Study Title

Assessment of the *in vitro* Digestibility of the Dicamba Mono-Oxygenase (DMO) Enzyme in Simulated Gastric and Simulated Intestinal Fluids

Authors

Jamie J. Burge, B.S., Luis A. Burzio, Ph. D., and John J. Finnessy, M.S.

Report Completed On

March 25, 2010

Sponsor

Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, MO 63167
Sponsor Representative: Global Regulatory Pipeline, Agronomic Traits Team
Primary Contact: Melinda McCann
Phone: (314) 694-7556

Performing Laboratory

Monsanto Company
Protein Sciences and Safety Platform
800 North Lindbergh Boulevard
St. Louis, Missouri 63167

Laboratory Project ID

Monsanto Study Number: REG-09-512
MSL: 0022502

The text below applies only to use of the data by the United States Environmental Protection Agency (U.S. EPA) in connection with the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

The inclusion of this page in all studies is for quality assurance purposes and does not necessarily indicate that this study has been submitted to the U.S. EPA

Statement of No Data Confidentiality Claims

No claim of data confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10 (d) (1) (A), (B) or (C).

We submit this material to the U.S. EPA specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by the EPA strictly in accordance with FIFRA. By submitting this material to EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by the company notwithstanding this submission to EPA.

Company: Monsanto Company

Company Agent: _____

Title: _____

Signature: _____ Date: _____

Statement of Compliance

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

Submitter — Date

Melinda C. McCann — 25 Mar 2010

Melinda McCann
Sponsor Representative — Date

Jamie J. Burge — 3/25/10

Jamie J. Burge
Study Director — Date

Quality Assurance Unit Statement

Study Title: Assessment of the *in vitro* Digestibility of the Dicamba Mono-Oxygenase (DMO) Enzyme in Simulated Gastric and Simulated Intestinal Fluids

Study Number: REG-09-512

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

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

Dates of Inspection / Audit	Phase	Date Reported To:	
		Study Director	Management
1/4/2010	Digestive Fate	1/4/2010	1/4/2010
2/1/2010	Raw Data Audit	2/8/2010	2/8/2010
3/10/2010	Draft Report Audit	3/10/2010	3/10/2010

Michael Finkes

3/25/2010

Michael Finkes
Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

Date

Study Certification Page

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

Signatures of Final Report Approval:

Jamie J. Burge
Study Director

3/25/10
Date

John J. Finnessy, M.S.
Protein Sciences and Safety Team Lead

3/25/2010
Date

Study Information

Study Number:	REG-09-512
MSL Number:	0022502
Title:	Assessment of the *in vitro* Digestibility of the Dicamba Mono-Oxygenase (DMO) Enzyme in Simulated Gastric and Simulated Intestinal Fluids.
Testing Facility:	Monsanto Company Protein Sciences and Safety Platform 800 North Lindbergh Boulevard St. Louis, Missouri 63167
Team Lead:	John J. Finnessy, M.S.
Study Director:	Jamie J. Burge
Contributors:	Richard S. Thoma
Study Initiation Date:	December 11, 2009
Study Completion Date:	March 25, 2010
Specimen Retention:	Specimens will be retained at Monsanto Company, St. Louis, as specified in Section 7.0
Records Retention:	All study specific raw data, electronic files, study protocol, amendments, and final report will be retained at Monsanto Company, St. Louis.

Table of Contents

List of Figures

List of Appendices

Abbreviations and Definitions[1]

BLAST	Basic local alignment search tool
CFR	Code of Federal Regulations
DF	Dilution factor
DMO	Dicamba mono-oxygenase protein
DMO+27	DMO protein plus 27 amino acids originating from the pea Rubisco small subunit on the N-terminus
DMO enzyme	Trimer containing DMO and DMO+27
DMO proteins	Both forms of the proteins: DMO and DMO+27
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
GLP	Good Laboratory Practice
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
ILSI	International Life Science Institute
LB	Laemmli buffer [62.5mM Tris-HCl, 5% (v/v) 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, 0.005% (w/v) bromophenol blue, 10% (v/v) glycerol, pH 6.8.
5× LB	Five times concentrated 1× LB
LOD	Limit of Detection
MWM	Molecular Weight Markers
MSL	Monsanto Scientific Literature
NFDM	Non-fat dry milk
PBST	Phosphate buffered saline - Tween® 20
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SOP	Standard Operating Procedure
T	Time
TCA	Trichloroacetic acid
U.S.	United States

[1] Standard abbreviations, e.g. units of measure, concentration, mass, time etc., are used without definition according to the format described in “Instructions to Authors” in The Journal of Biological Chemistry.

1.0 Summary

Monsanto Company has developed herbicide-tolerant soybean MON 87708 that is tolerant to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide. MON 87708 contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses the dicamba mono-oxygenase (DMO) enzyme to confer tolerance to the herbicide.

The purpose of this study was to assess the *in vitro* digestibility of the DMO enzyme produced in MON 87708. Structurally, the DMO enzyme functions as a trimer and consists of two forms of the DMO protein, namely DMO and DMO+27. The DMO protein is the mature form of the protein while the DMO+27 protein contains an additional 27 amino acids on its amino terminus, originating from the pea (*Pisum sativum*)Rubisco small subunit. The digestibility of the DMO proteins was assessed in simulated gastric fluid (SGF) containing a proteolytic enzyme, pepsin, and also in simulated intestinal fluid (SIF) containing a mixture of the enzymes called pancreatin. Digestibility of the DMO proteins was assessed using sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and western blot methods. The extent of digestion of the DMO proteins was evaluated by visual analysis of Colloidal Brilliant Blue G stained polyacrylamide gels or by visual analysis of developed western blot X-ray films.

The results of the study demonstrated that 98% of the DMO proteins were digested within 30 s of incubation in SGF when analyzed using a stained gel, and greater than 98% was digested when analyzed using western blot with an anti-DMO antibody. Visual examination of the X-ray films showed that no immuno-reactive fragments were observed at any time points in SGF. On the stained gel, a transiently stable fragment with a molecular weight of ~21 kDa was observed in SGF throughout the digestion. This band was not recognized by anti-DMO antibody, and was N-terminally sequenced in an attempt to establish its identity. The sequences obtained for the ~21 kDa band did not match the predicted sequence of DMO or DMO+27 protein and the identity could not be established. Most likely, the fragment originated from an endogenous soybean protein co-purified with the DMO enzyme.

Visual examination of the X-ray films showed that no proteolytic fragments were detected at any time points in SIF using an anti-DMO antibody. Results of this study demonstrated that greater than 95% of the full-length DMO proteins were digested within 5 min of incubation in SIF.

The results of this study demonstrate that the DMO enzyme is readily digestible in SGF and SIF. Rapid digestion of the full-length DMO enzyme in either SGF or SIF indicates that it is highly unlikely that the DMO enzyme will pose any safety concern to human or animal health since it would be completely digested before absorption in the gastrointestinal tract would occur.

2.0 Introduction

Monsanto Company has developed herbicide-tolerant soybean MON 87708 that is tolerant to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide. MON 87708 contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses the DMO protein oto confer tolerance to dicamba herbicide.

The DMO protein produced in MON 87708 is targeted to chloroplasts for co-localization with the endogenous reductase and ferredoxin proteins that can supply electrons for the DMO oxidative reaction (Chakraborty et al., 2005). The MON 87708-produced DMO contains a chloroplast transit peptide (CTP) from pea (*Pisum sativum*) and 27 amino acids from the N-terminal coding region of the pea Rubisco small subunit that were located between the CTP and the amino terminal end of the coding region of DMO to potentially stabilize expression of this protein *in planta* (Feng and Malven, 2008; Song et al., 2009). It was anticipated that during translocation into chloroplasts the CTP and the additional 27 amino acids would be cleaved, resulting in the appropriate amino terminus for mature DMO. However, analysis of leaf and mature seed tissue by western blot shows the presence of two bands (Feng and Malven, 2008; Morey and Niemeyer, 2009a and 2009b). One band corresponds to the DMO protein, whereas the second, larger band contains the additional 27 amino acids originating from the pea Rubisco small subunit (Feng and Malven, 2008). This form of the protein is designated DMO+27.

The DMO enzyme functions as a trimer (D'Ordine et al., 2009; Dumitru et al., 2009) and in the case of MON 87708, the DMO enzyme is comprised of DMO and DMO+27 (Feng and Malven, 2008). Therefore, this study was conducted to analyze the digestibility of the proteins found in the DMO enzyme from MON 87708.

Proteins introduced into commercial food crops using biotechnology are evaluated for their safety for human and animal consumption. One aspect of this assessment includes an evaluation of a protein's intrinsic sensitivity to proteolytic digestion with enzymes of the gastrointestinal tract. One characteristic of many allergens is their ability to withstand proteolytic digestion by enzymes present in the gastrointestinal tract (Astwood et al., 1996; Vassilopoulou et al., 2006; Moreno et al., 2005; Vieths et al., 1999). Allergenic proteins or their fragments, when presented to the intestinal immune system, can lead to a variety of gastrointestinal and systemic manifestations of immune-mediated allergy.

A relationship between protein digestibility with pepsin at an acidic pH in SGF and the likelihood of the protein being an allergen has been previously reported (Astwood et al., 1996), but this correlation is not absolute (Fu et al., 2000). The SGF assay protocol has been standardized based on results obtained from an international, multi-laboratory ring study (Thomas et al., 2004). The study showed that the results of *in vitro* pepsin digestion assays were reproducible when a standard protocol was followed. The susceptibility of the DMO proteins was assessed using this standardized *in vitro* pepsin digestion protocol.

To reach the intestinal mucosa where antigen processing cells reside, protein or protein fragment(s) must first pass through the stomach where they are exposed to pepsin, and then the duodenum where they are exposed to pancreatic fluid containing a mixture of enzymes called pancreatin. The digestion of a protein by pepsin in the gastric system greatly reduces the possibility that any intact protein or protein fragment(s) will reach the absorptive epithelium of the small intestine. In instances where transient stability of the protein or protein fragment(s) is observed in SGF, further degradation of these fragments in SIF *in vitro* can be evaluated to better understand the fate of the protein during *in vivo* digestion. For example, following exposure to SGF, if a protein or a protein fragment(s) is completely digested during short exposure to SIF, then the probability of the protein or the protein fragment(s) reaching the epithelial cells of the small intestine would be extremely low.

Finally, *in vitro* digestibility of protein in SIF is also used as a stand alone independent test system to assess the digestibility of food components (Yagami et al., 2000; Okunuki et al., 2002). The relationship between protein allergenicity and protein stability in the *in vitro* stand alone SIF study is limited, because the protein has not first been exposed to the acidic, denaturing conditions of the stomach, as would be the case with *in vivo* digestion (FAO/WHO, 2001).

3.0 Purpose

The purpose of this study was to assess the *in vitro* digestibility of the proteins found in the DMO enzyme in SGF and SIF.

4.0 Materials

4.1 Test Substance

The DMO enzyme (Orion lot11261646) was produced and purified from soybean seed of MON 87708, which had been generated to express the coding region for the DMO enzyme. The DMO enzyme is in a storage buffer containing: 50 mM potassium phosphate pH 8.0, 100 mM NaCl, 5% Glycerol (v/v), and 1 mM DTT.

4.2 Characterization of Test Substance

The characterization of the physicochemical properties of the test substance was performed concurrently under characterization plan REG-09-576 and is summarized in the Certificate of Analysis. The DMO enzyme preparation had a total protein concentration of 0.18 mg/ml, with a purity of 81 %. The apparent molecular weights of DMO and DMO+27 were 39.8 & 42.0 kDa as determined by SDS- PAGE, respectively. The N-terminal sequence of both DMO and DMO+27 were also confirmed during characterization.

5.0 Test Systems

Two test systems, SGF and SIF, were utilized independently to test the digestibility of the DMO proteins.

5.1 Simulated Gastric Fluid (SGF)

SGF contained the proteolytic enzyme pepsin in a buffer adjusted to an acidic pH of 1-2. The SGF was prepared using a highly purified form of pepsin (catalog number P-6887, Sigma Company, St. Louis, MO). The SGF was formulated so that ten units of pepsin activity per μg of the DMO proteins would be present in the digestion reactions. The amount of pepsin powder used to prepare SGF was calculated from the specific activity of pepsin reported on the product label. Activity was assessed using a SGF activity assay, where one unit of activity is defined as an increase in absorbance at 280nm of 0.001 per min at 37 °C, measured as trichloroacetic acid (TCA) soluble products using hemoglobin as the substrate. The activity of SGF preparation was confirmed prior to initiating the DMO digestion assay. The digestion of the DMO proteins was monitored by SDS-PAGE stained gels and western blot analysis using a DMO specific antibody.

5.1.1 Justification for Selection of the SGF Test System

In vitro digestion models are used widely to assess the nutritional value of ingested proteins based on their amino acid bioavailability. The correlation between protein allergenicity and protein stability in an *in vitro* pepsin digestion assay has been previously established (Astwood et al., 1996). The pepsin digestibility assay protocol that was used in this study was standardized by the International Life Sciences Institute (ILSI) in a multi-laboratory test and the results demonstrated that the *in vitro* pepsin digestion assay is reproducible when a common protocol is followed (Thomas et al., 2004).

5.2 Simulated Intestinal Fluid (SIF)

SIF contained a mixture of proteolytic enzymes known as pancreatin in a buffer adjusted to pH of ~7.5. SIF was prepared according to the current version of SOP BR-ME-0461 which is based on the method described in The United States Pharmacopoeia (USP 23, 1995). The pancreatin used for the preparation of SIF was obtained from Sigma Company (catalog number P-1500, St. Louis, MO). The SIF was formulated so that 55.3 μg of pancreatin powder would be present per μg of DMO enzyme in the digestion reactions. One unit of pancreatin activity in the SIF assay is defined as an increase in the absorbance at 574 nm of 0.001 per min at 37 ºC. The assay is based on the estimation of the amount of soluble peptides present in a trichloroacetic acid (TCA) solution after pancreatin digestion of resorufin-labeled casein. The activity of the SIF preparation was confirmed prior to initiating the DMO digestion assay. The digestion of the DMO proteins in SIF was assessed by western blot analysis using a DMO specific antibody.

5.2.1 Justification for selection of the SIF Test System

In vitro digestion models are used widely to assess the digestibility of ingested substances. SIF is frequently used for *in vitro* studies to assess the digestibility of food components (Yagami et al., 2000; Okunuki et al., 2002).

6.0 Experimental Design

6.1 Digestibility of the DMO Proteins in SGF

Digestibility of the DMO proteins in SGF was evaluated over time by analyzing specimens from targeted incubation time points. A numerical code using the numbers 0 through 7 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Specimen Code
0 min	SGF T0, SGF P0, SGF N0
30 s	SGF T1
2 min	SGF T2
5 min	SGF T3
10 min	SGF T4
20 min	SGF T5
30 min	SGF T6
60 min	SGF T7, SGF P7, SGF N7

SGF for the digestion was prepared to contain approximately 2632 U/ml of pepsin activity, in a buffer containing 10 mM HCl, 2 mg/ml NaCl, pH ~1.2. The digestion mixture was prepared by adding 580 µl of the DMO enzyme preparation to a tube containing 397 µl of pre-heated (37.2 ºC, 5 min) SGF which corresponds to 104 µg of the DMO proteins and 1044 U of pepsin, respectively. The tube contents were mixed by vortexing and immediately placed in a 37.2 ºC water bath. Specimens (109 µl) were removed at 0.5, 2, 5, 10, 20, 30 and 60 min (corresponding to specimen time points SGF T1 through SGF T7). Each 109 µl specimen was immediately placed in a tube containing the quenching mixture, consisting of 38.0 µl of 0.7 M sodium carbonate buffer (0.7 M Na_2CO_3, pH 11), and 36.6 µl of 5× Laemmli Buffer (LB) [5× LB, 312.5 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) Bromophenol Blue, and 50% (v/v) glycerol, pH 6.8)].

The SGF T0 incubation specimen was prepared in a separate tube. The SGF solution (44.1 µl) was quenched by the addition of 0.7 M sodium carbonate buffer (38.0 µl), and 5× LB (36.6 µl) prior to the addition of the DMO enzyme (64.4 µl).

All quenched specimens were heated to 75-100 ºC for 5-10 min, frozen on dry ice, and stored in a –80 ºC freezer until analyzed.

6.1.1 SGF Experimental Controls

Experimental control specimens were prepared to determine the stability of the DMO proteins in the test system buffer lacking pepsin (10 mM HCl, 2 mg/ml NaCl, pH ~1.2). These experimental control specimens were prepared in a similar manner as described in Section 6.1 for SGF T0, but the targeted incubation times were limited to 0 min (SGF P0) and 60 min (SGF P7).

Experimental control specimens were also prepared to determine the stability of the test system lacking the DMO proteins. Test substance storage buffer (50 mM potassium phosphate pH 8.0, 100 mM NaCl, 5% (v/v) glycerol, and 1 mM DTT) was added to SGF in place of the DMO enzyme. These experimental control specimens were prepared in a similar manner as described above in Section 6.1 for SGF T0, but the targeted incubation times were limited to 0 min (SGF N0) and 60 min (SGF N7).

All quenched specimens, were heated to 75-100 ºC for 5-10 min, frozen on dry ice, and stored in a –80 ºC freezer until analyzed.

6.2 Digestibility of the DMO Proteins in SIF

Digestibility of the DMO proteins in SIF was evaluated over time by analyzing specimens at targeted incubation time points. A numerical code using the numbers 0 through 8 was used to distinguish incubation time points according to the following:

Targeted Incubation Time Point	Designation(s)
0 min	SIF T0, SIF P0, SIF N0
5 min	SIF T1
15 min	SIF T2
30 min	SIF T3
1 h	SIF T4
2 h	SIF T5
4 h	SIF T6
8 h	SIF T7
24 h	SIF T8, SIF P8, SIF N8

The digestion was prepared by adding 400 µl of the DMO enzyme preparation to a tube containing 398 µl of pre-heated (37.2 ºC, 5 min) SIF, corresponding to 72.0 µg of the DMO proteins and 3.98 mg of pancreatin, respectively. The tube contents were mixed by vortexing and immediately placed in a 37.2 ºC water bath. Digestion specimens (60 µl) were removed at 5, 15, 30 min, 1, 2, 4, 8, and 24 h (corresponding to specimen time points SIF T1 through SIF T8) and immediately placed in a tube containing 15 µl of 5× LB, heated to 75-100 ºC for 5-10 min, and frozen on dry ice for complete quenching.

The zero time incubation specimen (SIF T0) was prepared in a separate tube by first quenching 29.9 µl of SIF (0.30 mg) with 15 µl of 5× LB buffer and heating to 75-100 ºC for 5-10 min prior to the addition of 30.0 µl (5.40 µg) of the DMO proteins.

6.2.1 SIF Experimental Controls

Experimental control specimens were prepared to determine the stability of the DMO proteins in the test system buffer lacking pancreatin (50 mM potassium phosphate, pH 7.5). These experimental control specimens were prepared in a similar manner as described in Section 6.2 for SIF T0, but the targeted incubation times were limited to 0 h (SIF P0) and 24 h (SIF P8).

Experimental control specimens were also prepared to characterize the test system (SIF) lacking the DMO proteins. Test substance storage buffer (50 mM potassium phosphate pH 8.0, 100 mM NaCl, 5% Glycerol, and 1 mM DTT) was added to SIF in place of the DMO proteins. These experimental control specimens were prepared in a similar manner as described above in Section 6.2 for SIF T0, but the targeted incubation times were limited to 0 h (SIF N0) and 24 h (SIF N8).

All quenched specimens, were heated to 75-100 ºC for 5-10 min, frozen on dry ice, and stored in a –80 ºC freezer until analyzed.

7.0 Specimen Retention

All specimens will be retained in a –80 °C freezer for one year, after which they will no longer afford analytical evaluation and may be discarded.

8.0 Analytical Methods

Activities of the SGF and SIF were assessed using pepsin and pancreatin activity assays, respectively. The digestibility of the DMO proteins in SGF was assessed using stained SDS-PAGE and western blot analysis. The digestibility of the DMO proteins in SIF was assessed using western blot analysis. The lower limit of detection (LOD) of the DMO proteins was determined for stained SDS-PAGE and western blots. A fragment with a molecular weight of ~21 kDa was observed on the stained gel and was not recognized with DMO specific antibody, the identity was assessed by N-terminal sequencing.

8.1 SGF Activity Assays

The SGF activity assay was used to confirm the suitability of the test system before its use with the DMO proteins according to the current version of SOP BR-ME-0460. The assay is based on the ability of pepsin to digest denatured hemoglobin. Undigested hemoglobin was precipitated with TCA, and the amount of soluble peptides was estimated by measuring the absorbance at 280 nm. The amount of soluble peptides is directly proportional to the amount of protease activity. One unit of pepsin activity in this assay is defined as the amount of pepsin that will produce a

change in absorbance at 280 nm of 0.001 per min at pH 1.2-2.0 at 37 ± 2 ºC. The SGF solution was formulated to contain 0.03 mg of powder per ml of SGF buffer. Acceptable specific activity (units/mg pepsin powder) for the SGF was equal to the specific activity determined by the manufacturer, ±1000 units/mg.

SGF solution was diluted to 0.03 mg of solid material (pepsin) per ml of SGF [the dilution factor (DF) was 27.3]. Acidified hemoglobin [2% (w/v), 5 ml] was added to each of three replicates of the test sample and blank sample and pre-warmed at 37 ± 2 °C for 5-10 min prior to starting the reactions. Diluted SGF (1 ml) was added to each replicate of test samples and both test and blank samples were incubated at 37.2 °C for an additional 10 min. The reactions were stopped by the addition of 10 ml of 5% (v/v) chilled TCA to the test and blank samples. Diluted SGF (1 ml) was then added to the blank samples. Samples were mixed and then incubated for another 5-10 min at 37.2 °C. Precipitated protein was removed by centrifugation of the test and the blank samples. Samples of the clarified test and blank samples were read at 280 nm in a Beckman DU-650 Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) The activity of pepsin was calculated using the following equation:

$$\frac{MeanTest_{A280nm} - MeanBlank_{A280nm}}{0.001 \times 10\,\text{min} \times 1ml} \times DF$$

where 0.001 is the change in the absorbance at 280 nm per min at pH 1.2-2.0 and 37 ± 2 ºC produced by one unit of pepsin activity; 10 min is the reaction time, 1 ml is the amount of SGF added to the reaction; and, DF is the dilution factor for the SGF.

8.2 SIF Activity Assay

The SIF activity assay was used to confirm the suitability of the test system before its use with the DMO proteins according to the current version of SOP BR-ME-0461. One unit of pancreatin activity in this assay is defined as an increase in the absorbance at 574 nm of 0.001 per min at 37 ± 2 ºC. An acceptable specific activity for the SIF was defined as 11,000 ± 3,000 U/ml.

The assay is based on the estimation of the amount of soluble peptides present in a trichloroacetic acid (TCA) solution after pancreatin digestion of resorufin-labeled casein (Roche Molecular Biochemicals, Mannheim, Germany). Undigested resorufin-labeled casein is precipitated with TCA and the amount of soluble peptide is estimated in the supernatant by measuring the absorbance at 574 nm. The amount of soluble peptide is directly proportional to the amount of proteolytic activity.

For the activity assays, three activity replicates were incubated with 0.05× SIF (1× SIF was diluted to 0.05× SIF before the activity assay was initiated) for 15 min at 37.2 ºC. Three blank replicates were incubated with 50 mM KH_2PO_4, pH 7.5 in place of SIF. The reactions was quenched by addition of chilled 5% (v/v) TCA to activity and blank replicates. The supernatants recovered after centrifugation were

neutralized by the addition of assay buffer (500 mM Tris-HCl, pH 8.8), and the absorbance of the clarified activity and blank replicates was read at 574 nm using a Beckman DU-650 spectrophotometer. The activities of SIF solutions was calculated using the following equation:

$$\frac{Mean\,Activity_{A574nm} - Mean\,Blank_{A574nm}}{0.001 \times 15\,\text{min} \times 0.1ml \times 0.05}$$

where 0.001 is the change in the absorbance at 574 nm per min at 37 ± 2 ºC produced by one unit of pancreatin activity, 15 min is the reaction time, 0.1 ml is the amount of 0.05× SIF added to the reaction, and 0.05 is the SIF dilution factor.

8.3 SDS-PAGE and Colloidal Brilliant Blue G Staining

Specimens containing 1× LB from the SGF *in vitro* digestions of the DMO proteins were separated by SDS-PAGE using pre-cast Tricine 10-20% polyacrylamide gradient gels with Tricine running buffer (Invitrogen, Carlsbad, CA) according to the current version of SOP AG-ME-0388. To improve the resolution of pepsin and the DMO proteins a pre-cast Tris-glycine 8% polyacrylamide gels with Tris-glycine running buffer (Invitrogen, Carlsbad, CA) system was also utilized; mainly to confirm the digestion of the full-length DMO proteins. The DMO proteins were loaded at 1.0 µg per lane based on pre-digestion total protein concentration. All experimental controls were loaded at the same volumes as those containing the DMO proteins so that they would be comparable. Mark 12 molecular weight markers (Invitrogen, Carlsbad, CA) was loaded in parallel to estimate the relative molecular weight of proteins and peptides visualized by staining. Electrophoresis was performed at a constant voltage of 125 V for 75 min and 100 min for Tricine 10-20% gels and Tris-glycine 8% gel, respectively. After separation by electrophoresis, proteins were visualized by staining the gel with colloidal Brilliant Blue G (Sigma, St. Louis, MO).

The colloidal Brilliant Blue G staining method was selected because it is an effective method for detecting nanogram quantities of a protein in a gel (Neuhoff et al., 1988). After separation of the proteins by electrophoresis, the gels were fixed in a solution containing 7% (v/v) acetic acid and 40% (v/v) methanol for 30 min and stained for 17 h in 1× Brilliant Blue G-colloidal stain solution containing 20% (v/v) methanol. The gels were destained for 30 s in 10% (v/v) acetic acid, 25% (v/v) methanol and then completely destained for 7 h in a 25% (v/v) methanol solution. Images were captured using a Bio-Rad GS-800 densitometer (BioRad, Hercules, CA). The results of the *in vitro* digestibility of DMO proteins in SGF was determined by visual examination of both bands, corresponding to DMO and DMO+27, on the stained gels.

The LOD of the DMO proteins using the colloidal Brilliant Blue G staining procedure was determined using a Tris-glycine 8% gel to ensure that there would be no interference from the pepsin in establishing the LOD. Various dilutions of the SGF

zero time point (SGF T0) digestion specimen were loaded onto a separate Tris-glycine 8% gel that was run concurrently with the Tris-glycine 8% gel used to assess the DMO proteins digestibility in SGF. Aliquots of the SGF T0 digestion specimen representing approximately 0.25, 0.13, 0.06, 0.03, 0.02, and 0.01 µg total DMO proteins per lane were used for the stained LOD gel.

8.4 Western Blot Analysis

Specimens from the SGF and SIF *in vitro* digestions of the DMO proteins were separated by SDS-PAGE using pre-cast Tricine 10-20% polyacrylamide gradient mini-gels with Tricine running buffer, and pre-cast Tris-glycine 8% polyacrylamide gels with Tris-glycine running buffer. The protein loaded in each lane was based on pre-digestion concentrations of the DMO proteins. The digestion samples were diluted with 1× LB to a concentration of ~3.2 and ~3.6 ng/µl. Approximately 20 ng of the DMO proteins digestion specimens and were loaded in each lane for SGF and SIF digestion specimens, respectively. The experimental controls were loaded in the same volumes as the digestion specimens. All samples were heated to 100.1 °C and 98.6 °C for 5 min each for the Tricine 10-20% gels and Tris-glycine 8% gel, respectively, prior to loading on the gels. Electrophoresis was performed at a constant voltage of 125 V for 75 min and 100 min for Tricine 10-20% gels and Tris-glycine 8% gel, respectively.

After electrophoresis, the proteins were electrotransferred onto PVDF membranes with a pore size of 0.45 µm (Invitrogen, Carlsbad, CA) for 90 min at a constant voltage of 25 V. Prestained molecular weight markers (Precision Plus Dual color Protein Standards, Bio-Rad, Hercules, CA) were used to verify electrotransfer of the proteins to the membranes. Mark 12 unstained molecular weight markers (MWM) (Invitrogen, Carlsbad, CA) were also loaded on the Tricine 10-20% gel for SGF digestions of the DMO proteins to estimate the relative molecular weight of the proteins and peptides visualized by western blot analysis and compare their size to the proteins and peptides visualized by staining. To visualize the Mark 12 unstained MWM on the blot, the blot was washed with Milli Q water 3 times (2-5 min for each wash), stained with Ponceau S (Sigma, St. Louis, MO) stain for 30 s to 2 min. The visualized markers were designated on the blot, and then the blot was washed with Milli Q water for 30-60 s with several changes to remove the Ponceau S stain.

Proteins transferred to PVDF membranes were analyzed by western blot. All membrane incubations were performed at room temperature. The membranes were blocked for 60 min with 5% (w/v) non-fat dry milk (NFDM) in a phosphate buffered saline - Tween® 20 (PBST) buffer. Anti–DMO antibody (lot 11223358) was incubated with the membranes for 60 min at a dilution of 1:2000 in 2% (w/v) NFDM in PBST. Excess antibody was removed by three 10 min washes with PBST. The membranes were incubated with HRP-conjugated rabbit anti-goat IgG (lot JH1162411, Thermo Scientific, Rockford, IL) at a dilution of 1:10,000 in 2% (w/v)

NFDM in PBST for 45 min, and washed three times for 10 min with PBST. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ) and exposed to Hyperfilm ECL high performance chemiluminescence film (GE Healthcare, Piscataway, NJ). Films were developed using a Konica SRX101A automated film processor (Konica, Tokyo, Japan). The films were scanned using a Bio-Rad GS-800 densitometer (Bio-Rad, Hercules, CA) to produce electronic images to be used as figures for reporting purposes.

The approximate molecular weights of the proteins observed on the western blots were visually determined relative to the positions of the molecular weight markers.

The LOD for the western blot analysis procedure was determined for the DMO proteins by loading various dilutions of the SGF and SIF zero time point (SGF T0 and SIF T0, respectively) digestion specimens on separate gels. These gels were run concurrently with the SGF and SIF digestion western blot gels, respectively, and subjected to the same western blot procedure as described above. The following approximate total DMO proteins loadings of the SGF T0 were used for the western blot LOD analysis: 8.0, 4.0, 2.0, 1.0, 0.5, 0.3, and 0.1 ng per lane. The following approximate total DMO proteins loadings of the SIF T0 were used for the western blot LOD analysis: 8.1, 4.0, 2.0, 1.0, 0.5, and 0.3 ng per lane. In both cases the bands corresponding to the DMO proteins (DMO and DMO+27) were analyzed together to determine an LOD for the combined DMO proteins.

8.5 N-Terminal Sequencing

N-terminal sequencing by Edman degradation was used to assess the N-terminal sequence of the SGF protein band with apparent molecular weight of ~21 kDa.

8.5.1 Protein Blot for N-Terminal Sequence Analysis

The specimen SGF T4 was used to further characterize a protein fragment with an apparent molecular weight of ~21 kDa. This specimen corresponds to the 10 min digestion time point of the DMO proteins and provided sufficient amount of the fragments for sequencing.

The SGF T4 specimen was loaded in 4 lanes at 2.1 µg per lane onto a Tricine 10-20% polyacrylamide gradient 10-well gel. Precision Plus prestained molecular weight markers (Bio-Rad, Hercules, CA)were loaded in parallel to verify electrotransfer of the protein to the membranes. Samples were heated to 99.8 °C for 5 min each prior to loading on the gel. Electrophoresis was performed at a constant voltage of 125 V for 75 min. Electrotransfer to 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 90 min at a constant voltage of 25 V. The blots were stained with Coomassie Blue R-250 stain (Bio-Rad, Hercules, CA) and then destained for $\geq$ 5 min with Coomassie Blue R-250 destain (Bio-Rad, Hercules, CA) to visualize the markers, and the fragments from

digestion of DMO proteins in SGF. The blots were scanned using a Bio-Rad GS-800 densitometer (Bio-Rad, Hercules, CA) to produce an electronic image.

8.5.2 N-Terminal Sequencing

The band corresponding to the fragment of ~21 kDa was excised from the blot and N-terminally sequenced. N-terminal sequence analysis for the blot was performed for 15 cycles using automated Edman degradation chemistry (Hunkapillar and Hood, 1983). An Applied Biosystems (ABI) 494 Procise Sequencing System (Carlsbad, CA) and a Perkin Elmer 200 (Waltham, MA) detector were used. Chromatographic data were collected using ABI SequencePro software. A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to chromatographically calibrate the instrument for the analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 picomole β-lactoglobulin, Applied Biosystems, Foster City, CA) was analyzed before and after the analysis of the protein band to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

9.0 Control of Bias

Measures taken to control bias in this study were the inclusion of both stability and test system experimental controls to account for any effects due to the model in the absence of the pepsin and pancreatin enzymes and the absence of the test substance. Digestion specimens and LOD samples were analyzed concurrently to eliminate run-to-run variation.

10.0 Rejected Data

There were no rejected data in this study.

11.0 Deviations

There was one study-specific SOP deviation. SOP BR-ME-0461 states that the 1X SIF solution is prepared by adjusting the pH with 0.2 N NaOH. The 1X SIF solution was prepared by adjusting the pH with 2.5 N NaOH instead. The pH was adjusted to 7.5 as described in the SOP, and there was no impact on the proteolytic activity of pancreatin in SIF.

There was also one protocol deviation. The protocol stated that N-terminal sequencing would be performed following SOP BR-EQ-0265-02. However, due to technical challenges, the N-terminal sequence analysis was performed using a draft of the newest version of the same SOP (AG-EQ-0265-03). Instead of using Atlas™, data was collected and processed with the ABI SequencePro (Carlsbad, CA) software. A new UV/Vis detector was installed on the N-terminal sequencing system replacing a broken UV/Vis detector, resulting in Atlas™ not being available to collect the N-terminal sequence data

for this study. The latest version of "Applied Biosystems 494 Procise Protein Sequencing System" SOP AG-EQ-0265-03 was used in place of SOP BR-EQ-0265-02. This change in the protocol did not have a negative impact on the study. This draft SOP is the newest version of the SOP, and is considered to be an improved version from BR-EQ-0265-02.

12.0 Results and Discussion

12.1 Pepsin Activities is SGF

To assess the suitability of the SGF test system used in this study, pepsin activity in SGF was evaluated prior to its use. The experimentally observed activity of 3010 units per mg of pepsin powder was within the acceptable range of pepsin activity (i.e., 2200 to 4200 units per mg pepsin powder) and, therefore, suitable for the use in this study.

12.2 Pancreatin Activity in SIF

To assess the suitability of the SIF test systems used in this study, pancreatin activity in SIF was evaluated prior to each assay The experimentally observed activity was 13120 U/ml in the preparation used for the SIF digestion assay. The SIF preparation was within the acceptable range of SIF activity (i.e., 8,000 to 14,000 U/ml of SIF), and, therefore, suitable for the use in this study.

12.3 Digestibility of the DMO Proteins in SGF

12.3.1 Assessment of the Digestibility of the DMO Proteins in SGF by Colloidal Brilliant Blue G Gel Staining of SDS-PAGE

The digestibility of the DMO proteins in SGF was evaluated by visual analysis of a colloidal Brilliant Blue G stained Tricine 10-20% polyacrylamide gradient gel to evaluate the presence of digestion fragments (Figure 1). The full-length DMO proteins were completely digested within 30 s. A ~21 kDa fragment was observed in SGF digest specimens throughout the digest, and is also present in the DMO enzyme preparation represented by SGF P0 (Figure1, lane 3). Therefore, it was unlikely that this fragment originated from any of the DMO proteins. This band was analyzed by N-terminal sequencing (see Section 12.4) and it was confirmed that no stable fragments of the DMO proteins were observed.

The pepsin and DMO proteins migrate to similar positions in this gel system, but are still visible. To further confirm that that the full-length DMO proteins are being digested and not being masked by pepsin, the digestibility of the DMO proteins in SGF was also evaluated by visual analysis of a colloidal Brilliant Blue G stained Tris-glycine 8% polyacrylamide gel (Figure 2, panel A). Due to the improved resolution of pepsin and DMO proteins, a separate Tris-glycine 8% polyacrylamide gel was run concurrently to determine the LOD of the DMO proteins (Figure 2, panel B). The migration of pepsin relative to the DMO proteins is different in each gel system. Changes in protein mobility in different

gel systems is due to a variety of factors including changes in acrylamide percentage and pH of each gel system (Makowski and Ramsby, 1997). The LOD of the intact DMO proteins (~39 kDa for DMO and ~42 kDa for DMO+27) was visually estimated to be 0.03 µg. The LOD estimated for the DMO proteins was used to calculate the maximum amount of DMO proteins that could remain visually undetected after digestion, which corresponded to approximately 2.0% of the total protein loaded:

$$\frac{Estimated\ LOD \times 100\%}{Protein\ loaded\ \ per\ lane} \cong \%\ of\ \ protein\ remaining$$

$$\frac{0.02\,\mu g \times 100\%}{1.0\,\mu g} \cong 2.0\%$$

The gel used to assess the stability of the DMO proteins in SGF (Figure 2, panel A) was loaded with ~1.0 µg of total protein (based on pre-digestion concentrations) for each of the digestion specimens. Visual examination of the stained gel confirmed that the full-length DMO and DMO+27 proteins were digested within 30 s of incubation in SGF (Figure 1A, lane 5) as observed in the initial gel system utilized. Therefore, based on the limit of detection, 98% (100%-2.0% = 98%) of the full-length DMO proteins were digested within 30 s of incubation in SGF.

No changes in the full-length DMO proteins band intensity were observed in the absence of pepsin in the experimental control specimens SGF P0 and SGF P7 (Figures 1 and 2A, lanes 3 and 12) indicating that the digestion of the DMO proteins was due to the proteolytic activity of pepsin present in SGF and not due to instability of the proteins while incubated at pH ~1.2 at ~37°C for 60 min.

The experimental control specimens SGF N0 and SGF N7 (Figures 1 and 2A, lanes 2 and 13), which evaluate the stability of the pepsin in the test system (SGF) lacking the DMO proteins demonstrated that the pepsin was observed as a stained protein band at ~38 kDa throughout the experimental phase.

12.3.2 Assessment of the Digestibility of the DMO Proteins in SGF by Western Blot Analysis

The digestibility of the DMO proteins in SGF was also evaluated by western blotting. Again two gel systems were employed and proteins were separated by SDS-PAGE using a Tricine 10-20% polyacrylamide gradient gel (Figure 3) to determine if any fragments were detected, and a Tris-glycine 8% polyacrylamide gel (Figure 4) to confirm that the full-length DMO proteins were digested and not masked due to co-migration with pepsin. In both cases, the results demonstrate that the full-length DMO proteins are digested within 30 s of exposure to SGF. The western blot of the 8% polyacrylamide gel used to assess the stability of the DMO proteins to pepsin digestion (Figure 4, panel A) was run concurrently with a

western blot to determine the LOD of the DMO proteins (Figure 4, panel B). The LOD of the full-length DMO proteins was visually estimated to be 0.3 ng. The LOD estimated for the intact proteins present in the DMO enzyme (~39 kDa for DMO and ~42 kDa for DMO+27) was used to calculate the maximum amount of DMO proteins that could remain visually undetected after digestion, which corresponded to 1.5% of the total protein loaded:

$$\frac{Estimated\ LOD \times 100\%}{Protein\ loaded\ \ per\ lane} \cong \%\ of\ \ protein\ remaining$$

$$\frac{0.3\,ng \times 100\%}{20ng} = 1.5\%$$

The gel was used to assess the DMO proteins *in vitro* digestibility by western blot, and was loaded with 20 ng per lane of total protein (based on pre-digestion concentrations) for each of the digestion specimens. Western blot analysis demonstrated that the DMO proteins were digested to levels below the LOD within 30 s of incubation in SGF (Figures 3 and 4A, lane 5). Based on the western blot LOD for the DMO proteins it was concluded that more than 98% (100% - 1.5% = 98.5%) of the DMO proteins were digested within 30 s. No immunoreactive bands were detected in any lanes corresponding to the digest specimens, indicating that the DMO proteins were digested in SGF within 30 s.

No change in the full-length DMO protein bands intensity was observed in the absence of pepsin in the experimental control specimens SGF P0 and P7 (Figures 3 and 4A, lanes 3 and 12), reaffirming that the DMO proteins were stable in the test system without pepsin.

No immunoreactive bands were observed in control specimens SGF N0 and SGF N7 that represent test system experimental controls (Figure s 3 and 4A, lanes 2 and 13). This indicates that non-specific interactions between the test system components and the antibodies were not observed under these experimental conditions.

12.4 Identification of SGF Digest Fragments by N-Terminal Sequencing

A fragment with a molecular weight of ~21 kDa observed on the stained SGF gel throughout the digest, which was not detected with a DMO specific antibody, was N-terminally sequenced.

To establish identity of the fragment, the acquired sequence data was compared to the predicted DMO and DMO+27 proteins sequences. Typically, 4 to 5 consecutive residues are required to compare sequence data to the predicted protein sequence, and 8 to 9 unambiguous consecutive residues are required for identification of protein

using the BLAST (basic local alignment search tool) algorithm and the National Center for Biotechnology Information (NCBI) protein database to avoid false positives. For the ~21 kDa fragment, only 4 consecutive amino acid residues were clearly identified, which did not match the predicted DMO proteins sequences. Because less than 8 unambiguous consecutive amino acids were identified for the ~21 kDa fragment, the identity could not be unequivocally established using BLAST search and a protein database. It is likely that this fragment originated from soybean proteins which co-purified with the DMO enzyme.

12.5 Assessment of the Digestibility of the DMO Proteins in SIF by Western Blot Analysis

The digestibility of the full-length DMO proteins in SIF was evaluated by western blot (Figure 5). The western blot used to assess the *in vitro* digestibility of the DMO proteins in SIF (Figure 5, panel A) was run concurrently with the western blot to determine the LOD (Figure 5, panel B) of the DMO proteins. The LOD was visually estimated to be 1.0 ng. The LOD estimated for the DMO proteins was used to calculate the maximum amount of DMO proteins that could remain visually undetected after digestion, which corresponded to 5% of the total protein loaded:

$$\frac{Estimated\ LOD \times 100\%}{Protein\ loaded\ \ per\ lane} \cong \%\ of\ \ protein\ remaining$$

$$\frac{1\ ng \times 100\%}{20\ ng} = 5\%$$

The gel used to assess *in vitro* digestibility of the DMO proteins by western blot was loaded with 20 ng total protein (based on pre-digestion concentrations) for each of the digestion specimens. Western blot analysis demonstrated that bands corresponding to the DMO and the DMO+27 proteins were digested below the LOD within 5 min of incubation in SIF (Figure 5A, lane 5). Therefore, based on the LOD, at least 95% (100% - 5% = 95%) of the DMO proteins were digested within 5 min. No proteolytic fragments of the DMO proteins were detected in any digestion specimens. These data suggest that the DMO enzyme degrades rapidly when exposed to pancreatin at neutral pH.

No change in the DMO protein bands intensity was observed in the absence of pancreatin in the experimental control specimens SIF P0 and SIF P9 (Figure 5A, lanes 3 and 13). This indicates that the DMO proteins were stable in the test system without pancreatin at ~37 ºC over the course of the experiment. Higher order aggregates were observed in the experimental control SIF P9 (Figure 5A, lane 13). This is most likely due to protein aggregation during the course of the incubation.

No immunoreactive bands were observed in specimens SIF N0 and SIF N9 that represent SIF test system experimental controls (Figure 5A, lanes 2 and 14).

13.0 Conclusions

The results of the study demonstrate that at least 98% of the DMO proteins were digested within 30 s of incubation in SGF when analyzed using stained gel, and greater than 98% was digested when analyzed using western blot with an anti-DMO antibody. On the stained gel, a fragment with a molecular weight of ~21 kDa was observed in SGF throughout the digest. This band was not observed on the western blot X-ray films and N-terminal sequencing data determined that it did not match any part of the DMO proteins. Most likely the fragment originated from one of the endogenous soybean proteins co-purified with the DMO enzyme.

Results of this study also demonstrated that greater than 95% of the DMO proteins were digested within 5 min of incubation in SIF with no proteolytic fragment(s) detected by western blot using a DMO–specific antibody.

The results of this study show that the DMO enzyme is readily digestible in either SGF or SIF. Rapid digestion of the DMO enzyme in SGF and SIF indicates that it is highly unlikely that the DMO enzyme will pose any safety concern to human health since it would be completely digested before absorption in the gastrointestinal tract would occur.

14.0 References

Astwood, J.D., Leach, J.N., and Fuchs, R.L. 1996. Allergenicity of foods derived from transgenic plants. *Nature Biotech*. 14: 1269-1273.

Chakraborty, S., M. Behrens, P.L. Herman, A.F. Andersen, W.R. Hagen, D.L. Carlson, X-Z. Wang, and D.P. Weeks. 2005. A three-component dicamba O-demethylase from Pseudomonas maltophilia, strain DI-6: Purification and characterization. *Arch. Biochem. Biophys*. 437: 20-28

D'Ordine, R.L., T.J. Rydel, M.J. Storek, E.J. Sturman, F. Moshiri, R.K. Bartlett, G.R Brown, R.J. Eilers, C. Dart, Y. Qi, S. Flasinski, and S.J. Franklin. 2009. Dicamba monooxygenase: structural insights into a dynamic Rieske oxygenase that catalyzes an exocyclic monooxygenation. J. Mol. Biol. 392:481-97.

Dumitru R, W.Z. Jiang, D.P. Weeks, and M.A. Wilson. 2009. Crystal Structure of Dicamba Monooxygenase: A Rieske Nonheme Oxygenase that Catalyzes Oxidative Demethylation. J. Mol. Biol. 392:498–510.

FAO/WHO 2001. Stability of known allergens (digestive and heat stability) Document Biotech 01/07. Joint FAO/WHO expert consultation on foods derived from biotechnology, 22 – 25 January 2001, Rome, Italy. Available: ftp://ftp.fao.org/es/esn/food/bi07al.pdf.

Feng, P. and M. Malven. 2008. Engineering soybean tolerance to dicamba via a degradation mechanism using a bacterial dicamba mono-oxygenase (DMO). Monsanto Technical Report MSL0020633. St. Louis, MO.

Fu, T-J., Abbott, U.R., and Hatzos, C. 2002. Digestibility of food allergens and non-allergenic proteins in simulated gastric fluid and simulated intestinal fluid-a comparative study. *J. Agric Food Chem*. 50: 7154-7160.

Hunkapillar, M.W., and Hood, L.E. 1983. Protein sequence analysis: automated microsequencing. *Science* 219 (4585)**:** 650-659.

Makowski, G.S. and Ramsby, M.L. 1997. Protein molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Pages 1-27 in Protein Structure A Practical Approach. Creighton, T.E (ed.) Oxford University press, New York, NY.

McGregor, C., Chen, L., Pomroy, N.C., Hwang, P., Go, S., Chakrabarrty, A., and Prive, G.G. 2003. Lipopeptide detergents designed for the structural study of membrane protein. *Nature Biotechnology* 21:171-176.

Moreno, F.J., Mellon, F.A., Wickham, M.S.J., Bottrill, A.R., and Mills C.E.N. 2005. Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant *in vitro* gastrointestinal digestion. *FEBS Journal*. 272: 341-352.

Morey, J.M. and K.E. Niemeyer. 2009a. Assessment of DMO Protein Levels in Leaf and Mature Seed from Dicamba-Tolerant Soybean MON 87708 Grown in a Greenhouse during 2007 in Support of a Japan Stage III Application. Monsanto Technical Report MSL0021458. St. Louis, MO.

Morey, J.M. and K.E. Niemeyer. 2009b. Western Blot Analysis of DMO Protein in Dicamba-Tolerant Soybean MON 87708 Leaf Across Multiple Generations Produced in the Greenhouse During 2007 and 2008. Monsanto Technical Report MSL0021459. St. Louis, MO.

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.

Okunuki, H., Techima, R., Shigeta, T., Sakushima, J., Akiyama, H., Yukihiro, G., Toyoda, M., and Sawada, J. 2002. Increased digestibility of two products in genetically modified food (CP4-EPSPS and Cry1Ab) after preheating. *J. Food Hyg. Soc. Japan* 43: 68-73.

Song, Z., K.D. Lawry, J.F. Rice, and Q. Tian. 2009. Amended Report for MSL0021418: Molecular Analysis of Dicamba-Tolerant Soybean MON 87708. Monsanto Technical Report MSL0022109. St. Louis, MO.

Thomas, K., Aalbers, M., Bannon, G.A., Bartels, M., Dearman, R.J., Esdaile, D.J., Fu, T.J., Glatt, C.M., Hadeld, N., Hatzos, C., Hee, S.L., Heylings, J.R., Goodman, R.E., Henry, B., Herouet, C., Holsapple, M., Ladics, G.S., Landry, T.D., MacIntosh, S.C., Rice, E.A., Privalle, L.S., Steiner, H.Y., Teshima, R., van Ree, R., Woolhiser, M., and Zawodny, J. 2004. A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology and Pharmacology* 39: 87–98.

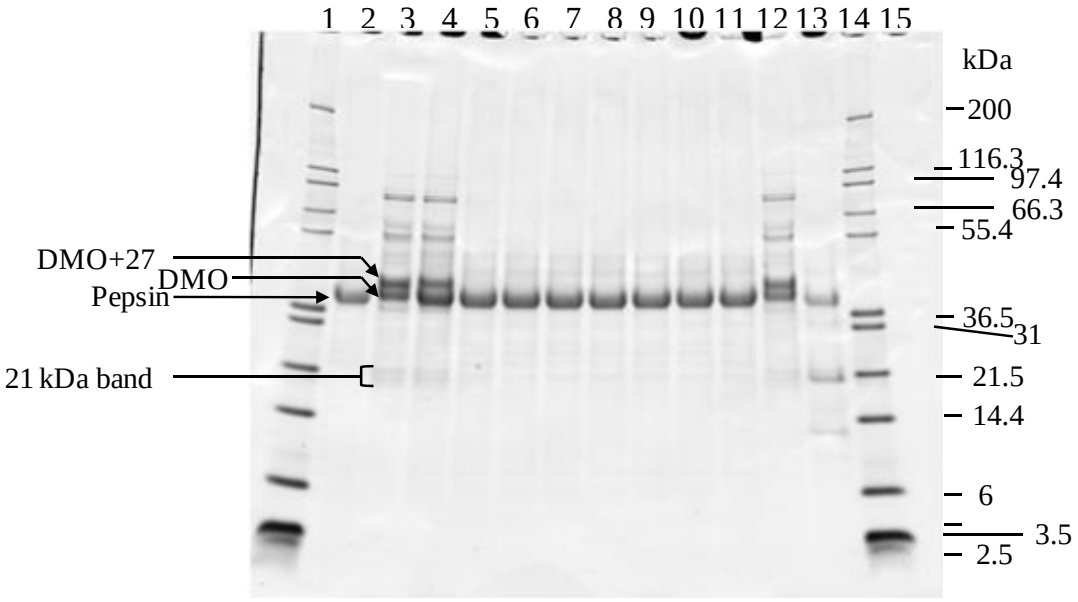
USP 23 NF 18. 1995. pp. 2053. Published by United States Pharmacopeial

Von Jagow, G., Link, T., and Schagger, H. 1994. A Practical Guide to membrane Protein Purification. Academic Press.

Vassilopoulou, E., Rigby, N., Moreno, F.J., Zuidmeer, L., akkerdaas, J., tassios, I., Papadopoulos, N.G., Saxoni-Papageorgiou, P., Ree, R.v., and Mills, C. 2006. Effect of *in vitro* gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. *J. Allergy Clin. Immunol.* 118: 473-480.

Vieths, S., Reindl, J., Hoffmann, A., and Haustein, D. 1999. Digestibility of peanut and hazelnut allergens investigated by a simple in vitro procedure. *Eur Food Res Technol*. 209: 379-388.

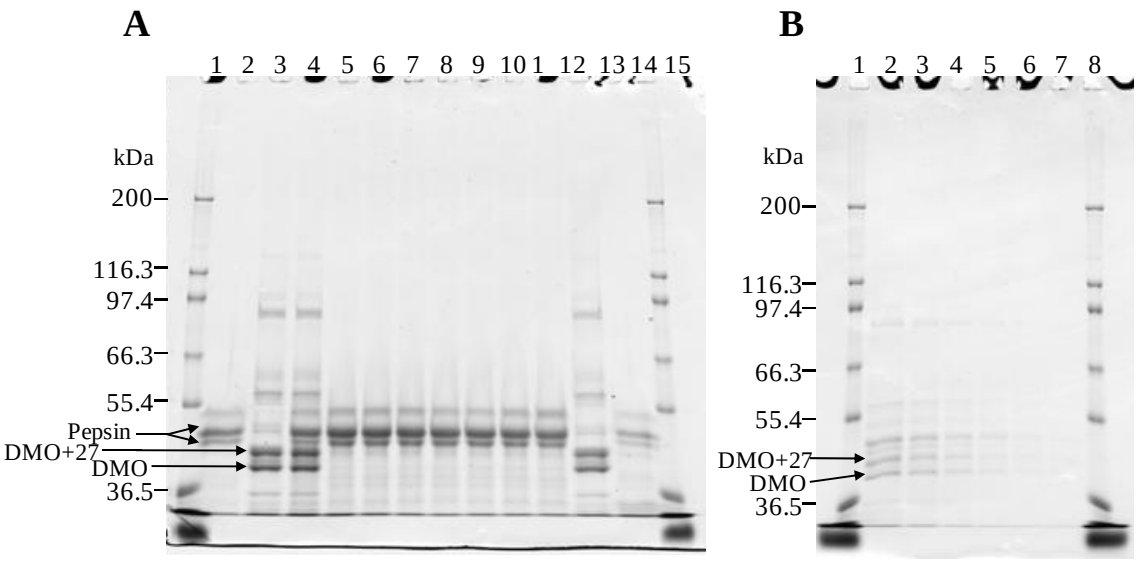
Yagami, T., Haishima, Y., Nakamura, A., Hiroyuki, O., and Ikesawa, Z. 2000. Digestibility of allergens extracted from natural rubber latex and vegetable foods. *J. Allergy Clin. Immunol.* 106**:** 752-762



Lane	Sample	Incubation Time (min)
1	Mark 12 MWM	-
2	SGF N0	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Mark 12 MWM	-
15	Blank	-

Figure 1. Tricine 10-20% SDS-PAGE Analysis of the Digestion of the DMO Proteins in SGF

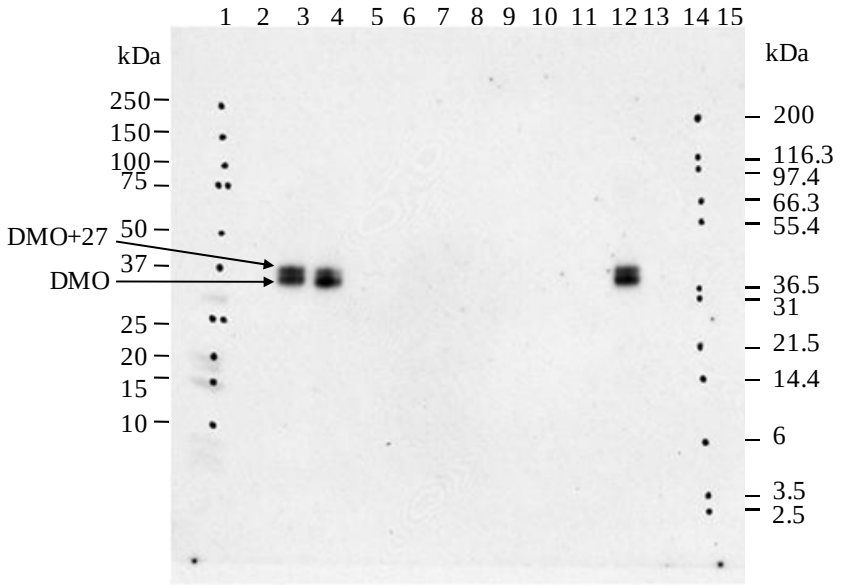
Colloidal Brilliant Blue G stained Tricine 10-20% polyacrylamide gel was used to analyze the digestibility of the DMO proteins in SGF. The figure corresponds to the digestion of DMO proteins in SGF and analyzed by SDS-PAGE. Based on pre-digestion protein concentrations, 1.0 µg of total protein was loaded in each lane containing the DMO enzyme. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel.



Lane	Sample	Incubation Time (min)	Lane	Sample	Amount (µg)
1	Mark 12 MWM	-	1	Mark 12 MWM	-
2	SGF N0	0	2	T0, protein+SGF	0.25
3	SGF P0	0	3	T0, protein+SGF	0.13
4	SGF T0	0	4	T0, protein+SGF	0.06
5	SGF T1	0.5	5	T0, protein+SGF	0.03
6	SGF T2	2	6	T0, protein+SGF	0.02
7	SGF T3	5	7	T0, protein+SGF	0.01
8	SGF T4	10	8	Mark 12 MWM	-
9	SGF T5	20			
10	SGF T6	30			
11	SGF T7	60			
12	SGF P7	60			
13	SGF N7	60			
14	Mark 12 MWM	-			
15	Blank	-			

Figure 2. Tris-Glycine 8% SDS-PAGE Analysis of the Digestion of the DMO Proteins in SGF

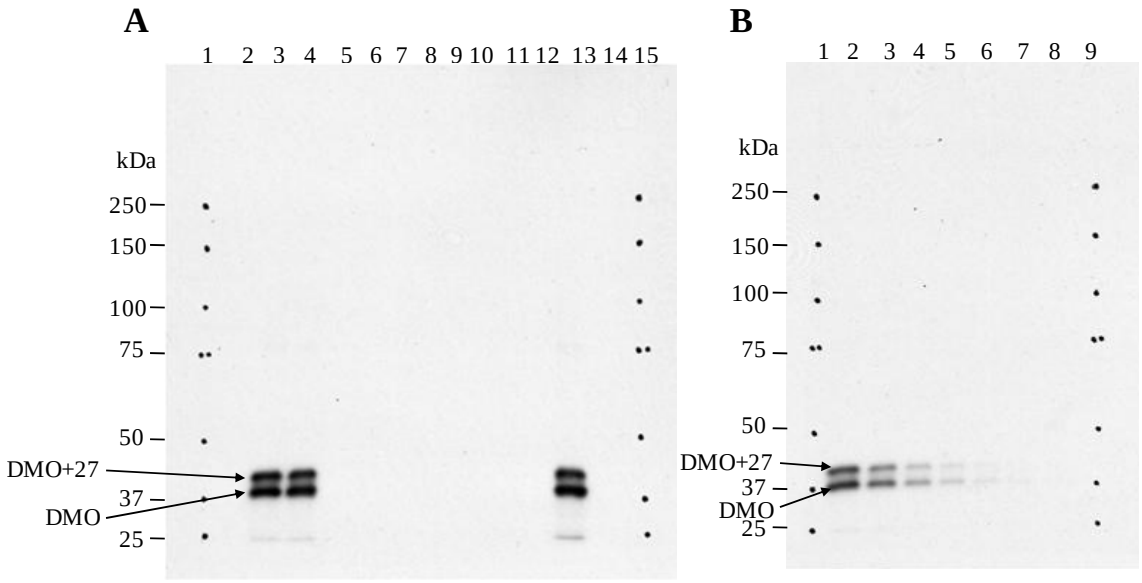
Colloidal Brilliant Blue G stained 8% Tris-glycine polyacrylamide gels were used to analyze the digestibility of the DMO proteins in SGF. **Panel A** corresponds to the DMO enzyme digestion in SGF. Based on pre-digestion protein concentrations, 1.0 µg of total protein was loaded in each lane containing the DMO proteins. **Panel B** corresponds to the limit of detection of the DMO proteins. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel.



Lane	Sample	Incubation Time (min)
1	Precision Plus MWM	-
2	SGF N0	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Precision Plus MWM	-
15	Blank	-

Figure 3. Tricine 10-20% SDS-PAGE/Western Blot Analysis of the Digestion of the DMO Proteins in SGF

The figure corresponds to the DMO proteins digestion in SGF separated by SDS-PAGE using a Tricine 10-20% polyacrylamide gradient gel. Based on pre-digestion protein concentrations, 20 ng (total protein) was loaded in the lanes containing the DMO enzyme. Approximate molecular weights (kDa) are shown on the left and right of the blot. A 30 s exposure is shown.

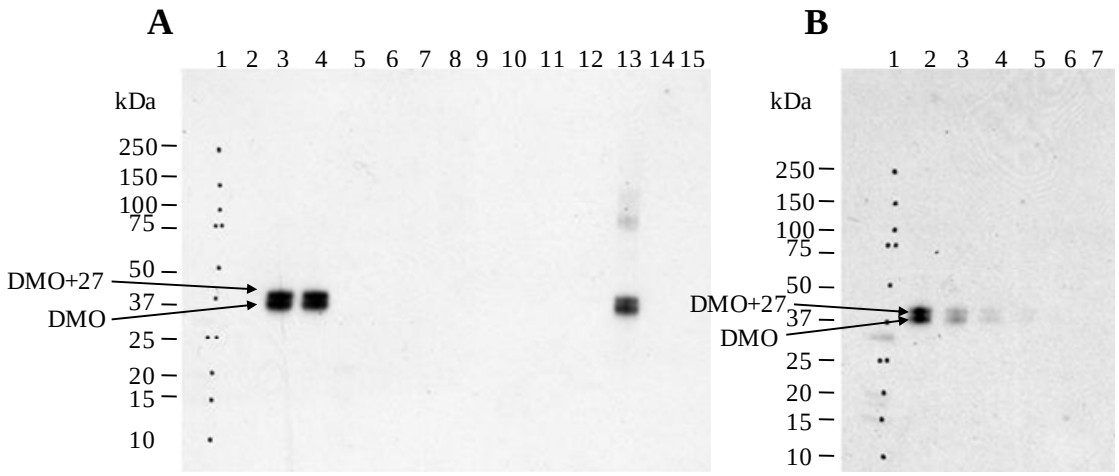


Lane	Sample	Incubation Time (min)
1	Precision Plus MWM	-
2	SGF N0	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Precision Plus MWM	-
15	Blank	-

Lane	Sample	Amount (ng)
1	Precision Plus MWM	-
2	T0, protein+SGF	8.0
3	T0, protein+SGF	4.0
4	T0, protein+SGF	2.0
5	T0, protein+SGF	1.0
6	T0, protein+SGF	0.5
7	T0, protein+SGF	0.3
8	T0, protein+SGF	0.1
9	Precision Plus MWM	-

Figure 4. Tris-Glycine 8% SDS-PAGE/Western Blot Analysis of the Digestion of the DMO Proteins in SGF

Panel A corresponds to the DMO proteins digestion in SGF separated by SDS-PAGE using Tris-glycine 8% polyacrylamide gels. Based on pre-digestion protein concentrations, 20 ng (total protein) was loaded in the lanes containing the DMO proteins. **Panel B** corresponds to the limit of detection of the DMO proteins. The lanes have been cropped and re-numbered. Approximate molecular weights (kDa) are shown on the left, and correspond to the markers loaded in each gel. A 15 s exposure is shown.



Lane	Sample	Incubation Time	Lane	Sample	Amount (ng)
1	Precision Plus MWM	-	1	Precision Plus MWM	-
2	SIF N0	0	2	T0, protein+SIF	15
3	SIF P0	0	3	T0, protein+SIF	10
4	SIF T0	0	4	T0, protein+SIF	5
5	SIF T1	5 min	5	T0, protein+SIF	2.5
6	SIF T2	15 min	6	T0, protein+SIF	1
7	SIF T3	30 min	7	Mark 12 MWM	-
8	SIF T4	1 h			
9	SIF T5	2 h			
10	SIF T6	4 h			
11	SIF T7	8 h			
12	SIF T8	24 h			
13	SIF P8	24 h			
14	SIF N8	24 h			
15	Mark 12 MWM	-			

Figure 5. Western Blot Analysis of the Digestion of the DMO Proteins in SIF

Panel A corresponds to the DMO proteins digestion in SIF. Based on pre-digestion protein concentrations, 20 ng (total protein) was loaded in the lanes containing the DMO proteins. **Panel B** corresponds to the limit of detection of the DMO proteins, lanes were cropped. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. A 15 s exposure is shown.

Appendix 1. List of Applicable SOPs

SOP Number	Title
BR-ME-0460-02	Preparation of Simulated Gastric Fluid and Assay of the Proteolytic Activity
BR-ME-0461-03	Preparation of Simulated Intestinal Fluid and Assay of the Proteolytic Activity
AG-ME-0388-03	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-ME-0924-01	Electrotransfer of Proteins to Membranes
BR-ME-0392-01	Western Blot Analysis (Immunoblotting)
BR-EQ-0935-01	Konica SRX X-Ray Film Processors
BR-ME-0926-01	Staining of Proteins on Blot Membranes
BR-EQ-0265-03	Applied Biosystems 494 Procise™ Protein Sequencing System (Draft)
BR-EQ-0599-04	Bio-Rad GS-800 Densitometer System