

Study Title

Amended Report for MSL 0022391: Characterization of the CP4 EPSPS Protein Purified from the Grain of MON 87427 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. coli*-Produced CP4 EPSPS Proteins.

Authors

Dilip Chandu, Ph.D., Kathleen S. Crowley, Ph.D., Thomas C. Lee, Ph.D., and John J. Finnessy, M.S.

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Sponsor and Performing Laboratory

Monsanto Company
Regulatory Product Characterization Center
800 North Lindbergh Boulevard
St. Louis, Missouri 63167

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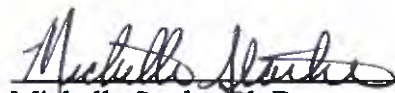
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Statement of Compliance

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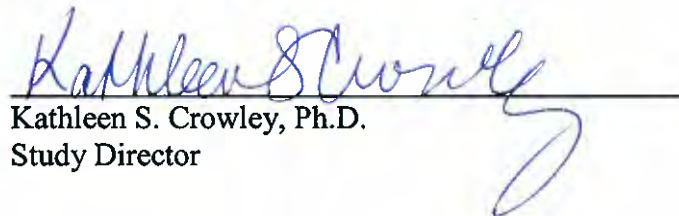


Michelle Starke, Ph.D.

Sponsor Representative

11/19/10

Date



Kathleen S. Crowley, Ph.D.

Study Director

11/29/10

Date

Quality Assurance Unit Statement

Study Title: Characterization of the CP4 EPSPS Protein Purified from the Grain of MON 87427 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. coli*-Produced CP4 EPSPS Proteins.

Study Number: REG-09-237

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on the study reported herein.

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
09/18/2009	Bio-Rad Assay (Total Protein Concentration)	9/21/2009	9/21/2009
12/14/2009	Raw Data Audit	12/15/2009	12/15/2009
02/16/2010	Draft Report Audit	02/16/2010	02/17/2010
10/18/2010	Amended Report Review (Amendment 1)	10/18/2010	10/18/2010

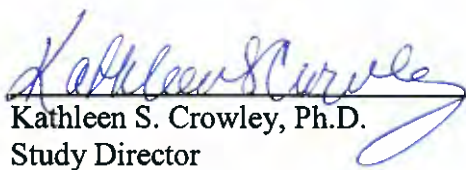

 Todd S. Butzlaff
 Quality Assurance Specialist
 Monsanto Regulatory
 Monsanto Company

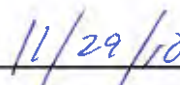

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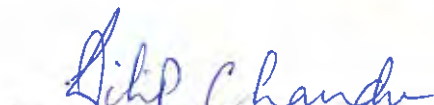
Study Certification Page


The results reported in this report accurately reflect the data generated under Study Number REG-09-237.


Signatures of Final Report Approval:



Kathleen S. Crowley, Ph.D.
Study Director


Date


Dilip Chandu, Ph.D.
Principal Investigator


Date


John J. Finnessy, M.S.
Protein Sciences and Safety Team Lead


Date

Study Information

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Testing Facility: Monsanto Company
Regulatory Product Characterization Center
800 North Lindbergh Boulevard
St. Louis, Missouri 63167

Authors: Dilip Chandu, Ph.D., Kathleen S. Crowley, Ph.D., Thomas C. Lee, Ph.D., and John J. Finnessy, M.S.

Study Director: Kathleen S. Crowley, Ph.D.

Contributors: Jing Berry, Chris Dalton, Shefalee Kapadia, and Richard Thoma

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Abbreviations and Definitions¹

AA	Amino Acid
APS	Analytical Protein Standard
BSA	Bovine Serum Albumin
CFR	Code of Federal Regulations
COA	Certificate of Analysis
CP4	<i>Agrobacterium sp.</i> strain CP4
<i>cp4 epsps</i>	Coding sequence for the CP4 EPSPS Protein
α -Cyano	α -Cyano-4-hydroxycinnamic acid
Da	Dalton
DHB	2,5-Dihydroxybenzoic Acid
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EPA	Environmental Protection Agency
EPSPS	5-Enolpyruvylshikimate-3-phosphate Synthase
<i>E. coli</i>	<i>Escherichia coli</i>
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act (U.S.)
GLP	Good Laboratory Practice
HRP	Horseradish Peroxidase
LB	Loading buffer [62.5mM Tris-HCl, 4% (v/v) 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, 0.005% (w/v) bromophenol blue, 10% (v/v) glycerol, pH 6.8]
MALDI-TOF	Matrix Assisted Laser Desorption and Ionization - Time of Flight
MH+	Protonated mass ion
MS	Mass Spectrometry
MW	Molecular Weight
MWCO	Molecular Weight Cutoff
NFDM	Non-Fat Dried Milk
PAGE	Polyacrylamide Gel Electrophoresis
PBST	Phosphate Buffered Saline containing 0.05% (v/v) Tween-20
PEP	Phosphoenolpyruvate
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene Difluoride
SDS	Sodium Dodecyl Sulfate
S3P	Shikimate-3-phosphate
Sinapinic Acid	3,5-dimethoxy-4-hydroxycinnamic acid
SOP	Standard Operating Procedure
TFA	Trifluoroacetic Acid

¹ Standard abbreviations, e.g. units of measure, concentration, mass, time etc., are used without definition according to the format described in "Instructions to Authors" in The Journal of Biological Chemistry.

U	Unit (of enzyme activity)
U.S.	United States
VOI	Verification of Identity

1.0 Summary

Monsanto Company has developed MON 87427, an inducible male sterile and glyphosate tolerant corn, to facilitate the production of viable hybrid corn seed. MON 87427 produces the same 5-enolpyruvyl shikimate-3-phosphate synthase (CP4 EPSPS) protein that is produced in commercial Roundup Ready corn products, via the incorporation of a *cp4 epsps* coding sequence. However, MON 87427 utilizes a specific promoter and intron combination (*e35S-hsp70*) to drive CP4 EPSPS protein expression in only vegetative and female reproductive tissues. Little to no CP4 EPSPS protein is expected to be produced in the pollen of MON 87427, thus pollen from MON 87427 is not tolerant to glyphosate. Appropriately timed glyphosate applications produce a non-viable pollen phenotype and allow for specific cross pollinations to be made in corn without using traditional methods to control self pollination.

The MON 87427-produced CP4 EPSPS protein was engineered to match the *E. coli*-produced CP4 EPSPS protein (Orion lot 10000739) contained in other RoundupReady® products. The *E. coli*-produced CP4 EPSPS protein has been used previously in a number of safety assessment studies. Demonstration of the equivalence between the *E. coli*- and MON 87427-produced CP4 EPSPS proteins justifies the utilization of the existing data to confirm the safety of the CP4 EPSPS protein in MON 87427. Hence, the purpose of this study was to characterize the CP4 EPSPS protein isolated from grain of MON 87427 and demonstrate the equivalence of the MON 87427-produced CP4 EPSPS to the previously characterized *E. coli*-produced CP4 EPSPS.

The following analyses were performed to characterize the MON 87427-produced CP4 EPSPS protein. The total protein concentration of the MON 87427-produced CP4 EPSPS protein was measured using the Bio-Rad protein assay and determined to be 0.1 mg/ml. The identity of the MON 87427-produced CP4 EPSPS protein was confirmed by immunoblot analysis carried out with previously characterized anti-CP4 EPSPS antibodies. Anti-CP4 EPSPS antibodies specifically detected the MON 87427-produced CP4 EPSPS protein. Additionally, identity was confirmed by N-terminal sequence analysis and matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analysis of the trypsin digested CP4 EPSPS protein. The expected N-terminal sequence was observed and peptide masses consistent with the expected peptide masses for the trypsin digested CP4 EPSPS protein were observed. For the latter, the identified peptide masses yielded coverage of more than 70% of the expected peptide sequence (320 out of 455 amino acids) of the CP4 EPSPS protein. Purity and apparent molecular weight of the MON 87427-produced CP4 EPSPS protein were determined using densitometric analysis of a Colloidal Brilliant Blue G stained SDS-PAGE gel. Purity of the MON 87427-produced CP4 EPSPS protein was 96% and the apparent molecular weight was 44.1 kDa. The average mass of the intact MON 87427-produced protein was 47552 Da as measured by MALDI-TOF MS analysis. This measurement was consistent with the theoretical mass of the CP4 EPSPS protein (47481 Da). Analysis of the glycosylation status using a fluorescent glycoprotein detection assay indicated that

the MON 87427-produced CP4 EPSPS protein was not glycosylated. The functional activity of the MON 87427-produced CP4 EPSPS protein was determined using a phosphate release assay. The MON 87427-produced CP4 EPSPS protein was shown to be active, with a specific activity of 8.67 ± 0.23 U/mg of CP4 EPSPS. Finally, SDS-PAGE was used to determine the purity and molecular weight (MW) of the protein after storage at ~ -80 °C. This analysis demonstrated that the MON 87427-produced CP4 EPSPS protein was stable throughout the experimental phase (14 days).

The equivalence of the MON 87427- and *E. coli*-produced CP4 EPSPS proteins was assessed by comparing their apparent molecular weight, immunoreactivity, functional activity, and glycosylation status. Based on the previously set acceptance criteria and the results obtained, the MON 87427-produced CP4 EPSPS protein was shown to be equivalent to the *E. coli*-produced CP4 EPSPS protein.

These data provide a detailed characterization of the CP4 EPSPS protein isolated from MON 87427 and establish the equivalence of the MON 87427-produced CP4 EPSPS protein to the *E. coli*-produced CP4 EPSPS proteins.

2.0 Introduction

Monsanto Company has developed MON 87427, an inducible male sterile and glyphosate tolerant corn, to facilitate the production of viable hybrid corn seed. MON 87427 produces the same CP4 EPSPS protein that is produced in commercial Roundup Ready corn products, via the incorporation of a *cp4 epsps* coding sequence. However, MON 87427 utilizes a specific promoter and intron combination (*e35S-hsp70*) to drive CP4 EPSPS protein expression in only vegetative and female reproductive tissues. Little to no CP4 EPSPS protein is expected to be produced in pollen of MON 87427, thus pollen from MON 87427 is not tolerant to glyphosate. Appropriately timed glyphosate applications produce a non-viable pollen phenotype and allow for specific cross pollinations to be made in corn without using traditional methods to control self pollination.

MON 87427 was produced by the stable incorporation of a *cp4 epsps* coding sequence derived from the common soil bacterium *Agrobacterium* sp. strain CP4 using the transformation vector PV-ZMAP1043. Molecular analysis of MON 87427 show that it contains a single intact copy of the coding sequence for *cp4 epsps*. The integrity of the inserted *cp4 epsps* gene cassette has been confirmed by molecular characterization (Arackal et al., 2009).

The CP4 EPSPS protein is present in a number of biotechnology-derived crops and the results of protein safety assessment studies have been summarized (Harrison et al., 1996). CP4 EPSPS protein degrades rapidly in simulated gastric and intestinal fluids, and is safe when tested by acute oral gavage in mice (Harrison et al., 1996). Thus, it was concluded that the CP4 EPSPS protein poses no risk to human and animal health.

3.0 Purpose

The purpose of this study was to characterize the CP4 EPSPS protein purified from the grain of MON 87427 (Orion lot 11243843) and compare the physicochemical and functional properties to the previously characterized *E. coli*-produced CP4 EPSPS reference protein (Orion lot 10000739, historical APS lot 20-100015). Demonstration of the physicochemical and functional equivalence between the MON 87427- and *E. coli*-produced CP4 EPSPS proteins justifies the application of existing protein safety data for the CP4 EPSPS protein produced in MON 87427.

4.0 Materials

4.1 MON 87427-Produced CP4 EPSPS Protein (Test substance)

The MON 87427-produced CP4 EPSPS protein (Orion lot 11243843) purified from MON 87427 grain was used as the test substance. The grain was produced under production plan REG-08-069. A copy of the verification of identity (VOI) for the harvested grain has been archived under the Orion lot 10007650 (Source ID 10001857). The MON 87427-produced CP4 EPSPS protein was stored in a -80 °C freezer in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM

DTT, 1 mM benzamidine-HCl, and 25% glycerol. The purification records for the MON 87427-produced protein have been archived under the Orion lot 11243843 according to the current version of SOP BR-PO-0722.

4.2 *E. coli*-Produced CP4 EPSPS Protein (Reference Substance)

The *E. coli*-produced CP4 EPSPS protein (Orion lot 10000739, historical APS lot 20-100015) was used as the reference substance. The CP4 EPSPS protein reference substance was generated from cell paste produced by large-scale fermentation of *E. coli* containing the pMON21104 expression plasmid. The coding sequence for *cp4 epsps* contained on the expression plasmid (pMON21104) was confirmed prior to and after fermentation. The *E. coli*-produced CP4 EPSPS protein was previously characterized and a copy of the certificate of analysis (COA) is shown in Appendix 2.

4.3 Assay Controls

Protein molecular weight standards (Precision Plus Protein™ Standards Dual color; Bio-Rad, Hercules, CA) were used to calibrate some SDS-PAGE gels and verify protein transfer to Polyvinylidene difluoride (PVDF) membranes. Broad range SDS-PAGE molecular weight standards (Bio-Rad, Hercules, CA) were used to generate a standard curve for the apparent molecular weight estimation of the MON 87427-produced CP4 EPSPS protein. The *E. coli*-produced CP4 EPSPS reference standard was used to construct a standard curve for the estimation of total protein concentration using a Bio-Rad protein assay. A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to calibrate the instrument for each analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass and a BSA standard (NIST, Gaithersburg, MD) was used to calibrate the MALDI-TOF mass spectrometer for intact mass analysis. Transferrin (Amersham Biosciences, Piscataway, NJ) and horseradish peroxidase (Sigma-Aldrich, St. Louis, MO) were used as positive controls for glycosylation analysis. CandyCane™ glycoprotein molecular weight standards (Molecular Probes, Eugene, OR) were used as molecular weight markers, as well as, additional positive and negative controls for glycosylation analysis.

5.0 Methods

5.1 Protein Purification

The plant-produced CP4 EPSPS protein was purified from grain of MON 87427 prior to initiation of this equivalence study. The purification procedure was not performed under a GLP plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed. The CP4 EPSPS protein was purified at ~4 °C from an extract of ground grain using a combination of ammonium

sulfate fractionation, hydrophobic interaction chromatography, anion exchange chromatography, and cellulose phosphate affinity chromatography. The purification procedure is briefly described below and a detailed description of the purification procedure was filed under Orion Lot 11243843.

Approximately 400 g of grain of MON 87427 was mixed with 400 g of dry ice and then ground using a laboratory mill (Perten Instruments, model 3100). The ground powder (~ 400 g) was stored in a -80 °C freezer until used for extraction of the CP4 EPSPS protein. The ground powder was mixed with extraction buffer (100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM benzamidinium-HCl, 4 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 1% polyvinylpyrrolidone and 10% glycerol) for ~1 hr at a sample weight (g) to buffer volume (ml) of approximately 1:10. The slurry was centrifuged at 15,000 x g for 60 min at ~ 4 °C. The supernatant (~3.8 L) was collected and brought to 40% ammonium sulfate saturation by slow addition of 859 g of ammonium sulfate in a cold room (~ 4 °C). The solution was stirred for ~1 hr at ~ 4 °C and then centrifuged at 15,000 x g for 45 min. The supernatant (~3.8 L) was again collected and 710 g of ammonium sulfate was added to bring the solution to 70% ammonium sulfate saturation. The solution was stirred for ~ 1 hr in a cold room and the pellet was collected by centrifugation at 15,000 x g for 60 min. The pellet was re-suspended in 750 ml of PS(A) buffer [50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% glycerol (v/v), 1.5 M ammonium sulfate]. The sample was loaded onto a 471 ml column (5 cm x 24 cm) of Phenyl Sepharose Fast Flow (GE Healthcare, Piscataway, NJ) equilibrated with PS(A) buffer. Proteins were eluted with a linear salt gradient that decreased from 1.5 M to 0 M ammonium sulfate over a volume of 2400 ml. Fractions containing the CP4 EPSPS protein, identified based on Western blot analysis, were pooled to a final volume of ~225 ml. The pooled sample was desalted by dialysis against 4 L of QS(A) buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM benzamidinium-HCl, 4 mM DTT) at ~4 °C with one additional 4 L buffer change using a dialysis tubing [Spectrum Laboratories, Inc., Rancho Dominguez, CA; Molecular Weight Cutoff (MWCO): 3.5 kDa] for a total of 24 hr.

The desalted sample (310 ml) was loaded onto a 48 ml column (2.6 cm x 9 cm) of Q Sepharose Fast Flow anion exchange resin (GE Healthcare, Piscataway, NJ) equilibrated with QS(A) buffer. The bound CP4 EPSPS was eluted with a linear salt gradient that increased from 0 M to 0.4 M KCl in QS(A) buffer over 600 ml. Fractions containing CP4 EPSPS, identified by Western blot analysis, were pooled to a final volume of ~ 110 ml. The pooled sample was dialyzed against 2 L CP2(A) buffer (10 mM sodium citrate, pH 5.0, 1 mM benzamidinium-HCl, 2 mM DTT) for a total of 36 h at ~4 °C with 2 additional 2 L buffer changes using a dialysis tubing (Spectrum Laboratories, Inc. Rancho Dominguez, CA; MWCO: 3.5 kDa).

The dialyzed sample (120 ml) was then loaded onto a 32 ml column (2.6 x 6 cm) of cellulose phosphate P11 cation exchange (Whatman) pre-equilibrated with CP2(A) buffer. After an initial wash with 300 ml of CP2(A), the column was washed with a

linear gradient that increased from 0 to 100% UGN50 buffer (10 mM sodium citrate, 1 mM benzamidine, 50 mM NaCl, 0.3 mM UTP, 0.3 mM glucose-1-phosphate, and 4 mM DTT, pH 5.0) over 32 ml and was held at 100% for ~70 ml. The column was further washed with a linear gradient that increased from 0 to 100% PEP buffer (10 mM sodium citrate, 1 mM benzamidine, 50 mM NaCl, 0.3 mM phosphoenolpyruvate (PEP), 4 mM DTT, pH 5.3) over 32 ml and was held at 100% for ~140 ml. The bound CP4 EPSPS protein was eluted with a linear gradient that increased from 0-100% PEP/S3P buffer (10 mM sodium citrate, 1 mM benzamidine, 50 mM NaCl, 0.5 mM PEP, and 0.5 mM shikimate-3-phosphate (S3P), 4 mM DTT, pH 5.7) over 32 ml and was held at 100% for ~130 ml. Fractions containing CP4 EPSPS protein, based on SDS PAGE analysis and confirmed by Western blot analysis, were pooled (~27 ml). The pooled sample was divided between four iCon Concentrators (MWCO: 20 kDa; size: 7 ml; Pierce, Rockford, IL) and concentrated by centrifugation at 4,000 \times g for 30 min at ~4 °C. Buffer exchange was carried out in the same units by the addition of ~6.5 ml FSB buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine-HCl) followed by centrifugation at 4,000 \times g for 30 min at ~4 °C. The exchange was conducted a total of four times, and during the final exchange, the sample was concentrated to ~0.2 ml per unit. The samples were pooled (~0.8 ml) and mixed with 0.8 ml FSB buffer (containing 50% glycerol) to final volume of 1.6 ml. Final buffer composition of the sample was: 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine-HCl and 25% glycerol. This CP4 EPSPS protein purified from the grain of MON 87427 was aliquoted, assigned APS lot 11243843, and stored at in a -80°C freezer.

5.2 Protein Concentration

The total protein concentration of the MON 87427-produced CP4 EPSPS protein sample was determined using a Bio-Rad protein assay. *E. coli*-produced CP4 EPSPS reference standard protein was used to prepare a standard curve ranging from 0.05 to 0.6 mg total protein/ml. Using a best-fit linear regression line through the standard points, the total protein concentration of the MON 87427-produced CP4 EPSPS protein stock solution was calculated by interpolation of the blank-corrected absorbance values at 595 nm obtained for the test sample. Data were collected using a PowerWave Xi microplate scanning spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT) employing KC4 software version 3.3 revision 10.

5.3 Immunoblot Analysis

Immunoblot analysis was performed to confirm the identity of the CP4 EPSPS protein purified from grain of MON 87427 and to compare the immunoreactivity of the MON 87427- and *E. coli*-produced proteins.

The MON 87427- and *E. coli*-produced CP4 EPSPS proteins were analyzed concurrently on the same gel using three loadings of 1, 2 and 3 ng. Loadings of the three concentrations of the test and reference proteins were made in duplicate on the

gel. Aliquots of each protein were diluted in water and 5× loading buffer (LB) containing 312 mM Tris-HCl, 20% (v/v) 2-mercaptoethanol, 10% (w/v) SDS, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8), heated at ~99°C for 3 min, and applied to a 15 well pre-cast Tris-glycine 4 - 20% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA). Pre-stained molecular weight markers (Precision Plus Protein™ Standards Dual color; Bio-Rad, Hercules, CA) were loaded in parallel to verify electrotransfer of the proteins to the membrane and to estimate the size of the immunoreactive bands observed. Electrophoresis was performed at a constant voltage of 150 V for 90 min. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 100 min at a constant voltage of 25 V. After electrotransfer, the membrane was blocked for 1 hour with 5% (w/v) non-fat dried milk (NFDM) in 1× phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBST). The membrane was then probed with a 1:1,000 dilution of goat anti-CP4 EPSPS antibody (lot 10000787, aliquot # 39) in 5% (w/v) NFDM in PBST for 1 h. Excess antibody was removed using three 10 min washes with PBST. Finally, the membrane was probed with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Thermo, Rockford, IL) at a dilution of 1:10,000 in 5% (w/v) NFDM in PBST for 1 h. Excess HRP-conjugate was removed using three 10 min washes with PBST. All incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (GE, Healthcare, Piscataway, NJ) with exposure (1, 3, and 5 min) to Amersham Hyperfilm (GE, Healthcare, Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

Quantification of the bands on the blot was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA) using the lane finding and contour tool. The signal intensities of the immunoreactive bands observed for the test and reference proteins migrating at the expected position on the blot film were quantified as “contour quantity” values. The raw data was exported to a Microsoft Excel [2007 (12.0.6504.5001) SP1 MSO (12.0.6320.5000)] file for the pair wise comparison of the average of the load replicates. An average difference was calculated for each comparison to assess the immunoreactivity equivalence.

5.4 N-Terminal Sequencing

N-terminal sequencing, carried out by automated Edman degradation chemistry, was used to confirm the identity of the MON 87427-produced CP4 EPSPS.

MON 87427-produced CP4 EPSPS was separated by SDS-PAGE and transferred to PVDF membrane. The blot was stained using Coomassie Blue R-250. The major band at ~ 44 kDa containing the test protein was excised from the blot and was used for N-terminal sequence analysis. The analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied

Biosystems 494 Procise Sequencing System with 140C Microgradient pump and 785 Programmable Absorbance Detector was controlled with Procise™ Control (version 1.1a) software. Chromatographic data were collected using Atlas 2003 software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A control protein (10 picomoles of β -lactoglobulin, Applied Biosystems, Foster City, CA) was analyzed before and after the sequence analysis of the CP4 EPSPS protein to verify that the sequencer met performance criteria for repetitive yield and sequence identity. Identity was established if ≥ 8 amino acids, consistent with the predicted sequence of the N-terminus of the MON 87427-produced CP4 EPSPS, were observed during analysis.

5.5 MALDI-TOF Tryptic Mass Fingerprint Analysis

MALDI-TOF tryptic mass fingerprint analysis was used to confirm the identity of the MON 87427-produced CP4 EPSPS protein. MON 87427-produced CP4 EPSPS protein was subjected to SDS-PAGE and the gel was stained using Brilliant Blue G Colloidal stain. Each ~44 kDa band was excised, transferred to a microcentrifuge tube, and destained with 40% methanol/10% glacial acetic acid followed by 10% acetonitrile in 25 mM ammonium bicarbonate. The gel bands were washed in 100 mM ammonium bicarbonate and then, to reduce the protein in each, gel bands were incubated in 100 μ l of 10 mM dithiothreitol at ~37°C for 2 hr. The protein was then alkylated in the dark for 2 hr with 100 μ l of 20 mM iodoacetic acid and washed with 200 μ l of 25 mM ammonium bicarbonate for 1 hr once and for 15 min twice. Gel bands were dried with a Speed-Vac concentrator and then rehydrated with 20 μ l of trypsin solution (20 μ g/ml). After 1 hr, excess liquid was removed and the gel was incubated at 37.6 °C for 16 h in 40 μ l of 10% acetonitrile in 25 mM ammonium bicarbonate. To elute proteolytic fragments, gel bands were sonicated for 5 min. The resulting extracts were transferred to new microcentrifuge tubes labeled Extract 1 and dried using speed-Vac concentrator. The gel bands were re-extracted twice with 30 μ l of a 60% acetonitrile, 0.1% trifluoroacetic acid, 0.1% β -octyl-glucopyranoside solution and sonicated for 5 min. Both 60% acetonitrile, 0.1% trifluoroacetic acid, 0.1% β -octyl-glucopyranoside extracts were pooled into a new tube labeled Extract 2 and dried with a Speed-Vac concentrator. A solution of 0.1% TFA was added to all Extract 1 and 2 tubes and they were dried as before. To acidify the extracts, a solution of 50% acetonitrile, 0.1% trifluoroacetic acid was added to each tube and all were sonicated for 5 min. Each extract (0.3 μ l) was spotted to three wells on an analysis plate. For each extract 0.75 μ l of 2, 5-dihydroxybenzoic acid (DHB), α -cyano-4-hydroxycinnamic acid (α -Cyano), or 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid) (Waters Corp., Milford, MA) was added to one of the spots. The samples in DHB matrix were analyzed in the 300 to 7500 Dalton range. Samples in α -Cyano and Sinapinic acid were analyzed in the 500 to 5000 and 500 to 7500 Dalton range, respectively. Protonated (MH⁺) peptide masses were monoisotopically resolved in reflector mode (Aebersold, 1993; Billeci & Stults, 1993). Calmix 2 was

used as the external calibrant (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) for the analysis. GPMW32 software (Lighthouse Data, Odense M, Denmark) was used to generate a theoretical trypsin digest of the CP4 EPSPS protein sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Known autocatalytic fragments from trypsin digestion and apparent modifications were identified in the raw data. The list of experimental masses was then compared to the theoretical list from the GPMW32 software. Those experimental masses within 1 Da of a theoretical mass were matched. All matching masses were tallied and a coverage map was generated for the mass fingerprint. The tryptic mass fingerprint coverage was considered acceptable if $\geq 40\%$ of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments.

5.6 Molecular Weight and Purity Estimation by SDS-PAGE

An aliquot of the test substance was mixed with 5 \times LB to a final total protein concentration of 0.08 $\mu\text{g}/\mu\text{l}$. Molecular weight markers (Bio-Rad broad-range) and reference substance were diluted to a final total protein concentration of 0.9 and 0.15 $\mu\text{g}/\mu\text{l}$, respectively. The test substance was analyzed in duplicate at 0.75, 1.5, and 2.25 μg protein per lane. The *E. coli*-produced CP4 EPSPS reference standard was analyzed at 0.75 μg total protein in a single lane. All samples were heated at $\sim 100^\circ\text{C}$ for 3 min and loaded onto a 10-well pre-cast Tris glycine 4 - 20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at a constant voltage of 150 V for 95 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) acetic acid for 30 min, stained for 16 hr and 40 min with Brilliant Blue G-Colloidal stain (Sigma-Aldrich, St. Louis, MO). Gels were destained for 30 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and for 6 hr and 15 min with 25% (v/v) methanol. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). The apparent molecular weight of each observed band was estimated from a standard curve generated by the Quantity One software which was based on the molecular weights of the markers and their migration distance on the gel. All visible bands within each lane were quantified using Quantity One software. Apparent molecular weight and purity were reported as an average of all six lanes containing the MON 87427-produced CP4 EPSPS protein.

5.7 MALDI-TOF Intact Mass Analysis

MALDI-TOF mass spectrometry was used to further characterize the MON 87427-produced CP4 EPSPS. Prior to MALDI-TOF MS analysis, an ethanol precipitation was performed to concentrate the MON 87427-produced CP4 EPSPS sample and remove buffer components that interfere with the MALDI-TOF MS analysis. The precipitated protein was re-suspended in 5 μl 60% formic acid. A portion of the MON 87427-produced CP4 EPSPS protein sample, and a BSA protein standard

(0.3 µl each), were spotted on an analysis plate, mixed with 0.75 µl of Sinapinic acid solution containing 0.3% Trifluoroacetic acid (TFA) and air-dried. Mass spectral analysis of the MON 87427-produced CP4 EPSPS protein was performed using an Applied Biosystems Voyager DE-Pro Biospectrometry Workstation MALDI-TOF MS instrument with the supplied Data Explorer software (version 4.0.0.0, Foster City, CA). Mass calibration of the instrument was performed using the BSA protein standard. The sample was analyzed in the 2,000 to 100,000 Dalton range using 150 shots at a laser intensity setting of 3316 (a unit-less MALDI-TOF instrument specific value). Average protonated (MH⁺) protein masses were observed in linear mode (Aebersold, 1993; Billeci & Stults, 1993). GPMW32 software (Lighthouse Data, Odense M, Denmark) was used to generate a theoretical mass of the expected CP4 EPSPS protein sequence based upon the nucleotide sequence. The mass of the MON 87427-produced CP4 EPSPS protein was reported as an average of three separate mass spectral acquisitions.

5.8 Glycosylation Analysis

Glycosylation analysis was used to determine whether the MON 87427-produced CP4 EPSPS was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the MON 87427-produced CP4 EPSPS protein, the *E. coli*-produced CP4 EPSPS reference standard, and the positive controls, transferrin (GE Healthcare, Piscataway, NJ) and horseradish peroxidase (Sigma-Aldrich, St Louis, MO), were each diluted with water and mixed with 5 × LB. These samples were heated at ~98 °C for 3 min, cooled, and each was loaded at approximately 30 and 60 ng per lane on a Tris-glycine 10-well 4 - 20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA). Precision Plus ProteinTM Dual color Standards (Bio-Rad, Hercules, CA) were also loaded to verify electrotransfer of the proteins to the membrane and CandyCaneTM Glycoprotein Molecular Weight Standards (Molecular Probes, Eugene, OR) were loaded as positive controls and markers for molecular weight. Electrophoresis was performed at a constant voltage of 150 V for 80 min. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 105 min at a constant voltage of 25 V.

Carbohydrate detection was performed directly on the PVDF membrane at room temperature using the Pro-Q® Emerald 488 Glycoprotein Gel and Blot Stain Kit (Molecular Probes, Eugene, OR). With this kit, carbohydrate moieties are detected by fluorescence which is produced when Pro-Q Emerald 488 glycoprotein stain reacts with periodate oxidation carbohydrates conjugated to proteins. An image of the final blot containing the fluorescent-labeled glycoproteins was captured using the Bio-Rad Pharos FX Molecular Imager System using the Alexa 488 band pass setting and equipped with Quantity One® software (version 4.6).

After glycosylation analysis the blot was stained to visualize the proteins present on the membrane. Proteins were stained for 30 sec to 2 min using Coomassie Brilliant

Blue R-250 staining solution (Bio-Rad, Hercules, CA) and then destained with 1× destain solution (Bio-Rad, Hercules, CA) for 5 min. After washing with water, the blot was scanned using Bio-Rad GS-800 densitometer with the supplied Quantity One® software (version 4.4.0, Hercules, CA).

5.9 Functional Activity Assay

Prior to functional activity analysis, both test and reference proteins were diluted to a purity corrected concentration of ~50 µg/ml with a 50 mM HEPES, pH 7.0 buffer. Assays for both proteins were conducted in triplicate. The reactions were performed in 50 mM HEPES (pH 7.0), 0.1 mM ammonium molybdate, 1 mM PEP and 5 mM potassium fluoride with or without 2 mM S3P for 2 min at ~25 °C. The reactions were initiated by the addition of PEP. After 2 min, the reactions were quenched with malachite green (phosphate assay reagent) and then fixed with 33% (w/v) sodium citrate. A standard curve was prepared using 0 to 10 nmoles of inorganic phosphate in water treated with the malachite green (phosphate assay) reagent and 33% (w/v) sodium citrate. The absorbance of each reaction and each standard was measured in duplicate at 660 nm using a PowerWave Xi (Bio-Tek, Richmond, VA) microplate reader. The amount of inorganic phosphate released from PEP in each reaction was determined using the standard curve. For CP4 EPSPS, the specific activity was defined in unit per mg of protein (U/mg), where a unit (U) is defined as 1 µmole of inorganic phosphate released from PEP per min at 25 °C. Calculations of the specific activities were performed using Microsoft Excel 2007 (12.0.6504.5001) SP1 MSO (12.0.6320.5000).

5.10 Storage Stability

The short-term storage stability of the MON 87427-produced CP4 EPSPS protein in a -80 °C freezer over the experimental phase of the study was evaluated by comparing the purity and molecular weight values obtained on day 0 to the purity and molecular weight values obtained on day 14 of storage. Day 0 stability analysis corresponds to the purity and molecular weight determination described in Section 5.6. On day 14, an aliquot was removed from a ~-80 °C freezer and mixed with 5× LB to a final protein concentration of 0.08 µg/µl, heated at ~100 °C for 3 min, and loaded in duplicate at three amounts (0.75, 1.5, and 2.25 µg per lane) onto a 10-well Tris-glycine 4 - 20% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at a constant voltage of 150 V for 85 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) acetic acid for 30 min, stained for 18 hr and 1 min with Brilliant Blue G-Colloidal stain (Sigma-Aldrich, St. Louis, MO). Gels were destained for 30 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and again for 6 hr and 45 min with 25% (v/v) methanol. The protein sample was considered to be stable if purity and molecular weight of the test protein is within ± 10% of the reference protein.

5.11 Equivalence Criteria

The equivalence of the MON 87427-produced and *E. coli*-produced CP4 EPSPS proteins was determined based on four characteristics. The two proteins were considered equivalent if they meet the following criteria:

1. Immunoreactivity with CP4-specific antibodies: the immunoreactive signal of the test protein should be within $\pm 35\%$ of the reference protein.
2. Molecular weight: the apparent molecular weight, by SDS-PAGE, of the test protein should be within $\pm 10\%$ of the reference protein as reported in its COA.
3. Activity assay: the functional activity of the test protein should be within 2-fold of the functional activity of the reference protein.
4. Glycosylation status: both test and reference proteins are not glycosylated.

6.0 Data Rejected or Not Reported

Data from two experiments were rejected.

1. One blot for N-terminal sequence analysis was rejected because the criterion for effective transfer of the protein markers to blot membrane mentioned in the SOP BR-ME-0924-01 was not met.
2. A positively stained band in the Plant CP4 EPSPS lanes at ~30 kDa was observed on the blot processed for glycoprotein detection (lower MW than that expected for CP4 EPSPS). The blot was stained for total protein with Coomassie Blue R-250. In addition to the expected band at ~44kDa, a protein band (corresponding to the glycosylation signal at ~30 kDa) was observed in plant CP4 EPSPS lanes. The additional band was not observed in the previous blots and gels performed for N-terminal sequence analysis, immuno-staining, or purity. Based on these data, it is most likely that the aliquot of plant CP4 EPSPS used for glycosylation analysis was contaminated with unknown protein and hence, this set of data was rejected.

7.0 Deviations

There were two SOP deviations.

1. During MALDI-TOF-MS tryptic mass fingerprinting, the analyst used a lower concentration of acetonitrile and trifluoroacetic acid to solubilize sinapinic acid than that stated in the SOP BR-EQ-783-02. As high quality ion data was observed from the mass data acquired from this sinapinic acid solution, the results of this study have not been impacted by using a different solubilization buffer for the sinapinic acid.
2. The cartridge blocks were not washed in $\geq 70\%$ nitric acid for more than 2 months prior to the N-terminal sequence analysis as mentioned in the SOP BR-EQ-0265-02. There is no observable effect from inadvertently missing the cartridge block wash routine

maintenance and the results from the sequence analysis for this study were considered valid.

8.0 Control of Bias and Quality Measures

Controls and standards were included with each analysis. A protein standard (β -lactoglobulin) was sequenced before and after N-terminal sequence analysis of the MON 87427-produced CP4 EPSPS protein sample to ensure instrument performance. CalMix #2 from the Sequazyme Peptide Mass Standards kit (Applied Biosystems, Foster City, CA) was used to perform an external mass calibration of the MALDI-TOF MS. Replicate analyses were used for the quantitative immunoblot, purity and MW determination, stability, and glycosylation analyses. For the glycosylation analysis, transferrin and HRP were used as the positive controls while the *E. coli*-produced CP4 EPSPS protein served as negative control. The *E. coli*-produced CP4 EPSPS was also used as a reference standard for the CP4 EPSPS functional activity assay and for the Bio-Rad protein assay.

9.0 Results and Discussion

9.1 Protein Concentration

The concentration of the MON 87427-produced CP4 EPSPS was determined to be 0.1 mg/ml based on the Bio-Rad protein assay (Table 1).

9.2 Protein Identity

The identity of the MON 87427-produced CP4 EPSPS protein was confirmed using three analytical methods: immunoblot analysis, N-terminal sequencing, and MALDI-TOF MS tryptic mass map analysis. In addition, immunoblot analysis using anti-CP4 EPSPS antibody was used to confirm equivalent immunoreactivity of the MON 87427- and *E. coli*-produced proteins.

9.2.1 Immunoblot Analysis Using an Anti-CP4 EPSPS Antibody

Immunoreactive bands of comparable intensity migrating at the expected apparent MW were observed for lanes loaded with either the MON 87427-produced (Figure 1, lanes 9-14) or *E. coli*-produced CP4 EPSPS proteins (Figure 1, lanes 2-7). As expected, the signal intensity increased with increasing amounts of the MON 87427- and *E. coli*-produced proteins loaded on the gel. No additional bands were observed in either protein sample. Hence, the immunoblot analysis confirmed the identity of the MON 87427-produced CP4 EPSPS protein. Densitometric analysis of the bands showed an average difference of 9.6% between the intensity of the signals from the MON 87427-produced CP4 EPSPS protein and the signals from the *E. coli*-produced CP4 EPSPS reference standard (Table 2). Because the difference was within the previously set acceptance criterion of $\pm 35\%$, MON 87427- and *E. coli*-produced proteins are considered to have equivalent immunoreactivity.

9.2.2 N-Terminal Sequencing

N-terminal sequencing performed on the MON 87427-produced CP4 EPSPS resulted in 15 amino acid residues being determined (Figure 2). The sequence obtained is identical to that of the mature CP4 EPSPS protein deduced from the *cp4 epsps* gene present in grain of MON 87427 (Arackal et al., 2009) after processing of the chloroplast transit protein and the N-terminal methionine (Arfin & Bradshaw, 1988; Bradshaw et al., 1998). The N-terminal sequence information, therefore, confirms the identity of the CP4 EPSPS protein isolated from the grain of MON 87427.

9.2.3 MALDI-TOF Tryptic Mass Fingerprint Analysis

The identity of the MON 87427-produced CP4 EPSPS protein was also confirmed by MALDI-TOF mass spectrometry analysis of tryptic peptide fragments prepared from the MON 87427-produced CP4 EPSPS protein. The ability to identify a protein using this method is dependent upon matching a sufficient number of observed tryptic peptide fragment masses with predicted tryptic peptide fragment masses. In general, protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen et al., 1997).

There were 26 unique peptides identified that corresponded to the expected masses of peptides produced from trypsin-digested CP4 EPSPS (Table 3). The identified masses were used to assemble a mass fingerprint map of the entire CP4 EPSPS protein (Figure 3). The experimentally determined mass coverage of the CP4 EPSPS protein was 70.3% (320 out of 455 amino acids). This analysis serves as additional identity confirmation for the MON 87427-produced CP4 EPSPS protein.

9.3 Molecular Weight and Purity Determination

For molecular weight and purity analysis, the MON 87427-produced CP4 EPSPS protein was separated using SDS-PAGE, the gel stained with Brilliant Blue G Colloidal stain (Sigma-Aldrich, St. Louis, MO), and analyzed by densitometry (Figure 4). The data are summarized in Table 4. The MON 87427-produced CP4 EPSPS protein (Figure 4, lanes 3-8) migrated to the same position on the gel as the *E. coli*-produced CP4 EPSPS reference standard (Figure 4, lanes 2) and had an apparent molecular weight of 44.1 kDa (Table 4). The apparent molecular weight of the *E. coli*-produced CP4 EPSPS reference standard, as reported on the COA (Appendix 2), is 43.8 kDa. The difference in apparent molecular weight between the MON 87427- and *E. coli*-produced CP4 EPSPS proteins was 0.7% (Table 5). Because this difference met the previously set acceptance criteria ($\leq 10\%$ difference), the MON 87427- and *E. coli*-produced CP4 EPSPS proteins are considered equivalent based on their experimentally estimated apparent molecular weights.

The purity of the MON 87427-produced CP4 EPSPS protein was calculated based on the six loads on the gel (Figure 4, lanes 3 to 8). The average purity was determined to be 96% (Table 4).

The intact mass of the MON 87427-produced CP4 EPSPS protein was also determined by MALDI-TOF MS analysis. The average obtained from three measurements of the intact mass of the MON 87427-produced CP4 EPSPS protein was 47552 Da. This value is comparable to the theoretical mass of the full-length protein without N-terminal methionine (47481 Da) within the accuracy window of the MALDI-TOF MS instrument.

9.4 Glycosylation Analysis

Many eukaryotic proteins are post-translationally modified with carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex, branched polysaccharide structures or simple monosaccharides. In contrast, glycosylation in prokaryotes is uncommon. In *E. coli*, the organism used to produce the reference protein used in this study, only a few specific proteins have been confirmed to be glycosylated (Sherlock et al., 2006). To test whether potential post-translational glycosylation of the MON 87427-produced CP4 EPSPS occurred, it was analyzed for the presence of covalently bound carbohydrate moieties. The *E. coli*-produced CP4 EPSPS reference standard was used as a negative control since it has previously been shown to be free of glycosylation (e.g., Bonner et al., 2003). Horseradish peroxidase and transferrin were both used as positive controls. Both the negative and positive controls were analyzed concurrently with the MON 87427-produced CP4 EPSPS protein. The results of this analysis are shown in Figure 5A. The positive controls were clearly detected at the expected molecular weights, in a concentration-dependent manner (Figure 5A, lanes 2-5). Faint signals at a level slightly above the background noise were observed for the reference protein as well as the test protein at the molecular weight expected for CP4 EPSPS (Figure 5A, lanes 6-9). While the reference protein has previously been shown to be free of glycosylation (e.g., Bonner et al., 2003), it is not unprecedented for a faint signal to be observed for this protein in a glycosylation assay (e.g. Karunanandaa et al., 2003). The *E. coli*-produced CP4 EPSPS is not glycosylated, therefore, the weak signal observed for both this protein as well as the MON 87427-produced CP4 EPSPS test protein are not indicative of a glycosylated species. Other data collected under this study demonstrated the absence of glycosylation of the MON 87427-produced CP4 EPSPS. In particular, glycosylation would result in an increase in the protein mass relative to the theoretically calculated mass. The agreement of the observed protein mass of the MON 87427-produced CP4 EPSPS protein (47552 Da) to the theoretical mass (47481Da) does not support the existence of a glycosylated species, as the addition of even a single sugar would increase the mass by at least 160 Da. Finally, to confirm that the proteins were transferred to the membrane, the same membrane was stained with Coomassie Blue R 250 and scanned again (Figure 5B). The image

demonstrates that CP4 EPSPS was efficiently transferred to the membrane. Thus, the data cited above demonstrate that MON 87427-produced protein is not glycosylated and is equivalent to the *E. coli*-produced CP4 EPSPS reference standard.

9.5 Functional Activity

The results of the specific activity assay are presented in Table 6. The specific activity of MON 87427- and *E. coli*-produced CP4 EPSPS proteins were measured to be 8.67 U/mg and 5.41 U/mg of CP4 EPSPS, respectively. Because the value of MON 87427-produced CP4EPSPS specific activity falls within 2-fold of the *E. coli*-produced CP4 EPSPS value (between 2.71 U/mg and 10.82 U/mg), the previously set acceptance criteria was met and the MON 87427-produced CP4 EPSPS protein is considered to have equivalent functional activity to that of the *E. coli*-produced protein.

9.6 Storage Stability

Stability of the MON 87427-produced CP4 EPSPS protein stored in a -80 °C freezer for a 14 day period was assessed (Tables 7 and 8, Figure 6). Stability was evaluated by comparison of the apparent molecular weight and purity of the protein after storage (day 14) to the initial apparent molecular weight and purity values determined on day 0. The molecular weight of the CP4 EPSPS protein was determined to be 44.0 kDa on day 14. This value differed from the molecular weight obtained on day 0 (44.1 kDa) by 0.23% (Table 8). The purity value for the MON 87427-produced CP4 EPSPS protein (Figure 6, Lanes 2-7) was determined to be 99% by densitometric analysis. This value differed from the purity obtained on day 0 (96%) by 3% (Table 8). Therefore, based on the apparent molecular weight and purity, the MON 87427-produced CP4 EPSPS protein was stable when stored in a ~-80 °C freezer for the duration of the experimental phase of this study (14 days).

10.0 Conclusions

A panel of analytical techniques was used to characterize the MON 87427-produced CP4 EPSPS protein purified from grain of MON 87427. Identity of the MON 87427-produced CP4 EPSPS was confirmed by recognition with anti-CP4 EPSPS antibodies, identification of the first 15 amino acids of the N-terminus by amino acid sequencing, and mapping of tryptic peptides that yielded a 70.3% overall coverage of the expected protein sequence. The concentration of the MON 87427-produced CP4 EPSPS was 0.1 mg/ml. The purity and apparent molecular weight of the MON 87427-produced CP4 EPSPS was 96% and 44.1 kDa, respectively. MALDI-TOF mass spectrometry analysis of the intact protein resulted in an average mass of 47552 Da, reflecting the expected mass of the protein minus the N-terminal methionine. The MON 87427-produced CP4 EPSPS protein was not glycosylated and had a specific activity of 8.67 U/mg of CP4 EPSPS. Finally, the MON 87427-produced CP4 EPSPS protein was stable for at least 14 days while stored in a ~-80 °C freezer, which encompassed the duration of the experimental phase of this study.

The equivalence of the MON 87427- and *E. coli*-produced CP4 EPSPS proteins was evaluated by comparing their apparent molecular weight, immunoreactivity with anti-CP4 EPSPS antibodies, glycosylation status, and functional activity. The results obtained demonstrate that the MON 87427-produced CP4 EPSPS protein is equivalent to the *E. coli*-produced CP4 EPSPS protein. This equivalence justifies the use of the previously conducted protein safety studies whereby the *E. coli*-produced CP4 EPSPS protein was used as a test substance.

11.0 References

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Table 1. Concentration of the MON 87427-Produced CP4 EPSPS Protein.

The concentration of the MON 87427-produced CP4 EPSPS protein was determined by Bio-Rad protein assay.

Sample	Concentration (mg/ml) ¹	RSD ²
MON 87427-produced CP4 EPSPS	0.1	4%

¹Value refers to mean calculated based on n=3. The result of the concentration determination was rounded to one decimal place.

²Relative standard deviation about the mean.

Table 2. Immunoreactivity of the MON 87427-Produced and *E. coli*-Produced CP4 EPSPS Proteins with CP4 EPSPS-Specific Antibodies.

The immunoreactivity of the MON 87427-produced CP4 EPSPS was compared to that of the *E. coli*-produced reference standard. The average difference in the band intensities obtained from Figure 1 is reported. Calculations are shown below. The 5 min exposure was used for this analysis.

Sample	Gel lane	Amount (ng)	Contour Qty (OD x mm ²)	Average Contour Qty	Percent difference ² (%)	Average Difference ³ (%)	
<i>E. coli</i> CP4 EPSPS	2	1	1.201	1.106	14.96	9.6	
<i>E. coli</i> CP4 EPSPS	3	1	1.011				
Plant CP4 EPSPS	9	1	1.346				
Plant CP4 EPSPS	10	1	1.255				
<i>E. coli</i> CP4 EPSPS	4	2	2.130	2.308	6.46		9.6
<i>E. coli</i> CP4 EPSPS	5	2	2.486				
Plant CP4 EPSPS	11	2	2.829				
Plant CP4 EPSPS	12	2	2.106				
<i>E. coli</i> CP4 EPSPS	6	3	3.310	3.388	7.37	9.6	
<i>E. coli</i> CP4 EPSPS	7	3	3.466				
Plant CP4 EPSPS	13	3	3.433				
Plant CP4 EPSPS	14	3	3.882				

¹Average Density = $\sum[\text{Density}]/2$

²Percent Difference (%) = $\frac{|\text{Average Density plant} - \text{Average Density E.coli}|}{\text{Average Density plant}} \times 100\%$

³Average difference (%) = $\sum[\% \text{ difference}]/3$. The standard deviation was calculated using Microsoft Office Excel 2007 (12.0.6504.5001) SP1 MSO (12.0.6320.5000).

Table 3. Summary of the Tryptic Masses Identified for the MON 87427-Produced CP4 EPSPS Using MALDI-TOF MS.

Matrix									
α -Cyano	α -Cyano	DHB	DHB	Sinapinic acid	Sinapinic acid	Expected Mass ¹	Diff. ²	AA Position ³	Fragment
Extract 1	Extract 2	Extract 1	Extract 2	Extract 1	Extract 2				
506.08						506.22	0.14	354-357	ESDR
599.17						599.33	0.16	29-33	SISHR
616.17	616.32				615.67	616.34	0.17	128-132	RPMGR
629.16						629.29	0.13	201-205	DHTEK
629.16						629.34	0.18	383-388	GRPDGK
711.26	711.43	711.30				711.45	0.19	133-138	VLNPLR
835.17						835.39	0.22	62-69	AMQAMGAR
863.23						863.46	0.23	15-23	SSGLSGTVR
872.21		872.29				872.45	0.24	313-320	GVTVPEDR
872.21		872.29				872.52	0.31	358-366	LSAVANGLK
948.26	948.48	948.32	948.44			948.52	0.26	161-168	TPTPIYR
991.29						991.55	0.26	14-23	KSSGLSGTVR
1115.27		1115.36		1114.83		1115.57	0.30	295-305	LAGEDVADLR
1357.32	1357.65	1357.44				1357.71	0.39	146-157	SEDGDRLPVTLR
1359.27	1359.58	1359.39	1359.56	1358.90		1359.72	0.45	354-366	ESDRLSAVANGLK
1359.27	1359.58	1359.39	1359.56	1358.90		1359.64	0.37	34-46	SFMFGGLASGETR
		1558.50	1558.65			1558.83	0.35	47-61	ITGLLEGDVINTGK
1646.34	1646.70	1646.52	1646.92			1646.84	0.50	389-405	GLGNASGAATHLDHR
1763.29						1763.81	0.52	367-382	LNGVDCDEGETSLVVR
1993.38	1993.80	1993.60	1993.68	1993.21		1993.97	0.59	206-224	MLQGFGANLTVETDADGVR
2182.54	2183.00	2182.77	2182.92	2182.40	2182.84	2183.17	0.63	275-294	TGLILTLQEMGADIEVINPR
2366.61	2367.14	2366.86	2366.96	2366.66		2367.33	0.72	178-200	SAVLLAGLNTPGITTVIEPIMTR
				2449.44		2450.23	0.79	24-46	IPGDKSISHRSFMFGGLASGETR
				2449.44		2450.22	0.78	105-127	LTMGLGVVYDFDSTFIGDASLTK
3250.78(AVE)		3251.23(AVE)		3250.80(AVE)	3252.37(AVE)	3251.75	0.97	321-351	APSMIDEYPIAVAAAAFAGATVMNGLEELR
		4190.17(AVE)		4190.98(AVE)	4190.14(AVE)	4180.89	0.72	234-274	LTGQVIDVPGDPSSTAFPLVAALLVPGSDVTILNVLMPNTR

¹Only experimental masses that matched expected masses are listed in the table.

²The numbers represent the difference between the expected mass and the experimental mass listed within the first row. Other experimental masses shown within a row also met the criteria of being within 1 Da of the expected mass.

³AA position refers to amino acid position within the predicted CP4 EPSPS sequence as depicted in Figure 3.

AVE indicates that the experimental mass average of the observed peptide was compared to the expected peptide masses. For larger peptides the monoisotopic mass is, in general, poorly resolved and therefore the mass average is used for comparison.

Table 4. Apparent Molecular Weight and Purity Analysis of the MON 87427-Produced CP4 EPSPS Protein.

The apparent molecular weight and the purity of the MON 87427-produced CP4 EPSPS protein were determined by densitometric analysis of SDS polyacrylamide gel (Day 0) shown in Figure 4. Final molecular weight was rounded to one decimal place.

Total protein loaded	Apparent MW (kDa)	Purity (%)
0.75 µg in lane 3	43.90	95.3
0.75 µg in lane 4	43.97	96.0
1.5 µg in lane 5	44.41	97.2
1.5 µg in lane 6	44.17	97.6
2.25 µg in lane 7	44.21	97.8
2.25 µg in lane 8	44.16	92.3
Average	44.1	96

Table 5. Molecular Weight Difference Between the MON 87427- and *E. coli*-Produced CP4 EPSPS Proteins.

Molecular Weight of MON 87427-Produced CP4 EPSPS Protein ¹	Molecular Weight of <i>E. coli</i> -Produced CP4 EPSPS Protein ²	% Difference from <i>E. coli</i> -Produced CP4 EPSPS Protein ³
44.1 kDa	43.8 kDa	0.7%

¹See Table 4 for the MW of the MON 87427-produced CP4 EPSPS.

²See COA (Appendix 2) for the MW of the *E. coli*-produced reference standard.

³% Difference = $\left| \frac{(\text{MW plant} - \text{MW } E \text{ coli})}{\text{MW plant}} \right| \times 100\%$

Table 6. Specific Activity of MON 87427-Produced CP4 EPSPS.

The specific activity of the MON 87427-produced CP4 EPSPS protein was determined using a phosphate release assay. This end-point type colorimetric assay measures the release of inorganic phosphate from PEP by the action of the CP4 EPSPS enzyme.

MON 87427-produced CP4 EPSPS¹ (U/mg)	<i>E. coli</i>-produced CP4 EPSPS¹ (U/mg)	Previously set acceptance limits² (U/mg)
8.67 ± 0.23	5.41 ± 0.37	2.71-10.82

¹Value refers to mean and standard deviation calculated based on n = 6 which includes three replicate assays spectrophotometrically analysed at 660 nm in duplicate.

²Within 2-fold of the *E.coli*-produced CP4 EPSPS specific activity (5.41 ÷ 2 U/mg to 5.41 x 2 U/mg)

Table 7. Molecular Weight and Purity Estimation for the MON 87427-Produced CP4 EPSPS after 14 Days of Storage in a -80 °C Freezer.

The day 14 purity and molecular weight values for the MON 87427-produced CP4 EPSPS protein were determined by densitometric analysis of the SDS polyacrylamide gel shown in Figure 6. Molecular weight was rounded to one decimal place.

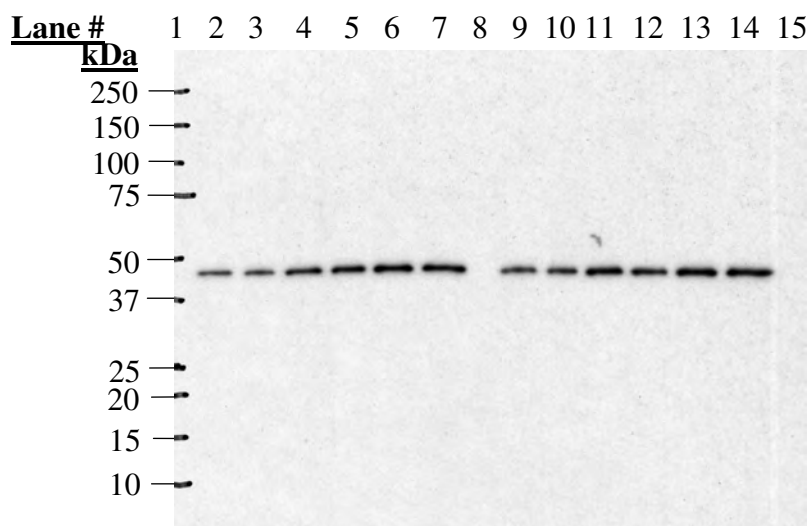
Total protein loaded	Apparent MW (kDa)	Purity (%)
0.75 µg in lane 3	44.19	98.4
0.75 µg in lane 4	44.20	98.3
1.5 µg in lane 5	43.82	98.8
1.5 µg in lane 6	43.71	98.0
2.25 µg in lane 7	43.69	98.6
2.25 µg in lane 8	44.12	99.1
Average	44.0	99

Table 8. Storage Stability (Day 14 vs. Day 0) of the MON 87427-Produced CP4 EPSPS Protein at ~ -80 °C.

The purity and molecular weight values of the MON 87427-produced CP4 EPSPS protein are shown in Table 4 at day 0 and in Table 7 at day 14.

Apparent MW of CP4 EPSPS (kDa)			Purity (%)		
Day 0	Day 14	% Difference ¹ (Day 14 vs. 0)	Day 0	Day 14	% Difference ¹ (Day 14 vs. 0)
44.1	44.0	0.23	96	99	3

$$^1\% \text{ Difference} = \left| \frac{(\text{Day 0} - \text{Day 14})}{\text{Day 0}} \right| \times 100\%$$



<u>Lane</u>	<u>Sample</u>	<u>Amount (ng)</u>
1	Precision Plus Protein™ Standards Dual color	—
2	<i>E. coli</i> -produced CP4 EPSPS reference standard	1
3	<i>E. coli</i> -produced CP4 EPSPS reference standard	1
4	<i>E. coli</i> -produced CP4 EPSPS reference standard	2
5	<i>E. coli</i> -produced CP4 EPSPS reference standard	2
6	<i>E. coli</i> -produced CP4 EPSPS reference standard	3
7	<i>E. coli</i> -produced CP4 EPSPS reference standard	3
8	Empty	
9	MON 87427-produced CP4 EPSPS protein	1
10	MON 87427-produced CP4 EPSPS protein	1
11	MON 87427-produced CP4 EPSPS protein	2
12	MON 87427-produced CP4 EPSPS protein	2
13	MON 87427-produced CP4 EPSPS protein	3
14	MON 87427-produced CP4 EPSPS protein	3
15	Empty	

Figure 1. Western Blot Analysis and Immunoreactivity of MON 87427- and *E. coli*-Produced CP4 EPSPS.

Aliquots of the MON 87427-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS reference standard were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with anti-CP4 EPSPS antibodies and immunoreactive bands were visualized using an ECL system and film. Approximate MWs (kDa) are shown on the left and correspond to the markers loaded in lane 1. The 5 min exposure is shown.

Amino acid residue # from the N-terminus ¹ →	1				5					10					15		
Expected Sequence	M	L	H	G	A	S	S	R	P	A	T	A	R	K	S	S	
Experimental Sequence		L	H	G	A	S	S	R	P	A	T	A	R	K	S	S	

Figure 2. N-Terminal Sequence of the MON 87427-Produced CP4 EPSPS.

The expected amino acid sequence of the N-terminus of the mature CP4 EPSPS protein was deduced from the *cp4 epsps* gene present in MON 87427. The experimental sequence obtained from the MON 87427-produced CP4 EPSPS was compared to the expected sequence beginning at position 2.

¹ The single letter IUPAC-IUB amino acid code is **M**, methionine; **L**, leucine; **H**, histidine; **G**, glycine; **A**, alanine; **S**, serine; **R**, arginine; **P**, proline; **T**, threonine; **K**, lysine.

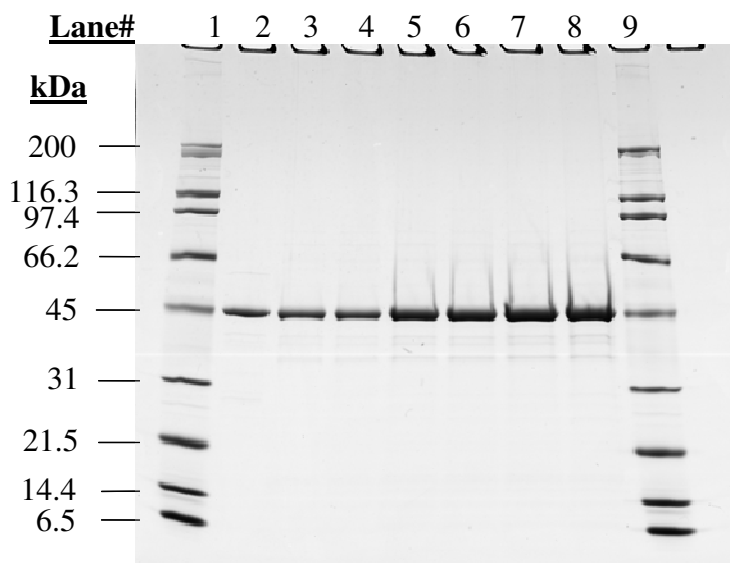
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001  MLHGASSRPA TAR[KSSGLSG TVRIPGDKSI SHRSFMFGGL ASGETRITGL
051  [LEGEDVINTG KAMQAMGAR]I RKEGDTWIID GVGNGGLLAP EAPLDFGNAA
101  TGCR[LTMGLV GYDFDSTFI GDASLTRKPM GRVLNPLR]EM GVQVK[SEDGD
151  [RLPVTLR]GPK [TPTPITYR]VP MASAQVK[SAV LLAGLNTPGI TTVIEPIMTR
201  [DHTEKMLQGF GANLTVETDA DGVR]TIRLEG RGK[LTGQVID VPGDPSSTAF
251  [PLVAALLVPG SDVTILNVLM NPTRTGLILT LQEMGADIEV INPRLAGGED
301  [VADLR]VRSST LK[GVTVPEDR APSMIDEYPI LAVAAFAEG ATVMNGLEEL
351  [RVK]ESDRLSA VANGLKLNGV DCDEGETSLV VRGRPDGKGL GNASGAAVAT
401  [HLDHR]IAMSF LVMGLVSENP VTVDDATMIA TSFPEFMDLM AGLGAKIELS
451  DTKAA

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Figure 3. MALDI-TOF MS Coverage Map of the MON 87427-Produced CP4 EPSPS.

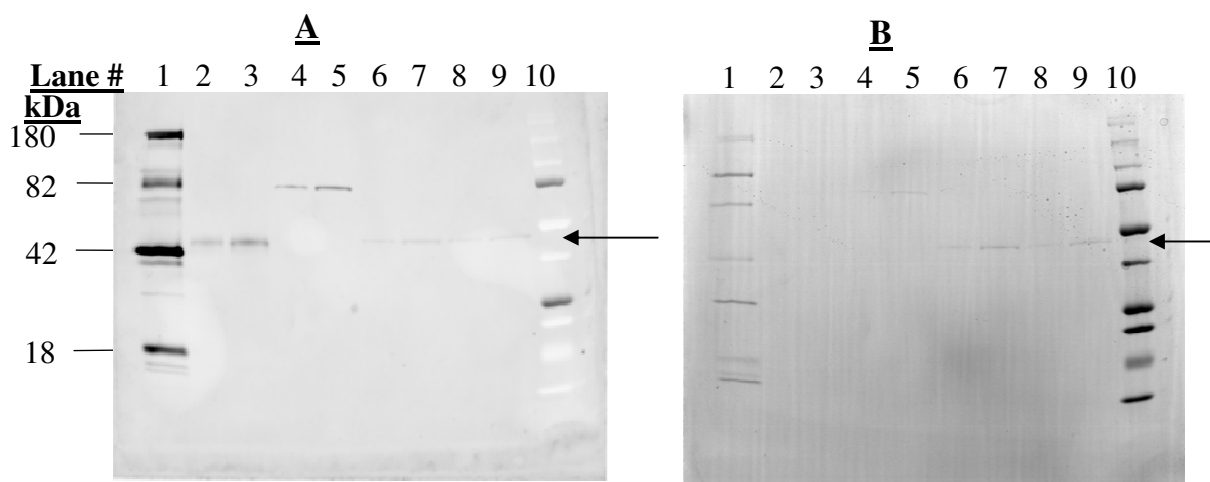
The amino acid sequence of the mature CP4 EPSPS protein was deduced from the *cp4 epsps* gene present in MON 87427. Boxed regions correspond to tryptic peptides that were identified from the MON 87427-produced CP4 EPSPS protein sample using MALDI-TOF MS. In total, 70.3% (320 of 455 total amino acids) of the expected protein sequence was identified.



<u>Lane</u>	<u>Sample</u>	<u>Amount (µg)</u>
1	Broad Range MW markers	4.5
2	<i>E. coli</i> -produced CP4 EPSPS reference standard	0.75
3	MON 87427-produced CP4 EPSPS protein	0.75
4	MON 87427-produced CP4 EPSPS protein	0.75
5	MON 87427-produced CP4 EPSPS protein	1.5
6	MON 87427-produced CP4 EPSPS protein	1.5
7	MON 87427-produced CP4 EPSPS protein	2.25
8	MON 87427-produced CP4 EPSPS protein	2.25
9	Broad Range MW markers	4.5

Figure 4. Purity and Molecular Weight Analysis of the MON 87427-Produced CP4 EPSPS Protein.

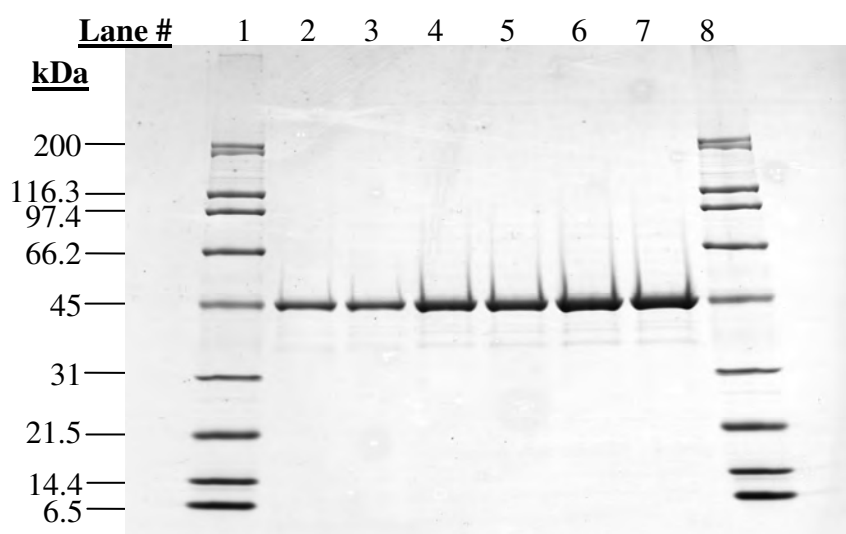
Aliquots of the MON 87427- and the *E. coli*-produced CP4 EPSPS proteins were separated on a 4-20% Tris glycine polyacrylamide gradient gel and then stained with Brilliant Blue G-Colloidal stain. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 9. Empty lane was partially cropped.



<u>Lane</u>	<u>Sample</u>	<u>Amount (ng)</u>
1	CandyCane Glycoprotein MW standards	—
2	Horseradish Peroxidase (positive control)	30
3	Horseradish Peroxidase (positive control)	60
4	Transferrin (positive control)	30
5	Transferrin (positive control)	60
6	MON 87427-produced CP4 EPSPS	30
7	MON 87427-produced CP4 EPSPS	60
8	<i>E. coli</i> -produced CP4 EPSPS (negative control)	30
9	<i>E. coli</i> -produced CP4 EPSPS (negative control)	60
10	Precision Plus Protein™ Standards Dual color	—

Figure 5. Glycosylation Analysis of the MON 87427-Produced CP4 EPSPS Protein.

Aliquots of the MON 87427-produced CP4 EPSPS protein, *E. coli*-produced CP4 EPSPS reference standard (negative control), horseradish peroxidase (positive control) and transferrin (positive control) were separated by SDS-PAGE (4-20%) and electrotransferred to a PVDF membrane. (A) Where present, periodate-oxidized protein-bound carbohydrate moieties reacted with Pro-Q Emerald 488 glycoprotein stain and emitted a fluorescent signal at 488 nm. The signal was captured using a Bio-Rad Molecular Imager FX. (B) The same blot was stained with Coomassie Blue R250 to confirm the presence of proteins. The signal was captured using a Bio-Rad GS800 with quantity one software (version 4.4.0). Approximate MWs (kDa) correspond to the glycosylated markers loaded in Lane 1 and the dual color markers (used to verify transfer) in Lane 10. Arrows indicate the band corresponding to CP4 EPSPS proteins.



<u>Lane</u>	<u>Sample</u>	<u>Amount (µg)</u>
1	Broad Range MW markers	4.5
2	MON 87427-produced CP4 EPSPS protein	0.75
3	MON 87427-produced CP4 EPSPS protein	0.75
4	MON 87427-produced CP4 EPSPS protein	1.5
5	MON 87427-produced CP4 EPSPS protein	1.5
6	MON 87427-produced CP4 EPSPS protein	2.25
7	MON 87427-produced CP4 EPSPS protein	2.25
8	Broad Range MW markers	4.5

Figure 6. Storage Stability of the MON 87427-Produced CP4 EPSPS.

SDS-PAGE analysis was performed on the MON 87427-Produced CP4 EPSPS sample stored in a -80 °C freezer for 14 days. The gel was stained with a Brilliant Blue G-Colloidal stain. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Empty lanes were partially cropped.

Appendix 1. List of Applicable SOP

<u>SOP Number</u>	<u>Title</u>
AG-ME-0388-03	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-EQ-0265-02	Applied Biosystems 494 Procise™ Protein Sequencing System
BR-EQ-0599-05	Bio-Rad GS-800 Densitometer
BR-EQ-0600-04	Bio-Tek PowerWave X _i Microplate Reader System
BR-EQ-0783-02	Applied Biosystems Voyager DE Pro Biospectrometry Workstation
BR-EQ-0935-01	Konica SRX X-Ray Film Processors
BR-EQ-1155-01	Bio-Rad PharosFX plus Molecular Imager System
BR-ME-0392-01	Western Blot Analysis (Immunoblotting)
BR-ME-0408-02	Phosphate Release Assay for Functionally Active EPSPS
BR-ME-0525-02	Bio-Rad protein assay
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-ME-0924-01	Electrotransfer of Proteins to Membranes
BR-ME-0926-01	Staining of Proteins on Blot Membranes
BR-ME-0932-03	Assessment of Immunoreactive Bands from Western Blots Exposed to X-Ray Films Using Bio-Rad GS-800 Densitometer
BR-ME-0956-03	Protein Percent Purity and Apparent Molecular Weight Determination
BR-ME-1252-01	Removal of Proteins from Complex Buffer Systems by Means of Ethanol Precipitation Prior to Analytical Analyses
BR-ME-1266-01	Staining of Carbohydrate Moieties on Blots Using the Pro-Q® Emerald 488 Glycoprotein Gel and Blot Stain Kit

Appendix 2. Certificate of Analysis APS Lot number 20-100015 (Orion Lot# 10000739)

**Analytical Protein Standard
Certificate of Analysis**

MONSANTO

ANALYTICAL PROTEIN STANDARDS

Re-characterization No. 8

Sample Information:

Name of APS E. coli-produced CP4 EPSPS	Orion Lot Number 10000739	Expiration Date July 31, 2010
Common or Alias Name(s) None	Historical APS Lot Number(s) 20-100015	Storage Requirements (until use) -80 °C
Source: Fermentation of <i>Escherichia coli</i> containing the pMON21134 expression plasmid.		Comment(s) None
Additional Background Information: None		

Re-characterization Information

Characteristic	Method	Assay Date
Concentration	Amino Acid analysis	21 May 2009
Purity/Molecular weight	SDS-PAGE/Densitometry	3 June 2009
Activity	Phosphate release assay	15 June 2009

Characteristic	Method	Assay Date	Result
Concentration	Amino Acid analysis	10 March 2003	3.8 mg/mL (total protein)
Purity	SDS-PAGE/Densitometry	10 April 2003	97%
Molecular weight	SDS-PAGE/Densitometry	10 April 2003	45.0 kDa
Molecular weight	MALDI-TOF MS	17 March 2003	47466.1 Da
Identity	Immunoblot	3 April 2003	Confirmed
Identity	N-terminal sequence	19 March 2003	Confirmed MLI-CASSHPATA(N)KS
Identity	MALDI-TOF MS	18 March 2003	Confirmed (64% coverage of expected sequence)
Activity	Phosphate release assay	26 March 2003	4.86 Units/mg CP4 EPSPS

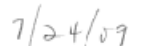
Buffer composition: 50 mM Tris-Cl, pH 7.5, 50 mM KCl, 2 mM DTT, 25% (v/v) glycerol and 1 mM benzamide-HCl
Physical description: Clear colorless solution

Purity corrected concentration is 3.7 mg/mL (3.8 mg/mL × 0.97 = 3.7 mg/mL)


Quality Assurance Specialist


Date


Analytical Protein Standards Officer


Date

Appendix 3. Notes for Reviewer

MSL 0022391 was amended to reword the summary in order to make the flow similar to the summaries found in previous protein characterization and equivalence study reports. This change had no impact on the study because it only changed the writing style used in the report.

Page Number in MSL0022391	Change
1	Added “Amended Report for MSL0022391:” to Study Title, “Amended Report Completed on”, and revised report completion date.
4	Added “Amended Report for MSL0022391:” to Study Title, and added “Draft Report Amendment 1 Review” to list of reviews and date for audit.
6	Changed MSL number, added “Amended Report for MSL0022391:” to Title, added “Original” to Study Completion Date, and added “Amendment 1 Report Completion Date”
8	Added “Appendix 3. Notes for Reviewer” to List of Appendices.
12	Removed: “The following analyses were performed to characterize the MON 87427-produced CP4 EPSPS protein. The total protein concentration of the MON 87427-produced CP4 EPSPS protein was measured using the Bio-Rad protein assay and determined to be 0.1 mg/ml. The identity of the MON 87427-produced CP4 EPSPS protein was confirmed by immunoblot analysis carried out with previously characterized anti-CP4 EPSPS antibodies. Anti-CP4 EPSPS antibodies specifically detected the MON 87427-produced CP4 EPSPS protein. Additionally, identity was confirmed by N-terminal sequence analysis and matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analysis of the trypsin digested CP4 EPSPS protein. The expected N-terminal sequence was observed and peptide masses consistent with the expected peptide masses for the trypsin digested CP4 EPSPS protein were observed. For the latter, the identified peptide masses yielded coverage of more than 70% of the expected peptide sequence (320 out of 455 amino acids) of the CP4 EPSPS protein. Purity and apparent molecular weight of the

MON 87427-produced CP4 EPSPS protein were determined using densitometric analysis of a Colloidal Brilliant Blue G stained SDS-PAGE gel. Purity of the MON 87427-produced CP4 EPSPS protein was 96% and the apparent molecular weight was 44.1 kDa. The average mass of the intact MON 87427-produced protein was 47552 Da as measured by MALDI-TOF MS analysis. This measurement was consistent with the theoretical mass of the CP4 EPSPS protein (47481 Da). Finally, SDS-PAGE was used to determine the purity and molecular weight (MW) of the protein after storage at ~ -80 °C, and the MON 87427-produced CP4 EPSPS protein was shown to be stable throughout the experimental phase (14 days).

The equivalence of the MON 87427- and *E. coli*-produced CP4 EPSPS proteins was assessed by comparing their apparent molecular weight, immunoreactivity, functional activity, and glycosylation status. The difference in MW between the MON 87427- and *E. coli*-produced protein on a SDS polyacrylamide gel was 0.7%. The difference in immunoreactivity between the MON 87427- and *E. coli*-produced proteins was 9.6% based on densitometric analysis of a Western blot. The specific activity of the MON 87427- and *E. coli*-produced CP4 EPSPS proteins were determined to be 8.67 ± 0.23 and 5.41 ± 0.37 U/mg of CP4 EPSPS, respectively, as measured by a validated enzyme assay based on the release of inorganic phosphate from phosphoenolpyruvate. Hence, the difference in functional activity was 1.6-fold. Finally, the MON 87427- and *E. coli*-produced CP4 EPSPS protein demonstrated equivalence in a fluorescent glycoprotein detection assay indicating that like the *E. coli*-produced CP4 EPSPS protein, the MON 87427-produced CP4 EPSPS protein was not glycosylated. Based on the previously set acceptance criteria and the results obtained, the MON 87427-produced CP4 EPSPS protein was shown to be equivalent to the *E. coli*-produced CP4 EPSPS protein.”

Replaced with:

“The following analyses were performed to characterize the MON 87427-produced CP4 EPSPS protein. The total protein concentration of the MON 87427-produced CP4 EPSPS protein was measured using the Bio-Rad protein assay and determined to be 0.1 mg/ml. The identity of the MON 87427-produced CP4 EPSPS protein was confirmed by immunoblot analysis

carried out with previously characterized anti-CP4 EPSPS antibodies. Anti-CP4 EPSPS antibodies specifically detected the MON 87427-produced CP4 EPSPS protein. Additionally, identity was confirmed by N-terminal sequence analysis and matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analysis of the trypsin digested CP4 EPSPS protein. The expected N-terminal sequence was observed and peptide masses consistent with the expected peptide masses for the trypsin digested CP4 EPSPS protein were observed. For the latter, the identified peptide masses yielded coverage of more than 70% of the expected peptide sequence (320 out of 455 amino acids) of the CP4 EPSPS protein. Purity and apparent molecular weight of the MON 87427-produced CP4 EPSPS protein were determined using densitometric analysis of a Colloidal Brilliant Blue G stained SDS-PAGE gel. Purity of the MON 87427-produced CP4 EPSPS protein was 96% and the apparent molecular weight was 44.1 kDa. The average mass of the intact MON 87427-produced protein was 47552 Da as measured by MALDI-TOF MS analysis. This measurement was consistent with the theoretical mass of the CP4 EPSPS protein (47481 Da). Analysis of the glycosylation status using a fluorescent glycoprotein detection assay indicated that the MON 87427-produced CP4 EPSPS protein was not glycosylated. The functional activity of the MON 87427-produced CP4 EPSPS protein was determined using a phosphate release assay. The MON 87427-produced CP4 EPSPS protein was shown to be active, with a specific activity of 8.67 ± 0.23 U/mg of CP4 EPSPS. Finally, SDS-PAGE was used to determine the purity and molecular weight (MW) of the protein after storage at ~ -80 °C. This analysis demonstrated that the MON 87427-produced CP4 EPSPS protein was stable throughout the experimental phase (14 days).

The equivalence of the MON 87427- and *E. coli*-produced CP4 EPSPS proteins was assessed by comparing their apparent molecular weight, immunoreactivity, functional activity, and glycosylation status. Based on the previously set acceptance criteria and the results obtained, the MON 87427-produced CP4 EPSPS protein was shown to be equivalent to the *E. coli*-produced CP4 EPSPS protein.”

43	Added “Appendix 3. Notes for reviewer” detailing the contents of Amendment 1.