

**Title**

**Assessment of the *in vitro* digestibility of purified *E. coli*-produced CP4 EPSPS protein in simulated gastric fluid**

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**Study Completed On**

**May 31, 2002**

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**Laboratory Project ID**

**MSL Number: 17566  
Study Number: 01-01-62-09**

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This study meets the GLP requirements for 40CFR Part 160 (EPA), except for the following.

An SOP was not in place and calibration and maintenance records were not kept for the spectrophotometer used in this study.

The original stained SDS-PAGE gels were not retained, however, photographs and scans of the gels were used to obtain a permanent image of the results.

These deviations from the Good Laboratory Practice standards did not adversely affect the scientific integrity of this study.

Submitter: \_\_\_\_\_ Date: \_\_\_\_\_

Sponsor  
Representative: Thomas C. Lee Date: 5/31/02

Study Director: John Teasdale Date: 5/31/02

## Quality Assurance Statement

**Study Title:** Assessment of the *in vitro* digestibility of purified *E. coli*-produced CP4 EPSPS protein in simulated gastric fluid

**Study Number:** 01-01-62-09

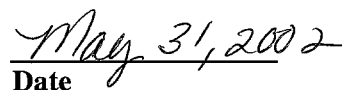
Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflect the raw data of the study.

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

<b>Dates of Inspection / Audit</b>	<b>Phase</b>	<b>Date Reported To:</b>	
		<b>Study Director</b>	<b>Management</b>
November 27, 2001	Digestive Fate	December 5, 2001	December 5, 2001
May 30, 2002	Raw Data Audit	May 31, 2002	May 31, 2002
May 30, 2002	Draft Report Review	May 31, 2002	May 31, 2002



**Paula A. Price**  
Quality Assurance Unit  
Monsanto Regulatory, Monsanto Company

  
Date

### Signatures of Approval

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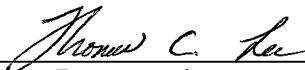
**Study Start Date:** November 20, 2001

**Study Completion Date:** May 31, 2002

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

**Sample Retention:** Any study samples, which are to be retained, will be stored at Monsanto, St. Louis.

  
Study Director \_\_\_\_\_ Date 5/31/02

  
Sponsor Representative \_\_\_\_\_ Date 5/31/02

  
Director of Product Safety Center \_\_\_\_\_ Date May 31, 2002

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### Abbreviations

aa	amino acid
A <sub>280 nm</sub>	Absorbance of light at a wavelength of 280 nm
CFR	Code of Federal Regulations
ECL	Enhanced Chemiluminescence
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
GLP	Good Laboratory Practice
HRP	Horseradish peroxidase
kDa	kilodalton
LOD	Lower limit of detection
mA	milliampere
mM	millimolar
N0	Experimental control without the CP4 EPSPS protein at zero incubation time
N9	Experimental control without the CP4 EPSPS protein, incubated for as long as T=9
NFDM	Nonfat dried milk
P0	Experimental control without pepsin at zero incubation time
P9	Experimental control without pepsin, incubated for as long as T=9
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
P/N	Product number, same as catalog number
SDS	Sodium dodecyl sulfate
SGF	Simulated gastric fluid
SGF-p	Simulated gastric fluid formulated without pepsin
SOP	Standard operating procedure
T	Time point
TCA	Trichloroacetic acid
Tricine	N-[tris(hydroxymethyl)methyl]glycine
Tris	Tris(hydroxymethyl)aminomethane
v/v	solute volume to solution volume
w/v	solute weight to solution volume

## 1.0 Summary

Monsanto Company has developed Roundup Ready<sup>®</sup> crops that are tolerant to glyphosate, the active ingredient in the Roundup<sup>®</sup> family of agricultural herbicides. Glyphosate tolerance is conferred by insertion of the *cp4 epsps* coding sequence, isolated from *Agrobacterium* sp. strain CP4, into the plant; *cp4 epsps* encodes for the production of the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein.

The purpose of this study was to assess the *in vitro* digestibility of the CP4 EPSPS protein in simulated gastric fluid (SGF), which contains the enzyme, pepsin. The CP4 EPSPS protein used in this study was produced in and purified from *E. coli*. Digestibility was assessed by SDS-PAGE gel staining, western blot analysis, and EPSPS enzymatic activity assay.

The results of this study demonstrate that the *E. coli*-produced CP4 EPSPS protein was rapidly digested after incubation in SGF. The SDS-PAGE colloidal blue gel staining method demonstrated that at least 98% of the *E. coli*-produced CP4 EPSPS protein was digested in SGF within 15 seconds. No degenerative bands due to digestion were observed. Western blot analysis confirmed that greater than 95% of the *E. coli*-produced CP4 EPSPS protein was digested in SGF within 15 seconds. Likewise, it was demonstrated that the EPSPS activity was reduced by >90% within 15 seconds of incubation of the CP4 EPSPS protein in SGF. In summary, the three detection methods all demonstrate that the *E. coli*-produced CP4 EPSPS protein is rapidly degraded in simulated gastric fluid.

## 2.0 Introduction

Monsanto Company has developed Roundup Ready crop products that produce 5-enolpyruvyl-shikimate-3-phosphate synthase, a protein endogenous to *Agrobacterium* sp. strain CP4 (CP4 EPSPS). The CP4 EPSPS protein confers tolerance to glyphosate, the active ingredient in the Roundup family of agricultural herbicides. Glyphosate exerts its herbicidal activity through the inhibition of a plant's endogenous EPSPS, which catalyzes an essential step in the shikimate pathway for aromatic amino acid biosynthesis in plants and microorganisms (Haslam, 1993). The CP4 EPSPS protein is functionally similar to the plant's endogenous EPSPS, but is highly resistant to inhibition by glyphosate and has a high catalytic efficiency compared to most glyphosate tolerant EPSPS proteins (Barry *et al.*, 1992, and Padgett *et al.*, 1993). Upon application of glyphosate, plants expressing the CP4 EPSPS protein are unaffected, since the continued action of the glyphosate-tolerant CP4 EPSPS protein meets the plant's need for aromatic amino acids.

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<sup>®</sup> Roundup and Roundup Ready are registered trademarks of Monsanto Technology, LLC.

### **3.0 Purpose**

The purpose of this study was to assess the *in vitro* digestibility of *E. coli*-produced CP4 EPSPS protein in SGF. *In vitro* digestion of CP4 EPSPS protein was evaluated by SDS-PAGE with colloidal blue gel staining, by western blot analysis probing with anti-CP4 EPSPS serum, and by an EPSPS activity assay.

### **4.0 Materials**

#### **4.1 Test Substance**

The test substance for this study was CP4 EPSPS protein (Lot 5192245) isolated from a large-scale fermentation of *E. coli*. This material was characterized and found to have a total protein concentration of 3.96 mg/ml in buffer [50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT and 50% (v/v) glycerol], with a purity of 90% (Harrison *et al.*, 1993). The test substance was stored in a -80 °C freezer until transferred to the study director and was stored in a -20 °C freezer thereafter. At the start of this study, this material was diluted to 1.68 mg/ml with phosphate buffered saline (PBS).

#### **4.2 Control Substance**

There was no control substance for this study.

#### **4.3 Reference Substance**

There was no reference substance for this study.

#### **4.4 Characterization of Test, Control, and Reference Substances**

Characterization of the *E. coli*-produced CP4 EPSPS protein was conducted prior to this study (Harrison *et al.*, 1993) to assess the identity, concentration, purity, composition, and activity.

#### **4.5 Characterization of Analytical References**

Certificates of analysis for pepsin, hemoglobin, and bovine serum albumin used in this study were added to the study data file. Product specification information for molecular weight markers and running buffers used in this study were also added to the study data file.

### **5.0 Test System**

The test system for this study was simulated gastric fluid (SGF), which contains the enzyme pepsin. SGF preparation was based on SOP BR-ME-0460-01 with the exception that a highly purified form of pepsin (Sigma P/N P-6887, Lot 99H7665) was used. SOP BR-ME-0460-01 describes the use of pepsin (Sigma P/N P-7000), which is only about 50-60% pure and contains less units of activity per mg of solid. Therefore, the amount of

pepsin powder used to formulate SGF, using the highly purified form of pepsin (Sigma P/N P-6887) was reduced to provide a digestion environment of 10 pepsin activity units per  $\mu\text{g}$  of test substance total protein. The amount of pepsin powder used to prepare SGF was calculated from the specific activity on the product label, which was 100% protein with an activity of 3,460 units/mg protein. One unit of activity is defined as a change in  $A_{280\text{ nm}}$  of 0.001 at 37 °C, measured as trichloroacetic acid (TCA)-soluble products using hemoglobin as the substrate. The final SGF formulation contained 0.20% (w/v) sodium chloride and 0.2557 mg/ml pepsin powder, adjusted with HCl to a pH of 1.2.

### 5.1 Justification for Selection of the Test System

*In vitro* digestion models are used widely to assess the digestibility of ingested substances. Previous studies have demonstrated that digestibility is a factor relevant to dietary exposure assessments for proteins (Astwood *et al.*, 1996 and del Val *et al.*, 1999). The activity of SGF was assessed following the procedure described by SOP BR-ME-0460-01, with the exceptions described in Section 7.1.

The time course and experimental conditions used in this study are similar to conditions used in a previously published study (Astwood *et al.*, 1996).

### 5.2 Experimental Controls

Experimental controls were prepared to characterize the stability of the test substance in the test system (SGF-p) formulated without pepsin. These experimental controls were incubated for 0 and 60 minutes and were designated with the letter "P". Additionally, experimental controls were prepared to characterize the test system (SGF) without the CP4 EPSPS protein test substance, also for 0 and 60 minutes. These experimental controls were prepared by substituting phosphate buffered saline (PBS) for the CP4 EPSPS protein test substance, and were designated with the letter "N".

### 5.3 Specimens

Digestive fate specimens were generated by incubating the test substance in the test system for various lengths of time at 37 °C. Experimental tubes were numbered to distinguish assay time points, and labeled with colored dots for easy recognition. Specimens were stored in a -20 °C freezer and will be discarded approximately one year after the completion of the study.

### 6.0 Experimental Design

A schematic of the digestion experiment is shown in the protocol, which is attached to the end of this report.

## 6.1 Digestibility of the Test Substance in SGF

Digestions of the test material were prepared by adding the *E. coli*-produced CP4 EPSPS protein to tubes containing SGF. The digestions were conducted such that 10 pepsin activity units were used per  $\mu\text{g}$  of test substance total protein and were incubated at 37 °C in separate tubes for each of the incubation time points.

At the specified time, the SGF activity was quenched by the addition of 0.2 M sodium carbonate to the test system. This has been shown in pre-study experiments to be an appropriate method of quenching SGF activity (Astwood *et al.*, 1996). After quenching, all trials were frozen on dry ice and then transferred to a -20 °C freezer for long-term storage.

The zero time point digestions (T = 0) were quenched by addition of 0.2 M sodium carbonate to SGF prior to addition of the test substance.

The incubation time points generated in this study were 0, 15, and 30 seconds, and 1, 2, 4, 8, 15, 30, and 60 minutes. These are the time points 0-9 referenced to in Figures 1 and 3, with time point T = 0 corresponding to 0 seconds, and time point T = 9 corresponding to 60 minutes.

## 6.2 Experimental Controls

Experimental controls were prepared to characterize the stability of the test substance in the test system (SGF-p) formulated without pepsin. These experimental controls were prepared in a manner similar to that described in Section 6.1. The volumes used were the same as those used to prepare the digestion assays of CP4 EPSPS protein in SGF. However, only incubation time points of 0 and 60 minutes (T = 0 and T = 9) were generated for the experimental controls.

Experimental controls were also prepared to characterize the test system (SGF) without the test substance. The same buffer as that used to store *E. coli*-produced CP4 EPSPS protein [50 mM Tris-HCl, pH 7.5, 150 mM KCl, 2 mM DTT and 25% (v/v) glycerol] was added to SGF. These experimental controls were prepared in a manner similar to that described in Section 6.1. The volumes were the same as those used to prepare digestions of CP4 EPSPS protein in SGF. However, only incubation time points of 0 and 60 minutes (T = 0 and T = 9) were generated for these controls.

### **6.3 Control of Bias**

Measures taken to control bias in this study included appropriate experimental controls to account for any effects due to the model in the absence of CP4 EPSPS protein and experimental controls to account for any effects due to CP4 EPSPS protein in SGF-p.

### **7.0 Analytical Methods**

The SGF was assayed before and after conducting the timed incubations to demonstrate that pepsin remained active throughout the experiment. The digestibility of purified *E. coli*-produced CP4 EPSPS protein in SGF was assessed using SDS-PAGE gel staining, western blot, and an EPSPS enzyme activity assay. Lower limits of detection (LOD) were determined for the gel staining and western blot methods.

#### **7.1 Digestive Fluid Activity Assay**

The activity of SGF was confirmed following the procedure described by SOP BR-ME-0460-01. Since SGF was prepared with a different formulation than that described by SOP BR-ME-0460-01, the specified dilution, calculation of activity, and the acceptable range of activity were not appropriate. Therefore, this assay was used as a qualitative assessment of SGF activity. Acceptable activity for this study was defined in the protocol to be a value greater than 1000 units/ml.

Following SOP BR-ME-0460-01, with the exceptions mentioned above, the activity of SGF was assessed before and after generating the digestion trials. Both assessments of SGF activity demonstrated that the test system was active and acceptable for use in this study.

#### **7.2 SDS-PAGE**

Samples from the SGF *in vitro* digestion of purified *E. coli*-produced CP4 EPSPS protein were analyzed by SDS-PAGE using pre-cast 10-20% tricine mini-gels (NOVEX, P/N EC66255) as described in SOP BtC-PRO-026-01 with the following modifications. For tricine gels, the upper buffer reservoir was filled with 100 mM tricine, 100 mM Tris and 0.1% (w/v) SDS, pH  $\approx$  8.25 and the lower buffer reservoir was filled with 200 mM Tris, pH  $\approx$  8.9. Tricine gels were used because they have been shown to provide optimum resolution of low molecular weight proteins (Schägger and von Jagow, 1987).

#### **7.3 Colloidal Blue Gel Staining**

Gel staining methods are commonly used and extensively referenced in the scientific literature relevant to the purposes for which they are being employed in this study (Deutcher, 1990; Schägger and von Jagow, 1987; Neuhoff *et al.*, 1988).

After separation of proteins by SDS-PAGE, the gels were incubated in fix solution [40% (v/v) methanol, 7% (v/v) acetic acid] for approximately 35 minutes at room temperature. Gels were then stained overnight with Brilliant Blue G Colloidal dye (Sigma P/N B-2025) diluted 4:1 with methanol. Background from excess dye was removed by washing the gels in 25% (v/v) methanol.

The colloidal blue gel staining method was selected because it is sensitive to low amounts of protein (Neuhoff *et al.*, 1988). A lower limit of detection for this method specific to *E. coli*-produced CP4 EPSPS protein was determined by loading samples of serial dilutions of the zero incubation time point onto a gel that was run concurrently with the gel used to assess digestibility.

#### 7.4 Western Blot Analysis

Samples from the SGF *in vitro* digestions were analyzed by western blot according to SOP GEN-PRO-002-03. After the proteins were separated by SDS-PAGE (Section 7.2), they were electroblotted onto a 0.45 µm pore size PVDF membrane (Sigma P/N P-2813) at approximately 300 mA constant current for 1 hour and 18 minutes at 4 °C. Non-specific binding sites on the blots were blocked by incubating the blots overnight at 4 °C in phosphate buffered saline with Tween 20 (PBST) that contained 5% (w/v) nonfat dried milk (NFDM). CP4 EPSPS protein was detected using a sandwich probe methodology. First, for specific binding to the CP4 EPSPS protein, goat antiserum (Lot JB6313149) developed against the CP4 EPSPS protein was used at a 1:2000 dilution in 20 ml/blot of PBST that contained 1% (w/v) NFDM. After incubation for 1.5 hours at room temperature, unbound goat antibody was removed by three washes in PBST for 5-15 minutes each. Second, to identify the bound goat antibody, biotinylated protein G (Pierce P/N 29988) was used at a 1:2000 dilution in 20 ml/blot of PBST that contained 1% (w/v) NFDM. After a 1-hour incubation at room temperature, unbound protein G was removed by three washes in PBST for 5-15 minutes each. Finally, to probe for protein G bound to the blot, neutravidin conjugated to horseradish peroxidase (HRP; Pierce P/N 31001) was used at a 1:10,000 dilution in 30 ml/blot of PBST that contained 1% (w/v) NFDM. After incubation for 1.5 hours at room temperature, unbound neutravidin was removed by three washes in PBST for 5-15 minutes each. Enhanced chemiluminescent (ECL) reagents (Amersham P/N RPN 2106) and Hyperfilm™ (Amersham P/N RPN 3114K) were used for detection of the antibody-bound CP4 EPSPS proteins.

A lower limit of detection for this method specific to *E. coli*-produced CP4 EPSPS protein was determined by loading samples of serial dilutions of the zero incubation time point onto a gel that was run concurrently with the gel used to assess digestibility.

Molecular weight standards (Invitrogen P/N LC5677) were used to assess transfer of proteins from the SDS-PAGE gel to the PVDF membrane and to provide a relative molecular weight scale. Since these molecular weight standards were not pre-stained, these lanes were cut from the blot after transfer and stained with Ponceau S (Sigma P/N P-7170). The relative molecular weight scale was later added to the ECL film images by overlaying the ECL film onto the reassembled blots and marking the position of the molecular weight standards. To replace the markings on the film with the molecular weight labels seen in Figures 1 and 2, the labels were aligned to the markings on the scanned image. The markings were later cropped from the image to provide the figures shown in this report.

### **7.5 EPSPS Activity Assay**

Selected samples (incubations of 0, 15, 30 and 60 seconds, and all experimental controls) from the SGF *in vitro* digestion of purified *E. coli*-produced CP4 EPSPS protein were analyzed by an EPSPS enzyme assay conducted in accordance with SOP GS-PRO-035-00. A standard curve to determine the concentration of inorganic phosphate released from the reaction was prepared using potassium phosphate monobasic (Fisher Scientific P/N P285-500). An additional buffer blank prepared by adding CP4 EPSPS storage buffer to SGF-p was added to the assay to subtract SGF background before quantitation.

### **7.6 Statistical Methods**

No statistical analysis was performed.

## **8.0 Results and Discussion**

### **8.1 Assessment of Digestibility by Colloidal Blue Gel Staining of SDS-PAGE**

The digestibility of *E. coli*-produced CP4 EPSPS protein was evaluated by staining SDS-PAGE gels with colloidal blue dye using a method described in greater detail in Section 7.3. Each lane was loaded with 500 ng of *E. coli*-produced CP4 EPSPS protein. Experimental controls without CP4 EPSPS protein were loaded in the same volume as those containing CP4 EPSPS protein so that all other components of the digestion sample were loaded at the same level. The resulting stained gels demonstrate that the *E. coli*-produced CP4 EPSPS protein was digested to a level below the limit of detection within 15 seconds in SGF (Figure 1).

Experimental controls using SGF-p, which was formulated without pepsin (Figure 1, lanes 2 and 15), demonstrated that degradation of *E. coli*-produced CP4 EPSPS protein was due to digestion by SGF and not instability of the test

substance in pH 1.2 while incubating at 37 °C. This conclusion was based on the similar band intensity of CP4 EPSPS protein in lanes 2 and 15.

The activity assay of SGF conducted before and after the digestion experiment demonstrated the stability of pepsin activity throughout the experimental phase of this study. Stability of pepsin was also demonstrated by similar pepsin band intensities in all of the incubation times (Figure 1).

An SDS-PAGE gel was run and stained concurrently with the gel for assessment of digestibility by colloidal blue gel staining in order to determine a lower limit of detection for this method specific to the samples being analyzed (Figure 2). The limit of detection was determined by loading various dilutions of the zero incubation time point of *E. coli*-produced CP4 EPSPS protein in SGF (T = 0) and observing which dilutions could be detected by visual inspection of the stained gel. The lower limit of detection was estimated to be a 1/50 dilution of the zero incubation time point, which is representative of the *E. coli*-produced CP4 EPSPS protein level at 98% digestion. Since 500 ng of the CP4 EPSPS protein (prior to digestion) was loaded per lane, the lower LOD was approximately 10 ng of CP4 EPSPS protein per lane.

## 8.2 Assessment of Digestibility by Western Blot Analysis

The digestibility of *E. coli*-produced CP4 EPSPS protein was also assessed by a western blot method described in greater detail in Section 7.4. Each lane was loaded with 1 ng of *E. coli*-produced CP4 EPSPS protein. Experimental controls without CP4 EPSPS protein were loaded in the same volume as those containing CP4 EPSPS protein, so that all other components of the digestion sample were equally loaded. *E. coli*-produced CP4 EPSPS protein was digested below the limit of detection within 15 seconds in SGF (Figure 3, lane 5).

Experimental controls using SGF-p, which was formulated without pepsin (Figure 3, lanes 2 and 15) demonstrated that degradation of *E. coli*-produced CP4 EPSPS protein was attributed to digestion by SGF and not instability of the test substance in pH 1.2 while incubating at 37 °C. Experimental controls without *E. coli*-produced CP4 EPSPS protein (Figure 3, lanes 3 and 14) demonstrate consistent background detection for digestion samples incubated from 0 to 60 minutes.

A western blot was run concurrently with the western blot for assessment of digestibility, to determine a lower LOD for this method specific to the samples being analyzed (Figure 4). The limit of detection was determined by loading various dilutions of the zero incubation time point of *E. coli*-produced CP4 EPSPS protein in SGF (T=0) and demonstrating which dilutions could be

detected. The lower LOD was estimated to be a 1/20 dilution of the zero incubation time point, which is representative of the *E. coli*-produced CP4 EPSPS protein level at 95% digestion. Since 1 ng of the CP4 EPSPS protein (prior to digestion) was loaded per lane, the lower LOD was approximately 0.05 ng of CP4 EPSPS protein per lane.

### 8.3 Assessment of Digestibility by EPSPS Activity Assay

Selected samples (incubations of 0, 15, 30 and 60 seconds, and all experimental controls) from the SGF *in vitro* digestion of purified *E. coli*-produced CP4 EPSPS protein were assessed by an EPSPS enzyme activity assay described in greater detail in Section 7.5 (Table 1). As expected, no significant amount of EPSPS activity was observed in samples incubated for 15 seconds or longer in SGF with pepsin. EPSPS activity was observed in samples where no digestion was expected (0 incubation and experimental controls without pepsin). However, comparison of the experimental controls incubated in SGF prepared without pepsin, pH 1.2 (P0 and P9), show that EPSPS activity was reduced after incubation at 37 °C for 60 minutes. Since the western blot and stained gel results show that *E. coli*-produced CP4 EPSPS protein is stable after incubation for 60 minutes without pepsin (Figures 1 and 3, lanes 2 and 15), loss of EPSPS activity is attributed to denaturing of the CP4 protein at pH 1.2 while incubating at 37 °C.

Experimental controls without *E. coli*-produced CP4 EPSPS protein, a buffer control containing CP4 EPSPS storage buffer, and experimental controls without pepsin demonstrated that components of the digestion samples other than *E. coli*-produced CP4 EPSPS protein produce a low and consistent background in the EPSPS activity assay. Therefore, a horizontal baseline is appropriate for background subtraction.

### 8.4 Rejected Data / Data Not Reported

There were instances where data was not used due to gel or buffer contamination, inaccurate molecular weight values, or poor image quality. Records for rejected data or data not reported under this study have been archived in the study file.

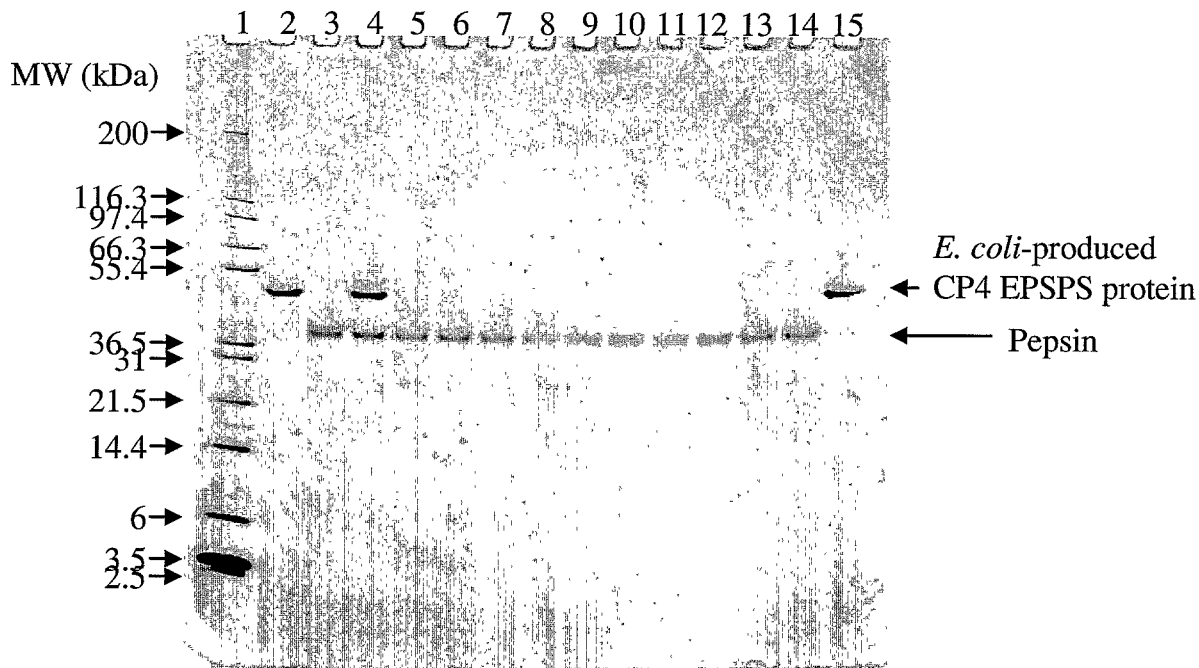
### 9.0 Conclusions

The results of this study demonstrated that *E. coli*-produced CP4 EPSPS protein was rapidly digested after incubation in SGF at 37 °C. At least 98% of the *E. coli*-produced CP4 EPSPS protein was digested within 15 seconds in SGF as determined by colloidal blue gel staining. Likewise, greater than 95% of the *E. coli*-produced CP4 EPSPS protein was digested in SGF within 15 seconds as determined western blot analysis. Moreover, it was demonstrated that the EPSPS activity was reduced by >90% within 15 seconds of

incubation of the CP4 EPSPS protein in SGF. In summary, the three detection methods all demonstrate that the *E. coli*-produced CP4 EPSPS protein is rapidly degraded in simulated gastric fluid.

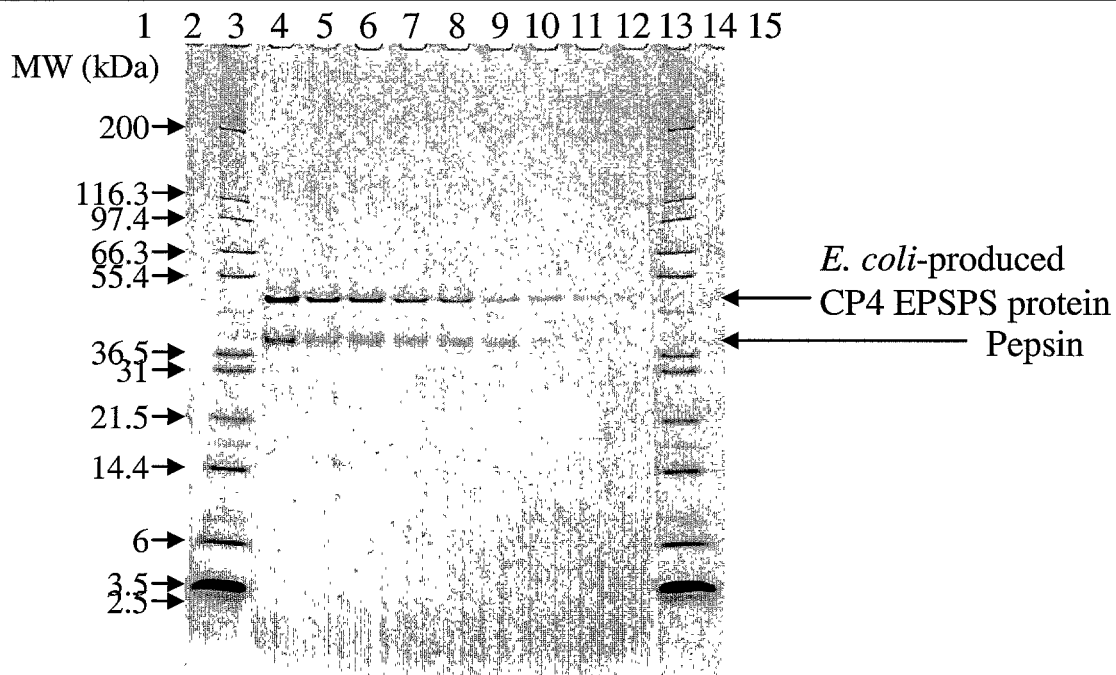
## 10.0 References

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- del Val, G., Yee, B. C., Lozano, R. M., Buchanan, B. B., Ermel, R. W., Lee, Y. M. and Frick, O. L. (1999). Thioredoxin treatment increases digestibility and lowers allergenicity of milk. *J Allergy Clin Immunol* **103**: 690-697.
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- Padgette, S. R., Barry, G. F., Re, D. B., Weldon, M., Eichholtz, D. A., Kolacz, K. and Kishore, G. M. (1993). Purification, cloning, and characterization of a highly glyphosate-tolerant EPSP synthase from *Agrobacterium* sp. strain CP4. Monsanto Technical Report, St. Louis, MO, MSL-12738.
- Schägger, H. and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry*, **166**: 368-379.



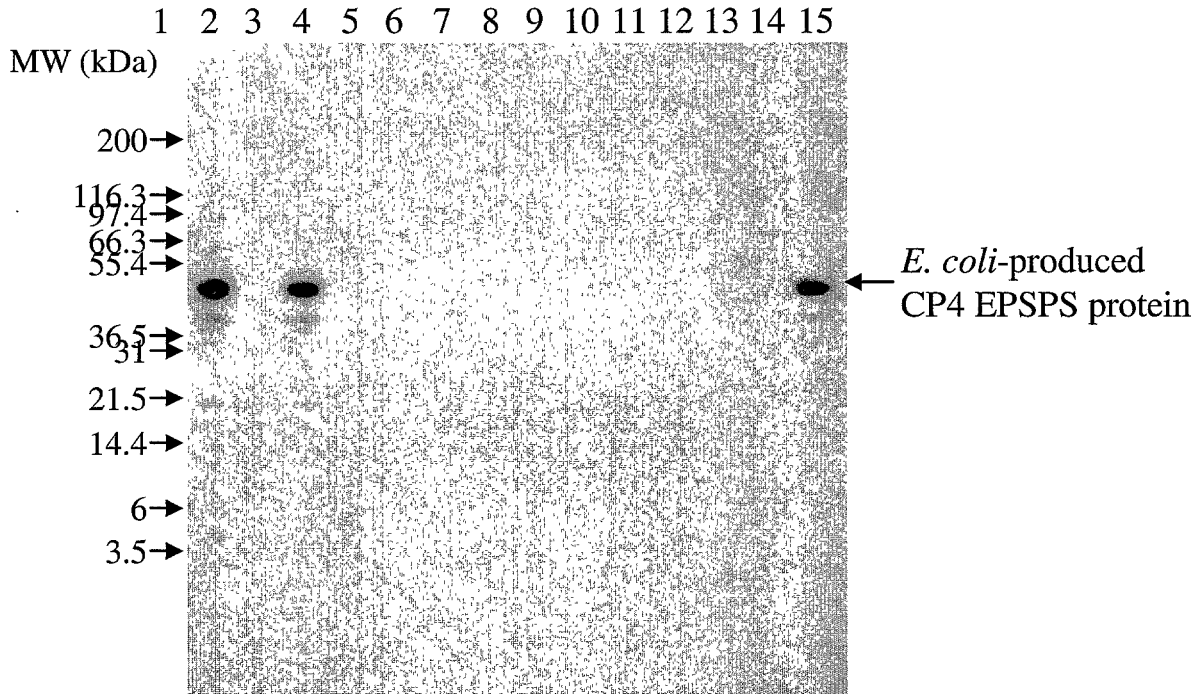
**Figure 1. Colloidal Blue Stained SDS-PAGE Gel Showing the Digestion of Purified *E. coli*-produced CP4 EPSPS Protein in Simulated Gastric Fluid.** Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G stain. *E. coli*-produced CP4 EPSPS protein was loaded at 500 ng per lane based on pre-digestion concentration.

<u>Lane</u>	<u>Description</u>	<u>Incubation time</u>
1	Molecular weight markers	
2	Experimental control without pepsin (P0)	0 s
3	Experimental control without CP4 EPSPS (N0)	0 s
4	CP4 EPSPS protein in SGF, T = 0	0 s
5	CP4 EPSPS protein in SGF, T = 1	15 s
6	CP4 EPSPS protein in SGF, T = 2	30 s
7	CP4 EPSPS protein in SGF, T = 3	1 min
8	CP4 EPSPS protein in SGF, T = 4	2 min
9	CP4 EPSPS protein in SGF, T = 5	4 min
10	CP4 EPSPS protein in SGF, T = 6	8 min
11	CP4 EPSPS protein in SGF, T = 7	15 min
12	CP4 EPSPS protein in SGF, T = 8	30 min
13	CP4 EPSPS protein in SGF, T = 9	60 min
14	Experimental control without CP4 EPSPS (N9)	60 min
15	Experimental control without pepsin (P9)	60 min



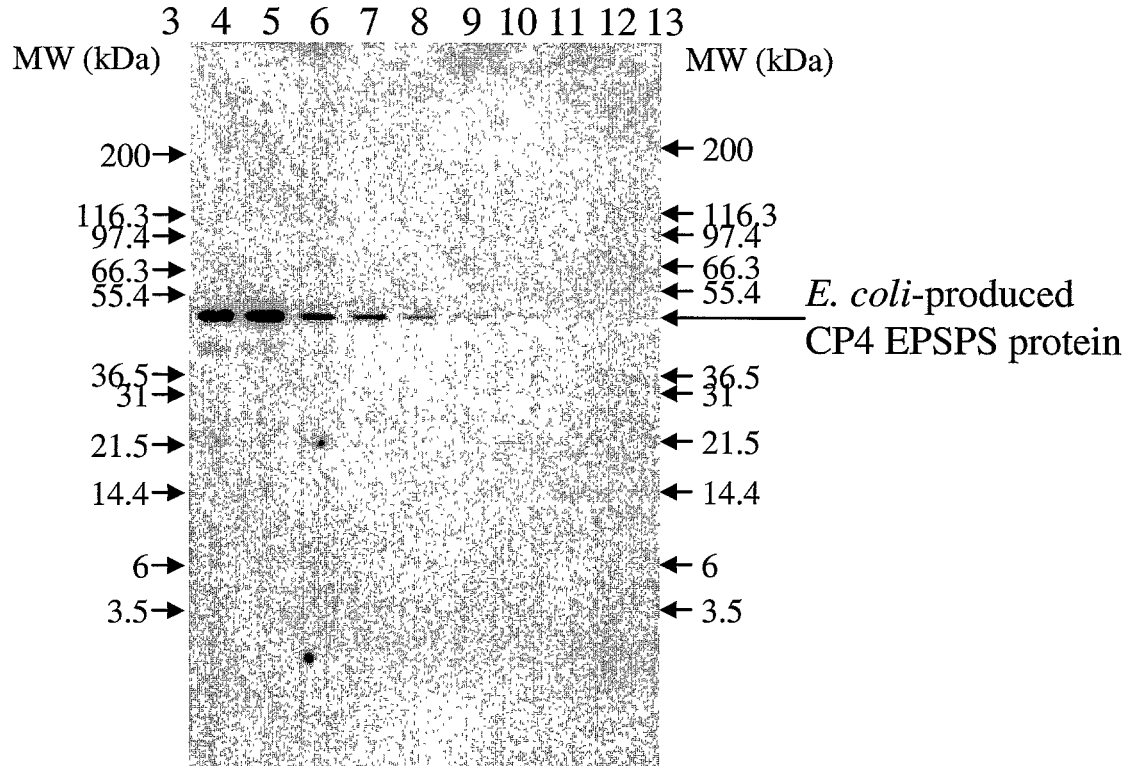
**Figure 2. Colloidal Blue Stained SDS-PAGE Gel Showing the LOD Specific to *E. coli*-produced CP4 EPSPS protein in Simulated Gastric Fluid.** Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G stain.

<u>Lane</u>	<u>Description</u>	<u>Loading of CP4 EPSPS protein</u>
1	Empty	
2	Empty	
3	Molecular weight markers	
4	CP4 EPSPS protein in SGF, T = 0	500 ng
5	CP4 EPSPS protein in SGF, T = 0	250 ng
6	CP4 EPSPS protein in SGF, T = 0	200 ng
7	CP4 EPSPS protein in SGF, T = 0	150 ng
8	CP4 EPSPS protein in SGF, T = 0	100 ng
9	CP4 EPSPS protein in SGF, T = 0	50 ng
10	CP4 EPSPS protein in SGF, T = 0	25 ng
11	CP4 EPSPS protein in SGF, T = 0	10 ng
12	CP4 EPSPS protein in SGF, T = 0	5 ng
13	Molecular weight markers	
14	Empty	
15	Empty	



**Figure 3. Western Blot Showing the Digestion of Purified *E. coli*-produced CP4 EPSPS Protein in Simulated Gastric Fluid.** Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. *E. coli*-produced CP4 EPSPS protein was loaded at 1 ng per lane based on pre-digestion concentration.

<u>Lane</u>	<u>Description</u>	<u>Incubation time</u>
1	Molecular weight markers	
2	Experimental control without pepsin (P0)	0 s
3	Experimental control without CP4 EPSPS (N0)	0 s
4	CP4 EPSPS protein in SGF, T = 0	0 s
5	CP4 EPSPS protein in SGF, T = 1	15 s
6	CP4 EPSPS protein in SGF, T = 2	30 s
7	CP4 EPSPS protein in SGF, T = 3	1 min
8	CP4 EPSPS protein in SGF, T = 4	2 min
9	CP4 EPSPS protein in SGF, T = 5	4 min
10	CP4 EPSPS protein in SGF, T = 6	8 min
11	CP4 EPSPS protein in SGF, T = 7	15 min
12	CP4 EPSPS protein in SGF, T = 8	30 min
13	CP4 EPSPS protein in SGF, T = 9	60 min
14	Experimental control without CP4 EPSPS (N9)	60 min
15	Experimental control without pepsin (P9)	60 min



**Figure 4. Western Blot Showing the LOD Specific to *E. coli*-produced CP4 EPSPS protein in Simulated Gastric Fluid.** Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel.

<u>Lane</u>	<u>Description</u>	<u>Loading of CP4 EPSPS protein</u>
1	Empty	
2	Empty	
3	Molecular weight markers	
4	CP4 EPSPS protein in SGF, T = 0	1 ng
5	CP4 EPSPS protein in SGF, T = 0	0.5 ng
6	CP4 EPSPS protein in SGF, T = 0	0.4 ng
7	CP4 EPSPS protein in SGF, T = 0	0.3 ng
8	CP4 EPSPS protein in SGF, T = 0	0.2 ng
9	CP4 EPSPS protein in SGF, T = 0	0.1 ng
10	CP4 EPSPS protein in SGF, T = 0	0.05 ng
11	CP4 EPSPS protein in SGF, T = 0	0.02 ng
12	CP4 EPSPS protein in SGF, T = 0	0.01 ng
13	Molecular weight markers	
14	Empty	
15	Empty	

**Table 1. Specific Activity of *E. coli*-produced CP4 EPSPS Protein After Digestion in Simulated Gastric Fluid.**

Sample	Specific Activity (Units/mg protein)
Experimental control without pepsin incubated for 0 seconds (P0)	4.92
Experimental control without pepsin incubated for 60 minutes (P9)	2.10
<i>E. coli</i> -produced CP4 EPSPS protein in SGF incubated for 0 seconds (T=0)	5.63
<i>E. coli</i> -produced CP4 EPSPS protein in SGF incubated for 15 seconds (T=1)	0.27
<i>E. coli</i> -produced CP4 EPSPS protein in SGF incubated for 30 seconds (T=2)	0.15
<i>E. coli</i> -produced CP4 EPSPS protein in SGF incubated for 60 seconds (T=3)	0.15
Experimental control without CP4 EPSPS incubated for 0 seconds (N0)	0.02
Experimental control without CP4 EPSPS incubated for 60 minutes (N9)	0.05
Buffer Blank	0.01

## **Appendix 1**

### **Protocol and Deviation Attachments**

Protocol is attached as Pages 26 - 38

and

Protocol deviation is attached as Page 39

**Monsanto Study #:** 01-01-62-09

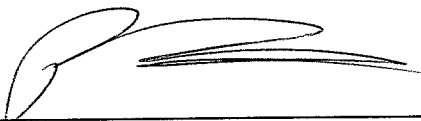
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produced CP4 EPSPS protein in simulated gastric fluid

**Sponsor:** Monsanto Company  
700 Chesterfield Parkway North  
St. Louis, MO 63198

**Primary Testing Facility:** Monsanto Company  
Product Safety Center  
800 North Lindbergh  
St. Louis, MO 63167

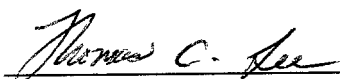
**Study Director:** John N. Leach  
Monsanto Company  
Product Safety Center  
800 North Lindbergh  
St. Louis, MO 63167

**Approved By:**



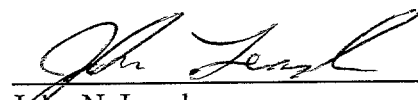
Patrick T. Weston  
**Testing Facility Management Representative**  
Monsanto Company - BB5B  
700 Chesterfield Parkway North  
St. Louis, MO 63198  
Phone: (636) 737-5407

Nov 20, 2001  
Date



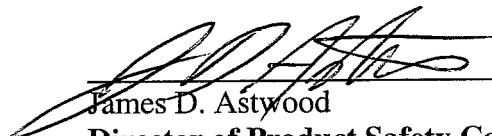
Thomas C. Lee  
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11/20/01  
Date



John N. Leach  
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Phone: (314) 694-8452  
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e-mail: john.n.leach@monsanto.com

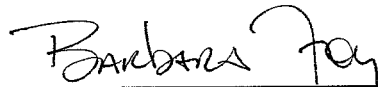
Nov. 20, 2001  
Date



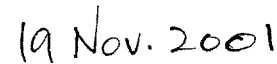
James D. Astwood  
**Director of Product Safety Center**  
Monsanto Company - O3E  
800 North Lindbergh  
St. Louis, MO 63167  
Phone: (314) 694-8396

Nov 19/01  
Date

**Reviewed By:**



\_\_\_\_\_  
**Quality Assurance Specialist**  
Monsanto Company  
Monsanto Regulatory



\_\_\_\_\_  
Date

## 1.0 Regulatory Compliance

### 1.1 GLP Compliance

This study will be conducted in compliance with the United States EPA FIFRA Good Laboratory Practice Regulations (40 CFR Part 160). Monsanto Regulatory QAU will provide QA oversight for this study, and will distribute QA reports according to Monsanto Regulatory QAU SOPs.

## 2.0 Purpose

The purpose of this study is to assess the *in vitro* digestibility in simulated gastric fluid of purified *E. coli*-produced CP4 EPSPS protein.

## 3.0 Timelines

3.1 Proposed Experimental Start Date: November 2001

3.2 Proposed Experimental Termination Date: December 2001

## 4.0 Test, Control and Reference Substances

### 4.1 Test Substances

The test substance for this study is *E. coli*-produced CP4 EPSPS protein.

*E. coli*-produced CP4 EPSPS protein (lot no. 5192245) was produced in and isolated from a large-scale fermentation of *E. coli* containing the pET23b(+)/30139 expression plasmid. This material was characterized to have a total protein concentration of 3.96 mg/ml in buffer [50 mM Tris-HCl, pH 7.5, 150 mM KCl, 2 mM DTT and 25% (v/v) glycerol], with a purity of 90% (Lee et al., 2001). The test substance will be stored in a 4 °C refrigerator, or colder, until use in this study.

### 4.2 Control Substance

There is no control substance for this study.

### 4.3 Reference Substance

There is no reference substance for this study.

Appropriate reference standards will be documented in the results and will be described in the final report for each analytical procedure employed. Reference standards will include, but are not limited to, molecular weight markers and inorganic phosphate.

### 4.4 Characterization of Test, Control and Reference Substances

Characterization of the *E. coli*-produced CP4 EPSPS protein was conducted prior to this study (Harrison et al., 1993) to assess the identity, concentration, purity, composition, and activity. Any further preparation

of these substances before use in this study will be documented and discussed in the final report.

The stability of *E. coli*-produced CP4 EPSPS protein was assessed prior to conducting this study (Harrison et al., 1993).

#### **4.5 Characterization of Analytical References**

When available, certificates of analysis confirming the characterization of reference standards and other materials used in this study will be copied and filed with this study.

### **5.0 Test System**

The test system is simulated gastric fluid (SGF).

SGF will be prepared according to SOP No. BR-ME-0460-01 with the exception that a highly purified form of pepsin (Sigma Company catalog number P-6887) will be used. The amount of pepsin used to prepare SGF will also be changed to maintain a digestion environment of 10 units pepsin activity per 1  $\mu$ g of total protein from the digested sample. The pH will be adjusted to 1.2 with hydrochloric acid.

#### **5.1 Justification for Selection of the Test System**

*In vitro* digestion models are used widely to assess the digestibility of ingested substances. A previous study has demonstrated that digestibility is a factor relevant to dietary exposure assessments for proteins (Astwood et al., 1996). The activity of SGF will be confirmed according to SOP No. BR-ME-0460-01, with the exception that before conducting the assay, SGF will not be diluted to 0.05X and all calculations will be conducted as if 0.05X SGF had been used in the assay. Since SGF is prepared with a different formulation than described by this SOP, the acceptable range of activity specified in the SOP may not be appropriate. Acceptable activity will be defined as an activity greater than 1000 units/mL.

The time course and experimental parameters proposed in this study are similar to conditions used in a previously published study (Astwood et al., 1996).

#### **5.2 Experimental Controls**

Experimental controls will be prepared to characterize the stability of the test substance in the test system (SGF) lacking pepsin for the duration of the longest digestion time. These experimental controls will be designated with the letter "P". Conversely, experimental controls will be prepared to characterize the test system (SGF) lacking the test substance for the

duration of the digestion time. These experimental controls will be designated with the letter "N".

### **5.3 Specimens**

Specimens will be generated to represent various lengths of time at which the test substance will be incubated in the test system. See sections 7.0 through 7.6 for details on the preparation and analysis of specimens. Specimens will be retained for approximately one year, after which they will no longer afford analytical value and will be disposed.

### **5.4 Procedure for Identification of Specimens**

A numerical code using the numbers 0 through 9 will be used to distinguish assay time points.

## **6.0 Experimental Design**

A schematic of the digestibility experimental procedure is shown in Attachment 3. All assay tubes will be frozen on dry ice and transferred to a -20 °C freezer until analyzed.

### **6.1 Digestibility of the Test Substances in SGF**

The test substances will be used in this study to generate an incubation time course of CP4 EPSPS protein in SGF.

Digestions will be prepared by adding CP4 EPSPS protein to tubes containing SGF. Digestions will be conducted so that 10 units of pepsin activity will be used per 1 µg of total protein from the test substance. Digestions will be incubated at  $37 \pm 2$  °C in separate tubes for each of the targeted incubation times. SGF digestions will be quenched by addition of a sodium carbonate solution to the test system. This has been shown in prestudy experiments to be an appropriate method of quenching SGF activity (Astwood et al., 1996).

Zero incubation time points ( $T = 0$ ) will be quenched by addition of sodium carbonate solution to SGF prior to addition of the test substance.

The targeted incubation times will be 0, 15, 30 sec, and 1, 2, 4, 8, 15, 30, and 60 min. Actual incubation times will be recorded in the data file.

### **6.2 Experimental Controls**

Experimental controls will be prepared to characterize the stability of the test substance in the test system (SGF) lacking pepsin. These experimental controls will be prepared in a similar manner as described above in section 6.1. Volumes will be the same as those used to prepare

digestions of CP4 EPSPS protein in SGF, but the targeted incubation times will be 0 and 60 minutes. Additional time points will be used if necessary.

Experimental controls will also be prepared to characterize the test system (SGF) lacking the test substance. The buffer used to store *E. coli*-produced CP4 EPSPS protein [50 mM Tris-HCl, pH 7.5, 150 mM KCl, 2 mM DTT and 25% (v/v) glycerol] will be added to SGF. These experimental controls will be prepared in a similar manner as described above in section 7.1. Volumes will be the same as those used to prepare digestions of CP4 EPSPS protein in SGF, but the targeted incubation times will be 0 and 60 minutes. Additional time points will be used if necessary.

All trials will be frozen on dry ice until they can be stored in a -20 °C freezer or colder, where they will remain until analysis.

## 7.0 Analytical Methods

The digestibility of purified *E. coli*-produced CP4 EPSPS protein in SGF will be assessed using SDS-PAGE gel staining, western blot, and enzyme assay. Gel staining methods are commonly used and extensively referenced in the scientific literature relevant to the purposes for which they are being employed (Deutcher, 1990; Schägger and von Jagow, 1987; Neuhoff et al., 1988). Other methods for detection of *E. coli*-produced CP4 EPSPS protein may be employed as needed or to increase sensitivity. Lower limits of detection will be determined for the gel staining and western blot methods. If observed, digestion products will be characterized.

### 7.1 Digestive fluid activity assays

The activity of SGF will be assessed before and after preparing digestions according to SOP No. BR-ME-0460-01. This will demonstrate that the test system is appropriate for use in this study.

### 7.2 SDS-PAGE

Samples from the SGF *in vitro* digestion of purified *E. coli*-produced CP4 EPSPS protein will be analyzed by SDS-PAGE using pre-cast 10-20% tricine mini-gels (NOVEX, P/N EC66255). This procedure is described in SOP No. BtC-PRO-026-01 with the following modifications. All SDS-PAGE runs conducted during this study will use NOVEX brand tricine gels run with tricine buffers. For tricine gels, the upper buffer reservoir will contain 100 mM tricine, 100 mM Tris and 0.1% (w/v) SDS, pH  $\approx$  8.25 and the lower buffer reservoir will contain 200 mM Tris, pH  $\approx$  8.9. Tricine SDS-PAGE gels will be used because they have been shown to provide optimum resolution of low molecular weight proteins (Schägger and von Jagow, 1987).

Based on predigestion concentrations and purity corrections, approximately 500 ng of *E. coli*-produced CP4 EPSPS protein will be loaded per lane. Experimental controls will be loaded with the same volumes used to load the corresponding digestion trials.

### **7.3 Colloidal blue staining**

After separation of proteins by SDS-PAGE, gels will be stained using a colloidal blue dye. Prior to staining, the gels are incubated in fix solution [40% (v/v) methanol, 7% (v/v) acetic acid] for at least 30 minutes at room temperature. Gels are then stained for at least one hour with Brilliant Blue G Colloidal dye (Sigma P/N B-2025) diluted 4:1 with methanol. Excess background is removed by washing the gels in 25% (v/v) methanol.

This method was selected because it is sensitive to low amounts of protein (Neuhoff et al., 1988). A limit of detection specific to *E. coli*-produced CP4 EPSPS protein will be demonstrated within this study to assess the amount of digestion.

### **7.4 Western blot analysis**

Samples from the SGF *in vitro* digestions will be analyzed by western blot according to SOP No. GEN-PRO-002-03.

### **7.5 EPSPS activity assay**

Selected samples from the SGF *in vitro* digestion of purified *E. coli*-produced CP4 EPSPS protein will be analyzed by an EPSPS enzyme assay conducted in accordance with SOP No. GS-PRO-035-00. Additional controls will be added to the assay to subtract SGF background before quantitation.

### **7.6 Statistical methods**

No statistical analysis will be performed.

## **8.0 Control of Bias**

Measures taken to control bias in this study will include, but are not limited to, the analysis of samples in sets to eliminate run-to-run variations and the inclusion of appropriate controls to account for any effects due to the model in the absence of test substance. Trials generated from the incubation time course will serve as replicates that fall within a certain range.

## **9.0 Records to be Maintained**

Records will be maintained of all sample transfers, analyses, the protocol and all deviations and amendments thereto and copies of all letters memoranda and other correspondence related to this study. These documents may include: photocopies,

computer generated hard copies or hand-written notes that describe the procedures used to generate data for this study. Upon completion of the study, all study records and final report will be archived.

#### **10.0 Changes to the Protocol**

Planned changes to the protocol will be documented in the form of written protocol amendments and signed by the Study Director. Amendments become part of the protocol and will be archived with the protocol. All other changes will be in the form of written protocol deviations and will be filed with the raw data. All changes to the protocol will be addressed in the final report.

## 11.0 References

- Astwood, J. D., Leach, J. N., and Fuchs, R. L. (1996). Stability of food allergens to digestion *in vitro*. *Nature Biotechnology*, **14**: 1269-1273.
- Chua, N. H. and Schmidt, G. W. (1978). Post-translational transport to intact chloroplast of a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase. *Proc Natl Acad Sci USA* **75**: 6110-6114.
- Deutcher, M.P. (1990). Guide to Protein Purification, in: *Methods in Enzymology* 182. Academic Press, Inc., Harcourt Brace Jovanovich, Publishers, New York.
- Harrison, L. A. Bailey, M. R., Leimgruber, R. M., Smith, C. E., Nida, D. L., Taylor, M. L., Gustafson, M., Geeren, B. and Padgett, S. R. (1993). Characterization of Microbially-Expressed Protein: CP4 EPSPS. Monsanto Technical Report, St. Louis, MSL-12901.
- Highfield, P. E. and Ellis, R. J. (1978). Synthesis and transport of the small subunit of chloroplast ribulose bisphosphate carboxylase. *Nature* **271**: 420-424.
- Neuhoff, V., Norbert, A., Taube, D. and Wolfgang, E. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9**: 255-262.
- Oblong, J. E. and Lamppa, G. K. (1992). Identification of two structurally related proteins involved in proteolytic processing of precursors targeted to the chloroplast. *EMBO J.* **11**: 4401-4409.
- Schägger, H. and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry*, **166**: 368-379.

**Attachment 1: Abbreviations**

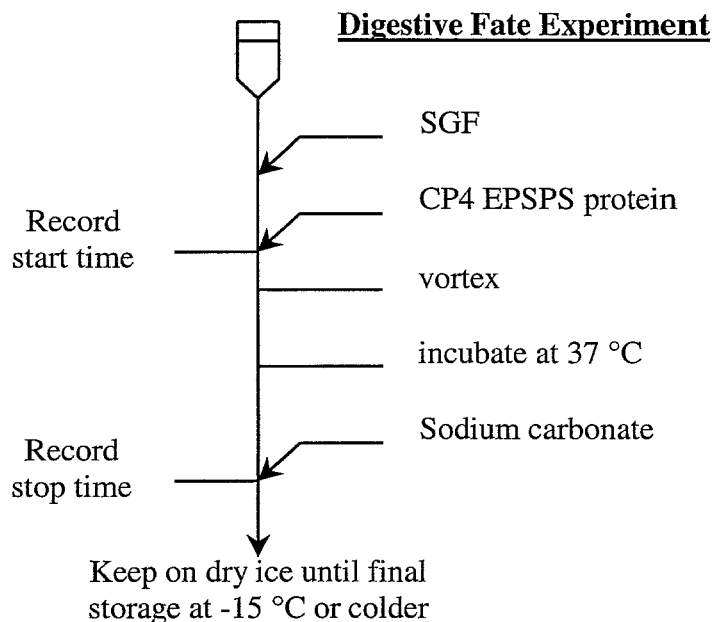
CFR	Code of Federal Regulations
EPSP	5- <i>enol</i> -pyruvyl-shikimate-3-phosphate
EPSPS	5- <i>enol</i> -pyruvyl-shikimate-3-phosphate synthase
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
GLP	Good Laboratory Practice
PAGE	Polyacrylamide gel electrophoresis
P/N	Product number, same as catalog number
purified water	Water prepared using a Milli-Q filter purification system
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecylsulfate
SOP	Standard Operating Procedure
SGF	Simulated gastric fluid
T	Time
TCA	Trichloroacetic acid
Tricine	N-tris[hydroxymethyl]methyl glycine
Tris	Tris(hydroxymethyl)aminomethane
w/o	Without

**Attachment 2: List of Applicable Method SOPs**

SOPs cited in this protocol will be used in this study unless superseded by newer versions. In this event, the actual SOPs followed in this study will be reflected in the final report.

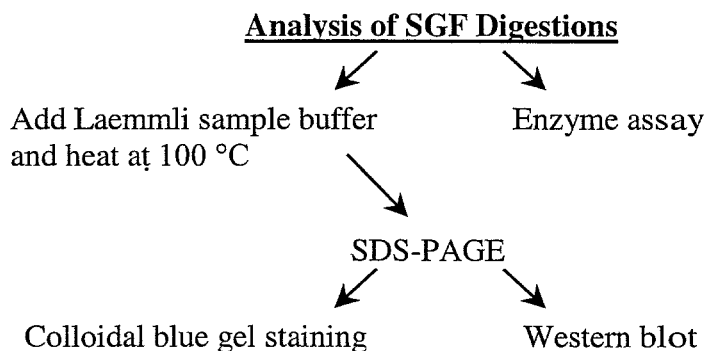
<b>SOP</b>	<b>Title</b>
BR-ME-0460-01	Assay for Pepsin Activity in Simulated Gastric Fluid
BtC-PRO-026-01	SDS Polyacrylamide Gel Electrophoresis (PAGE) using Pre-Cast Gels in Mini Gel Electrophoresis Apparatus
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)
GS-PRO-035-00	Phosphate Release Enzymatic Assay for the Quantitation of Functionally Active EPSP Synthase

**Attachment 3: Example Schematic of Experimental Procedure**



Experimental controls are prepared by adding protein buffer to SGF in place of the test substance. Additional experimental controls are prepared by adding the test substance to SGF lacking pepsin.

Pepsin activity assay is conducted on SGF before and after the digestive fate experiment.



**Protocol Deviation Form**

**Monsanto Study #:** 01-01-62-09

**Date(s) deviation occurred:** November 2001


**Page number(s) and section(s):** Section 4.1 on page 4 of the protocol

**Description of deviation:** The buffer used to store the test substance was described in the protocol as being 50 mM Tris-HCl, pH 7.5, 150 mM KCl, 2 mM DTT and 25% (v/v) glycerol. According to the certificate of analysis records, the test substance was actually stored in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT and 50% (v/v) glycerol

**Reason for deviation, how was deviation addressed and what impact will result from this deviation:**

When the protocol was written, it was unknown that researchers were using two different buffers to contain CP4 EPSPS protein. The buffer currently being used was entered into the protocol and was used in this study to represent the test substance without CP4 EPSPS protein. Since the two buffers are similar, no impact was expected or observed.

**Acknowledged By:**

  
\_\_\_\_\_  
John N. Leach  
Study Director

5-31-02  
\_\_\_\_\_  
Date