Study Title

Characterization of the CP4 EPSPS Protein Purified from the Seed of MON 88302 and Comparison of the Physicochemical and Functional Properties of the MON 88302-produced and *E. coli*-Produced CP4 EPSPS Proteins.

Authors

[redacted]

September 20th, 2010

Sponsor and Performing Laboratory

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Laboratory Project ID

Monsanto Study Number: REG-10-152
MSL Number: MSL0022841

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Statement of Compliance

This study meets the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160.

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Sponsor Representative Date

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Study Director Date

Quality Assurance Unit Statement

Study Title: Characterization of the CP4 EPSPS Protein Purified from the Seed of MON 88302 and Comparison of the Physicochemical and Functional Properties of the MON 88302-produced and *E. coli*-Produced CP4 EPSPS Proteins.

Study Number: REG-10-152

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
05/13/2010	MALDI analysis	05/13/2010	05/13/2010
08/30/2010	Raw Data and Draft Report Review	08/31/2010	08/31/2010

[redacted]
Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

Study Certification Page

The results reported in this report accurately reflect the data generated under Study Number REG-10-152.

Signatures of Final Report Approval:

[redacted]

Study Director Date

[redacted]

Protein Sciences and Safety, Lead Date

Study Information

Study Number:	REG-10-152
MSL Number:	MSL 0022841
Title:	Characterization of the CP4 EPSPS Protein Purified from the Seed of MON 88302 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. coli*-Produced CP4 EPSPS Proteins
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Authors:	[redacted]
Study Director:	[redacted]
Contributors:	[redacted]
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Records Retention:	All characterization 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
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
E. coli	*Escherichia coli*
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 /Ionization - Time of Flight
MS	Mass Spectrometry
MW	Molecular Weight
MWCO	Molecular Weight Cutoff
NFDM	Non-Fat Dried Milk
PBST	Phosphate Buffered Saline containing 0.05% (v/v) Tween-20
PEP	Phosphoenolpyruvate
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene Difluoride
SDS-PAGE	Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
S3P	Shikimate-3-phosphate
Sinapinic Acid	3,5-dimethoxy-4-hydroxycinnamic acid
SOP	Standard Operating Procedure
TFA	Trifluoroacetic Acid
U	Unit (of enzyme activity)

[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.S.	United States
V	Volts
v/v	Volume to volume ratio
w/v	Weight to volume ratio

1.0 Summary

Monsanto Company has developed a second generation herbicide-tolerant canola product, MON 88302 that allows a glyphosate application from emergence to first flowering at a rate up to 1.8 kg a.e. per hectare. With an increased window of application and higher spray rates, MON 88302 will provide superior weed control compared to the commercial first generation Roundup Ready® canola product RT73 (also referred to as GT73). The increased level of glyphosate tolerance in MON 88302 is achieved through the use of improved promoter sequences that regulate the expression of the *cp4 epsps* gene conferring glyphosate tolerance.

MON 88302 contains the 5-enolpyruvylshikimate-3-phosphate synthase (*cp4 epsps*) gene derived from *Agrobacterium sp*. strain CP4. The MON 88302-produced CP4 EPSPS protein matches the *E. coli*-produced CP4 EPSPS protein (Orion lot 10000739) and the CP4 EPSPS protein 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 88302-produced CP4 EPSPS proteins justifies the uses of the existing data to demonstrate the safety of the CP4 EPSPS protein in MON 88302. Hence, the purpose of this study was to characterize the CP4 EPSPS protein isolated from seed of MON 88302 and demonstrate the equivalence of the MON 88302-produced CP4 EPSPS to the previously characterized *E. coli*-produced CP4 EPSPS.

MON 88302-produced CP4 EPSPS protein was characterized as follows. The total protein concentration of the purified MON 88302-produced CP4 EPSPS protein was measured using the Bio-Rad protein assay and was determined to be 0.26 mg/ml. The identity of the MON 88302-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 88302-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 expected peptide masses for the trypsin digested CP4 EPSPS protein were observed. For the latter, the identified peptide masses yielded coverage of 85.5% of the expected peptide sequence (389 out of 455 amino acids) of the CP4 EPSPS protein. Purity and apparent molecular weight (MW) of the MON 88302-produced CP4 EPSPS protein were determined using densitometric analysis of a Colloidal Brilliant Blue G stained SDS-PAGE gel. Purity of the MON 88302-produced CP4 EPSPS protein was 99% and the apparent MW was 43.1 kDa. The average mass of the intact MON 88302-produced protein was 47,351 Da as measured by MALDI-TOF MS analysis. This measurement was consistent with the theoretical mass of the CP4 EPSPS protein starting at position 2 (47481 Da). Analysis of the glycosylation status using the Amersham ECL glycoprotein detection system showed that the MON 88302-produced CP4 EPSPS protein was not glycosylated. The functional activity of the MON 88302-produced protein was

determined using a phosphate release assay. The MON 88302-produced CP4 EPSPS protein was shown to be active, with a specific activity of 4.93 ± 0.36 U/mg of CP4 EPSPS. Finally, determination of the purity and MW of the protein after storage at ~ -80 °C (98 days) by densitometric analysis of a Colloidal Brilliant Blue G stained SDS-PAGE gel, demonstrated that the MON 88302-produced CP4 EPSPS protein was stable throughout the experimental phase.

These data provide a detailed characterization of the CP4 EPSPS protein isolated from MON 88302 and establish the equivalence of the MON 88302-produced CP4 EPSPS protein to the *E. coli*-produced CP4 EPSPS protein.

2.0 Introduction

Monsanto Company has developed a second generation herbicide-tolerant canola product, MON 88302 that allows a glyphosate application from emergence to first flowering at a rate up to 1.8 kg a.e. per hectare. With an increased window of application and higher spray rates, MON 88302 will provide superior weed control compared to the commercial first generation Roundup Ready® canola product RT73. The increased level of glyphosate tolerance in MON 88302 is achieved through the use of improved promoter sequences that regulate the expression of the *cp4 epsps* gene.

MON 88302 was produced via stable insertion of a *cp4 epsps* coding sequence derived from the common soil bacterium *Agrobacterium sp.* strain CP4 using the transformation vector PV-BNHT2672. The *cp4 epsps* coding sequence directs the synthesis of a 5-enolpyruvylshikimate-3-phosphate synthase protein (CP4 EPSPS) which renders the plant tolerant to glyphosate, the active ingredient in the Roundup® family of agricultural herbicides.

The CP4 EPSPS protein is present in a number of biotechnology-derived crops. The results of protein safety assessment studies have been summarized and it has been concluded that the CP4 EPSPS protein poses no risk to human and animal health (Harrison et al., 1996).

3.0 Purpose

The purpose of this study was to characterize the CP4 EPSPS protein purified from the seed of MON 88302 (Orion lot 11266369) 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 88302- and *E. coli*- produced CP4 EPSPS proteins justifies the application of existing safety data for the CP4 EPSPS protein to the CP4 EPSPS protein produced in MON 88302.

4.0 Materials

4.1 MON 88302-Produced CP4 EPSPS Protein (Test substance)

The MON 88302-produced CP4 EPSPS protein (Orion lot 11266369) purified from seed of MON 88302 was used as the test substance. The seed was produced by Monsanto Biotech. A copy of the certificate of analysis (COA) for the seed has been archived under the Orion lot 11266369. The MON 88302-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 dithiothreitol (DTT), 1 mM benzamidine-HCl, and 25 % glycerol. The purification records for the MON 88302-produced protein have been archived under the Orion lot 11266369 according to the current version of SOP BR-PO-0722.

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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 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 in pMON21104 was confirmed prior to and following fermentation. The *E. coli*-produced CP4 EPSPS protein was previously characterized and copies of the certificate of analysis (COA) are shown in Appendix 2.

4.3 Assay Controls

Protein MW 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) as well as nitrocellulose membranes. Broad Range SDS-PAGE molecular weight standards (Bio-Rad, Hercules, CA) were used to generate a standard curve for the apparent MW estimation,. 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 phenylthiohydantoin (PTH) amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to calibrate the instrument for each analysis. 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 bovine serum albumin (BSA) standard (NIST, Gaithersburg, MD) was used to calibrate the MALDI-TOF mass spectrometer for intact mass analysis. Transferrin (Sigma-Aldrich, St. Louis, MO) was used as positive control for glycosylation analysis.

5.0 Methods

5.1 Protein Purification

The plant-produced CP4 EPSPS protein was purified from seed of MON 88302 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 seed 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 11266369.

Approximately 498 g of MON 88302 seed was frozen with liquid nitrogen in a mortar and ground with a pestle. The partially crushed seed was further ground using a Magic Bullet grinder. The ground seed was then defatted by extraction with heated hexane (~50 °C)

followed by vacuum filtration. This was repeated three times at a ground seed (g) to hexane volume (ml) ratio of approximately 1:5. The defatted ground seed was allowed to dry overnight at room temperature in a fume hood. The following day the defatted ground seed was mixed with extraction buffer (100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM benzamidine-HCl, 4 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1% polyvinylpolypyrrolidone, and 10% glycerol) for ~1.5 h at a sample weight (g) to buffer volume (ml) ratio of approximately 1:10. The slurry was centrifuged at 15,182 x *g* for 1 h at ~ 4 ºC. The supernatant (~3.5 liters) was collected and brought to 45% ammonium sulfate saturation by slow addition of 903 g of ammonium sulfate in a cold room (~ 4 ºC). The solution was stirred for ~1 h at ~ 4 ºC and then centrifuged at 15,182 x *g* for 1 h. The supernatant (~3.8 liters) was again collected and 592 g of ammonium sulfate was added to bring the solution to 70% ammonium sulfate saturation. The solution was stirred for ~ 1 h in a cold room and the pellet was collected by centrifugation at 15,182 x *g* for 1 h. The pellet was re-suspended in 1 liter 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 460 ml column (5 cm x 23 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 2.3 liters. Fractions containing the CP4 EPSPS protein, identified based on immunoblot analysis and SDS-PAGE analysis, were pooled to a final volume of ~440 ml. The pooled sample was desalted by dialysis against 20 liters of QS(A) buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM benzamidine-HCl, 4 mM DTT) at ~4 °C using a dialysis tubing [Spectrum Laboratories, Inc., Rancho Dominguez, CA; Molecular Weight Cutoff (MWCO): 3.5 kDa] for a total of 16 h.

The desalted sample (600 ml) was loaded onto a 180 ml column (5 cm x 9.2 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 2.1 liters. Fractions containing CP4 EPSPS, identified by immunoblot analysis, were pooled to a final volume of ~ 280 ml. The pooled sample was dialyzed against 20 liters CP(A) buffer (10 mM sodium citrate, pH 5.0, 1 mM benzamidine-HCl, 2 mM DTT) for a total of 18 h at ~4 °C using a dialysis tubing (Spectrum Laboratories, Inc. Rancho Dominguez, CA; MWCO: 3.5 kDa).

Of the 350 ml recovered after dialysis, approximately 50 ml of the dialyzed sample was loaded onto a 5 ml column (1.6 x 2.5 cm) of cellulose phosphate P11 cation exchange (Whatman, Kent, UK) pre-equilibrated with CP(A) buffer. After an initial wash with 40 ml of CP(A) buffer, the column was washed with 50 ml of CP(B) buffer [CP(A) buffer with pH adjusted to 5.2 and supplemented with 0.5 mM phosphoenolpyruvate (PEP)]. The column was further washed with CP(C) buffer [CP(A) buffer with pH adjusted to 5.4 and supplemented with 0.5 mM PEP]. The bound CP4 EPSPS protein was eluted with of

CP(D) buffer [CP(A) buffer with pH adjusted to 5.7 and supplemented with 0.5 mM PEP and 0.5 mM shikimate-3-phosphate (S3P)] over 90 ml. Fractions containing CP4 EPSPS protein, based on SDS-PAGE analysis and confirmed by immunoblot analysis, were pooled (~22 ml), supplemented with 10% glycerol, labeled Pool 1, and stored at -20°C. Approximately 200 ml of the remaining dialyzed sample was then loaded onto a freshly prepared 20 ml column (2.6 x 3.7 cm) of cellulose phosphate P11 cation exchange (Whatman, Kent, UK) pre-equilibrated with freshly prepared CP(A) buffer. After an initial wash with 200 ml of CP(A) buffer, the column was washed with 160 ml of freshly prepared CP(B) buffer. The column was further washed with freshly prepared CP(C) buffer. The bound CP4 EPSPS protein was eluted with freshly prepared CP(D) buffer. Fractions containing CP4 EPSPS protein, based on SDS-PAGE analysis and confirmed by immunoblot, were pooled (Pool 2). Pool 1 and Pool 2 were combined (~82 ml) and divided between four iCon concentrators (MWCO: 20 kDa; size: 20 ml; Pierce, Rockford, IL) and concentrated by centrifugation at 4,000 x *g* for 30 min at ~4 °C. Buffer exchange was carried out in the same concentrators by the addition of ~19 ml an initial buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine-HCl followed by centrifugation at 4,000 x *g* for 30 min at ~4 °C repeated four times. After the fourth buffer exchange the remaining sample (~10 ml) was transferred to a new iCon concentrator (MWCO: 20 kDa; size: 20 ml; Pierce, Rockford, IL), supplemented with equal volume of the buffer containing 50 mM Tris-HCl pH 7.5, 50 mM KCl, 2 mM DTT, 50% glycerol and 1 mM benzamidine-HCl, and the sample was concentrated to ~2.4 ml. The 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 seed of MON 88302 was aliquoted, assigned APS lot 11266369, and stored at in a -80°C freezer.

5.2 Protein Concentration

The total protein concentration of the MON 88302-produced CP4 EPSPS protein sample was determined using a Bio-Rad protein assay. *E. coli*-produced CP4 EPSPS reference 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 88302-produced CP4 EPSPS protein solution was calculated by interpolation of the blank-corrected absorbance values at 595 nm obtained for the test substance. 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 seed of MON 88302 and to compare the immunoreactivity of the MON 88302- and *E. coli*-produced proteins.

The MON 88302- 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× Laemmli 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 130 V for 90 min. Electrotransfer to a 0.45 µm nitrocellulose membrane (Invitrogen, Carlsbad, CA) was performed for 90 min at a constant voltage of 30 V. After electrotransfer, the membrane was blocked for 1 h 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 1:1000 dilution of goat anti-CP4 EPSPS antibody (lot 10000787) in 5% (w/v) NFDM in PBST overnight at 4°C. 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 at room temperature. Excess HRP-conjugate was removed using three 10 min washes with PBST. All washes were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (GE, Healthcare, Piscataway, NJ) with exposure (1 and 3 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 tools. The signal intensities of the immunoreactive bands observed for the test (MON 88302-produced) and reference (*E. coli*-produced) 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 (12.0.6535.5002) SP2 MSO (12.0.6535.5002)] 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 88302-produced CP4 EPSPS.

MON 88302-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 a 140C Microgradient pump and a 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, 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 88302-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 88302-produced CP4 EPSPS protein. MON 88302-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 and transferred to a microcentrifuge tube. The gel bands were washed in 100 mM ammonium bicarbonate and then, to reduce the protein in each, gel bands were incubated in 100 µl of 10 mM DTT at ~37°C for 2 h. The protein was then alkylated in the dark for 25 min with 100 µl of 20 mM iodoacetic acid and washed with 200 µl of 25 mM ammonium bicarbonate for 3 x 20 min washes. Gel bands were dried with a Speed-Vac concentrator (Thermo Fisher Scientific, Waltham, MA) and then rehydrated with 20 µl of trypsin solution (20 µg/ml). After 1 h, excess liquid was removed and the gel was incubated at ~37 °C for 16 h in 40 µl of 10% acetonitrile in 25 mM ammonium bicarbonate. Gel bands were sonicated for 5 min to further elute proteolytic fragments. 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 µl 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% trifluoroacetic acid (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% TFA was added to

each tube and all were sonicated for 5 min. Each extract (0.3 µl) was spotted to three wells on an analysis plate. For each extract 0.75 µl 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 7000 Dalton range. Samples in α-Cyano and Sinapinic acid were analyzed in the 500 to 5000 and 500 to 7000 Dalton range, respectively. Protonated peptide masses were monoisotopically resolved 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 (Lighthouse Data, Odense M, Denmark) was used to generate a theoretical trypsin digest of the CP4 EPSPS protein sequence. 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 ≥ 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 and reference substances were mixed with 5× LB and diluted with H_2O to a final total protein concentration of 0.2 µg/µl. Biorad Molecular Weight Standards, Broad Range (Hercules, CA) were diluted to a final total protein concentration of 0.9 units. The test substance was analyzed in duplicate at 1, 2, and 3 µg protein per lane. The *E. coli*-produced CP4 EPSPS reference standard was analyzed at 1 µg total protein in a single lane. The samples were loaded onto a 10-well pre-cast Tris glycine 4 - 20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA) and electrophoresis was performed at a constant voltage of 130 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 h with Brilliant Blue G-Colloidal stain (Sigma-Aldrich, St. Louis, MO). Gels were destained for 30-45 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and for ~7 h 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 MW of each observed band was estimated from a standard curve generated by the Quantity One® software which was based on the MWs of the markers and their migration distance on the gel. All visible bands within each lane were quantified using Quantity One® software. Apparent MW and purity were reported as an average of all six lanes containing the MON 88302-produced CP4 EPSPS protein.

5.7 MALDI-TOF Intact Mass Analysis

MALDI-TOF mass spectrometry was used to further characterize the MON 88302-produced CP4 EPSPS. Prior to MALDI-TOF MS analysis, an ethanol precipitation was

performed to concentrate the MON 88302-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 88302-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% TFA and air-dried. Mass spectral analysis of the MON 88302-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 1,000 to 120,000 Dalton range using 150 shots at a laser intensity setting of 3245 (a unitless MALDI-TOF instrument specific value). Average protonated protein masses were observed in linear mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW32 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 88302-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 88302-produced CP4 EPSPS was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the MON 88302-produced CP4 EPSPS protein, the *E. coli*-produced CP4 EPSPS (negative control) and the positive control, transferrin (Sigma-Aldrich, St Louis, MO), were each diluted in 1 × LB. These samples were heated at ~95 °C for 3 min. The MON 88302- and the *E. coli*- produced CP4 proteins were loaded at approximately 100 and 200 ng per lane and transferrin was loaded at approximately 50, 100, 150 and 200 ng on a Tris-glycine 10-well 4 - 20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA). Precision Plus Protein™ Dual color Standards (Bio-Rad, Hercules, CA) were also loaded to verify electrotransfer of the proteins to the membrane and as markers for molecular weight. Electrophoresis was performed at a constant voltage of 155 V for 75 min. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 35 min at a constant voltage of 100 V.

Carbohydrate detection was performed directly on the PVDF membrane at room temperature using the Amersham ECL™ glycoprotein Detection Module (GE, Healthcare, Piscataway, NJ). With this module, carbohydrate moieties of proteins are oxidized with sodium metaperiodate and are then biotinylated with biotin-X-hydrazide. The biotinylated proteins can be detected on the blot by addition of streptavidin conjugated to HRP for luminol-based detection using ECL ™ reagents (GE, Healthcare, Piscataway, NJ) and with subsequent exposure (1, 2and 3 min) to Amersham Hyperfilm (GE, Healthcare,

Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

A second identical blot run in parallel to that used for the glycosylation analysis 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× Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad, Hercules, CA) for 5 min. After washing with water, the blot was dried and 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 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 phosphate assay reagent (0.033 % malachite green, 1.1 % ammonium molybdate) 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 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 (12.0.6524.5003) SP2 MSO (12.0.6425.1000).

5.10 Storage Stability

The short-term storage stability of the MON 88302-produced CP4 EPSPS protein in a -80 °C freezer over the experimental phase of the study was evaluated by comparing the purity and MW values obtained on day 0 to the purity and MW values obtained on day 98 of storage. Day 0 stability analysis corresponds to the purity and molecular weight determination described in Section 5.6. On day 98, several aliquots were removed from a ~-80 °C freezer and purity and MW analysis was conducted as in section 5.6. The protein sample was considered to be stable if purity and molecular weight of the test protein is within ± 10% of the purity and molecular weight observed at day 0 of the experimental phase.

5.11 Equivalence Criteria

The equivalence of the MON 88302-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 ± 35% 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 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

Carbohydrate detection experiments performed using the Pro-Q® Emerald 488 Glycoprotein Gel and Blot Stain Kit (Molecular Probes, Eugene, OR) were rejected. In one case, staining of the CandyCane ™ molecular weight standards (Molecular Probes, Eugene, OR) was anomalous. In a second case, staining of one of the positive controls was anomalous. In both cases, staining of the negative control was inconsistent with glycosylation status.

7.0 Protocol Amendment

The protocol was amended to change the method used to assess the glycosylation status of the MON 88302-produced CP4 EPSPS protein. In this study the original method using the Molecular Probes Pro-Q® Emerald 488 kit produced results on two occasions that did not meet the acceptance criteria pre-set for the assay, while on two other occasions in pre-study method development, it did meet these same criteria. It was therefore necessary to investigate and implement an alternative, more reproducible method to evaluate the glycosylation status of the plant-produced CP4 EPSPS protein. The Amersham ECL glycoprotein detection system was the final method to assess the glycosylation status of the MON 88302-produced CP4 EPSPS protein. This change had a positive impact on the study execution and results obtained.

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 88302-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 was used as the positive control while the *E. coli*-

produced CP4 EPSPS protein served as a 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 total protein concentration of the MON 88302-produced CP4 EPSPS was determined to be 0.26 mg/ml based on the Bio-Rad protein assay (Table 1).

9.2 Protein Identity

The identity of the MON 88302-produced CP4 EPSPS protein was confirmed using the following analytical methods: immunoblot analysis, N-terminal sequencing, and MALDI-TOF MS tryptic mass fingerprint analysis. In addition, immunoblot analysis using anti-CP4 EPSPS antibody was used to confirm equivalent immunoreactivity of the MON 88302- 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 88302-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 88302- 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 88302-produced CP4 EPSPS protein. Densitometric analysis of the bands showed an average difference of 24.1% between the intensity of the signals from the MON 88302-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 ± 35%, MON 88302- and *E. coli*-produced proteins are considered to have equivalent immunoreactivity.

9.2.2 N-Terminal Sequencing

N-terminal sequencing performed on the MON 88302-produced CP4 EPSPS resulted in 15 amino acid residues being determined (Figure 2). The experimental results yielded two observable protein sequences both of which are consistent with the predicted N-terminus of the CP4 EPSPS protein produced in MON 88302. The two sequences match the expected sequence for CP4 EPSPS (MON88302) beginning at either position 2 or position 4. The observation of a staggered N-

terminal sequence for the plant-produced CP4 EPSPS protein has previously been reported in canola (Thorp and Silvanovich, 2004). This finding is not uncommon since the N-terminal methionine is typically processed from proteins in eukaryotic organisms by an endogenous methionine aminopeptidase (Arfin and Bradshaw, 1988; Bradshaw et al., 1998). The loss of several additional N-terminal residues may be due to endogenous protease activity that is released upon cell disruption. Despite the staggered N-terminus, the sequence information confirms the identity of the CP4 EPSPS protein isolated from the seed of MON 88302.

9.2.3 MALDI-TOF Tryptic Mass Fingerprint Analysis

The identity of the MON 88302-produced CP4 EPSPS protein was also confirmed by MALDI-TOF mass spectrometry analysis of tryptic peptide fragments prepared from the MON 88302-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 34 unique peptides identified that corresponded to the expected masses (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 85.5% (389 out of 455 amino acids). This analysis serves as additional identity confirmation for the MON 88302-produced CP4 EPSPS protein.

9.2.4 MALDI-TOF Intact Mass Analysis

The intact mass of the MON 88302-produced CP4 EPSPS protein was determined by MALDI-TOF MS analysis. The MON 88302-produced protein was shown to have a MW of 47351 Da. This value compares well with the theoretical mass of the full-length protein starting at position 2 (47481 Da). The 130 Da difference is likely a result of the staggered N-terminus (see section 9.2.2). While two species were noted during N-terminal analysis, the abundance of each species could not be confirmed and the MALDI-TOF signal likely represents the most abundant species.

9.3 Molecular Weight and Purity Determination

For MW and purity analysis, the MON 88302-produced CP4 EPSPS protein was separated using SDS-PAGE. Following electrophoresis the gel was 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 88302-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, lane 2) and had an apparent MW of 43.1 kDa (Table 4). The apparent MW 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 88302- and *E. coli*-produced CP4 EPSPS proteins was 1.6% (Table 5). Because this difference met the previously set acceptance criteria (≤10% difference), the MON 88302- and *E. coli*-produced CP4 EPSPS proteins are considered equivalent based on their experimentally estimated apparent MW.

The purity of the MON 88302-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 99% (Table 4).

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. MON 88302-produced CP4 EPSPS 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 been shown previously to be free of glycosylation (Harrison *et al*, 1996) while transferrin was used as the positive control. Both the negative and positive controls were analyzed concurrently with the MON 88302-produced CP4 EPSPS protein. The results of this analysis are shown in Figure 5A. The positive control is clearly detected at the expected molecular weight, in a concentration-dependent manner (Figure 5A, lanes 2-5). In contrast, signals were not observed in the lanes containing the reference protein or the test protein at the molecular weight expected for CP4 EPSPS (Figure 5A, lanes 6-9). Other data collected under this study also demonstrated the absence of glycosylation of the MON 88302-produced CP4 EPSPS. For example glycosylation of the CP4 EPSPS protein 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 88302-produced CP4 EPSPS protein (47351 Da) to the theoretical mass starting at position 2 does not support the existence of a glycosylated species, as the addition of even a single sugar moiety would increase the mass by at least 160 Da. Finally, to confirm that the proteins were transferred to the membrane, a second membrane (with identical loadings and transfer times) was stained

with Coomassie Blue R 250 and scanned (Figure 5B). Thus, the data cited above demonstrate that MON 88302-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 88302- and *E. coli*-produced CP4 EPSPS proteins was measured to be 4.93 U/mg and 2.79 U/mg of CP4 EPSPS, respectively. Because the value of MON 88302-produced CP4EPSPS specific activity falls within 2-fold of the *E. coli*-produced CP4 EPSPS value (between 1.40 U/mg and 5.58 U/mg), the previously set acceptance criteria was met and the MON 88302-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 88302-produced CP4 EPSPS protein stored in a -80 °C freezer for a 98 day period was assessed (Table 7, Table 8 and Figure 6). Stability was evaluated by comparison of the apparent MW and purity of the protein after storage (day 98) to the initial apparent MW and purity values determined on day 0. The MW of the CP4 EPSPS protein was determined to be 43.5 kDa on day 98. This value differed by 0.9 % to the MW value obtained on day 0 (Table 8). The purity value for the MON 88302-produced CP4 EPSPS protein (Figure 6, Lanes 3-8) was determined to be 99% by densitometric analysis. This value was identical to the purity obtained on day 0 (Table 8). Therefore, based on the apparent MW and purity, the MON 88302-produced CP4 EPSPS protein was stable when stored and aliquoted in a ~-80 °C freezer for the duration of the experimental phase of this study (98 days).

10.0 Conclusions

A panel of analytical techniques was used to characterize the MON 88302-produced CP4 EPSPS protein purified from seed of MON 88302. Identity of the MON 88302-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 85.5% overall coverage of the expected protein sequence. The concentration of the MON 88302-produced CP4 EPSPS was 0.26 mg/ml. The purity and MW weight of the MON 88302-produced CP4 EPSPS was 99 % and 43.1 kDa, respectively. MALDI-TOF mass spectrometry analysis of the intact protein resulted in an average mass of 47481 Da. The MON 88302-produced CP4 EPSPS protein was not glycosylated and had a specific activity of 4.93 U/mg of CP4 EPSPS. Finally, the MON 88302-produced CP4 EPSPS protein was stable for at least 98 days while stored in a ~-80 °C freezer, which encompassed the duration of the experimental phase of this study.

The equivalence of the MON 88302- and *E. coli*-produced CP4 EPSPS proteins was evaluated by comparing their apparent MW, immunoreactivity with anti-CP4 EPSPS antibodies, glycosylation status, and functional activity. The results obtained demonstrate that the MON 88302-produced CP4 EPSPS protein is equivalent to the *E. coli*-produced CP4 EPSPS protein. This equivalence justifies the of the previously conducted protein safety studies in which 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 88302-Produced CP4 EPSPS Protein

The concentration of the MON 88302-produced CP4 EPSPS protein was determined by Bio-Rad protein assay.

Sample	Concentration (mg/ml)[1]	RSD[2]
MON 88302-produced CP4 EPSPS	0.26	8%

[1]Value refers to mean calculated based on n=6. The result of the concentration determination was rounded to two decimal places.

[2] Relative standard deviation about the mean.

Table 2. Immunoreactivity of the MON 88302-Produced and *E. coli*-Produced CP4 EPSPS Proteins with CP4 EPSPS-Specific Antibodies

The immunoreactivity of the MON 88302-produced CP4 EPSPS (plant CP4 EPSPS) was compared to that of the *E. coli*-produced reference protein. The average difference in the band intensities obtained from Figure 1 is reported. Calculations are shown below. The 1 min exposure was used for this analysis.

Sample	Gel lane	Amount (ng)	Contour Qty (OD x mm^2)	Average Contour Qty[1]	Percent difference[2] (%)	Average Difference[3] (%)
E. coli CP4 EPSPS	2	1	1.257	1.408	30.8	24.1
E. coli CP4 EPSPS	3	1	1.558			
Plant CP4 EPSPS	9	1	2.064	2.033		
Plant CP4 EPSPS	10	1	2.002			
E. coli CP4 EPSPS	4	2	3.296	3.748	26.5	
E. coli CP4 EPSPS	5	2	4.199			
Plant CP4 EPSPS	11	2	4.979	5.101		
Plant CP4 EPSPS	12	2	5.222			
E. coli CP4 EPSPS	6	3	6.264	6.407	14.9	
E. coli CP4 EPSPS	7	3	6.549			
Plant CP4 EPSPS	13	3	7.737	7.527		
Plant CP4 EPSPS	14	3	7.317			

[1]Average Density =∑ 〚Contour Quantity〛 /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 (12.0.6504.5001) SP1 MSO (12.0.6320.5000).

Table 3. Summary of the Tryptic Masses Identified for the MON 88302-Produced CP4 EPSPS Using MALDI-TOF MS

a-Cyano	a-Cyano	DHB	DHB	Sinapinic acid	Sinapinic acid	**Expected Mass**	**Diff.[1]**	**Fragment**	**Sequence**
Extract 1	Extract 2	Extract 1	Extract 2	Extract 1	Extract 2				
		389 28				389 25	0 03	225-227	TIR
		474 32				474 27	0 05	228-231	LEGR
506 29						506 22	0 07	354-357	ESDR
		529 36				529 30	0 06	24-28	IPGDK
599 43	599 51	599 41				599 33	0 10	29-33	SISHR
616 44	616 48	616 41		616 24		616 34	0 10	128-132	RPMGR
629 44		629 45				629 29	0 15	201-205	DHTEK
629 44		629 45				629 34	0 10	383-388	GRPDGK
711 57	711 62	711 56	711 61			711 45	0 12	133-138	VLNPLR
		790 55				790 48	0 07	306-312	VRSSTLK
		790 55				790 41	0 14	139-145	EMGVQVK
		805 54				805 43	0 11	447-453	IELSDTK
835 54	835 58	835 53				835 39	0 15	62-69	AMQAMGAR
863 61	863 68	863 60				863 46	0 15	15-23	SSGLSGTVR
872 61	872 66	872 61	872 67	872 53		872 45	0 16	313-320	GVTVPEDR
872 61	872 66	872 61	872 67	872 53		872 52	0 09	358-366	LSAVANGLK
		930 66				930 51	0 15	169-177	VPMASAQVK
948 68	948 74	948 68	948 75			948 52	0 16	161-168	TPTPITYR
991 72		991 71				991 55	0 17	14-23	KSSGLSGTVR
1115 75	1115 83	1115 77	1115 86	1115 69		1115 57	0 18	295-305	LAGGEDVADLR
1357 94	1358 01	1357 97	1358 05	1357 89		1357 71	0 23	146-157	SEDGDRLPVTLR
1359 88	1359 96	1359 91	1360 00	1359 81	1359 87	1359 72	0 16	354-366	ESDRLSAVANGLK
1359 88	1359 96	1359 91	1360 00	1359 81	1359 87	1359 64	0 24	34-46	SFMFGGLASGETR
1559 11	1559 18	1559 13	1559 01			1558 83	0 28	47-61	ITGLLEGEDVINTGK
1647 10	1647 24	1647 16	1647 24			1646 84	0 26	389-405	GLGNASGAAVATHLDHR
1764 10	1764 26	1764 16		1764 06		1763 81	0 29	367-382	LNGVDCDEGETSLVVR
1994 31	1994 43	1994 35	1994 55	1994 21	1994 35	1993 97	0 34	206-224	MLQGFGANLTVETDADGVR
2183 54	2183 67	2183 57	2183 80	2183 45	2183 53	2183 17	0 37	275-294	TGLILTLQEMGADIEVINPR
2367 73	2367 87	2367 77	2367 85	2367 65	2367 8	2367 33	0 40	178-200	SAVLLAGLNTPGITTVIEPIMTR
2450 65	2450 83	2450 80		2450 51	2450 6	2450 23	0 42	24-46	IPGDKSISHRSFMFGGLASGETR
2450 65	2450 83	2450 80		2450 51	2450 6	2450 22	0 43	105-127	LTMGLVGVYDFDSTFIGDASLTK
3247 10 (Ave)	3247 05 (Ave)			3246 89 (Ave)	3246 97 (Ave)	3246 54 (Ave)	0 56	73-104	EGDTWIIDGVGNGGLLAPEAPLDFGNAATGCR
3251 94 (Ave)	3252 18 (Ave)	3252 06 (Ave)	3253 42 (Ave)	3252 58 (Ave)	3252 04 (Ave)	3251 75 (Ave)	0 19	321-351	APSMIDEYPILAVAAAFAEGATVMNGLEELR
		4191 34 (Ave)	4191 48 (Ave)	4191 89 (Ave)	4191 63 (Ave)	4190 89 (Ave)	0 37	234-274	LTGQVIDVPGDPSSTAFPLVAALLVPGSDVTILNVLMNPTR

Only experimental masses that matched expected masses are listed in the table. [1] The 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. 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 88302-Produced CP4 EPSPS Protein

The apparent molecular weight and the purity of the MON 88302-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	42.84	99.5
1 µg in lane 4	42.72	99.5
2 µg in lane 5	42.87	99.3
2 µg in lane 6	43.13	99.0
3 µg in lane 7	43.31	99.1
3 µg in lane 8	43.54	99.2
Average	43.1	99

Table 5. Molecular Weight Difference Between the MON 88302- and *E. coli*-Produced CP4 EPSPS Proteins

Molecular Weight of MON 88302-Produced CP4 EPSPS Protein[1]	Molecular Weight of *E. coli*-Produced CP4 EPSPS Protein[2]	% Difference from *E. coli*-Produced CP4 EPSPS Protein[3]
43.1 kDa	43.8 kDa	1.6 %

[1]See Table 4 for the MW of the MON 88302-produced CP4 EPSPS.

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

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

Table 6. Specific Activity of MON 88302-Produced CP4 EPSPS

The specific activity of the MON 88302-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 88302-produced CP4 EPSPS[1] (U/mg)	***E. coli*-produced CP4 EPSPS[1] (U/mg)**	**Previously set acceptance limits[2] (U/mg)**
4.93 ± 0.36	2.79 ± 0.26	1.40 – 5.58

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

[2]Within 2-fold of the *E.coli*-produced CP4 EPSPS specific activity (2.79 ÷ 2 U/mg to 2.79 x 2 U/mg)

Table 7. Molecular Weight and Purity Estimation for the MON 88302-Produced CP4 EPSPS after 98 Days of Storage in a -80 °C Freezer

The day 98 purity and molecular weight values for the MON 88302-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.

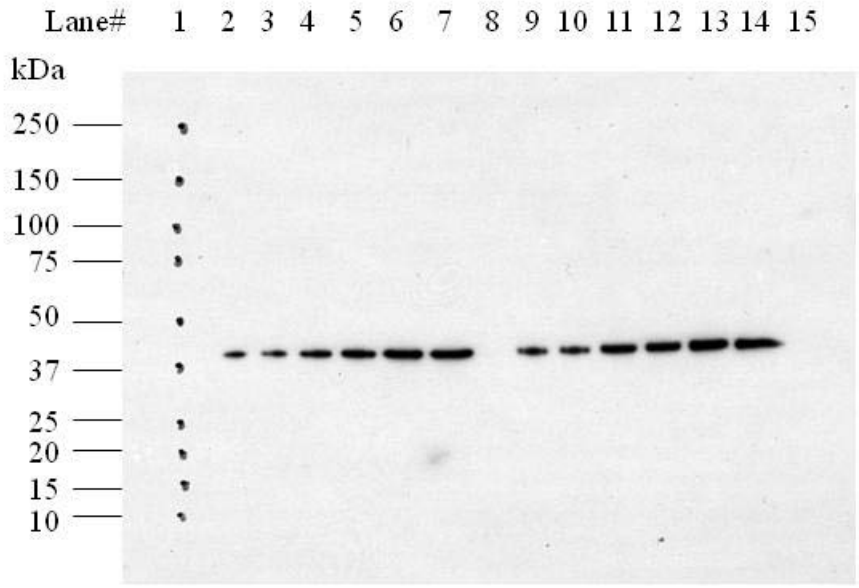
Total protein loaded	Apparent MW (kDa)	Purity (%)
1 µg in lane 3	44.18	99.3
1 µg in lane 4	43.62	99.3
2 µg in lane 5	43.32	99.1
2 µg in lane 6	43.44	99.1
3 µg in lane 7	43.18	99.0
3 µg in lane 8	43.35	99.1
Average	43.5	99

Table 8. Storage Stability (Day 98 vs. Day 0) of the MON 88302-Produced CP4 EPSPS Protein at ~ -80 °C

The purity and molecular weight values of the MON 88302-produced CP4 EPSPS protein are shown in Table 4 at day 0 and in Table 7 at day 98.

Apparent MW of CP4 EPSPS (kDa)			Purity (%)		
Day 0	Day 98	% Difference[1] (Day 98 vs. 0)	Day 0	Day 98	% Difference[1] (Day 98 vs. 0)
43.1	43.5	0.9	99	99	0.0

[1] $\% \text{ Difference} = \left| \frac{(\text{Day } 0 - \text{Day } 98)}{\text{Day } 0} \right| \text{X } 100\%$



Lane	Sample	Amount (ng)
1	Precision Plus Protein™ Standards Dual color	-
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 88302-produced CP4 EPSPS protein	1
10	MON 88302-produced CP4 EPSPS protein	1
11	MON 88302-produced CP4 EPSPS protein	2
12	MON 88302-produced CP4 EPSPS protein	2
13	MON 88302-produced CP4 EPSPS protein	3
14	MON 88302-produced CP4 EPSPS protein	3
15	Empty	-

Figure 1. Immunoblot Analysis and Immunoreactivity of MON 88302- and *E. coli*-Produced CP4 EPSPS

Aliquots of the MON 88302-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 1 min exposure is shown.

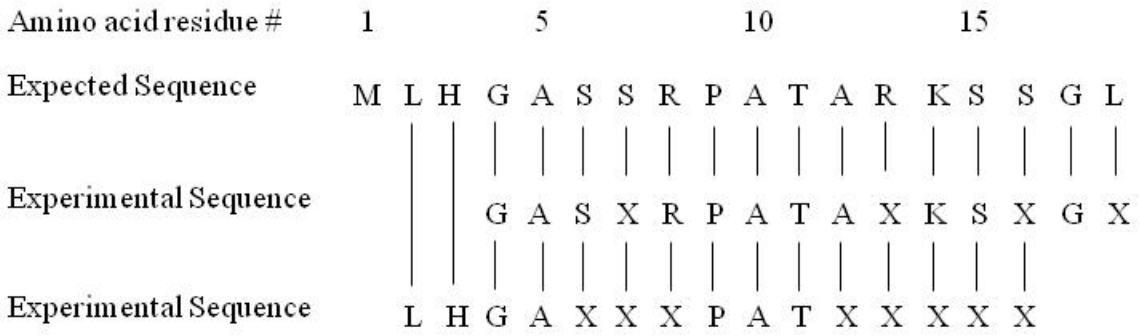


Figure 2. N-Terminal Sequence of the MON 88302-Produced CP4 EPSPS

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 88302. The experimental sequences obtained from the MON 88302-produced CP4 EPSPS were compared to the expected sequence.

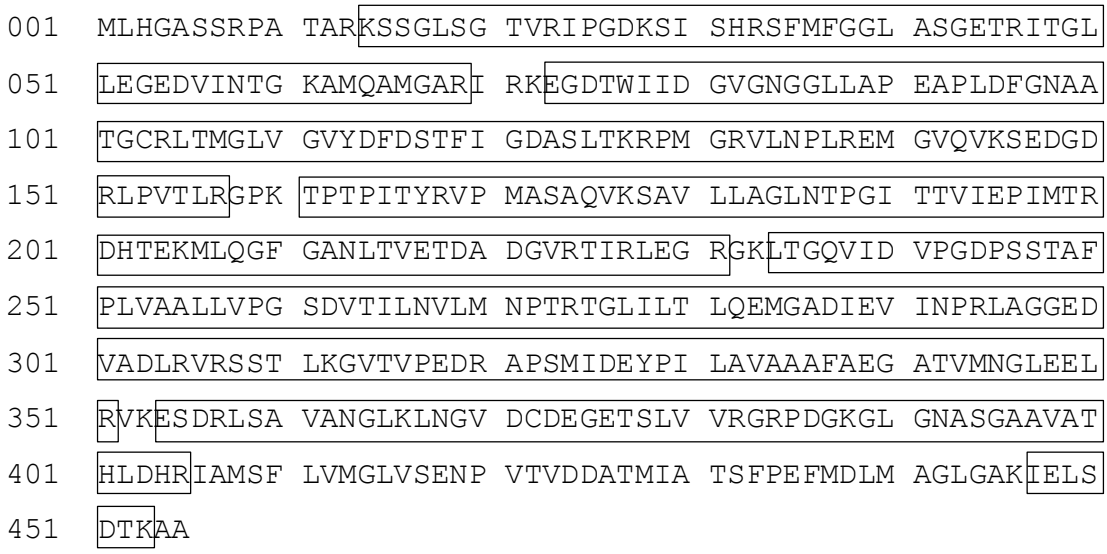
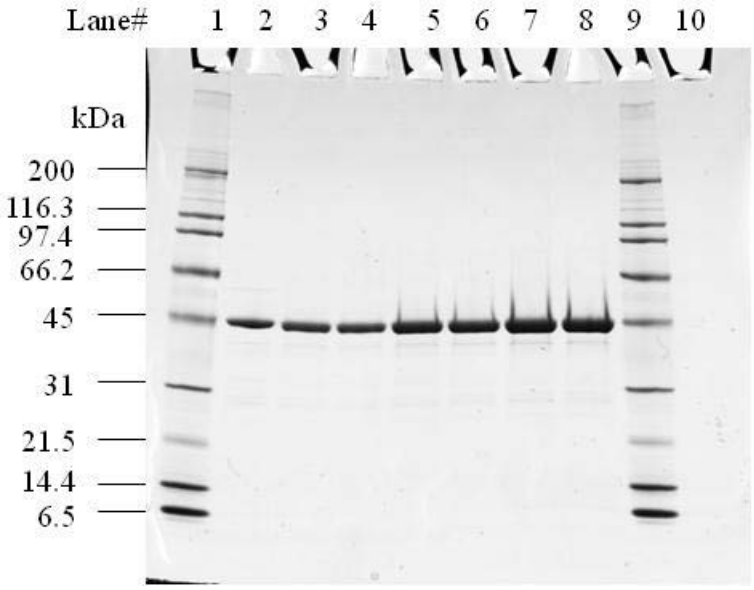


Figure 3. MALDI-TOF MS Coverage Map of the MON 88302-Produced CP4 EPSPS

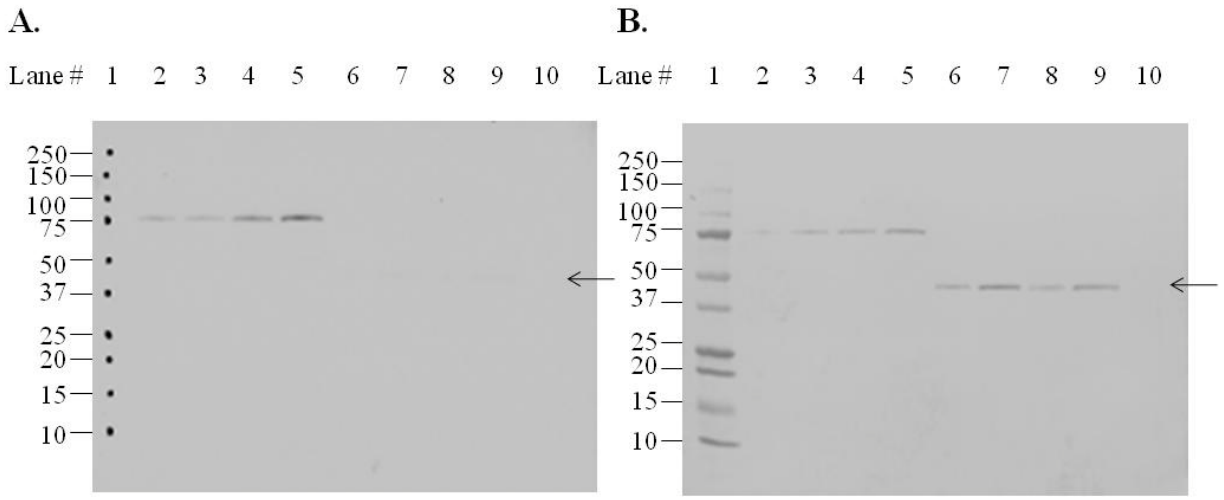
The amino acid sequence of the mature CP4 EPSPS protein was deduced from the *cp4 epsps* gene present in MON 88302. Boxed regions correspond to tryptic peptides that were identified from the MON 88302-produced CP4 EPSPS protein sample using MALDI-TOF MS. In total, 85.5 % (389 of 455 total amino acids) of the expected protein sequence was identified.



Lane	Sample	Amount (µg)
1	Broad Range MW markers	4.5
2	*E. coli*-produced CP4 EPSPS protein	1
3	MON 88302-produced CP4 EPSPS protein	1
4	MON 88302-produced CP4 EPSPS protein	1
5	MON 88302-produced CP4 EPSPS protein	2
6	MON 88302-produced CP4 EPSPS protein	2
7	MON 88302-produced CP4 EPSPS protein	3
8	MON 88302-produced CP4 EPSPS protein	3
9	Broad Range MW markers	4.5
10	Empty	-

Figure 4. Purity and Molecular Weight Analysis of the MON 88302-Produced CP4 EPSPS Protein

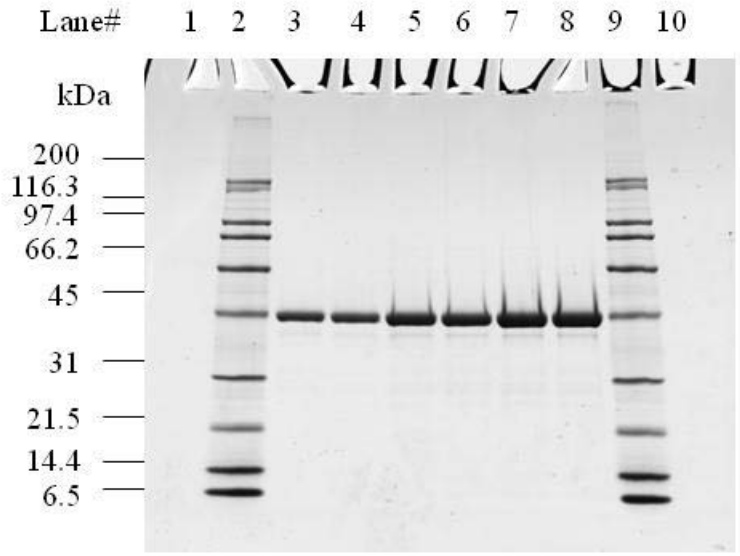
Aliquots of the MON 88302- and the *E. coli*-produced CP4 EPSPS proteins were separated on a 4-20% Tris glycine polyacrylamide gradient gel and stained with Brilliant Blue G-Colloidal stain. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 9.



Lane	Sample	Amount (ng)
1	Precision Plus, dual color MW markers	-
2	Transferrin (positive control)	50
3	Transferrin (positive control)	100
4	Transferrin (positive control)	150
5	Transferrin (positive control)	200
6	*E. coli*-produced CP4 EPSPS (negative control)	100
7	*E. coli*-produced CP4 EPSPS (negative control)	200
8	MON 88302-produced CP4 EPSPS	100
9	MON 88302-produced CP4 EPSPS	200
10	Empty	-

Figure 5. Glycosylation Analysis of the MON 88302-Produced CP4 EPSPS Protein

Aliquots of the transferrin (positive control), *E. coli*-produced CP4 EPSPS reference standard (negative control) and MON 88302-produced CP4 EPSPS protein were separated by SDS-PAGE (4-20%) and electrotransferred to PVDF membranes. (A) Where present, the labeled carbohydrate moieties were detected by addition of streptavidin conjugated to HRP followed by a luminol-based the detection using ECL reagents and exposure to Hyperfilm®. A 2 min exposure time is shown. (B) An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. The signal was captured using a Bio-Rad GS-800 with Quantity One® software (version 4.4.0). Approximate MWs (kDa) correspond to the dual color markers (used to verify transfer and MW) in Lane 1. The arrows show the expected migration of the *E. coli*- and CP4 EPSPS MON 88302- produced proteins.



Lane	Sample	Amount (µg)
1	Empty	-
2	Broad Range MW markers	4.5
3	MON 88302-produced CP4 EPSPS protein	1
4	MON 88302-produced CP4 EPSPS protein	1
5	MON 88302-produced CP4 EPSPS protein	2
6	MON 88302-produced CP4 EPSPS protein	2
7	MON 88302-produced CP4 EPSPS protein	3
8	MON 88302-produced CP4 EPSPS protein	3
9	Broad Range MW markers	4.5
10	Empty	-

Figure 6. Storage Stability of the MON 88302-Produced CP4 EPSPS Protein

SDS-PAGE analysis was performed on the MON 88302-Produced CP4 EPSPS sample stored in a -80 °C freezer for 98 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 2 and 9.

Appendix 1. List of Applicable SOP

SOP Number	Title
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	Immunoblot 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 Immumoblot 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. Certificates 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	Orion Lot Number	Expiration Date
E. coli-produced CP4 EPSPS	10000739	July 31, 2010
Common or Alias Name(s)	**Historical APS Lot Number(s)**	**Storage Requirements (until use)**
None	20-100015	–80 °C
Source: Fermentation of *Escherichia coli* containing the pMON21104 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	43.8 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 MLHGASSRPATA(R)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 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)

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Page 1 of 1

Analytical Protein Standard Certificate of Analysis

MONSANTO ANALYTICAL PROTEIN STANDARDS

Re-characterization No. 9

Sample Information:

Name of APS	Orion Lot Number	Expiration Date
E. coli-produced CP4 EPSPS	10000739	July 31, 2012
Common or Alias Name(s)	**Historical APS Lot Number(s)**	**Storage Requirements (until use)**
None	20-100015	–80 °C
Source: Fermentation of *Escherichia coli* containing the pMON21104 expression plasmid.		**Comment(s)** None
Additional Background Information: None		

Re-characterization Information		
Characteristic	**Method**	**Assay Date**
Concentration	Amino Acid analysis	5 June 2010
Purity/Molecular weight	SDS-PAGE/Densitometry	3 June 2010
Activity	Phosphate release assay	4 June 2010

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	43.8 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 MLHGASSRPATA(R)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 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)

[redacted]

[redacted]