

**Study Title:**

Amended Report for MSL0022432: Effect of Temperature Treatment on the Functional Activity of CP4 EPSPS

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03/09/2011

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**Laboratory Project ID**

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MSL0023307

Regulatory Product Characterization Center

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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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Submitter

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Date

**Sponsor Representative**

**Study Director**

**Quality Assurance Unit Statement**

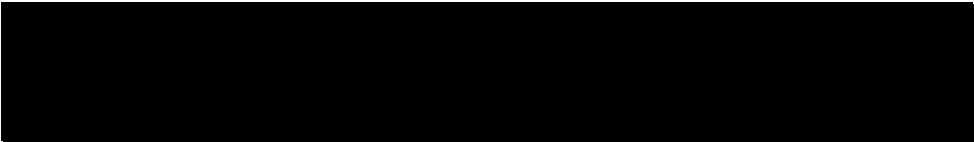
Study Title: Amended Report for MSL0022432: Effect of Temperature Treatment on the Functional Activity of CP4 EPSPS

Study Number REG 09-457

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.

Date of Inspection Audit	Phase	Date Reported to Study Director	Date Reported to Management
10/14/2009	CP4 EPSPS Functional Assay	11/16/2009	11/16/2009
11/24/2009	Raw Data Audit	12/01/2009	12/01/2009
02/26/2010	Draft Report Review	02/26/2010	02/26/2010
03/03/2011	Raw Data Audit and Amended Draft Report Review	03/03/2011	03/03/2011

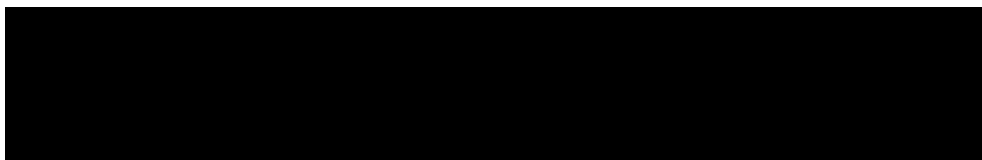


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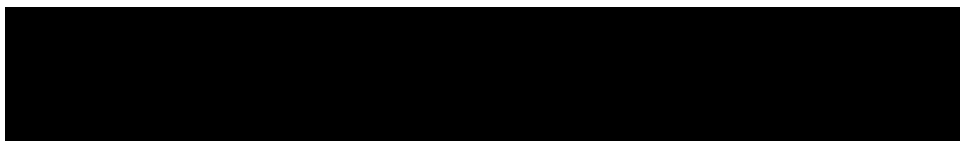
**Study Certification**

This report is an accurate and complete representation of the study/project activities.

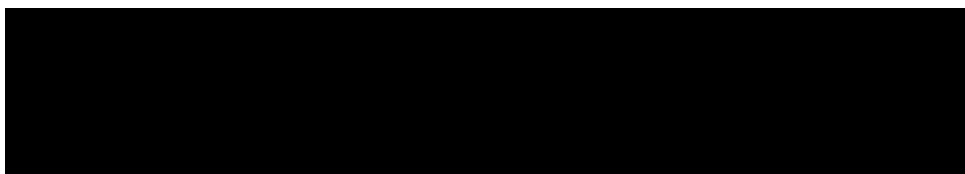
**Signatures of Final Report Approval**



Study Director



Principal Investigator



Protein Sciences and Safety Lead\

## Study Information

**Study Number:** REG-09-457

**Title:** Amended Report for MSL0022432: Effect of Temperature Treatment on the Functional Activity of CP4 EPSPS

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**Study Initiation Date:** 10/15/2009

**Study Completion Date:** 03/09/2011

**Amended Report Completion:** 03/09/2011

**Records Retention:** The protocol, all raw data, documentation, records, and the final report for this study are retained at Monsanto Company.

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**Abbreviations and Definitions**

APS	Analytical Protein Standard
CFR	Code of Federal Regulations
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EPA	Environmental Protection Agency
CP4 EPSPS	5-Enol-pyruvylshikimate-3-phosphate synthase protein <i>Agrobacterium sp.</i> strain CP4
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
HCl	Hydrochloric Acid
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
kDa	Kilodalton
MSL	Monsanto Scientific Literature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard Operating Procedure
Tris	Tris(hydroxymethyl)aminomethane

## 1.0 Summary

Monsanto Company has developed biotechnology-derived glyphosate-protected crops which produce CP4 EPSPS protein, a 5-enolpyruvylshikimate-3-phosphatase synthase (EPSPS) from *Agrobacterium sp.* strain CP4 (CP4 EPSPS). Expression of CP4 EPSPS renders the crops tolerant to glyphosate. The CP4 EPSPS protein used in this study is identical to that found in the Roundup Ready<sup>®</sup> family of crops.

The purpose of this study was to assess the effect of heating on the functional activity of the CP4 EPSPS protein. The purified CP4 EPSPS protein was used as the test substance at a final total protein concentration of 1.0 mg/ml. Aliquots of the test substance were heated to 25, 37, 55, 75, and 95 °C for either 15 or 30 minutes, while a single aliquot of the control substance was maintained on wet ice for the duration of the heat treatments. Heated test substances and unheated temperature control substance were analyzed by a CP4 EPSPS activity assay to assess the impact of temperature on the functional activity of CP4 EPSPS protein. Additionally, the protein resulting from each temperature treatment was analyzed by SDS-PAGE to assess CP4 EPSPS degradation.

Results of this study demonstrate that the CP4 EPSPS protein is functionally active at 25 °C and 37 °C. At 55 °C, a decrease in functional activity was observed, specifically the functional activity decreased to 70% of the control when treated for 15 min and to 25 % of control activity when treated for 30 min. The amount of CP4 EPSPS activity remaining following heat treatment for both 15 and 30 min at 75 °C and 95 °C was below the limit of detection. SDS-PAGE analysis showed no significant change in band intensity of the CP4 EPSPS protein due heat treatment.

## 2.0 Introduction

Monsanto Company has developed a biotechnology-derived glyphosate-protected crop which produces CP4 EPSPS protein, a 5-enolpyruvylshikimate-3-phosphatase synthase (EPSPS) from *Agrobacterium sp.* strain CP4 (CP4 EPSPS). Expression of CP4 EPSPS renders a crop tolerant to glyphosate. The CP4 EPSPS protein used in this study is identical to that found in many of the Roundup Ready<sup>®</sup> family of crops.

This study was performed to evaluate the thermal stability of purified CP4 EPSPS in an aqueous solution at five temperatures ranging from 25 °C to 95 °C and at two incubation times of 15 and 30 minutes. CP4 EPSPS protein stability was assessed by a CP4 EPSPS activity assay and SDS-PAGE analysis.

### 3.0 Purpose

The purpose of this study was to assess the thermal stability of the CP4 EPSPS enzyme using a CP4 EPSPS functional activity assay and SDS-PAGE analysis following heat treatment.

### 4.0 Materials

#### 4.1 Test substance

The test substance is the CP4 EPSPS protein (Orion lot 10000739) purified from *Escherichia coli* (*E. coli*) cells transformed with plasmid pMON21104. The DNA sequence encoding this CP4 EPSPS protein was confirmed both prior to and following fermentation of *E. coli*. Records pertaining to the production and isolation of the *E. coli*-produced test protein are archived in the Monsanto Regulatory Archives. The test protein was stored in a -80 °C freezer, in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 25% (v/v) glycerol and 1 mM benzamidine. This CP4 EPSPS protein has been shown to be equivalent to plant produced CP4 EPSPS in canola (Thorp and Silvanovich, 2004), corn (Bonner, et al., 2003), cotton (Karunanandaa, et al., 2003a), soybean (Karunanandaa, et al., 2003b), and sugar beet (Silvanovich and Lee, 2003).

#### 4.2 Control Substance

The control substance is the CP4 EPSPS protein (Orion lot 10000739) purified from *Escherichia coli* (*E. coli*) cells transformed with plasmid pMON21104. As control substance, an aliquot was maintained on wet ice throughout the heat treatment incubation period. Records pertaining to the production and isolation of the *E. coli*-produced control substance are archived in the Monsanto Regulatory. The control substance was stored in a -80 °C freezer, in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 25% (v/v) glycerol and 1 mM benzamidine.

#### 4.3 Reference Substance

The CP4 EPSPS listed above as a test substance was also used as reference substance. As reference substance, a vial of CP4 EPSPS protein was maintained at -80 °C until the heat treatment samples were ready for analysis. The reference substance was evaluated along with the heat treatment samples in the functional assay and the SDS-PAGE analysis.

### 5.0 Characterization of the Test, Control, and Reference Substances

The CP4 EPSPS protein (Orion lot 10000739) was previously characterized under the Analytical Protein Standard (APS) characterization plan 20-100015. The CP4 EPSPS

protein has a purity of 97 %, a total protein concentration of 3.8 mg/ml, and an apparent molecular weight of 43.8 kDa. A copy of the certificate of analysis for the CP4 EPSPS reference substance is archived with the study file in the Monsanto Regulatory Archives.

## **6.0 Methods**

### **6.1 Heat Treatment**

The CP4 EPSPS protein was thawed on wet ice and diluted in 50 mM Tris HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine HCl to a final total protein concentration of 1 mg/ml. For each incubation time period, 15 minutes or 30 minutes, aliquots of 200  $\mu$ l of the diluted CP4 EPSPS protein were transferred to six tubes. The six aliquots in tubes were maintained on wet ice until the heat treatments were initiated. Five tubes were placed in the appropriate heat treatment conditions (25, 37, 55, 75, or 95  $^{\circ}$ C, each  $\pm$  2  $^{\circ}$ C) and incubated for 15  $\pm$  1 minutes. Five tubes were placed in the appropriate heat treatment conditions (25, 37, 55, 75, or 95  $^{\circ}$ C, each  $\pm$  2  $^{\circ}$ C) and incubated for 30  $\pm$  1 minutes. All heat treated test substances were returned immediately to wet ice following the heat treatment incubation period. The sixth tube (i.e., the control substance) for each incubation period was maintained on wet ice throughout the heat treatment incubation period.

Following the heat treatments, 20  $\mu$ l of each heat treated test substance and the control substance was transferred to a clean tube and mixed with 5  $\mu$ l of 5 $\times$  SDS-PAGE loading buffer (0.312 M Tris HCl pH 6.8, 10% SDS, 50% glycerol, 3.6 M 2-mercaptoethanol, 0.025% Bromophenol Blue) as preparation for use in SDS-PAGE analysis. The 20  $\mu$ l samples were heated at 95  $\pm$  5  $^{\circ}$ C for 3-5 minutes, quick frozen by placement on dry ice, and stored at -80  $^{\circ}$ C until analysis. The remainder of each temperature-treated sample (approximately 180  $\mu$ l each) was maintained on wet ice and used for functional activity assessment, as described in section 6.2.

### **6.2 Functional Activity Assay**

The CP4 EPSPS functional activity of the heat treatment samples, the control substance, and the reference substance were determined according to the current version of SOP BR-ME-0408. All samples were diluted to total protein concentration of 0.05 mg/ml in 50 mM HEPES, pH 7.0 prior to analysis. Two replicates of each diluted protein sample were used for the analysis.

### **6.3 SDS-PAGE**

The samples prepared for SDS-PAGE analysis, described in section 6.1, were thawed, heated at 95  $\pm$  5  $^{\circ}$ C for 3-5 min, and loaded onto their respective 4-20% polyacrylamide gradient gel at 0.8 mg total protein/ml. The reference substance was

loaded on the same gel at 0.8 mg/ml (100% reference standard equivalent) and at 0.08 mg/ml (10% reference standard equivalent). The electrophoretic separation of the proteins was conducted according to the current version of SOP AG-ME-0388. Following electrophoresis, gels were stained with Brilliant Blue G Colloidal (Sigma, St. Louis, MO) according to SOP BR-ME-0527.

After staining, the CP4 EPSPS protein at each heat treatment was evaluated qualitatively. The intensity of the major protein band at 43.8 kDa in the heat treatment lanes was compared visually to the same band in the lanes with the control treatment, 100% reference substance equivalent, and 10% reference substance equivalent.

## **7.0 Control of Bias**

Appropriate controls and standards were included with each analysis.

## **8.0 Rejected Data or Data Not Used**

For the 30 minute heat treatment one complete set of phosphate release assay data was rejected because duplicate wells of each sample could not be analyzed on a single plate, as described in the relevant SOP. This was subsequently addressed by amendment 01. Also for the 30 minute heat treatment two additional phosphate release assays were rejected because the standard deviation of replicate samples was outside the acceptance criteria (%CV < 15%).

## **9.0 Protocol Amendments**

Four amendments were written to the original protocol. Amendment 01 - Section 6.2 was amended to allow two replicates of each diluted sample to be used for the functional activity assay for CP4 EPSPS, rather than the three replicates specified in the protocol. This change was introduced to allow for all heat treatment samples, control samples, standards, assay blanks, and reference proteins to be read on a single microtiter plate per SOP-BR-ME-0408. There was no impact on the conclusions as the assay was still conducted in accordance with the established SOP. Amendment 02 - Sections 2.2 and 6.2 were amended to remove the names of government agencies to broaden the utility of the study. Amendment 03 - Section 6.2 was amended to report temperature treatment as a percentage of the functional activity of the control treatment. Amendment 04 - Added a 15 minute heat treatment of test and control substances at 25, 37, 55, 75, and 95 °C. Included one additional SDS-PAGE gel and activity assay for the 15 minute incubation at 25, 37, 55, 75, and 95 °C for the test and control substance

## 10.0 Results and Discussion

Results of the activity assay for CP4 EPSPS protein incubated for 15 and 30 min at the indicated temperatures are represented in Tables 1 and 2, respectively. Activity of the control substance for the 15 minute incubation time (Table 1) and 30 minute incubation (Table 2) met the acceptance criteria demonstrating that CP4 EPSPS activity was maintained during incubation on ice. When heated at a temperature of 25 °C, 37 °C and 55 °C with an incubation time of 15 minutes, a small reduction in CP4 EPSPS activity was observed to 81%, 84 %, and 70% of control respectively. The test substance heated for 30 minutes showed no negative change in CP4 EPSPS activity at 25 °C and a small reduction in activity to 88% of control at 37°C. The test substance heated to 55 °C demonstrated a reduction in CP4 EPSPS activity with 25 % activity remaining relative to the control substance after the 30 minute incubation. . The level of CP4EPSPS activity following incubation at temperatures of 75 and 95 °C was below the limit of detection for incubations at both time points.

Analysis by SDS-PAGE stained with Brilliant Blue G Colloidal dye (Figure 1 and 2) demonstrated that the reference standard and control substance contain one major band with an apparent molecular weight of approximately 43.8 kDa corresponding to the CP4 EPSPS protein. Results of the SDS-PAGE data for the heat treatment of the test substances incubated for 15 minutes and 30 minutes are illustrated in Figures 1 and 2, respectively. The control substance loaded on each respective gel (lane 2, Figures 1 and lane 7, Figure 2) showed equivalent band intensity at 43.8 kDa to the 100 % reference standard (lane 8, Figures 1 and 2); demonstrating that the CP4 EPSPS protein was stable on wet ice during the incubation period. No apparent decrease in band intensity of the 43.8 kDa CP4 EPSPS protein band was observed in the test substance when heated at all temperatures for 15 minutes (lanes 3-7, Figure 1) or 30 minutes (lanes 2-6, Figure 2).

## 11.0 Conclusion

The purpose of this study was to examine the thermal stability of purified CP4 EPSPS. At temperatures of 75 °C and above CP4 EPSPS functional activity was below the limit of detection. SDS-PAGE demonstrated that heat treated test substance after incubation for 15 or 30 minutes at all temperatures investigated had no effect on the band intensity of CP4 EPSPS protein. These data demonstrate that CP4 EPSPS behaves with a predictable tendency toward enzyme denaturation at elevated temperatures.

## 12.0 References

Bonner, H.K.S., T. Ganguly, A.P. Vaughn, J.L. Lee and R.E. Hileman. 2003. Assessment of the physicochemical and functional equivalence of the CP4 EPSPS protein produced in grain of MON 88017 corn to the *E.coli*- produced CP4 EPSPS protein. Monsanto Technical Report MSL-18815. St. Louis, Missouri.

Karunanandaa, K., J.J. Thorp, J.L. Lee and A. Silvanovich. 2003a. Characterization of the CP4 EPSPS protein purified from the seed of Roundup Ready flex cotton MON 88913 produced in year 2002 and assessment of the physicochemical and functional equivalence of the plant- and *E. coli*-produced CP4 EPSPS proteins. Monsanto Technical Report MSL-18859. St. Louis, Missouri.

Karunanandaa, K., J.J. Thorp, J.L. Lee and A. Silvanovich. 2003b. Characterization of the CP4 EPSPS protein purified from the seed of Roundup Ready soybean 40-3-2 and assessment of the physicochemical and functional equivalence of the plant- and *E. coli*-produced CP4 EPSPS proteins. Monsanto Technical Report MSL-18871. St. Louis, Missouri.

Silvanovich, A. and T.C. Lee. 2003. Characterization of the CP4 EPSPS protein purified from the leaf tissue of Roundup Ready sugar beet event H7-1 and assessment of the physicochemical and functional equivalence of the plant- and *E. coli*-produced CP4 EPSPS proteins. Monsanto Technical Report MSL-18734. St. Louis, Missouri.

Thorp, J.J. and A. Silvanovich. 2004. Characterization of the CP4 EPSPS Protein from Roundup Ready Canola RT73 and Assessment of the Physicochemical and Functional Equivalence of the Plant- and *E. coli*-Produced CP4 EPSPS Proteins. Monsanto Technical Report MSL-19362. St. Louis, Missouri.

**Table 1 Activity of CP4EPSPS After 15 Minutes at Elevated Temperatures**

Temperature	Specific Activity Units/mg CP4 EPSPS <sup>1</sup>	Relative activity <sup>2</sup>
0 °C (control substance)	6.03±0.29	100%
25 °C	4.88±0.24	81%
37 °C	5.08±0.33	84%
55 °C	4.22±0.12	70%
75 °C	Below LOD <sup>3</sup>	<3%
95 °C	Below LOD <sup>3</sup>	<3%

<sup>1</sup> Mean specific activity determined from n=2.<sup>2</sup> CP4EPSP activity of control substance was assigned 100 % active.

% CP4 EPSP activity remaining = [specific activity of sample/specific activity of control substance] x 100

<sup>3</sup> LOD is defined as the value that is three times the assay blank standard deviation plus the mean of the assay blank.**Table 2 Activity of CP4EPSPS After 30 Minutes at Elevated Temperatures**

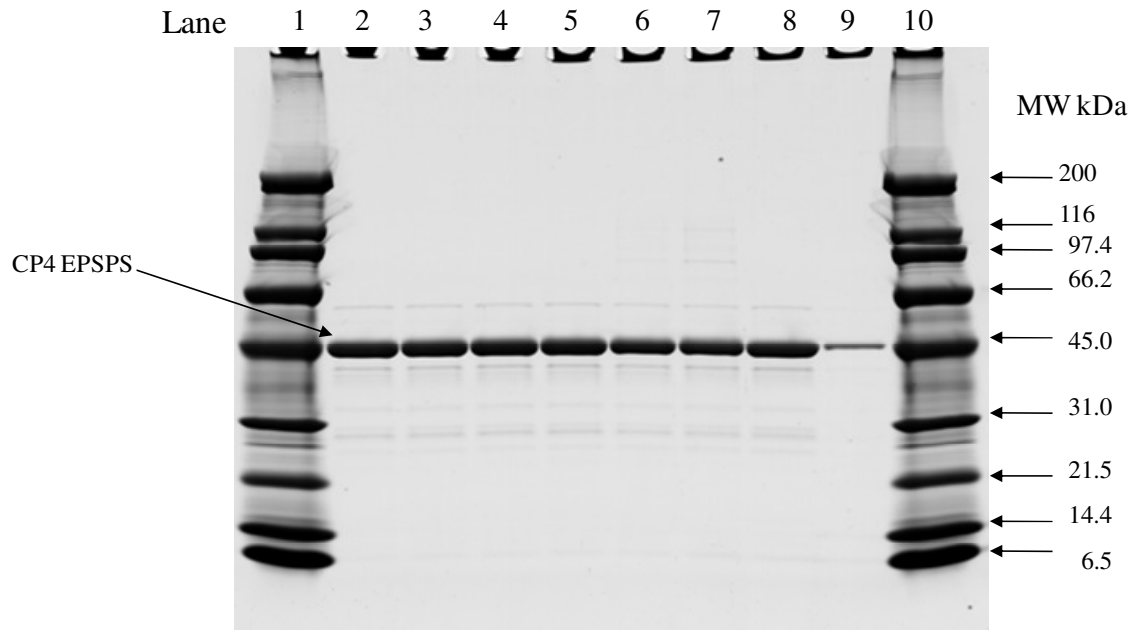
Temperature	Specific Activity Units/mg CP4 EPSPS	Relative activity <sup>2</sup>
0 °C (control substance)	2.8 ± 0.26	100%
25 °C	3.1 ± 0.23	110%
37 °C	2.5 ± 0.05	88%
55 °C	0.70 ± 0.09	25%
75 °C	Below LOD <sup>3</sup>	<8%
95 °C	Below LOD <sup>3</sup>	<8%

<sup>1</sup> Mean specific activity determined from n=2<sup>2</sup> CP4EPSP activity of control substance was assigned 100 % active.

% CP4 EPSP activity remaining = [specific activity of sample/specific activity of control substance] x 100

<sup>3</sup> LOD is defined as the value that is three times the assay blank standard deviation plus the mean of the assay blank.

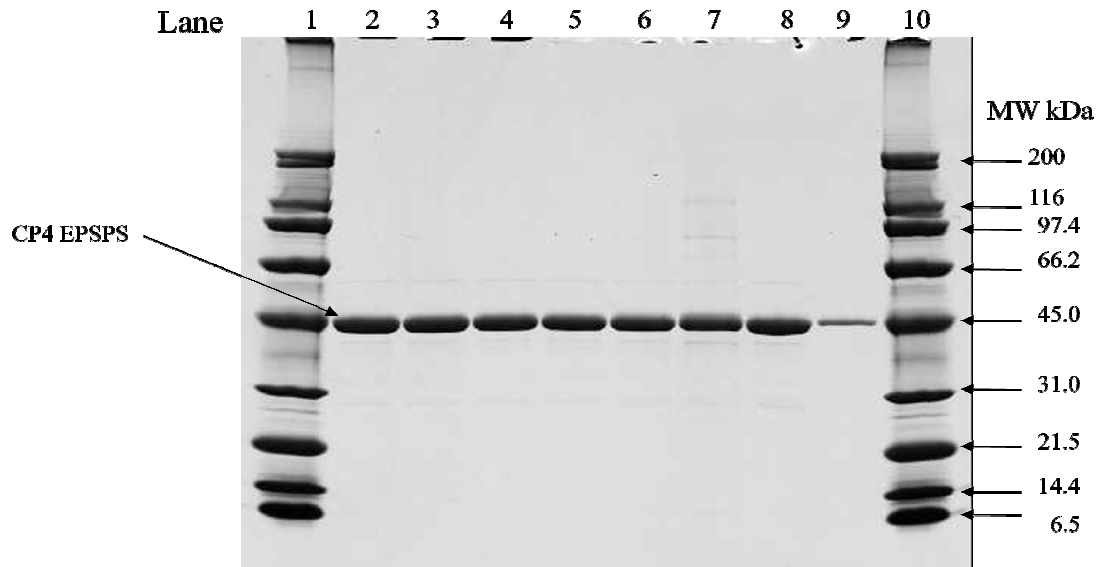




**Figure 1 SDS-PAGE of CP4 EPSPS Protein Following Heat Treatment for 15 Minutes.**

Heated-treated samples of CP4 EPSPS (3.2 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10.

Lane	Description	Amount
1	Broad Range Molecular Weight Markers	4.5 µg
2	CP4 EPSPS Temperature Control Substance	3.2 µg
3	CP4 EPSPS 25 °C	3.2 µg
4	CP4 EPSPS 37 °C	3.2 µg
5	CP4 EPSPS 55 °C	3.2 µg
6	CP4 EPSPS 75 °C	3.2 µg
7	CP4 EPSPS 95 °C	3.2 µg
8	CP4 EPSPS Reference 100% Equivalence	3.2 µg
9	CP4 EPSPS Reference 10% Equivalence	0.32 µg
10	Broad Range Molecular Weight Markers	4.5 µg



**Figure 2 SDS-PAGE of CP4 EPSPS Protein Following Heat Treatment for 30 Minutes.**

Heated-treated samples of CP4 EPSPS (3.2 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10.

Lane	Description	Amount
1	Broad Range Molecular Weight Markers	4.5 µg
2	CP4 EPSPS 25 °C	3.2 µg
3	CP4 EPSPS 37 °C	3.2 µg
4	CP4 EPSPS 55 °C	3.2 µg
5	CP4 EPSPS 75 °C	3.2 µg
6	CP4 EPSPS 95 °C	3.2 µg
7	CP4 EPSPS Temperature Control Substance	3.2 µg
8	CP4 EPSPS Reference 100% Equivalence	3.2 µg
9	CP4 EPSPS Reference 10% Equivalence	0.32 µg
10	Broad Range Molecular Weight Markers	4.5 µg

**Appendix 1. List of Current SOPs Used in the Study**

SOP AG-ME-0388-03	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SOP BR-ME-0408-02	Phosphate Release Assay for Functionally Active EPSPS
SOP BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels

**Appendix 2 Notes for Reviewer**

MSL 0022432 was amended to include an assess of the thermostability of CP4 EPSPS when heat treated for 15 min. This change had no impact on the study because it only changed the writing style used in the report.

Page Number in MSL0022432	Change
1	Added “Amended Report for MSL0022432:” to Study Title, revised report completion date, added Bin Chen, Ph.D to author string Changed Report number.
4	Added “Amended Report for MSL0022432:” to Study Title, and added “Amended Draft Report Review” to list of reviews and date for audit.
5	Added [REDACTED] to Signature Approval
6	Added “Amended Report for MSL0022391:” to Title, added [REDACTED] to author string ,added “Principal Investigator [REDACTED]” changed to Study Completion Date, and added “Amendment Report Completion Date”
8	Added “CP4 EPSPS Activity Assay of Heat-Treated CP4EPSPS After 15 Minutes at Elevated Temperatures” to list of Tables, SDS-PAGE CP4 EPSPS Demonstrating the Effect After 15 Minutes at Elevated Temperatures on Protein Structural Stability to list of Figures, and “Appendix 2. Notes for Reviewer” to List of Appendices.
10	Added “The purpose of this study was to assess the effect of heating on the functional activity of the CP4 EPSPS protein. The purified CP4 EPSPS protein was used as the test substance at a final total protein concentration of 1.0 mg/ml. Aliquots of the test substance were heated to 25, 37, 55, 75, and 95 °C for either 15 or 30 minutes, while a single aliquot of the control substance was maintained on wet ice for the duration of the heat treatments. Heated test substances and unheated temperature control substance were analyzed by a CP4 EPSPS activity assay to assess the impact of temperature on the functional activity of CP4 EPSPS protein. Additionally, the protein resulting from each temperature treatment was analyzed by SDS-PAGE to assess CP4 EPSPS degradation.”Added “and at two incubation times of 15 and 30 minutes. CP4 EPSPS protein stability was assessed by a CP4 EPSPS activity assay and SDS-PAGE analysis.”

	<p>Removed “The purpose of this study was to assess the thermal stability of CP4 EPSPS functional activity and protein degradation after heat treatment.”</p> <p>Replaced with “The purpose of this study was to assess the thermal stability of the CP4 EPSPS enzyme using a CP4 EPSPS functional activity assay and SDS-PAGE analysis following heat treatment.”</p>
11	<p>Added “The control substance is the CP4 EPSPS protein (Orion lot 10000739) purified from <i>Escherichia coli</i> (<i>E. coli</i>) cells transformed with plasmid pMON21104. As control substance, an aliquot was maintained on wet ice throughout the heat treatment incubation period. Records pertaining to the production and isolation of the <i>E. coli</i>-produced controls substance are archived in the Monsanto Regulatory. The control substance was stored in a -80 °C freezer, in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 25% (v/v) glycerol and 1 mM benzamidine.”</p> <p>Changed “reference protein” to “reference substance”</p>
12	<p>Changed “control treatment samples” to “control substance,</p> <p>Changed “reference protein” to “reference substance”</p>
13	<p>Changed “reference protein” to “reference substance”,</p> <p>Replaced “Three amendments” with “Four amendments,” added Added a 15 minute heat treatment of test and control substances at 25, 37, 55, 75, and 95 °C. Included one additional SDS-PAGE gel and activity assay for the 15 minute incubation at 25, 37, 55, 75, and 95 °C for the test and control substance.</p> <p>Removed “Results of the activity assay for CP4 EPSPS protein are listed in Table 1. Activity of the reference protein was measured as 4.7 units/mg CP4 EPSPS protein. The control sample had activity of 2.8 units/mg of CP4 EPSPS protein, thus demonstrating that protein activity was maintained during storage on ice. A minimal decrease in the activity of the CP4 EPSPS was observed when protein was incubated at 25 °C or 37 °C with activity measured as 3.1 and 2.5 units/mg CP4 EPSPS, respectively. Samples treated at higher temperatures demonstrate a substantial reduction in enzymatic activity; at 55 °C only 25 % of enzymatic activity was detected in the heat-treated sample, and at either 75 °C or 95 °C enzymatic activity of CP4 EPSPS was below the limit of detection . Subsequent analysis by SDS-PAGE stained with Brilliant Blue G Colloidal (Figure 1) demonstrated that the reference, control, and all</p>

	<p>temperature treated samples contain a major band with an apparent molecular weight of approximately 44 kDa corresponding to the CP4 EPSPS protein. No detectable loss of this band intensity was visually observed in the temperature treated samples under all conditions investigated.”</p> <p>Replaced with;</p> <p>“Results of the activity assay for CP4 EPSPS protein incubated for 15 and 30 min at the indicated temperatures are represented in Tables 1 and 2, respectively. Activity of the control substance for the 15 minute incubation time (Table 1) and 30 minute incubation (Table 2) met the acceptance criteria demonstrating that CP4 EPSPS activity was maintained during incubation on ice. When heated at a temperature of 25 °C, 37 °C and 55 °C with an incubation time of 15 minutes, a small reduction in CP4 EPSPS activity was observed to 81%, 84 %, and 70% of control respectively. The test substance heated for 30 minutes showed no negative change in CP4 EPSPS activity at 25 °C and a small reduction in activity to 88% of control at 37°C. The test substance heated to 55 °C demonstrated a reduction in CP4 EPSPS activity with 25 % activity remaining relative to the control substance after the 30 minute incubation. . The level of CP4EPSPS activity following incubation at temperatures of 75 and 95 °C was below the limit of detection for incubations at both time points.</p> <p>Analysis by SDS-PAGE stained with Brilliant Blue G Colloidal dye (Figure 1 and 2) demonstrated that the reference standard and control substance contain one major band with an apparent molecular weight of approximately 43.8 kDa corresponding to the CP4 EPSPS protein. Results of the SDS-PAGE data for the heat treatment of the test substances incubated for 15 minutes and 30 minutes are illustrated in Figures 1 and 2, respectively. The control substance loaded on each respective gel (lane 2, Figures 1 and lane 7, Figure 2) showed equivalent band intensity at 43.8 kDa to the 100 % reference standard (lane 8, Figures 1 and 2); demonstrating that the CP4 EPSPS protein was stable on wet ice during the incubation period. No apparent decrease in band intensity of the 43.8 kDa CP4 EPSPS protein band was observed in the test substance when heated at all temperatures for 15 minutes (lanes 3-7, Figure 1) or 30 minutes (lanes 2-6, Figure 2).”</p>
14	Removed “From this study, CP4 EPSPS protein at 25 °C and at 37 °C is stable, as determined by phosphate release assay. A reduction

	<p>in enzyme activity at 55 °C to 25% of control was observed indicating at least partial stability of CP4 EPSPS protein at this temperature. However, at temperatures of 75 °C and above a complete loss of functional activity was observed. SDS-PAGE of heat treated samples illustrated electrophoretic mobility equivalent to both the reference and control samples with no significant loss in stained band intensity”</p> <p>Replaced with “At temperatures of 75 °C and above CP4 EPSPS functional activity was below the limit of detection. SDS-PAGE demonstrated that heat treated test substance after incubation for 15 or 30 minutes at all temperatures investigated had no effect on the band intensity of CP4 EPSPS protein.”</p>
16	Changed Table 1 to Table 2, Added Table 1
18	Changed Figure 1 to Figure 2 and added Figure 1