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FINAL REPORT

Report No.: MSL-12901

Job/Project No.: 393773

Date: 10/5/93

Title: Characterization of Microbially-Expressed Protein: CP4 EPSPS

Authors: Leslie A. Harrison, Michele R. Bailey, Richard M. Leimgruber, Christine E. Smith, Debbie L. Nida, Mary L. Taylor, Mark E. Gustafson, Bob Heeren and Stephen R. Padgette

Abstract:

Purpose

The protein being investigated in this study is 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). This enzyme has been expressed in crop plants to confer tolerance to glyphosate, the active ingredient of Roundup® herbicide, and has also been used for glyphosate selection in a variety of plant species. Glyphosate exerts its herbicidal activity due to inhibition of EPSPS, an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in plants and microorganisms (but not mammals). The EPSPS from *Agrobacterium* sp. strain CP4 is highly tolerant to inhibition by glyphosate and has high catalytic efficiency, compared to most glyphosate-tolerant EPSPSs (1,2). Upon glyphosate treatment, plants or plant cells expressing the CP4 EPSPS are unaffected since the continued action of the glyphosate-tolerant EPSPS enzyme meets the plant's need for aromatic compounds.

In order to perform an oral toxicity study in mice with a protein, it is necessary to produce relatively large amounts of the test protein. Since the expression levels of introduced proteins (such as CP4 EPSPS) in plants are very low, it is not feasible to use plant material as a source of the protein test material.

Alternatively, proteins can be expressed in *E. coli* using an over-expression vector, purified if needed, and used for ingestion, providing the plant-expressed and *E. coli*-expressed proteins meet equivalence criteria.

The purpose of this study is to characterize the *E. coli*-produced CP4 EPSPS used in an acute oral toxicity study in mice (3) and other studies. Studies to address the equivalence of plant-produced CP4 EPSPSs with the *E.coli*-produced enzyme are described separately (see, for instance, references (4,5)).

The information contained in this study is being supplied to the U.S. Food and Drug Administration (FDA) as part of a food safety assessment program by Monsanto Company, in accord with the FDA Policy: Foods Derived from New Plant Varieties, May 29, 1992.

Justification of Test System

The test systems are the analytical techniques used for characterization and assessment of each of the proteins, and are standard analytical methods used to identify proteins. The panel of techniques were chosen by consensus among a group of protein biochemistry experts within the testing facility and in concurrence with FDA scientific consultations. The test system for each analysis is described in Materials and Methods.

Test Material

E. coli-expressed CP4 EPSPS (lot #5192245) was produced by fermentation in *E. coli* GB100 pMON21104 (2,6), and purified to greater than 90% purity (7). Stability of the test material during the time of gavage is addressed another study (8).

Materials, Methods and Results

Standard methods referenced in the literature (9) were identified to characterize the CP4 EPSPS purified from *E. coli*. The analytical methods utilized and a brief summary of the results are presented in the following table:

Summary of Protein Characterization: *E. coli*-produced CP4 EPSPS

Analytical Method	Function	Results
SDS-PAGE	Molecular weight (MW) and purity	Single band at the predicted apparent MW
Western blot	Immunological response and confirm MW	Immunological recognition at predicted apparent MW
Glycosylation	Detects carbohydrate moieties	No carbohydrate moieties detected
N-terminal amino acid Sequence	Corresponds to pMON21104 DNA-predicted CP4 EPSPS amino acid sequence	Correct N-terminus through 15 positions
CP4 EPSPS enzymatic activity	Functional identity and catalytic activity	Specific activity = 3.0 U/mg
CP4 EPSPS ELISA reactivity	Immunological dose response	Positive correlation between quantity and ELISA response

Conclusions

The data in this study provides necessary information for the characterization of *E. coli*-expressed CP4 EPSPS used in an acute oral toxicity study in mice (3) and other studies. The fermentation of *E. coli* GB100 pMON provided sufficient material for the isolation of CP4 EPSPS at a purity level of greater than 90% purity. The purified CP4 EPSPS has 1) the correct chemical identity as determined by SDS-PAGE, Western blot, and N-terminal amino acid sequence, 2) the expected functional identity as determined by enzymatic activity and 3) sample integrity as determined by Western blot, SDS-PAGE, enzymatic activity and ELISA. In addition, there were no carbohydrate moieties detected in association with the CP4 EPSPS. In conclusion, the test material was shown to be of acceptable identity, strength, purity and composition.

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J. Ream/GG4K
P. Keck/GG4K
P. Sanders/AA2G
P. Lavrik/GG4C
A. Reed/GG4F

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Study Title

Characterization of Microbially-Expressed Protein: CP4 EPSPS

Study Director
Stephen R. Padgette

Contributing Scientists
Leslie A. Harrison, Michele R. Bailey, Richard M. Leimgruber, Christine E. Smith, Debbie L. Nida, Mary L. Taylor, Mark Gustafson, and Bob Heeren

Study Completed on
10/5/93

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Laboratory Project ID

Study 92-01-30-14

Experiment 92-419-720

Statement of compliance

This study meets the requirements for 40 CFR Part 160 and 21 CFR 58.

Items not performed under GLP:

1) The reference material, *E. coli*-produced CP4 EPSPS from pMON17101, was purified and sequenced according to standard protein purification techniques, but not under GLP guidelines. This includes the specific activity value reported in this study.

2) The N-terminal amino acid sequencing experiments were performed according to standard techniques, but were not performed under GLP guidelines.

Submitter _____ 10/6/93

Sponsor _____ 10/6/93

Study Director _____ 10/5/93

Quality assurance statement

Study Number: 92-01-30-14

This signed statement indicates that the ESH Q&CA Quality Assurance Unit has monitored this study and reviewed the study data and final report. These reviews indicate that the final report accurately presents the raw data as developed during the study.

Dates of reviews as well as dates that findings were reported to testing facility management and the study director are listed below.

Dates of Quality Assurance reviews:

December 2, 9, 1992
March 19, 1993
June 1, 15, 1993
July 12, 1993
August 17, 26, 1993
September 27, 1993

Dates findings were reported to management and study director:

December 2,9, 1992
March 19, 1993
June 1, 15, 1993
July 12, 29, 1993
August 17, 26, 1993
September 28, 1993

Quality Assurance reviews conducted by:

J.W. Greer
K. F. Yount

Quality Assurance Representative
Monsanto Company

October 4, 1993
Date

Study Number: 92-01-30-14
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Title: Characterization of Microbially-Expressed Protein: CP4 EPSPS

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Study Start Date: December 14, 1992

Records Retention: All study specific raw data, protocols, final reports and facility records will be retained at Monsanto - St. Louis

Signatures of Approval

Study Director — Date: 10/5/93

Principal Investigator — Date: 10/5/93

Sponsor — Date: 10/6/93

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I. Summary

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II. Purpose

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The purpose of this study is to characterize the *E. coli*-produced CP4 EPSPS used in an acute oral toxicity study in mice (3). Studies to address the equivalence of plant-produced CP4 EPSPS with the *E. coli*-produced enzyme are described separately (see, for instance, references (4,5)).

III. Samples for Analysis

A. Test substance (CP4 EPSPS from *E. coli*, lot #5192245): The test substance was microbially-expressed CP4 EPSPS, produced in *E. coli* GB100 from plasmid pMON21104 (2,6) and purified to greater than 90% purity (7), based on SDS-PAGE analysis (Figures 1 and 2). Stability of the test substance is addressed in another study (8).

B. Reference substance (CP4 EPSPS from *E. coli*, lot#22291): The reference substance was microbially-expressed CP4 EPSPS produced on an analytical scale in strains of *E. coli* GB100 from plasmid pMON17101 (2) and purified to greater than 90% purity. The initial characterization of this reference material is summarized on Monsanto notebook page 4703831.

C. Control substance: The appropriate control substances are described in each of the individual analytical methods.

IV. Justification of the Test System:

The test systems are the analytical techniques used for characterization and assessment of each of the proteins, and are standard analytical methods used

to identify proteins (9). The panel of techniques were chosen by consensus among a group of protein biochemistry experts within the testing facility and in concurrence with FDA scientific consultations. The test system for each analysis is described in the following Materials and Methods (Section V).

V. Materials and Methods:

This characterization study was run concurrently with a separate study designed to assess the equivalence of the *E. coli*-produced CP4 EPSPS to the glyphosate-tolerant soybean seed-produced CP4 EPSPS (4). The location of original data utilized for both studies is cross-referenced in each data file.

The *E. coli*-expressed CP4 EPSPS protein was chemically and functionally characterized using the following analytical methods:

A. SDS-PAGE: One dimensional SDS-PAGE gels containing the *E. coli*-expressed CP4 EPSPS protein and appropriate molecular weight markers (Bio-Rad #161-0304 SDS-PAGE molecular weight standards, low range) were run. Protein bands in the polyacrylamide gels were detected with Coomassie blue. This analysis was used to assess apparent molecular weight. To estimate purity, the Coomassie-stained gels were scanned by laser densitometry using an LKB Ultrascan XL Enhanced Laser Densitometer with GelScan software.

B. Western blot: *E. coli*-expressed CP4 EPSPS was run on an SDS-PAGE gel, electrophoretically transferred to PVDF Flourotrans membrane (VWR 28152-454), a specific antibody was hybridized to the blots, then the blots were reacted with ^{125}I-Protein G and developed with autoradiography to assess immunological response and apparent molecular weight.

C. Glycosylation: SDS-PAGE of the *E. coli* CP4 EPSPS and controls was followed by electrophoretic transfer of proteins onto PVDF. Horseradish peroxidase and transferrin were used as positive controls. Carbohydrate moieties associated with the blotted protein bands were detected using the Glycotrack kit (Oxford Systems, Rosedale, NY) according to the manufacturers instructions for Protocol 1a (10).

D. Amino-terminal amino acid sequence: Amino-terminal amino acid sequencing of *E. coli*-expressed CP4 EPSPS was used to assess primary sequence at the amino terminus. Automated Edman degradation chemistry was used to determine the NH_2-terminal protein sequence. An Applied Biosystems, Inc. model 470A gas phase sequencer (Foster City, CA) was employed for the degradations (11) using the standard sequencer cycle (03RPTH). The respective PTH-aa derivatives were identified by RP-HPLC analysis in an on-line fashion employing an Applied Biosystems, Inc., Model 120A PTH Analyzer fitted with a Brownlee 2.1 mm I.D. PTH-C_{18} column.

E. Enzymatic activity: The procedure utilized for determining the amount of functionally active CP4 EPSPS entailed the use of the phosphate release

assay for EPSPS, which has been described elsewhere (12). Reaction mixtures contained enzyme and 50 mM HEPES, 2.0 mM shikimate-3-phosphate (S3P), 1.0 mM phosphoenolpyruvate (PEP), 0.1 mM ammonium molybdate, and 5 mM potassium fluoride. Reactions were run for 2 to 5 minutes at approximately 25°C, quenched with malachite green reagent, then fixed with sodium citrate. The EPSPS-catalyzed release of phosphate from S3P was determined spectrophotometrically versus a standard curve of inorganic phosphate treated with the same malachite green reagent protocol. For EPSPS, 1 unit (U) is defined to be 1 µmol phosphate (or EPSP) produced/ min at approximately 25°C, under the assay conditions described. The specific activity of each sample, in U/mg, was calculated based on the units of activity present in each sample as determined by the enzymatic assay and the amount of protein as determined by the Bio-Rad protein assay (13,14)

F. ELISA of *E. coli*-expressed CP4 EPSPS: A double antibody indirect enzyme-linked immunosorbent assay (ELISA) was developed and validated (15) for detection of CP4 EPSPS. Quantitation of CP4 EPSPS levels is estimated by extrapolation from the logistic curve fit of the purified CP4 EPSPS standard curve. The ELISA utilizes two antibodies from two different animal species raised against the native CP4 EPSPS protein. The double antibody sandwich is detected with donkey anti-rabbit alkaline phosphatase conjugate followed by development with pNPP, an alkaline phosphatase substrate that yields a soluble yellow product that can be measured by optical density to quantitate analyte levels. This assay, which recognizes the non-denatured CP4 EPSPS was used to assess the immunological epitope (three-dimensional conformation). Dilutions of the protein were run in order to evaluate the dose response curve. Results were evaluated graphically.

G. Deviations to the protocol or SOPs: No standard operating procedure (SOP) or protocol deviations occurred which impacted the results of this study. Minor deviations are given in the raw data file.

VI. Results and Discussion:

A. Apparent molecular weight and purity: SDS-PAGE was performed to evaluate the apparent molecular weight and purity of the CP4 EPSPS samples. Dilutions of the test and reference proteins were run on an SDS-PAGE gel and stained with Coomassie Blue, as shown in Figure 1. This gel also contains protein samples from plant material for equivalence studies (4,5). Both the test and reference CP4 EPSPS protein samples migrated the same on the gel. The purity of the test sample was determined by densitometry of the stained gel. As shown in Figure 2A, the test material corresponded to 95% purity, based on the integrated protein bands in the sample. However, since there were several very small non-integrated signals in the sample, the purity is estimated at >90%. The reference sample, CP4 EPSPS from pMON17101 in *E. coli*, corresponded to 83% purity based on the integrated protein bands in the sample.

The protein encoded by the CP4 EPSPS gene in pMON21104 has a predicted subunit molecular weight of 47.6 kD (2). As shown in Figure 3, the experimentally determined molecular weight of CP4 EPSPS was determined to be approximately 45.0 kD. The theoretical and experimentally-determined apparent molecular weights for the CP4 EPSPS test material are thus in good agreement. These results show that the test material has a high degree of purity and migrates with the anticipated molecular weight relative to CP4 EPSPS reference material.

B. Immunological recognition and apparent molecular weight: By Western blot, comparison of the immunoreactive protein bands of the test material, CP4 EPSPS from pMON21104 expression in *E. coli*, with that of the reference material (purified CP4 EPSPS from pMON17101 in *E. coli*) shows that both have equivalent apparent molecular weights (Figure 4). These results substantiate the results from the stained SDS-PAGE gel (Figure 1), indicating that the apparent molecular weight of CP4 EPSPS by SDS-PAGE is approximately 45 to 47 kD. Differences in the absolute intensities of the bands from the test and reference material in Figure 4 are most likely due to variability in the Bio-Rad protein assay (14) used to calculate loading quantities, since the Coomassie-stained gel (Figure 1) also shows non-identical band intensities. In any event, there is no indication of significant differences in immunological reactivities between the test and reference materials by comparing data from Figures 1 and 4.

C. Protein glycosylation: The presence or absence of glycosylation was determined by detecting the presence of carbohydrate moieties associated with the blotted protein bands. Historically, proteins expressed in prokaryotes, such as *E. coli*, are not glycosylated (16). As shown in Figure 5, the glycoproteins used as positive controls, horseradish peroxidase and transferrin, both exhibited strong signals. However, *E. coli*-expressed CP4 EPSPS protein was negative for glycosylation with this detection system (Figure 5). These results indicate that within the limits of sensitivity of the Glycotrack kit, the *E. coli*-expressed CP4 EPSPS test material is not glycosylated.

D. Amino-terminal amino acid sequence: CP4 EPSPS was subjected to N-terminal sequence analysis. The sequence data obtained verified the N-terminus for the first 15 positions of protein as compared to the predicted protein sequence from the cloned gene in pMON21104 (2,17) (Figure 6). In addition, no clipped or contaminating species were detected.

E. CP4 EPSPS enzymatic activity: The functional EPSPS activity in the purified sample of CP4 EPSPS test material was determined based on protein concentration (mg/ml) and EPSPS activity (U/ml). The specific activity of the *E. coli*-expressed CP4 EPSPS test material was found to be 3.0 U/mg. These results demonstrate that the test material is functionally active. The specific

activity of the reference material had been previously found to be 1.4 U/mg (Monsanto Notebook Page 4703832).

F. ELISA dilution response: Dilutions of CP4 EPSPS test material were run in the ELISA to assess quantity verses immunological response in the ELISA. A response curve comparable to that of the CP4 EPSPS reference material was obtained by graphical analysis, as shown in Figure 7.

VII. Conclusions:

The data in this study provides necessary information for characterization of *E. coli*-expressed CP4 EPSPS. The fermentation of *E. coli* GB100 pMON provided sufficient material for the purification of CP4 EPSPS at a purity level of greater than 90%. The purified CP4 EPSPS has the correct 1) chemical identity as determined by SDS-PAGE, Western blot, and N-terminal amino acid sequence; 2) functional identity as determined by enzymatic activity; and 3) integrity as determined by Western blot, enzymatic activity and ELISA. In addition, there were no carbohydrate moieties detected in association with the CP4 EPSPS. In conclusion, the test material was shown to be of acceptable identity, strength, purity and composition.

VIII. References:

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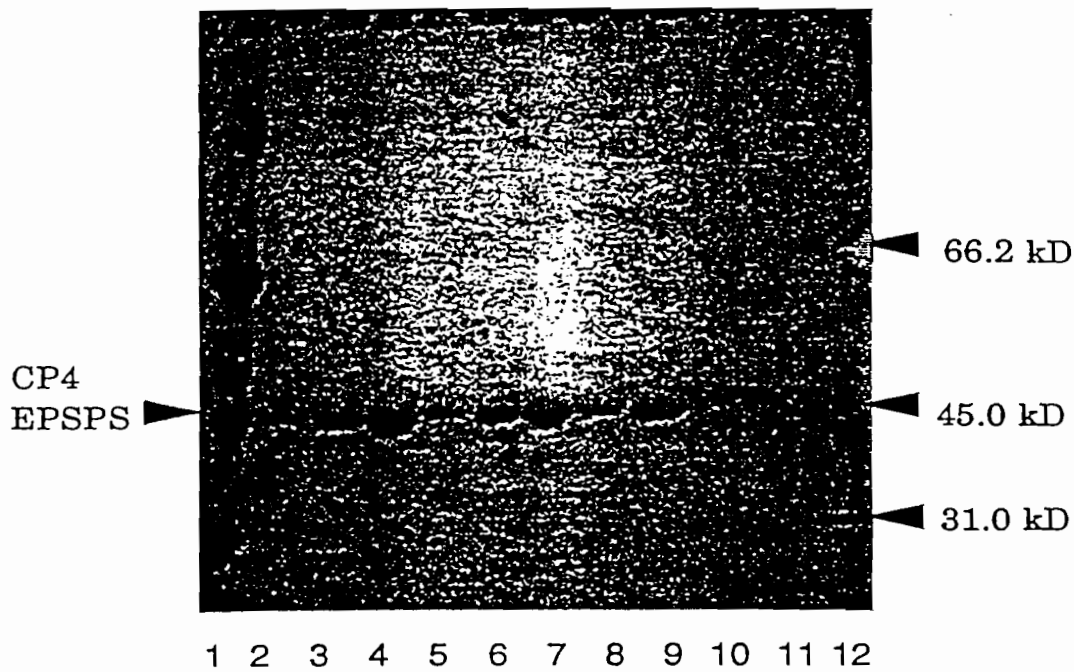
IX. List of abbreviations:

CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4
CTP	chloroplast transit peptide
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbant assay
EPSP	5-enolpyruvylshikimate-3-phosphate
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FDA	Food and Drug Administration
GTS	glyphosate tolerant soybeans
hr	hour
I.D.	internal diameter
kD	kilodaltons (10^3 daltons)
kg	kilogram (10^3 gram)
L	liter
mg	milligram (10^{-3} gram)
mL	milliliter (10^{-3} liter)
mM	millimolar (10^{-3} molar)
MW	molecular weight
ng	nanogram (10^{-9} gram)
N-terminus	amino terminus
nmoles	nanomoles (10^{-9})
PEP	phosphoenol pyruvate
PTH-aa	phenylthiohydantoin-amino acid
PVDF	polyvinylidene fluoride
RP-HPLC	reverse phase high pressure liquid chromatography
S3P	shikimate-3-phosphate
SA	specific activity
SDS-PAGE	Sodium Doedecyl Sulfate-Polyacrylamide Gel Electrophoresis
U	units
µg	microgram (10^{-6} gram)

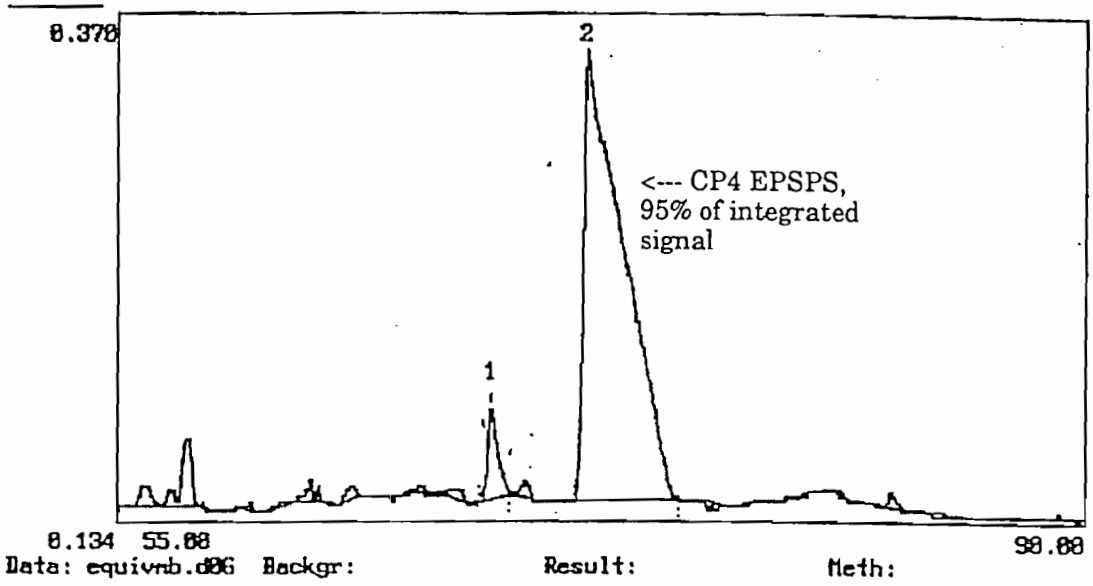
Figure 1. Coomassie blue stained SDS-PAGE gel. Shown is the Coomassie blue stained SDS-PAGE gel (1.0 mm, 4 to 20%) demonstrating predicted apparent molecular weights of *E. coli*-expressed CP4 EPSPS. The gel shown in this figure is denoted as gel #2 in the raw data file. Lanes 8 through 11 contain CP4 EPSPS from glyphosate-tolerant soybean and canola which is discussed in separate studies (4,5).



Lane #	Sample Description	Amount loaded (µg)
1	Rainbow Molecular Weight Markers #33-4	NA
2	CP4 standard lot# SRP22291 - reference substance	1
3	CP4 standard lot# SRP22291 - reference substance	2.5
4	CP4 standard lot# SRP22291 - reference substance	5
5	CP4 bioprocess standard lot# 5192245 - test substance	1
6	CP4 bioprocess standard lot#5192245 - test substance	2.5
7	CP4 bioprocess standard lot# 5192245 - test substance	5
8	CP4 purified from soybean seed lot# 485183	1
9	CP4 purified from soybean seed lot# 485183	2.5
10	CP4 purified from canola seed lot# 470388	1
11	CP4 purified from canola seed lot# 470388	2.5
12	Biorad Molecular Weight Markers #17135	NA

Figure 2. Densitometer scans of SDS-PAGE gels to estimate purity of the CP4 EPSPS test and reference materials. Gels were scanned with an LKB Ultrascan XL Enhanced Laser Densitometer with GelScan software. The gel position is shown on the x-axes, and the band absorbance is shown on the y-axes.

A. Test material: CP4 EPSPS purified from pMON21104-expressing *E. coli*, lot 5192245. Lane 6 from the gel in Figure 1 was scanned.



B. Reference substance: CP4 EPSPS purified from pMON17101-expressing *E. coli*, lot SRP22291. Lane 3 from the gel in Figure 1 was scanned.

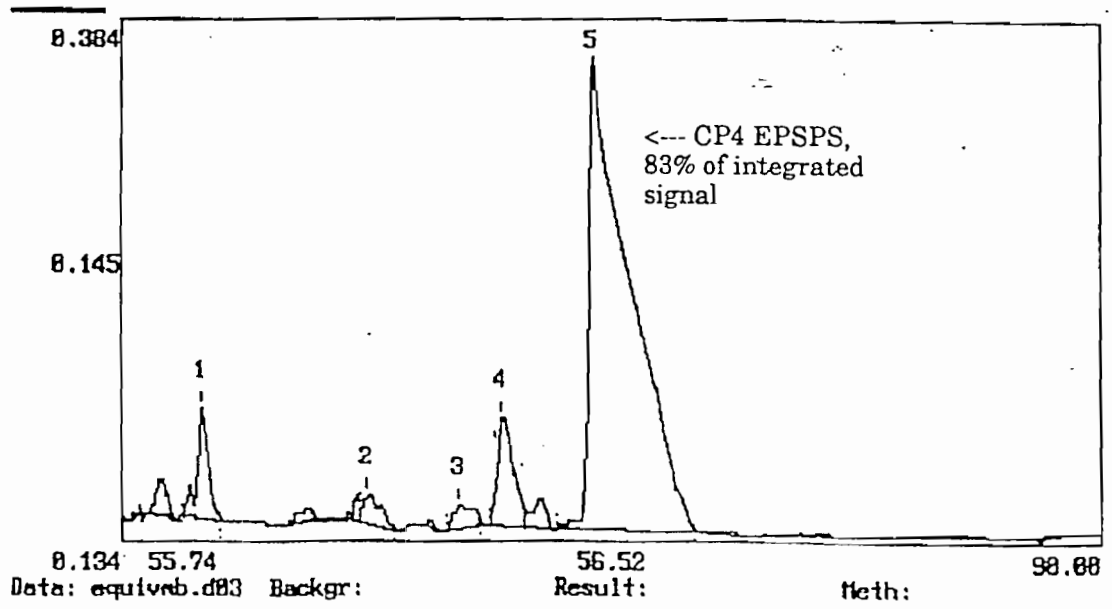


Figure 3. Molecular weight calibration curve for SDS-PAGE gel using Bio-Rad low range molecular weight markers. By linear regression;, y = 5.1012-0.74641 x R^2 R^2 = 0.996. The R_f of CP4 EPSPS = 0.6, so 5.1012-0.74641(0.6) = 4.653354. The experimental molecular weight of CP4 EPSPS is therefore $10^{4.653354}$ = 45,015 MW.

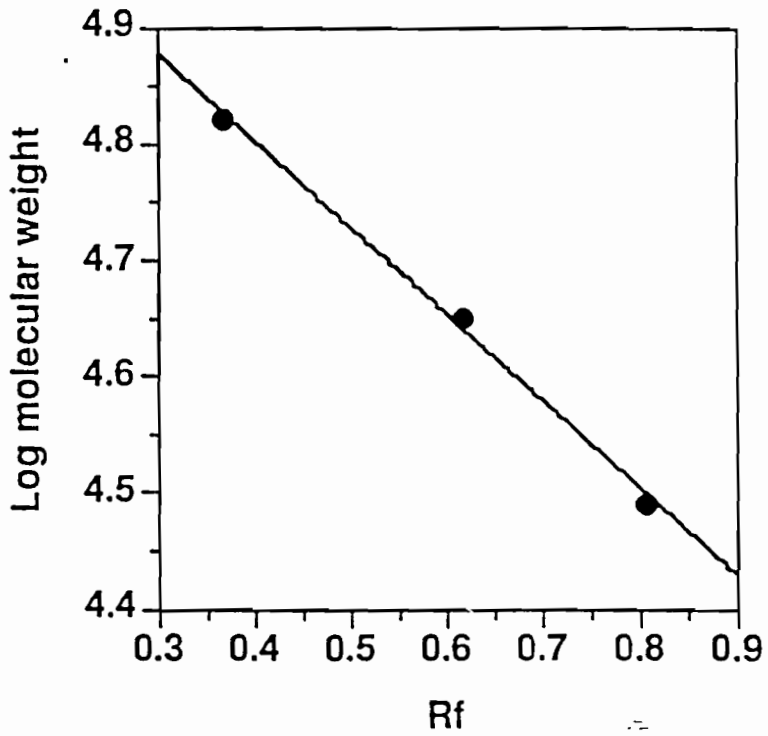
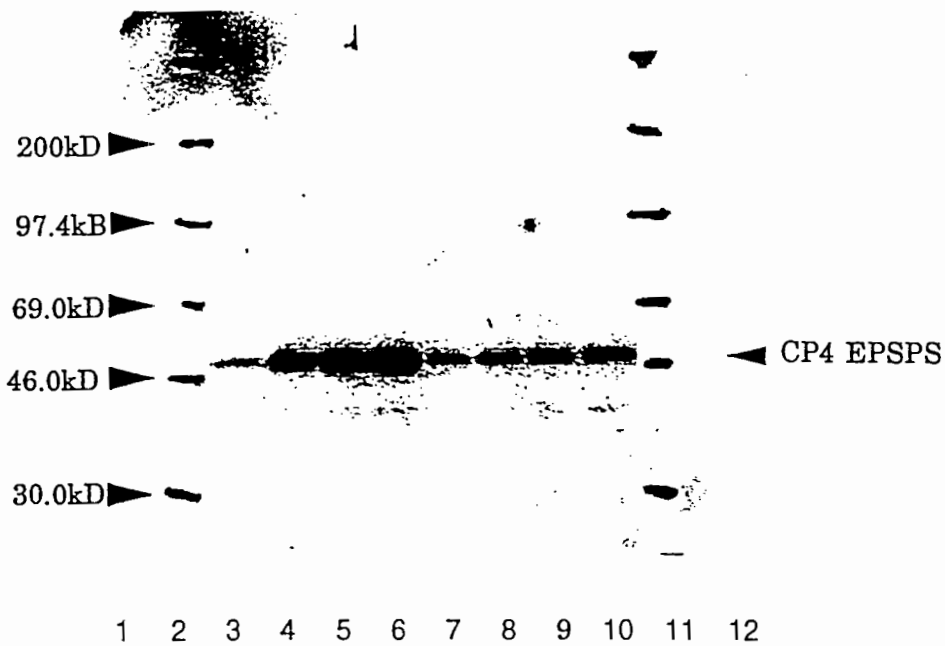
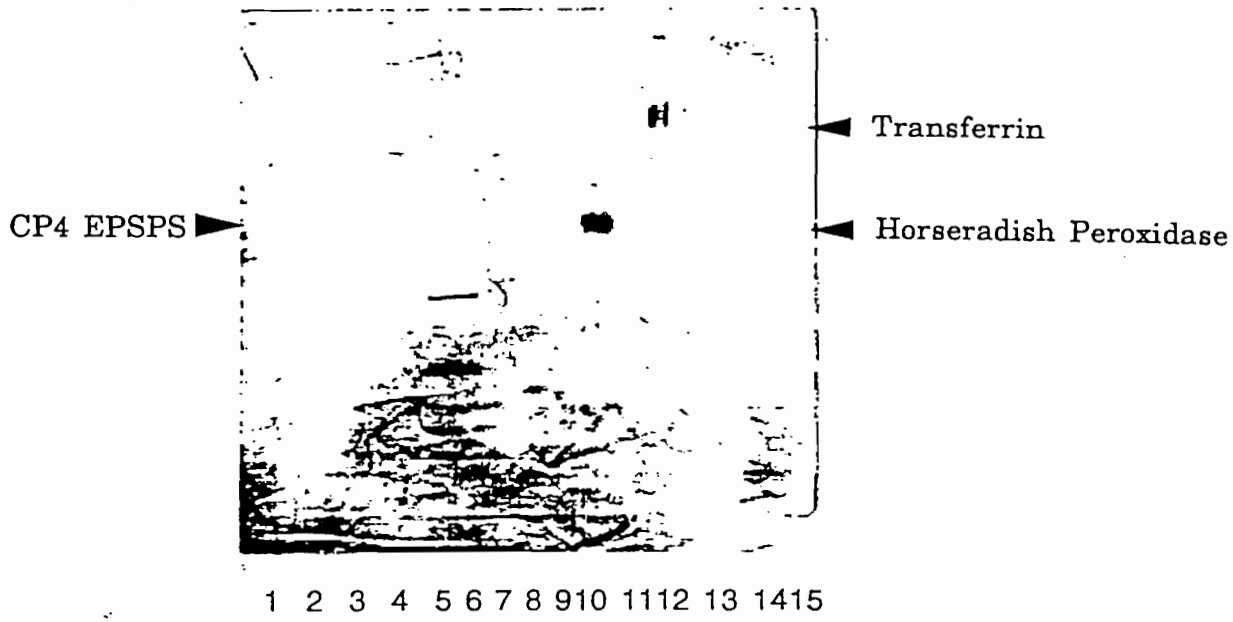


Figure 4. I^{125} Western blot of an SDS-PAGE gel. Shown is the I^{125} Western blot of an SDS-PAGE gel comparing the immunological response and apparent molecular weight of the CP4-EPSPS standard and the *E. coli*-expressed CP4-EPSPS. Blotted molecular weight markers were marked on the blot for visualization on the autoradiogram. This Western blot is labelled Western #1 in the data file, and was exposed for 18 h.



Lane #	Sample Description	Amount loaded(ng)
1	Empty	NA
2	Rainbow Molecular Weight Markers #33-4	NA
3	CP4 standard lot # SRP22291 - reference substance	2.5
4	CP4 standard lot # SRP22291 - reference substance	5
5	CP4 standard lot # SRP22291 - reference substance	7.5
6	CP4 standard lot # SRP22291 - reference substance	10
7	CP4 bioprocess standard lot #5192245 - test substance	2.5
8	CP4 bioprocess standard lot #5192245 - test substance	5
9	CP4 bioprocess standard lot #5192245 - test substance	7.5
10	CP4 bioprocess standard lot #5192245 - test substance	10
11	Rainbow Molecular Weight Markers #33-4	NA
12	Empty	NA

Figure 5. Test for glycosylation. SDS-PAGE of *E. coli*--expressed CP4 EPSPS and controls, followed by electrophoretic transfer of proteins onto PVDF. Carbohydrate moities associated with the blotted protein bands were detected using the Glycotrack kit (Oxford Systems, Rosedale, NY)(10). CP4 EPSPS can be visualized as a negative staining band.



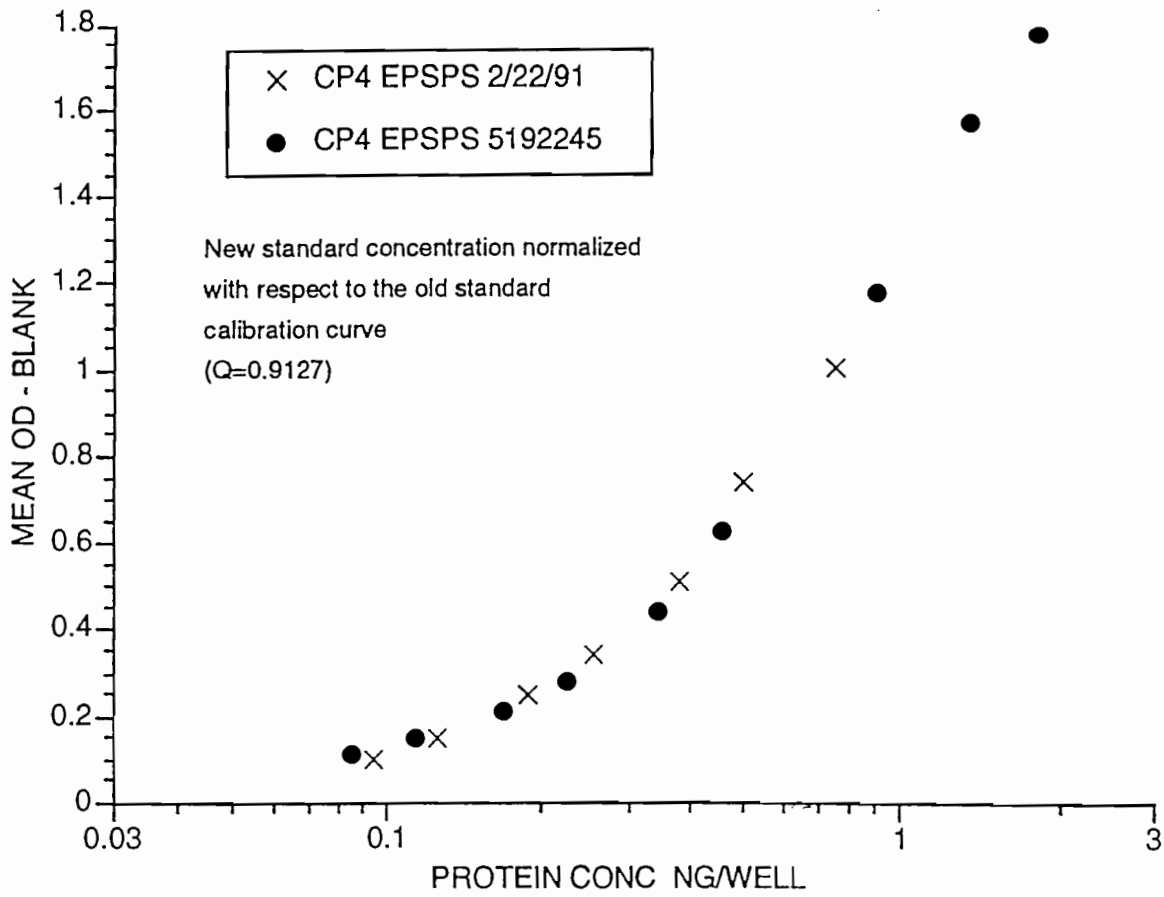
Lane #	Sample Description	Amount Loaded (µg)
1	Blank	NA
2	CP4 EPSPS from E. coli #5192245 - test substance	5.0
3	Blank	NA
4	Blank	NA
5	Biorad Low Molecular Weight Markers	NA
6	Blank	NA
7	Blank	NA
8	Blank	NA
9	Blank	NA
10	Horseradish Peroxidase	1.0
11	Blank	NA
12	Transferrin	0.5
13	Blank	NA
14	Blank	NA
15	Blank	NA

Figure 6. N-terminal amino acid sequence of the first fifteen amino acids of CP4 EPSPS. (a) Test material: *E. coli pMON21104*-expressed CP4 EPSPS; (b) amino acid sequence coded by cloned DNA sequence in *E. coli* pMON21104 (2). Parentheses denote tenuous designations.

(a) (M) L (H) G A S (S) (R) P A T A R K S

(b) M L H G A S S R P A T A R K S

Figure 7. ELISA dose response curve of test material (5192245) vs. reference material (SRP22291). Calculation of Q factor (normalization factor based on concentration) is described in the data file.



Attachment 1. Protocol for Characterization of Microbially-expressed Protein: CP4 EPSPS. Study #92-01-30-14, Experiment #92-419-720.

Study Number:	92-01-30-14
Experiment Number:	92-419-720
Study Title:	Characterization of Microbially-Expressed Protein: CP4 EPSPS
Sponsor:	Monsanto Company 800 N. Lindbergh Blvd. St. Louis, MO. 63167
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Co-investigators:	Monsanto Company: Richard Leimgruber -- BB4G Christine Smith -- AA2E Mark Gustafson -- GG3N Bob Heeron -- GG3N Debbie Nida -- AA3I Mary Taylor -- AA3I Michele Bailey -- AA3I

[redacted]

SPONSOR APPROVAL

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Kevin Yount
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Quality Control: Robin King Date: 12/8/92

Robin King
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1. Purpose:

The purpose of this study is to characterize microbially-produced CP4 EPSPS (5-enol-pyruvylshikimate-3-phosphate synthase). This protein is a glyphosate-tolerant EPSP synthase originally isolated from *Agrobacterium* sp. strain CP4.

2. Confidentiality:

No raw data, worksheets, data or information summaries, reports, or other information related to this study may be revealed or released to any third party without prior notification and authorization of the Agricultural Group of Monsanto Company.

3. Records and data retention requirements:

A. Records

All data and information generated either internally or via contract laboratories will be recorded directly and promptly onto appropriate forms. The exceptions are electronically captured data, for which a printout will be generated and included with other study data. All data and information generated during the conduct of this study will be written legibly in indelible ink, preferably black. Data and information shall never be recorded in pencil. All entries must be dated on the day of entry and signed or initialed by the person entering the data. Computer printouts will be dated and signed or initialed by the person responsible for their generation. Any change in entries must be made so as not to obscure the original entry, must indicate the reason for such change, and must be dated and signed (or initialed) at the time of the change. All raw data such as ELISA reader printouts, chromatograms, western blots, photos of SDS-PAGE gels, etc., will be saved. All raw and final data will be archived upon completion of the tests. Excess samples will be retained (archived) until notified of final disposition by the sponsor.

B. Retention.

Records will be retained of all sampling and observational raw data, the protocol and all deviations and amendments thereto, and copies of all letters, memoranda, and other correspondence related to this study. Upon completion of the study, all original documentation, records, and raw data will be transferred to the archives of the Sponsor.

[redacted]

For this study, "raw data" is defined as any laboratory or field worksheets, records, memoranda, notes, or exact copies thereof, that are the result of original observations and activities of a study and are necessary for the reconstruction and evaluation of the study of that report. In the event that exact transcripts or copies of raw data have been prepared, the certified exact copy or exact transcript may be substituted for the original source as raw data. An exact copy must be certified by stamping "This is an exact copy of the original document" or its equivalent on each page and must be signed and dated by the person making the copy.

C. Signatures.

The names, titles, signatures, and initials of all individuals recording data and/or observations for this study must be supplied in the data package.

4. Proposed experimental start date: December, 1992

5. Proposed experimental termination date: December, 1992

6. Samples for Analysis:

A. Test substance (CP4 EPSPS from *E. coli*): Microbially-expressed CP4 EPSPS will be produced on a large scale in strains of GB100 *E. coli* and purified to greater than 90% purity, based on SDS-PAGE analysis. A purity level, and lot number will be provided.

B. Reference substance (CP4 EPSPS from *E. coli*): Microbially-expressed CP4 EPSPS produced on an analytical scale in strains of GB100 *E. coli* and purified to greater than 90% purity. A lot number will be provided.

C. Control substance: The appropriate control substance will be described in each of the individual analytical methods.

Sample transfers, including distribution of the appropriate forms, will be coordinated by the Principal Investigator.

7. Experimental design:

The characterization of the microbially-produced CP4 EPSPSs will be evaluated using the following criteria:

[redacted]

A. One dimensional SDS-PAGE: One gel containing both the purified microbially-expressed proteins and appropriate molecular weight markers will be run and stained by Coomassie blue or silver according to the appropriate SOP(s). This method will be used to assess molecular weight and purity.

B. Western blot analysis from a one dimensional SDS-PAGE gel containing the above proteins and appropriate standards will be performed according to the appropriate SOP(s) to assess immunological response and confirm molecular weight.

C. Glycosylation staining of a protein blot from a one dimensional electrophoretic gel containing the above mentioned proteins, according to the appropriate SOP(s), will be used to assess post-translational glycosylation.

D. Amino-terminal amino acid sequencing of each protein isolated will be used to assess primary sequence at the amino terminus.

E. The enzymatic activity of the CP4 EPSPSs will be used to assess functional properties according to the appropriate SOP(s).

F. An ELISA of the above proteins run side-by-side according to the appropriate SOP(s) will be used to assess immunological dose response.

8. Justification of the test system:

While there is no test system (by definition), the analytical techniques used for characterization and to assess the similarity of the microbially-produced proteins are standard analytical methods used to identify proteins. The panel of techniques were chosen by consensus among a group of protein biochemistry experts within the testing facility.

9. Proposed statistical methods:

Statistical evaluation is not needed for the following analytical techniques used in this study. Evaluation of the similarity between the test and reference substances is qualitative for these techniques:

SDS-PAGE
Western blot
Amino-terminal amino acid sequencing

[redacted]

Glycosylation staining
Enzyme activity assay

Regression analysis and graphical procedures will be used to determine if the response curve of the above proteins in ELISA are equivalent.

10. General practices (adherence to protocol):

All deviations from this protocol must be documented in writing and signed by the Study Director. His telephone number is listed on the title page of this protocol.

If this trial is negatively affected by environmental or other conditions, notify the Study Director immediately. His telephone number is listed on the title page of this protocol.

11. Protocol amendments:

All changes in or deviations from this approved protocol and the associated reasons must be documented, signed by the Study Director, dated, and maintained with the protocol.

12. Quality assurance:

This characterization study will be performed in compliance with FDA 21CFR58, EPA 40CFR160, and OECD Good Laboratory Practice Standards and principles.

The Sponser's Quality Assurance Unit will visit at appropriate intervals during the duration of the study and prepare a written report of its findings.

[redacted]