Study Title

Characterization of the Dicamba Mono-Oxygenase (DMO) Enzyme Isolated from the Seed of MON 87708

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Sponsor and Performing Laboratory

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Laboratory Project ID

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MSL Number: MSL0022497

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

Submitter — Date

Melinda C. McCann
Sponsor Representative — 22 Feb 2010 — Date

Cunxi Wang, Ph.D.
Study Director — 02/22/2010 — Date

Quality Assurance Unit Statement

Study Title: Characterization of the Dicamba Mono-Oxygenase (DMO) Enzyme Isolated from the Seed of MON 87708

Study Number: REG-09-576
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	Date Reported to Management
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02/04/2010	Draft Report Review	02/04/2010	02/04/2010
02/04/2010	Raw Data Audit	02/04/2010	02/04/2010

Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

02/22/2010
Date

Study Certification Page

The results reported in this report accurately reflect the data generated under Study Number REG-09-576.

Signatures of Final Report Approval:

Cunxi Wang, Ph.D.
Study Director

02/22/2010
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Stephen R. Hill, M.S.
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Study Information

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Abbreviations and Definitions[1]

AA	Amino Acid
APS	Analytical Protein Standard
BSA	Bovine Serum Albumin
CHT	Ceramic Hydroxyapatite
CFR	Code of Federal Regulations
Con A	Concanavalin A
CTP	Chloroplast Transit Peptide
α-Cyano	α-Cyano-4-hydroxycinnamic acid
Da	Dalton
DCSA	3,6-dichlorosalicylic acid
Dicamba	3,6-dichloro-2-methoxybenzoic acid
DHB	2,5-Dihydroxybenzoic Acid
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
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act (U.S.)
GLP	Good Laboratory Practice
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
LB	Loading buffer 1X: 62.5mM Tris-HCl, 4% (v/v) 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, 0.005% (w/v) bromophenol blue, 10% (v/v) glycerol, pH 6.8
L	Liter
MALDI-TOF	Matrix Assisted Laser Desorption and Ionization - Time of Flight
MH+	Protonated mass ion
MS	Mass Spectrometry
MW	Molecular Weight
MWCO	Molecular Weight Cutoff
NFDM	Non-Fat Dried Milk
PAGE	Polyacrylamide Gel Electrophoresis
PBST	Phosphate Buffered Saline containing 0.05% (v/v) Tween-20
PEP	Phosphoenolpyruvate

[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.

PMSF	Phenylmethylsulfonyl fluoride
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene Difluoride
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
Sinapinic Acid	3,5-dimethoxy-4-hydroxycinnamic acid
SOP	Standard Operating Procedure
TFA	Trifluoroacetic Acid
U.S.	United States
VOI	Verification of Identity

1.0 Summary

Monsanto Company has developed a 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) protein to confer tolerance to dicamba herbicide.

In order to produce protein for a safety assessment, the DMO enzyme was purified from the seed of MON 87708 and its physicochemical properties were characterized. Structurally, the MON 87708-produced 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 Rubisco small subunit. This report describes the results of the characterization of the DMO enzyme present in MON 87708.

A panel of analytical techniques was used to characterize the DMO enzyme. These techniques included: 1) total protein concentration determination using amino acid analysis, 2) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry to determine protein purity and apparent molecular weight (MW), 3) N-terminal sequence analysis, 4) matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) peptide fingerprint analysis, 5) immunoblotting, 6) DMO enzyme specific activity assay, and 7) glycosylation analysis. The stability of the DMO proteins during the experimental phase of the study was also assessed using SDS-PAGE by estimating the purity and apparent molecular weight of the proteins after storage at 4, - 20 and -80 °C.

Identities of DMO proteins (DMO and DMO+27) were confirmed using immunoblot analysis with anti-DMO specific antibody, N-terminal sequencing, and MALDI-TOF MS analysis of peptides produced after trypsin digestion. The antibody specifically detected both DMO and DMO+27 on an immunoblot. The N-terminal sequence of the first 15 amino acid residues of both DMO and DMO+27 was confirmed with the exception of the N-terminal methionine, which was missing in DMO and methylated in DMO+27. MALDI-TOF MS analyses of the DMO proteins yielded peptide masses consistent with their expected peptide masses. The identified tryptic peptide masses from DMO yielded 77.4% overall coverage of the entire sequence while the observed tryptic peptide fragments generated from DMO+27 covered 82.0% of the expected protein sequence. Additionally, the missing N-terminal methionine in DMO and methylation of the N-terminal methionine in DMO+27 were further confirmed by MALDI-TOF MS analysis. The total protein concentration of the DMO enzyme preparation was determined to be 0.18 mg/ml. Purity of the DMO proteins was 81%. The apparent molecular weights of DMO and DMO+27 were 39.8 and 42.0 kDa, respectively. Neither DMO nor DMO+27 was glycosylated. The DMO enzyme was active, with a specific activity of 62.21 nmol DCSA/min/mg. The DMO proteins were stable for at least 12 days when stored in a -20 °C or -80 °C freezer, but was not stable when stored in a 4 °C refrigerator for 12

days. Taken together, these data provide a detailed characterization of the DMO enzyme isolated from the seed of MON 87708.

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 to 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 terminus 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 fully 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 B). One band corresponds to the mature DMO protein (referred as to DMO), whereas the second band is DMO plus 27 amino acids originating from the pea Rubisco small subunit on its N-terminus (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).

3.0 Purpose

The purpose of this study was to characterize the physicochemical and functional properties of the DMO enzyme isolated from the seed of MON 87708.

4.0 Materials

4.1 MON 87708-Produced DMO enzyme (Test substance)

The DMO enzyme (Orion lot 11261646) purified from the seed of MON 87708 was used as the test substance. The seed was harvested under production plan PPN-08-183 from seed lot 11214058 and 11214059. Copies of the VOI for the seed have been archived under the Orion lot 11214058 and 11214059. The DMO enzyme purified from the seed of MON 87708 was stored in a - 80°C freezer in a buffer solution containing 50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 1 mM DTT and 5% glycerol. The purification records for this enzyme have been archived under the Orion lot 11261646 according to the current version of SOP BR-PO-0722.

4.2 Assay Controls

Protein molecular weight standards (SeeBlue Plus2 Pre-stained, Invitrogen, Carlsbad, CA) were used to calibrate SDS-PAGE gels and verify protein transfer to Polyvinylidene difluoride (PVDF) membranes. The broad range SDS-PAGE molecular weight standards (Bio-Rad, Hercules, CA) were used to determine the apparent molecular weight of the DMO proteins. A peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass and intact mass analysis. Transferrin provided with the kit (GE healthcare, Piscataway, NJ) was used as a positive control for glycosylation analysis.

5.0 Methods

5.1 Protein Purification

The DMO enzyme was purified from defatted flour made from the seed of MON 87708 prior to the initiation of this characterization plan. The purification procedure was not performed under a GLP plan; however, all procedures were documented on worksheets or notebooks and, where applicable, SOPs were followed. The DMO enzyme was purified using a combination of extraction, filtration and diafiltration, and various chromatographic separations. A detailed description of the purification process was filed under Orion lot 11261646, and is briefly described below.

Defatting of seed from MON 87708 was done at Pilot Plant Corporation in Saskatoon, Canada (Production Plan number: PPN-09-131). The seed was cracked, dehulled, and then ground to meal in the presence of dry ice. The meal was then solvent extracted, dried, and returned to the Creve Coeur site and stored in a -20°C cold room. Aliquots of the de-fatted flour were used as starting material in the purification process.

Approximately 7.5 kg of defatted MON 87708 flour were extracted with 75 liters (L) of extraction buffer [25 mM potassium phosphate, pH 7.2, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM E-64, and 0.1 μM bestatin]. The extraction was done at room temperature (RT) for 2 hours using a lightnin mixer with slow stirring (The speed was set up at 5. Graham Transmissions Inc, Menomonee Falls , WI). The slurry was filtered using an Ertel Alsop filter press (Kingston, NY) with Die 42 micro media filter pads and a Cuno filter (Hagedorn and Gannon Co., Inc) after the addition of 7.5 kg of diatomaceous earth [5.6 kg fine hy-flo (Celite Corporation, Lompoc, CA) and 1.9 kg Celite 560 coarse (Sigma-Aldrich, St Louis, MO)]. The pads on the press were precoated with 1.8 kg of fine hy-flo prior to the filtration of the extract. After washing the press with extraction buffer, the final volume collected was 150 L.

The extract was then concentrated to 75 L using a hollow fiber cartridge with a 30,000 kDa molecular weight cutoff (MWCO) (GE Healthcare, Piscataway, NJ) at RT. Solid KCl was added to a final concentration of 0.15 M. Seventy-five L of the concentrated extract was dialfiltered with 4 changes of 25 L each of a phenyl sepharose equilibration buffer (25 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 μM E-64, 0.1 μM bestatin, and 0.15 M KCl).

The first chromatographic step was run at RT. A 30 L phenyl sepharose (GE Healthcare, Piscataway, NJ) column equilibrated with phenyl sepharose equilibration buffer was charged with the dialfiltered extract and then washed with 3 column volumes (CV) of the same buffer. A single CV of elution buffer (50 mM triethanolamine, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 μM E-64, 0.1 μM bestatin, and 100 μM dicamba) was loaded onto the column, the flow stopped and the column incubated for 1 h. The released protein was eluted with an additional volume of elution buffer and stored at 4°C.

Solid potassium phosphate was added to the phenyl column elution to a final concentration of 25 mM, the pH adjusted to 8.0. Fresh DTT and protease inhibitors were added to the same concentrations as described above. A 3 L ceramic hydroxyapatite column (CHT) (Bio-Rad, Hercules, CA) was packed at 4°C and equilibrated in a buffer containing 25 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 μM E-64, 0.1 μM bestatin, and 100 μM dicamba. Half of the adjusted phenyl elution was charged on the column. The column was washed with 2 CV of the CHT equilibration buffer. The bound proteins were then eluted with 400 mM potassium phosphate, pH 8.0. The flow-through containing the DMO enzyme, detected by immunoblot analysis, was collected. The eluted fractions without the DMO enzyme were discarded and the column was reequilibrated. The second half of the phenyl elution was processed with the CHT column in the same manner. The flow-through collected from each CHT column run was combined into a single pool.

The flow-through pool from the CHT step was then charged on a 5 L DEAE macroprep (Bio-Rad, Hercules, CA) column at 4 °C and equilibrated in a buffer containing 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 μM E-64, 0.1 μM bestatin, and 100 μM dicamba. Before loading, fresh, solid DTT and protease inhibitors were added to the CHT column flow-through pool. The DEAE column was then washed with 5 CV of the DEAE equilibration buffer and then 5 CV of the equilibration buffer plus 100 mM NaCl. The DMO enzyme was eluted with a 20 CV linear NaCl gradient from 100 mM to 350 mM in the equilibration buffer. The DEAE column was then washed with the equilibration buffer containing 1 M NaCl. The fractions collected throughout the gradient were analyzed by immunoblot and those fractions containing the DMO enzyme were pooled.

To concentrate the DEAE macroprep pool, it was diluted with the DEAE equilibration buffer (to reduce the conductivity) and then charged onto a 1 L DEAE macroprep column. After charging, the column was washed with 3 CV of equilibration buffer and then eluted with minimal volume of the equilibration buffer plus 1 M NaCl. This concentrated the DEAE pool from 16 to 1.6 L.

The concentrated DEAE macroprep pool was mixed with 1 L of concanavalin A (Con A) sepharose 4B (Sigma-Aldrich, St Louis, MO) that was previously equilibrated with the DEAE equilibration buffer with fresh, solid DTT and protease inhibitors added. The chromatography was run in batch mode at RT. The DMO enzyme did not bind to the resin. After stirring the concentrated DEAE macroprep pool with the equilibrated Con A resin for 1 hour, the resin was filtered out using a Buchner funnel and Whatman filter paper (GE healthcare, Piscataway, NJ). The resin was washed with 3 L of equilibration buffer. All filtrates were combined.

The Con A treated DMO enzyme preparation was then concentrated on ice for ~ 4 h using a tangential flow membrane (Sartorius-Stedim, Goettingen, Germany) with a 100 kDa MWCO. After a 10x concentration step, the DMO enzyme was dialfiltered with 10 exchanges of DEAE macroprep equilibration buffer containing fresh DTT and protease inhibitors as described above.

The concentrated DMO enzyme was further purified on CHT at RT. A 1.0 L column was packed and equilibrated with the DEAE macroprep equilibration buffer with fresh DTT and protease inhibitors. In the complete absence of phosphate, the DMO enzyme bound to the column. The column was washed with 3 CV of equilibration buffer. The protein was eluted with a linear phosphate gradient using an elution buffer (400 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 μM E-64, 0.1 μM bestatin, and 100 μM dicamba) increasing from 0% to 50 % over 10 CV. The fractions containing at least 80% pure DMO enzyme as estimated by gel densitometry of fractions analyzed by SDS-PAGE were pooled.

This procedure was repeated with 2 batches of 7.5 kg of defatted flour from MON 87708. After analysis, all CHT pools were combined into a single final pool that was concentrated on ice for ~ 2 h to 370 ml with a tangential flow membrane with a 30 kDa MWCO. The concentrated pool was dialyzed versus enzyme storage buffer (50 mM potassium phosphate, pH 8.0, 100 mM sodium chloride, 5% glycerol, and 1 mM DTT). There were 2 exchanges of 4 L each over 2 days and the dialysis was conducted at 4°C. The dialysate was aliquoted, assigned APS lot 11261646 and stored at -80°C.

5.2 Protein Concentration

The total protein concentration of the DMO enzyme preparation was determined by amino acid analysis (AAA) using AccQ-Tag™ derivatization (Waters Corporation, Milford, MA), which allows for high sensitivity fluorescent detection of amino acids. In order to avoid the interference from buffer components during protein hydrolysis,

protein samples were precipitated using ethanol. The DMO enzyme preparation was diluted to approximately 0.1 µg/µl with water, based on pre-characterization estimates. In a hydrolysis tube, approximately 1 µg of protein samples (~10 µl) were mixed with 200 µl of chilled 95% ethanol. After incubation overnight at -20 ºC, samples were centrifuged at 12,000 rpm in a microcentrifuge for at least 45 min at 2-8°C. The supernatant was removed and discarded. Precipitates were then washed twice with 200 µl of chilled acetone. Along with replicates of the test sample, a hydrolysis blank, 4 dilutions of a calibration standard (NIST), and Bovine Serum Albumin (BSA) control were also analyzed. An internal calibrant, α-aminobutyric acid, was included in all non-blank samples. All samples were evaporated to dryness in hydrolysis tubes using a Speed-Vac concentrator, then 500 µl of hydrolysis solution (6N HCl/1% phenol) was added and the tubes were transferred to a vacuum chamber. Samples were hydrolyzed for 90 min at 150±2 °C under vacuum. After cooling, the vacuum was released and the hydrolysates were evaporated to dryness using a Speed-Vac concentrator and then reconstituted in 20 µl of 20 mM HCl by vortexing. Sixty µl of AccQ-Fluor Borate Buffer and 20 µl of AccQ-Fluor reagent were added sequentially to each vial, vortexing after each addition. The samples were transferred individually to autosampler vials, capped, and heated to 55 °C for 10 min. Samples were analyzed using a 2695 Separation Module (Waters Corporation, Milford, MA) equipped with a reverse-phase C-18 column for separation of AccQ-Tag derivatized amino acids. Chromatographic data were collected and concentrations were calculated using Atlas™ 2003 software (Thermo Fisher Scientific Inc, Waltham, MA).

5.3 Protein Identity

5.3.1 Immunoblot Analysis

An aliquot of the DMO enzyme preparation was diluted with water and mixed with 5x loading buffer (LB) [312 mM Tris-HCl, 20% (v/v) 2-mercaptoethanol, 10% (w/v) SDS, 0.025% (w/v) bromophenol blue, and 50% (v/v) glycerol, pH 6.8)], heated at 99 °C for 3 min, and applied on a pre-cast Tris glycine 4 - 20% polyacrylamide gradient 10-well gel (Invitrogen, Carlsbad, CA). Three amounts (20, 30, and 40 ng) of the DMO enzyme preparation were loaded in duplicate on the gel. Electrophoresis was performed at a constant voltage of 125 V for 90 min. Pre-stained molecular weight markers (SeeBlue Plus2 Pre-stained, Invitrogen, Carlsbad, CA) were loaded in parallel to verify electrotransfer of the proteins to the membrane and estimate the size of the immunoreactive bands observed. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 90 min at a constant voltage of 25 V.

For immunodetection, the membrane was blocked for 1 h with 10% (w/v) Non-Fat Dried Milk (NFDM) in 1× Phosphate Buffered Saline containing 0.05% (v/v) Tween-20 (PBST). The membrane was then probed with a 1:3,000 dilution of

goat anti-DMO antibody in 5% (w/v) NFDM in PBST for 1 h. Excess antibody was removed using three 10 min washes with PBST. Finally, the membrane was probed with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Thermo, Rockford, IL) at a dilution of 1:10,000 in 5% (w/v) NFDM in PBST for 1 h. Excess HRP-conjugate was removed using three 10 min washes with PBST. All incubations were performed at RT. Immunoreactive bands were visualized using the ECL (Enhanced Chemiluminescence) detection system (GE Healthcare, Piscataway, NJ) and exposed to Amersham Hyperfilm (GE, Healthcare, Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan). Three exposures (20, 30 and 60 s) were taken and the 20 s exposure was scanned using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA).

5.3.2 N-Terminal Sequencing

N-terminal sequencing using automated Edman degradation chemistry was used to confirm the identity of the DMO proteins.

Ninety µl of the DMO enzyme preparation were mixed with 22.5 µl of 5x LB, heated at 99 °C for 3 min and loaded in 4 lanes (10 µl/lane) onto a pre-cast Tris glycine 4 - 20% polyacrylamide gradient 10-well gel (Invitrogen, Carlsbad, CA). Electrophoresis was carried out at constant voltage of 150 V for 80 min. Proteins in the gel were electrotransferred to a PVDF (Invitrogen, Carlsbad, CA) membrane for 90 min in a buffer containing 10 mM CAPS, pH 11 and 10% methanol at a constant voltage of 25 V. Pre-stained molecular weight markers (SeeBlue Plus2 Pre-stained, Invitrogen, Carlsbad, CA) were loaded in parallel to verify the electrotransfer of protein to the membrane and estimate the size of the stained bands observed. The blot was stained with Ponceau S (Sigma-Aldrich, St Louis, MO).

Following electrotransfer and staining, the bands corresponding to DMO and DMO+27 were excised based on apparent molecular weight from the blot and N-terminal sequence analyses were performed for 15 cycles using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied Biosystems 494 Procise Protein Sequencing System with 140C Microgradient HPLC pump, ABI 785A Programmable Absorbance Detector and Procise™ Control Software (version 2.1) were used. Chromatographic data were collected using Atlas™ 2003 software (Thermo Fisher Scientific Inc, Waltham, MA). A PTH (Phenylthiohydantoin) -amino acid standard mixture (Applied Biosystems, Foster City, CA) was used as the calibration standard in the chromatographic 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 pmol β-lactoglobulin (Applied Biosystems, Foster City, CA), was analyzed before and after the analysis to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

5.3.3 MALDI-TOF Tryptic Mass Fingerprint Analysis

MALDI-TOF MS was used to confirm the identity of the DMO proteins.

An aliquot (89.5 µl) of the DMO enzyme preparation was mixed with 22.5 µl of 5x LB, heated at 99 °C for 3 min and loaded in 4 lanes (3 lanes each loaded for 4.2 µg and 1 lane for 3.1 µg of total protein) onto a pre-cast Tris glycine 4 - 20% polyacrylamide gradient 10-well gel (Invitrogen, Carlsbad, CA). Pre-stained MW markers (SeeBlue Plus2 Pre-stained, Invitrogen, Carlsbad, CA) were loaded in parallel to estimate the size of the stained bands observed. Electrophoresis was carried out at constant voltage of 150 V for 80 min. Following electrophoresis, the gel was stained with Brilliant Blue G Colloidal (Sigma-Aldrich, St Louis, MO). The bands corresponding to DMO and DMO+27 were excised from 4 lanes of the gel, destained, reduced, and alkylated. Briefly, each gel band was destained for 30 min by incubation in 100 µl of destain solution (40% methanol, 50% water, and 10% glacial acetic acid) in a microfuge tube. This step was repeated twice for 60 min each, removing all visible Brilliant Blue G Colloidal stain. Following destaining, the gel bands were incubated in 100 µl per band of 100 mM ammonium bicarbonate buffer for 16 hrs at RT. The protein was reduced in 100 µl of 10 mM DTT solution for 2 h at 37 °C. After removing the reducing solution, the protein in the gel was alkylated by incubating in 100 µl of 20 mM iodoacetic acid. The alkylation reaction was allowed to proceed at RT for 20 min in the dark. The gel containing the protein band was incubated in 200 µl of 25 mM ammonium bicarbonate buffer for 15 min at RT. This step was repeated two additional times for 15 min each, following then the gel band was dried using a Savant Speed Vac concentrator (Holbrook, NY). Each gel band was rehydrated with 20 µl of 0.02 µg/µl trypsin in 25 mM ammonium bicarbonate and 10% acetonitrile, and incubated for about 1 h at RT. Following the incubation, the excess solution was removed and the gel/trypsin reaction mixture was incubated overnight at 37 °C in 40 µl of 25 mM ammonium bicarbonate and 10% acetonitrile. The following day, the sample was sonicated for 5 min, and the supernatant transferred to a new tube and dried using a Speed Vac concentrator (Extract 1). The gel band was resuspended in 30 µl of a solution consisting of 60% acetonitrile, 0.1% trifluoroacetic acid (TFA) and 0.1% octyl-β-D-glucopyranoside, and sonicated for 5 min. After transfer of the supernatant to a new tube, this step was repeated once and the combined supernatants were dried using the Speed Vac concentrator (Extract 2). Extracts 1 and 2 were separately dissolved in 20 µl 0.1% TFA and then dried using a Speed Vac concentrator. Finally, Extract 1 was dissolved in 5 µl of 50% acetonitrile/0.1% TFA, while Extract 2 was dissolved in 10 µl of the same solution. To maximize the solubilization, each sample was sonicated for 5 min.

Mass spectral analyses were performed as follows: Mass calibration of the instrument was performed using an external peptide mixture (Sequazyme™

Peptide Mass Standards Kit, Calibration Mixture 2, Applied Biosystems, Foster City, CA). The samples Extract 1 and Extract 2 (0.3 µl) were co-crystallized with 0.75 µl each of the following matrix solutions: dihydroxybenzoic acid (DHB), α-cyano-4-hydroxy cinnamic acid (α-cyano), and 3, 5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) at separate locations on the analysis plate. The samples in DHB matrix were analyzed in the 300 to 5,000 Dalton (Da) range. The samples in α-cyano matrix were analyzed in the 500 to 5,000 Da range. The samples in sinapinic acid matrix were analyzed in the 500 to 7,000 Da range. Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993), except above 3,000 Da, where mass-averaged values were used. GPMAW32 software (Lighthouse data, Denmark) was used to generate a theoretical trypsin digestion of the deduced DMO and DMO+27 amino acid sequences. Masses were calculated for each theoretical peptide and compared to the raw experimental mass data. Below 1000 Da, experimental masses (MH+) were assigned to peaks when two or more isotopically resolved peaks were observed. Above 1000 Da, experimental masses (MH+) were assigned to peaks when three or more isotopically resolved peaks were observed. Peaks were not assessed if the peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger mass signal.

5.4 Apparent Molecular Weight and Purity Estimation by SDS-PAGE

An aliquot of the DMO enzyme preparation was mixed with 5x LB to a final total protein concentration of 0.09 µg/µl and heated at 99 °C for 3 min. Molecular weight markers (Broad Range MW Marker, Bio-Rad, Hercules, CA) was diluted to a final total protein concentration of 0.9 µg/µl. The DMO enzyme preparation was loaded in duplicate at 0.5, 1.0 and 1.5 µg of total protein per lane onto a pre-cast Tris glycine 4 - 20% polyacrylamide gradient 10-well gel (Invitrogen, Carlsbad, CA). The molecular weight markers were loaded in parallel at 4.5 µg protein per lane. Electrophoresis was performed at a constant voltage of 125 V for 90 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) acetic acid for 30 min, stained for 16 h with Brilliant Blue G Colloidal stain (Sigma-Aldrich, St. Louis, MO), destained 30 s with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and finally destained with 25% (v/v) methanol for 6.0 h. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). Molecular weight markers were used to estimate the apparent molecular weight of each observed band. All visible bands within each lane were quantified using Quantity One software. Apparent molecular weight was obtained for the DMO proteins while the purity was calculated based on the addition of the average purity of both proteins. The results were reported as an average of all six loadings containing the DMO proteins.

5.5 Glycosylation Analysis

Glycosylation analysis was used to determine whether the DMO proteins were post-translationally modified with covalently bound carbohydrate moieties. An aliquot of the DMO enzyme preparation, the positive control, transferrin provided by the kit (GE Healthcare, Piscataway, NJ) were each diluted with water and mixed with 5x LB. These samples were heated at 101.0 °C for 3 min, cooled, and loaded on a Tris glycine 4 - 20% polyacrylamide gradient 10-well mini-gel (Invitrogen, Carlsbad, CA). Three amounts of transferrin (50, 100 and 200 ng) and two amounts (100 and 200 ng) of the purity-corrected DMO proteins were loaded in the gel. SeeBlue Plus2 Pre-stained protein molecular weight markers (Invitrogen, Carlsbad, CA) were loaded to verify electrotransfer of the proteins to the membrane. Electrophoresis was performed at a constant voltage of 150 V for 87 min. Electrotransfer to a 0.45 μm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 60 min at a constant voltage of 25 V followed by 30 min at 30V.

Carbohydrate detection was performed directly on the PVDF membrane using the GE Healthcare Glycosylation Detection Module (Cat. No. RPN 2190, Piscataway, NJ). The manufacturer's protocol was followed and all the reagents except phosphate buffered saline (PBS) were provided with the kit. All steps were performed at RT. Following electrotransfer to PVDF membrane, the blot was incubated first in 30 ml of PBS for 10 min, then in 20 ml of 10 mM $NaIO_4$ for 20 min in darkness. Following the treatment, the membrane was rinsed twice with PBS and washed three times with 20 ml PBS for 10 min each. The membrane was incubated with 20 ml solution consisting of 0.125 mM biotin-hydrazide, 100 mM acetate, pH 5.5 for 60 min followed by two PBS rinses and three 10 min washes with PBS. The membrane was blocked for 60 min with 5% blocking reagent in PBS followed by two PBS rinses and three 10 min washes with PBS. The membrane was incubated with strepavidin-HRP at 1:6000 dilution for 30 min. After two PBS rinses and three 10 min washes with PBS, the membrane was then developed with ECL detection reagents by mixing 1 ml of Reagent 1 and 1 ml of Reagent 2. After a 1 min incubation, the excess detection solution was removed by blotting with paper towels and the blot was exposed to Hyperfilm ECL (GE Healthcare, Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan). Three exposures (30 s, 1 min and 2 min) were performed and the 30 s exposure was shown in this report. The image was captured using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA).

5.6 Specific Activity Assay

The specific activity of the DMO enzyme was determined by quantifying the conversion of 3,6-dichloro-2-methoxybenzoic acid (dicamba) to 3,6 dichlorosalicylic acid (DCSA) via HPLC (Agilent Technologies 1100 series, Santa Clara, CA) separation and fluorescence detection (Agilent Technologies 1200 series, G1321A). The assay was performed according to the draft of the SOP BR-ME-1244. Briefly,

standard assays were conducted in 200 µl solution consisting of 25 mM potassium phosphate, pH 7.2, 3.4 µg Ferredoxin, 3.4 µg Reductase, 0.5 mM $FeSO_4$, 10 mM $MgCl_2$, 0.7 mM NADH, 0.3 mM dicamba, 2 µl (42.48 U/ml) of formaldehyde dehydrogenase and either 2 µg DMO enzyme or 1 µg HIS-DMO enzyme as an assay positive control. The reactions were performed in PCR tubes (Sorenson, Salt Lake City, UT) and incubated at 30°C for 15 min. The reactions were initiated by the addition of dicamba and quenched with the addition of 50 µl of 5% H_2SO_4. The reactions were then filtered using Whatman Anotop 10 filters (0.2 µm, GE healthcare, Piscataway, NJ), and 40 µl was transferred to a HPLC sample vial (200 µl, Agilent, Santa Clara, CA) for analysis. Twenty-five µl of the filtered reaction was injected onto a Phenomenex® Synergi 4 µm C18/ODS Hydro-RP column (150 x 4.6 mm ID, Torrance, CA). The mobile phase was: solvent A: 21.5 mM phosphoric acid and solvent B: 100% acetonitrile running at 1.5 ml/min. DCSA was eluted from the column using a linear gradient from 90% to 40% solvent A for the first 14 min, followed by a step to 10% solvent A for 1 min and then re-equilibration at 90% solvent A for 10 min before the next injection. DCSA was monitored by the detection of fluorescent emission at 424 nm (excitation 306 nm) and quantified relative to a standard curve of DCSA generated using 0.1, 0.3, 0.6, 0.9, 1.2, 2.4, and 4.8 nmol/250 µl. Chromatographic data were collected using Atlas™ 2003 software (Thermo Fisher Scientific Inc, Waltham, MA). The specific activity was calculated based on the amount of purity corrected DMO enzyme added to the reaction mixture and expressed as nmol of DCSA produced per minute per mg of the DMO enzyme (nmol/min/mg).

5.7 Storage Stability

The short-term stability of the DMO proteins was evaluated by comparing the purity and MW values before and after storage for 12 days (the duration of the experimental phase) in a 4 ºC refrigerator, and -20 ºC and -80 ºC freezers. Day 0 of the stability analysis corresponds to the purity and molecular weight determination described in Section 5.4. On day 12, an aliquot was removed from a 4 °C refrigerator, and -20 and -80 °C freezers, respectively, and mixed with 5x LB to a final protein concentration of 0.09 µg/µl, heated at ~100 °C for 3 min, and loaded in duplicate at three amounts (0.5, 1.0 and 1.5 µg total protein per lane) onto a Tris glycine 4 - 20% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA), respectively. Staining and densitometric analysis were performed as described in section 5.4. The protein sample was considered to be unstable if a >10% change in purity and/or molecular weight was observed relative to the value determined on Day 0.

6.0 Data Rejected or Not Reported

One MALDI-TOF-MS analysis was rejected because less protein than planned was used for the analysis which resulted in high background and low mass signal. One specific

activity analysis for an assay control was rejected because the Atlas program incorrectly analyzed the baseline position.

7.0 Study Protocol Amendment

There was one amendment to the original protocol. The protocol was amended to change the term grain to the term seed. This change makes the text consistent with the terminology Monsanto Company uses to describe the harvested soybean material which has been used in previous regulatory submission. There was no impact on the study because no data were changed.

8.0 Deviation

There was one SOP-related deviation. The SOP for amino acid analysis using AccQ-Tag (SOP BR-ME-1139) has a defined criterion that all test sample replicates must be within 20% of the average protein concentration. One of the replicates (A3) did not meet this criterion, but it was established that the replicate of this sample was contaminated as evidenced by the large amino acid yields of glycine, which was ~5 fold that of the other replicates. In addition, the composition of test sample replicate A3 was substantially different from that of the other test sample replicates and the DMO theoretical amino acid composition, which removes the possibility that an incorrect volume of test sample was loaded into the hydrolysis tube. A jackknife residual statistical test, which identifies outliers, was performed for the five test sample replicates and identified A3 as an outlier. Since test sample A3 was determined to be an outlier it was removed from the average concentration calculation and the remaining four replicates soundly met the established criterion. The analysis was not rejected as specified in the SOP. There was no adverse impact on the study by removing the outlier replicate (A3). The removal of the outlier produced a reliable and accurate average protein concentration using the 4 remaining replicates.

9.0 Control of Bias and Quality Measures

Controls and standards were included with each analysis. A protein standard (β-lactoglobulin) was sequenced before and after N-terminal sequence analysis of DMO and DMO+27 to ensure instrument performance. A four-peptide mixture from the Sequazyme Peptide Mass Standards kit (Applied Biosystems, Foster City, CA) was used to calibrate the MALDI-TOF mass spectrometer. Replicate analyses were used for the immunoblot, purity and MW determination, stability, and glycosylation analyses. For the glycosylation analysis, transferrin provided with the kit (GE healthcare, Piscataway, NJ) was used as the positive control.

10.0 Results and Discussion

10.1 Protein Concentration

The concentration of the DMO enzyme preparation was 0.18 mg/ml based on amino acid analysis (Table 1).

10.2 Protein Identity

Identities of the DMO proteins were confirmed using three analytical methods: immunoblot analysis, N-terminal sequencing, and tryptic-mapping with MALDI-TOF MS analysis.

10.2.1 Immunoblot Analysis Using an Anti-DMO Antibody

On the immunoblot, the anti-DMO antibody recognized two bands migrating at the expected apparent molecular weights of approximately 39 kDa (DMO) and 42 kDa (DMO+27), respectively (Figure 1). As expected, the intensity of the immunoreactive bands increased with increasing amount of protein loaded. No additional bands were observed in the DMO enzyme preparation. This immunoblot analysis confirmed identities of the DMO proteins. The 20 s exposure was used for this analysis.

10.2.2 N-Terminal Sequencing

N-terminal sequence analysis of the two major protein bands on the PVDF membrane corresponding to ones observed on the immunoblot returned a sequence of 15 amino acids per band that matched the expected N-terminal sequences of DMO and DMO+27 (Figures 2 and 3), respectively, which were deduced from the *dmo* gene present in the seed of MON 87708 (Song et al., 2009). The N-terminal methionine residue in DMO was not observed, indicating that it was removed during posttranslational processing of the precursor protein. Processing of the N-terminal methionine occurs through methionine aminopeptidase (Arfin and Bradshaw, 1988; Bradshaw et al., 1998; Polevoda and Sherman, 2000) and is common in many organisms.

In the case of DMO+27, the first cycle of N-terminal sequence analysis resulted in a PTH-amino acid derivative which corresponds to a methylated modification of the N-terminal methionine. It is well-known that the amino-terminal methionine of the Rubisco small subunit is post-translationally modified to N-methyl-methionine *in vivo* in pea and other plant species (Grimm et al., 1997; Whitney and Andrews, 2001). The N-terminal sequencing results clearly confirm the identity of DMO and DMO+27 isolated from the seed of MON 87708.

10.2.3 MALDI-TOF Tryptic Mass Fingerprint Analysis

The identity of the DMO proteins was further confirmed by tryptic mapping with MALDI-TOF MS analysis of the fragments. Prior to analysis, DMO and DMO+27 were separated by SDS-PAGE, reduced, alkylated and digested with trypsin. The ability to identify a protein using this method is dependent upon matching a sufficient number of observed tryptic peptide fragment masses with predicted tryptic peptide fragment masses. In general, protein identification made by peptide mapping is considered to be reliable if the measured coverage of the

sequence is 15% or higher with a minimum of five matched peptides (Jensen et al., 1997).

There were 26 unique peptides identified from DMO that corresponded to the expected masses of the DMO trypsin-digested peptides while 29 fragments from DMO+27 were found to match the expected peptides (Tables 2 and 3). The identified peptides were used to assemble a coverage map indicating the matched peptide sequences for the entire DMO and DMO+27 protein sequences, resulting in 77.4% (263/340) and 82.0% (301/367) coverage of the amino acid sequence, respectively (Figures 4 and 5). Notably, N-terminal peptides of both DMO and DMO+27 were identified. The data were consistent with the N-terminal sequencing data that determined the N-terminal methionine was missing in DMO and methylated in DMO+27 (Tables 2 and 3; Figures 2 and 3). These results confirm the identity of the DMO proteins.

10.3 Apparent Molecular Weight and Purity Determination

The DMO enzyme preparation was separated using SDS-PAGE and the gel stained using Brilliant Blue G Colloidal stain (Sigma-Aldrich, St. Louis, MO). Purity and apparent molecular weight of DMO and DMO+27 were determined using densitometric analysis of the gel (Figure 6). As summarized in Table 4, apparent molecular weight values were averaged from duplicated loads of 0.5, 1.0 and 1.5 μg of total protein (Figure 6, lanes 2-7). The predominant bands identified as DMO and DMO+27 were estimated to have apparent molecular weights of 39.8 kDa and 42.0 kDa, respectively. The average purity of the DMO proteins was determined to be 81%. Hence, after purity-based correction, the final concentration of the DMO proteins in the preparation is 0.15 mg/ml.

10.4 Glycosylation Analysis

Many eukaryotic proteins are post-translationally modified with carbohydrate moieties (Rademacher et al., 1988). There are several forms of glycosylation (Caragea et al., 2007). The first occurs on the nitrogen of asparagine side chains, and is known as N-glycosylation. The other form is O-glycosylation, which is the addition of the glycan such as N-acetyl-galactosamine to the β-hydroxyl of either serine or threonine residues. O-glycosylation sites are less well defined (Thanka et al., 2001). The third form is C-glycosylation, a rare form of glycosylation where a sugar is added to a carbon on a tryptophan side chain (Julenius, 2007). The fourth form is GPI (glycosylphosphatidylinositol) anchors. A hydrophobic phosphatidylinositol group is linked to a residue at or near the C-terminus of a protein through a carbohydrate-containing linker (Caragea et al., 2007). To test whether DMO or DMO+27 was glycosylated when expressed in the seed of MON 87708, the proteins were analyzed for the presence of covalently bound carbohydrate moieties using a GE Glycoprotein Detection Module (GE healthcare, Piscataway, NJ). Transferrin, a naturally glycosylated protein, was used as a positive control in the

assay. The results of this analysis are presented in Figure 7. The positive control was clearly detected at the expected molecular weight and in a concentration-dependent manner (Figure 7, lanes 2-4). No detectable glycosylation signal was observed for DMO or DMO+27 at their expected molecular weight positions (39.8 and 42 kDa) (Figure 7, lane 5 and 6). Thus, these results demonstrate that the DMO proteins are not glycosylated.

10.5 Specific Activity

The DMO enzyme is a Rieske oxygenase that catalyzes the formation of DCSA using dicamba as a substrate. In this study, the DMO enzyme activity was determined by measuring the production of DCSA. The specific activity was determined to be 62.21 nmol/min/mg of the DMO enzyme (Table 5). The value represents an average of three independent assays. This result demonstrates that the DMO enzyme isolated from the seed of MON 87708 is active.

10.6 Storage Stability

Storage stability of the DMO proteins was assessed after the protein preparations were stored in a 4°C refrigerator, and -20 and -80 °C freezers for 12 days (Figures 8, 9 and 10). Stability was evaluated by comparison of the apparent molecular weight and purity of the proteins after storage (day 12) to the initial apparent molecular weight and purity values determined on day 0. Apparent molecular weights of DMO and DMO+27 were averaged based on the six loadings summarized in Table 6. None of these values differ in the apparent molecular weights obtained on day 0 by more than 1.2%, suggesting that no significant molecular weight change of either DMO or DMO+27 occurred under these storage conditions.

The purity values for the DMO proteins stored at three different temperatures for 12 days were obtained (Table 7) by densitometric analysis of SDS-polyacrylamide gels stained by Brilliant Blue G Colloidal stain. Purity of the DMO proteins (DMO and DMO+27) was calculated as the average of the six loadings. The difference between day 0 and day 12 in average purity of the DMO proteins was determined to be 23.4%, 6.2% and 9.9% respectively for 4 °C, -20 °C and -80 °C on day 12 (Table 7). The results for storage at -20 °C and -80 °C are within the preset acceptance criterion of less than 10% , but storage at 4 °C did not meet the criterion. These results indicate that the DMO proteins were stable for at least 12 days when stored at -20 °C or -80 °C, but it was not stable at 4 °C for the same period of time.

11.0 Conclusions

A panel of analytical techniques was used to characterize the MON 87708-produced DMO enzyme. Identity of the DMO proteins was confirmed by detection of DMO and DMO+27 with anti-DMO antibodies, identification of the first 15 amino acids of DMO and DMO+27 N-terminus by amino acid sequencing, and mapping of tryptic peptides that yielded 77.4% and 82.0% overall coverage of the expected DMO and DMO+27

protein sequences, respectively. The concentration of the DMO enzyme preparation was 0.18 mg/ml. The purity of the DMO proteins was 81%. Apparent molecular weights of DMO and DMO+27 were 39.8 kDa and 42.0 kDa, respectively. Neither DMO nor DMO+27 was glycosylated. The DMO enzyme had a specific activity of 62.21 nmol DCSA/ min/mg. Finally, the DMO proteins were stable for 12 days when stored in a -20 °C or −80 °C freezer, but were not stable for that time period during storage in a 4 °C refrigerator.

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Table 1. Total Protein Concentration of the DMO Enzyme Preparation

The total protein concentration of the DMO enzyme preparation was determined by amino acid analysis.

Sample #	Concentration (mg/ml)
1	0.1804
2	0.1788
3	0.1709
4	0.2074
Average	0.18 ± 0.02*

* the standard deviation.

Table 2. Summary of the Tryptic Masses Identified for the DMO Protein Using MALDI-TOF MS

Matrix						Expected Mass[1]	Difference[2]	AA position[3]	Fragment
α-Cyano		DHB		Sinapinic acid					
Ext.1	Ext.2	Ext.1	Ext.2	Ext.1	Ext.2				
			331.20			331.22	0.02	304-305	RR
		391.34				391.18	0.16	293-295	EDK
		435.38	435.38			435.27	0.11	206-208	FLR
593.61	593.61	593.51	593.53			593.34	0.27	2-6	ATFVR
720.67	720.68	720.60	720.60			720.37	0.30	131-136	VDPAYR
833.78	833.80	833.74	833.77			833.45	0.33	99-105	SFPVVER
856.77	856.78	856.72	856.75			856.43	0.34	242-248	EQSIHSR
914.89	914.91	914.84	914.88			914.53	0.36	296-303	VVVEAIER
1030.96	1030.97	1030.92	1030.92			1030.57	0.39	284-292	SWQAQALVK
1108.93	1108.95	1108.89	1108.94			1108.50	0.43	167-176	ANAQTDAFDR
		1171.08				1170.63	0.45	194-205	IPGGTPSVLMAK
1276.17	1276.20	1276.19	1276.21	1276.19		1275.73	0.44	26-36	TILDTPLALYR
1287.14		1287.19				1286.70	0.44	293-303	EDKVVVEAIER
1429.18	1429.20	1429.23	1429.26	1429.20		1428.69	0.49	209-221	GANTPVDAWNDIR
		1502.35	1502.37	1502.34		1501.79	0.56	180-193	EVIVGDGEIQALMK
1507.27	1507.27	1507.32				1506.73	0.54	167-179	ANAQTDAFDRLER
1578.24	1578.27	1578.32	1578.33	1578.30		1577.73	0.51	270-283	NFGIDDPEMDGVLR
1745.42	1745.51	1745.59		1745.56		1744.93	0.49	225-241	VSAMLNFIAVAPEGTPK
1762.48	1762.48	1762.62		1762.54		1761.90	0.58	37-52	QPDGVVAALLDICPHR
1994.65	1994.67	1994.76		1994.78		1994.03	0.62	150-166	LLVDNLMDLGHAQYVHR
2143.78	2143.84	2143.94		2143.96	2143.95	2143.12	0.66	7-25	NAWYVAALPEELSEKPLGR
2294.97				2294.93		2294.09	0.88	306-326	AYVEANGIRPAMLSCDEAAVR
2398.86	2398.77			2399.15		2398.08	0.78	249-269	GTHILTPETEASCHYFFGSSR
2581.87				2582.22		2582.34	0.47	225-248	VSAMLNFIAVAPEGTPKEQSIHSR
2700.08				2700.31		2699.25	0.83	106-130	DALIWICPGDPALADPGAIPDFGCR
				4218.47[4]		4217.77[4]	0.70	99-136	SFPVVERDALIWICPGDPALADPGAIPDFGCRVDPAYR

[1]Only experimental masses that matched expected masses are listed in the table.
[2]The number represents the difference between the expected mass and the first column which has the corresponding numbers.
[3]AA position refers to amino acid position within the predicted DMO sequence as depicted in Figure 4.
[4]Mass average

Table 3. Summary of the Tryptic Masses Identified for the DMO+27 Protein Using MALDI-TOF MS

Matrix						Expected Mass[1]	Difference[2]	AA position[3]	Fragment
α-Cyano		DHB		Sinapinic acid					
Ext.1	Ext.2	Ext.1	Ext.2	Ext.1	Ext.2				
			331.19			331.22	0.03	331-332	RR
		435.30				435.27	0.03	233-235	FLR
720.55	720.64	720.47	720.55			720.37	0.18	158-163	VDPAYR
795.61	795.71	795.54	795.66			795.42	0.19	27-33	AMATFVR
833.65	833.73	833.59	833.69			833.45	0.20	126-132	SFPVVER
856.64	856.72	856.58	856.64			856.43	0.21	269-275	EQSIHSR
914.74		914.69				914.53	0.21	323-330	VVVEAIER
1030.80	1030.89	1030.75				1030.57	0.23	311-319	SWQAQALVK
1069.78	1069.92					1069.57	0.21	1-9	M*QVWPPIGK
1108.75	1108.90	1108.71				1108.50	0.25	194-203	ANAQTDAFDR
			1170.98			1170.66	0.32	221-232	IPGGTPSVLMAK
1275.97	1276.12	1275.98	1276.14	1275.97		1275.73	0.24	53-63	TILDTPLALYR
1286.95		1286.97				1286.70	0.25	320-330	EDKVVVEAIER
1428.95	1429.10	1429.00	1429.26	1428.93		1428.69	0.26	236-248	GANTPVDAWNDIR
		1470.93		1469.94		1470.63	0.30	164-176	TVGGYGHVDCNYK
				1502.10		1501.79	0.31	207-220	EVIVGDGEIQALMK
1507.01	1507.18	1507.03				1506.73	0.28	194-206	ANAQTDAFDRLER
1565.13	1565.30	1565.22	1565.34			1564.87	0.26	11-23	KFETLSYLPPLTR
1578.02	1578.14	1578.06	1578.29	1578.04		1577.73	0.29	297-310	NFGIDDPEMDGVLR
1693.26	1693.38	1693.29				1692.97	0.29	10-23	KKFETLSYLPPLTR
1745.17	1745.36	1745.28	1745.51	1745.22		1744.93	0.24	252-268	VSAMLNFIAVAPEGTPK
1762.17	1762.37	1762.29				1761.90	0.27	64-79	QPDGVVAALLDICPHR
1994.34	1994.55	1994.48		1994.42		1994.03	0.31	177-193	LLVDNLMDLGHAQYVHR
2143.46	2143.63	2143.57	2143.98	2143.57		2143.12	0.34	34-52	NAWYVAALPEELSEKPLGR
				2294.62		2294.09	0.53	333-353	AYVEANGIRPAMLSCDEAAVR
	2398.72	2398.49		2398.52		2398.08	0.64	276-296	GTHILTPETEASCHYFFGSSR
				2581.78		2582.34	0.56	252-275	VSAMLNFIAVAPEGTPKEQSIHSR
		2699.73		2699.84		2699.25	0.48	133-157	DALIWICPGDPALADPGAIPDFGCR
				4215.70		4215.03	0.67	126-163	SFPVVERDALIWICPGDPALADPGAIPDFGCRVDPAYR

[1]Only experimental masses that matched expected masses are listed in the table. *methylated Methionine.
[2]The number represents the difference between the expected mass and the first column which has the corresponding numbers.
[3]AA position refers to amino acid position within the predicted DMO+27 sequence as depicted in Figure 5.

Table 4. Apparent Molecular Weight and Purity Analysis of the DMO Proteins

The apparent molecular weight and the purity of DMO and DMO+27 were determined by densitometric analysis of SDS polyacrylamide gel (Day 0) shown in Figure 6. Final molecular weight was rounded to one decimal place and purity was reported as a whole number percentage. Purity of the DMO proteins equals the average purity of DMO plus the average purity of DMO+27.

Total Protein Loaded	Apparent Molecular Weight (kDa)		Purity (%)		
	DMO	DMO+27	DMO	DMO+27	DMO Proteins
0.5 µg in lane 3	39.2	41.6	34	44	
0.5 µg in lane 4	39.2	41.5	33	43	
1.0 µg in lane 5	39.5	41.7	36	46	
1.0 µg in lane 6	39.8	42.0	34	46	
1.5 µg in lane 7	40.3	42.4	37	47	
1.5 µg in lane 8	40.7	42.8	35	47	
Average	39.8	42.0	35	46	81

Table 5. Specific Activity of the DMO Enzyme

The DMO enzyme activity was determined by measuring the production of DCSA.

Assay#	Specific activity (DCSA nmol/min/mg)	Average (nmol/min/mg)
1	61.92	
2	51.33	62.21 ± 11.03*
3	73.39	

* the standard deviation

Table 6. Storage Stability (Day 12 vs. Day 0) of the DMO Proteins Stored in a 4 °C Refrigerator, and in -20 °C and -80 °C Freezers: **Apparent MW**

The apparent MW values for DMO and DMO+27 proteins at Day 0 are shown in Table 4.

Apparent MW	Temperature	Day 12[1](kDa)	Percent difference (%)[2]
DMO	4°C	40.0	0.5
	-20°C	39.4	1.0
	-80°C	39.5	0.8
DMO+27	4°C	42.5	1.2
	-20°C	41.9	0.2
	-80°C	42.0	0

[1]Apparent molecular weights at Day 12 were determined based on SDS-PAGE gels shown in Figures 8-10.

[2]$\%\ \text{Difference} = \frac{(\text{Day 0} - \text{Day12})}{\text{Day 0}}\ \text{X}\ 100\%$

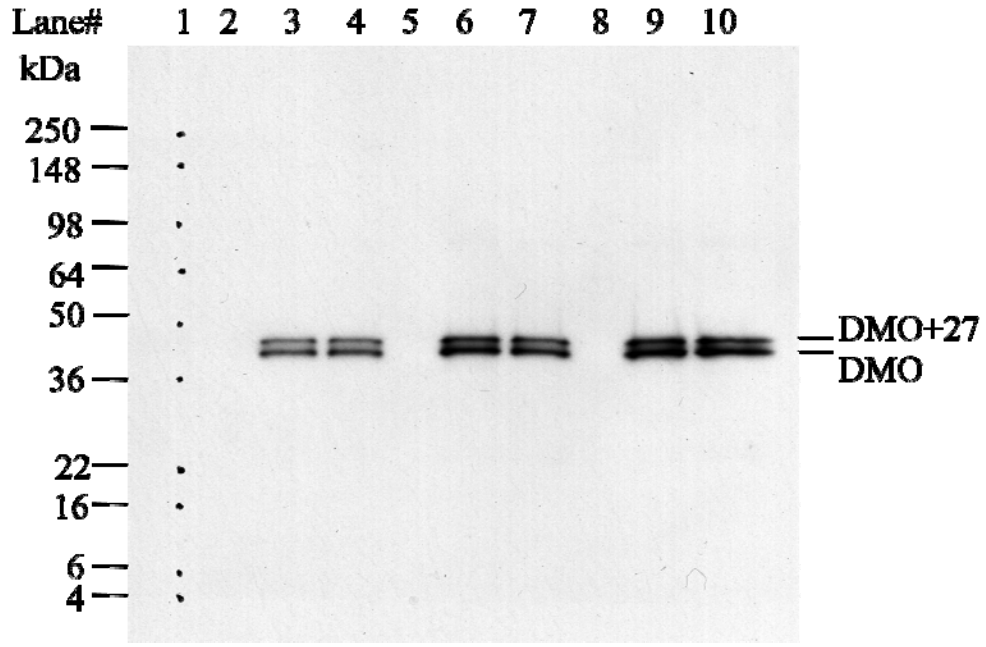
Table 7. Storage Stability (Day 12 vs. Day 0) of the DMO Proteins Stored in a 4 °C Refrigerator, and in -20 °C and -80 °C Freezers: P**urity**

The purity value of the DMO proteins at Day 0 is shown in Table 4.

Purity	Temperature	Day 12[1] (%)	Percent difference (%)[2]
DMO proteins	4°C	62	23.5
	-20°C	76	6.2
	-80°C	73	9.9

[1]Apparent molecular weights at Day 12 were determined based on SDS-PAGE gels shown in Figures 8-10.

[2]$\%\ \text{Difference} = \frac{(\text{Day } 0 - \text{Day} 12)}{\text{Day } 0}\ \text{X } 100\%$



Lane	Sample	Amount (ng)
1	See Blue Plus2 Pre-Stained MW markers	—
2	empty	
3	DMO proteins	20
4	DMO proteins	20
5	empty	
6	DMO proteins	30
7	DMO proteins	30
8	empty	
9	DMO proteins	40
10	DMO proteins	40

Figure 1. Immunoblot Analysis of the DMO Proteins

An aliquot of the DMO enzyme preparation and molecular weight markers were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with anti-DMO antibodies and immunoreactive bands were visualized using an ECL system. Approximate MWs (kDa) are shown on the left and correspond to the markers loaded in lane 1. The 20 second exposure is shown.

Amino acid residue # from the N-terminus[1] →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Expected Sequence	M	A	T	F	V	R	N	A	W	Y	V	A	A	L	P	E
	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence	-	A	T	F	V	R	N	A	W	Y	V	A	A	L	P	E

Figure 2. N-Terminal Sequence of DMO protein

The expected amino acid sequence of the N-terminus of DMO was deduced from the *dmo* gene present in MON 87708. The experimental sequence obtained from DMO was compared to the expected sequence.

[1] The single letter IUPAC-IUB amino acid code is **M**, Methionine; **A**, Alanine; **T**, Threonine; **F**, phenylalanine**; V**, Valine; **R**, Arginine; **N**, Asparagine; **W**, Tryptophan; **Y**, Tyrosine; **V**, Valine; **L**, Leucine; **P**, Proline; **E**, Glutamic acid and (-) Indicates the residue was not observed.

Amino acid residue # from the N-terminus[1] →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Expected Sequence	M	Q	V	W	P	P	I	G	K	K	K	F	E	T	L
	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence	M*	Q	V	W	P	P	I	G	K	K	K	F	E	T	L

Figure 3. N-Terminal Sequence of DMO+27 protein

The expected amino acid sequence of the N-terminus of DMO+27 was deduced from the *dmo* gene present in MON 87708. The experimental sequence obtained from DMO+27 was compared to the expected sequence.

[1] The single letter IUPAC-IUB amino acid code is **M**, Methionine; **Q**, Glutamine; **V**, Valine; W, Tryptophan; **P**, Proline; **I**, Isoleucine; **G**, Glycine; **K**, Lysine; **F,** Phenylalanine; **E**, Glutamic acid; **T**, Threonine; **L**, Leucine; **M***, methylated Methionine.

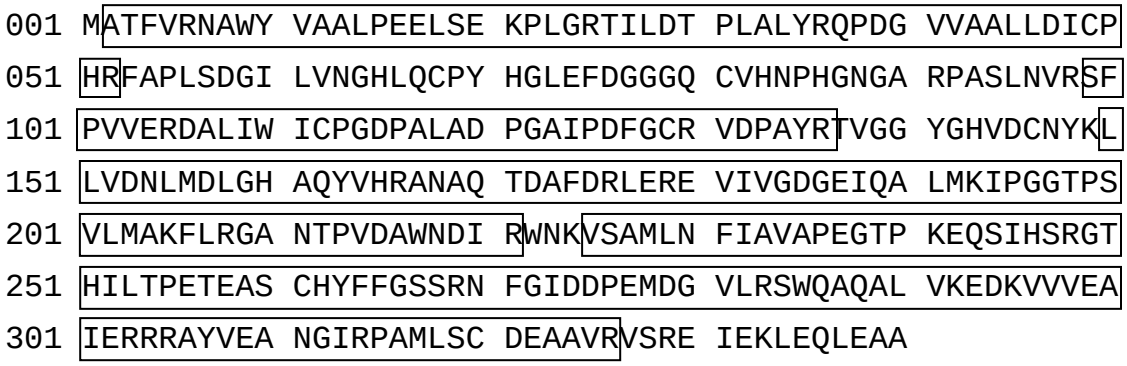


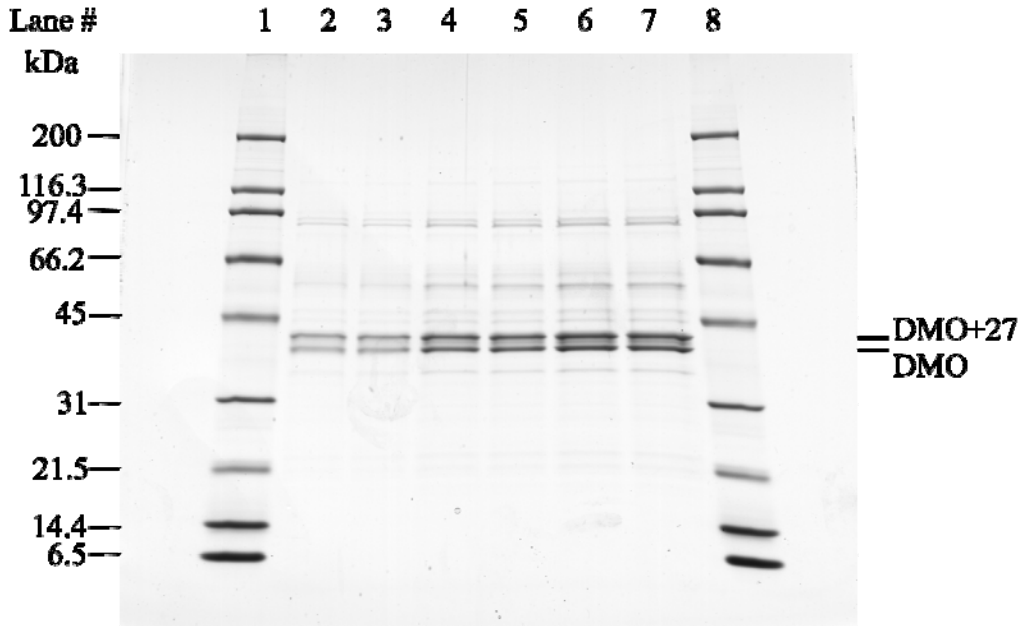
Figure 4. MALDI-TOF MS Coverage Map of DMO protein

The amino acid sequence of DMO was deduced from the *dmo* gene present in MON 87708. Boxed regions correspond to tryptic peptides that were identified from DMO using MALDI-TOF MS. In total, 77.4% (263 of 340 total amino acids) of the expected protein sequence was identified.

001 MQVWPPIGKK KFETLSYLPP LTRDSRAMAT FVRNAWYVAA LPEELSEKPL
051 GRTILDTPLA LYRQPDGVVA ALLDICPHRF APLSDGILVN GHLQCPYHGL
101 EFDGGGQCVH NPHGNGARPA SLNVRSFPVV ERDALIWICP GDPALADPGA
151 IPDFGCRVDP AYRTVGGYGH VDCNYKLLVD NLMDLGHAQY VHRANAQTDA
201 FDRLEREVIV GDGEIQALMK IPGGTPSVLM AKFLRGANTP VDAWNDIRWN
251 KVSAMLNFIA VAPEGTPKEQ SIHSRGTHIL TPETEASCHY FFGSSRNFGI
301 DDPEMDGVLR SWQAQALVKE DKVVVEAIER RRAYVEANGI RPAMLSCDEA
351 AVRVSREIEK LEQLEAA

Figure 5. MALDI-TOF MS Coverage Map of DMO+27 Protein

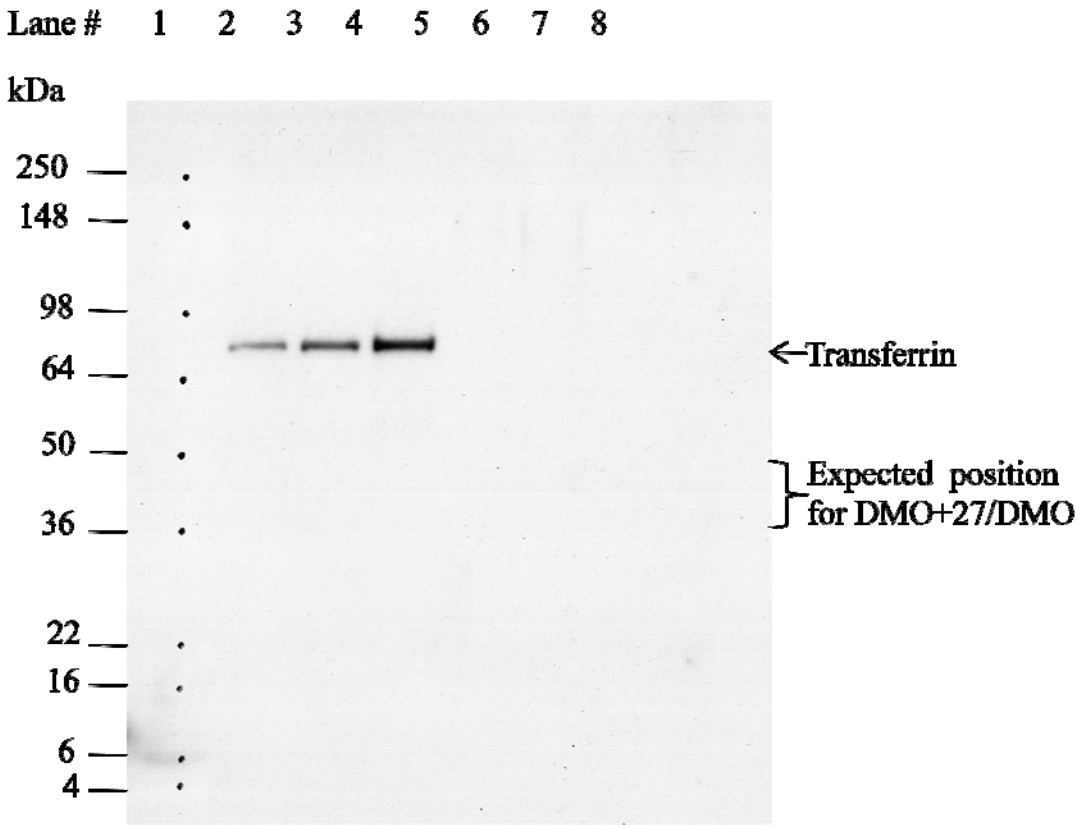
The amino acid sequence of DMO+27 was deduced from the *dmo* gene present in MON 87708. Boxed regions correspond to tryptic peptides that were identified from DMO+27 using MALDI-TOF MS. In total, 82.0% (301 of 367 total amino acids) of the expected protein sequence was identified.



Lane	Sample	Amount (µg)
1	Broad Range MW markers	4.5
2	DMO proteins	0.5
3	DMO proteins	0.5
4	DMO proteins	1.0
5	DMO proteins	1.0
6	DMO proteins	1.5
7	DMO proteins	1.5
8	Broad Range MW markers	4.5

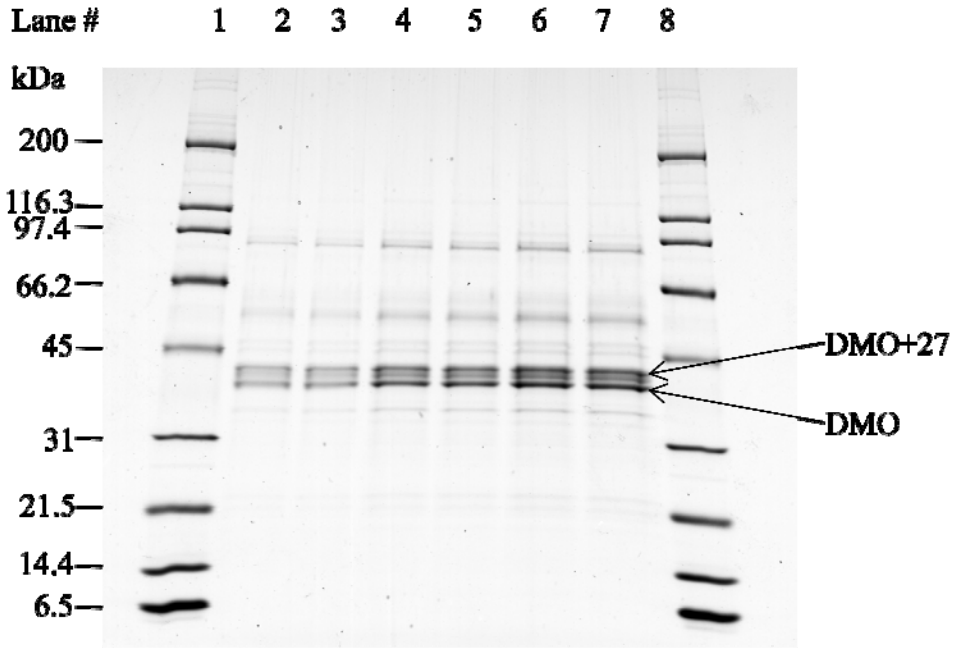
Figure 6. Purity and Molecular Weight Analysis of the DMO Proteins

An aliquot of the DMO enzyme preparation was separated on a 4 - 20% Tris glycine polyacrylamide gradient gel and then stained with Brilliant Blue G Colloidal stain. Approximate apparent molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Empty lanes were cropped.



Lane	Sample	Amount (ng)
1	See Blue Plus2 Pre-Stained MW markers	—
2	Transferrin	50
3	Transferrin	100
4	Transferrin	200
5	DMO proteins	100
6	DMO proteins	200
7	Empty lane	
8	Empty lane	

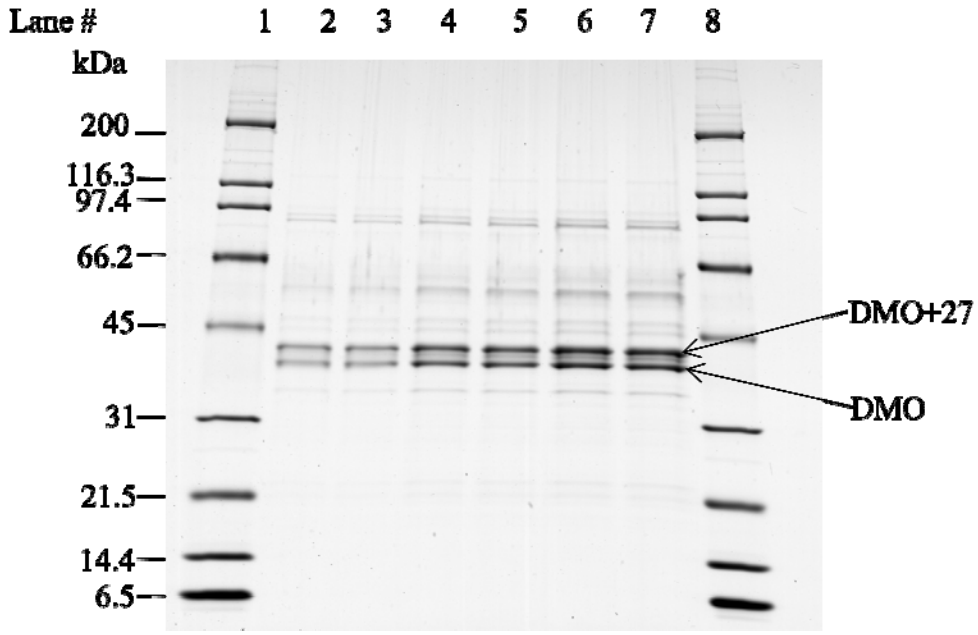
Figure 7. Glycosylation Analysis of the DMO Proteins
Molecular weight markers, transferrin (positive control) and an aliquot of the DMO enzyme preparation were separated by SDS-PAGE (4 - 20%) and electrotransferred to a PVDF membrane. The image was captured using a Bio-Rad GS800 with Quantity One software (version 4.4.0). The 30 second exposure is shown.



Lane	Sample	Amount (µg)
1	Broad Range MW markers	4.5
2	DMO proteins	0.5
3	DMO proteins	0.5
4	DMO proteins	1.0
5	DMO proteins	1.0
6	DMO proteins	1.5
7	DMO proteins	1.5
8	Broad Range MW markers	4.5

Figure 8. Storage Stability (Day 12) of the DMO Proteins Stored at 4 °C

An aliquot of the DMO enzyme preparation stored at 4 °C was separated on a 4 - 20% Tris glycine polyacrylamide gradient gel and then stained with Brilliant Blue G Colloidal stain. Approximate apparent molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Empty lanes were cropped.



Lane	Sample	Amount (µg)
1	Broad Range MW markers	4.5
2	DMO proteins	0.5
3	DMO proteins	0.5
4	DMO proteins	1.0
5	DMO proteins	1.0
6	DMO proteins	1.5
7	DMO proteins	1.5
8	Broad Range MW markers	4.5

Figure 9. Storage Stability (Day 12) of the DMO Proteins Stored at -20 °C

An aliquot of the DMO enzyme preparation stored at -20 °C was separated on a 4 - 20% Tris glycine polyacrylamide gradient gel and then stained with Brilliant Blue G Colloidal stain. Approximate apparent molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Empty lanes were cropped.

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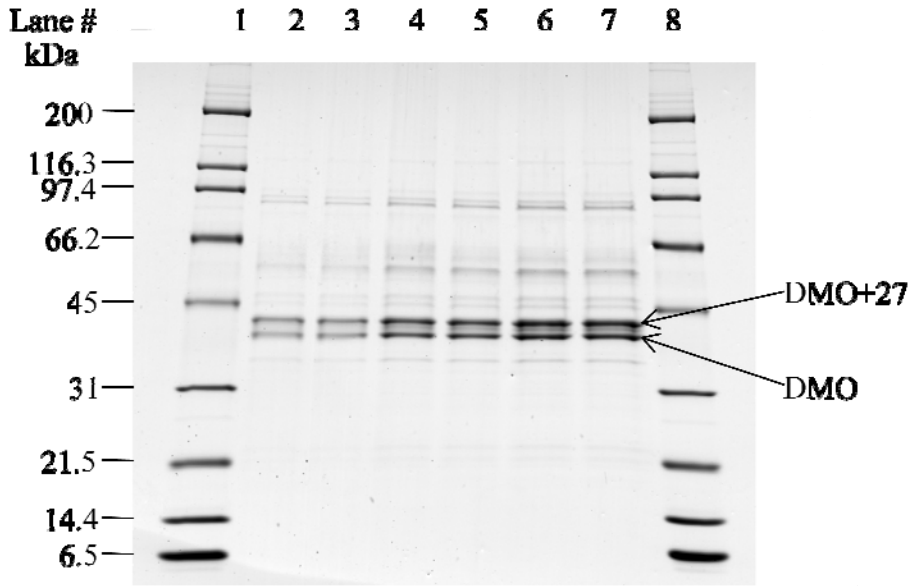

Lane	Sample	Amount (µg)
1	Broad Range MW markers	4.5
2	DMO proteins	0.5
3	DMO proteins	0.5
4	DMO proteins	1.0
5	DMO proteins	1.0
6	DMO proteins	1.5
7	DMO proteins	1.5
8	Broad Range MW markers	4.5

Figure 10. Storage Stability (Day 12) of the DMO Proteins Stored at -80 °C

An aliquot of the DMO enzyme preparation stored at -80 °C was separated on a 4 - 20% Tris glycine polyacrylamide gradient gel and then stained with Brilliant Blue G Colloidal stain. Approximate apparent molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Empty lanes were cropped.

Appendix 1. List of Applicable SOP

SOP Number	Title
AG-ME-0388-03	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-EQ-0265-02	Applied Biosystems 494 Procise™ Protein Sequencing System
BR-EQ-0599-05	Bio-Rad GS-800 Densitometer System
BR-EQ-0783-02	Applied Biosystems Voyager DE Pro Biospectrometry Workstation
BR-EQ-0935-01	Konica SRX X-Ray Film Processors
BR-ME-0392-01	Western Blot Analysis (Immunoblotting)
BR-ME-0527-01	Brilliant Blue G Colloidal Staining of Polyacrylamide Gels
BR-ME-0924-01	Electrotransfer of Proteins to Membranes
BR-ME-0926-01	Staining of Proteins on Blot Membranes
BR-ME-0956-03	Protein Percent Purity and Apparent Molecular Weight Determination
BR-ME-1139-01	Vapor Phase Acid Hydrolysis Using 6 N HCl and Subsequent Amino Acid analysis using AccQ-Tag derivatization
BR-ME-1252-01	Removal of Proteins from Complex Buffer Systems by Means of Ethanol Precipitation Prior to Analytical Analyses
BR-ME-1244-draft	Dicamba Mono-oxygenase Activity Assay