.12**

Upper rows = theoretical ions.
Lower rows (in **bold**) = observed fragment ions.

Table 10. Amino acid sequence obtained for internal T12 peptide of 2m-EPSPS from tryptic digest (a.a. 106-127; m/z 1053.11; retention time 23.93 min) containing P to S mutation.

Sequence: **S**LTAAVTAAGGNATYVLDGVPR
Fragment ion masses: monoisotopic
Peptide mass $[M+H]^{2+}$(monoisotopic): 1052.56

	S	*L*	*T*	*A*	*A*	*V*	*T*	*A*	*A*	*G*	*G*	*N*	*A*	*T*	*Y*	*V*	*L*	*D*	*G*	*V*	*P*	*R*
a(+1)	60.04	173.13	274.18	345.21	416.25	515.32	616.37	687.40	758.44	815.46	872.48	986.53	1057.56	1158.61	1321.68	1420.74	1533.83	1648.85	1705.88	1804.94	1902.00	
		173.11			**416.24**	**515.29**																
b(+1)	88.04	201.12	302.17	373.21	444.25	543.31	644.36	715.40	786.44	843.46	900.48	1014.52	1085.56	1186.61	1349.67	1448.74	1561.82	1676.85	1733.87	1832.94	1929.99	
		201.13	**302.17**	**373.21**	**444.24**	**543.28**	**644.34**		**786.41**								**1561.77**					
y(+1)		2017.07	1903.99	1802.94	1731.90	1660.87	1561.80	1460.75	1389.71	1318.68	1261.65	1204.63	1090.59	1019.55	918.50	755.44	656.37	543.29	428.26	371.24	272.17	175.12
					1731.71	**1660.79**	**1561.77**	**1460.78**	**1389.75**	**1318.64**	**1261.65**		**1090.57**	**1019.55**	**918.54**	**755.45**	**656.36**	**543.28**	**428.26**		**272.17**	**175.12**

Upper rows = theoretical ions.
Lower rows (in **bold**) = observed fragment ions.

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Table 11. Amino acid sequence obtained for internal T11 peptide of 2m-EPSPS from tryptic digest (a.a. 91-105; m/z 724.99; retention time 25.56 min) containing T to I mutation.

Sequence: EEVQLFLGNAG**I**AMR
Fragment ion masses: monoisotopic
Peptide mass $[M+H]^{2+}$(monoisotopic): 824.43

	E	E	V	Q	L	F	L	G	N	A	G	I	A	M	R
a(+1)	102.06	231.10	330.17	458.23	571.31	718.38	831.46	888.48	1002.53	1073.56	1130.58	1243.67	1314.71	1445.75	
	102.06	**231.10**			**571.29**										
b(+1)	130.05	259.09	358.16	486.22	599.30	746.37	859.46	916.48	1030.52	1101.56	1158.58	1271.66	1342.70	1473.74	
		259.09	**358.16**	**486.23**	**599.30**	**746.38**	**859.49**								
y(+1)		1518.81	1389.77	1290.70	1162.64	1049.56	902.49	789.40	732.38	618.34	547.30	490.28	377.20	306.16	175.12
				1290.63	**1162.62**	**1049.55**	**902.49**	**789.40**	**732.37**	**618.33**	**547.29**	**490.28**	**377.19**	**306.16**	**175.12**

Upper rows = theoretical ions.
Lower rows (in **bold**) = observed fragment ions.

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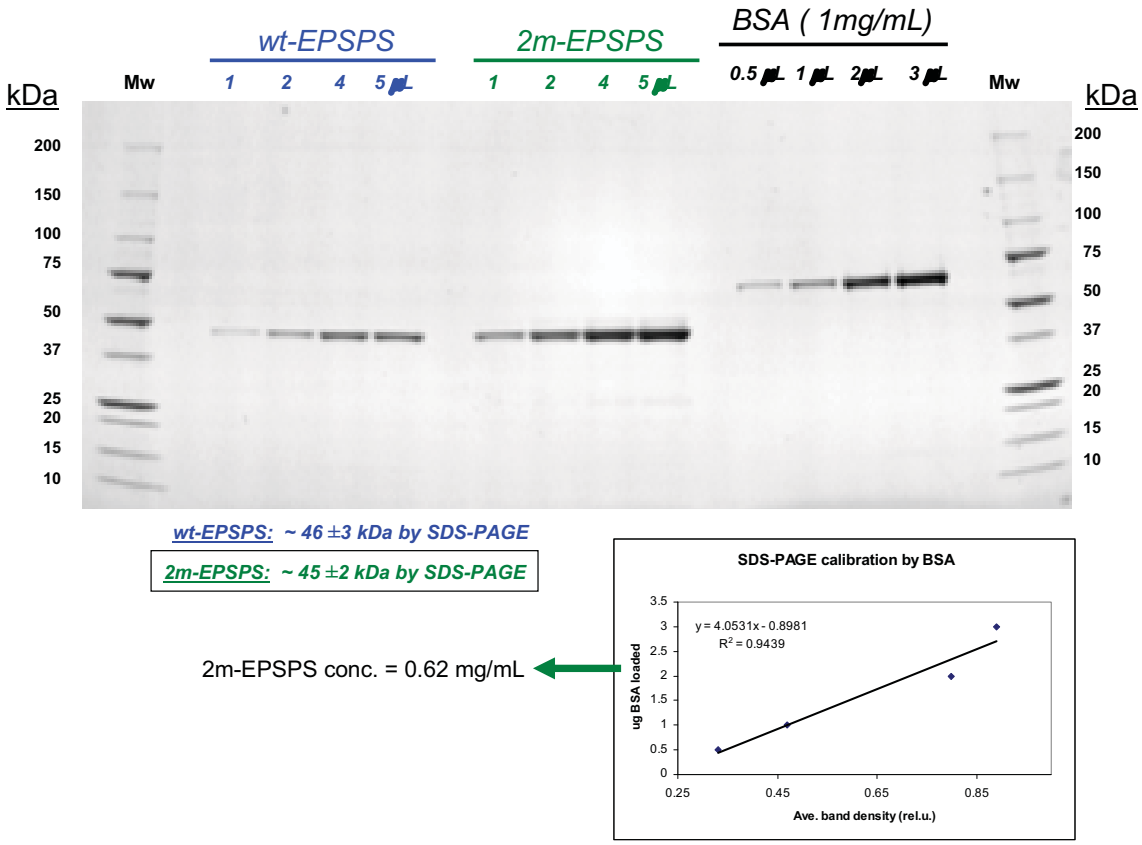
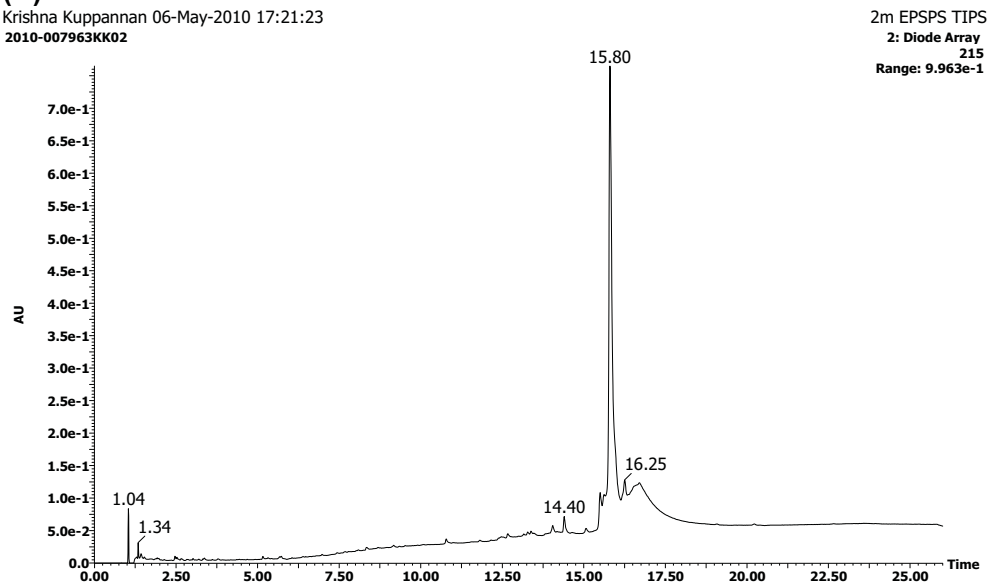


Figure 1. SDS-PAGE analysis of 2m-EPSPS protein sample. Lanes on the left of gel: wt-EPSPS reference material. Lanes in the center of gel: 2m-EPSPS sample. Lanes on the right of gel: BSA protein standard. Bottom panel: estimate of 2m-EPSPS protein quantity based on known amounts of loaded BSA standard. MW = molecular weight standard (Bio-Rad). BSA = bovine serum albumin standard (Sigma). wt-EPSPS = wild-type EPSPS reference [2].

(A)



(B)

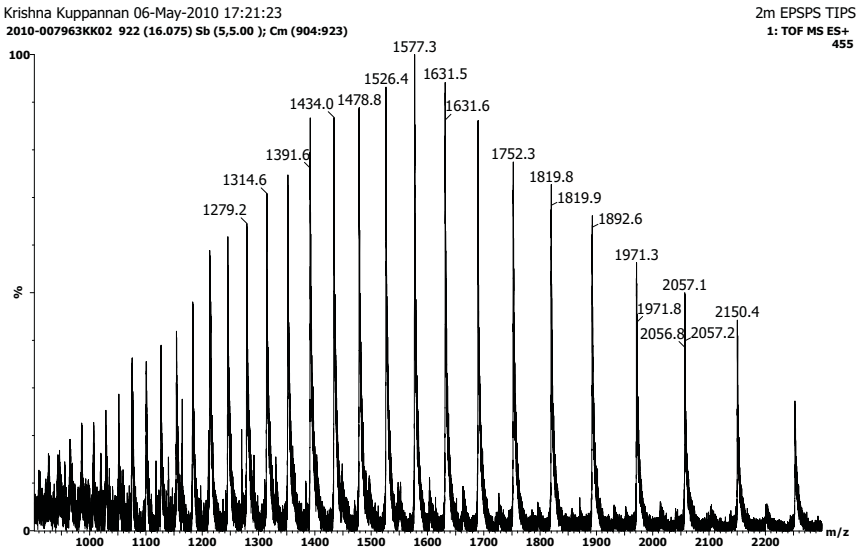


Figure 2 (continued on next page).

(C)

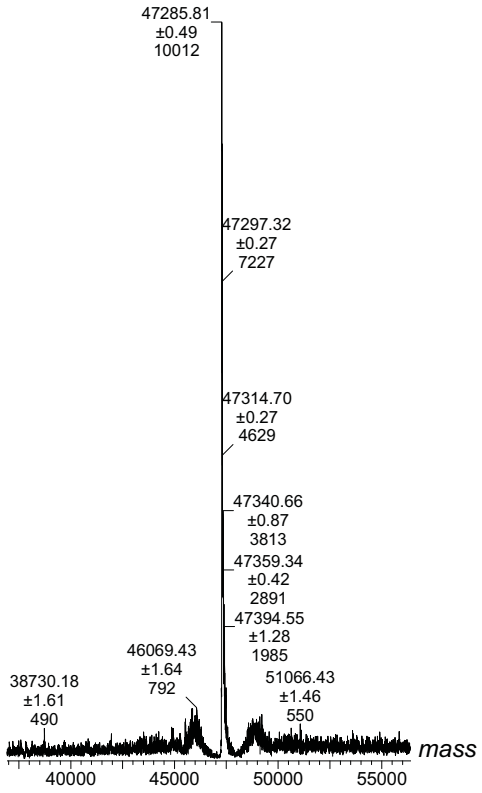


Figure 2 (continued). (**A**) 2m-EPSPS intact MW analysis: LC/MS chromatogram (UV at 215 nm). (**B**) 2m-EPSPS intact MW analysis: raw ESI mass spectrum (multiple charge envelope). (**C**) 2m-EPSPS intact MW analysis: Deconvoluted mass-spectrum corresponding to the principal sample component eluting at 15.80 min.

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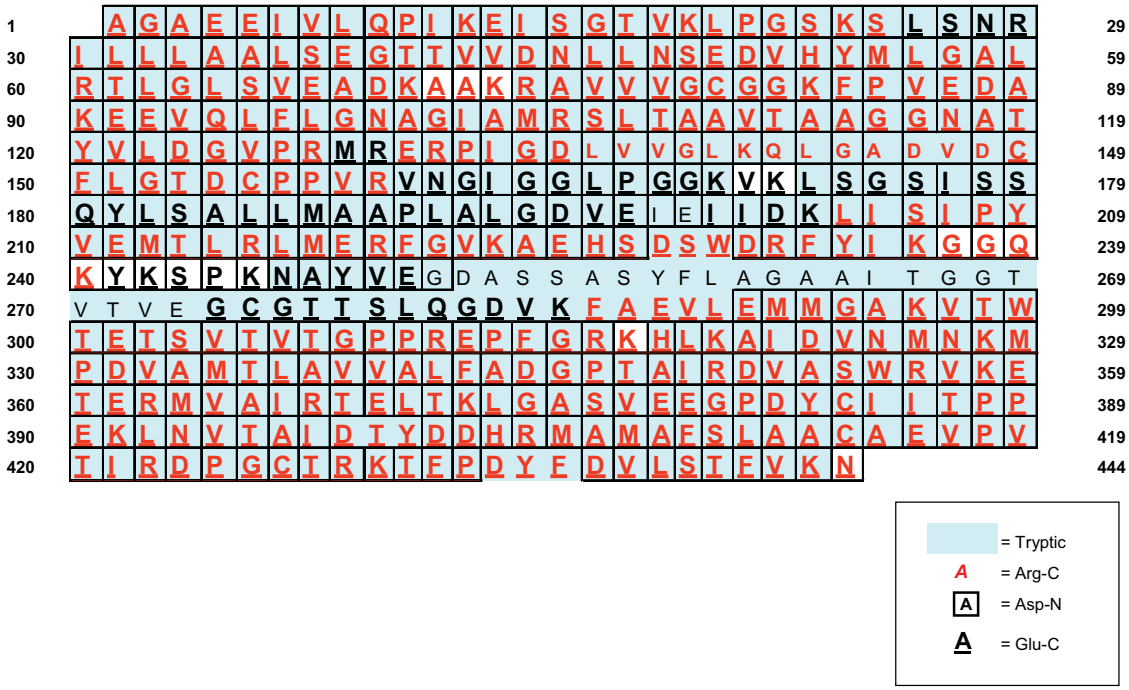

= Tryptic
A = Arg-C
A = Asp-N
A = Glu-C

Figure 3. Theoretical amino acid sequence of 2m-EPSPS, with Tryptic, Arg-C, Asp-N, and Glu-C fragments detected by mass-spectrometry highlighted. Sequence coverage is complete (100%). The corresponding observed proteolytic fragments are listed in **Tables 1** through **4**.

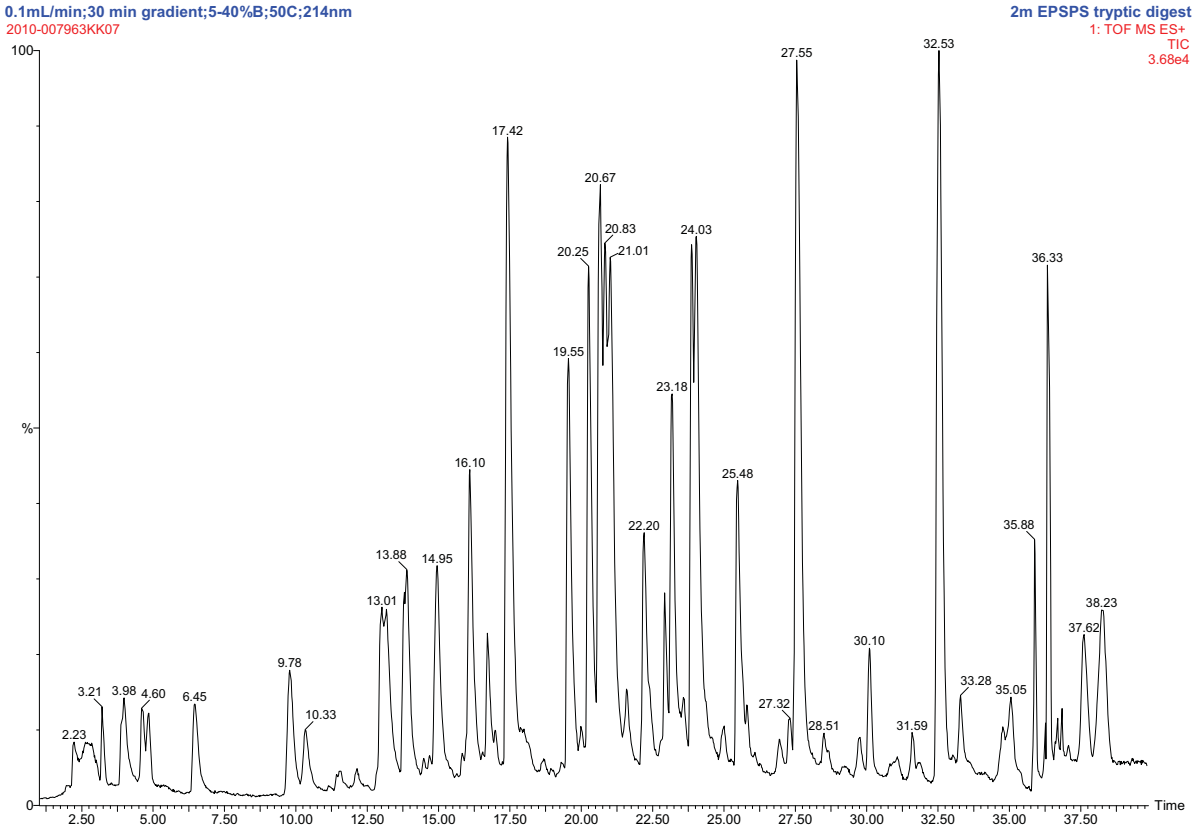

Figure 4. LC/MS chromatogram (TIC) of 2m-EPSPS Tryptic digest. The corresponding observed proteolytic fragments are listed in **Table 1**.

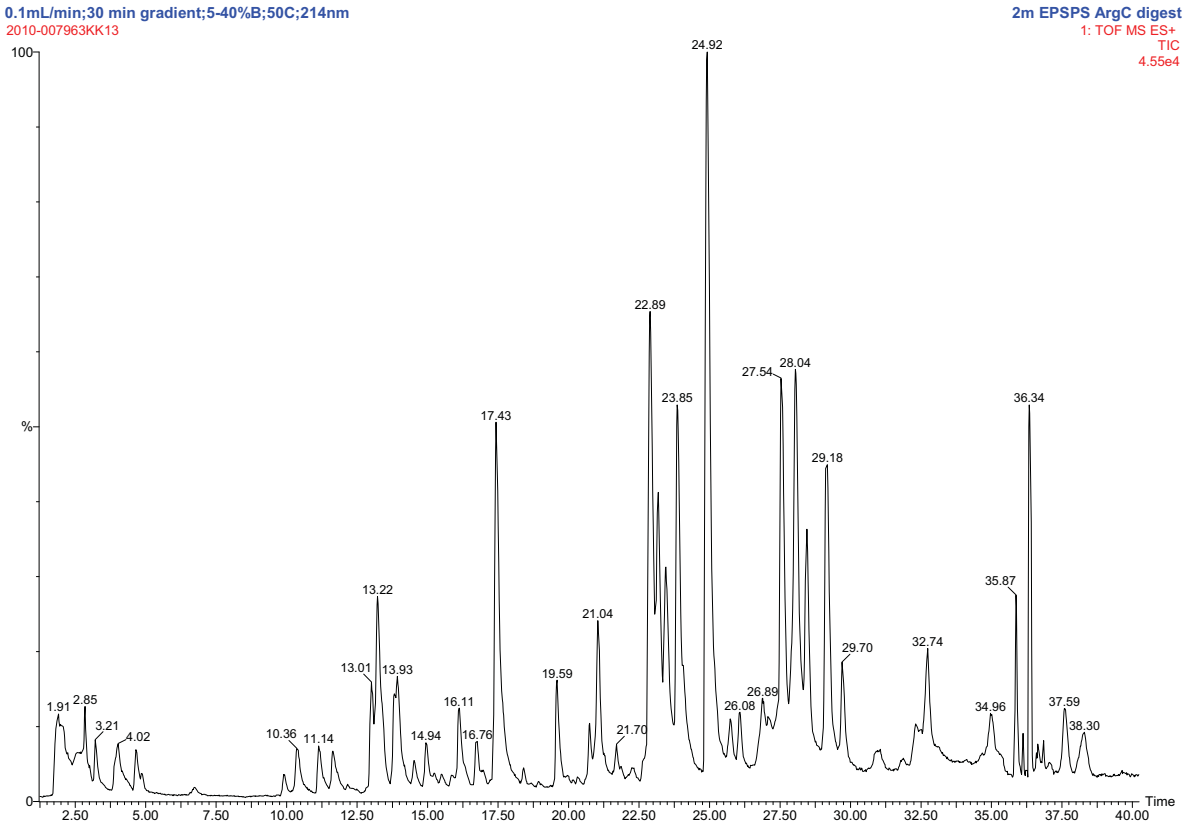

Figure 5. LC/MS chromatogram (TIC) of 2m-EPSPS Arg-C digest. The corresponding observed proteolytic fragments are listed in **Table 2**.

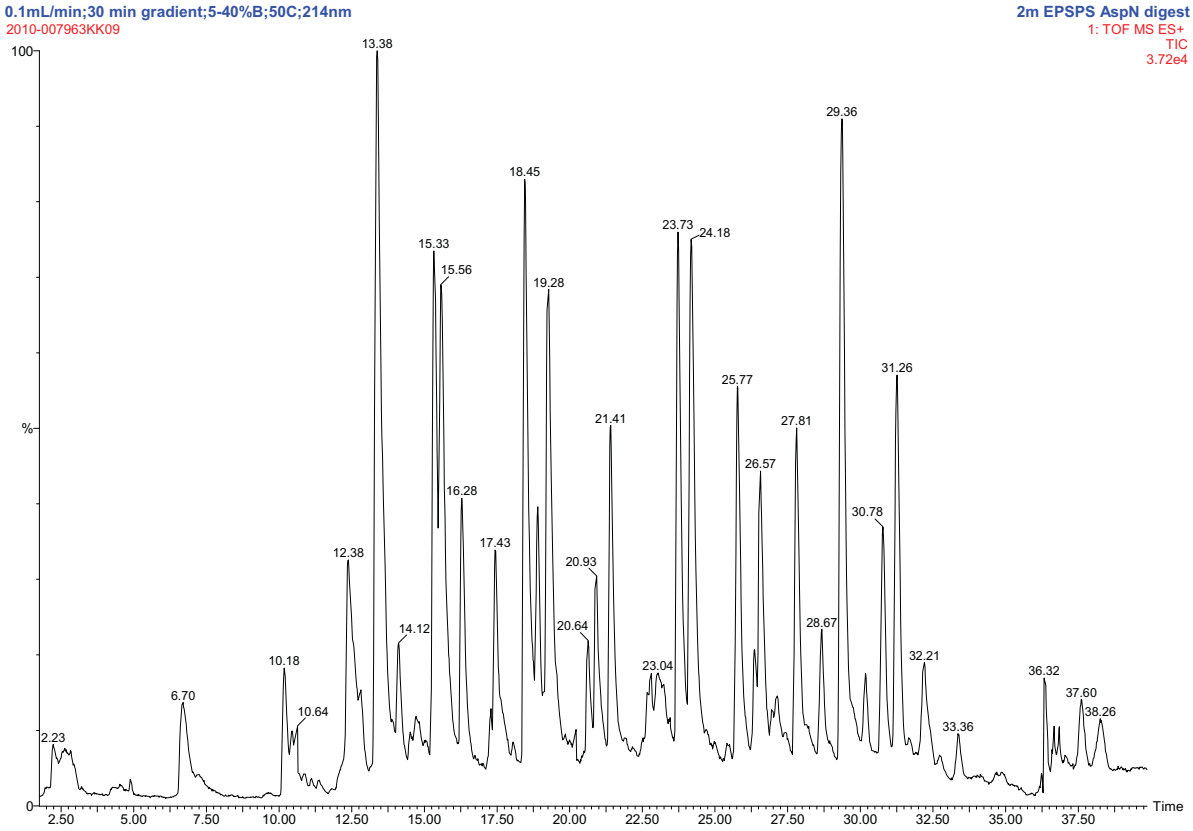

Figure 6. LC/MS chromatogram (TIC) of 2m-EPSPS Asp-N digest. The corresponding observed proteolytic fragments are listed in **Table 3**.

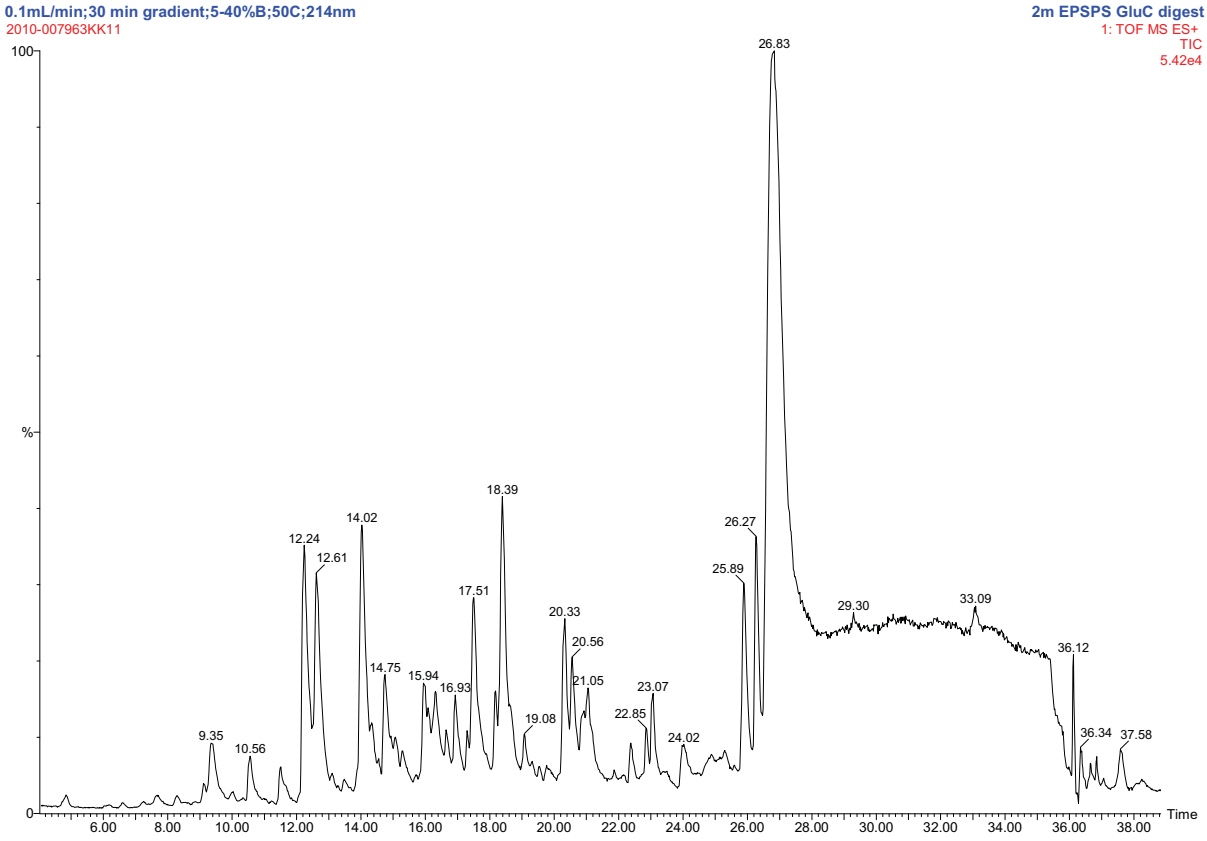

Figure 7. LC/MS chromatogram (TIC) of 2m-EPSPS Glu-C (pH8) digest. The corresponding observed proteolytic fragments are listed in **Table 4**.

ACCESSION #: 2010003338

Study Director: Karnoup, Anton (AS) (U369292)

****

Study: 2010-007963

Date Result Required: 6/30/2010
Client Project: Other R&D Support - DOW AGROSCIENCES
Charge Number: 83941000; Strain and Process Improvements; 010/16/YF
Description: Detailed characterization of 2mEPSPS with TIPS mut
Client:
Schafer, Barry (BW) (U097380)

Sample Data:
Sample-174582: 2010-007963-001
Required Date: 6/30/2010
Description:
Operator: Karnoup, Anton (AS) (U369292)

Aliquot Data:
Aliquot-146226: 2010-007963-001-001
Description: 2mEPSPS -TIPS mutation
Operator: Karnoup, Anton (AS) (U369292)

Test Data:
Test-
Operator:
Description:
Method or SOP Ref:
Method or SOP Ref:
Aliquot-146227: 2010-007963-001-002
Description: 2mEPSPS -TIPS mutation
Operator: Kuppannan, Krishna (K) (U386368)

Test Data:
Test-
Operator:
Description:
Method or SOP Ref:
Method or SOP Ref:

APPENDIX 5

Mass Spectrometry Analysis of Soybean-Derived 2mEPSPS Protein (Event DAS-444Ø6-6)

Mass Spectrometry Report Dow AgroSciences LLC		
Department	Geographic Location	
Biotechnology Regulatory Sciences	Indianapolis	
Page Count	Protocol Study Number	Report Status
22	101707	Final
Title		
Characterization of 2mEPSPS partially purified from soybean event DAS-44406-6		
Author(s): Last Name and Initials (Master Numbers)	Author(s) Signature / Date	
Harpham, Nicholas (NJ) (U407575)	20-JULY-2011	
Reviewer(s): Last Name and Initials (Master Numbers)	Reviewer Signature(s)/Date	
Singletary, Linda (L) (U411575) Karnoup, Anton (AS) (U369292) Schafer, Barry (BW) (U097380)	Linda Singletary 20-Jul-11 AKarnoup 06/24/2011 20-JUL-2011	
Patent Status		
Disclosure Submitted	Case Filed	No Action Required
[ ]	[ ]	[x]

Descriptive Summary and Conclusions

A partially purified 2mEPSPS herbicide tolerance protein from soybean event DAS-44406-6 was submitted as a band on a Coomassie stained gel by Brian Barnett, on behalf of Barry Schafer of Dow AgroSciences Biotechnology Regulatory Sciences Group for characterization. Comprehensive peptide mapping, analysis of N- and C-terminal sequences and characterization of possible post-translational modifications by MALDI MS, and MALDI MS-MS were requested. The quantity of SDS-PAGE separated 2mEPSPS protein submitted was approximately 500 ng per band. In addition a liquid sample was submitted for in-solution analysis. This sample was estimated to contain 0.05 mg/mL of protein.

The sequence coverage obtained from a tryptic digest of the 2mEPSPS (DAS-44406-6) protein analyzed by MALDI Peptide Mass Fingerprint (PMF) MS measurements was 57.2% and coverage by MALDI MS-MS measurements was 43.7%. The sequence coverage obtained from a chymotryptic digest of the 2mEPSPS protein analyzed by MALDI PMF MS measurements was 69.8% and coverage by MALDI MS-MS measurements was 48.9%. The combined MALDI PMF MS sequence coverage obtained from all methods of measurement was 86.3% with MS-MS data covering 70.0% of the peptides in the protein sequence. The methods used included MALDI-TOF MS and MALDI-TOF MS-MS of in-gel and in-solution tryptic and chymotryptic digests of the 2mEPSPS protein.

The N-terminal peptide ^{1}AGAEEIVL^{8} (from the chymotryptic digest) were determined to be present, as was the N-terminal peptide ^{1}AGAEEIVLQPIKEISGTVKLPGSKSL^{26} which derives from a single missed cleavage in the chymotryptic digest. These peptides exhibited collision induced dissociation fragmentation patterns (in MALDI TOF-TOF MS-MS) that were consistent with their expected theoretical amino acid sequences.

The C-terminal fragment ^{442}VKN^{444} from the chymotryptic digest was determined to be present, as was the C-terminal peptide ^{439}STFVKN^{444} which derives from a single missed cleavage in the chymotryptic digest. In addition the C-terminal peptide ^{429}KTFPDYFDVLSTFVKN^{444} from the tryptic digest was determined to be present. All peptides were confirmed by MALDI TOF-TOF MS-MS following collision induced dissociation.

No other post-translational or in-process modifications were detected for the 2mEPSPS protein.

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INTRODUCTION

A sample of partially purified in-solution and SDS-PAGE resolved 2mEPSPS herbicide tolerance protein (DAS-44406-6) was submitted by Brian Barnett, on behalf of Barry Schafer, of Dow AgroSciences for characterization. Analytical data on peptide mass fingerprinting and N- and C-terminal sequencing was requested.

EXPERIMENTAL

In-solution protein processing and enzymatic digests:

Equipment:

a) AB Sciex 4800 MALDI ToF-ToF
b) Fisher Scientific Dual channel pH/Ion meter
c) Mettler AT200 analytical balance
d) Eppendorf Centrifuge, Model 5417R5D
e) Eppendorf, Thermomixer R
f) Centrifugal evaporator (Jouan Speed Vac)
g) Gilson and Eppendorf adjustable pipettes
h) Siliconized microcentrifuge tubes, 0.5 and 1.5mL, (Fisher)
i) Zeba Spin Columns, 2mL (Thermo Scientific)

Reagents and Standards:

1. Acetonitrile, Fluka (LC-MS grade)
2. Ammonium bicarbonate, (Sigma)
3. Dithiothreitol (DTT), GE Healthcare
4. Iodoacetamide (IAA), GE Healthcare
5. Trypsin, (Roche)
6. Chymotrypsin, (Roche)
7. Formic Acid (FA), Fluka
8. Trifluoroacetic Acid (TFA), Pierce
9. Milli-Q deionized water (freshly drawn)
10. 10N Sodium Hydroxide (Fluka)
11. Guanidine hydrochloride (Sigma)

Reagent Solution Preparation:

a. 25 mM Ammonium Bicarbonate buffer: dissolved 98.8 mg NH_4HCO_3 in 50 mL of Milli-Q water; filtered through 0.22 μm sterile syringe filter.
b. 100 mM Tris buffer: was prepared from liquid stock and filtered through 0.22 μm sterile syringe filter.
c. Protein dissolution buffer (6M guanidine hydrochloride (Gu:HCl), 400 mM ammonium bicarbonate, pH 7.8): to 948 mg of ammonium bicarbonate, 22.5 mL of 6M Gu:HCl solution and 7.5 mL of water were added. pH was adjusted to 7.8 with NaOH. Buffer was filtered through 0.22 μm sterile syringe filter.
d. DTT solution (100 mM; prepared fresh): dissolved 15.4 mg DTT in 1 mL of water.
e. Alkylating reagent (IAA) (200 mM; prepared fresh): dissolved 37 mg IAA in 1 mL of water.
f. Trypsin solution: Dissolved 25 μg of dried trypsin in 400 μL of 25 mM NH_4CO_3 buffer immediately prior to digestion procedure.
g. Chymotrypsin solution: Dissolved 25 μg of dried chymotrypsin in 400 μL of 1 mM HCl immediately prior to digestion procedure.

In-solution protein processing procedure (Reduction/ alkylation/ digestion):

a. Original 2mEPSPS protein solution (~50 μL) was dispensed in a 0.5 mL siliconized microcentrifuge tube and dried in a centrifugal evaporator to completeness.
b. Reduction and carboxyamidomethylation (alkylation) of protein: approximately 100 μL of protein dissolution buffer, 6 M Gu:HCl/ 0.4 M ammonium bicarbonate, pH 7.8, was added to the dry 2mEPSPS sample, and the sample was mixed by pipette action. Sample reduction was achieved by adding 11.1 μL of 100 mM DTT (reducing reagent) solution to the microfuge tube. The microfuge tube was sealed, vortexed, and incubated at 65 ºC for 30 min in a thermomixer at 1100 rpm. The microfuge tube was cooled to room temperature, centrifuged for 30 seconds and 22.2 μL of 200 mM IOA (alkylating reagent) solution was added to the tube. The microfuge tube was incubated in the dark at room temperature for 1 hour. In order to quench the IOA 44.5 μL of DTT solution was added to consume unreacted IAA and the tube was allowed to incubate for 20 min at room temperature.
c. Desalting of the reduced/alkylated protein sample was performed using a 2 mL Zeba Spin columns (Thermo Scientific) as per the manufacturer's procedure. Zeba Spin columns were pre-equilibrated with 100 mM Tris buffer, pH 8.0, and protein elution was performed with the same buffer. The reduced and alkylated protein sample was made up to 350 μL with 172 μL of 100 mM Tris buffer pH 8.0 prior to application on the column.

d. After desalting the samples were dried in a centrifugal evaporator for 30 minutes, resuspended in 50 μL of 100 mM Tris buffer pH8.0 and then split into two 25 μL fractions in separate tubes to be digested with various enzymes.
e. In-solution Tryptic digestion of reduced/alkylated protein:5 μL of trypsin solution (a 1:5 enzyme:substrate weight ratio) was added to the 325 μL of reduced/alkylated protein 2mEPSPS sample in 100 mM Tris buffer, pH8.0. The digest was incubated for 16 hours (overnight) at 37 ºC in a thermomixer at 900 rpm.
f. In-solution Chymotryptic digestion of reduced/alkylated protein: 5 μL of chymotrypsin solution (a 1:5 enzyme:substrate weight ratio) was added to the 325 μL of reduced/alkylated protein 2mEPSPS sample. The digest was incubated for 16 hours (overnight) at 25 ºC in a thermomixer at 900 rpm.
g. Prior to mass-spectrometry analyses, digested samples were concentrated in a centrifugal evaporator: dried to completeness and reconstituted in 25 μL of 0.1% aqueous TFA. The samples were centrifuged, purified for MALDI MS analysis using C18 zip-tips (procedure is described separately under "*MALDI-TOF MS analysis of 2mEPSPS proteolytic digests*").

In-gel protein processing and enzymatic digests:

In-gel Protein Processing Procedure:

a. For each enzyme digestion SDS-PAGE bands at ~ 47 kDa corresponding to 2mEPSPS protein (approximately 500 ng) were excised with a sterile scalpel from the gel, and processed as follows (a blank section of the gel at approximately the same height in the gel was processed alongside with the protein sample, in a separate tube, using identical procedure):
b. Gel pieces were crushed with a sterile micro-pestle in a siliconized microcentrifuge tube, and destained as follows: 0.4 mL of 50% ACN and 0.4 mL of ammonium bicarbonate buffer were added, the tube was sealed and shaken at room temperature for 30 min in a Thermomixer R at 1100 rpm; the tube was centrifuged to settle the gel pieces, and the supernatant was removed with a pipette tip and discarded; the destain procedure was repeated 3 times.
c. The protein was reduced and alkylated in-gel as follows: (1) 0.2 mL of DTT solution was added to gel pieces, and the tube was incubated at room temperature for 30 min in a Thermomixer R; (2) the tube was centrifuged, and the DTT solution was removed by a pipette tip; (3) the gel pieces were washed with 0.5 mL of 25 mM ammonium bicarbonate buffer, the tube was centrifuged, and the buffer was removed; (4) 0.2 mL of IAA solution was added to the gel pieces, and the tube was incubated in darkness at ambient

temperature for 1 hour; (5) the gel pieces were washed twice with 0.5 mL of 25 mM ammonium bicarbonate buffer (the tube was centrifuged, and the buffer was removed after each wash).

d. After the destaining/ reduction/ alkylation procedures, the gel pieces were dried in a centrifugal evaporator for 30 min.
e. In-gel digestion with Trypsin: the dried gel pieces were re-hydrated with trypsin solution (25 μg in 400 μL of 25 mM ammonium bicarbonate buffer, pH 7.8; prepared fresh) and incubated in a Thermomixer R at 900 rpm and 37 ºC for 16 hours (overnight).
f. In-gel digestion with Chymotrypsin: the dried gel pieces were re-hydrated with chymotrypsin solution (25 μg in 400 μL of 1mM HCl; prepared fresh) and incubated in a Thermomixer R at 900 rpm and 25 ºC for 16 hours (overnight).
g. Extraction of peptides from gel: peptides were extracted sequentially with 0.4 mL of 50% ACN/ 0.1% TFA, then 0.4 mL of 50% ACN/ 5% FA, and then 0.4 mL of 75% ACN/ 5% FA (30 min per extraction; in a Thermomixer R at room temperature, shaking at 1100 rpm). The extracts for each sample were combined and dried in a centrifugal evaporator.
h. Dried peptides were reconstituted in 25 μL of 0.1% TFA and were purified for MALDI MS analysis using C18 zip-tips, as described below.

MALDI-TOF MS analysis of 2mEPSPS proteolytic digests:

Reagents and Standards:

1. Trifluoroacetic Acid (TFA), Pierce
2. Deionized water, 18.2 MΩ/cm, MilliQ, Millipore, freshly drawn
3. Acetonitrile, Fluka (LC-MS grade)
4. 4700 Proteomics Analyzer Mass Standards Kit, (ABSciex)
5. CHCA (α-Cyano-4-hydroxycinnamic acid), Sigma

Analytical Procedure:

Sample preparation and deposition: Tryptic (in-gel and in-solution digest), and Chymotryptic (in-gel and in-solution digest) peptides were purified using Millipore C18 zip-tips as shown on the relevant run sheet. Purified peptides were eluted directly onto MALDI plate, sequentially, with aqueous 10%, 25%, 50%, and 70% ACN (supplemented with 0.1% TFA). The C18 zip-tip fractions were mixed on-plate with 1 μL of CHCA matrix and air-dried.

MALDI-TOF MS: The sample preparations were analyzed directly by MALDI-TOF mass spectrometry. All mass spectra were acquired on an AB Sciex 4800 MALDI-TOF-TOF mass spectrometer (S/N AK011030605H). Mass calibration was performed with peptide standards utilizing Applied Biosystems 4700 Proteomics Analyzer Mass Standards Kit, consisting of the calibration mixture (monoisotopic $(M+H)^+$

m/z values used): des-Arg[1]-Bradykinin, m/z 904.4681; Angiotensin I, m/z 1296.6853; Glu[1]-Fibrinopeptide B, m/z 1570.6774; ACTH (fragment 1-17), m/z 2093.8532; ACTH (fragment 18-39), m/z 2465.1989; ACTH (fragment 7-38) m/z 3657.9294. The plate wide calibration model was used for MS calibration.

Peak matching for plate wide calibration were as follows;

Minimum signal to noise set to 10
Mass tolerance ± 0.3 m/z
Minimum peaks to match set to 5
Max outlier error set to 10 ppm
Only monoisotopic peaks were used
Weighted fit was set to S/N

The following mass spectrometer settings were used for MS spectral acquisitions:

Mode of operation: reflector
Extraction mode: delayed
Polarity: positive
Acquisition control: manual
Accelerating voltage: 20000 V
Grid 1 voltage 16000 V
Source Lens voltage 10000 V
Lens 1 voltage 4000
Grid to Source 1 voltage ratio: 0.8000
Mirror 2 to Mirrior 1 voltage ratio: 1.4639
Mirror 2 to Source 1 voltage ratio: 1.1200
Extraction delay time: 390 nsec
Acquisition mass range: 300-6000 Da (in segments 300 – 1000, 600 – 4000 Da, and 1900 – 6000 Da)
Number of laser shots: 400-500/spectrum
Laser intensity: 2700 - 3200
Calibration matrix: α-cyano-4-hydroxycinnamic acid (CHCA)
Timed ion selector: enabled
Bin size: 0.5 nsec
Vertical scale: 0.50 mV
Vertical offset: 0.00%
Input bandwidth: 500 MHz

A total of 3 to 6 spectra were summed per MS analysis and the following settings used to process the spectra;

Minimum signal to noise filter of 20
Local noise window width set to 250
Min peak width at full width half max (bins) set to 2.9

MALDI-TOF-TOF MS-MS: The sample preparations were analyzed directly by MALDI-TOF-TOF tandem mass spectrometry. All mass spectra were acquired on an AB Sciex 4800 MALDI-TOF-TOF mass spectrometer (S/N AK011030605H). Mass calibration was performed with peptide standards utilizing Applied Biosystems 4700 Proteomics Analyzer Mass Standards Kit, consisting of the calibration mixture (monoisotopic $(M+H)^+$ m/z values used): des-Arg[1]-Bradykinin, m/z 904.4681; Angiotensin I, m/z 1296.6853; Glu[1]-Fibrinopeptide B, m/z 1570.6774; ACTH (fragment 1-17), m/z 2093.0867; ACTH (fragment 18-39), m/z 2465.1989; ACTH (fragment 7-38) m/z 3657.9294. For MS-MS calibration b- and

y-ions derived from the Angiotensin I peptide were used to produce the calibration values for MS-MS data. The following is a list of the monoisitopic (M+H)+ m/z values used in MS-MS calibration; His immonium ion, m/z 110.072; y2, m/z 269.161; b2, m/z 272.136; y3, m/z 416.230; y4, m/z 513.283; b4-17, m/z 517.241; y5, m/z 650.341; b6-17 m/z 767.384; b6, m/z 784.411; b8+H2O, m/z 1046.542; b9, m/z 1165.591; y9, m/z 1181.658; y9+H2O, 1183.601. Peak matching for MS-MS calibration were as follows;

Minimum signal to noise set to 7
Mass tolerance ± 0.3 m/z
Minimum peaks to match set to 5
Max outlier error set to 0.05 m/z
Only monoisotopic peaks were used
Weighted fit was set to S/N

Masses obtained from the MS experiment were used to manually set the parent mass to acquire MS-MS data. The following mass spectrometer settings were used for MS-MS spectral acquisitions:

Mode of operation: reflector
Extraction mode: delayed
Polarity: positive
Acquisition control: manual
Accelerating voltage: 8000 V
Grid 1 voltage 7300 V
Source 1 focus voltage 4200 V
Source 1 Lens voltage 3200
Lens 1 voltage 2700
Deceleration stack voltage 6300
Collision cell offset -40 V
Source 2 voltage 15000 V
Lens 3 voltage 4000 V
Mirror 2 voltage 18000 V
Reflector detector voltage 2190 V
Combined precursor 1650 V
Grid to Source 1 voltage ratio: 0.9189
Mirror 2 to Mirror 1 voltage ratio: 1.7192
Mirror 2 to Source 2 voltage ratio: 1.2000
Number of laser shots: 400-500/spectrum

A total of 3 to 6 spectra were summed per MS analysis and the following settings used to process the spectra;

Minimum signal to noise filter of 20
Local noise window width set to 250
Min peak width at full width half max (bins) set to 2.9

The mass-spectra of proteolytic fragments were analyzed using Applied Biosystems Data Explorer v 4.9 (Build 115) and GPMAW v. 9.02 (Lighthouse Data) software.

RESULTS AND DISCUSSION

In this study, the numbering of the amino acid residues is in accordance with the theoretical sequence of the recombinant 2mEPSPS protein starting with Ala^{1} and containing a total of 444 residues.

Band Excision:

The purified 2mEPSPS (DAS-44406-6) was analyzed by SDS-PAGE in order to confirm sample integrity, and to estimate protein quantity (needed for subsequent digest preparations). The annotated SDS-PAGE gel image is presented in **Figure 2** and the post excision gel is shown in **Figure 3**.

Peptide Mass Fingerprinting and MS-MS analysis:

In order to generate a peptide (sequence) coverage map, N-terminal and C-terminal sequences, and examine possible presence of post-translational modifications, in-gel and in-solution trypsin and chymotrypsin digests of reduced and alkylated (carboxyamidomethylated) 2mEPSPS (DAS-44406-6) were generated and analyzed by MALDI-TOF MS and MALDI TOF-TOF MS-MS. A summary of the mass spectral data of in-gel digested 2mEPSPS (DAS-44406-6) with assignments from MALDI-TOF MS and MALDI TOF-TOF MS-MS analyses are presented in **Table I** and **Table II** for the tryptic and chymotryptic digests, respectively. The peptide mass fingerprint (PMF) sequence coverage for the tryptic digest was 57.2% (**Figure 4**) and the PMF coverage for the chymotryptic digest was 69.8% (**Figure 5**). The combined PMF sequence coverage from both analyses is extensive at 86.3% (**Figure 6**).

Most peptides observed in the proteolytic digests of 2mEPSPS (DAS-44406-6) exhibited collision induced fragmentation patterns (MALDI TOF-TOF, MS-MS) consistent with their expected theoretical amino acid sequences. Tandem MS analysis of the PMF ions showed MS-MS sequence coverage for the tryptic digest was 43.7% (**Figure 4**) and the MS-MS coverage for the chymotryptic digest was 48.9% (**Figure 5**). The combined MS-MS sequence coverage from both analyses was extensive at 70.0% (**Figure 6**).

The N-terminal peptide ^{1}AGAEEIVL^{8} (from the chymotryptic digest) was determined to be present, as was the N-terminal peptide ^{1}AGAEEIVLQPIKEISGTVKLPGSKSL^{26}, which derives from a single missed cleavage in the chymotryptic digest. These peptides exhibited collision induced dissociation fragmentation patterns (in MALDI TOF-TOF MS-MS) that were consistent with their expected theoretical amino acid sequences (**Table III** and **Table IV)**.

The C-terminal fragment ^{442}VKN^{444} from the chymotryptic digest was determined to be present, as was the C-terminal peptide ^{439}STFVKN^{444}, which derives from a single missed cleavage in the chymotryptic digest. In addition, the C-terminal peptide ^{429}KTFPDYFDVLSTFVKN^{444} from the tryptic digest was determined to be present. Amino acid sequences for all of these peptides were confirmed by MALDI TOF-TOF MS-MS following collision induced dissociation **(Table V** and **Table VI)**.

Table I Tryptic digest coverage map of 2mEPSPS (DAS-44406-6): Proteolytic fragments observed by MALDI-TOF MS and MALDI TOF-TOF MS-MS from in-gel and in-solution tryptic digests.

Fragment #	# of missed cleavages	Start	End	Sequence	Charged State	Theor. mono. m/z	% ACN (elution from C18 zt; MALDI)	Observed m/z MALDI-MS	Comment	Mass range identifier	Base peak intensty	Mass difference (ppm)	MALDI MS-MS data (all fragments are in a singularly charged)
T1	0	1	12	AGAEEIVLQPIK	1	1267.7256		ND					
T2	0	13	19	EISGTVK	1	733.4090	**10%**, 25%	733.4232	In-gel detected with T30-31, T35	001	97	-19	b1=130.10, b5=488.30, b6=587.34, y2=246.18, y6=604.37
T10	0	84	90	FPVEDAK	1	805.4090	**10%**, 25%, 50%, 70%	805.4829	**In-gel,** in-sol	**001**, 002	422	-92	b2=245.13, b4=473.26, b5=588.32, y1=147.10, y2=218.15, y3=333.19, y4=462.25, y5=561.32, y6=658.31
T11	0	91	105	EEVQLFLGNAGIAMR	1	1647.8523	25%, **50%,** 70%	1647.9211	in-gel	001	42	-42	b2=259.09, b3=358.17, b4=486.22, b5=599.32, b6=746.44, b7=859.52, b8=916.55, b10=1101.78, y1=175.10, y3=377.19, y4=490.28, y5=547.32, y6=618.37, y8=789.46, y9=902.57, y10=1049.68, y11=1162.84, y12=1290.84, y13=1389.93, y14=1518.91
T12	0	106	127	SLTAAVTAAGGNATYVLDGVPR	1	2104.1033	**25%,** 50%, 70%	2104.2361	**in-gel,** in-sol	**001,** 003	79	-63	b1=210.12, b3=302.17, b5=444.23, b6=543.28, b7=644.34, b10=843.46, b13=1085.66, b17=1561.94, y1=175.11, y2=272.15, y3=371.20, y4=428.23, y5=543.28, y7=755.47, y8=918.59, y9=1019.62, y10=1090.67, y11=1204.74, y12=1261.77, y13=1318.81, y16=1460.95, y19=1803.03, y20=1904.01
T14	0	130	141	ERPIGDLVVGLK	1	1295.7682	**25%,** 70%	1295.8612	In-gel, in-sol	001	73	-72	b2=286.15, b3=383.22, b4=496.33, b6=668.39, b11=1149.73, y1=147.10, y3=317.25, y4=416.32, y5=515.39, y8=800.63, y10=1010.71
T15	0	142	159	QLGADVDCFLGTDCPPVR	1	2019.9263	25%, **50%,** 70%	2020.1023	**in-gel,** in-sol	**001,** 003	179	-87	b3=299.17, b5=485.23, b6=584.31, b7=699.43, b12=1277.73, b14=1552.89, y1=175.11, y4=468.28, y5=628.33, y6=743.40, y7=884.46, y8=901.50, y9=1014.58, y10=1161.69, y11=1321.77, y12=1436.79, y16=1779.02
T16	0	160	170	VNGIGGLPGGK	1	968.5524	25%	968.6297	In gel	001	37	-80	y4=358.21, y9=755.57
T19	0	204	215	LISIPYVEMTLR	1	1434.8025	**50%,** 70%	1434.8264	**in-gel,** in-sol	001	50	-17	b2=227.18, b3=314.21, b4=427.30, b6=687.40, b7=785.57, y1=175.12, y3=389.26, y4=520.33, y5=649.40, y6=748.48, y8=1008.66, y9=1121.76, y10=1208.80, y11=1321.85
T22	0	224	232	AEHSDSWDR	1	1102.4548	10%	1102.5530	In-gel	001	127	-89	b1=72.09, b2=201.11, b3=338.16, b4=425.20, b5=540.25, b6=627.30, b7=813.38, b8=928.41, y1=175.11, y2=290.15, y3=476.26, y4=563.30, y5=678.33, y6=765.37, y7=902.43, y8=1031.50
T24	0	237	240	GGQK	1	389.2143	**25%,** 50%	389.1750	in-sol	002	33	101	b1=58.08, b3=243.19, y1=147.13
T25	0	241	242	YK	1	310.1761	**10%,** 25%	310.1288	in-sol	002	59	152	b1=164.12, y1=147.11
T26	0	243	245	SPK	1	331.1976	10%, 25%, **50%**	331.2551	in-sol, **in-sol**	002	315	-174	b1=88.09, y1=147.08, y2=44.28
T29	0	297	311	VTWTETSVTVTGPPR	1	1630.8435	**25%,** 70%	1630.9565	in-gel	001	182	-69	b2=201.14, b4=488.29, b5=617.35, b6=718.41, b7=805.48, b8=904.62, b9=1005.60, b13=1359.94, y1=175.14, y3=369.24, y4=426.27, y5=527.33, y6=626.44, y7=727.48, y9=913.63, y10=1014.67, y11=1143.78, y12=1244.79, y13=1430.87
T30	0	312	316	EPFGR	1	605.3042	**10%**, 25%	605.3510	**in-gel**, in-sol	**001**, 002	272	-77	b2=227.13, b3=374.22, y1=175.15, y2=232.17, y3=379.26, y4=476.30
T32	0	318	320	HLK	1	397.2558	**10%,** 50%, 70%	397.2026	in-sol	002	38	134	b2=251.12, y1=147.09, y2=260.13
T34	0	329	350	MPDVAMTLAVVALFADGPTAIR	1	2259.1876	50%, **70%**	2259.3191	**in-gel**, in-sol	003	80	-58	
T35	0	351	356	DVASWR	1	733.3628	**10%**, 25%	733.4232	In-gel detected with T30-31, T2	**001**, 002	97	-82	b2=215.12, b3=286.16, b4=373.21, y6=175.13, y5=361.23, y4=519.31, y5=618.37
T39	0	368	372	TELTK	1	591.3348	10%	591.2098	in-sol	002	34	211	
T40	0	373	391	LGASVEEGPDYCIITPPEK	1	2075.0001	25%	2075.1357	in-gel	**001,** 003	56	-65	b3=242.13, b4=329.17, b5=428.33, b6=557.27, b8=743.35, b11=1118.55, b12=1278.61, b13=1391.71, b14=1504.76, b15=1605.82, b16=1702.80, y3=373.18, y4=470.24, y6=684.40, y7=797.50, y8=957.55, y9=1120.61, y11=1332.74, y13=1518.80, y14=1647.86
T41	0	392	404	LNVTAIDTYDDHR	1	1532.7340	10%, **25%,** 70%	1532.8400	**in-gel**, in-sol	001	104	-69	b1=86.11, y1=175.12, y2=312.18, y3=427.12, y4=542.27, y5=705.36, y6=806.41, y7=921.43, y8=1034.51, y9=1105.61, y10=1206.64, y11=1305.72
T45	0	430	443	TFPDYFDVLSTFVK	1	1678.8363	**50%,** 70%	1678.8497	in-gel	001	28	-8	b2=221.16, b3=318.13, y1=147.11, y2=246.20, y4=494.31, y5=581.37, y6=694.49, y7=793.58, y8=908.62, y9=1055.75, y10=1218.86, y12=1430.92
T7-T8	1	71	74	AAKR	1	445.2881	50%, **70%**	445.2899	in-sol	002	59	-4	b1=44.07, y1=175.13
T10-T11	1	84	105	FPVEDAKEEVQLFLGNAGIAMR	1	2434.2435	**50%,** 70%	2434.4431	in-gel	003	34	-82	b2=245.11, b3=344.15, b4=473.22, b5=588.24, b7=787.44, b8=916.45, b9=1045.58, b10=1144.63, b11=1272.68, b15=1702.31, y1=175.11, y2=306.15, y3=377.16, y5=547.28, y6=618.32, y7=732.38, y8=789.39, y9=902.53, y10=1049.65, y11=1162.73, y12=1290.84, y13=1389.93, y16=1776.21, y18=1962.31, y20=2190.34
T25-T26	1	241	245	YKSPK	1	622.3559	**10%**, 25%, 50%, 70%	622.0853	in-sol	002	195	435	b2=292.19, y1=147.09, y2=244.05, y3=331.19
T29-T30	1	297	316	VTWTETSVTVTGPPREPFGR	1	2217.1299	50%, **70%**	2217.1123	in-gel	001, **003**	100	8	b2=201.13, b9=1005.66, y3=379.09, y12=1313.69
T30-T31	1	312	317	EPFGRK	1	733.3991	**10%**, 25%	733.4232	In-gel detected with T2, T35	001	97	-33	b1=130.10, b4=431.25, b5=587.34, y5=604.37
T44-T45	1	429	443	KTFPDYFDVLSTFVK	1	1806.9313	70%	1806.9402	**in-gel,** in-sol	001	34	-5	b1=129.12, b6=752.40, b8=1014.67, b9=1113.72, b10=1226.83, b11=1313.91, b12=1414.90, b13=1562.08, y2=246.18, y5=581.42, y6=694.51, y7=793.61, y8=908.64, y9=1055.79, y12=1430.67
T12-T14	2	106	141	SLTAAVTAAGGNATYVLDGVPRMRERPIGDLVVGLK	1	3667.9952	25%, **70%**	3667.8367	In-gel	003	46	43	y3=317.13, y11=1166.18
T30-32	2	312	320	EPFGRKHLK	1	1111.6371	10%, **25%,** 50%	1111.6373	In-gel	001	137	0	b1=130.09, b2=227.12, b6=715.35, b7=852.45, b8=965.58, y1=147.12, y2=260.21, y5=681.34, y7=885.58
T35-T37	2	351	362	DVASWRVKETER	1	1475.7601	25%	1475.8564	In-gel	001	45	-65	
T37-T39	2	359	372	ETERMVAIRTELTK	1	1676.9000	70%	1676.7732	in-gel	001	30	76	
T44-T46	2	429	444	KTFPDYFDVLSTFVKN	1	1920.9742	50%	1921.0364	in-sol	003	38	-32	b6=752.48, b7=899.50, b8=1014.56, b9=1113.68, b10=1226.86, y2=261.17, y6=695.43, y8=907.65, y9=1022.72, y11=1332.95
T22-T25	3	224	242	AEHSDSWDRFYIKGGQKYK	1	2315.1204	70%	2315.2966	in-gel	001	53	-76	
T43-T46	3	423	444	DPGCTRKTFPDYFDVLSTFVKN	1	2607.2548	70%	2607.3967	in-gel	003	57	-54	
T35-T39	4	351	372	DVASWRVKETERMVAIRTELTK	1	2618.4083	70%	2618.3362	in-gel	001	36	28	

Cys residues were alkylated with Iodoacetamide. Background and auto-proteolytic peaks were removed. ND = not detected.

Table II Chymotryptic digest coverage map of 2mEPSPS (DAS-44406-6): Proteolytic fragments observed by MALDI-TOF MS and MALDI TOF-TOF MS-MS from in-gel and in-solution chymotryptic digests.

Fragment #	# of missed cleavages	Start	End	Sequence	Charged State	Theor. mono. m/z	% ACN (elution from C18-zt; MALDI)	Observed m/z MALDI-MS	Comment	Mass range identifier	Base peak intensty	Mass difference (ppm)	MALDI MS-MS data (all fragments are in a singularly charged)
Y1	0	1	8	AGAEEIVL	1	801.4353	**25%,** 50%	801.4942	**in-gel,** in-sol	**001,** 002	53	-73	b1=72.10, b2=129.11, b7=670.44
Y3	0	27	31	SNRIL	1	602.3620	**25%,** 50%	602.3719	**in-gel,** in-sol	001, **002**	126	-16	b2=202.14, b4=471.35, y1=132.11, y3=401.34
Y9	0	48	54	NSEDVHY	1	863.3530	10%	863.4109	in-gel	**001,** 002	68	-67	b1=115.09, b2=202.11, b3=331.18, b4=446.22, b5=545.31, b6=682.38, y2=319.18, y3=418.28, y4=533.32, y5=662.36, y6=749.39
Y20	0	123	136	DGVPRMRERPIGDL	1	1610.8431	25%	1610.8142	in-gel	001	37	18	
Y21	0	137	140	VVGL	1	387.2602	50%	387.1909	in-gel; detected with Y5-6	002	113	179	b1=100.09, b3=256.30, y3=288.33
Y23	0	144	150	GADVDCF	1	783.2978	**10%,** 25%	783.3431	in-gel	**001,** 002	134	-58	b2=129.10, b3=244.14, b4=343.22, b5=458.27, b6=618.33, y2=326.17, y3=441.23, y4=540.33, y5=655.35
Y25	0	152	173	GTDCPPVRVNGIGGLPGGKVKL	1	2191.2016	25%	2191.2493	**in-gel,** in-sol	002	41	-22	
Y26	0	174	181	SGSISSQY	1	828.3734	10%, 25%, **50%**	828.4150	in-gel; detected with Y3-5 and Y63-65	**001,** 002	35	-50	b3=232.16, y7=741.53
Y30	0	187	191	MAAPL	1	502.2694	25%, **50%**	502.3544	in-gel	002	50	-169	b3=272.21
Y37	0	221	230	GVKAEHSDSW	1	1115.5116	10%	1115.5928	in-gel	001	32	-73	b4=356.27, b5=485.36, b6=622.42, b7=709.48, b8=824.51, b9=911.55, y6=631.35, y7=760.42
Y38	0	231	233	DRF	1	437.2143	**10%,** 25%, 50%	437.1647	**in-gel,** in-sol	002	122	113	b1=116.09, b2=271.22, y1=166.14, y2=322.24
Y40	0	235	241	IKGGQKY	1	793.4567	10%, **25%**	793.3873	**in-gel,** in-sol	002	50	87	b1=114.11, b2=242.16, b3=299.19, b6=612.43, y5=552.31, y6=680.43
Y41	0	242	248	KSPKNAY	1	807.4359	10%	807.4839	in-gel	**001,** 002	35	-59	b1=129.12, b3=313.24, b4=441.36, b5=555.42, y2=253.18, y5=592.41, y6=679.44
Y46	0	281	286	QGDVKF	1	693.3566	10%	693.2233	**in-gel,** in-sol	**001,** 002	41	192	b1=129.12, b3=301.16, y1=166.11, y2=294.20, y4=508.30
Y49	0	300	314	TETSVTVTGPPREPF	1	1617.8119	**25%,** 50%	1617.8801	in-sol	001	168	-42	b2=231.12, b3=332.19, b4=419.22, b5=518.32, b13=1355.94, b14=1453.01, y2=263.17, y6=742.53, y7=799.59, y8=900.61, y9=999.77, y10=1100.77, y11=1199.91, y12=1286.97, y13=1387.99
Y54	0	343	355	ADGPTAIRDVASW	1	1358.6699	25%	1358.7408	**in-gel,** in-sol	001	129	-52	b1=72.10, b2=187.10, b3=244.13, b5=442.25, b6=513.27, b7=626.41, b8=782.54, b9=897.57, b12=1154.76, y7=846.57, y8=917.67, y10=1115.72, y12=1172.74
Y58	0	384	392	CIITPPEKL	1	1070.5914	25%	1070.5851	in-gel, detected with Y28-31	001	41	6	b5=585.42, b7=811.53, y5=583.43, y6=684.56
Y64	0	436	438	DVL	1	346.1973	50%	346.2659	in-sol	002	144	-198	b1=116.11, y1=132.11
Y66	0	442	444	VKN	1	360.2241	10%	360.2131	in-sol	002	109	31	b1=100.07, b2=228.14, y1=133.09, y2=261.14
Y1-2	1	1	26	AGAEEIVLQPIKEISGTVKLPGSKSL	1	2664.5182	50%	2664.5735	in-sol	003	37	-21	b1=72.10, b2=129.11, b4=329.19, b5=458.24, b6=571.32, b7=670.40, b8=783.51, b9=911.63, b18=1836.57, y20=2077.74, y1=132.34, y12=1173.75, y17=1754.54
Y3-4	1	27	32	SNRILL	1	715.4461	**50%,** 70%	715.4267	in-sol	001, **002**	45	27	b1=202.12, b3=358.26, b5=584.46, y1=132.10, y3=358.26, y4=514.45
Y5-6	1	33	36	LAAL	1	387.2602	50%	387.1909	in-gel; detected with Y21	002	113	179	b1=114.10, b3=256.30, y2=302.16
Y10-11	1	55	59	MLGAL	1	504.2850	25%	504.3478	in-gel	002	77	-125	b3=302.22, b4=373.26, y2=373.26
Y11-12	1	57	62	GALRTL	1	630.3933	10%, **25%,**	630.4132	**in-gel,** in-sol	**001,** 002	140	-32	b1=58.08, b3=242.29, y1=132.09, y2=233.12
Y21-22	1	137	143	VVGLKQL	1	756.4978	10%, **25%**	756.4637	in-gel	002	38	45	y2=260.16, y4=501.36
Y22-23	1	141	150	KQLGADVDCF	1	1152.5354	**25%,** 50%, 70%	1152.5848	**in-gel,** in-sol	001	904	-43	b1=129.12, b2=257.19, b3=370.29, b4=427.34, b5=498.40, b6=613.46, b7=712.55, b8=827.59, b9=987.62, y2=326.17, y7=783.47, y8=896.54, y9=1024.61
Y29-30	1	186	191	LMAAPL	1	615.3534	70%	615.3393	in-sol	001	39	23	
Y30-31	1	187	193	MAAPLAL	1	686.3906	70%	686.3797	in-sol	001	41	16	
Y31-32	1	192	204	ALGDVEIEIIDKL	1	1427.7992	**50%**, 70%	1427.8224	**in-gel,** in-sol	001	40	-16	b1=72.10, b2=185.114, b4=357.23, b5=456.28, b6=585.40, b7=698.41, b8=827.57, b9=940.73, b11=1168.77, y2=260.22, y5=601.50, y6=730.57, y7=843.71, y8=972.77, y9=1071.87, y11=1243.91
Y32-33	1	194	209	GDVEIEIIDKLISIPY	1	1816.9943	25%, **50%,** 70%	1816.8356	in-gel	001	94	87	
Y34-35	1	210	216	VEMTLRL	1	861.4862	25%	861.5051	in-gel	002	32	-22	b1=100.10, y1=132.09, y3=401.28
Y35-36	1	215	220	RLMERF	1	851.4556	10%, **25%**	851.4983	in-gel	**001,** 002	73	-50	b2=270.23, b3=401.27, b4=530.37, b5=686.47, y1=166.16, y2=322.23, y3=451.38, y4=582.40, y5=695.44
Y38-39	1	231	234	DRFY	1	600.2776	25%	600.3195	in-gel	002	65	-70	b1=272.21, y1=182.15, y3=485.34
Y39-40	1	234	241	YIKGGQKY	1	956.5200	10%	956.6207	in-gel, **in-sol**	**001,** 002	33	-105	b2=277.20, b4=462.37, b5=519.41, b6=647.50, b7=775.61, y2= 310.23, y3=428.35, y4=495.37, y5=552.40, y6=680.52, y7=793.60
Y57-58	1	374	392	GASVEEGPDYCIITPPEKL	1	2075.0001	50%	2075.0547	in-sol	003	61	-26	b2=129.10, b9=842.60, b11=1165.80, y6=684.47, y8=910.76, y9=1070.74, y10=1233.91, y16=1859.52
Y58-59	1	384	400	CIITPPEKLNVTAIDTY	1	1948.0096	**25%,** 70%	1948.0138	in-gel: deteced with Y35-37	001	47	-2	
Y65-66	1	439	444	STFVKN	1	695.3723	10%, **25%,** 70%	695.3156	**in-gel,** in-sol	001, **002**	139	82	b2=189.13, y1=133.09
Y3-5	2	27	33	SNRILLL	1	828.5302	10%, 25%, **50%**	828.4150	in-gel; detected with Y26 and Y63-65	**001,** 002	35	139	b4=471.36
Y7-9	2	37	54	SEGTTVVDNLLNSEDVHY	1	1991.9193	50%	1991.9684	in-sol	003	58	-25	b6=575.39, b9=903.54, b10=1016.68, y2=319.15, y3=418.25, y5=622.32, y6=749.44, y7=863.47, y8=976.71, y9=1089.72, y10=1203.83, y11=1318.81
Y10-12	2	55	62	MLGALRTL	1	874.5179	**25%,** 50%, 70%	874.5398	**in-gel,** in-sol	**001,** 002	60	-25	b3=302.22, b5=486.40
Y28-30	2	183	191	SALLMAAPL	1	886.5067	70%	886.4531	in-sol	001	33	60	b4=385.35, b5=516.35, y3=300.25
Y33-35	2	205	216	ISIPYVEMTLRL	1	1434.8025	25%	1434.9641	in-gel	001	45	-113	b1=114.11, b5=574.34
Y35-37	2	215	230	RLMERFGVKAEHSDSW	1	1947.9494	**25%,** 70%	1948.0138	in-gel; detected with Y58-59	001	88	-33	
Y43-45	2	259	280	FLAGAAITGGTVTVEQCGTTSL	1	2083.0376	25%	2083.1897	in-gel, **in-sol**	001, **003**	50	-73	
Y56-58	2	371	392	TKLGASVEEGPDYCIITPPEKL	1	2417.2269	50%	2417.2712	in-sol	003	55	-18	y1=132.09, y19=1751.41
Y63-65	2	435	441	FDVLSTF	1	828.4138	10%, 25%, **50%**	828.4150	in-gel; detectd with Y26 and Y3-5	**001,** 002	35	-1	
Y6-9	3	34	54	AALSEGTTVVDNLLNSEDVHY	1	2247.0776	**50%,** 70%	2247.1243	in-sol	003	100	-21	b2=256.10, b8=731.34, y2=319.16, y7=863.53, y8=976.64, y9=1089.58, y10=1203.81, y11=1318.03, y13=1516.97
Y15-18	3	96	120	FLGNAGIAMRSLTAAVTAAGGNATY	1	2398.2184	50%	2398.1794	in-sol	003	35	16	
Y27-Y30	3	182	191	LSALLMAAPL	1	999.5907	10%, **25%,** 50%, 70%	999.5765	in-sol	**001**, 002	1578	14	b1=114.11, b4=385.30, y6=615.56, y8=799.59
Y28-Y31	3	183	193	SALLMAAPLAL	1	1070.6278	25%	1070.5851	**in-gel,** detected with Y58	001	41	40	b4=385.31, b5=516.38, b9=868.74, y3=316.20
Y5-9	4	33	54	LAALSEGTTVVDNLLNSEDVHY	1	2360.1616	50%	2360.2097	in-sol	003	83	-20	b2=184.14, b3=256.19, b5=456.27, b21=2179.63, y2=319.15, y4=533.27, y6=749.40, y7=863.46, y8=976.56, y9=1089.73, y11=1318.88, y12=1417.98, y13=1517.07
Y8-13	5	47	64	LNSEDVHYMLGALRTLGL	1	2002.0426	25%	2002.1512	in-gel, **in-sol**	001, **003**	75	-54	y4=403.19, y10=1207.78

Cys residues were alkylated with Iodoacetamide. Background and auto-proteolytic peaks were removed.

Table III Amino acid sequence obtained for N-terminal peptide identified from the chymotryptic digest of 2mEPSPS (DAS-44406-6) (1-8; m/z 801.4942). Fragment ions positively identified from the MS-MS spectrum are shown in bold.

N-terminal peptide from chymotryptic digest 1-8 (theoretical m/z = 801.4353)
Sequence: AGAEEIVL
Parent peptide mass $[M+H]^{1+}$(monoisotopic): 801.4942

	A	***G***	***A***	***E***	***E***	***I***	***V***	***L***
a	44.05	101.07	172.11	301.15	430.19	543.28	642.35	
	44.06		**172.11**	**301.18**	**430.27**	**543.39**	**642.48**	
b	72.05	129.07	200.10	329.15	458.19	571.27	670.34	
	72.10	**129.11**		**329.24**	**458.34**		**670.44**	
c	89.07	146.09	217.13	346.17	475.22	588.30	687.37	
		146.09					**687.39**	
x		756.38	699.36	628.32	499.28	370.23	257.15	158.08
		756.53			**488.30**	**370.30**		
y		730.40	673.38	602.34	473.30	344.25	231.17	132.10
			673.44	**602.41**		**344.24**	**231.13**	**132.11**
z		714.38	657.36	586.32	457.28	328.24	215.15	116.08
				586.40		**328.26**		

Table IV Amino acid sequence obtained for N-terminal peptide identified from chymotryptic digest of 2mEPSPS (DAS-44406-6) with a single missed cleavage (1-26; m/z 2664.5735). Fragment ions positively identified from the MS-MS spectrum are shown in bold.

N-terminal peptide from chymotryptic digest 1-26 (theoretical m/z = 2664.5182)
Sequence: AGAEEIVLQPIKEISGTVKLPGSKSL
Parent peptide mass $[M+H]^{1+}$(monoisotopic): 2664.5735

	A	***G***	***A***	***E***	***E***	***I***	***V***	***L***	***Q***	***P***	***I***	***K***	***E***
a	44.05	101.07	172.11	301.15	430.19	543.28	642.35	755.43	883.49	980.54	1093.63	1221.72	1350.76
	44.07	**101.12**	**172.12**		**430.23**	**543.34**							
b	72.05	129.07	200.10	329.15	458.19	571.27	670.34	783.43	911.48	1008.54	1121.62	1249.72	1378.76
	72.11	**129.11**		**329.19**	**458.24**	**571.32**	**670.40**	**783.51**	**911.63**				
c	89.07	146.09	217.13	346.17	475.22	588.30	687.37	800.45	928.51	1025.56	1138.65	1266.74	1395.78
						588.39							
x		2619.46	2562.44	2491.40	2362.36	2233.32	2120.23	2021.17	1908.08	1780.02	1682.97	1569.89	1441.79
y		2593.48	2536.46	2465.42	2336.38	2207.34	2094.25	1995.19	1882.10	1754.04	1656.99	1543.91	1415.81
z		2577.46	2520.44	2449.40	2320.36	2191.32	2078.24	1979.17	1866.08	1738.02	1640.97	1527.89	1399.79

Sequence continues below

	I	***S***	***G***	***T***	***V***	***K***	***L***	***P***	***G***	***S***	***K***	***S***	***L***
a	1463.85	1550.88	1607.90	1708.95	1808.02	1936.11	2049.20	2146.25	2203.27	2290.30	2418.40	2505.43	
b	1491.84	1578.87	1635.90	1736.94	1836.01	1964.11	2077.19	2174.24	2231.27	2318.30	2446.39	2533.42	
							2077.74						
c	1508.87	1595.90	1652.92	1753.97	1853.04	1981.13	2094.22	2191.27	2248.29	2335.32	2463.42	2550.45	
		1595.10											
x	1312.75	1199.66	1112.63	1055.61	954.56	855.49	727.40	614.32	517.26	460.24	373.21	245.11	158.08
												245.14	
y	1286.77	1173.68	1086.65	1029.63	928.58	829.51	701.42	588.34	491.28	434.26	347.23	219.13	132.10
		1173.75					**701.40**	**588.39**					**132.34**
z	1270.75	1157.67	1070.63	1013.61	912.56	813.50	685.40	572.32	475.26	418.24	331.21	203.12	116.08
			1070.75								**331.07**		

Table V Amino acid sequence obtained for C-terminal peptide from tryptic digest (429-444; m/z 1921.0364) of 2mEPSPS (DAS-44406-6). Fragment ions positively identified are shown in bold.

C-terminal peptide from tryptic digest 429-444 (theoretical m/z = 1920.9742)

Sequence: KTFPDYFDVLSTFVKN

Parent peptide mass $[M+H]^{1+}$(monoisotopic): 1921.0364

	K	***T***	***F***	***P***	***D***	***Y***	***F***	***D***	***V***	***L***	***S***	***T***	***F***	***V***	***K***	***N***
a	101.11	202.16	349.22	446.28	561.30	724.37	871.44	986.46	1085.53	1198.62	1285.65	1386.70	1533.76	1632.83	1760.93	
	101.10					**724.47**	**871.51**	**986.47**	**1085.60**						**1760.64**	
b	129.10	230.15	377.22	474.27	589.30	752.36	899.43	1014.46	1113.53	1226.61	1313.64	1414.69	1561.76	1660.83	1788.92	
	129.11	**230.11**	**377.17**	**474.30**	**589.40**	**752.48**	**899.50**	**1014.56**	**1113.68**	**1226.86**						
c	146.13	247.18	394.25	491.30	606.33	769.39	916.46	1031.48	1130.55	1243.64	1330.67	1431.72	1578.78	1677.85	1805.95	
	146.08						**916.68**					**1431.94**				
x		1818.86	1717.81	1570.74	1473.69	1358.66	1195.60	1048.53	933.51	834.44	721.35	634.32	533.27	386.20	287.14	159.04
																159.10
y		1792.88	1691.83	1544.76	1447.71	1332.68	1169.62	1022.55	907.53	808.46	695.37	608.34	507.29	360.22	261.16	133.06
		1792.96		**1544.98**				**1022.72**	**907.65**			**608.36**		**360.17**	**261.17**	**133.07**
z		1776.86	1675.81	1528.74	1431.69	1316.67	1153.60	1006.53	891.51	792.44	679.35	592.32	491.27	344.21	245.14	117.04
			1675.21							**792.21**						

Table VI Amino acid sequence obtained for C-terminal peptide from chymotryptic digest (442-444; m/z 360.2131 and 439-444, m/z 695.3156) of 2mEPSPS (DAS-44406-6). Fragment ions positively identified are shown in bold.

C-terminal peptide from chymotryptic digest 442-444 (theoretical m/z = 360.2241)
Sequence: VKN
Parent peptide mass $[M+H]^{1+}$(monoisotopic): 360.2131

	V	***K***	***N***
a	72.08	200.18	
		200.08	
b	100.08	228.17	
	100.07	**228.14**	
c	117.10	245.20	
	117.08		
x		287.14	159.04
		287.16	**159.11**
y		261.16	133.06
		261.14	
z		245.14	117.04
			117.08

C-terminal peptide from chymotryptic digest 439-444 (theoretical m/z = 695.3723)
Sequence: STFVKN
Parent peptide mass $[M+H]^{1+}$(monoisotopic): 695.3156

	S	***T***	***F***	***V***	***K***	***N***
a	60.05	161.09	308.16	407.23	535.32	
	60.07		**308.22**			
b	88.04	189.09	336.16	435.22	563.32	
	88.08	**189.13**			**563.33**	
c	105.07	206.11	353.18	452.25	580.35	
	105.09	**206.12**	**353.29**			
x		634.32	533.27	386.20	287.14	159.04
		634.39			**287.21**	**159.11**
y		608.34	507.29	360.22	261.16	133.06
			507.32		**260.16**	
z		592.32	491.27	344.21	245.14	117.04
					245.19	

Figure 1 Theoretical amino acid sequence of 2mEPSPS.

2mEPSPS

[1-444] mass = 47284.5

Small polar:	D(26)	E(28)	N(11)	Q(6)			
Large polar:	K(26)	R(20)	H(4)				
Small non-polar:	S(26)	T(31)	A(46)	G(42)			
Large non-polar:	L(42)	I(22)	V(44)	M(14)	F(13)	Y(11)	W(3)
Special:	C(7)	P(22)					

1	A	G	A	E	E	I	V	L	Q	P	I	K	E	I	S	G	T	V	K	L	P	G	S	K	S	L	S	N	R	I	30
31	L	L	L	A	A	L	S	E	G	T	T	V	V	D	N	L	L	N	S	E	D	V	H	Y	M	L	G	A	L	R	60
61	T	L	G	L	S	V	E	A	D	K	A	A	K	R	A	V	V	V	G	C	G	G	K	F	P	V	E	D	A	K	90
91	E	E	V	Q	L	F	L	G	N	A	G	I	A	M	R	S	L	T	A	A	V	T	A	A	G	G	N	A	T	Y	120
121	V	L	D	G	V	P	R	M	R	E	R	P	I	G	D	L	V	V	G	L	K	Q	L	G	A	D	V	D	C	F	150
151	L	G	T	D	C	P	P	V	R	V	N	G	I	G	G	L	P	G	G	K	V	K	L	S	G	S	I	S	S	Q	180
181	Y	L	S	A	L	L	M	A	A	P	L	A	L	G	D	V	E	I	E	I	I	D	K	L	I	S	I	P	Y	V	210
211	E	M	T	L	R	L	M	E	R	F	G	V	K	A	E	H	S	D	S	W	D	R	F	Y	I	K	G	G	Q	K	240
241	Y	K	S	P	K	N	A	Y	V	E	G	D	A	S	S	A	S	Y	F	L	A	G	A	A	I	T	G	G	T	V	270
271	T	V	E	G	C	G	T	T	S	L	Q	G	D	V	K	F	A	E	V	L	E	M	M	G	A	K	V	T	W	T	300
301	E	T	S	V	T	V	T	G	P	P	R	E	P	F	G	R	K	H	L	K	A	I	D	V	N	M	N	K	M	P	330
331	D	V	A	M	T	L	A	V	V	A	L	F	A	D	G	P	T	A	I	R	D	V	A	S	W	R	V	K	E	T	360
361	E	R	M	V	A	I	R	T	E	L	T	K	L	G	A	S	V	E	E	G	P	D	Y	C	I	I	T	P	P	E	390
391	K	L	N	V	T	A	I	D	T	Y	D	D	H	R	M	A	M	A	F	S	L	A	A	C	A	E	V	P	V	T	420
421	I	R	D	P	G	C	T	R	K	T	F	P	D	Y	F	D	V	L	S	T	F	V	K	N							444

Figure 2 SDS-PAGE analysis of 2mEPSPS (DAS-44406-6). SDS-PAGE image (gel supplied by Brian Barnett) of affinity enriched fraction for 2mEPSPS loaded in lanes 3 and 5. To aid band excision and estimate the amount of protein per band a bacterially expressed standard 2mEPSPS was loaded in lane 7 at x520 ng.

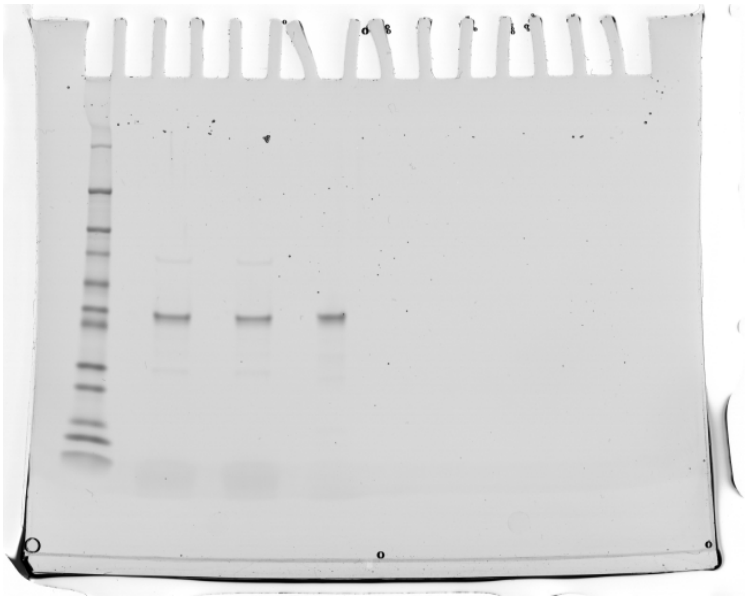
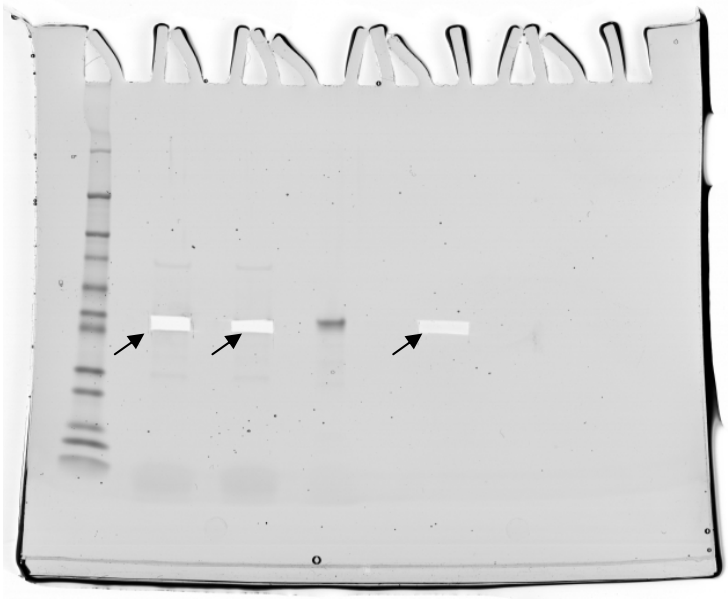


Figure 3 SDS-PAGE analysis of 2mEPSPS (DAS-44406-6) after excision of the putative 2mEPSPS bands and the blank control bands. (arrowed)



* 2mEPSPS bands (~ 47 kDa) were excised from gel, digested with trypsin or chymotrypsin and analyzed by MALDI-TOF MS and MALDI TOF-TOF MS-MS. A band was removed from the blank part of the gel at a similar height to provide control samples for processing

Figure 4 Tryptic digest sequence coverage for 2mEPSPS (DAS-44406-6) by MALDI-TOF MS and MALDI TOF-TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 57.2% with PMF data and 43.7% by tandem MS.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
1	A	G	A	E	E	I	V	L	Q	P	I	K	**E**	**I**	**S**	**G**	**T**	**V**	**K**	L	P	G	S	K	S	L	S	N	R	I	30	7	7
31	L	L	L	A	A	L	S	E	G	T	T	V	V	D	N	L	L	N	S	E	D	V	H	Y	M	L	G	A	L	R	60	0	0
61	T	L	G	L	S	V	E	A	D	K	**A**	**A**	**K**	**R**	A	V	V	V	G	C	G	G	K	**F**	**P**	**V**	**E**	**D**	**A**	**K**	90	11	11
91	**E**	**E**	**V**	**Q**	**L**	**F**	**L**	**G**	**N**	**A**	**G**	**I**	**A**	**M**	**R**	**S**	**L**	**T**	**A**	**A**	**V**	**T**	**A**	**A**	**G**	**G**	**N**	**A**	**T**	**Y**	120	30	30
121	**V**	**L**	**D**	**G**	**V**	**P**	**R**	**M**	**R**	**E**	**R**	**P**	**I**	**G**	**D**	**L**	**V**	**V**	**G**	**L**	**K**	**Q**	**L**	**G**	**A**	**D**	**V**	**D**	**C**	**F**	150	30	30
151	**L**	**G**	**T**	**D**	**C**	**P**	**P**	**V**	**R**	**V**	**N**	**G**	**I**	**G**	**G**	**L**	**P**	**G**	**G**	**K**	V	K	L	S	G	S	I	S	S	Q	180	20	20
181	Y	L	S	A	L	L	M	A	A	P	L	A	L	G	D	V	E	I	E	I	I	D	K	**L**	**I**	**S**	**I**	**P**	**Y**	**V**	210	7	7
211	**E**	**M**	**T**	**L**	**R**	L	M	E	R	F	G	V	K	**A**	**E**	**H**	**S**	**D**	**S**	**W**	**D**	**R**	**F**	**Y**	**I**	**K**	**G**	**G**	**Q**	**K**	240	22	18
241	**Y**	**K**	**S**	**P**	**K**	N	A	Y	V	E	G	D	A	S	S	A	S	Y	F	L	A	G	A	A	I	T	G	G	T	V	270	5	5
271	T	V	E	G	C	G	T	T	S	L	Q	G	D	V	K	F	A	E	V	L	E	M	M	G	A	K	**V**	**T**	**W**	**T**	300	4	4
301	**E**	**T**	**S**	**V**	**T**	**V**	**T**	**G**	**P**	**P**	**R**	**E**	**P**	**F**	**G**	**R**	**K**	**H**	**L**	**K**	A	I	D	V	N	M	N	K	**M**	**P**	330	22	9
331	**D**	**V**	**A**	**M**	**T**	**L**	**A**	**V**	**V**	**A**	**L**	**F**	**A**	**D**	**G**	**P**	**T**	**A**	**I**	**R**	**D**	**V**	**A**	**S**	**W**	**R**	**V**	**K**	**E**	**T**	360	30	0
361	**E**	**R**	**M**	**V**	**A**	**I**	**R**	**T**	**E**	**L**	**T**	**K**	**L**	**G**	**A**	**S**	**V**	**E**	**E**	**G**	**P**	**D**	**Y**	**C**	**I**	**I**	**T**	**P**	**P**	**E**	390	30	23
391	**K**	**L**	**N**	**V**	**T**	**A**	**I**	**D**	**T**	**Y**	**D**	**D**	**H**	**R**	M	A	M	A	F	S	L	A	A	C	A	E	V	P	V	T	420	14	14
421	I	R	**D**	**P**	**G**	**C**	**T**	**R**	**K**	**T**	**F**	**P**	**D**	**Y**	**F**	**D**	**V**	**L**	**S**	**T**	**F**	**V**	**K**	**N**							444	22	16
																															Tot. a.a. covered =	***254***	***194***

A = Tryptic MS coverage

A = Tryptic MS-MS data

MALDI PMF sequence coverage = 57.2 %

MALDI MS-MS sequence coverage = 43.7 %

Figure 5 Chymotryptic digest sequence coverage for 2mEPSPS (DAS-44406-6) by MALDI-TOF MS and MALDI TOF-TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 69.8% with PMF data and 48.9% by tandem MS.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
1	**A**	**G**	**A**	**E**	**E**	**I**	**V**	**L**	**Q**	**P**	**I**	**K**	**E**	**I**	**S**	**G**	**T**	**V**	**K**	**L**	**P**	**G**	**S**	**K**	**S**	**L**	**S**	**N**	**R**	**I**	30	30	30
31	**L**	**L**	**L**	**A**	**A**	**L**	**S**	**E**	**G**	**T**	**T**	**V**	**V**	**D**	**N**	**L**	**L**	**N**	**S**	**E**	**D**	**V**	**H**	**Y**	**M**	**L**	**G**	**A**	**L**	**R**	60	30	29
61	**T**	**L**	**G**	**L**	S	V	E	A	D	K	A	A	K	R	A	V	V	V	G	C	G	G	K	F	P	V	E	D	A	K	90	4	4
91	E	E	V	Q	L	**F**	**L**	**G**	**N**	**A**	**G**	**I**	**A**	**M**	**R**	**S**	**L**	**T**	**A**	**A**	**V**	**T**	**A**	**A**	**G**	**G**	**N**	**A**	**T**	**Y**	120	25	0
121	V	L	**D**	**G**	**V**	**P**	**R**	**M**	**R**	**E**	**R**	**P**	**I**	**G**	**D**	**L**	**V**	**V**	**G**	**L**	**K**	**Q**	**L**	**G**	**A**	**D**	**V**	**D**	**C**	**F**	150	28	14
151	L	**G**	**T**	**D**	**C**	**P**	**P**	**V**	**R**	**V**	**N**	**G**	**I**	**G**	**G**	**L**	**P**	**G**	**G**	**K**	**V**	**K**	**L**	**S**	**G**	**S**	**I**	**S**	**S**	**Q**	180	29	7
181	**Y**	**L**	**S**	**A**	**L**	**L**	**M**	**A**	**A**	**P**	**L**	**A**	**L**	**G**	**D**	**V**	**E**	**I**	**E**	**I**	**I**	**D**	**K**	**L**	**I**	**S**	**I**	**P**	**Y**	**V**	210	30	30
211	**E**	**M**	**T**	**L**	**R**	**L**	**M**	**E**	**R**	**F**	**G**	**V**	**K**	**A**	**E**	**H**	**S**	**D**	**S**	**W**	**D**	**R**	**F**	**Y**	**I**	**K**	**G**	**G**	**Q**	**K**	240	30	30
241	**Y**	**K**	**S**	**P**	**K**	**N**	**A**	**Y**	V	E	G	D	A	S	S	A	S	Y	**F**	**L**	**A**	**G**	**A**	**A**	**I**	**T**	**G**	**G**	**T**	**V**	270	20	8
271	**T**	**V**	**E**	**G**	**C**	**G**	**T**	**T**	**S**	**L**	**Q**	**G**	**D**	**V**	**K**	**F**	A	E	V	L	E	M	M	G	A	K	V	T	W	**T**	300	17	7
301	**E**	**T**	**S**	**V**	**T**	**V**	**T**	**G**	**P**	**P**	**R**	**E**	**P**	**F**	G	R	K	H	L	K	A	I	D	V	N	M	N	K	M	P	330	14	14
331	D	V	A	M	T	L	A	V	V	A	L	F	**A**	**D**	**G**	**P**	**T**	**A**	**I**	**R**	**D**	**V**	**A**	**S**	**W**	R	V	K	E	T	360	13	13
361	E	R	M	V	A	I	R	T	E	L	**T**	**K**	**L**	**G**	**A**	**S**	**V**	**E**	**E**	**G**	**P**	**D**	**Y**	**C**	**I**	**I**	**T**	**P**	**P**	**E**	390	20	20
391	**K**	**L**	**N**	**V**	**T**	**A**	**I**	**D**	**T**	**Y**	D	D	H	R	M	A	M	A	F	S	L	A	A	C	A	E	V	P	V	T	420	10	2
421	I	R	D	P	G	C	T	R	K	T	F	P	D	Y	**F**	**D**	**V**	**L**	**S**	**T**	**F**	**V**	**K**	**N**							444	10	9
																															Tot. a.a. covered =	***310***	***217***

A = Chymotryptic MS coverage

A = Chymotryptic MS-MS data

MALDI PMF sequence coverage = 69.8 %

MALDI MS-MS sequence coverage = 48.9 %

Figure 6 Overall sequence coverage of trypsin and chymotrypsin digests for 2mEPSPS (DAS-44406-6) by MALDI-TOF MS and MALDI TOF-TOF. Cys residues were alkylated with iodoacetamide. Overall sequence coverage was 86.3% with PMF data and 70.0% by tandem MS.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
1	A	G	A	E	E	I	V	L	Q	P	I	K	E	I	S	G	T	V	K	L	P	G	S	K	S	L	S	N	R	I	30	30	30
31	L	L	L	A	A	L	S	E	G	T	T	V	V	D	N	L	L	N	S	E	D	V	H	Y	M	L	G	A	L	R	60	30	29
61	T	L	G	L	S	V	E	A	D	K	A	A	K	R	A	V	V	V	G	C	G	G	K	F	P	V	E	D	A	K	90	15	15
91	E	E	V	Q	L	F	L	G	N	A	G	I	A	M	R	S	L	T	A	A	V	T	A	A	G	G	N	A	T	Y	120	30	30
121	V	L	D	G	V	P	R	M	R	E	R	P	I	G	D	L	V	V	G	L	K	Q	L	G	A	D	V	D	C	F	150	30	30
151	L	G	T	D	C	P	P	V	R	V	N	G	I	G	G	L	P	G	G	K	V	K	L	S	G	S	I	S	S	Q	180	30	27
181	Y	L	S	A	L	L	M	A	A	P	L	A	L	G	D	V	E	I	E	I	I	D	K	L	I	S	I	P	Y	V	210	30	30
211	E	M	T	L	R	L	M	E	R	F	G	V	K	A	E	H	S	D	S	W	D	R	F	Y	I	K	G	G	Q	K	240	30	30
241	Y	K	S	P	K	N	A	Y	V	E	G	D	A	S	S	A	S	Y	F	L	A	G	A	A	I	T	G	G	T	V	270	20	8
271	T	V	E	G	C	G	T	T	S	L	Q	G	D	V	K	F	A	E	V	L	E	M	M	G	A	K	V	T	W	T	300	20	7
301	E	T	S	V	T	V	T	G	P	P	R	E	P	F	G	R	K	H	L	K	A	I	D	V	N	M	N	K	M	P	330	22	20
331	D	V	A	M	T	L	A	V	V	A	L	F	A	D	G	P	T	A	I	R	D	V	A	S	W	R	V	K	E	T	360	30	14
361	E	R	M	V	A	I	R	T	E	L	T	K	L	G	A	S	V	E	E	G	P	D	Y	C	I	I	T	P	P	E	390	30	23
391	K	L	N	V	T	A	I	D	T	Y	D	D	H	R	M	A	M	A	F	S	L	A	A	C	A	E	V	P	V	T	420	14	2
421	I	R	D	P	G	C	T	R	K	T	F	P	D	Y	F	D	V	L	S	T	F	V	K	N							444	22	16
																															Tot. a.a. covered =	*383*	*311*

A = Tryptic MS coverage

A = Tryptic MS-MS data

A = Chymotryptic MS coverage

A = Chymotryptic MS-MS data

MALDI PMF sequence coverage = 86.3 %

MALDI MS-MS sequence coverage = 70.0 %

APPENDICES (AS CONTAINED IN THE STUDY FILE)

Appendix A: SDS-PAGE data, scan of gels, scan of post excision gel and sequences data
Appendix B: Experimental run-sheets
Appendix C: Sample and calibration logs
Appendix D: MS calibration and settings
Appendix E: MS-MS calibration and settings
Appendix F: MS data record sheets and coverage maps for tryptic and chymotryptic digests
Appendix G: Tryptic digest MS spectral print outs
Appendix H: Chymotrypyic digest MS spectral print outs
Appendix I: Tryptic digest MS-MS spectral print outs
Appendix J: Chymotrypyic digest MS-MS spectral print outs
Appendix K: N- and C-terminal peptide coverage maps and MS-MS spectral print outs
Appendix L: Data not used

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