Study Title:
The Effect of Heat Treatment on Dicamba Mono-Oxygenase (DMO) Enzyme Functional Activity

Authors
Ronald Hernan, Ph.D., Robert Heeren, B.S and John Finnessy, M.S.

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Sponsor:
Monsanto Company
800 North Lindbergh Boulevard
St. Louis, MO 63167
Sponsor Representative: Global Regulatory Pipeline
Primary Contact: Melinda McCann
Phone: (314) 694-3080

Primary Testing Facility:
Monsanto Company
Regulatory Product Characterization Center
Protein Sciences and Safety
800 North Lindbergh Boulevard
St. Louis, MO 63167

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Statement of Compliance

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

Submitter — Date

Melinda C. McCann — 8 Oct 2010

Melinda McCann
Sponsor Representative — Date

Ronald Hernan — 10/8/2010

Ronald Hernan, Ph.D.
Study Director — Date

Quality Assurance Unit Statement

Study Title: The Effect of Heat Treatment on Dicamba Mono-Oxygenase (DMO) Enzyme Functional Activity

Study Number: REG-10-318

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

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

Date of Inspection Audit	Phase	Date Reported to Study Director	Date Reported to Management
08/04/2010	Enzyme Assay	08/06/2010	08/06/2010
10/01/2010	Raw Data and Draft Report Audit	10/01/2010	10/01/2010

Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

10/08/10
Date

Study Certification

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

Signatures of Final Report Approval

Ronald Hernan, Ph.D.
Study Director

10/8/2010
Date

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

10/8/2010
Date

Study Information

Study Number:	REG-10-318
Title:	The Effect of Heat Treatment on Dicamba Mono-Oxygenase (DMO) Enzyme Functional Activity
Testing Facility:	Monsanto Company Regulatory Product Characterization Center Protein Sciences and Safety 800 North Lindbergh Boulevard St. Louis, MO 63167
Authors:	Ronald Hernan, Ph.D., Robert Heeeren, B.S. and John Finnessy, M.S.
Study Director:	Ronald Hernan
Study Initiation Date:	6/16/2010
Study Completion Date:	10/08/2010
Records Retention:	The protocol, all raw data, documentation, records, and the final report for this study are retained at Monsanto Company.

Table of Contents

List of Tables

List of Figures

List of Appendices

Abbreviations and Definitions

APS	Analytical Protein Standard
CFR	Code of Federal Regulations
DMO	Dicamba mono-oxygenase
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
DTT	Dithiothreitol
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
GLP	Good Laboratory Practice
kDa	Kilodalton
QA	Quality Assurance
QAU	Quality Assurance Unit
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard Operating Procedure

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 dicamba mono-oxygenase (*dmo*) gene from *Stenotrophomonas maltophilia* that expresses the DMO enzyme to confer tolerance to dicamba herbicide.

The purpose of this study was to assess the effect of heating on the functional activity of the DMO enzyme from 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 full length 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 and intervening sequence. The purified DMO enzyme was used as the test substance at a final total protein concentration of 0.18 mg/ml. Aliquots of the test substance were heated to 25, 37, 55, 75, and 95 °C for either 15 or 30 minutes, while a single aliquot of the control substance was maintained on wet ice for the duration of the heat treatments. Heated test substances and unheated temperature control substance were analyzed by a dicamba mono-oxygenase activity assay to assess the impact of temperature on the functional activity of DMO enzyme. Additionally, the protein resulting from each temperature treatment was analyzed by SDS-PAGE to assess DMO degradation.

Results of this study demonstrate that the DMO enzyme is functionally active at 25 ºC and 37 ºC. The amount of the DMO enzyme activity remaining following heat treatment at 55, 75, or 95 ºC was below the limit of quantification. DMO enzyme test substances analyzed by SDS-PAGE showed no visible change in band intensity of the heat-treated substance at temperatures up to 55 °C. The test substance heated at 75 ºC for 15 minutes showed no visible change in band intensity ; however incubation for 30 minutes resulted in a visible change in band intensity although the band intensity was greater than the 10% DMO enzyme reference equivalent. The test substance treated at 95 ºC for both 15 and 30 minutes resulted in a visible band intensity less than or equal to the 10% DMO enzyme reference equivalent. These data demonstrate that the DMO enzyme behaves with a predictable tendency towards loss of functional activity at elevated temperatures.

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 *dmo* gene from *Stenotrophomonas maltophilia* that expresses the DMO enzyme to confer tolerance to dicamba herbicide.

The DMO 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* (Song et al., 2010). 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 mature seed tissue by western blot shows the presence of two bands (Wang et al., 2010). 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 and intervening sequence. 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.

This study was performed to evaluate the thermal stability of purified DMO in an aqueous buffered solution after treatments at five temperatures ranging from 25 ºC to 95 ºC and at two incubation times of 15 and 30 minutes. DMO enzyme stability was assessed by a dicamba mono-oxygenase activity assay and SDS-PAGE analysis.

3.0 Purpose

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

4.0 Materials

4.1. Test Substance

The test substance was the DMO enzyme (Orion Lot 11271156) purified from soybean seed of MON 87708. Records pertaining to the production and isolation of the plant-produced DMO enzyme are archived in the Monsanto Regulatory Archives. The test substance was stored in a -80 °C freezer, in a buffer solution containing 50 mM potassium phosphate buffer pH 8.0, 100 mM NaCl, 5 % glycerol, 1 mM DTT.

4.2. Control Substance

The control substance was DMO enzyme (Orion Lot 11271156) purified from soybean seed of MON 87708. As control substance, an aliquot was maintained on wet ice throughout the heat treatment incubation period. Records pertaining to the production and isolation of the plant-produced DMO enzyme are archived in the Monsanto Regulatory Archives. The control substance was stored in a -80 °C freezer, in a buffer solution containing 50 mM potassium phosphate buffer pH 8.0, 100 mM NaCl, 5 % glycerol, 1 mM DTT.

4.3. Reference Standard

The DMO enzyme listed above as a test substance was also used as reference standard. The reference standard was maintained at -80 °C until the heat treatment test substances were ready for analysis and was evaluated along with the heat treatment test substances in the dicamba mono-oxygenase activity assay and in the SDS-PAGE analysis.

5.0 Characterization of the Test and Control Substances and Reference Standard

The test substance, control substance, and reference standard (Orion lot 11271156) has a purity of 81%, a total protein concentration of 0.18 mg/ml and apparent molecular weights of 39.8 and 42.0 kDa for DMO and DMO+27, respectively. A copy of the certificate of analysis for the test substance, control substance, and reference standard are archived with the study file.

6.0 Methods

6.1. Heat Treatment

The DMO enzyme was thawed on wet ice at final total protein concentration of 0.18 mg/ml in 50 mM potassium phosphate buffer pH8.0, 100 mM NaCl, 5% glycerol, 1 mM DTT. Aliquots of 100 µl of the DMO enzyme were transferred to eleven tubes. The eleven aliquots in tubes were maintained on wet ice until the heat treatments were initiated. Five tubes were placed in the appropriate heat treatment conditions (25, 37, 55, 75, or 95 °C, each ± 2 °C) and incubated for 15 ± 1 minutes. Five tubes were placed in the appropriate heat treatment conditions (25, 37, 55, 75, or 95 °C, each ± 2 °C) and incubated for 30 ± 1 minutes. All heat treated test substances were returned immediately to wet ice following the heat treatment incubation period. The eleventh tube (i.e., the control substance) was maintained on wet ice throughout the heat treatment incubation period.

Following the heat treatments, 40 µl of each heat treated test substance and the control substance was transferred to a clean tube and mixed with 10 µl of 5× SDS-PAGE loading buffer (0.312 M Tris HCl pH 6.8, 10% SDS, 50% glycerol, 3.6 M 2-mercaptoethanol, 0.025% Bromophenol Blue) as preparation for use in SDS-PAGE analysis. The 50 µl samples were heated at 95 ± 5 °C for 3-5 minutes, quick frozen by placement on dry ice, and stored at -80 °C until analysis. The remainder of each sample, approximately 60 µl, will be maintained on wet ice and transferred as quickly as practical into the functional activity assay described in section 6.2.

6.2. Dicamba Mono-oxygenase Activity Assay

The DMO functional activity of the heat-treated test substance, control substance, and the reference standard were determined according to an approved draft of SOP BR-ME-1244 (hereto referred to as the “draft activity SOP”). All samples (including the reference standard) were at a total protein concentration of 0.18 mg/ml in 50 mM potassium phosphate buffer pH8.0, 100mM NaCl, 5% glycerol, and 1 mM DTT prior to analysis. Three replicates of each diluted sample were used for the analysis. Unused sample(s) remaining after the assay were discarded.

For the assay to be considered valid, all acceptance criteria specified in the draft activity SOP must be met. In addition, the activity results of the reference standard must fall within the acceptance range specified for the most recent characterization for Orion lot 11271156 in the APS program.

6.3. SDS-PAGE Analysis

The samples prepared for SDS-PAGE analysis, described in section 6.1, were thawed, heated at 95 ± 5 °C for 3-5 min, and loaded onto their respective 4-20% polyacrylamide gradient gel at 0.14 mg total protein/ml. The reference standard was loaded on each respective gel at 0.14 mg/ml (100% reference standard equivalent) and at 0.014 mg/ml (10% reference standard equivalent). The electrophoretic separation of the proteins was conducted according to the current version of SOP AG-ME-0388. Following electrophoresis, gels were stained with Brilliant Blue G Colloidal (Sigma, St. Louis, MO) according to SOP BR-ME-0527.

After staining, the stability of the DMO enzyme at each heat treatment was evaluated qualitatively. The intensity of the major protein bands at 39.8 and 42.0 kDa in the heat treatment lanes was compared visually to the same band in the lanes with the control substance, 100% reference standard equivalent, and 10% reference standard equivalent.

7.0 Control of Bias

Appropriate controls and standards were included with each analysis.

8.0 Rejected Data/Data Not Used

All data in this study was used

9.0 Protocol Amendments and Deviations

One protocol amendment was required as Orion Lot number for the test substance had changed prior to its use in the study. The lot number change is reflected in the COA for the test substance. Because the study's experimental design is unchanged, this change will have no negative impact on the study.

10.0 Results and Discussion

Results of the activity assay for DMO enzyme incubated for 15 and 30 minutes are listed in Tables 1 and 2 respectively. Activity of the reference standard was measured as 17.2 nmole DSCA/min/mg DMO enzyme. The control substance had activity of 16.6 nmole DSCA/min/mg of DMO enzyme, thus demonstrating that protein activity was maintained during incubation on ice. When heated at a temperature of 25 ºC with incubation times of 15 and 30 minutes and 37 ºC with an incubation time of 15 minutes, no effect on the activity of DMO enzyme was observed. The test substance heated to 37 ºC demonstrated a small reduction in DMO enzyme activity with 72 % activity remaining relative to the control substance after the 30 minute incubation. The level of DMO enzyme activity following incubation at temperatures of 55, 75 and 95 ºC was below the limit of quantification for incubations at both time points.

Analysis by SDS-PAGE stained with Brilliant Blue G Colloidal (Figure 1 and 2) demonstrated that the reference standard and control substance contain two major bands with an apparent molecular weight of approximately 39.8 and 42.0 kDa corresponding to

the DMO enzyme (DMO and DMO+27). Results of the SDS-PAGE data for the heat treatment of the test substances incubated for 15 minutes and 30 minutes are illustrated in Figures 1 and 2, respectively. The control substance loaded on each respective gel (lane 7, Figures 1 and 2) showed equivalent band intensity at 39.8 and 42.0 kDa to the 100 % reference standard (lane 8, Figures 1 and 2); demonstrating that the DMO enzyme was stable on wet ice during the incubation period.

No apparent decrease in band intensity of the 39.8 and 42.0 kDa DMO enzyme bands were observed in the test substance when heated at temperatures of 25, 37 and 55 ºC for 15 minutes (lanes 2-4, Figure 1) or 30 minutes (lanes 2-4, Figure 2). The test substance heated to 75 ºC for 15 minutes show no visible change in band intensity at 39.8 and 42.0 kDa (Lane 5, Figure 1). The test substance heated to 75 ºC for 30 minutes demonstrated a visible change in band intensity at 39.8 and 42.0 kDa (lane 5, Figures 2), greater than the DMO enzyme 10% reference equivalence. The test substance heated to 95 ºC for 15 minutes resulted in a visible change of band intensity at 39.8 kDa greater than the band intensity of the DMO enzyme 10% reference equivalence and a change in band intensity at 42.0 kDa similar to the DMO enzyme 10% reference equivalence (lane 6, Figure 1). The test substance heated to 95 ºC for 30 minutes resulted in a visible change in band intensity at 39.8 and 42.0 kDa less than the DMO enzyme 10% reference equivalence (lane 6, Figure 2).

11.0 Conclusion

The purpose of this study was to examine the thermal stability of the purified DMO enzyme from MON 87708. From this study, DMO enzyme incubated at 25 ºC and at 37 ºC for 15 and 30 minutes is stable, as determined by dicamba mono-oxygenase activity assay. However, at temperatures of 55 ºC and above a complete loss of functional activity was observed. SDS-PAGE demonstrated that heat treated test substance with incubation at 25, 37 and 55 ºC had no effect on the thermal stability of DMO enzyme. The test substance heated at 75 ºC for 15 minutes had no effect on the thermal stability; however incubation for 30 minutes resulted in a visible change in DMO enzyme protein band intensity although greater than the 10% DMO enzyme reference equivalent. The test substance treated at 95 ºC for both 15 and 30 minutes resulted in a visible DMO enzyme protein band intensity less than or equal to the 10% DMO enzyme reference equivalent. These data demonstrate that DMO enzyme behaves with a predictable tendency towards enzyme denaturation at elevated temperatures.

12.0 Reference

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Wang, C., S.R. Hill, L.A. Burzio, and J.J. Finnessy. 2010. Characterization of the Dicamba Mono-Oxygenase (DMO) Enzyme Isolated from the Seed of MON 87708. Monsanto Technical Report MSL0022497. St. Louis, Missouri.

Table 1. Dicamba Mono-oxygenase Activity Assay of Heat-Treated DMO Enzyme After 15 Minutes at Elevated Temperatures

Temperature	Specific Activity (nmole DSCA /min/mg DMO enzyme) [1]	% DMO Activity Remaining (% of control substance) [2]
0 ºC (control substance)	16.6	100 %
25 ºC	17.9	107 %
37 ºC	17.0	102 %
55 ºC	Below LOQ[3]	<25 %
75 ºC	Below LOQ[3]	<25 %
95 ºC	Below LOQ[3]	<25 %

[1] Mean specific activity determined from n=3.
[2]DMO enzyme activity of control substance was assigned 100 % active.
% DMO activity remaining = [specific activity of sample/specific activity of control substance] x 100
[3] The LOQ is 4.4 nmole DSCA /min/mg DMO enzyme.

Table 2. Dicamba Mono-oxygenase Activity Assay of Heat-Treated DMO Enzyme After 30 Minutes at Elevated Temperatures

Temperature	Specific Activity (nmole DSCA /min/mg DMO enzyme) [1]	% DMO Activity Remaining (% of control substance) [2]
0 ºC (control substance)	16.6	100 %
25 ºC	17.8	107 %
37 ºC	12.0	72 %
55 ºC	Below LOQ[3]	<25 %
75 ºC	Below LOQ[3]	<25 %
95 ºC	Below LOQ[3]	<25 %

[1] Mean specific activity determined from n=3.
[2]DMO enzyme activity of control substance was assigned 100 % active.
% DMO activity remaining = [specific activity of sample/specific activity of control substance] x 100
[3] The LOQ is 4.4 nmole DSCA /min/mg DMO enzyme.

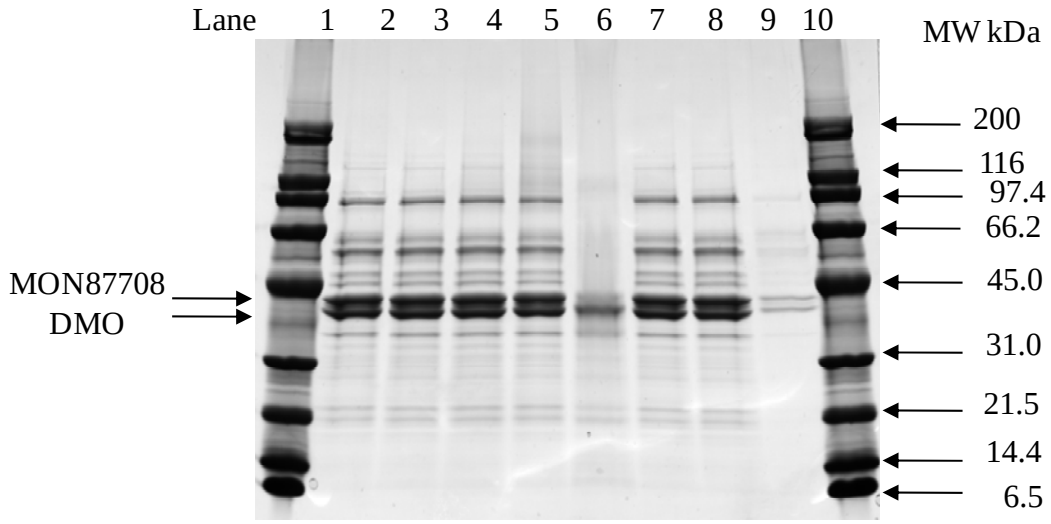

Figure 1. SDS-PAGE of DMO Enzyme Demonstrating the Effect After 15 Minutes at Elevated Temperatures on Protein Structural Stability

Heat-treated samples of DMO enzyme (2.8 µg total protein) separated on a Tris-glycine 4-20 % polyacrylamide gel under denaturing and reducing conditions. Gel was stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10.

Lane	Description	Total Amount
1	Broad Range Molecular Weight Markers	40.5 µg
2	DMO Enzyme 25 ºC	2.8 µg
3	DMO Enzyme 37 ºC	2.8 µg
4	DMO Enzyme 55 ºC	2.8 µg
5	DMO Enzyme 75 ºC	2.8 µg
6	DMO Enzyme 95 ºC	2.8 µg
7	DMO Enzyme Control Substance	2.8 µg
8	DMO Enzyme Reference 100 % Equivalence	2.8 µg
9	DMO Enzyme Reference 10 % Equivalence	0.28 µg
10	Broad Range Molecular Weight Markers	40.5 µg

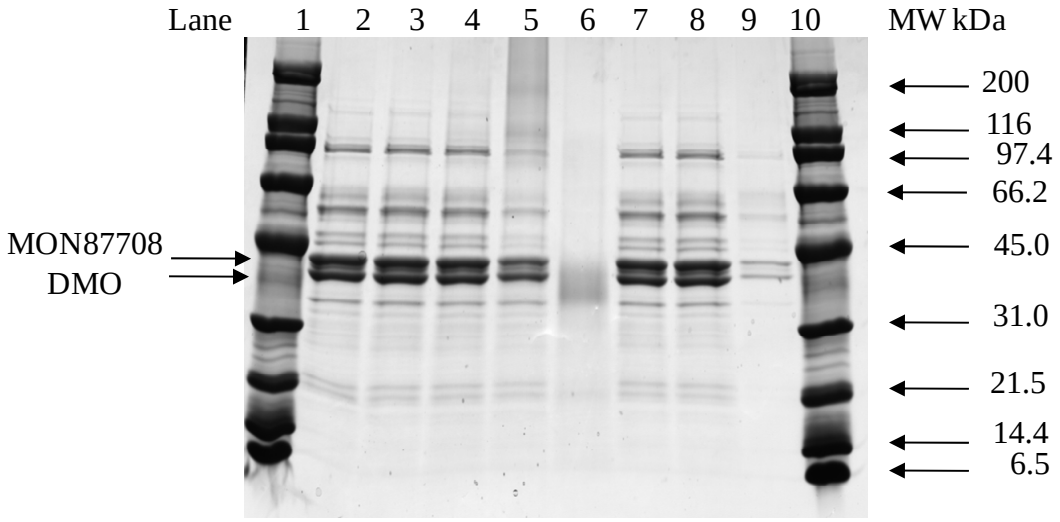

Figure 2. SDS-PAGE of DMO Enzyme Demonstrating the Effect After 30 Minutes at Elevated Temperatures on Protein Structural Stability

Heat-treated samples of DMO enzyme (2.8 µg total protein) separated on a Tris-glycine 4-20 % polyacrylamide gel under denaturing and reducing conditions. Gel was stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10.

Lane	Description	Total Amount
1	Broad Range Molecular Weight Markers	40.5 µg
2	DMO Enzyme 25 ºC	2.8 µg
3	DMO Enzyme 37 ºC	2.8 µg
4	DMO Enzyme 55 ºC	2.8 µg
5	DMO Enzyme 75 ºC	2.8 µg
6	DMO Enzyme 95 ºC	2.8 µg
7	DMO Enzyme Control Substance	2.8 µg
8	DMO Enzyme Reference 100 % Equivalence	2.8 µg
9	DMO Enzyme Reference 10 % Equivalence	0.28 µg
10	Broad Range Molecular Weight Markers	40.5 µg

Appendix 1 List of Current SOPs Used in the Study

AG-ME-0388-03	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-EQ-0599-05	Bio-Rad GS-800 Densitometer System
BR-ME-1244-01	Dicamba Mono-oxygenase Activity Assay