Characterization Title

Characterization of *Primula juliae* Δ6 Desaturase Protein Isolated from Immature Seeds of Soybean MON 87769

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Lead Scientist	Date

Quality Assurance Unit Statement

Characterization Title: Characterization of *Primula juliae* Δ6 Desaturase Protein Isolated from Immature Seeds of Soybean MON 87769

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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 characterization protocol.

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

Dates of Inspection/Audit	Phase	Date Reported to Technical Lead	Date Reported to Management
4/24-25/2008, 4/28/2008	Raw Data Audit	4/29/2008	4/29/2008
8/13-14/2008	Draft Report Review	8/14/2008	8/14/2008

Todd Butzlaff
Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

09/26/08
Date

Characterization Plan Certification

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

Signatures of Final Report Approval:

John J. Finnessy, M.S., Lead Scientist — Date: 9/30/08

Susan H. Elliott, APSO, B.S. — Date: 9/30/08

Thomas C. Lee, Ph.D., Project Lead — Date: 9/30/08

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Characterization Plan Information

Characterization Plan Number: 10001532

Title: Characterization of *Primula juliae* Δ6 Desaturase Protein Isolated from Immature Seeds of Soybean MON 87769

Testing Facility: Monsanto Company
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Records Retention: All characterization plan specific raw data, electronically stored Atlas files, the characterization plan, amendments, and final report will be retained at Monsanto Company, St. Louis.

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Abbreviations and Definitions[1]

AA	Amino Acid
AAA	Amino Acid Analysis
APS	Analytical Protein Standard
COA	Certificate of Analysis
ECL	Enhanced chemiluminescence
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
GLP	Good Laboratory Practice
HRP	Horseradish peroxidase
I.A.	Immuno-affinity
LB	Loading 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 LB
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization - Time of Flight Mass spectrometry
MH+	Protonated Mass Ion
MSL	Monsanto Scientific Literature
MW	Molecular Weight
MWCO	Molecular Weight Cut-Off
NcΔ15D	*Neurospora crassa* Δ15 Desaturase Protein
NFDM	Non-Fat Dried Milk
NIST	National Institute of Standards and Technology
PBST	Phosphate Buffered Saline - Tween® 20
PCR	Polymerase Chain Reaction
PjΔ6D	*Primula juliae* Δ6 Desaturase Protein
PTH	Phenyl thiohydantoin
PVDF	Polyvinylidene difluoride
SDA	Stearidonic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard Operating Procedure
SP-Buffer A	50 mM sodium acetate, pH 5.6, 10% glycerol (v/v), 0.1% Fos-choline 12 (w/v).
TFA	Trifluoroacetic Acid
Tris-HCl	Trishydroxymethylaminomethane – Hydrochloride
U.S.	United States
VOI	Verification of Identity

[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 biotechnology-derived soybean MON 87769 that produces stearidonic acid (SDA), an omega-3 fatty acid. Production of SDA in soybean seed was achieved through the introduction of genes encoding *Neurospora crassa* delta-15 desaturase (NcΔ15D) and *Primula juliae* delta-6 desaturase (PjΔ6D). These two genes are driven by seed-specific promoters, resulting in the production of SDA in soybean seeds.

In order to produce proteins for safety testing, the PjΔ6D protein was purified from immature soybean seeds of MON 87769 and its physicochemical properties have been characterized. This report describes the results of the characterization of PjΔ6D protein purified from immature seeds of MON 87769.

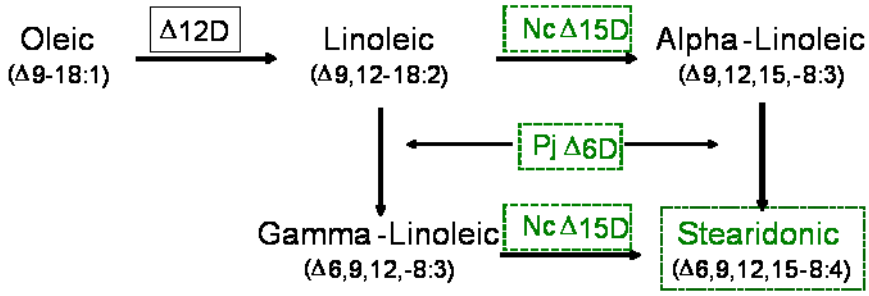
A panel of analytical techniques was used to characterize the MON 87769-produced PjΔ6D protein. These analytical techniques included sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining, western blot analysis, densitometry, matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, N-terminal sequencing, amino acid analysis (AAA) and glycosylation analysis. The short-term stability of the MON 87769-produced PjΔ6D protein was assessed using SDS-PAGE by estimating the purity and molecular weight of the protein present after storage in a 4°C refrigerator, and -20°C and −80°C freezers.

The identity of the MON 87769-produced PjΔ6D protein was confirmed by its recognition with anti-PjΔ6D antibodies in western blot analysis, identification of the first 15 amino acids of the N-terminus by amino acid sequencing, and identification of tryptic peptide fragments masses that cover 42.2% (188 out of 446 amino acids) of the expected PjΔ6D protein sequence. The total protein concentration of the MON 87769-produced PjΔ6D protein preparation was 0.52 mg/ml. Purity of the MON 87769-produced PjΔ6D protein was 47% and the apparent molecular weight of the MON 87769-produced PjΔ6D protein was estimated to be 45.9 kDa. The MON 87769-produced PjΔ6D protein was not glycosylated. Finally, the MON 87769-produced PjΔ6D protein was stable for at least 29 days while stored in a -20 °C or −80 °C freezer, which encompasses the duration of the experimental phase of this study. Taken together, these data provide a detailed characterization of the PjΔ6D protein purified from MON 87769.

2.0 Introduction

Stearidonic acid (SDA), a C-18:4 omega-3 fatty acid, is a metabolic precursor to EPA (James *et al*., 2003; Harris *et al*., 2007). As depicted below, Δ6 desaturase converts linoleic acid (LA) to gamma-linoleic acid (GLA) and alpha-linoleic acid (ALA) to SDA, while Δ15 desaturase catalyzes the desaturation of LA to ALA and GLA to SDA. Monsanto has developed a biotechnology-modified soybean that accumulates SDA in

seeds by introducing two desaturase genes encoding *Primula juliae* (PjΔ6D) and *Neurospora crassa* (NcΔ15D) proteins.



Metabolic pathway engineering to enhance polyunsaturated fatty acid biosynthesis.
The introduced enzymes and their products are represented by dash-lined boxes.

PjΔ6D and NcΔ15D proteins belong to a family of membrane fatty acid desaturases that catalyze the NAD(P)H- and O_2-dependent introduction of double bonds into methylene-interrupted fatty acyl chains (Shanklin and Cahoon, 1998). Both PjΔ6D and NcΔ15D desaturases are integral membrane proteins which contain three conservative histidine box motifs that coordinate iron atoms and act as the catalytic center of desaturases (Hashimoto *et al*., 2008). Although the activity of the desaturases can be demonstrated in crude extracts when appropriate substrates are supplied (Stymne and Appelqvist, 1980; Griffiths, *et al.,* 1996), it has not been possible, thus far, to assay their enzymatic activities following their solubilization and purification away from the membranes. This is most likely due to the desaturases' requirement for the requisite electron transfer proteins co-localized in the membrane. Hence, functional characterization of the integral membrane desaturases is not currently possible using isolated enzyme preparations.

Purification of integral membrane desaturases is a challenging task that requires removal of membranes and replacement of the lipids surrounding the protein's hydrophobic membrane-spanning regions with the appropriate detergent that will keep the protein in solution. To purify integral membrane NcΔ15D protein from immature seeds as the source material for safety testing, the membranes were isolated and a panel of detergents was tested for their ability to release the protein from the seed membranes and maintain in solution. As a result, the zwitterionic detergent Fos-choline 12 was selected. Fos-choline 12 is a member of a relatively new class of detergents that are phospholipid analogs and it has been rapidly adopted in the refolding and structure studies of integral membrane proteins due to its structure-stabilizing properties (Gorzelle *et al*., 1999; Li *et al*., 2001; Choowongkomon *et al*., 2005; Oxenoid and Chou, 2005; Fares *et al*., 2006; Narayanan *et al,*. 2007). After the protein was solubilized from the membranes, multiple chromatographic steps were applied to further purify the PjΔ6D. Standard precautions to retain the structural integrity of the solubilized protein during purification were undertaken including conducting all chromatographic steps in a cold room, inclusion of

protease inhibitors at critical steps, and the addition of reducing agent and glycerol to buffers. This approach allowed for the isolation of structurally-intact desaturase which enabled the characterization of its physicochemical properties.

3.0 Purpose

The purpose of this study was to describe the physicochemical properties of the PjΔ6D protein (Orion lot 10001532) purified from immature seeds of soybean MON 87769.

4.0 Materials

4.1 MON 87769-Produced PjΔ6D Protein

The PjΔ6D protein (Orion lot 10001532) was purified from immature seeds of soybean MON 87769. The MON 87769 immature seed was produced at a Trait Development Site in Wyoming, Illinois (Trial ID, M0322). The serial number for the starting seed was 60087467986. This harvested seed was assigned Orion ID 10002214. The identity of this batch of MON 87769 seed was confirmed by event-specific PCR and the data are archived in the Monsanto Regulatory Archive. A brief description of the isolation and purification of the protein is described in Section 5.1. Additional information regarding the protein preparation has been archived under Orion lot number 10001532. The final protein preparation is stored at –80 °C in a buffer solution containing 50 mM sodium acetate, pH 5.6, 1 mM $MgCl_2$, 0.1% Fos-choline 12, 0.5M NaCl and 10% glycerol.

4.2 Assay Controls

Prestained and non-stained protein molecular weight standards (BioRad, Hercules, CA) were used to calibrate SDS-PAGE gels or to verify protein transfer to polyvinylidene difluoride (PVDF) membranes. A peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems) was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass and intact mass analysis. β-Lactoglobulin (Applied Biosystems, Foster City, CA) was used as control to confirm N-terminal sequencing performance. Transferrin (GE Healthcare, Piscataway, NJ) was used as a positive control for glycosylation analysis.

5.0 Methods

5.1 Protein Purification

The PjΔ6D protein was purified from the immature seeds of MON 87769 prior to initiation of this characterization plan. The purification of PjΔ6D from the immature seeds of MON 87769 was not performed under a GLP study or plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed. All operations were carried out at 4 °C unless otherwise indicated.

A total of 11 kg of immature soybean seeds (Orion Lot No. 10002214) were used for the isolation of PjΔ6D. The seeds were homogenized for membrane isolation at a

scale of ~1000-2500 g immature seed per extraction (6 runs). The membranes were solubilized with 2% Fos-choline 12 and separated on a 3,000-ml cation exchange chromatography column (3 runs) followed by immuno-affinity purification (30 runs). The MON 87769-produced PjΔ6D protein isolated from the different batches were combined as a final preparation. After preliminary values for purity and protein concentration were obtained, the preparation was submitted to Monsanto's APS program under the Orion lot # 10001532.

Each run included the following series of purification steps:

Homogenization and membrane isolation - Immature soybean seeds were homogenized with a Viking blender (Waring Commercial®, Torrington, CT) for 3 min using a tissue to buffer ratio of 1g/10ml of ice cold homogenization buffer consisting of 100 mM Tris-HCl, pH 8.0, 350 mM NaCl, 5.0 mM DTT, 0.5 mM PMSF, 1 µM leupeptin, and 1.0 mM sodium benzamidine hydrochloride. After filtration of the homogenate through four layers of cheese cloth, the filtrate was centrifuged at 2,000 × *g* to remove cell debris. The supernatant was centrifuged at 37,000 × *g* for 1 hr. After the centrifugation, the supernatant was discarded and the pellet re-suspended in ice cold deionized H_2O at a total protein concentration of approximately 40 mg/ml. The membrane suspension was mixed with an equal volume of 2× Carbonate Wash Buffer (200 mM sodium carbonate, pH 11.5, 2 mM $MgCl_2$, 0.5 mM PMSF, 1 µM leupeptin, and 1.0 mM benzamidine hydrochloride). Immediately after mixing, the mixture was centrifuged at 37,000 × *g* at 4 °C for 30 min. The supernatant was discarded and the membrane pellets were stored at -80 °C until use. Extraction was repeated until all the remaining immature seeds were homogenized.

Solubilization and cation exchange chromatography – The frozen membrane pellets (~100 g fresh weight) were thawed and re-suspended in 5 L of the solubilization buffer [50 mM sodium acetate, pH 5.6, 100 mM NaCl, 1.0 mM DTT, 10% Glycerol (v/v), 0.5 mM PMSF, and 1 µM leupeptin]. Solid Fos-choline 12 was added to a final concentration of 2% and the membrane/detergent solution was incubated at 4 °C for 2 hr with stirring. The mixture was centrifuged at 37,000 × *g* at 4 °C for 30 min to remove the insoluble fractions. The solubilized supernatant was applied onto a SP-Sepharose column (bed volume ~3.0 L, GE Healthcare, Piscataway, NJ) which had been previously equilibrated with SP-Buffer A consisting of 50 mM sodium acetate, pH 5.6, 10% glycerol (v/v), and 0.1% Fos-choline 12 (w/v). After washing the column with SP-Buffer A, the column was further washed with 0.1 M NaCl in SP-Buffer A. The bound proteins were then eluted stepwise with 0.5 M NaCl in SP-Buffer A with fractions containing protein pooled based on OD_{280}, and stored at -80 °C.

Immuno-affinity chromatography - Immuno-affinity (IA) resins used in the purification were prepared by immobilizing anti-PjΔ6D IgG to agarose resins using an IgG-orientation kit from Pierce (Cat. No. 44990, Rockford, IL) according to

manufacturer's instruction. The anti-PjΔ6D antibody (Lot Numbers: 7580967 and 7580957) was used for immuno-affinity resin preparation (total vol. of resin ~28 ml). The immuno-affinity column was equilibrated with Immuno-affinity Binding and Washing (IABW) Buffer consisting of 1× PBS, pH7.2, 1mM $MgCl_2$, 10% glycerol (v/v) and 0.1% Fos-choline 12 (w/v). Before applying the samples to the immuno-affinity column, the pH of the SP-Sepharose-purified protein preparation was adjusted to pH 7.4 with 2.0 M potassium phosphate, pH 8.5. The flow through fraction was re-applied to the column and the loading was repeated three times. The column was washed extensively with the IA washing buffer and PjΔ6D was eluted with 0.1 M Glycine, pH 2.8 containing 1mM $MgCl_2$, 10% glycerol (v/v) and 0.1% Fos-choline 12 (w/v). The eluted protein fractions (~140 mL) were pooled and brought to pH 5.5 with 2.0 M MES, pH 10, and stored at -80 °C.

Concentration of Protein solution - To concentrate the IA-purified protein preparations, about 250 – 500 ml of the eluted fractions were loaded onto a 2-ml SP-Sepharose column (GE Healthcare, Piscataway, NJ) which had been previously equilibrated with SP-Buffer A. The flow rate was typically 4.0 ml/min. The protein was eluted with 0.5 M NaCl in SP-Buffer A and the fractions containing PjΔ6D protein were pooled and stored at -80°C. A total of eight batches were prepared.
Final purification by Hydroxyapatite chromatography – All eight batches of the concentrated PjΔ6D preparations were combined and applied onto a 5-ml hydroxyapatite column (Type I ceramic hydroxyapatite, BioRad, Hercules, CA). While the majority of the non-PjΔ6D proteins bound to the column, the flow through fraction was collected and saved as the final PjΔ6D protein preparation. After preliminary values for purity and concentration were obtained, the final preparation was submitted to APS program under the Orion lot # 10001532.

5.2 Determination of Protein Concentration

The total protein concentration of the purified MON 87769-produced PjΔ6D protein sample was determined by amino acid analysis (AAA) using AccQ-Tag® derivatization (Waters Corporation, Milford, MA), a precolumn derivatization method 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 sample preparation was as follows: in a hydrolysis tube (~300 µl), approximately 1µg of a protein sample was mixed with 200 µl of chilled 95% ethanol. After incubation overnight at -20 ºC, each sample was spun for 45 min at 2-4 °C at a setting of 12,000 rpm in a microcentrifuge to precipitate proteins. The supernatant was removed and discarded. Each sample was then washed twice sequentially with 100 µl of chilled acetone followed by water. Along with replicates of the test sample, a hydrolysis blank, 4 dilutions of a calibration standard (NIST), and a 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. A 500 µl volume 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 tube contents were evaporated to dryness using a Speed-Vac concentrator. A 20 µl volume of reconstitution solution (20 mM HCl) was added and tubes were vortexed to resuspend the sample. A 60 µl volume of AccQ-Fluor Borate Buffer and a 20 µl volume of AccQ-Fluor reagent were added sequentially to each vial with vortexing after each addition. The samples were transferred individually to autosampler vials, capped, and heated at 55 °C for 10 min. Samples were analyzed using a 2695 Separation Module (Waters Corp.) in conjunction with reverse-phase C-18 column for separation of AccQ-Tag derivatized amino acids. Chromatographic data were collected using Atlas software (Thermo Electron Corp.).

5.3 Protein Identity

5.3.1 Immunoblot Analysis

Immunoblot analysis was performed to confirm the identity of PjΔ6D protein purified from MON 87769. Based on the concentration and purity, aliquots of the purified PjΔ6D protein preparation were diluted in 50 mM sodium acetate, pH 5.6, 10% glycerol and 0.1% Fos-choline 12 to give final amounts of 4, 6, 8, 10 and 12 ng, respectively. Samples were mixed with 5× Loading Buffer (312.5mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8.) and loaded directly onto gels without boiling.

Membrane proteins in the presence of detergents tend to self-associate via hydrophobic contact surfaces, forming irreversible nonspecific aggregates (McGregor *et al.*, 2003; Sagne *et al.,* 1996). This results in a smeared appearance on SDS-PAGE. Heating of the samples causes an increase in this behavior. As a result, no PjΔ6D samples were heated before analysis using SDS-PAGE. The samples were then separated on SDS-PAGE using 4-20% precast polyacrylamide gels (Invitrogen, Carlsbad, CA) at a constant voltage of 50 V for 25 min followed by 160 V for 70 min. Proteins in the gel were electrotransferred to PVDF membrane (Invitrogen, Carlsbad, CA) at a constant voltage of 25 V for 90 min. Pre-stained molecular weight markers (Precision Plus protein standard, BioRad, Hercules, CA) were used to verify the electrotransfer of proteins and estimate the size of the immunoreactive bands observed.

Following the electrotransfer, the membrane was blocked for 30 min with 4% (w/v) NFDM in PBST. The membrane was then probed with a 1:5000 dilution of goat anti- PjΔ6D antibody (Lot No. 7580957) in PBST containing 2% (w/v) NFDM for 1 hr. Rabbit anti-goat IgG conjugated with horse radish peroxidase

(Pierce, Rockford, IL) was used as secondary antibody at a dilution of 1:10,000. Excess antibody was removed using 5 × 10 min washes with PBST. Finally, the membrane was probed with horseradish peroxidase-conjugated rabbit anti-goat IgG (Pierce, Rockford, IL) at a dilution of 1:10,000 in PBST containing 2% (w/v) NFDM for 60 min. Following 5 × 10 min washes with PBST, immunoreactive bands were visualized using the ECL detection system (GE Healthcare, Piscataway, NJ) and exposed (30 and 60 sec) to Hyperfilm ECL (G. E. Healthcare, Piscataway, NJ). Films were developed using a Konica SRX-101A automated film processor (Tokyo, Japan). The MW of the immunoreactive bands was estimated 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

Edman degradation was used to confirm the N-terminal amino acid sequence identity of the MON 87769-produced PjΔ6D.

5.3.2.1 Protein Blot for N-Terminal Analysis

An aliquot of MON 87769-produced PjΔ6D protein was removed from storage and mixed with 5× LB to a final concentration of 0.42 µg/µl and loaded in 5 lanes at 10.5 µg of total protein per lane. Samples were loaded directly onto gels without boiling for the reasons mentioned in section 5.3.1. SDS-PAGE was conducted using 4-20% precast polyacrylamide gels (Invitrogen, Carlsbad, CA). Electrophoresis was carried out at constant voltage of 50 V for 25 min followed by 160 V for 70 min. Proteins in the gel were electrotransferred to PVDF (Invitrogen, Carlsbad, CA) membrane in the electrotransfer buffer containing 10 mM CAPS, pH 11 and 10% methanol at a constant voltage of 26 V for 120 min. Pre-stained molecular weight markers (Precision Plus protein standard, BioRad, Hercules, 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 Coomassie Blue R-250 (BioRad, Hercules, CA) to visualize the protein of interest.

5.3.2.2 N-Terminal Sequencing

Following electroblotting and staining, the band corresponding to MON 87769-produced PjΔ6D protein was excised based on MW from the blot and N-terminal sequence analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller and Hood, 1983). A second band of approximate molecular weight 7 kDa was also sequenced as it comprised 10.6 % of the total protein seen in the purity gel (see Figure 4) and, therefore, it must be analyzed according to the characterization plan criteria. An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and 785

Programmable Absorbance Detector and Procise™ Control Software (version 1.1a) were used. Chromatographic data were collected using Atlas software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used as the calibration standard in the chromatographic analysis. A control protein (10 pmole β-lactoglobulin, Applied Biosystems) was analyzed before and after the analysis of the PjΔ6D protein, to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

5.3.3 MALDI-TOF Tryptic Mass Map Analysis

MALDI-TOF mass spectrometry was used to confirm the identity of the MON 87769-produced PjΔ6D protein.

5.3.3.1 Protein Gel for Tryptic Mass Map Analysis

A 100 µl aliquot of MON 87769-produced PjΔ6D protein was removed from storage, mixed with 5× LB to a final concentration of 0.42 µg/µl and loaded in 5 lanes at 10.5 µg per lane. Samples were loaded directly onto gels without boiling. SDS-PAGE was conducted using 4-20% precast polyacrylamide gels (Invitrogen, Carlsbad, CA). Electrophoresis was carried out at constant voltage of 50 V for 25 min followed by 160 V for 70 min. The gel was stained with Coomassie blue dye R-250 (BioRad, Hercules, CA) to visualize the protein of interest. The band corresponding to the MON 87769-produced PjΔ6D protein was excised from 5 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, 10% acetic acid in water) in a microfuge tube. This step was repeated 4 times with the final destain step proceeding for 60 minutes. Following destaining, the gel band was incubated in 100 µl of 100 mM ammonium bicarbonate buffer for 30 min at room temperature. The protein was reduced in 100 µl of 10 mM dithiothreitol solution for 2 h at 37°C. The protein was alkylated by the addition of 100 µl of 20 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. The gel band was incubated in 200 µl of 25 mM ammonium bicarbonate buffer for 15-25 min at room temperature. This step was repeated twice, following which the gel bands were dried using a Speed Vac concentrator (Ramsey, MN). A single gel band was rehydrated with 20 µl of 0.02 µg/µl trypsin in 25 mM ammonium bicarbonate, 10% acetonitrile, and was incubated for about 1 h at room temperature, after which excess liquid was removed and the sample was incubated overnight at 37°C in 40 µl of 25 mM ammonium bicarbonate, 10% acetonitrile. The following day, the sample was

sonicated for 5 min, and the supernatant was transferred to a new tube and dried using a Speed Vac concentrator (Extract 1). The gel band was resuspended in 30 µl 60% acetonitrile, 0.1% trifluoroacetic acid, 0.1% octyl-β-D-glucopyranoside solution, and sonicated for 5-10 min. After transfer of the supernatant to a new tube, this step was repeated one time, and the combined supernatants were dried using a Speed Vac concentrator (Extract 2). Extracts 1 and 2 were each resuspended in 20 µl 0.1% TFA and then dried using a Speed Vac concentrator. Extract 1 was resuspended in 5 µl of 50% acetonitrile, 0.1% TFA, while Extract 2 was resuspended in 10 µl of the same solution. Each was sonicated for 5 min.

5.3.3.2 MALDI-TOF Mass Analysis

Mass spectral analyses were performed as follows. Mass calibration of the instrument was performed using an external peptide mixture (CalMix 2; Applied Biosystems). Extract 1 and Extract 2 samples (0.3 µl) were co-crystallized with 0.70 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 7500 Dalton range using 200 shots at a laser intensity setting of 3001. The laser intensity setting is a unit-less MALDI-TOF instrument-specific value. The samples in α-cyano matrix were analyzed in the 300 to 7500 Dalton range using 200 shots at a laser intensity setting of 2601. The samples in sinapinic acid matrix were analyzed in the 850 to 7500 Dalton range using 200 shots at a laser intensity setting of 3200. Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993), except above 5000 Daltons, where mass-averaged values were used. GPMAW32 software (Applied Biosystems) was used to generate a theoretical trypsin digest of the expected PjΔ6D protein sequence based upon the nucleotide sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks in the 500 to 1000 Da range if there were two or more isotopically resolved peaks, and in the 1000 to 8000 Da range if there were three or more isotopically resolved peaks in the spectra. 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 signal ±2 Daltons from the mass analyzed.

5.4 Molecular Weight and Purity Estimation – SDS-PAGE

Aliquots of the MON 87769-produced PjΔ6D protein were diluted with Milli-Q water and mixed with 5× LB to a final protein concentration of 0.2 µg/ µl. The MON 87769-produced PjΔ6D protein was analyzed in duplicate at 2, 3, and 4 µg of total protein per lane. Molecular weight standards were heated, though the test samples were not and all samples were applied to a pre-cast 4-20% polyacrylamide gradient 10-well gel (Invitrogen, Carlsbad, CA). Molecular weight markers (BioRad, Hercules, CA) were loaded in parallel. Electrophoresis was performed at a constant voltage of 120 V for 60 min followed by 180 V for 30 minutes. Proteins were stained using Colloidal Coomassie Brilliant Blue stain (Sigma, Saint Louis, MO).

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). The molecular weight markers were used to estimate the apparent molecular weight of the MON 87769-produced PjΔ6D protein. For the purity evaluation, all visible bands within each lane were quantified. The purity and estimated MW of the MON 87769-produced PjΔ6D protein were reported as the average of the six values obtained by densitometric analysis.

5.5 Glycosylation Analysis

The GE Healthcare Glycosylation Detection Module (Piscataway, NJ) was used to detect carbohydrate covalently bound to MON 87769-produced PjΔ6D after the proteins were resolved by SDS-PAGE and electrotransferred onto PVDF membrane. The kit utilizes the specific labeling of biotin to the bound carbohydrate moiety followed by probing with streptavidin-conjugated horse radish peroxidase (Strep-HRP) and detection by ECL. The biotin labeling consists of two steps: pretreatment of the glycoprotein on the membrane and covalent conjugation of biotin to the carbohydrate moiety of the glycoprotein. The pre-treatment oxidizes hydroxyl groups of the carbohydrate moieties in the glycoproteins to aldehydes which is then reacts with biotin, resulting in covalent conjugation of biotin. This biotin-labeling procedure is specific for the detection of conjugation with carbohydrates and sensitive due to the use of biotin's high affinity binding partner, avidin, in an enzyme linked assay.

Aliquots of 0.5 µg and 1.0 µg of the purity-corrected PjΔ6D protein in SP-buffer A were mixed with 5× LB and loaded onto a 4-20% precast polyacrylamide gel (Invitrogen, Carlsbad, CA). Along with the PjΔ6D samples, 25, 50 100 and 200 ng of the glycosylated control protein, transferrin, were loaded in parallel. The PjΔ6D samples were not heated, but the transferrin controls were boiled prior to the loading. Electrophoresis was carried out at constant voltage of 90 V for 30 min followed by 159 V for 60 min. Proteins in the gel were electrotransferred to a PVDF membrane (Invitrogen, Carlsbad, CA) at a constant voltage of 25 V for 90 min. Pre-stained molecular weight markers (Precision Plus Dual Color protein standard, BioRad,

Hercules, CA) were loaded in parallel to verify electrotransfer of protein to the membrane and estimate the size of the stained bands observed. Transferrin (~ 76 – 81 kDa, Sigma-Aldrich, St. Louis, MO) was loaded in parallel as a positive control of glycosylated protein in series of dilution.

The labeling and detection of carbohydrate was carried out according to the manufacturer's instructions and all the reagents except PBS were provided in the kit. Following electrotransfer to 0.45 μm PVDF membrane, the blot was incubated first in 30 ml of PBS for 10 min, then in 10 mM $NaIO_4$ for 20 min in darkness. The membrane blot was then rinsed twice with 15 ml PBS followed by 3 washes of PBS for 10 min each. To label the carbohydrate moiety on the protein with biotin, the membrane was incubated with biotin-hydrazide for 45 min followed by two rinses and three 10 min PBS washes as described above. The membrane was further blocked for 60 minutes with 5% blocking reagent in PBS followed two rinses and three 10 min PBS washes. Streptavidin-HRP at 1:6000 dilution was overlaid onto the membrane and incubate at room temperature for 30 min. After two PBS rinses and three 10 min PBS washes, the membrane was then developed with ECL detection reagents by mixing 2 ml of Reagent 1 and 2 ml of Reagent 2. After 1 min incubation, the excess detection solution was removed by blotting with paper towel and the blot was exposed to Hyperfilm ECL (G. E. Healthcare, Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan). The blot images were captured using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA).

5.6 Storage Stability

The short-term stability of the MON 87769-produced PjΔ6D protein was evaluated by comparing the purity and molecular weight values before and after storage for 29 days in all of the following areas: a 4 ºC refrigerator, a -20 ºC and a -80 ºC freezer. At the end of storage, aliquots of the PjΔ6D protein samples were removed and subjected to SDS-PAGE followed by staining, and subsequent purity and MW estimation. The MON 87769-produced PjΔ6D protein was analyzed in duplicate at 2, 3, and 4 μg of total protein per lane. MW standards were heated, though the test samples were not and all samples were applied to a pre-cast 4-20% polyacrylamide gradient 10-well gel (Invitrogen, Carlsbad, CA). Pre-stained molecular weight markers (BioRad, Hercules, CA) were loaded in parallel. Electrophoresis was performed at a constant voltage of 120 V for 20 min followed by 180V for 60 minutes. Proteins were stained using colloidal Coomassie Brilliant Blue stain (Sigma, Saint Louis, MO).

Analysis of each gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). The molecular weight markers were used to estimate the apparent molecular weight of the MON 87769-produced PjΔ6D protein. The protein samples were considered to have undergone

degradation if a > 10% decrease in purity and/or molecular weight was observed relative to the value determined on Day 0.

6.0 Data Rejected or Not Reported

No data were rejected and all data were reported.

7.0 Characterization Protocol Amendments

There was 1 amendment to the original protocol.

1) The protocol was amended to include only transferrin as a positive control for identification of glycosylation status. The original plan included both transferrin and horse radish peroxidase (HRP). It was determined that HRP could interfere with the detection method used during glycosylation detection and thus it was eliminated as a control.

8.0 Control of Bias and Quality Measures

Controls and standards were included with each analysis. A four-peptide mixture from the Sequazyme Peptide Mass Standards kit (Applied Biosystems) was used to calibrate the MALDI-TOF mass spectrometer for masses observed between 500-5000 Daltons. A NIST amino acid standard (NIST Cat. No. 2389) was used to calibrate the retention times of the amino acids observed in amino acid analysis.

9.0 Results and Discussion

9.1 Protein Concentration

The total protein concentration of the purified PjΔ6D preparation from MON 87769 immature seed was estimated using AAA. The average concentration across five independently prepared dilutions was determined to be 0.52 mg/ml (Table 1). A total of 20 mg total protein in 39 ml was obtained and submitted to the Analytical Protein Standards Program of Monsanto.

9.2 Protein Identity

The identity of the MON 87769-produced PjΔ6D protein was confirmed using three independent methods: immuno-blot analysis using an anti-PjΔ6D antibody, N-terminal sequencing, and MALDI-TOF MS-generated tryptic map mass analysis.

9.2.1 Immunoblot Analysis

Purity determinations of the PjΔ6D protein were made as described below (Section 9.3). Based on purity values, an amount of MON 87769-produced PjΔ6D protein calculated to be 4, 6, 8, 10 or 12 ng was loaded. An immunoreactive band was observed in each lane migrating at approximately 46 kDa (Figure 1, lanes 3-7). As expected, the intensity of the immunoreactive band increased with increasing amount of protein loaded.

The immunoreactive profiles shown in Figure 1 reveal a diffuse signal in the high molecular area between 100 kDa and 250 kDa. When membrane proteins are separated on SDS-PAGE, they tend to aggregate and a portion tends to migrate as higher molecular weight aggregates (McGregor *et al*., 2003; Von Jagow *et al*., 1994). While all possible precautions were taken during electrophoresis (i.e., the samples were not heated to prevent further aggregation), it is reasonable to assume that the diffuse signal observed on these blots consisted mainly of PjΔ6D protein. It is also possible that the signal resulted from an endogenous cross-reacting with anti-PjΔ6D antibody protein that was co-purifed with PjΔ6D.

The immunoreactive profiles shown in Figure 1 also reveal a band of approximately 15 kDa. Most likely this fragment is the results of proteolytic degradation of the PjΔ6D protein or of an endogenous cross-reacting protein co-purified during the purification procedure.

9.2.2 N-Terminal Sequencing

N-terminal sequence analysis of the major protein band with molecular weight of ~46 kDa observed on stained SDS-PAGE and immuno-reacted with PjΔ6D antibody identified 15 amino acids that matched the expected N-terminal sequence for the PjΔ6D protein (Figure 2). The N-terminal methionine was not observed, indicating that it was removed during posttranslational processing of the protein. Processing of the N-terminal methionine occurs through an enzymatic digestion by aminopeptidase (Bradshaw *et al*., 1998) and is common in many organisms. The expected amino acid sequence of the N-terminus of the MON 87769-produced PjΔ6D protein was deduced from the coding region of the full-length PjΔ6D gene present in MON 87769 (Girault *et al*., 2008). A second band of approximate molecular weight 7 kDa was sequenced as it comprised 10.6 % of the total protein seen in the purity gel (see Figure 4) and, therefore, it must be analyzed according to the characterization plan criteria. The sequencing data from this band yielded multiple amino acid identifications during each cycle of Edman degradation suggesting the presence of multiple proteins of similar abundance. This confounds analysis and prevents clear sequence determination or identification of the components of this protein band. This result is not unexpected as lower molecular weight bands observed on the SDS polyacrylamide gel may contain low molecular weight proteins and cleavage products of larger molecular weight proteins. While there may be degradation products of PjΔ6D within this band, it was not possible to clearly identify them.

9.2.3 MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 87769-produced PjΔ6D protein was further confirmed by MALDI-TOF mass spectrometry. Prior to analysis, the protein sample was chemically reduced, alkylated and digested with trypsin. The ability to identify a protein using this method is dependent on matching a sufficient number of

observed mass fragments to expected (theoretical) mass fragments. In general, a 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 30 unique protein fragments identified that matched the expected tryptic masses of the PjΔ6D trypsin-digested protein (Table 2). The identified masses were used to assemble a coverage map that indicates the position of those matched peptide sequences within the protein sequence (Figure 3). A total of 188 out of 446 (42.2%) amino acid residues were identified and were mapped to the predicted positions within the PjΔ6D sequence. The protein is confirmed to be PjΔ6D.

9.3 Purity and Molecular Weight Estimation

Purity and molecular weight of the PjΔ6D protein were estimated using densitometric analysis after the proteins were separated by SDS-PAGE and stained with Colloidal Brilliant Blue G (Figure 4). As summarized in Table 3, molecular weight values were averaged from duplicated loads of approximately 2.0, 3.0 and 4.0 μg of total protein (Lanes 3-8, Figure 4). The predominant band identified as PjΔ6D (Section 9.2) was estimated to have molecular weight of approximately 46 kDa. The average purity of the PjΔ6D protein is estimated to be 47%. Hence, after purity-based correction, the final concentration of the PjΔ6D protein in the preparation is 0.24 mg/ml of the PjΔ6D protein.

9.4 Glycosylation Analysis

Many eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher *et al*., 1988). There are two forms of glycosylation. The first occurs on asparagine side chains, and is known as N-glycosylation. The other form is O-glycosylation, which is the addition of N-acetylglucosamine (N-GlcNAc) to the β-hydroxyl of either serine or threonine residue. O-glycosylation sites are less well defined (Thanka *et al*., 2001) and may occur at largely any serine or threonine residue. PjΔ6D is an integral membrane protein of higher plant origin (primrose) which contains two putative N-glycosylation sites (Asn-Xxx-Ser/Thr) (Marshall, 1972). The presence of a potential glycosylation site does not imply the presence of glycosylation. To test whether the MON 87769-produced PjΔ6D protein was glycosylated once expressed in seeds of soybean, it was analyzed for the presence of covalently bound carbohydrate moieties using a GE Glycoprotein Detection Module which detects N and O-linked carbohydrates. A naturally glycosylated protein, transferrin, was utilized as a positive control in the assay. The results of this analysis are present in Figure 5. A strong signal at the expected MW position of approximately 76-81 kDa was detected for the transferrin positive control in a concentration dependent manner (Lanes 2-5). No detectable signal was observed (lanes 7-8) at the expected molecular position of approximately 46 kDa for the PjΔ6D protein.

Three faint and slightly smeared were detected by the carbohydrate reaction in the high load lane (Lane 8, Figure 5) in the molecular weight range of 60-200 kDa. Taking into consideration that the purity of PjΔ6D protein is only 47% and that no signal was observed at the expected MW for PjΔ6D protein (~46 kDa), it was concluded that the faint signals observed on the blot are not derived from the PjΔ6D protein. Most likely, the observed faint bands originated from plant proteins that were co-purified with the PjΔ6D protein. Therefore, the above data demonstrate that the MON 87769-produced PjΔ6D is not glycosylated.

9.5 Storage Stability

Storage stability of the MON 87769-produced PjΔ6D protein was assessed after the protein aliquots were stored in a 4 ºC refrigerator, -20 ºC and a -80 ºC freezers for 29 days (Figures 6-8, Table 4). Stability was evaluated by comparison of the apparent molecular weight and purity of the MON 87769-produced PjΔ6D protein after storage to the initial apparent molecular weight and purity values determined on day 0 (Figure 1 and Table 2). As summarized in Table 4, the apparent molecular weight of MON 87769-produced PjΔ6D protein was estimated to be 44.4, 46.5 and 46.7 kDa respectively for 4 ^{o}C, -20 ^{o}C and -80 ^{o}C on day 29. The percent difference between the apparent molecular weight values obtained on day 0 and day 29 for each storage temperature (45.9 kDa) are 3, 1 and 1.7%, respectively, for 4 ^{o}C, -20 ^{o}C and -80 ^{o}C (Table 4), suggesting that no significant molecular weight changes occurred at these different temperatures.

The purity of the MON 87769-produced PjΔ6D protein stored for a 29 day period at different temperatures was assessed (Table 4 and Figures 6-8, Lanes 3-8) by densitometric analysis of a Coomassie stained SDS-polyacrylamide gel. The percent decrease between day 0 and day 29 in average purity of MON 87769-produced PjΔ6D protein was estimated to be 70%, 2% and 0% respectively for 4 ^{o}C, -20 ^{o}C and -80 ^{o}C (Table 4). These results are within the acceptance criterion of ± 10% for storage at -20 ^{o}C and -80 ^{o}C, but not for storage at 4 ^{o}C indicating that PjΔ6D protein is unstable during storage at 4 ^{o}C. Therefore, based on molecular weight and purity, the MON 87769-produced PjΔ6D protein was stable when stored in -20 °C or -80 ^{o}C freezers for 29 days but was unstable and became degraded when stored at approximately 4 ^{o}C.

10.0 Conclusions

A panel of analytical techniques was used to characterize the purified MON 87769-produced PjΔ6D protein. Identity of the MON 87769-produced PjΔ6D protein was confirmed by recognition with anti-PjΔ6D antibodies, identification of the first 15 amino acids of the expected N-terminus by amino acid sequencing, and identification of tryptic peptide masses that yielded 42.2% overall coverage of the expected protein sequence. The total protein concentration of the MON 87769-produced PjΔ6D protein preparation

was 0.52 mg/ml with a purity of 47%. The apparent molecular weight of the 87769-produced PjΔ6D protein was estimated to be 45.9 kDa. The MON 87769-produced PjΔ6D protein was confirmed to be a non-glycosylated protein. Finally, the MON 87769-produced PjΔ6D protein was stable for at least 29 days while stored in -20 ^{o}C or −80 °C freezers.

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Table 1. Total Protein Concentration of MON 87769-Produced PjΔ6D Protein Preparation by Amino Acid Analysis

Sample #	**Sample Analyzed** (µl)	**Calculated Concentration** (µg/µl)
1	10.4	0.5198
2	10.1	0.5287
3	10.0	0.5238
4	10.2	0.5168
5	10.2	0.5204
	Average Concentration	0.52 +/- 0.005

Table 2. Summary of the Tryptic Masses Identified for MON 87769-Produced PjΔ6D Protein Using MALDI-TOF Mass Spectrometry[1]

1 DHB-1	2 DHB-2	3 AC-1	4 AC-2	5 SA-1	6 SA-2	Expected	Diff[2]	Fragment	Sequence
507.36		507.31	507.35			507.27	0.09 (1)	383-386	GQFR
		572.38				572.27	0.11 (3)	230-233	FYDK
		588.37				588.31	0.06 (3)	276-280	EVANR
603.44		603.39				603.34	0.10 (1)	169-172	KWNR
657.40		657.35				657.29	0.11(1)	101-105	MGMFR
673.43		673.34	672.41			673.29	0.14(1)	101-105	MGMFR
718.58	718.59	718.51	718.59			718.43	0.15 (1)	388-393	ISPFVR
		744.50				744.41	0.09 (3)	275-280	REVANR
		774.50				774.41	0.09 (3)	421-427	NTAIEAR
846.73		846.64				846.52	0.21 (1)	387-393	KISPFVR
859.66	859.70	859.58				859.47	0.19 (1)	94-100	LLDSFHK
								235-242	LNFDGVSR
907.72	907.73	907.59	907.70	907.48		907.46	0.27 (1)	380-386	MPRGQFR
971.77	971.79	971.64	971.76	971.54		971.50	0.27 (1)	222-229	FFNSLTSR
	976.78					976.49	0.29 (2)	436-443	NMVWEAVK
992.75	992.76					992.49	0.26(1)	436-443	NMVWEAVK
						1035.55	0.33 (1)	380-387	MPRGQFRK
1035.88	1035.87	1035.72	1035.83			1035.56	0.32(1)	234-242	KLNFDGVSR
1129.99	1130.01	1129.84	1129.99	1129.75		1129.66	0.33 (1)	411-420	ANVLTLETLR
		1267.79				1267.56	0.23 (3)	82-92	HSVSETSSDYR
1284.04						1283.67	0.37 (1)	4-14	TIYITSSELEK
								387-397	KISPFVRDLCK
		1362.90				1363.74	0.84 (3)	388-398	ISPFVRDLCKK
1396.19		1395.87				1395.65	0.54 (1)	82-93	HSVSETSSDYRK
1409.16	1409.18					1408.72	0.44 (1)	399-410	HNLTYNIASFTK
1485.31	1485.35	1485.11	1485.23	1485.02	1485.2	1484.87	0.44 (1)	69-81	LLPPFSTNLLLEK
1537.34	1537.29					1536.82	0.52 (1)	398-410	KHNLTYNIASFTK
						1652.84	0.65 (2)	222-234	FFNSLTSRFYDKK
	1653.49		1653.37	1653.11	1653.29	1652.92	0.57 (2)	261-274	LNMLAQSFILLFSR
1669.40	1669.46	1669.18	1669.35	1669.08	1669.28	1668.92	0.48(1)	261-274	LNMLAQSFILLFSR
				1725.09		1724.88	0.21(5)	94-107	LLDSFHKMGMFRAR

1 DHB-1	2 DHB-2	3 AC-1	4 AC-2	5 SA-1	6 SA-2	Expected	Diff[2]	Fragment	Sequence
	2208.87					2209.11	0.24 (2)	173-191	FAQILSSNCLQGISIGWWK
					2331.74	2332.10	0.36 (6)	243-260	FLVQYQHWSFYPVMCVAR
				2347.47	2347.70	2348.10	0.63(5)	243-260	FLVQYQHWSFYPVMCVAR

[1] Only experimental masses that matched expected masses are listed in the table. All mass values shown were rounded to two decimal places. Columns 1-6 represent experimentally observed masses from Extract 1 or Extract 2 of trypsinized protein mixed with matrices dihydroxybenzoic acid (DHB), α-cyano-4-hydroxy cinnamic acid (AC), or 3,5-dimethoxy-4-hydroxycinnamic acid (SA).
[2] Diff represents the difference between the experimental mass and the expected mass; the number in parenthesis indicates the column containing the experimental mass used to calculate the difference.

Table 3. Molecular Weight and Purity of MON 87769-Produced PjΔ6D Protein

PjΔ6D Sample Loaded	**Apparent Mol. Wt.** (kDa)	**Purity**[1] (%)
2 μg Load (Figure 4, lane 3)	45.8	44.2
2 μg Load (Figure 4, lane 4)	45.7	42.4
3 μg Load (Figure 4, lane 5)	45.8	49.2
3 μg Load (Figure 4, lane 6)	45.9	46.3
4 μg Load (Figure 4, lane 7)	46.0	49.3
4 μg Load (Figure 4, lane 8)	46.1	49.6
Average Values[2] (Total for each lane / 6)	45.9 +/- 0.1	47 +/- 3

[1] Average purity is rounded to the nearest whole number.
[2] Total of six lanes/6, ± SD

Table 4. **Storage Stability (Day 29 vs. Day 0) of the MON 87769-Produced** PjΔ6D **Protein Stored in a 4 °C refrigerator, and in -20 °C and -80 °C freezers**

	Temperature, °C	**Day 0[1]**	**Day 29[2]**	**Percent Difference[3] (Day 29 vs. Day 0)**
Average Apparent Molecular Weight (kDa)	**4**	**45.9**	**44.4**	**3%**
	-20	**45.9**	**46.5**	**1%**
	-80	**45.9**	**46.7**	**1.7%**
Average Total PjΔ6D Purity (%)	**4**	**47**	**14**	**70%**
	-20	**47**	**48**	**2%**
	-80	**47**	**47**	**0%**

[1] See Table 3 for the apparent molecular weight and total purity of the Day 0 sample.
[2] Apparent molecular weight and total purity on Day 29 were estimated based on SDS-PAGE shown in Figures 6-8.
[3] Percent difference for apparent molecular weight or purity was calculated as follows:

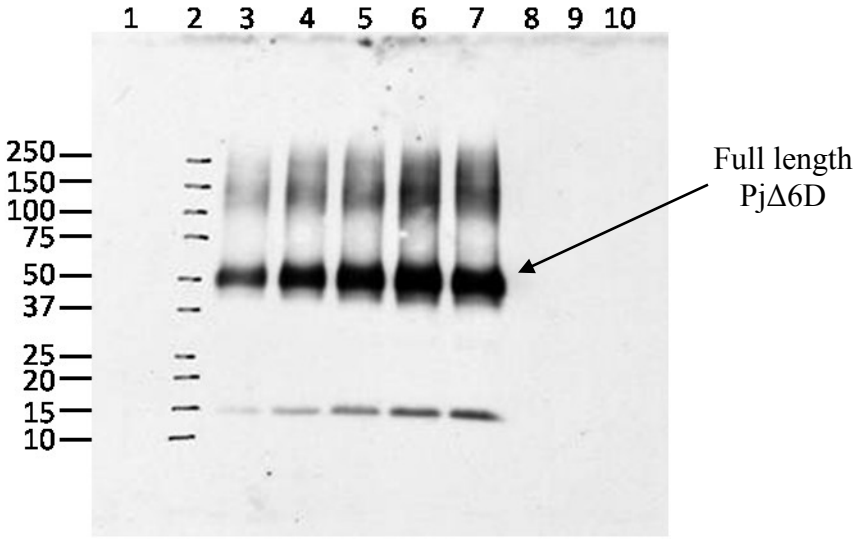

Figure 1. Immunoreactivity of MON 87769-Produced PjΔ6D Protein

Aliquots of the MON 87769 produced PjΔ6D proteins were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with an anti-PjΔ6D antibody, and immunoreactive bands were visualized using an ECL system. Approximate molecular weights (kDa) are shown on the right and correspond to the tick marks indicating the position of molecular weight markers loaded in lane 2. The 1 min exposure is shown. Arrow indicates PjΔ6D principal band.

Lane	Sample	Amount Loaded (ng)
1	Empty	-
2	Precision Plus Protein Standards	-
3	MON 87769-produced PjΔ6D protein	4
4	MON 87769-produced PjΔ6D protein	6
5	MON 87769-produced PjΔ6D protein	8
6	MON 87769-produced PjΔ6D protein	10
7	MON 87769-produced PjΔ6D protein	12
8	Empty	-
9	Empty	-
10	Empty	-

Amino acid residue # from the N-terminus	→	1	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Predicted PjΔ6D Sequence	→	M	T	K	T	I	Y	I	T	S	S	E	L	E	K	H	N
		|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Observed Sequence	→		T	K	T	I	Y	I	T	S	S	E	L	E	K	H	N

Figure 2. Summary of N-terminal Sequence Analysis of the MON 87769-Produced PjΔ6D Protein

Observed sequence obtained from the first fifteen cycles of Edman degradation was compared to that predicted from PjΔ6D. | indicates a match of the residues between the two sequences. The single letter IUPAC-IUB amino acid code is: E, glutamate; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; R, arginine; S, serine; T, threonine; Y, tyrosine.

```
1    MTKTIYITSS  ELEKHNKPGD  LWISIHGQVY  DVSSWAALHP  GGIAPLLALA
51   GHDVTDAFLA  YHPPSTSRLL  PPFSTNLLLE  KHSVSETSSD  YRKLLDSFHK
101  MGMFRARGHT  AYATFVIMIL  MLVSSVTGVL  CSENPWVHLV  CGAAMGFAWI
151  QCGWIGHDSG  HYRIMTDRKW  NRFAQILSSN  CLQGISIGWW  KWNHNAHHIA
201  CNSLEYDPDL  QYIPLLVVSP  KFFNSLTSRF  YDKKLNFDGV  SRFLVQYQHW
251  SFYPVMCVAR  LNMLAQSFIL  LFSRREVANR  VQEILGLAVF  WLWFPLLLSC
301  LPNWGERIMF  LLASYSVTGI  QHVQFSLNHF  SSDVYVGPPV  GNDWFKKQTA
351  GTLNISCPAW  MDWFHGGLQF  QVEHHLFPRM  PRGQFRKISP  FVRDLCKKHN
401  LTYNIASFTK  ANVLTLETLR  NTAIEARDLS  NPIPKNMVWE  AVKNVG
```

Figure 3. MALDI-TOF MS Coverage Map of the MON 87769-Produced PjΔ6D Protein

The amino acid sequence of the MON 87769-produced PjΔ6D protein was deduced from the coding region of the full-length PjΔ6D gene present in the grain of MON 87769 (Girault *et al*., 2008). Boxed regions correspond to tryptic peptide masses that were identified from the ~45.9 kDa protein band using MALDI-TOF MS. In total, 30 fragments covering 42.2% (188 of 446 total amino acids) of the expected protein sequence were matched to expected masses.

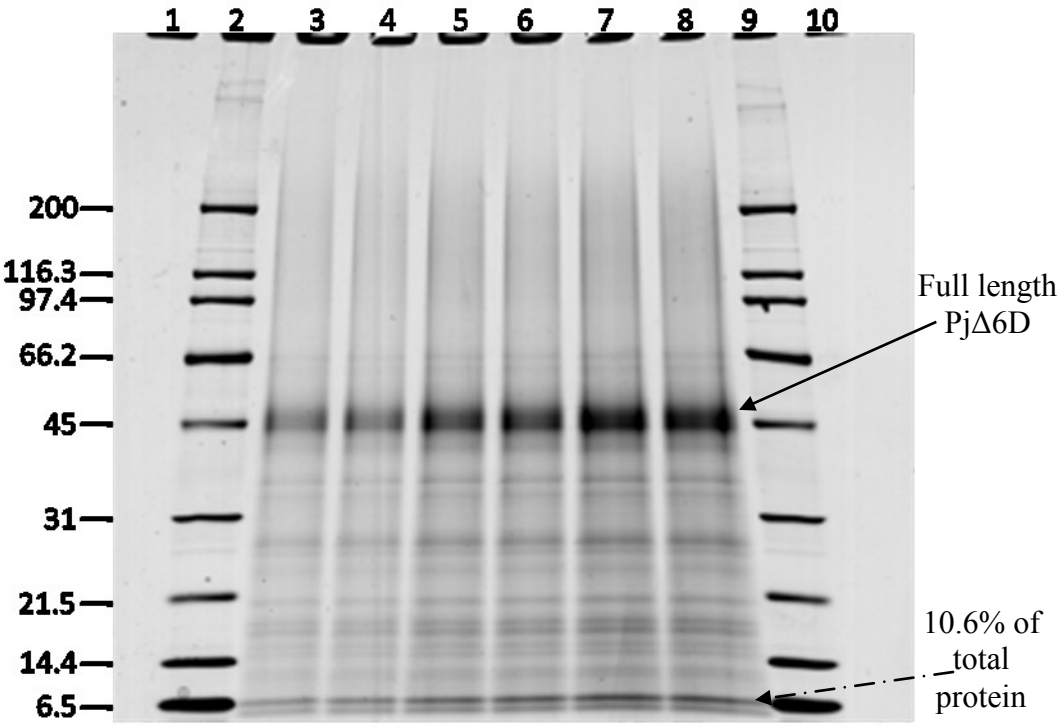

Figure 4. Molecular Weight and Purity Analysis of the MON 87769-Produced PjΔ6D Protein

Aliquots of the MON 87769-produced PjΔ6D protein were separated by SDS-PAGE, followed by Coomassie blue staining. Approximate molecular weights (kDa) are shown on the left and correspond to the marker loaded in lanes 2 and 9. Arrow indicates PjΔ6D principal band. Dotted arrow indicates band that constitutes 10.6% of the total protein and was subjected N-terminal sequence analysis.

Lane	Sample	Amount loaded (µg)
1	Blank	0
2	BioRad Broad Range Marker	4.5
3	MON 87769-produced PjΔ6D protein	2.0
4	MON 87769-produced PjΔ6D protein	2.0
5	MON 87769-produced PjΔ6D protein	3.0
6	MON 87769-produced PjΔ6D protein	3.0
7	MON 87769-produced PjΔ6D protein	4.0
8	MON 87769-produced PjΔ6D protein	4.0
9	BioRad Broad Range Marker	4.5
10	Blank	0

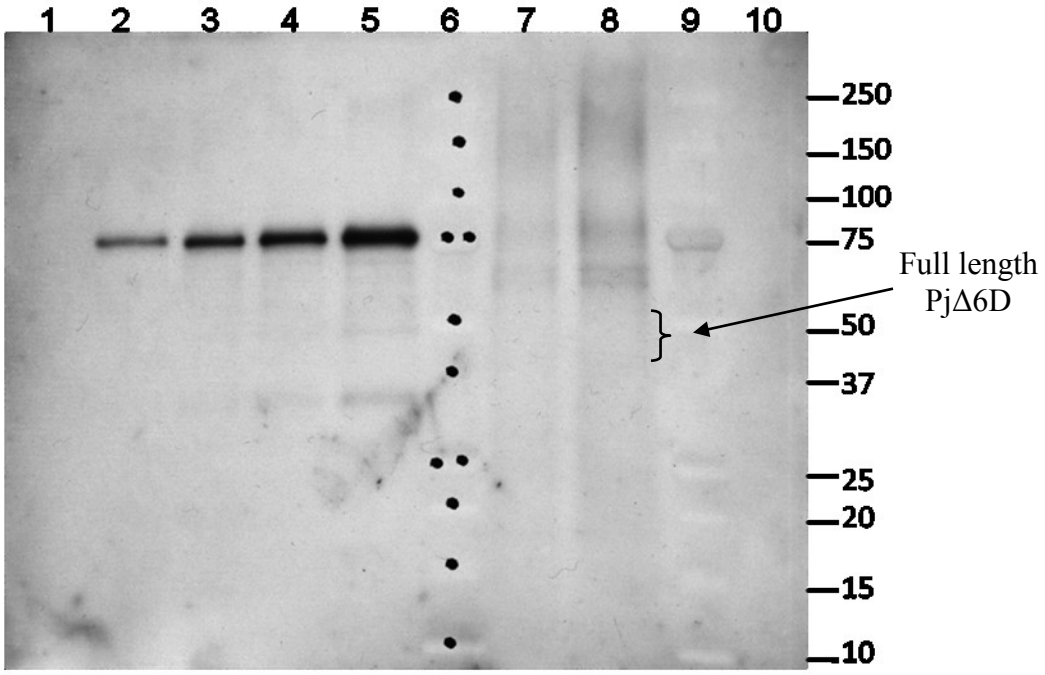

Figure 5. Glycosylation Analysis of the MON 87769-Produced PjΔ6D Protein

Aliquots of transferrin (positive control) and MON 87769-produced PjΔ6D protein were separated by SDS-PAGE and transferred to a PVDF membrane. Carbohydrate moieties were labeled with biotin-hydrazide and detected using a Streptavidin-HRP antibody. Chemiluminescent activity of the HRP tag was detected using Hyperfilm ECL. Approximate molecular weights indicated in kDa correspond to protein standard markers (lane 6 and 9). Bracket and arrow indicate expected position of PjΔ6D principal band.

Lane	Sample	Amount Loaded (µg)
1	Empty	-
2	Transferrin	0.025
3	Transferrin	0.050
4	Transferrin	0.100
5	Transferrin	0.200
6	Precision Plus Dual Color MW marker	na
7	MON 87769-produced PjΔ6D	0.500
8	MON 87769-produced PjΔ6D	1.0
9	Precision Plus Dual Color MW marker	na
10	Empty	-

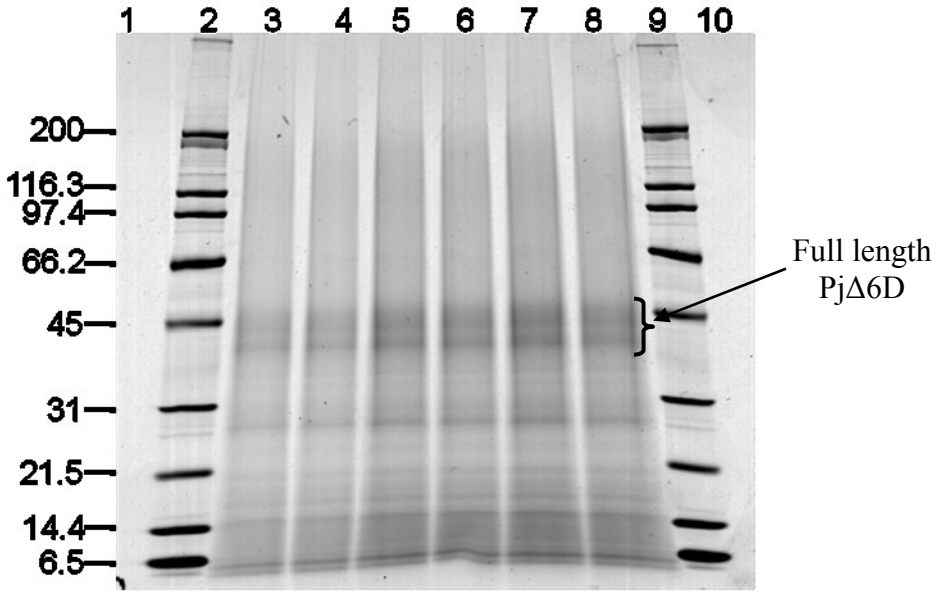

Figure 6. Storage Stability (Day 29) of the MON 87769-Produced PjΔ6D Protein Stored at 4°C. Arrow and bracket indicate PjΔ6D principal band. Figure 4 represents the Day 0 control for reference.

Lane	Sample	Amount loaded (µg)
1	Blank	-
2	BioRad Broad Range Marker	4.5
3	MON 87769-produced PjΔ6D protein	2.0
4	MON 87769-produced PjΔ6D protein	2.0
5	MON 87769-produced PjΔ6D protein	3.0
6	MON 87769-produced PjΔ6D protein	3.0
7	MON 87769-produced PjΔ6D protein	4.0
8	MON 87769-produced PjΔ6D protein	4.0
9	BioRad Broad Range Marker	4.5
10	Blank	-

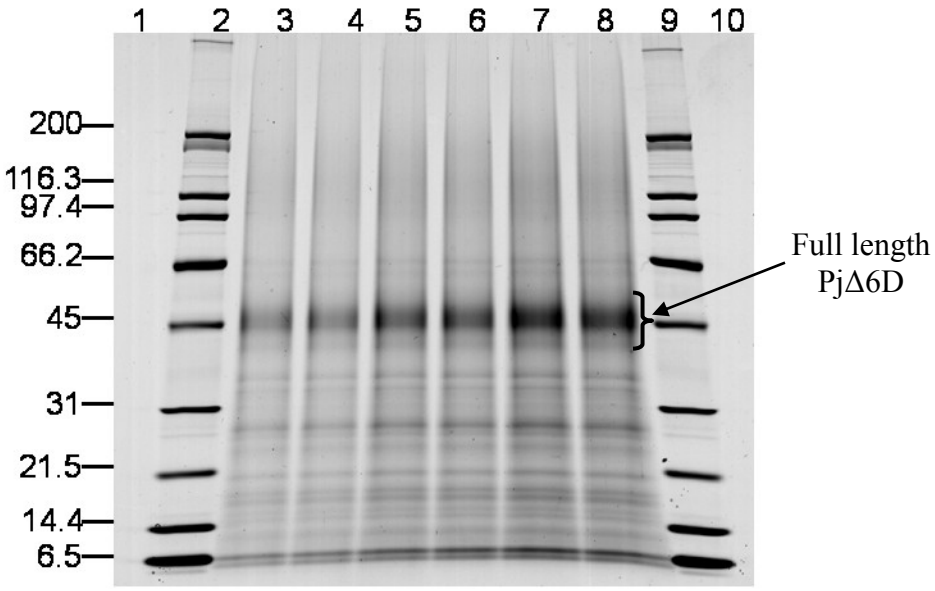

Figure 7. Storage Stability (Day 29) of the MON 87769-Produced PjΔ6D Protein Stored at -20°C. Arrow and bracket indicate PjΔ6D principal band. Figure 4 represents the Day 0 control for reference.

Lane	Sample	Amount loaded (µg)
1	Blank	-
2	BioRad Broad Range Marker	4.5
3	MON 87769-produced PjΔ6D protein	2.0
4	MON 87769-produced PjΔ6D protein	2.0
5	MON 87769-produced PjΔ6D protein	3.0
6	MON 87769-produced PjΔ6D protein	3.0
7	MON 87769-produced PjΔ6D protein	4.0
8	MON 87769-produced PjΔ6D protein	4.0
9	BioRad Broad Range Marker	4.5
10	Blank	-

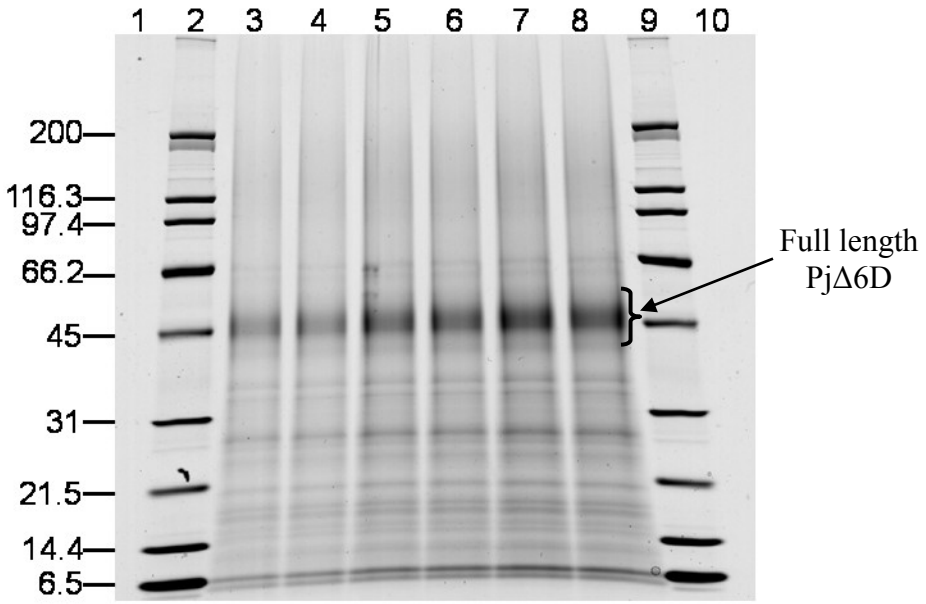

Figure 8. Storage Stability (Day 29) of the MON 87769-Produced PjΔ6D Protein Stored at -80°C. Arrow and bracket indicate PjΔ6D principal band. Figure 4 represents the Day 0 control for reference.

Lane	Sample	Amount loaded (µg)
1	Blank	-
2	BioRad Broad Range Marker	4.5
3	MON 87769-produced PjΔ6D protein	2.0
4	MON 87769-produced PjΔ6D protein	2.0
5	MON 87769-produced PjΔ6D protein	3.0
6	MON 87769-produced PjΔ6D protein	3.0
7	MON 87769-produced PjΔ6D protein	4.0
8	MON 87769-produced PjΔ6D protein	4.0
9	BioRad Broad Range Marker	4.5
10	Blank	-

Appendix 1. List of Applicable SOPs

SOP Number	Title
AG-EQ-1051-02	Atlas Chromatography Data System
BR-EQ-0265-02	Applied Biosystems 494 Procise™ Protein Sequencing System
BR-EQ-0599-03	Bio-Rad GS-800 Densitometer
BR-EQ-0783-02	Applied Biosystems Voyager DE Pro Biospectrometry Workstation
BR-EQ-0935-01	Konica SRX X-Ray Film Processors
BR-EQ-1138-01	Waters 2695 Separations Module for AccQ-Tag® Analysis
BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0392-01	Western Blot Analysis (Immunoblotting)
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-ME-0602-01	Staining of Carbohydrate Moieties Using Commercially Available Kits
BR-ME-0802-01	Protein Fragmentation via In-Gel Trypsin Digestion
BR-ME-0924-01	Electrotransfer of Proteins to Membranes
BR-ME-0926-01	Staining of Proteins on Blot Membranes
BR-ME-0932-03	Assessment of Immunoreactive Bands from Western Blots Exposed to X-Ray Films Using Bio-Rad GS-800 Densitometer
BR-ME-0956-03	Protein Percent Purity and Apparent Molecular Weight Determination
BR-ME-0994-01	Coomassie Blue Staining of Polyacrylamide Gels
BR-ME-1139-01	Vapor Phase Acid Hydrolysis Using 6 N HCl and Subsequent Amino Acid Analysis Using AccQ-Tag® Derivatization

Appendix 2. Deviations

There were 8 protocol deviations.

1) The protocol stated that the blot for N-terminal analysis would be stained following SOP BR-ME-0527-01, a colloidal Coomassie blue staining protocol. However, SOP BR-ME-0994-1 a non-colloidal protocol was followed. Both staining methods utilize Coomassie dye to visualize the proteins and the purpose of the staining was simply to locate the correct protein bands to be excised for analysis. Thus, there is no impact to the study.
2) The protocol for tryptic digestion states that destaining of the individual gel bands will be at most 3 separate events of not longer than 1 hour each. 4 events of destaining were done, 3 of 30 minutes and one of 60 minutes. Overall, staining time did not exceed 3 hours, but the number of events of staining was exceeded. There is no impact to the study.
3) The protocol for tryptic digestion states that 0.75 µl of matrix would be pipetted for analysis; 0.70 µl was used. The amount of matrix present in the analysis was suitable for the sample; therefore, there is no impact to the study.
4) There was deviation from SOP BR-ME-0956-03 in evaluation protein purity and molecular weight (Purity Gels from 02/015/08 and Stability Gels from 03/14/08). The SOP states: Image Acquisition, Page 3, step 5 From "Step III", select a resolution setting of 36.3 × 36.3 µm from the list of resolutions and then click "Done". In the actual image acquisition step, the resolution setting of 42.3 × 42.3 was chosen by system default, resulting in the deviation. To address the possible effect of this deviation, the -80 ^{o}C stability gel which had been stored in destain B solution was re-stained, destained again, and scanned at both the resolution setting of 36.3 × 36.3 as specified in BR-ME-0956-03 and the setting of 42.3 × 42.3. The purity of lanes 5 and 6 (the 2 µg loadings) were analyzed and compared at both settings. The results showed no difference in the purity when scanned with either setting. It was, therefore, concluded that this deviation had no impact on the study data. The results of this analysis have been archived with the study file.
5) The individual pmol amino acid (AA) yields of several AA's in the test sample, APS lot 10001532, did not fall within the range of the AA yields of the AA calibration standards. One of the acceptance criterion of BR-ME-1139-01 was not satisfied, but the data were accepted. This analysis was accepted due to the following four reasons: 1) The derivatization agent is present in significant excess to allow for complete derivatization of the amount of sample analyzed, 2) Based on manufacturer's data, the assay is able to linearly quantitate samples at the levels observed in this analysis and 3) The dynamic range of the detector used to quantitate the sample far exceeds the highest standard in the validated range of this SOP. Finally, 4) the estimated concentration based on pre-study work, when the sample was appropriately diluted to be within the range of the standards, agrees with the concentration obtained in this analysis. Taken together these four factors led to the conclusion that the sample has been

quantitatively derivatized, quantitated and detected. This deviation had no impact to the quality of the data returned by the analysis.

6) The protocol for total protein concentration determination states that precipitated protein will be spun at 12,000 rpm in a microcentrifuge for 30 minutes at 2-8 ^{o}C. The sample was spun for 45 minutes at 2-8 ^{o}C, which was incorrectly specified on the worksheet. Increased centrifugation will not cause increased precipitation or make the pellet more difficult to analyze as all protein would be completely precipitated within 30 min, therefore there is no impact to the study by this deviation.
7) SOP BR-ME-1139-01 requires the use of Atlas injection designations for final calculations in the final Excel spreadsheet. These designations were not used. Instead, the injection numbers defined initially within the HPLC system were carried forward. The Atlas system assigns an additional injection to a system conditioning run which is not designated as an injection by the HPLC system. As the two systems do not have identical designations, we have chosen to maintain the initial injection designation. There was no impact to the study data.
8) SOP BR-PO-0454-02 requires completely filled out logbooks for equipment and the use of black or blue ink to record handwritten data. The logbook for the film developer (PR-60) used to develop the immunoblot autoradiogram in this study was not completely or correctly filled out. As the film developed correctly thus verifying the solutions' activity, there is no impact to the data.

Appendix 3. Analytical Protein Standard Certificate of Analysis for MON 87769 Seeds of Soy (Orion Lot# 10001532)

Analytical Protein Standard
Certificate of Analysis

MONSANTO — ANALYTICAL PROTEIN STANDARDS

Sample Information:

Name of APS	APS Lot Number	Expiration Date
Seeds of Soy-produced *Primula juliae* Delta 6 Desaturase [MON 87769]	10001532	July 31, 2008

Common or Alias Name(s)	Historical APS Lot Number	Storage Requirements (until use)
PjD6D	—	–80 °C

Source	Comment(s)
Seeds of Soy MON 87769	None

Additional Background Information
None

Characteristic	Method	Assay Date	Result
Concentration	Amino Acid Composition	12 Feb 2008	0.52 mg/mL (total protein)
Purity	SDS-PAGE/Densitometry	14 Feb 2008	47%
Molecular weight	SDS-PAGE/Densitometry	14 Feb 2008	45.9 kDa
Identity	Immunoblot	29 Feb 2008	Confirmed – immuno reactive band observed
Identity	N-terminal sequence	27 Feb 2008	Confirmed – TKTIYITSSELEKHN
Identity	MALDI-TOF MS (Trypsinized)	28 Feb 2008	Confirmed sequence 42.2 % coverage of expected sequence

Buffer composition: 50 mM sodium acetate, pH 5.6, 1 mM $MgCl_2$, 0.1% Fos-choline 12, 0.5M NaCl and 10% glycerol.
Physical description: Clear solution
Short-term storage stability (29 days) was evaluated during the certification process. Based upon the criteria provided in Characterization Plan 10001532, no significant degradation was observed for samples stored at –80°C or -20º C. However, the relative molecular weight of the PjD6D protein had changed when stored at 4ºC.

Purity corrected concentration is 0.24 mg/mL (0.52 mg/mL × 0.47 = 0.24 mg/mL)

Quality Assurance Specialist — 04/29/08 Date

Analytical Protein Standards Officer — 4/29/08 Date

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