

**The effect corn grain matrix on the in-vitro digestibility of
CP4EPSPS and CP4EPSPS L214P proteins from the grain
of Roundup Ready ® Corn Event NK603 in simulated
gastric fluid**

**Study No.
MSL-18000**

Title

The effect of corn grain matrix on the *in vitro* digestibility of CP4 EPSPS and CP4 EPSPS L214P proteins from the grain of Roundup Ready[®] corn event NK603 in simulated gastric fluid

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An SOP was not in place for the spectrophotometer used in this study. However, since the researchers were familiar with the operation of this equipment and calibration records were maintained, no impact on the scientific integrity of this study is expected.

Characterization of purified protein lot 7069887 was not complete prior to use in this study. However, protein concentration data was available prior to conduct of the digestions and test substance purity data prior to loading SDS-PAGE gels. Identity analyses were not complete prior to use in this study.

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Quality Assurance Statement

Study Title: The effect of corn grain matrix on the *in vitro* digestibility of CP4 EPSPS and CP4 EPSPS L214P proteins from the grain of Roundup Ready[®] corn event NK603 in simulated gastric fluid

Study Number: 02-01-46-23

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.

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

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
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Study Certification

The results reported in the final report accurately reflect the data generated in the study.


Study Director _____ Date September 23, 2002

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Abbreviations

$A_{280\text{ nm}}$	Absorbance of light at a wavelength of 280 nm
CFR	Code of Federal Regulations
CP4 EPSPS	EPSPS from <i>Agrobacterium tumefaciens</i> ssp. Strain CP4
CP4 EPSPS L214P	CP4 EPSPS protein containing a leucine to proline change at amino acid position 214
ECL	Enhanced chemiluminescence
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FIR-RA	Federal Insecticide, Fungicide and Rodenticide Act
GLP	Good Laboratory Practice
HRP	Horseradish peroxidase
kDa	kilodalton
LOD	Limit of detection
mA	milliamperere
mM	millimolar
N0	Experimental control without CP4 EPSPS L214P protein at zero incubation time
N9	Experimental control without CP4 EPSPS L214P protein incubated for 60 minutes
NFDM	Nonfat dried milk
P0	Experimental control without pepsin at zero incubation time
P9	Experimental control without pepsin incubated for 60 minutes
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
P/N	Product number, same as catalog number
SDS	Sodium dodecyl sulfate
SGF	Simulated gastric fluid formulated with pepsin
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[®] corn event NK603 that is tolerant to glyphosate, the active ingredient in the Roundup[®] family of agricultural herbicides. Roundup Ready corn event NK603 contains two *cp4 epsps* coding sequences (Kesterson *et al.*, 2002). One CP4 EPSPS is the same as that found in Roundup Ready soybeans, canola, cotton, and sugar beet. The other CP4 EPSPS coding sequence has two nucleotide changes, one of which results in an amino acid substitution of proline for leucine at amino acid position 214 (L214P).

The purpose of this study was to assess the effect of corn grain matrix on the *in vitro* digestibility in simulated gastric fluid (SGF) of CP4 EPSPS and CP4 EPSPS L214P proteins from the grain of Roundup Ready corn event NK603. Two test substances were used in this study: i) a crude protein extract from plants containing both CP4 EPSPS and CP4 EPSPS L214P proteins (Lot No. 7083606B) prepared by extracting protein from the grain of corn containing event NK603 (LIMS No. 00ZMGRO02886, Lot No. SCP71A9C1T) at a ratio of 1 g tissue to 5 ml phosphate buffered saline (PBS) and ii) purified CP4 EPSPS and CP4 EPSPS L214P proteins (Lot No. 7069887) from the grain of corn event NK603. Digestibility was assessed by SDS-PAGE and western blot analysis. The matrix effect on the digestibility of CP4 EPSPS and CP4 EPSPS L214P proteins was determined by comparing the rate of digestion from using the crude test substance to the rate of digestion from using the purified test substance.

The results of this study demonstrated that the CP4 EPSPS and CP4 EPSPS L214P proteins present in both the crude and purified test substances were rapidly digested after incubation in the SGF digestibility assay. At least 98% of the CP4 EPSPS and CP4 EPSPS L214P proteins from these test substances were digested within 15 seconds, based on the sensitivity of the analytical methods employed. The western blot method's lower limit of detection was estimated to be 0.007-0.008 ng of the CP4 EPSPS and CP4 EPSPS L214P proteins. Since CP4 EPSPS and CP4 EPSPS L214P proteins from both test substances were at least 98% digested within the shortest time point used (15 seconds), no difference in the rate of digestion was observed in this study. Therefore, this study demonstrated that the extracted corn grain matrix had no effect on the *in vitro* digestibility in simulated gastric fluid of CP4 EPSPS and CP4 EPSPS L214P proteins from the grain of Roundup Ready corn event NK603.

2.0 Introduction

Monsanto Company has developed Roundup Ready corn event NK603, which is tolerant to glyphosate, the active ingredient in the Roundup family of agricultural herbicides, by insertion of the *cp4 epsps* coding sequence from *Agrobacterium ssp.* strain CP4 into corn.

Studies have shown that glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in the shikimate pathway (Franz *et al.*, 1997). The CP4 EPSPS protein is functionally similar to plant EPSPS enzymes but has a much reduced affinity for glyphosate (Padgett *et al.*, 1993). In plants, glyphosate binds to the plant EPSPS enzyme and blocks the biosynthesis of aromatic amino acids, thereby starving plants of these essential nutrients (Steinrücken and Amrhein, 1980; Haslam, 1993). In Roundup Ready plants, the nutritional requirements for growth and development are met by the continued action of the glyphosate-tolerant CP4 EPSPS enzyme in the presence of glyphosate (Padgett *et al.*, 1996). A comprehensive safety assessment of the CP4 EPSPS protein has been described in the literature (Harrison *et al.*, 1996).

The *cp4 epsps* gene from *Agrobacterium ssp.* strain CP4 has been sequenced and it encodes a 47.6 kDa CP4 EPSPS protein consisting of a single polypeptide of 455 amino acids (Padgett *et al.*, 1993). Roundup Ready corn event NK603 contains two *cp4 epsps* coding sequences. One CP4 EPSPS is the same as that found in Roundup Ready soybeans, canola, cotton, and sugar beet. The other CP4 EPSPS coding sequence has two nucleotide changes, one of which results in an amino acid substitution of proline for leucine at amino acid position 214 (L214P).

3.0 Purpose

The purpose of this study was to assess the effect of corn grain matrix on the *in vitro* digestibility in simulated gastric fluid of CP4 EPSPS and CP4 EPSPS L214P proteins.

4.0 Materials

4.1 Test Substances

The test substances for this study were: i) a crude extract containing CP4 EPSPS and CP4 EPSPS L214P proteins (Lot No. 7083606B) prepared by extracting protein from the grain of corn containing event NK603 (LIMS No. 00ZMGRO02886, produced from seed Lot No. SCP71A9C1T) at a ratio of 1 g tissue to 5 ml phosphate buffered saline (PBS) and ii) purified CP4 EPSPS and CP4 EPSPS L214P proteins (Lot No. 7069887) from the grain of corn event NK603. These test substances were stored in a -80 °C freezer until they were used in this study.

4.2 Control Substance

The control substance for this study was a crude extract of proteins from the grain of non-transgenic control line CRW0589(RX670). The non-transgenic control line had a similar genetic background to corn event NK603. The extract (Lot No. 7083606A) prepared from this grain was used to represent the corn matrix

extracted from Roundup Ready corn event NK603 minus the CP4 EPSPS and CP4 EPSPS L214P proteins. Similar to preparation of the crude test substance, proteins in the non-transgenic control grain were extracted from corn grain (LJMS No. 00ZMGRO02889, produced from seed Lot No. 1746LDRP) using PBS at a ratio of 1:5 (w/v). The control substance was stored in a -80 °C freezer until it was used in this study.

4.3 Reference Substance

There was no reference substance for this study.

Analytical reference standards used for this study were documented in the study file and are also described in Sections 4.4 and 7.4.

4.4 Characterization of Test and Control Substances

Grain from Roundup Ready corn event NK603 and its non-transgenic control line were produced according to production plan 00-01-46-18, where the identity of the test and control materials was confirmed by event specific PCR analysis. Certificates of analysis for the test and control corn grains were archived with this study.

Prior to this study, the total protein concentration for the control substance and the crude test substance was determined using a Bio-Rad dye binding method with bovine serum albumin as the standard (SOP GEN-PRO-015-00). The crude test substance had a total protein concentration of 1.34 mg/ml and the control substance had a total protein concentration of 1.50 mg/ml, which was diluted to 1.34 mg/ml prior to use in this study. The concentration of the combined CP4 EPSPS and CP4 EPSPS L214P proteins in the crude extract of Roundup Ready corn event NK603 was estimated by western blot analysis to be 0.09% of the total protein or 1.2 µg/ml.

The stability of the crude protein extracts was assessed using SDS-PAGE followed by colloidal blue gel staining. Molecular weight standards (Invitrogen P/N LC5677) were run on the gel to provide a relative scale for molecular weight mobility. Assessment of the stability was made by comparing the band pattern of proteins from aliquots used to conduct this study to the band pattern of proteins from aliquots freshly thawed from storage in a -80 °C freezer. No degradation of protein was observed (Figure 1). Controls within the experimental design, which demonstrate the stability of CP4 EPSPS and CP4 EPSPS L214P proteins in crude extract and throughout the experiment, are described in Sections 5.2 and 6.2, and are discussed in Section 8.1 of this report.

The CP4 EPSPS and CP4 EPSPS L214P proteins purified from corn event NK603 grain have been characterized (Lee *et al.*, 2002) to determine the identity, concentration, purity, composition, and activity. The assessed purity of the combined CP4 EPSPS and CP4 EPSPS L214P proteins was 77.5% of the total protein concentration of 2.12 mg/ml (based on amino acid composition analysis), which was diluted prior to use in this study to 1.34 mg/ml.

The stability of the CP4 EPSPS and CP4 EPSPS L214P proteins purified from corn event NK603 grain was also assessed concurrently with this study (Lee *et al.*, 2002). Controls within the experimental design of this study also demonstrated the stability of CP4 EPSPS and CP4 EPSPS L214P proteins from the purified test substance (Sections 5.2, 6.2, and 8.1).

4.5 Characterization of Analytical References and Study Materials

Certificates of analysis and product specification data for the molecular weight markers, bovine serum albumin, and pepsin are archived with the study data.

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 No. 99H7665) was used. SOP BR-ME-0460-01 describes the use of pepsin (Sigma P/N P-7000) that is only about 50-60% pure and contains fewer units of enzyme activity per mg of protein. 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 1 µg 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 0.001 increase in $A_{280\text{ nm}}$ at 37 °C, measured as trichloroacetic acid (TCA)-soluble products using hemoglobin as the substrate. The final SGF formulation contained 0.2% (w/v) sodium chloride adjusted with HCl to a pH of 1.2 and 0.2039 mg pepsin powder/ml SGF.

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 5.0 and 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 CP4 EPSPS and CP4 EPSPS L214P proteins from the test substances in the test system formulated without pepsin (SGF-p). 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 and CP4 EPSPS L214P proteins from either of the test substances, also for 0 and 60 minutes, and were designated with the letter "N". These experimental controls were prepared by substituting 50 mM MES, pH 5.9 for the purified test substance, and substituting the crude protein extract of the non-transgenic control substance for the crude protein extract of Roundup Ready corn event NK603.

5.3 Specimens

Digestive fate specimens were generated by incubating the test substances 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 until analysis and will be discarded approximately one year after the completion of the study.

6.0 Experimental Design

An overview of the experimental design is provided in the following sections. A schematic of the digestion experiment is shown in the protocol (see Appendix 1).

6.1 Digestibility of the Test Substances in SGF

Each test substance was used separately to prepare digestions of CP4 EPSPS and CP4 EPSPS L214P proteins in SGF. Digestions were conducted using 10 units of pepsin activity per 1 µg of total protein from the test substance. Digestions were incubated at 37 ± 2 °C in separate tubes for each of the incubation time points. SGF activity was quenched by addition of 0.2 M sodium carbonate to the test system. After quenching, samples were further diluted with Laemmli sample loading buffer and heated at 100 °C for 5 minutes. The samples were then frozen on dry ice and stored in a -20 °C freezer.

The zero time point digestions (T=0 and P0) were quenched by addition of 0.2 M sodium carbonate to the test system prior to addition of the test substance.

Ten incubation time points were generated for each of the test substances. They were 0, 15, 30 sec, and 1, 2, 4, 8, 15, 30, and 60 min. These time points are referred to as 0-9 in Figures 2-5 with time point 0 corresponding to time zero and time point 9 corresponding to 60 minutes.

6.2 Experimental Controls

For each of the test substances, experimental controls were prepared to characterize stability of the CP4 EPSPS and CP4 EPSPS L214P proteins in the test system formulated without pepsin (SGF-p). These experimental controls were prepared in a manner similar to that described in Section 6.1 of this report. The volumes used were the same as those used to prepare SGF digestions of the CP4 EPSPS and CP4 EPSPS L214P proteins. However, only incubation time points of 0 and 60 minutes (P0 and P9) were generated for these experimental controls.

Experimental controls were also prepared for each of the test substances to characterize the test system (SGF) incubated without the CP4 EPSPS and CP4 EPSPS L214P proteins for 0 and 60 minutes. These experimental controls were prepared by substituting 50 mM MES, pH 5.9 for the purified test substance, and substituting the crude protein extract of the non-transgenic control substance for the crude protein extract of Roundup Ready corn event NK603. These experimental controls were prepared in a manner similar to that described in Section 6.1 of this report. The volumes used were the same as those used to prepare SGF digestions of the CP4 EPSPS and CP4 EPSPS L214P proteins. However, only incubation time points of 0 and 60 minutes (N0 and N9) were generated for these experimental controls.

After quenching, samples were further diluted with Laemmli sample loading buffer and heated at 100 °C for 5 minutes. They were then temporarily stored on dry ice for a short period until stored in a -20 °C freezer pending analysis.

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 the CP4 EPSPS and CP4 EPSPS L214P proteins and experimental controls to account for any effects due to the CP4 EPSPS and CP4 EPSPS L214P proteins in SGF-p.

7.0 Analytical Methods

The digestibility of CP4 EPSPS and CP4 EPSPS L214P proteins in SGF was assessed using SDS-PAGE and western blot analysis. Lower limits of detection (LOD) specific to both of the test substances in SGF were determined for the western blot detection

method. In this method of analysis, CP4 EPSPS and CP4 EPSPS L214P proteins are not resolved into separate bands.

7.1 Digestive Fluid Activity Assay

The activity of SGF was assessed before and after the SGF digestion assay by following SOP BR-ME-0460-01, with the exception that prior to conducting these activity assays, SGF was diluted to 0.19 \times . Since SGF was prepared with a different formulation than described by this SOP, this modification was necessary to dilute SGF so that it would fall within the working range of the activity assay.

Assessment of the SGF activity before and after the SGF digestion assay confirmed that the test system was stable and appropriate for the period of use in this study. The activity values for the before and after assessments were 7383 and 7000 units/ml, respectively (one unit is defined as a 0.001 increase in $A_{250\text{ nm}}$ per minute at 37 °C). As expected, these values are approximately one fourth of the acceptance values described in the SOP.

7.2 SDS-PAGE

Samples from the *in vitro* SGF digestions were analyzed by SDS-PAGE using pre-cast 10-20% polyacrylamide gradient in tricine buffered mini-gels (NOVEX, P/N EC66255) as described in SOP BR-ME-0388-01 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 8.3 and the lower buffer reservoir was filled with 200 mM Tris, pH 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). These gels were run at 50 mA per gel until the phenol red dye-front in the molecular weight standards was near the bottom edge of the gel.

7.3 Colloidal Blue Gel Staining

SDS-PAGE and colloidal blue gel staining were used to assess the stability of the PBS extracts used as the test and control substances in this study. Aliquots of these extracts that were thawed for the first time, during the experimental phase of this study and after the experimental phase of this study, were examined for protein degradation.

After separation of proteins by SDS-PAGE (section 7.2), the gel was incubated in fix solution [40% (v/v) methanol, 7% (v/v) acetic acid] for approximately 1 hour at room temperature. The gel was then stained overnight with reformulated Brilliant Blue G Colloidal dye (Sigma P/N B-2025) diluted 4:1 with methanol. Background from excess dye was removed by washing the gel in destaining

solution [10% (v/v) acetic acid, 25% (v/v) methanol] for 1 minute and then in 25% (v/v) methanol for several hours.

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 proteins were separated by SDS-PAGE (Section 7.2), they were electroblotted onto 0.45 µm pore size PVDF membrane (Sigma P/N P-2813) at approximately 300 mA constant current for 1-1.4 hours 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 (NFDN). The CP4 EPSPS and CP4 EPSPS L214P proteins were detected using a sandwich probe methodology. First, for specific binding to the CP4 EPSPS and CP4 EPSPS L214P proteins, 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) NFDN. After incubation for 1.5-2 hours at room temperature, excess goat antibody was removed by three washes in PBST for 5-15 minutes each. Biotinylated protein G (Pierce P/N 29988) at a 1:2000 dilution in 20 ml/blot of PBST that contained 1% (w/v) NFDN was used to bind to the goat antibody already bound to the test protein on the blot. 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 the bound protein G, NeutrAvidin™¹ 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) NFDN. After incubation for 1-1.3 hour 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™² (Amersham P/N RPN 3114K) were used for detection of the CP4 EPSPS and CP4 EPSPS L214P proteins.

A lower limit of detection for this method specific to the CP4 EPSPS and CP4 EPSPS L214P proteins was determined by loading diluted samples of the zero incubation time point onto a gel that was run concurrently with the blot used to assess digestibility.

Molecular weight standards (Invitrogen P/N LC5677) were used to assess transfer of proteins from the SDS-PAGE gel onto 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

¹ NeutrAvidin™ is a trademark of Pierce

² Hyperfilm™ is a trademark of Amersham.

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 2-5, 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 Statistical Methods

No statistical analysis was performed.

8.0 Results and Discussion

8.1 Assessment of Digestibility by Western Blot Analysis

Digestibility of the CP4 EPSPS and CP4 EPSPS L214P proteins was evaluated by western blot analysis, as described in Section 7.4 of this report. Loadings of the combined CP4 EPSPS and CP4 EPSPS L214P proteins from each test substance were based on purity and pre-digestion estimates. The loading of combined CP4 EPSPS and CP4 EPSPS L214P proteins from the purified test substance was 0.4 ng per lane, while the loading of combined CP4 EPSPS and CP4 EPSPS L214P proteins from the crude test substance was 0.34 ng per lane. Although the CP4 EPSPS and CP4 EPSPS L214P proteins from the crude test substance were loaded at a lower level than those from the purified test substance, the western blot signal was greater than that from the purified test substance. An enhanced western blot signal has been commonly observed for proteins detected in crude corn extracts, and is believed to be a matrix effect on the western blot detection method. The CP4 EPSPS and CP4 EPSPS L214P proteins from both test substances became undetectable by the western blot method after 15 seconds in the SGF digestibility assay (Figures 2 and 4, lane 5).

Resolution between the CP4 EPSPS and CP4 EPSPS L214P proteins is not possible by the SDS-PAGE gels used in this study. However, two bands can be observed in lanes containing CP4 EPSPS and CP4 EPSPS L214P proteins. The fainter lower band in figures 2-5 is possibly due to degradation of the CP4 EPSPS and CP4 EPSPS L214P proteins prior to digestion.

Experimental controls (P0 and P9) were prepared by incubating CP4 EPSPS and CP4 EPSPS L214P proteins in SGF-p (formulated without pepsin), demonstrating that the loss of immuno-detectability for CP4 EPSPS and CP4 EPSPS L214P proteins was due to digestion by SGF and not due to instability of the test proteins in a pH 1.2 solution at 37 °C. This was determined by visually comparing the

relative band intensities of the CP4 EPSPS and CP4 EPSPS L214P proteins in lanes 2 and 15 of Figures 2 and 4.

Likewise, experimental controls (N0 and N9) prepared by substituting 50 mM MES, pH 5.9 for the purified test substance and by substituting the extract of non-transgenic corn for the crude test substance, demonstrated a lack of detectable background from other components of the test system or corn extract (Figures 2 and 4, lanes 3 and 14).

To determine a lower limit of detection specific to the test substance in SGF, a LOD western blot was concurrently run with each western blot for the digestibility assessment of the test protein. The LOD was determined by loading various dilutions of the zero incubation time point of CP4 EPSPS and CP4 EPSPS L214P proteins in SGF (T=0), and observing which of the individual dilutions could be detected (Figure 3 and Figure 5). The LOD was estimated for both test substances to be 1/50th of the zero incubation time point loading, which is representative of the CP4 EPSPS and CP4 EPSPS L214P proteins level at 98% digestion. Since 0.4 ng of the purified CP4 EPSPS and CP4 EPSPS L214P proteins (before digestion) was loaded per lane, the lower limit of detection was approximately 0.008 ng of the undigested purified CP4 EPSPS and CP4 EPSPS L214P proteins. Likewise, since 0.34 ng of CP4 EPSPS and CP4 EPSPS L214P proteins from the crude test substance (before digestion) was loaded per lane, the lower limit of detection was approximately 0.007 ng of the undigested CP4 EPSPS and CP4 EPSPS L214P proteins.

8.2 Rejected Data

There were two instances where data for the purified test substance was rejected due to the quality of the results. Rejected data have been archived in the study file.

9.0 Conclusions

The results of this study demonstrated that the CP4 EPSPS and CP4 EPSPS L214P proteins present in both the crude and purified test substances were rapidly digested after incubation in the SGF digestibility assay. At least 98% of the CP4 EPSPS and CP4 EPSPS L214P proteins from these test substances were digested within 15 seconds, based on the sensitivity of the analytical methods employed. The western blot method's lower limit of detection was estimated to be 0.007-0.008 ng of the CP4 EPSPS and CP4 EPSPS L214P proteins. Since CP4 EPSPS and CP4 EPSPS L214P proteins from both test substances were at least 98% digested within the shortest time point used (15 seconds), no difference in the rate of digestion was observed in this study. Therefore, this study demonstrated that the extracted corn grain matrix had no effect on the *in vitro*

digestibility of CP4 EPSPS and CP4 EPSPS L214P proteins from the grain of Roundup Ready corn event NK603 in simulated gastric fluid.

10.0 References

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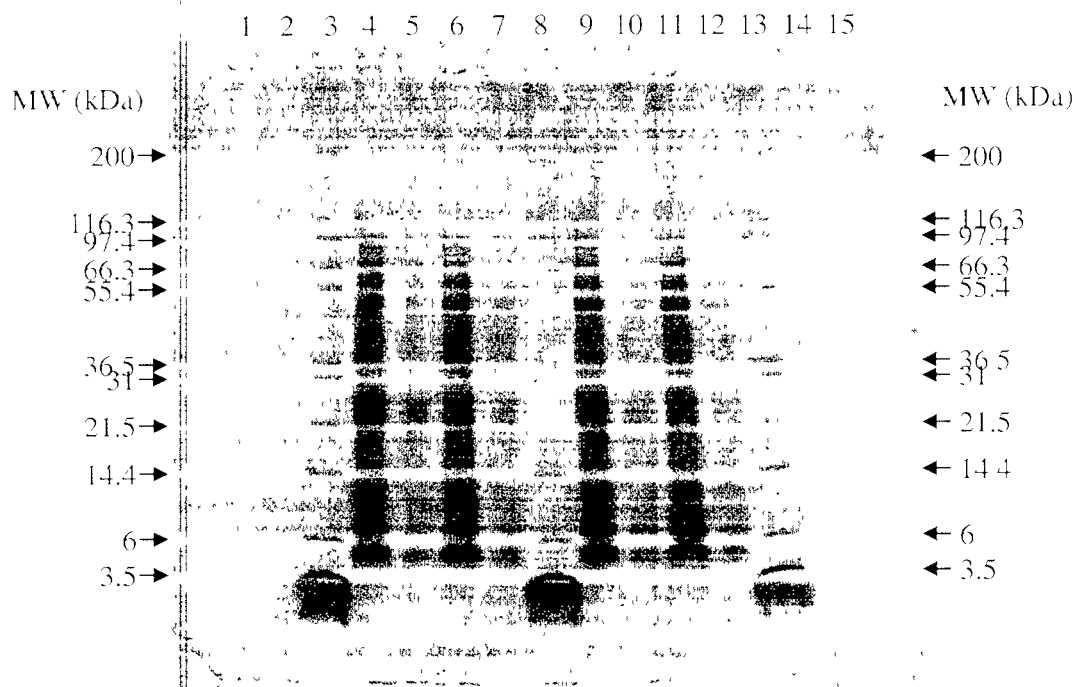


Figure 1. Colloidal Blue Stained Gel Showing the Stability of Matrix Proteins Extracted from the Grain of Roundup Ready Corn Event NK603 and Its Non-Transgenic Parental Control Line. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Note: The figure above may not be the actual size of the original dried gel.

<u>Lane</u>	<u>Description</u>	<u>Total Protein Loading</u>
1	Empty	
2	Empty	
3	Molecular weight markers (0.5 µg protein/band)	
4	Freshly thawed aliquot of crude test substance (NK603)	5 µg
5	Freshly thawed aliquot of crude test substance (NK603)	1 µg
6	Freshly thawed aliquot of control substance (non-transgenic)	5 µg
7	Freshly thawed aliquot of control substance (non-transgenic)	1 µg
8	Molecular weight markers (0.5 µg protein/band)	
9	Crude test substance aliquot that was used in study (NK603)	5 µg
10	Crude test substance aliquot that was used in study (NK603)	1 µg
11	Control substance aliquot that was used in study (non-transgenic)	5 µg
12	Control substance aliquot that was used in study (non-transgenic)	1 µg
13	Molecular weight markers (0.5 µg protein/band)	
14	Empty	
15	Empty	

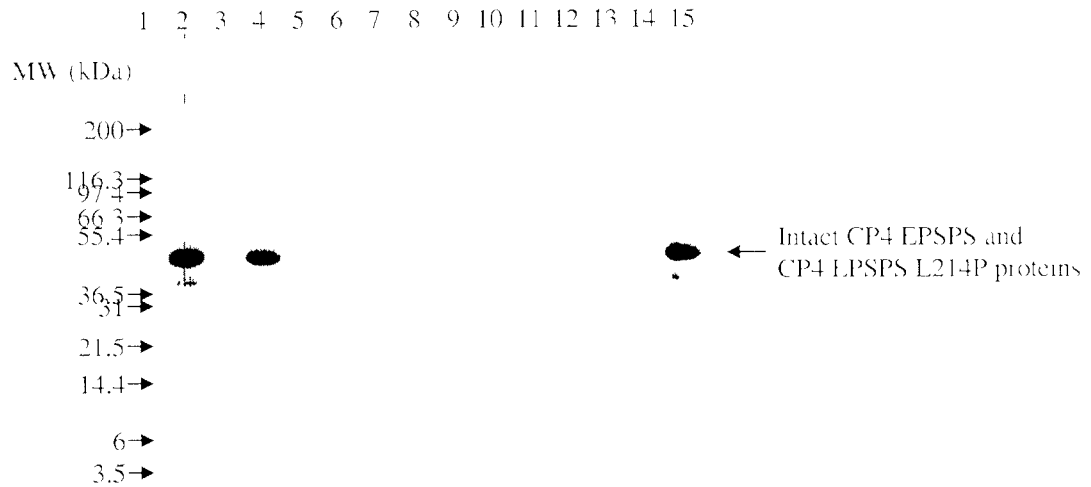


Figure 2. Western Blot Showing CP4 EPSPS and CP4 EPSPS L214P Proteins in a Crude Extract of Roundup Ready Corn Event NK603 Grain After Digestion by Simulated Gastric Fluid. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by ECL, where the image above is a ten-minute film exposure. CP4 EPSPS and CP4 EPSPS L214P proteins were loaded at 0.34 ng per lane. Note: The figure above may not be the actual size of the original film image.

<u>Lane</u>	<u>Description</u>	<u>Incubation time</u>
1	Molecular weight markers (1.5 µg protein/band)	
2	Experimental control, crude test substance in SGF-p (P0)	0 sec
3	Experimental control, non-transgenic corn extract in SGF (N0)	0 sec
4	Crude test substance in SGF, T = 0	0 sec
5	Crude test substance in SGF, T = 1	15 sec
6	Crude test substance in SGF, T = 2	30 sec
7	Crude test substance in SGF, T = 3	1 min
8	Crude test substance in SGF, T = 4	2 min
9	Crude test substance in SGF, T = 5	4 min
10	Crude test substance in SGF, T = 6	8 min
11	Crude test substance in SGF, T = 7	15 min
12	Crude test substance in SGF, T = 8	30 min
13	Crude test substance in SGF, T = 9	60 min
14	Experimental control, non-transgenic corn extract in SGF (N9)	60 min
15	Experimental control, crude test substance in SGF-p (P9)	60 min

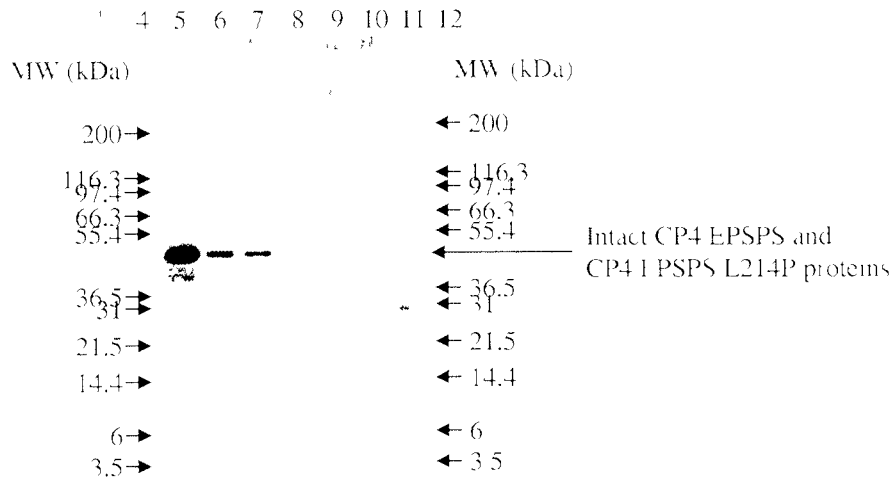


Figure 3. Western Blot Showing the Limit of Detection Specific to CP4 EPSPS and CP4 EPSPS L214P Proteins in a Crude Extract of Roundup Ready Corn Event NK603 Grain in Simulated Gastric Fluid. Proteins were separated by SDS-PAGE using a 10–20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by ECL, where the image above is a ten-minute film exposure. Note: The figure above may not be the actual size of the original film image. Lanes 1-3 and 13-15 were empty and are not shown in the figure.

Lane	Description	Amount of CP4 EPSPS and CP4 EPSPS L214P proteins
4	Molecular weight markers (1.5 µg protein/band)	
5	Experimental control, crude test substance in SGF-p (P0)	0.34 ng
6	Crude test substance in SGF, T = 0 (diluted 3/10)	0.10 ng
7	Crude test substance in SGF, T = 0 (diluted 1/5)	0.07 ng
8	Crude test substance in SGF, T = 0 (diluted 1/10)	0.03 ng
9	Crude test substance in SGF, T = 0 (diluted 1/20)	0.02 ng
10	Crude test substance in SGF, T = 0 (diluted 1/50)	0.007 ng
11	Molecular weight markers (1.5 µg protein/band)	
12	Molecular weight markers (1.5 µg protein/band)	

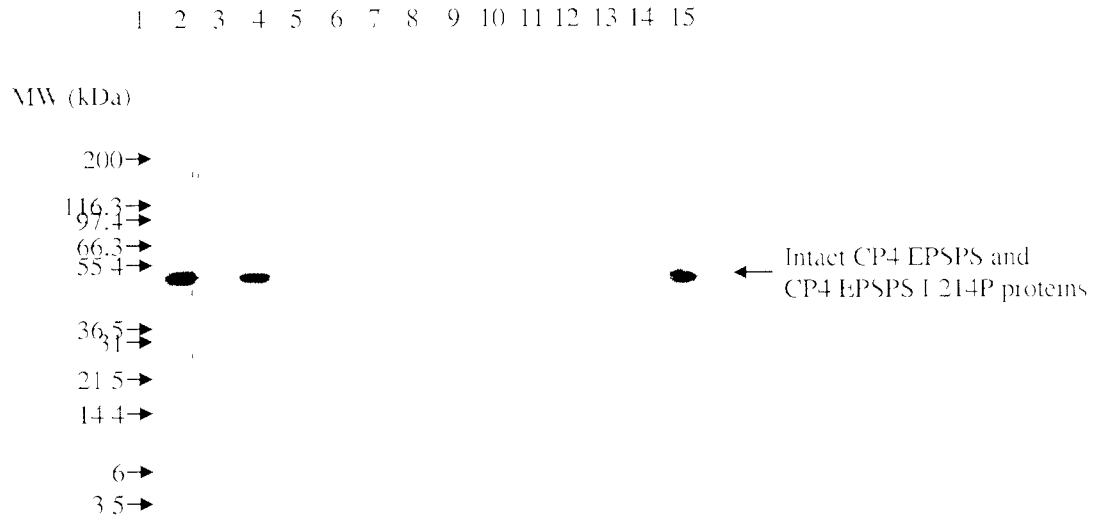


Figure 4. Western Blot Showing Purified CP4 EPSPS and CP4 EPSPS L214P Proteins from Roundup Ready Corn Event NK603 Grain After Digestion by Simulated Gastric Fluid. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by ECL, where the image above is a five-minute film exposure. The CP4 EPSPS and CP4 EPSPS L214P proteins were loaded at 0.40 ng per lane. Note: The figure above may not be the actual size of the original film image.

Lane	Description	Incubation time
1	Molecular weight markers (1.5 µg protein/band)	
2	Experimental control, purified test substance in SGF-p (P0)	0 sec
3	Experimental control, 50 mM MES, pH 5.9 in SGF (N0)	0 sec
4	Purified test substance in SGF, T = 0	0 sec
5	Purified test substance in SGF, T = 1	15 sec
6	Purified test substance in SGF, T = 2	30 sec
7	Purified test substance in SGF, T = 3	1 min
8	Purified test substance in SGF, T = 4	2 min
9	Purified test substance in SGF, T = 5	4 min
10	Purified test substance in SGF, T = 6	8 min
11	Purified test substance in SGF, T = 7	15 min
12	Purified test substance in SGF, T = 8	30 min
13	Purified test substance in SGF, T = 9	60 min
14	Experimental control, 50 mM MES, pH 5.9 in SGF (N9)	60 min
15	Experimental control, purified test substance in SGF-p (P9)	60 min

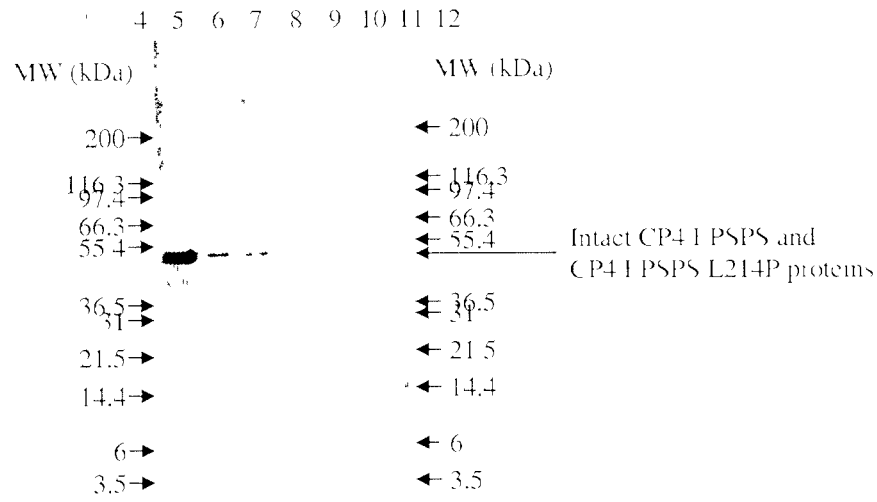


Figure 5. Western Blot Showing the Limit of Detection Specific to Purified CP4 EPSPS and CP4 EPSPS L214P Proteins from Roundup Ready Corn Event NK603 Event in Simulated Gastric Fluid. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by ECL, where the image above is a five-minute film exposure. Note: The figure above may not be the actual size of the original film image. Lanes 1-3 and 13-15 were empty and are not shown on the figure.

Lane	Description	Amount of CP4 EPSPS and CP4 EPSPS L214P proteins
4	Molecular weight markers (1.5 µg protein/band)	
5	Experimental control, purified test substance in SGF-p (P0)	0.40 ng
6	Purified test substance in SGF, T = 0 (diluted 3/10)	0.12 ng
7	Purified test substance in SGF, T = 0 (diluted 1/5)	0.08 ng
8	Purified test substance in SGF, T = 0 (diluted 1/10)	0.04 ng
9	Purified test substance in SGF, T = 0 (diluted 1/20)	0.02 ng
10	Purified test substance in SGF, T = 0 (diluted 1/50)	0.008 ng
11	Molecular weight markers (1.5 µg protein/band)	
12	Molecular weight markers (1.5 µg protein/band)	

Appendix 1.

Protocol and Protocol Deviations

Protocol is attached as Pages 29 - 42

Protocol deviations from

Method used to confirm the identity of the test and control substances

Lot number of test substance used to prepare a crude protein extract

Method used to destain SDS-PAGE gels stained with colloidal blue stain

and

Method used to assay SGF activity

are attached as Pages 43 - 46

Monsanto Study #: 02-01-46-23

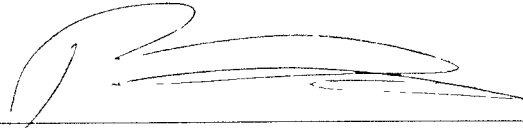
Study Title: The effect of corn grain matrix on the *in vitro* digestibility of CP4 EPSPS proteins present in the grain of Roundup Ready³ corn event NK603 in simulated gastric fluid

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

Primary Testing Facility: Monsanto Company
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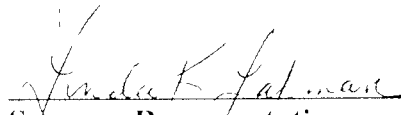
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Product Safety Center
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St. Louis, MO 63167

Approved By:



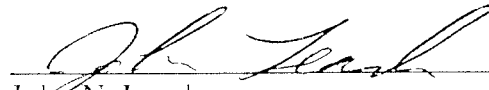
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Apr 17, 2002
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Apr 18, 2002
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April 18, 2002
Date



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April 17, 2002
Date

Reviewed By:



Quality Assurance Specialist
Monsanto Company
Monsanto Regulatory

7 April 2002
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 effect of corn grain matrix on the *in vitro* digestibility of CP4 EPSPS proteins in simulated gastric fluid. Effects on the rate of digestion will be determined by comparing the results from using purified CP4 EPSPS proteins to the results from using CP4 EPSPS proteins present in a crude protein extract from grain of corn event NK603.

3.0 Timelines

3.1 Proposed Experimental Start Date: April 2002

3.2 Proposed Experimental Termination Date: April 2002

4.0 Test, Control and Reference Substances

4.1 Test Substances

The test substances for this study are: 1) purified CP4 EPSPS proteins from the grain of corn event NK603 and 2) a crude protein extract from the grain of corn containing event NK603.

CP4 EPSPS proteins (Lot no. 7069887) were purified from the grain of corn event NK603 using chromatography methods (Lee *et al.*, 2002). The total protein concentration and purity was evaluated prior to this study (Lee *et al.*, 2002). This test substance will be stored in a -20 °C or -80 °C freezer, until its use in this study.

A crude protein extract (Lot No. 7083606B) containing CP4 EPSPS proteins was made from the grain of corn event NK603 (LIMS No. 00ZMGRO02886, Lot No. SCP71A9CIT) by extracting with PBS at a ratio of 1 g tissue: 5 ml buffer. The total protein concentration and purity was evaluated prior to this study. This test substance will be stored in a -80 °C freezer until its use in this study.

4.2 Control Substance

Proteins extracted from the non-transgenic control line with similar genetic background to grain containing the NK603 event, will be used as the control substance in this study. This extract (Lot No. 7083606A) will be used to represent the corn matrix extracted from Roundup Ready corn

event NK603 minus the CP4 EPSPS proteins. Proteins in the non-transgenic control line were extracted from corn grain (LIMS No. 00ZMGRO02889, Lot No. 1746LDRP) at a ratio of 1:5 in PBS. The total protein concentration was evaluated prior to this study. This control substance will be stored in a -80 °C freezer until use in this study.

4.3 Reference Substance

There is no reference substance for this study.

If used in this study, analytical reference standards will be documented and will be described in the final report. Reference standards will include, but may not be limited to, molecular weight markers.

4.4 Characterization of Test, Control and Reference Substances

Roundup Ready corn event NK603 and its non-transgenic control line, were produced according to production plan 00-01-46-18, where the test and control materials were identified by event-specific Southern blot analysis. Certificates of analysis for the test and control corn grains will be archived with this study.

Prior to this study, the total protein concentration and concentration of CP4 EPSPS proteins was determined for the crude extracts of test corn event NK603 and the control corn line. Copies of this data will be archived with this study. Any further preparation of these substances before use in this study will be documented and discussed in the final report.

The stability of the crude protein extracts will be assessed using SDS-PAGE followed by colloidal blue gel staining. Assessment of the stability will be made by comparing the band patterns of aliquots used to conduct this study to aliquots freshly thawed from storage in a -80 °C freezer.

Characterization of the CP4 EPSPS proteins purified from corn event NK603 will be conducted concurrently with this study (Lee *et al.*, 2002) to assess the identity, concentration, purity, composition, and activity.

Stability of the CP4 EPSPS proteins purified from corn event NK603 will also be assessed concurrently with this study (Lee *et al.*, 2002).

When available, certificates of analysis confirming the characterization of analytical 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 of pepsin activity (based on the activity value provided by Sigma Company on the reagent label) per 1 µg 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 exceptions described in section 7.1.

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 substances in the test system (SGF) lacking pepsin for the duration of the longest digestion time. These experimental controls will be identified 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 identified 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 6.0 through 7.5 for details on the preparation and analysis of specimens. Specimens will be retained for approximately one year, after which they 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

Two sets of digestions will be prepared to generate an SGF digestion time course for each of the test substances. For each set of digestions, two sets of experimental controls will be prepared as described in section 6.2.

Digestions will be prepared by adding 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 ± 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 pre-study experiments to be an appropriate method for 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

For each set of digestions, experimental controls will be prepared to determine the stability of the test substance within the test system (SGF) lacking pepsin. These experimental controls will be prepared in a similar manner as described above in section 6.1, but the targeted incubation times will be limited to 0 and 60 minutes. Additional time points will be used if necessary.

Experimental controls will also be prepared for each set of digestions to represent the test system (SGF) lacking either of the test substances. For assessment of the digestibility of purified CP4 EPSPS proteins in SGF, these controls will be prepared by adding the protein storage buffer to SGF in place of the purified CP4 EPSPS proteins. For assessment of the digestibility of CP4 EPSPS proteins in corn matrix, a crude extract of the control line will be added to SGF in place of the crude extract of corn event NK603. These experimental controls will be prepared in a similar manner as described above in section 6.1, but the targeted incubation times

will be limited to 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 CP4 EPSPS proteins in SGF will be assessed using SDS-PAGE and Western blot analysis. Lower limits of detection specific to both test substances will be determined for the Western blot method.

7.1 Digestive fluid activity assays

The activity of SGF will be confirmed according to SOP No. BR-ME-0460-01, with the exception that before conducting the assay, SGF may 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 10,000 units/mL.

SGF activity will be assessed before and after preparing digestions, to demonstrate the appropriateness and stability of the test system in this study.

7.2 SDS-PAGE

Proteins from the SGF *in vitro* digestion of CP4 EPSPS proteins will be separated by SDS-PAGE using pre-cast 10-20% tricine mini-gels (NOVEX, P/N EC66255). This procedure is described in SOP No. BR-ME-0388-01 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 ≈ 8.25 and the lower buffer reservoir will contain 200 mM Tris, pH ≈ 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, the gel loadings for analysis of both test substances will be adjusted to an equal amount of CP4 EPSPS proteins.

Experimental controls will be loaded with the same volumes used to load the corresponding digestion trials.

7.3 Colloidal blue staining

Stability of the aliquots of the corn grain extracts used to generate the digestion time course of CP4 EPSPS proteins in matrix and the experimental controls containing the non-transgenic corn line, will be assessed by SDS-PAGE described in SOP No. BR-ME-0388-01 followed by colloidal blue gel staining. Stability of these extracts will be assessed by comparing the banding patterns of the aliquots used in this study to aliquots thawed for the first time from storage in a -80 °C freezer.

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.

7.4 Western blot analysis

Samples from both sets of SGF *in vitro* digestions will be analyzed by Western blot according to SOP No. GEN-PRO-002-03.

A lower limit of detection for the western blot method specific to each test substance will be demonstrated by a western blot loaded with dilutions of the zero incubation time point (T=0) from each set of digestions.

7.5 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 inclusion of appropriate controls to account for any effects due to the model in the absence of test substance.

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

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- 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

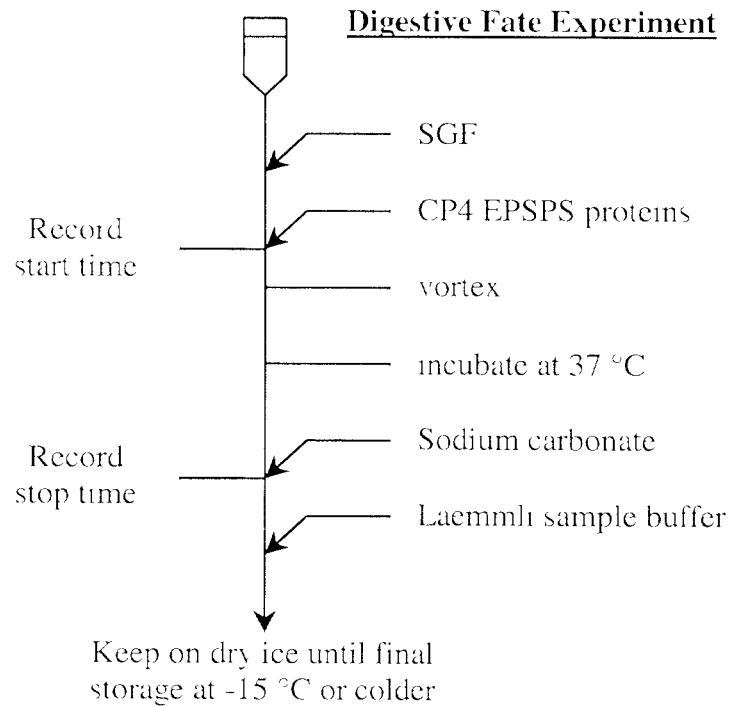
CFR	Code of Federal Regulations
EPSP	5- <i>enol</i> -pyruvyl-shikimate-3-phosphate
EPSPS	5- <i>enol</i> -pyruvyl-shikimate-3-phosphate synthase
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
GLP	Good Laboratory Practice
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
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.

SOP	Title
BR-ME-0460-01	Assay for Pepsin Activity in Simulated Gastric Fluid
BR-ME-0388-01	SDS Polyacrylamide Gel Electrophoresis (PAGE) using Pre-Cast Gels in Mini Gel Electrophoresis Apparatus
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)

Attachment 3: Example Schematic of Experimental Procedure



Experimental controls are prepared by adding protein buffer or an extract of the non-transgenic control line to SGF in place of the test substances. Additional experimental controls are prepared by adding the test substances to SGF lacking pepsin.

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

Protocol Deviation Form

Monsanto Study #: 02-01-46-23

Date(s) deviation occurred: April 18, 2002

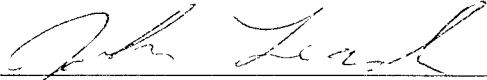
Page number(s) and section(s): Page 5 of 14 section 4.4

Description of deviation: The protocol says that the identification of the test and control materials had been done by event-specific Southern blot analysis. However, the certificates of analysis show that identification of these materials was done by event-specific PCR analysis.

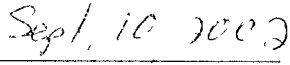
Reason for deviation, how was deviation addressed and what impact will result from this deviation:

This deviation was the result of miscommunication during the writing phase of the protocol. Since the identification of the test and control materials was confirmed by either method prior to beginning this study, there was no impact on the scientific integrity of this study.

Acknowledged By:



John N. Leach
Study Director



Date

Protocol Deviation Form

Monsanto Study #: 02-01-46-23

Date(s) deviation occurred: April 18, 2002

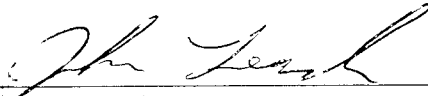
Page number(s) and section(s): Page 4 of 14, section 4.1

Description of deviation: The protocol says that a crude protein extract containing CP4 EPSPS proteins was made from the grain of corn event NK603 (LIMS No. 00ZMGRO02886, Lot No. SCP71APCIT).... The actual Lot No. was SCP71APCIT.

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

The Lot No. recorded in the protocol was the same Lot No. recorded on the certificate of analysis that was issued prior to beginning this study, however, it was later discovered that the certificate of analysis contained an error in the Lot No. A new certificate of analysis was issued and added to the study. There was no scientific impact on this study.

Acknowledged By:



John N. Leach
Study Director

Sept. 17, 2002
Date

Protocol Deviation Form

Monsanto Study #: 02-01-46-23

Date(s) deviation occurred: May 16, 2002

Page number(s) and section(s): Page 9 of 14, section 7.3 of the protocol

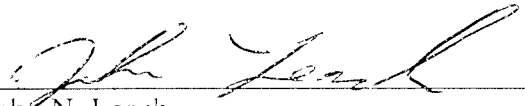
Description of deviation: The SDS-PAGE colloidal blue stained gel was washed in destaining solution for 1 minute prior to washing in 25% (v/v) methanol to remove excess background. This is an additional step not described in section 7.3 of the protocol.

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

The use of destain solution to wash the colloidal blue stained gel prior to washing the gel in 25% (v/v) methanol, was described in the draft SOP for staining gels with colloidal brilliant blue G stain. The destaining solution is commonly used in this technique to rapidly remove most of the excess stain from the gel.

The use of destain solution in this study was the result of confusing the protocol requirements with the draft SOP requirements, and may also be viewed as an oversight to include this step in section 7.3 of the protocol. It's use is not necessary nor does it alter the final results, so there was no impact on the study.

Acknowledged By:



John N. Leach
Study Director

Sept. 04, 2002
Date

Protocol Deviation Form

Monsanto Study #: 02-01-46-23

Date(s) deviation occurred: September 19, 2002

Page number(s) and section(s): Page 8 of 14 section 7.1

Description of deviation: The protocol says "...all calculations will be conducted as if 0.05X SGF had been used in the assay", and "Acceptable activity will be defined as an activity greater than 10,000 units/mL."


It was later decided that a better way to present the data, would be to calculate the final values based on the actual dilution of SGF used in the assay (0.19X), and to change to acceptable activity value to greater than 4211 units/mL. A discussion about how these values compare to the SOP acceptable range would be added to the report.

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

Modifications to the procedure described in SOP BR-ME-0460-01 were necessary to accommodate a formulation of SGF that was different from that described in the SOP. The protocol was written to describe modifications that would meet those accommodations. However, confusions that arose from the approach described in the protocol lead to a revisit of alternative accommodations that would be less confusing. These changes were made to provide a more straightforward approach to accommodating the formulation of SGF used in this study.

The impact on this study is expected to be a greater understanding of why modifications to the SOP were necessary, and how these modifications accommodate the use of SGF used in this study.

Acknowledged By:



John N. Leach
Study Director

Sept 20, 2002

Date