Study Title

Characterization of the CP4 EPSPS Protein Purified from the Seed of MON 87705 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. coli*-Produced CP4 EPSPS Proteins

Authors

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April 1, 2009

Sponsor and Performing Laboratory

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

Laboratory Project ID

Monsanto Study Number: REG-08-520
MSL Number: 0021863

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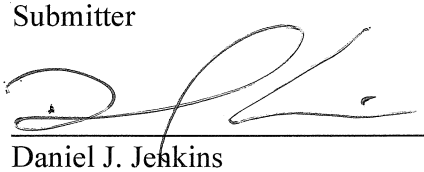

3/31/09

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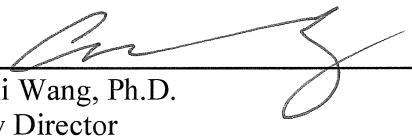

4/01/09

Cunxi Wang, Ph.D.
Study Director — Date

Quality Assurance Unit Statement

Study Title: Characterization of the CP4 EPSPS Protein Purified from the Seed of MON 87705 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. coli*-Produced CP4 EPSPS Proteins

Study Number: REG-08-520

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
12/05/2008	Specific activity	12/08/2008	12/08/2008
03/16-17/2009	Draft Report Review	03/17/2009	03/17/2009
02/02/2009	Raw Data Audit	03/10/2009	03/10/2009

Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

03/31/09
Date

Study Certification Page

The results reported in this report accurately reflect the data generated under Study Number REG-08-520.

Signatures of Final Report Approval:

Cunxi Wang, Ph.D.
Study Director — 4/01/2009 Date

Shefalee A. Kapadia, M.Sc
Principal Investigator — 3/31/2009 Date

Elena A. Rice, Ph.D.
Protein Team Lead — 4/01/2009 Date

Study Information

Study Number:	REG-08-520
MSL Number:	0021863
Title:	Characterization of the CP4 EPSPS Protein Purified from the Seed of MON 87705 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. coli*-Produced CP4 EPSPS Proteins
Testing Facility:	Monsanto Company Regulatory Product Characterization and Safety Center 800 North Lindbergh Boulevard St. Louis, Missouri 63167
Study Director:	Cunxi Wang, Ph.D.
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Team Lead:	Elena A. Rice, Ph.D.
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Records Retention:	All characterization plan specific raw data, electronically stored Atlas files, the characterization plan, amendment, and final report will be retained at Monsanto Company, St. Louis.

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Abbreviations and Definitions[1]

AA	Amino Acid
APS	Analytical Protein Standard
BSA	Bovine Serum Albumin
CFR	Code of Federal Regulations
COA	Certificate of Analysis
CP4	*Agrobacterium sp.* strain CP4
cp4 epsps	Coding sequence for the CP4 EPSPS Protein
CTP	Chloroplast Transit Peptide
α-Cyano	α-Cyano-4-hydroxycinnamic acid
Da	Dalton
DHB	2,5-Dihydroxybenzoic Acid
ECL	Enhanced Chemiluminescence
EPA	Environmental Protection Agency
EPSPS	5-Enolpyruvylshikimate-3-phosphate Synthase
E. coli	*Escherichia coli*
FATB	*Glycine max* gene for Palmitoyl-ACP Thioesterase
FAD2	*Glycine max* gene for Δ-12 Desaturase
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act (U.S.)
GLP	Good Laboratory Practice
HRP	Horseradish Peroxidase
LB	Laemmli 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
MES	2-[N-Morpholino] Ethanesulfonic Acid
MH+	Protonated mass ion
MS	Mass Spectrometry
MW	Molecular Weight
MWCO	Molecular Weight Cutoff
NFDM	Non-Fat Dried Milk
OECD	Organization for Economic Co-operation and Development
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

[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.

S3P	Shikimate-3-phosphate
Sinapinic Acid	3,5-dimethoxy-4-hydroxycinnamic acid
SOP	Standard Operating Procedure
TFA	Trifluoroacetic Acid
U	Unit (of enzyme activity)
U.S.	United States
VOI	Verification of Identity

1.0 Summary

Monsanto Company has developed a biotechnology-derived soybean, MON 87705, to generate nutritionally-improved soybean oil with decreased levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid) and increased levels of oleic acid (18:1) through suppression of two key oil biosynthetic enzymes, FATB and FAD2. MON 87705 also contains the 5-enolpyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium sp*. strain CP4 (*cp4 epsps*) encoding the CP4 EPSPS protein, which serves as a selection marker for the transformed plants.

The CP4 EPSPS protein is present in a number of biotechnology-derived crops and its safety has been previously addressed (Harrison et al., 1996). Studies utilizing the CP4 EPSPS protein revealed that it degrades rapidly in simulated gastric and intestinal fluids, and that consumption of a very high dose of CP4 EPSPS protein does not produce any hazardous effects on the health of mice (Harrison et al., 1996). It was concluded that the CP4 EPSPS protein poses no risk to human and animal health.

Since the CP4 EPSPS protein purified from *E. coli* has been used previously in a number of safety assessment studies, demonstration of the equivalence between the *E. coli*- and MON 87705-produced CP4 EPSPS proteins allows the utilization of the existing data to confirm the safety of the CP4 EPSPS protein in MON 87705. The purpose of this study was to characterize the CP4 EPSPS protein isolated from seed of MON 87705, and to demonstrate the equivalence of the MON 87705-produced CP4 EPSPS to the previously characterized *E. coli*-produced CP4 EPSPS.

The equivalence of the MON 87705- and *E. coli*-produced CP4 EPSPS proteins was evaluated by comparing their apparent molecular weight, immunoreactivity, glycosylation status, and functional activity. Based on preset acceptance criteria and the results obtained, the MON 87705-produced CP4 EPSPS protein is equivalent to the *E. coli*-produced CP4 EPSPS protein.

The CP4 EPSPS protein was purified from an extract of seed of MON 87705. Various analytical techniques were used to characterize the MON 87705-produced CP4 EPSPS protein. These techniques included: 1) total protein concentration determination using a Bio-Rad protein assay, 2) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry, 3) N-terminal sequence analysis, 4) matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry, 5) immunoblotting and densitometry, 6) CP4 EPSPS enzymatic activity assay, and 7) glycosylation analysis. The stability of the MON 87705-produced CP4 EPSPS protein during the experimental phase of the study was also assessed using SDS-PAGE by estimating the purity and molecular weight (MW) of the protein after storage at ~ -80 °C.

The identity of the MON 87705-produced CP4 EPSPS protein was confirmed using immunoblot analysis with anti-CP4 EPSPS antibody, N-terminal sequencing, and MALDI-TOF mass spectrometry analysis of the CP4 EPSPS protein after trypsin

digestion. The antibody specifically detected the protein on an immunoblot. The N-terminal sequence was confirmed, with the exception of methionine, which was removed enzymatically. MALDI-TOF analysis yielded peptide masses consistent with the expected peptide masses for the CP4 EPSPS protein. Together, the identified peptide masses yielded 79.6% overall coverage of the expected peptide sequence (362 out of 455 amino acids) of the MON 87705-produced CP4 EPSPS protein. The total protein concentration of the MON 87705-produced CP4 EPSPS protein was determined to be 0.21 mg/ml. Purity and apparent molecular weight of the MON 87705-produced CP4 EPSPS protein were determined using densitometric analysis of a Colloidal Brilliant Blue G stained SDS-PAGE gel. Purity of the MON 87705-produced CP4 EPSPS protein was 100% and the apparent molecular weight was 44.6 kDa. MALDI-TOF MS of the intact protein resulted in an average mass of 47396 Da, which is comparable to the theoretical mass of the full-length CP4 EPSPS protein minus the methionine (47481.48 Da). The MON 87705-produced CP4 EPSPS protein was not glycosylated. The functional activity of the MON 87705-produced CP4 EPSPS protein was determined using a phosphate release assay. The MON 87705-produced CP4 EPSPS protein was shown to be active, with a specific activity of 4.10 U/mg of CP4 EPSPS. The MON 87705-produced CP4 EPSPS protein was stable throughout the experimental phase (8 days) when stored in a -80 °C freezer.

These data provide a detailed characterization of the CP4 EPSPS protein isolated from MON 87705 and establish the equivalence of the MON 87705-produced CP4 EPSPS protein to the *E. coli*-produced CP4 EPSPS protein.

2.0 Introduction

Monsanto Company has developed a biotechnology-derived soybean, MON 87705, to generate nutritionally-improved soybean oil with decreased levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid) and increased levels of oleic acid (18:1) through the suppression of two key oil biosynthetic enzymes, FATB and FAD2. Suppression of the FATB enzyme results in a decrease in the levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid), while suppression of the FAD2 enzyme results in an increase of oleic acid (18:1). MON 87705 also contains a *cp4 epsps* gene. Expression of the CP4 EPSPS protein renders the soybean plant tolerant to glyphosate, which is the active ingredient in the Roundup® family of agricultural herbicides (Roundup is a registered trademark of Monsanto Technology, LLC). The CP4 EPSPS protein produced in MON 87705 is identical to that found in Roundup Ready soybean, Roundup Ready cotton, Roundup Ready corn 2, Roundup Ready Flex cotton, and Roundup RReady2Yield™ (Lee et al., 1995; Harrison et al., 1996; Karunanandaa et al., 2003; 2006). The CP4 EPSPS protein is structurally similar and functionally identical to endogenous plant EPSPS enzymes, but has a much lower affinity for glyphosate relative to the endogenous plant EPSPS (Padgette et al., 1996). In conventional plants, glyphosate binds to the endogenous plant EPSPS enzyme and blocks the biosynthesis of shikimate-3-phosphate (S3P), thereby depriving plants of aromatic amino acids (Steinrücken and Amrhein, 1980; Haslam

1993). Introduction of the *cp4 epsps* gene into soybean allows for plant growth and development in the presence of glyphosate (Padgette et al., 1996).

MON 87705 expresses a *cp4 epsps* gene fused with an *Arabidopsis* EPSPS chloroplast transit peptide (CTP) sequence at its 5' end. CTP is required for targeting CP4 EPSPS protein into chloroplasts where CP4 EPSPS is involved in the shikimate biosynthetic pathway. The *Arabidopsis* EPSPS CTP is 76 amino acids in length and is predicted to be cleaved by a transit peptidase upon import into chloroplasts. The integrity of the inserted *cp4 epsps* gene cassette has been confirmed by molecular characterization (Skipwith et al., 2009).

3.0 Purpose

The purpose of this study was to characterize the CP4 EPSPS protein purified from the seed of MON 87705, and compare the physicochemical and functional properties of this MON 87705-produced CP4 EPSPS protein (Orion lot 10002253) 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 87705- and *E. coli*-produced CP4 EPSPS proteins justifies the use of the existing data to confirm the safety of the CP4 EPSPS protein in MON 87705.

4.0 Materials

4.1 MON 87705-Produced CP4 EPSPS Protein (Test substance)

The MON 87705-produced CP4 EPSPS protein (Orion lot 10002253) purified from seed of MON 87705 was used as the test substance. The seeds were produced under production plan 07-01-83-18 from seed lot number GLP-0705-18715-S. A copy of the VOI for these harvested seeds has been archived under the Orion lot 10002253. The MON 87705-produced CP4 EPSPS protein was stored in a -80 ºC 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 same buffer as for *E. coli*-produced EPSPS (Orion lot 10000739)]. The purification records for the MON 87705-produced protein have been archived under the Orion lot 10002253 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 *E. coli*-produced CP4 EPSPS protein was generated using fermented *E. coli* paste containing the pMON21104 expression plasmid, the sequence of which was confirmed before and after fermentation. The *E. coli*-produced CP4 EPSPS protein was previously characterized and a copy of the certificate of analysis is included in Appendix 2.

4.3 Assay Controls

Protein molecular weight standards (SeeBlue Plus2) (Invitrogen, Carlsbad, CA) were used to calibrate SDS-PAGE gels and verify protein transfer to nitrocellulose or Polyvinylidene difluoride (PVDF) membranes. The broad range SDS-PAGE molecular weight standards (Bio-Rad, Hercules, CA) were used to determine the apparent molecular weight of the MON 87705-produced CP4 EPSPS. The *E. coli*-produced CP4 EPSPS reference standard (Orion lot 10000739) was used to create a standard curve to estimate the protein concentration using a Bio-Rad protein assay. 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 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 and positive and negative controls for glycosylation analysis.

5.0 Methods

5.1 Protein Purification

The MON 87705-produced CP4 EPSPS protein was purified from seed of MON 87705 prior to initiation of this characterization plan. 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 seed using a combination of ammonium sulfate fractionation, hydrophobic interaction chromatography, anion exchange chromatography, and cellulose phosphate affinity chromatography. A detailed description of the purification process was filed under Orion Lot 10002253, and is briefly described below.

Approximately 100 g of pre-chilled seed of MON 87705 were ground using a laboratory mill 3100. The ground powder (~ 100 g) was defatted 3 times with 500 ml each of Hexanes (EMD, Gibbstown, NJ) pre-warmed to 37 ºC, air-dried, and stored in a -80 ºC freezer prior to extraction of the CP4 EPSPS protein. A portion of the defatted seed powder (50 g) was mixed with an extraction buffer (100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM Benzamidine-HCl, 4 mM DTT, 2 mM Phenylmethylsulfonyl fluoride, 1% Polyvinylpolypyrrolidone and 10% glycerol) for 2 hours at approximately a 1:10 powder weight to extraction buffer volume ratio. The slurry was centrifuged at 23,500 x *g* for 20 min at ~ 4 ºC. The resultant 430 ml supernatant was subjected to 40% ammonium sulfate protein fractionation by addition of 97 g of ammonium sulfate over a 1 h time period in the cold room (2 to 8 ºC). The solution was stirred for 2 h at ~ 4 ºC and centrifuged at 23,500 x *g* for 20 min. Another 89.8 g of ammonium sulfate was added to the supernatant (480 ml) over a 1 h time period to a 70% saturation. The solution was stirred for 2 h in a 4 ºC cold

room and the pellet was collected by centrifugation at 23,500 x *g* for 30 min. The pellet was resuspended in 150 ml of a buffer designated as PS(A) [50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% glycerol (v/v), 1.5 M $(NH4)_2SO_4$]. The resuspended sample was loaded onto a 140 ml column of Phenyl Sepharose Fast Flow (5 cm x 7 cm column) (GE Healthcare, Piscataway, NJ) equilibrated with the PS(A) buffer. Proteins were eluted with a linear salt gradient that decreased from 1.5 to 0 M $(NH_4)_2SO_4$ in the PS(A) buffer over a volume of 1400 ml. Fractions containing the CP4 EPSPS protein, identified based on Western blot analysis, were pooled to a final volume of ~250 ml. The pooled sample was desalted by dialysis against 4 L of a buffer designated as QS(A) (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM benzamidine-HCl, 4 mM DTT) for 20 h at ~ 4 °C with 3 additional buffer changes using dialysis tubing [Molecular Weight Cutoff (MWCO), 12 to 14 kDa] (Spectrum Laboratories, Inc. Rancho Dominguez, CA).

The desalted sample (337 ml) was loaded onto a 60 ml column of Q Sepharose Fast Flow anion exchange resin (2 cm x 20 cm) (GE Healthcare, Piscataway, NJ), which was equilibrated with the QS(A) buffer. Bound CP4 EPSPS protein was eluted with a linear salt gradient that increased from 0 M to 0.4 M potassium chloride in the QS(A) buffer over 600 ml. Fractions containing CP4 EPSPS, identified by Western blot analysis, were pooled to a final volume of ~ 140 ml. The pooled sample was placed into dialysis tubing (MWCO, 12 to 14 kDa, Spectrum Laboratories, Inc. Rancho Dominguez, CA) and dialyzed against a buffer designated as CP(A) [50 mM MES, pH 5.8, 10% glycerol (v/v), 1 mM benzamidine-HCl and 1 mM DTT] for 18 h at ~ 4 °C with 2 additional buffer changes.

The dialyzed sample (100 ml) was then loaded onto a 19 ml cellulose phosphate P11 cation exchange column (1.6 x 9.5 cm) pre-equilibrated with the CP(A) buffer. Bound CP4 EPSPS protein was eluted with the CP(A) buffer containing 0.5 mM phosphoenolpyruvate (PEP) and 0.5 mM S3P. Fractions containing CP4 EPSPS protein, based on SDS PAGE analysis, were pooled (~14.5 ml). This pooled sample was concentrated to 10 ml at ~ 4 °C using a slide-A-lyzer dialysis cassette (MWCO: 10 kDa, size: 3 to 12 ml, Pierce, Rockford, IL) and covering it in a water absorbing polymer powder (Aquacide I, EMD, Gibbstown, NJ). After concentration, the cassette was placed into 2 L of dialysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine-HCl) and dialyzed for 20 h at ~ 4 °C with 2 additional buffer changes. The dialyzed sample in the cassette was further concentrated to 5 ml using Aquacide I as described above. This 5 ml sample was mixed with 5 ml dialysis buffer containing 50% glycerol to final volume of 10 ml. Final buffer composition of the sample was 50 mM Tris-HCl, pH7.5, 50 mM KCl, 2mM DTT, 1mM benzamidine-HCl and 25% glycerol. This CP4 EPSPS purified from seed of MON 87705 was aliquoted (100 µl each), assigned APS lot 10002253 and stored at ~ -80 °C.

5.2 Protein Concentration

The total protein concentration of the MON 87705-produced CP4 EPSPS protein sample was determined using a Bio-Rad protein assay. The *E. coli*-produced CP4 EPSPS reference standard protein (APS lot 10000739) was used to prepare a standard curve ranging from 0.05 to 0.6 mg/ml. The MON 87705-produced CP4 EPSPS protein concentration was determined by comparison of absorbance values at 595 nm obtained for the sample to the values of the *E. coli*-produced CP4 EPSPS standard curve. Data were collected using a Bio-Tek Instruments, Inc. PowerWave Xi microplate scanning spectrophotometer (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 seed of MON 87705 and to compare the immunoreactivity of the MON 87705- and *E. coli*-produced proteins.

The MON 87705- and *E. coli*-produced CP4 EPSPS proteins were both loaded onto the same gel at equal amounts of 1, 2, and 3 ng. Aliquots of each protein were diluted in water and 5 x loading buffer (LB) [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 100.5 °C for 3 min, and applied to a pre-cast Tris glycine 4 to 20% polyacrylamide gradient 15-well gel (Invitrogen, Carlsbad, CA). Three amounts of each protein were loaded in duplicate on the gel. Electrophoresis was performed at a constant voltage of 150 V for 88 min. Pre-stained molecular weight markers (SeeBlue Plus2 Prestained, Invitrogen, Carlsbad, CA) were loaded in parallel to verify electrotransfer of the proteins to the membrane and estimate the size of the immunoreactive bands observed. Electrotransfer to a 0.45 μm nitrocellulose membrane (Invitrogen, Carlsbad, CA) was performed for 90 min at a constant voltage of 25 V.

For immunodetection, 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 # 20) 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) and exposed (10, 30, and 60 s) to Amersham Hyperfilm (GE, Healthcare, Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

The immunoreactive bands of the MON 87705-produced CP4 EPSPS protein in each lane migrating to the same position as the reference standard were quantified and compared to the signals corresponding to the *E. coli* CP4 EPSPS reference substance. Quantification of 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 raw data was exported to a Microsoft Excel [2007 (12.0.6324.5001) 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 determine the immunoreactivity equivalence.

5.4 N-Terminal Sequencing

N-terminal sequencing using automated Edman degradation chemistry was used to confirm the identity of the MON 87705-produced CP4 EPSPS.

Because the protein was determined to be 100% pure based on pre-study data, it was not necessary to separate the protein by SDS-PAGE. Therefore, an aliquot of the MON 87705-produced CP4 EPSPS 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 system and 785 Programmable Absorbance Detector and Procise™ Control Software (version 1.1a) were used. Chromatographic data were collected using Atlas 99 software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to chromatographically 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 control protein (10 picomoles of β-lactoglobulin, Applied Biosystems, Foster City, CA) was analyzed before and after the 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 87705-produced CP4 EPSPS were observed during analysis.

5.5 MALDI-TOF Tryptic Mass Map Analysis

MALDI-TOF mass spectrometry was used to confirm the identity of the MON 87705-produced CP4 EPSPS. Since the protein was determined to be pure (100%) based on pre-study data, it was not deemed necessary to separate the protein by SDS-PAGE prior to trypsinization.

An ethanol precipitation was performed to concentrate the MON 87705-produced CP4 EPSPS sample and remove any buffer components that may interfere with the analysis. Twenty five µl of the MON 87705-produced CP4 EPSPS sample (0.21 mg/ml) was concentrated to approximately 20 µl with a Speed-Vac concentrator and then mixed with 200 µl prechilled 95% ethanol. After incubating overnight at -20 °C,

the mixture was centrifuged at 13,000 x g for 30 min at ~ 4 °C. The pellet was collected, washed twice with 200 µl of pre-chilled acetone and then twice with 200 µl of water. Ten µl of trypsin solution [20 µg/ml trypsin (Promega, Madison, WI) in a 25 mM ammonium bicarbonate buffer, pH 7.8] was added and incubated overnight at 37 °C. Trypsin digested samples (0.3 µl) were added directly onto the analysis plate in triplicate and followed by the addition of ~ 0.75 µl of three matrices, 2, 5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (α-Cyano) , and 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid) (Waters Corp., Milford, MA) on separate spots. The sample in DHB matrix was analyzed in the 300 to 7500 Dalton range using 100 shots at a laser intensity setting of 2480 (a unit-less MALDI-TOF instrument specific value) while samples in α-Cyano and Sinapinic acid were analyzed in the 500 to 7500 Dalton range using 100 shots at a laser intensity setting of 1980 and 2380, respectively. Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). Calmix 2 was used as the external calibrant (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) for the analysis. GPMAW32 software (Applied Biosystems, version 4.23) was used to generate a theoretical trypsin digest of the CP4 EPSPS protein sequence based upon the nucleotide sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks in the 300 to 7500 Da range if they met the following criteria: resolved monoisotopic peak; with at least 1 additional associated ion peak for masses < 1000 Da and at least 2 associated ion peaks for masses > 1000 Da; peak height greater than twice the baseline noise; and did not overlap with a stronger mass signal (±2 Daltons from the mass analyzed). Known autocatalytic fragments from trypsin digestion were identified in the raw data. The list of experimental masses was then compared to the theoretical list from the GPMAW software. Those experimental masses within 1 Da of a theoretical mass were matched. All matching masses were tallied and a coverage map was generated. The tryptic mass map coverage was considered acceptable if ≥ 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 x LB to a final total protein concentration of 0.168 µg/µl. Molecular weight markers (Bio-Rad broad-range) and reference substance were diluted to a final total protein concentration of 0.9 and 0.2 µg/µl, respectively. The test substance was analyzed in duplicate at 1, 2, and 3 µg protein per lane. The *E. coli*-produced CP4 EPSPS reference standard (Orion lot 10000739) was analyzed at 1 µg total protein. All samples were heated at ~ 100 °C for 3 min and loaded onto a pre-cast Tris glycine 4 to 20% polyacrylamide gradient 10-well 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 18 h and 35 min with Brilliant Blue G-Colloidal stain (Sigma-Aldrich, St. Louis, MO), destained 30 s with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and finally destained with 25% (v/v) methanol for 6.5 h. 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). Molecular weight markers were used to estimate the apparent molecular weight of each observed band. 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 loadings containing the MON 87705-produced CP4 EPSPS protein.

5.7 MALDI-TOF Mass Analysis

MALDI-TOF mass spectrometry was used to further characterize the molecular weight of the MON 87705-produced CP4 EPSPS. Prior to analysis, the MON 87705-produced CP4 EPSPS protein was dialyzed using drop dialysis (Görisch, 1988). Briefly, a 25 mm Millipore microdialysis disk (type VSWP, 0.025 µm pore size, Bedford, MA) was floated on HPLC-grade water, spotted with 2 µl of the sample, and dialyzed for 45 min. A portion of the MON 87705-produced CP4 EPSPS and BSA protein samples (0.125 and 0.25 µl) was spotted on an analysis plate, mixed with 0.375 and 0.75 µl of Sinapinic acid solution containing 0.3% Trifluoroacetic acid (TFA), respectively, and air-dried. Mass spectral analysis of the MON 87705-produced CP4 EPSPS protein was performed using an Applied Biosystems Voyager DE-Pro Biospectrometry Workstation MALDI-TOF instrument with the supplied Data Explorer software (version 4.0, Foster City, CA). Mass calibration of the instrument was performed using a BSA protein standard. The sample was analyzed in the 10,000 to 100,000 Dalton range using 100 shots at a laser intensity setting of 2983 (a unit-less MALDI-TOF instrument specific value). Average protonated (MH+) protein masses were observed in linear mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW32 software (Applied Biosystems, version 4.23, Foster City, CA) was used to generate a theoretical mass of the expected CP4 EPSPS protein sequence based upon the nucleotide sequence. The mass of the MON 87705-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 87705-produced CP4 EPSPS was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the MON 87705-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 97.6 °C for 5 min, cooled, and loaded on a Tris glycine 4 to 20%

polyacrylamide gradient 10-well mini-gel (Invitrogen, Carlsbad, CA). Each sample was loaded at 50 and 100 ng per lane. SeeBlue Plus2 pre-stained protein molecular weight markers (Invitrogen, Carlsbad, CA) were loaded to verify electrotransfer of the proteins to the membrane, and the CandyCane™ 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 90 min at a constant voltage of 25 V.

Carbohydrate detection was performed directly on the PVDF membrane using the Pro-Q® Emerald 488 Glycoprotein Gel and Blot Stain Kit (Molecular Probes, Eugene, OR). The manufacturer's protocol was followed and is briefly described. All steps were performed at room temperature. The PVDF membrane was fixed in 25 ml of a solution containing 50% methanol and 5% acetic acid for 1 h, the solution was then changed and the membrane was further fixed overnight. Two, 15 min washes (50 ml each) with 3% (v/v) acetic acid (wash solution), were followed by a 20 min oxidation in 25 ml of an oxidizing solution containing periodic acid (Component C from the kit). The membrane was washed three times (10 min each) in 50 ml of the wash solution. The membrane was then incubated in 25 ml of Pro-Q Emerald Staining Solution that was prepared using the kit reagents. After 40 min of staining in the dark, one 15 min, 50 ml wash was followed by two 30 min, 50 ml wash cycles. The further wash included two 50 ml, 1 min deionized water washes followed by three 5 min methanol washes (EMD, San Diego, CA). Finally the blot was washed with water for 10 min. The blot was then scanned using the Bio Rad Molecular Imager FX using the Alexa 488 illumination laser setting (488 nm excitation, 530 nm band pass) and Quantity One software (version 4.6, build 036) in order to visualize the fluorescently-labeled glycosylated proteins.

After glycosylation analysis the blot was stained to visualize the proteins present on the membrane. Proteins were stained for 1 min using Coomassie Brilliant Blue R-250 staining solution (Bio-Rad, Hercules, CA) and then destained with 1 x 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) in order to visualize total proteins.

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. Each assay replicate was subsequently analyzed spectrophotometrically in duplicate. Briefly, the reactions containing the CP4 EPSPS enzyme with S3P were initiated by the addition of PEP. The reactions were performed in a mixture of 50 mM HEPES (pH 7.0), 0.1 mM ammonium molybdate, 2 mM S3P, 1 mM PEP and 5 mM potassium fluoride for 2

min at 25.2 °C. The reactions were quenched with malachite green (phosphate assay reagent) and fixed after two min with 33% (w/v) sodium citrate. The release of inorganic phosphate from PEP was determined at a wavelength of 660 nm using a PowerWave Xi (Bio-Tek, Richmond, VA) microplate reader, and quantitated relative to a standard curve of inorganic phosphate treated with the malachite green (phosphate assay) reagent and 33% (w/v) sodium citrate. For CP4 EPSPS, the specific activity is 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 (12.0.6324.5001) SP1 MSO (12.0.6320.5000).

5.10 Storage Stability

The short-term stability over the experimental phase of the study of the MON 87705-produced CP4 EPSPS during storage in a -80 °C freezer was evaluated by comparing the purity and molecular weight values obtained on day 0 to the purity and molecular weight values obtained on day 8 of storage. Day 0 of the stability analysis corresponds to the purity and molecular weight determination described in Section 5.7. On day 8, an aliquot was removed from a -80 °C freezer and mixed with 5 x LB to a final protein concentration of 0.168 μg/μl, heated at ~100 °C for 3 min, and loaded in duplicate at three amounts (1, 2, and 3 μg per lane) onto a Tris glycine 4 to 20% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA). Staining and densitometric analysis were performed as described in section 5.7. The protein sample was considered to be unstable if a >10% change in purity and/or molecular weight was observed relative to the value determined on Day 0.

5.11 Equivalence Criteria

The equivalence of the MON 87705-produced and *E. coli*-produced CP4 EPSPS proteins was determined based on four characteristics. The two proteins are considered equivalent if they meet the following criteria:

1. Immunoreactivity with CP4-specific antibodies: the immunoreactive signal of the test protein should be within ± 30% of the reference protein.
2. Molecular weight: the apparent molecular weight, by SDS-PAGE, of the test protein should be within ± 10% of the reference protein.
3. Activity assay: the functional activity of the test protein should be within ± 50% of the reference protein.
4. Glycosylation status: both test and reference proteins are not glycosylated.

6.0 Data Rejected or Not Reported

All data was accepted and reported.

7.0 Deviations

There were two protocol and one facility-related deviations.

1: During MALDI-TOF-MS tryptic Mass fingerprinting, described in Section 6, the protocol stated that after the precipitation, the pellet will be overlaid with 10 µl of trypsin solution (20 µg/µl) and left at 37 °C overnight. The concentration of trypsin (20 µg/µl) was a typographical error. It should be 20 µg/ml. This error was recognized and corrected prior to performing this step of the analysis.

2: In MALDI-TOF-MS Mass analysis of Section 6, the protocol stated that the dried sample will be resuspended in 5 µl of 0.1% TFA and spotted on the plate with Sinapinic acid. The plate then will be spotted with ~0.5 µl of protein and ~1.0 µl of Sinapinic acid. The analyst inadvertently missed this instruction and the step was performed as follows: the sample was dried to ~ 1 µl. A mixture containing 0.5 µl sample and 1.5 µl Sinapinic acid was then prepared. Then 0.5 µl and 1.0 µl of this mixture were placed on the sample plate for MS analysis. This deviation had no impact on this study because: (1) the Sinapinic acid solution contains 0.3% TFA. Even dilution of the Sinapinic acid solution (1.5 µl, 0.3% TFA) by a non-acid containing analyte (0.5 µl) still resulted in 0.225% TFA, more than enough to provide an acidic environment for proper sample analysis; (2) the determined mass is correct because a mass calibration was performed prior to analyzing the test sample.

3: The required one point verification was not performed on the balance PR30 prior to the first use of the day between July 22, 2008 and September 3, 2008. The SOP required external mass check was not performed within 35 days prior to a weighting. However, the external mass checks performed on July 21, 2008 and September 4, 2008 satisfied the SOP acceptance criteria. This had no impact on this study.

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 87705-produced CP4 EPSPS protein sample to ensure instrument performance. A four-peptide mixture from the Sequazyme Peptide Mass Standards kit (Applied Biosystems, Foster City, CA) was used to calibrate the MALDI-TOF mass spectrometer. 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, respectively.

9.0 Results and Discussion

9.1 Protein Concentration

The concentration of the MON 87705-produced CP4 EPSPS was determined to be 0.21 mg/ml based on the Bio-Rad protein assay (Table 1).

9.2 Protein Identity

The identity of the MON 87705-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 87705- and *E. coli*-produced proteins.

9.2.1 Immunoblot Analysis Using an Anti-CP4 EPSPS Antibody

On the immunoblot, anti-CP4 EPSPS antibody recognized similar immunoreactive bands migrating to the identical molecular weight position for both MON 87705-produced CP4 EPSPS and *E. coli*-produced reference protein (Figure 1, lanes 2-7 and 9-14). As expected, the immunoreactive signal increased with increasing loads of both MON 87705- and *E. coli*-produced proteins. No additional bands were observed in either protein sample. The densitometric analysis of the intensity of the signals produced by both proteins showed there was 10.5% difference between the MON 87705-produced CP4 EPSPS protein and the *E. coli*-produced reference standard (Table 2), which is within the equivalence acceptance criterion of ± 30%. The immunoblot analysis confirmed the identity of the MON 87705-produced CP4 EPSPS protein and demonstrated that both the MON 87705- and *E. coli*-produced proteins had equivalent immunoreactivity. The 30s exposure was used for this analysis.

9.2.2 N-Terminal Sequencing

N-terminal sequencing performed on the MON 87705-produced CP4 EPSPS resulted in 10 amino acid residues assigned (Figure 2). The sequence obtained is identical to that of the mature CP4 EPSPS protein deduced from the *cp4 epsps* gene present in seed of MON 87705 (Skipwith, et al., 2009) with the exception of the missing methionine. The N-terminal methionine was likely cleaved from the mature CP4 EPSPS in plant by methionine aminopeptidase, a common modification, which has no effect on protein structure or activity (Arfin & Bradshaw, 1988; Bradshaw, *et al*., 1998; Polevoda & Sherman 2000). The N-terminal sequence information, therefore, confirms the identity of the CP4 EPSPS protein isolated from the seed of MON 87705.

9.2.3 MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 87705-produced CP4 EPSPS protein was also assessed by MALDI-TOF mass spectrometry analysis of tryptic fragments prepared from

the MON 87705-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). Matches were made without consideration for potential natural amino acid modifications.

There were 30 unique peptide fragments identified that corresponded to the expected masses of CP4 EPSPS trypsin-digested peptides (Table 3). The identified masses were used to assemble a coverage map indicating the matched peptide sequences for the entire CP4 EPSPS protein (Figure 3), resulting in 79.6% (362 out of 455 amino acids) coverage of the amino acid sequence of the CP4 EPSPS. This analysis confirms the identity of the MON 87705-produced CP4 EPSPS protein.

9.3 Molecular Weight and Purity Determination

The MON 87705-produced CP4 EPSPS protein was separated using SDS-PAGE and the gel stained using Brilliant Blue G Colloidal stain (Sigma-Aldrich, St. Louis, MO). Purity and apparent molecular weight of the MON 87705-produced CP4 EPSPS protein were determined using densitometric analysis of the gel (Figure 4) and the data are summarized in Table 4. The MON 87705-produced CP4 EPSPS protein migrated to the same position on the gel as the *E. coli*-produced CP4 EPSPS reference standard (Figure 4, lane 2 and 3) and had an apparent molecular weight of 44.6 kDa (Table 2). 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 87705- and *E. coli*-produced CP4 EPSPS proteins was 1.8% (Table 5). This difference in molecular weight is below the acceptance criterion (within 10% difference). Therefore, the MON 87705- and *E. coli*-produced CP4 EPSPS proteins are considered equivalent based on their apparent molecular weight.

The purity of the MON 87705-produced CP4 EPSPS protein was calculated based on the 6 loads on the gel (Figure 4, lanes 3 to 8). The average purity was determined to be 100% (Table 4).

The mass of the MON 87705-produced CP4 EPSPS was also determined by MALDI-TOF MS analysis. The average mass obtained for the MON 87705-produced CP4 EPSPS from three measurements was 47396 Da. This value is comparable to the theoretical mass of the full-length protein missing the methionine (47481.48 Da). The absence of the N-terminal methionine was confirmed by N-terminal sequencing (Section 9.2.2).

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, prokaryotic organisms such as *E. coli* lack the necessary biochemical "machinery" required for protein glycosylation. To test whether potential post-translational glycosylation of the MON 87705-produced CP4 EPSPS occurred, it was analyzed for the presence of covalently bound carbohydrate moieties. The *E. coli*-produced CP4 EPSPS reference standard (negative control), horseradish peroxidase (positive control), and transferrin (positive control) were analyzed concurrently with the MON 87705-produced CP4 EPSPS protein. The results of this analysis are presented 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 test and reference standard CP4 EPSPS proteins (Figure 5A, lanes 6-9) on the blot. This low level signal could be due to low level oxidation of amino acid residues of the protein and/or nonspecific binding of fluorescent reagents. Further evidences that the signals observed for MON 87705- and *E. coli*-purified CP4 EPSPS proteins are non-specific are listed below:

(1) The faint signal was also associated with the *E. coli*-produced CP4 EPSPS protein. However, the *E. coli*-produced CP4 EPSPS protein was shown not to be glycosylated (Harrison et al., 1996).

(2) Other data collected under this characterization plan further demonstrated the absence of glycosylation of the MON 87705-produced CP4 EPSPS. Glycosylation would result in an increase in the protein mass relative to the theoretically calculated mass. No increase in protein mass was observed for the MON 87705-produced CP4 EPSPS protein determined by MALDI-TOF mass spectrometry (47396 Da) as compared to its theoretical mass (47481.48 Da).

(3) Four potential glycosylation sites can be identified in the amino acid sequence of the CP4 EPSPS protein: one O linked at T248 and three N-linked at N213, N271 and N392 (see Fig 3 for amino acid positions). The tryptic fragments containing these amino acids were identified for the MON 87705-produced CP4 EPSPS protein by MALDI-TOF mass spectrometry. All identified masses matched the expected non-modified peptide masses (Table 3), indicating that no glycosylation had occurred.

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 87705-produced protein is not glycosylated and therefore, 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. Both MON 87705- and *E. coli*-produced CP4 EPSPS proteins exhibited similar specific activities of 4.10 and 4.38 U/mg of CP4 EPSPS, respectively. The difference between specific activities of the MON 87705-produced CP4 EPSPS protein and the *E. coli*-produced reference standard was 6.4%. Therefore, the pre-set acceptance criterion (within 50% difference) was met and the MON 87705-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 87705-produced CP4 EPSPS protein stored in a -80 °C freezer for a 8 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 8) 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 43.8 kDa on day 8. This value differed from the molecular weight obtained on day 0 (44.6 kDa) by 1.8% (Table 8). The purity value for the MON 87705-produced CP4 EPSPS protein (Figure 6, Lanes 2-7) was determined to be 100% by densitometric analysis, which had no difference as compared to Day 0 (Tables 7 and 8). Therefore, based on the apparent molecular weight and purity, the MON 87705-produced CP4 EPSPS protein was stable when stored in a -80 °C freezer for the duration of the experimental phase of this study (8 days).

10.0 Conclusions

A panel of analytical techniques was used to characterize the MON 87705-produced CP4 EPSPS protein purified from seed of MON 87705. Identity of the MON 87705-produced CP4 EPSPS was confirmed by recognition with anti-CP4 EPSPS antibodies, identification of the first 10 amino acids of the N-terminus by amino acid sequencing, and mapping of tryptic peptides that yielded a 79.6% overall coverage of the expected protein sequence. The concentration of the MON 87705-produced CP4 EPSPS was 0.21 mg/ml. The purity and apparent molecular weight of the MON 87705-produced CP4 EPSPS was 100% and 44.6 kDa, respectively. MALDI-TOF mass spectrometry analysis of the intact protein resulted in an average mass of 47396 Da, reflecting the expected mass of the protein minus the N-terminal methionine. The MON 87705-produced CP4 EPSPS protein was not glycosylated and had a specific activity of 4.10 U/mg of CP4 EPSP. Finally, the MON 87705-produced CP4 EPSPS protein was stable for at least 8 days while stored in a -80 °C freezer, which encompassed the duration of the experimental phase of this study.

The equivalence of the MON 87705- 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 87705-produced CP4 EPSPS protein is equivalent to the *E.*

coli-produced CP4 EPSPS protein. This equivalence justifies the use of the previously conducted studies where the *E. coli* CP4 EPSPS was used as a test substance.

11.0 References

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Table 1. Concentration of the MON 87705-Produced CP4 EPSPS Protein.

The concentration of the MON 87705-produced CP4 EPSPS protein was determined by Bio-Rad protein assay.

Sample	Concentration (mg/ml)[1]
MON 87705-produced CP4 EPSPS	0.21 ± 0.008

[1]Value refers to mean and standard deviation calculated based on n=3.

Table 2. Immunoreactivity of the MON 87705-Produced and *E. coli*-Produced CP4 EPSPS Proteins with CP4 EPSPS-specific antibodies.

The immunoreactivity of the MON 87705-produced CP4 EPSPS was compared to that of the *E. coli*-produced reference standard. The densities of each band were determined by image analysis of the quantitative immunoblot shown in Figure 1. Values were compared average density across the same concentrations and an average difference was determined. The 30s exposure was used for this analysis.

Sample	Gel lane	Amount (ng)	Density (OD x mm^2)	Average Density[1]	Percent difference[2] (%)	Average Difference[3] (%)
E. coli CP4 EPSPS	2	1	0.994	0.957	9.8	10.5 ± 1.8
E. coli CP4 EPSPS	3	1	0.920			
Plant CP4 EPSPS	9	1	1.027	1.062		
Plant CP4 EPSPS	10	1	1.096			
E. coli CP4 EPSPS	4	2	1.904	2.163	13.0	
E. coli CP4 EPSPS	5	2	2.421			
Plant CP4 EPSPS	11	2	2.584	2.485		
Plant CP4 EPSPS	12	2	2.386			
E. coli CP4 EPSPS	6	3	3.296	3.766	8.7	
E. coli CP4 EPSPS	7	3	4.236			
Plant CP4 EPSPS	13	3	4.039	4.124		
Plant CP4 EPSPS	14	3	4.208			

[1]Average Density =∑⟦Density⟧/2

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

[3]Average difference (%) =∑⟦% difference⟧/3. The standard deviation was calculated using Microsoft Office Excel 2007 (12.0.6324.5001) SP1 MSO (12.0.6320.5000).

Table 3. Summary of the Tryptic Masses Identified for the MON 87705-Produced CP4 EPSPS Using MALDI-TOF Mass Spectrometry.

Matrix			Expected Mass[1]	Difference[2]	AA position[3]	Fragment
α-Cyano	DHB	Sinapinic acid				
	389.18		389.25	0.07	225-227	TIR
	416.23		416.30	0.07	70-72	IRK
	474.20		474.27	0.07	228-231	LEGR
	506.17		506.22	0.05	354-357	ESDR
599.31	599.27		599.33	0.02	29-33	SISHR
616.32	616.29	616.10	616.34	0.02	128-132	RPMGR
629.32	629.28		629.29	0.03	201-205	DHTEK
629.32	629.28		629.34	0.02	383-388	GRPDGK
711.45	711.42		711.45	0	133-138	VLNPLR
835.39	835.37	835.29	835.39	0	62-69	AMQAMGAR
863.46	863.44		863.46	0	15-23	SSGLSGTVR
872.45	872.43		872.45	0	313-320	GVTVPEDR
872.45	872.43		872.52	0.07	358-366	LSAVANGLK
948.52	948.50		948.52	0	161-168	TPTPITYR
991.56			991.55	0.01	14-23	KSSGLSGTVR
1115.58	1115.58		1115.57	0.01	295-305	LAGGEDVADLR
1357.73	1357.73		1357.71	0.02	146-157	SEDGDRLPVTLR
1359.67	1359.69	1359.54	1359.72	0.05	354-366	ESDRLSAVANGLK
1359.67	1359.69	1359.54	1359.64	0.03	34-46	SFMFGGLASGETR
	1558.90	1558.73	1558.83	0.07	47-61	ITGLLEGEDVINTGK
1646.86	1646.89		1646.84	0.02	389-405	GLGNASGAAVATHLDHR
1705.82	1705.88		1705.81	0.01	367-382	LNGVDCDEGETSLVVR
1994.03	1994.07	1993.82	1993.97	0.06	206-224	MLQGFGANLTVETDADGVR
2183.24	2183.30	2183.05	2183.17	0.07	275-294	TGLILTLQEMGADIEVINPR
2367.43	2367.50	2367.21	2367.33	0.10	178-200	SAVLLAGLNTPGITTVIEPIMTR
		2450.13	2450.23	0.10	24-46	IPGDKSISHRSFMFGGLASGETR
		2450.13	2450.22	0.09	105-127	LTMGLVGVYDFDSTFIGDASLTK
	3186.35	3186.30	3186.52	0.17	73-104	EGDTWIIDGVGNGGLLAPEAPLDFGNAATGCR
	3249.77	3249.46	3249.62	0.15	321-351	APSMIDEYPILAVAAAFAEGATVMNGLEELR
		4188.82	4188.26	0.56	234-274	LTGQVIDVPGDPSSTAFPLVAALLVPGSDVTILNVLMNPTR

Cont'd

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

[2]The numbers represent the difference between the expected mass and the first column which has the corresponding numbers.

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

Table 4. Apparent Molecular Weight and Purity Analysis of the MON 87705-Produced CP4 EPSPS Protein.

The apparent molecular weight and the purity of the MON 87705-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 (%)
1 μg in lane 3	44.75	100
1 μg in lane 4	44.87	100
2 μg in lane 5	44.69	100
2 μg in lane 6	44.50	100
3 μg in lane 7	44.31	100
3 μg in lane 8	44.19	100
Average	44.6	100

Table 5. Molecular Weight Difference Between the MON 87705- and *E. coli*-Produced CP4 EPSPS Proteins.

Molecular Weight of MON 87705-Produced CP4 EPSPS Protein[1]	Molecular Weight of *E. coli*-Produced CP4 EPSPS Protein[2]	% Difference from *E. coli*-Produced CP4 EPSPS Protein[3]
44.6 kDa	43.8 kDa	1.8%

[1]See Table 4 for the MW of the MON 87705-produced CP4 EPSPS.

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

[3]$\%\ \text{Difference} = \frac{(\text{MW plant} - \text{MW E coli})}{\text{MW plant}}\ \text{X}\ 100\%$

Table 6. Specific Activity of MON 87705-Produced CP4 EPSPS.

The specific activity of the MON 87705-produced CP4 EPSPS protein was determined using a phosphate release assay. This end-point type colorimetric assay measures the release of inorganic phosphate from one of the substrates, PEP, by the action of the CP4 EPSPS enzyme.

MON 87705-produced CP4 EPSPS[1] (U/mg)	***E. coli*-produced CP4 EPSPS[1] (U/mg)**	**Difference (%, Plant vs *E. coli*)[2]**
4.10 ± 0.1	4.38 ± 0.33	6.4

[1]Value refers to mean and standard deviation calculated based on n = 6 (triplicate for CP4 EPSPS reaction and duplicate of each assay replicate for spectrophotometrical analysis at 660 nm).

[2]$\%\ \text{Difference} = \frac{(\text{Activity Ecoli} - \text{Activity plant})}{\text{Activity Ecoli}}\ \text{X}\ 100\%$

Table 7. Molecular Weight and Purity Estimation for the MON 87705-Produced CP4 EPSPS after 8 Days of Storage in a -80 °C Freezer.

The day 8 purity and molecular weight values for the MON 87705-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.

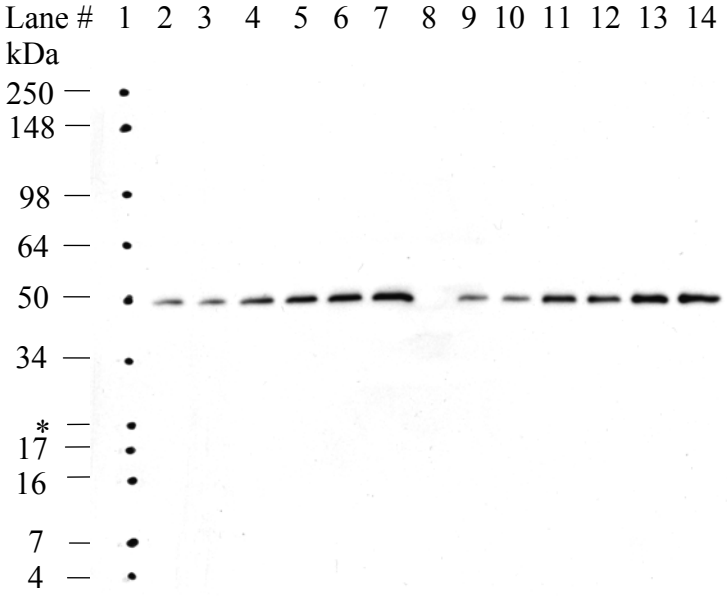
Total protein loaded	Apparent MW (kDa)	Purity (%)
1 µg in lane 3	44.34	100
1 µg in lane 4	44.12	100
2 µg in lane 5	43.71	100
2 µg in lane 6	43.62	100
3 µg in lane 7	43.46	100
3 µg in lane 8	43.67	100
Average	43.8	100

Table 8. Storage Stability (Day 8 vs. Day 0) of the MON 87705-Produced CP4 EPSPS Protein at ~ -80 °C.

The purity and molecular weight values of the MON 87705-produced CP4 EPSPS protein are shown in Table 4 at day 0 and in Table 7 at day 8.

Apparent MW of CP4 EPSPS (kDa)			Purity (%)		
Day 0	Day 8	% Difference[1] (Day 8 vs 0)	Day 0	Day 8	% Difference[1] (Day 8 vs 0)
44.6	43.8	1.8	100	100	0

[1] $\%\ \text{Difference} = \frac{(\text{Day 0} - \text{Day 8})}{\text{Day 0}}\ \text{X}\ 100\%$



Lane	Sample	Amount (ng)
1	See Blue® Plus2 Pre-Stained MW markers	—
2	*E. coli*-produced CP4 EPSPS reference standard	1
3	*E. coli*-produced CP4 EPSPS reference standard	1
4	*E. coli*-produced CP4 EPSPS reference standard	2
5	*E. coli*-produced CP4 EPSPS reference standard	2
6	*E. coli*-produced CP4 EPSPS reference standard	3
7	*E. coli*-produced CP4 EPSPS reference standard	3
8	Empty	
9	MON 87705-produced CP4 EPSPS protein	1
10	MON 87705-produced CP4 EPSPS protein	1
11	MON 87705-produced CP4 EPSPS protein	2
12	MON 87705-produced CP4 EPSPS protein	2
13	MON 87705-produced CP4 EPSPS protein	3
14	MON 87705-produced CP4 EPSPS protein	3

Figure 1. Western Blot Analysis and Immunoreactivity of MON 87705- and *E. coli*-Produced CP4 EPSPS.

Aliqouts of the MON 87705-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS reference standard were separated by SDS-PAGE and electrotransferred to a Nitrocellulose membrane. The membrane was incubated with anti-CP4 EPSPS antibodies and immunoreactive bands were visualized using an ECL system. Approximate MWs (kDa) are shown on the left and correspond to the markers loaded in lane 1. The 30 second exposure is shown. Empty lanes were cropped. *: non signed molecular weight marker.

Amino acid residue # from the N-terminus[1] →	1	2	3	4	5	6	7	8	9	10	11
Expected Sequence	M	L	H	G	A	S	S	R	P	A	T
		|	|	|	|	|	|	|	|	|	|
Experimental Sequence	-	L	H	G	A	S	S	R	P	A	T

Figure 2. N-Terminal Sequence of the MON 87705-Produced CP4 EPSPS.

The expected amino acid sequence of the N-terminus of the CP4 EPSPS protein was deduced from the *cp4 epsps* gene present in soybean MON 87705. The experimental sequence obtained from the MON 87705-produced CP4 EPSPS was compared to the expected sequence. [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; and (-) Indicates the **M** residue not observed.

001 MLHGASSRPA TARKSSGLSG TVRIPGDKSI SHRSFMFGGL ASGETRITGL

051 LEGEDVINTG KAMQAMGARI RKEGDTWIID GVGNGGLLAP EAPLDFGNAA

101 TGCRLTMGLV GVYDFDSTFI GDASLTKRPM GRVLNPLREM GVQVKSEDGD

151 RLPVTLRGPK TPTPITYRVP MASAQVKSAV LLAGLNTPGI TTVIEPIMTR

201 DHTEKMLQGF GANLTVETDA DGVRTIRLEG RGKLTGQVID VPGDPSSTAF

251 PLVAALLVPG SDVTILNVLM NPTRTGLILT LQEMGADIEV INPRLAGGED

301 VADLRVRSST LKGVTVPEDR APSMIDEYPI LAVAAAFAEG ATVMNGLEEL

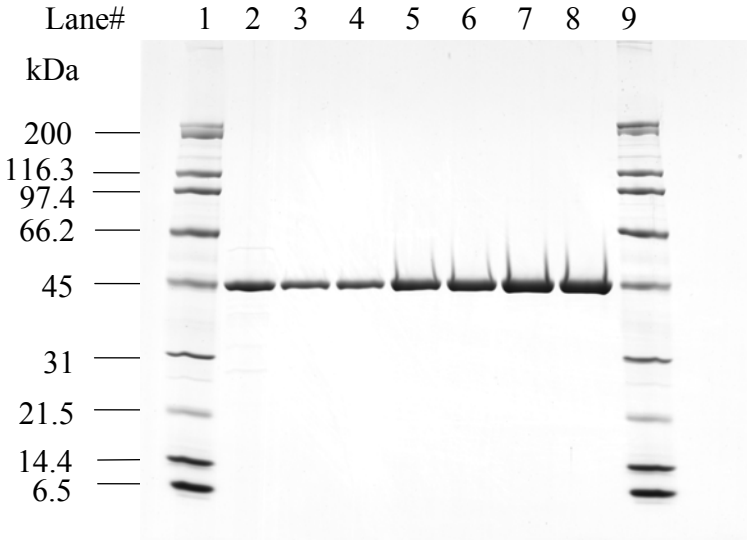
351 RVKESDRLSA VANGLKLNGV DCDEGETSLV VRGRPDGKGL GNASGAAVAT

401 HLDHRIAMSF LVMGLVSENP VTVDDATMIA TSFPEFMDLM AGLGAKIELS

451 DTKAA

Figure 3. MALDI-TOF MS Coverage Map of the MON 87705-Produced CP4 EPSPS.

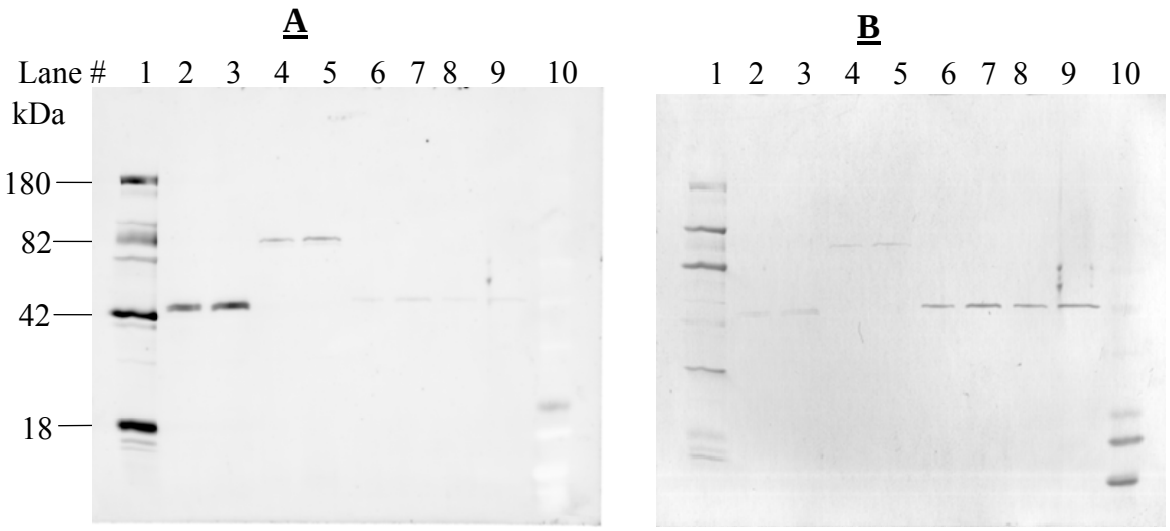
The amino acid sequence of the CP4 EPSPS protein was deduced from the *cp4 epsps* gene present in soybean MON 87705. Boxed regions correspond to tryptic peptides that were identified from the MON 87705-produced CP4 EPSPS protein sample using MALDI-TOF MS. In total, 79.6% (362 of 455 total amino acids) of the expected protein sequence was identified.



Lane	Sample	Amount (µg)
1	Broad Range MW markers	4.5
2	*E. coli*-produced CP4 EPSPS reference standard	1
3	MON 87705-produced CP4 EPSPS protein	1
4	MON 87705-produced CP4 EPSPS protein	1
5	MON 87705-produced CP4 EPSPS protein	2
6	MON 87705-produced CP4 EPSPS protein	2
7	MON 87705-produced CP4 EPSPS protein	3
8	MON 87705-produced CP4 EPSPS protein	3
9	Broad Range MW markers	4.5

Figure 4. Purity and Molecular Weight Analysis of the MON 87705-Produced CP4 EPSPS Protein.

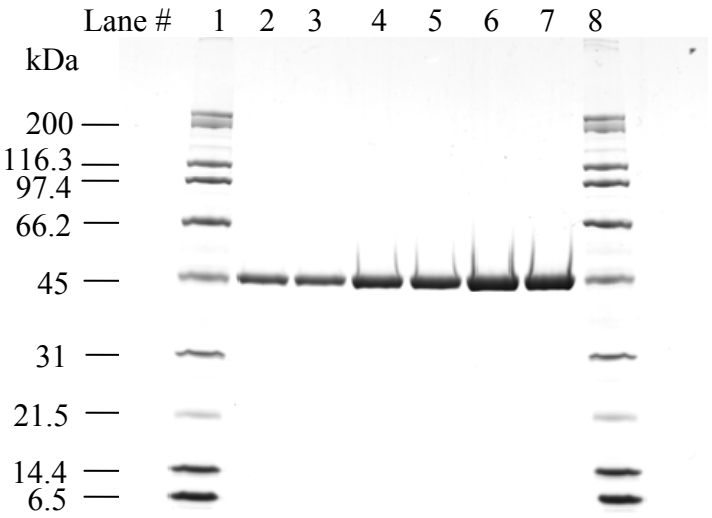
Aliquots of the MON 87705- and the *E. coli*-produced CP4 EPSPS proteins were separated on a 4 to 20% Tris glycine polyacrylamide gradient gel and then stained with Brilliant Blue G-Colloidal stain. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 9. Empty lanes were cropped.



Lane	Sample	Amount (ng)
1	CandyCane Glycoprotein MW standards	—
2	Horseradish Peroxidase (positive control)	50
3	Horseradish Peroxidase (positive control)	100
4	Transferrin (positive control)	50
5	Transferrin (positive control)	100
6	MON 87705-produced CP4 EPSPS	50
7	MON 87705-produced CP4 EPSPS	100
8	*E. coli*-produced CP4 EPSPS (negative control)	50
9	*E. coli*-produced CP4 EPSPS (negative control)	100
10	See Blue® Plus2 Pre-Stained MW markers	—

Figure 5. Glycosylation Analysis of the MON 87705-Produced CP4 EPSPS Protein.

Aliqouts of the MON 87705-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 to 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 4.40). Approximate MWs (kDa) correspond to the glycosylated markers loaded in Lane 1 and the dual color markers (used to verify transfer) in Lane 10.



Lane	Sample	Amount (µg)
1	Broad Range MW markers	4.5
2	MON 87705-produced CP4 EPSPS protein	1
3	MON 87705-produced CP4 EPSPS protein	1
4	MON 87705-produced CP4 EPSPS protein	2
5	MON 87705-produced CP4 EPSPS protein	2
6	MON 87705-produced CP4 EPSPS protein	3
7	MON 87705-produced CP4 EPSPS protein	3
8	Broad Range MW markers	4.5

Figure 6. Storage Stability of the MON 87705-Produced CP4 EPSPS.

SDS-PAGE analysis was performed on the sample stored in a -80 °C freezer for 8 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 cropped.

Appendix 1. List of Applicable SOP

SOP Number	Title
BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0392-01	Western Blot Analysis (Immunoblotting)
BR-ME-0924-01	Electrotransfer of Proteins to Membranes
BR-EQ-0599-03	Bio-Rad GS-800 Densitometer
BR-ME-0956-03	Protein Percent Purity and Apparent Molecular Weight Determination
BR-EQ-0935-02	Konica SRX X-Ray Film Processors
BR-EQ-0783-02	Applied Biosystems Voyager DE Pro Biospectrometry Workstation
BR-EQ-0265-02	Applied Biosystems 494 Procise™ Protein Sequencing System
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-0525-02	Bio-Rad protein assay
BR-ME-0986-01	Protein Drop Dialysis
BR-ME-0408-02	Phosphate Release Assay for Functionally Active EPSPS
BR-EQ-1155-01	Bio-Rad PharoseFX plus Molecular Imager System
BR-ME-1252-01	Removal of Proteins from Complex Buffer Systems by Means of Ethanol Precipitation Prior to Analytical Analyses

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

Analytical Protein Standard
Certificate of Analysis

MONSANTO ANALYTICAL PROTEIN STANDARDS

Sample Information:

Name of APS	APS Lot Number	Recertification Date
E. coli-produced CP4 EPSPS	20-100015	(3 months from APSO signature date)
Common or Alias Name(s)	**Historical APS Lot Number(s)**	**Storage Requirements (until use)**
n/a	n/a	–80 °C
Source: Fermentation of *Escherichia coli* containing the pMON21104 expression plasmid.		**Comment(s)** N/a
Additional Background Information: n/a		

Characteristic	Method	SOP(s)	Analysis Date	Result
Concentration	Amino Acid analysis	BR-EQ-0376-01	3/10/03	3.8 mg/mL (total protein)
Purity	SDS-PAGE Densitometry	BR-ME-0388-01 BR-ME-0527-01 BR-EQ-0599-01	4/10/03	97%
Molecular weight	SDS-PAGE Densitometry	BR-ME-0388-01 BR-ME-0527-01 BR-EQ-0599-01	4/10/03	43.8 kDa
Molecular weight	MALDI-TOF MS	BR-EQ-0783-01	3/17/03	47466.1 Da
Identity	Immunoblot	BR-ME-0388-01 GEN-PRO-002-03 BR-EQ-0599-01	4/3/03	Confirmed
Identity	N-terminal sequence	BR-ME-0388-01 AG-EQ-0379-01 BR-EQ-0265-01	3/19/03	Confirmed: MLHGASSRPATA(R)KS
Identity	MALDI-TOF MS	BR-ME-0388-01 BR-EQ-0783-01	3/18/03	Confirmed (64% coverage of expected sequence)
Activity	Phosphate release assay	GS-PRO-035-00 BR-EQ-0600-01	3/26/03	Units/mg total protein 4.57 (non-activated) 10.53 (activated)

Buffer composition: 50 mM Tris-Cl, pH 7.5, 50 mM KCl, 2 mM DTT, 25% (v/v) glycerol and 1 mM benzamidine-HCl
Physical description: Clear colorless solution
Purity corrected concentration is 3.7 mg/mL (3.8 mg/mL × 0.97 ≈ 3.7 mg/mL)

Stability Physically stable for 31 days at 4°C, –20 °C and –80 °C based on SDS-PAGE %purity and MW analysis.

Joan M. Rejda-Heath
Quality Assurance Specialist — May 7, 2003 Date

Thomas E Neumann
Testing Facility Management — 5/7/2003 Date

Analytical Protein Standards Officer — May 7, 2003 Date