Characterization Title

Characterization of *Neurospora crassa* Δ15 Desaturase Isolated from Immature Seeds of Soybean MON 87769

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Submitter	Date
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Lead Scientist	09-09-08 Date

Quality Assurance Unit Statement

Report Title: Characterization of *Neurospora crassa* Δ15 Desaturase Isolated from Immature Seeds of Soybean MON 87769

Characterization Plan Number: 10001516

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
4/17/2008, 4/21-22/2008	Raw Data Audit	04/29/2008	04/29/2008
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Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

09/09/2008
Date

Characterization Report Certification

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

Signatures of Final Report Approval:

Jian G. Dong, Ph.D., Lead Scientist — Date: 09-09-08

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Report Information

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Testing Facility: Monsanto Company
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Table of Contents

List of Tables

List of Figures

List of Appendices

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	SDS-Loading buffer [62.5mM Tris-HCl, pH6.8, 5% (v/v) 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, 0.005% (w/v) bromophenol blue, 10% (v/v) glycerol]
5x 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
NFDM	Non-fat dried milk
NcΔ15D	*Neurospora crassa* delta-15 desaturase
NIST	National Institute of Standards and Technology
PBST	Phosphate buffered saline - Tween® 20
PCR	Polymerase chain reaction
PjΔ6D	*Primula juliae* delta-6 desaturase
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 the *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.

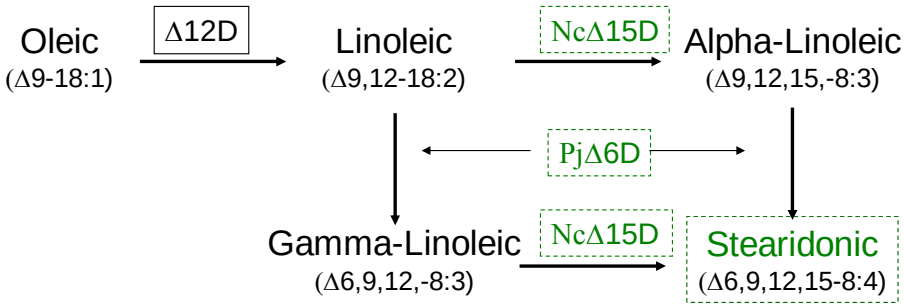
In order to produce proteins for safety testing, the NcΔ15D protein was purified from immature soybean seeds of MON 87769 and its physicochemical properties were characterized. This report describes the results of the characterization of NcΔ15D protein purified from MON 87769.

A panel of analytical techniques was used to characterize the MON 87769-produced NcΔ15D protein. These analytical techniques included sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Colloidal Brilliant Blue G 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 NcΔ15D protein was assessed using SDS-PAGE by estimating the purity and molecular weight of the protein after storage in a 4 °C refrigerator, and -20 °C and −80 °C freezers.

The identity of the purified MON 87769-produced protein was confirmed by its recognition with anti-NcΔ15D antibody in western blot analysis, identity of the first 15 amino acids of the expected N-terminus by amino acid sequencing, and identification of the tryptic peptide fragments that cover 45% (193 out of 429) of the expected NcΔ15D protein sequence. The total protein concentration of the final NcΔ15D protein preparation was determined to be 0.62 mg/ml. Purity of the purified NcΔ15D protein was 74% and the apparent molecular weight was estimated to be 46 kDa. The MON 87769-produced protein was not glycosylated. Finally, the MON 87769-produced NcΔ15D protein was stable for at least 29 days when stored in a -20 °C or −80 °C freezer, but was not stable when stored in a 4 °C refrigerator for 29 days. Taken together, these data provide a detailed characterization of the NcΔ15D 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 product are represented by dash-lined boxes.

PjΔ6D and NcΔ15D 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). They are involved in desaturation at the delta 6 and 15 positions of C-18 or longer fatty acids. Both PjΔ6D and NcΔ15D desaturases are integral membrane proteins which contain di-iron cofactors that are bound to three histidine box motifs (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 used to further purify the NcΔ15D. Standard precautions to

retain the structural integrity of the solubilized protein during purification were taken 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 the solubilized NcΔ15D which enabled the characterization of its physicochemical properties.

3.0 Purpose

The purpose of this report was to describe the physicochemical properties of the NcΔ15D protein purified from the seeds of soybean MON 87769 (Orion Lot No.: 10001516).

4.0 Materials

4.1 MON 87769-Produced Delta-15 Desaturase Protein

The plant-produced NcΔ15 protein was purified from immature seeds of soybean MON 87769. Two batches of immature seeds were used for the isolation. The first batch of immature seeds was obtained from MON 87769 that was grown in Hawaii under production plan 05-01-83-04. The LIMS ID for this seed is 05018304-00014 and the lot number is GLP-0509-16624-S. The identity of this batch of MON 87769 immature seeds was confirmed by event-specific polymerase chain reaction (PCR). A copy of the verification of identity (VOI) for this seed is archived with the synthesis file under Orion lot #: 10001516. The second source was immature seeds from MON 87769 grown at a Trait Development Site in Wyoming, Illinois (Trial ID, M0322, Lot # G-828102A). The serial number for this batch of seeds is 60087467986. The identity of this batch of seeds was confirmed by event-specific PCR and a copy of the VOI is archived with the synthesis records under Orion lot #:10001516. 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 10001516. The final protein preparation was 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

Molecular Weight (MW) standards (BioRad, Hercules, CA) were used to calibrate SDS-PAGE gels or to verify protein transfer to polyvinylidene difluoride (PVDF) membranes by Western blotting. A peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) was used to calibrate the MALDI-TOF mass spectrometer for mass determination of the tryptic fragments. β-Lactoglobulin (Applied Biosystems, Foster City, CA) was used as control to confirm N-terminal sequence determination. Transferrin (GE Healthcare, Piscataway, NJ) was used as a positive control in glycosylation analysis.

5.0 Methods

5.1 Protein Purification

The NcΔ15 protein was purified from the immature seeds of MON 87769 prior to initiation of this characterization plan. The purification 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 13.5 kg of immature soybean seeds were used for the isolation of NcΔ15D protein in two separate batches, a small batch and a large batch. For the isolation using the smaller batch, approximately 2.5 kg of seeds (Seed Lot No. GLP-0509-16624-S) were homogenized in 4 sub-batches (500-800 g seed per batch) for the isolation of membrane fractions. The membranes were then solubilized in 2% Fos-choline 12 and separated on a 500-ml cation exchange chromatography column followed by immuno-affinity purification. The solubilization and cation exchange chromatography were performed in 3 different runs. The remaining 11 kg of seeds (Seed Lot No. G-828102A) were homogenized for membrane isolation at a larger scale with 1,000-2,500 g of seeds per extraction (6 runs). The solubilized membranes were then separated on a 3,000-ml cation exchange chromatography column (3 runs) followed by immuno-affinity purification (30 runs). The NcΔ15D protein preparations by both small and large batches were combined. Although at different scales, similar conditions (e. g. buffer to sample ratio) were used to purify NcΔ15D protein at both the small and large scales, therefore, the protein preparations generated from both batches were practically identical. After a preliminary determination of the purity and concentration of the purified NcΔ15D preparation, the protein preparation was submitted to Monsanto's APS program under the Orion lot # 10001516.

Each run included the following series of purification steps:

Homogenization and membrane isolation – In a 5-L Viking Blender (Waring Commercial®, Torrington, CT), immature soybean seeds were homogenized at a setting of High for 3 min in 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 using a tissue to buffer ratio of 1g to 10 ml. The process was repeated until 1-2 kg of seeds were homogenized. After filtration of the homogenate through four layers of cheese cloth, the filtrate was centrifuged at 2,000 x *g* to remove cell debris. The supernatant was then centrifuged at 37,000 x *g* for 1 hr. After the centrifugation, the supernatant was discarded and the pellet re-suspended in ice-cold de-ionized H_2O at a membrane concentration of approximately 40 mg/ml (fw/v). The membrane suspension was then mixed with an equal volume of 2x 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 x *g* 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) were thawed and re-suspended in 5 liters 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% (w/v) and the membrane/detergent solution was incubated for 2 hr with stirring. The mixture was centrifuged at 37,000 x *g* for 30 min to remove the insoluble fractions. The solubilized supernatant was applied to 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. Fractions containing protein were pooled based on OD_{280}, and stored at -80 °C until further purification by immuno-affinity chromatography.

Immuno-affinity chromatography – Immuno-affinity (I.A.) resins used in the purification were prepared by immobilizing anti-NcΔ15D IgG to agarose resins using an IgG-orientation kit from Pierce (Cat. No. 44990, Rockford, IL) according to manufacture's instruction. The anti-NcΔ15D IgG (Lot Numbers: 7580958, 7580959, and 7580965) were used for I.A. resin preparation. The I.A. column (bed vol. ~28 ml) was equilibrated with Immuno-Affinity Washing Buffer consisting of 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 10% glycerol (v/v) and 0.1% Fos-choline 12 (w/v). Before applying the SP-Sepharose-purified protein preparation to the I.A. column, the pH of the sample 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 Immuno-Affinity Washing Buffer and the NcΔ15D protein 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 I.A.-purified NcΔ15D 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 the SP-Buffer A. The flow rate was typically at 4.0 ml/min. The protein was eluted with 0.5 M NaCl in the SP-Buffer A. Peak fractions containing protein were pooled and stored at -80 °C.

5.2 Determination of Protein Concentration

The total protein concentration of the purified MON 87769-produced NcΔ15D protein 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 sample preparation was as follows: in a hydrolysis tube (~300 µl), approximately 1µg 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 30 min at 2-8 °C. The supernatant was removed and discarded. Precipitates were then washed 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. 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 reconstituted in 20 µl of 20 mM HCl by vortexing. 60 µl of AccQ-Fluor Borate Buffer and 20 µl 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 to 55 °C for 10 min. Samples were analyzed using a 2695 Separation Module (Waters Corp.) equipped with a reverse-phase C-18 column for separation of AccQ-Tag derivatized amino acids. Chromatographic data were collected using Atlas software (Thermo Electron Corp. Waltham, MA).

5.3 Protein Identity

5.3.1 Immunoblot Analysis

Immunoblot analysis was performed to confirm the identity of NcΔ15D protein purified from MON 87769. Based on the concentration and purity, aliquots of the purified NcΔ15D protein preparation were diluted in 50 mM sodium acetate, pH 5.6, 10% glycerol and 0.1% Fos-choline 12 to give a final purity-corrected protein preparation of 2, 4, 6, 8 and 10 ng/10 µl, respectively. The samples (10 µl) were then mixed with 2.5 µl of 5x Loading Buffer (312 mM Tris-HCl, pH 6.8, 25% 2-mercaptoethanol, 10% sodium dodecyl sulfate, 0.025% bromophenol blue, 50% glycerol) 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 NcΔ15D sample was heated before analysis using SDS-PAGE. The samples were then separated on the SDS-PAGE at constant voltage of 50 V for 25 min followed by 160 V for 70 min. Proteins in the gel were electrotransferred to a PVDF membrane (Invitrogen, Carlsbad, CA) at a constant voltage of 25 V for 90 min in the electrotransfer buffer containing 12 mM Tris, pH 8.3, 96 mM glycine and 20% methanol. Pre-stained MW markers were loaded in parallel to verify the transfer of proteins and estimate the size of the immuno-reactive bands observed.

Following the electrotransfer, the membrane was blocked for 30 min with 4% (w/v) non-fat dry milk (NFDM) in phosphate-buffered saline-Tween 20 (PBST). The membrane was then probed with a 1:5,000 dilution of goat anti-NcΔ15D antibody (Lot No. 7580958) in PBST containing 2% (w/v) NFDM for 1 hr. Excess antibody was removed by 3x 15 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 5x 15 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 NcΔ15D.

5.3.2.1 Protein Blot for N-Terminal Analysis

Five 20 µl aliquots of MON 87769-produced NcΔ15D protein preparation were removed from storage, mixed with 5 µl of 5x LB and loaded in 5 lanes (25µl/lane) onto a 4-20% gradient polyacrylamide (10-well) gel. 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) membranefor 2 hr in the electrotransfer buffer containing 10 mM CAPS, pH 11 and 10% methanol at a constant voltage of 26 V. 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 NcΔ15D protein was excised based on molecular weight from the blot and N-terminal sequence analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller and Hood, 1983). 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[99] 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. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein, 10 pmole 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 Map Coverage Analysis

MALDI-TOF mass spectrometry was used to confirm the identity of the MON 87769-produced NcΔ15D protein.

5.3.3.1 Protein Gel for Tryptic Mass Map Analysis

Five 20 µl aliquots of MON 87769-produced NcΔ15D protein preparation were removed from storage, mixed with 5 µl of 5x LB and loaded in 5 lanes (25 µl/lane) onto a 4-20% gradient polyacrylamide (10-well) gel. Pre-stained MW markers (Precision Plus Protein Standard, BioRad, Hercules, CA) were loaded in parallel to estimate the size of the stained bands observed. 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. Following electrophoresis, the gel was stained with Coomassie blue dye R-250 (BioRad, Hercules, CA). The bands corresponding to the MON 87769-produced NcΔ15D protein were 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 in a microfuge tube. This step was repeated 3 times with the final destain step proceeding for 60 minutes. Following destaining, the gel bands were incubated in 100 µl per band 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. 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 room temperature for 20 min in the dark. The gel containing the protein band was incubated in 200 µl of 25 mM ammonium bicarbonate buffer for 15-45 min at room temperature. This step was repeated two additional times, following which the gel band was dried using a Savant Speed Vac concentrator (Ramsey, MN). Each gel band was rehydrated with 20 µl of 0.02 µg/µl trypsin in 25 mM ammonium bicarbonate and 10% acetonitrile, and was incubated for about 1 h at room temperature. 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 Speed Vac concentrator (Extract 1). The gel band was resuspended in 30 µl of a solution consisting of 60% acetonitrile, 0.1% trifluoroacetic acid and 0.1% octyl-β-D-glucopyranoside, 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 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.

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, Foster City, CA). The samples Extract 1 and Extract 2 (0.1-0.25 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 7,500 Dalton range using 100 shots at a laser intensity setting of 2974. 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 7,500 Dalton range using 100 shots at a laser intensity setting between 2,400 and 2,600. The samples in sinapinic acid matrix were analyzed in the 500 to 7,500 Dalton range using 100 shots at a laser intensity setting between 2,800 and 3,100. Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults,

1993), except above 3,000 Daltons, where mass-averaged values were used. GPMAW32 software (Applied Biosystems) was used to generate a theoretical trypsin digest of the deduced NcΔ15D amino acid 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 1,000 Da range if there were two or more isotopically resolved peaks, and in the 1,000 to 8,000 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 Analysis by SDS-PAGE

Aliquots of the MON 87769-produced NcΔ15D protein were diluted with the solubilization buffer (50 mM sodium acetate, pH 5.6, 0.1% Fos-choline 12. and 10% glycerol) and mixed with 5x LB to a final protein concentration of 0.2 μg/μl. The NcΔ15D protein was analyzed in duplicate at 1, 2, and 3 μg of total protein per lane. MW standards were heated, though the test samples were not for the reasons described in Section 5.3.1, and all samples were applied to a pre-cast 4-20% polyacrylamide gradient 10-well gel (Invitrogen, Carlsbad, CA). MW markers (Broad Range MW Marker, 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. The gel was stained using Colloidal Brilliant Blue G 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 MW markers were used to estimate the apparent MW of the NcΔ15D protein. For the purity evaluation, all visible bands within each lane were quantified. The purity and estimated MW of the MON 87769-produced NcΔ15D protein were reported as the average of the six values obtained by densitometric analysis.

5.5 Glycosylation Analysis

The GE Healthcare Glycosylation Detection Module (Cat. No. RPN 2190, Piscataway, NJ) was used to detect carbohydrate covalently bound to MON 87769-produced NcΔ15D after the proteins were resolved by SDS-PAGE and electrotransferred onto a 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. This biotin-labeling procedure is specific for the 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 NcΔ15D protein were mixed with 5x LB and loaded onto a 4-20% precast polyacrylamide gel (Invitrogen, Carlsbad, CA). Along with the NcΔ15D samples, 25, 50, 100 and 200 ng of the glycosylated control protein, transferrin, were loaded in parallel. The NcΔ15D 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. Following electrophoresis, proteins in the gel were electrotransferred to a 0.45 µM PVDF membrane (Invitrogen, Carlsbad, CA) at a constant voltage of 25 V for 90 min in the electrotransfer buffer containing 12 mM Tris, pH 8.3, 96 mM glycine and 20% methanol. Pre-stained MW markers were loaded to verify electrotransfer of proteins and estimate the size of the bands on the blot.

The labeling and detection of carbohydrate was carried out according to the Manufacturer's instruction and all the reagents except PBS were provided in the kit. Following electrotransfer to 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. Following the treatment, the membrane was then rinsed twice with 15 ml PBS and washed three times with 15 ml PBS for 10 min each. The membrane was incubated with biotin-hydrazide for 60 min followed by two PBS rinses and three 10 min washes with 15 ml PBS as described above. The membrane was blocked for 60 min with 5% blocking reagent in PBS followed by two PBS rinses and three 10 min washes with 15 ml PBS. Strepavidin-HRP at 1:6000 dilution was overlaid onto the membrane and incubated at room temperature for 30 min. After two PBS rinses and three 10 min washes with 15 ml PBS, 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 towels 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 NcΔ15D protein was evaluated by comparing the purity and MW values before and after storage for 29 days in the following areas: a 4 ºC refrigerator, a -20 ºC and a -80 ºC freezer. At the end of storage, aliquots of the NcΔ15D protein samples equivalent to 1, 2 and 3 µg of the purity-corrected NcΔ15D protein at Day 0 were removed and subjected to SDS-PAGE followed by staining, and subsequent purity and MW estimation. The MON 87769-produced NcΔ15D protein was analyzed in duplicate at 1, 2, and 3 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). Electrophoresis was performed at a constant voltage of 120 V for 20 min followed by 180 V for 60 minutes. The gels were stained using Colloidal Brilliant Blue G 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 NcΔ15D 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 was reported.

7.0 Characterization Plan 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.

9.0 Results and Discussion

9.1 Protein Concentration

The total protein concentration of the purified NcΔ15D preparation from MON 87769 immature seed was estimated using Accu-Tag AAA. The average across all five independently prepared dilutions was determined to be 0.62± 0.03 mg/ml (Table 1). A total of 43 mg total protein of the MON 87769-produced NcΔ15D protein in 69.5 ml was obtained and submitted to the Analytical Protein Standards program of Monsanto.

9.2 Protein Identity

The identity of the MON 87769-produced NcΔ15D protein was confirmed using three independent methods: immunoblot analysis using an anti-NcΔ15D peptide antibody, N-terminal sequencing, and MALDI-TOF MS-generated tryptic map mass analysis.

9.2.1 Immunoblot Analysis

The purity-corrected NcΔ15D protein was loaded at the amounts of 2, 4, 6, 8, and 10 ng per lane on a SDS-PAGE gel, blotted onto a PVDF membrane and probed with anti-NcΔ15D peptide antibody. A predominant immuno-reactive band was observed in each lane migrating at the expected molecular weight of approximately 46 kDa (Figure 1, lanes 2-6). As expected, the intensity of the immunoreactive band increased with increasing amount of protein loaded.

The immunoreactive profiles shown in Figure 1 reveal a slightly smeared staining in the high molecular area. When membrane proteins are separated on SDS-PAGE, a small portion tends to migrate as higher-order 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 smear seen in these blots consisted mainly of NcΔ15D protein or of endogenous cross-reacting proteins as the smear area is immuno-reactive with anti- NcΔ15D antibody.

In addition, two minor bands with molecular weight of approximately 27 kDa and 30 kDa (Figure 1) were recognized by the antibody. Most likely these fragments are the results of proteolytic degradation of the NcΔ15D protein or of endogenous cross-reacting proteins co-purified during the preparation of NcΔ15D protein.

9.2.2 N-Terminal Sequencing

N-terminal sequence analysis of the major protein band on PVDF membrane with molecular weight of ~46 kDa observed on stained SDS-PAGE and immuno-reacted with the NcΔ15D antibody revealed a sequence of 15 amino acids that matched the expected N-terminal sequence for the NcΔ15D 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 NcΔ15D protein was deduced from the coding region of the full-length NcΔ15D gene present in MON 87769 (Girault *et al*., 2008).

9.2.3 MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 87769-produced NcΔ15D 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 a sequence is 15% or higher with a minimum of five

matched peptides (Jensen *et al*., 1997). There were 15 unique protein fragments identified that matched the expected tryptic masses of the trypsin-digested NcΔ15D 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 2). A total of 193 out of 429 amino acid residues (45%) were identified and they were all mapped to the predicted positions within NcΔ15D sequence. The protein is confirmed to be NcΔ15D.

9.3 Purity and Molecular Weight Estimation

Purity and molecular weight of the purified NcΔ15D protein preparation were estimated using densitometric analysis after the proteins were separated by SDS-PAGE and stained with Brilliant Blue G (Figure 4). As summarized in Table 3, molecular weight values were averaged from duplicated loads of 1.0, 2.0, and 3.0 μg of total protein (Figure 4, Lanes 3-8). The predominant band that was identified as NcΔ15D was estimated to have a molecular weight of 46 kDa. The average purity of the NcΔ15D protein is estimated to be 74%. Hence, after purity-based correction, the final concentration of the NcΔ15D protein in the preparation is 0.46 mg/ml.

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 any serine or threonine residue. NcΔ15D is an integral membrane protein of microbial origin which contains two putative N-glycosylation sites, (Asn-Xxx-Ser/Thr) (Marshall, 1972). To test whether the MON 87769-produced NcΔ15D protein was glycosylated once expressed in soybean seeds, it was analyzed for the presence of covalently bound carbohydrate moieties using a GE Glycoprotein Detection Module which detects carbohydrate moieties conjugated through O- and N-linkages. A naturally, glycosylated protein transferrin was utilized as a positive control in the assay. The results of this analysis are presented in Figure 5. A strong signal at the expected MW position was detected for the transferrin positive control in a concentration-dependent manner (Lanes 2-5). No detectable signal was observed for the NcΔ15D protein (Lanes 7-8) at the expected molecular weight position of around 46 kDa.

Two faint bands were detected by the carbohydrate reaction at significantly higher molecular weight of ~70 kDa in lanes 6-7 containing purified NcΔ15D protein (Figure 5). An additional very weak signal was observed in the same lanes between 150 kDa and 250 kDa. Taking into consideration that the purity of NcΔ15D protein is

approximately 74% and that no signal was observed at the expected MW for NcΔ15D protein (~46 kDa), it was concluded that the faint signals observed on the blot are not derived from the NcΔ15D protein. Most likely, the observed faint bands originated from plant proteins present that co-purified during the preparation of NcΔ15D protein. Therefore, the above data demonstrate that the MON 87769-produced NcΔ15D is not glycosylated.

9.5 Storage Stability

Storage stability of the MON 87769-produced NcΔ15D protein was assessed after the protein preparation was stored in the following areas: in a 4 ºC refrigerator, in -20 ºC and a -80 ºC freezers for 29 days (Figures 6-8, Table 4). Stability was evaluated by comparing the apparent molecular weight and the purity after storage to those determined on day 0 (Figure 1 and Table 2). As summarized in Table 4, the apparent molecular weight was estimated to be 46.5, 47.0 and 46.7 kDa, respectively for 4 ^{o}C, -20 ^{o}C and -80 ^{o}C on day 29. These values differ in the apparent molecular weight of 46 kDa obtained on day 0 by 0.6%, 1.7% and 1.0%, suggesting that no significant molecular weight change occurred at these different temperature.

The purity for the MON 87769-produced NcΔ15D protein stored in all of the following areas: in a 4 ºC refrigerator, in -20 ºC and -80 ºC freezers for 29 days was also assessed (Table 4 and Figures 6-8, Lanes 3-8) by densitometric analysis of Coomassie stained SDS-polyacrylamide gels. The purity values were calculated as the average of the six loads. The percent between day 0 and day 29 in average purity of MON 87769-produced NcΔ15D protein was estimated to be 49%, 4% and 0% respectively for 4 ^{o}C, -20 ^{o}C and -80 ^{o}C on day 29 (Table 4). These results are within the preset acceptance criterion of ±10% for storage at -20 ^{o}C and -80 ^{o}C, but not for storage at 4 ^{o}C indicating that the NcΔ15D protein was unstable during storage at 4 ^{o}C.

10.0 Conclusions

A panel of analytical techniques was used to characterize the purified MON 87769-produced NcΔ15D protein. Identity of the purified NcΔ15D protein was confirmed by recognition with anti-NcΔ15D antibodies, identification of the first 15 amino acids of the expected N-terminus by amino acid sequencing, and identification of tryptic peptide masses that cover 45% of the expected protein sequence. The total protein concentration of the MON 87769-produced NcΔ15D protein preparation was 0.62 mg/ml with a purity of 74%. The apparent molecular weight of the NcΔ15D protein was estimated to be 46. kDa. The MON 87769-produced NcΔ15D protein was confirmed to be non-glycosylated. Finally, the MON 87769-produced NcΔ15D protein was stable for 29 days when stored in a -20 °C or −80 °C freezer, but was degraded during storage in a 4 °C refrigerator.

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Table 1. Total Protein Concentration of MON 87769-Produced NcΔ15D Protein Preparation by Amino Acid Analysis

Sample #	**Sample Analyzed** (µl)	**Calculated Concentration** (µg/µl)
1	1	0.6674
2	1	0.5786
3	1	0.6318
4	1	0.6174
5	1	0.6051
	Average Concentration	0.62 ± 0.03

Table 2. Summary of the Tryptic Masses Identified from the NcΔ15D Protein Using MALDI-TOF Mass Spectrometry

1 DHB-1	2 DHB-2	3 AC-1	4 AC-2	5 SA-1	6 SA-2	Expected Mass	Diff.[2]	Fragment	Sequence
		547.57				547.32	0.25	2-6	AVTTR
		683.65				683.34	0.31	158-162	FSHHR
776.46		776.67	776.70		776.39	776.39	0.28	243-249	GGMGWLR
783.49						783.40	0.09	343-349	DFGFIGK
1261.79						1261.63	0.16	42-52	SAEPIEYPDIK
1285.85			1286.40		1285.95	1285.68	0.72	67-76	VWISMAYFIR
1362.89	1363.00			1362.93		1362.68	0.25	250-261	VSHFEPSSAVFR
1383.84	1384.00	1384.25	1384.43	1384.65		1384.64	0.39	56-66	DAIPDHCFRPR
1567.07	1567.20	1567.47	1567.67	1567.14		1566.83	0.64	335-349	GALATVDRDFGFIGK
1721.01						1720.81	0.20	173-187	DMAFVPATEADRNQR
1864.21						1863.95	0.26	197-212	ETAEMFEDVPIVQLVK
					2360.76	2360.26	0.50	213-233	LIAHQLAGWQMYLLFNVSAGK
2583.52						2583.20	0.32	163-184	HHRFTGHMEKDMAFVPATEADR
2754.73						2754.38	0.35	368-390	IPFYYAEEATNSIRPMLGPLYHR
2936.76						2936.40	0.36	10-41	AAAATEPEVVSTGVDAVSAAAPSSSSSSSSQK

[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 NcΔ15D Protein

NcΔ15D Sample Loaded	Apparent Mol. Wt. (kDa)	Purity[1] (%)
1 μg Load (Figure 4, lane 2)	46.3	70.8
1 μg Load (Figure 4, lane 3)	46.2	74.4
2 μg Load (Figure 4, lane 4)	46.0	72.8
2 μg Load (Figure 4, lane 5)	46.1	75.2
3 μg Load (Figure 4, lane 6)	46.2	76.4
3 μg Load (Figure 4, lane 7)	46.4	72.4
Average Values [2]	46.2 ± 0.13	74 ± 0.13

[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 NcΔ15D Protein Stored in a 4 °C refrigerator, and in a -20 °C and -80 °C freezers

	Temperature, °C	Day 0[1]	Day 29[2]	Percent Difference[3] (Day 29 vs. Day 0)
Apparent MW (kDa)	4 °C	46.2	46.5	1%
	-20 °C	46.2	47.0	2%
	-80 °C	46.2	46.7	1%
Total NcΔ15D Purity (%)	4 °C	74	38	49%
	-20 °C	74	71	4%
	-80 °C	74	74	0%

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

$$\frac{\left|Day29 - Day0\right|}{Day0} \times 100 = \%Difference$$

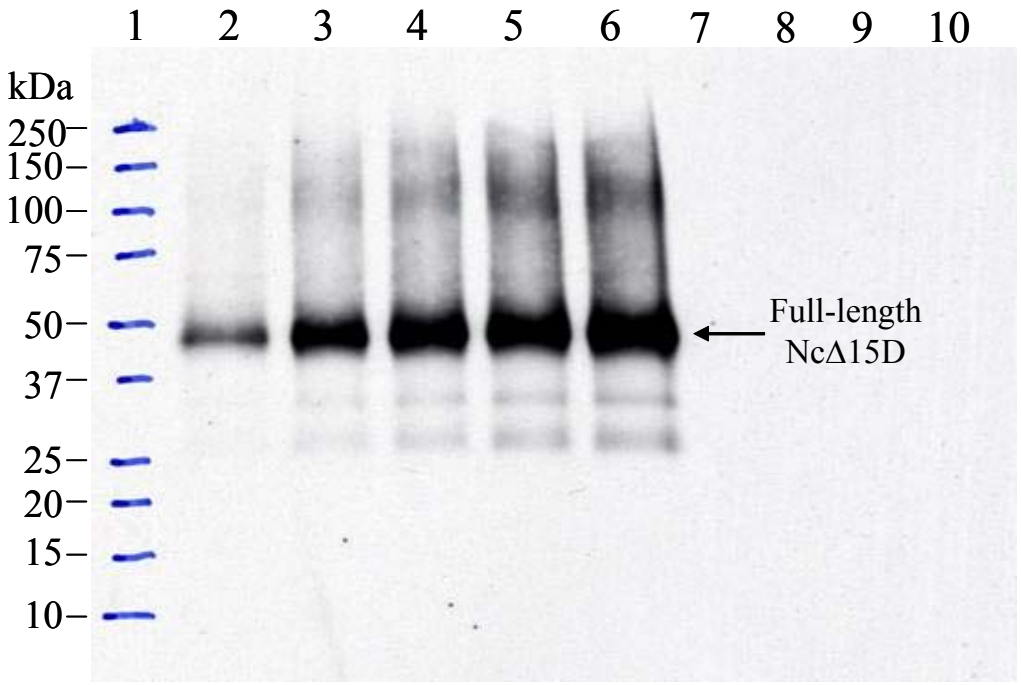

Figure 1. Immuno-reactivity of the MON 87769-Produced NcΔ15D Protein
Purity-corrected MON-87769-produced NcΔ15D were separated by SDS-PAGE and electrotransferred to PVDF membrane. The membrane was incubated with an anti-NcΔ15D antibody, and immuno-reactive bands were visualized using an ECL system. Approximate molecular weights (kDa) shown on the left correspond to the tick marks in Lane 1. The 30 sec exposure is shown. Arrow at right indicates the expected position of NcΔ15D.

Lane	Sample	Amount loaded (ng)
1	BioRad Precision Plus MWT Marker	
2	MON 87769-produced NcΔ15D protein	2.0
3	MON 87769-produced NcΔ15D protein	4.0
4	MON 87769-produced NcΔ15D protein	6.0
5	MON 87769-produced NcΔ15D protein	8.0
6	MON 87769-produced NcΔ15D protein	10.0
7	Empty	
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[1]	→	M	A	V	T	T	R	S	H	K	A	A	A	A	T	E	P
		|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Observed Sequence	→		A	V	T	T	R	S	H	K	A	A	A	A	T	E	P

Figure 2. Summary of N-terminal Sequence Analysis of the MON 87769-Produced NcΔ15D Protein

Observed sequence obtained from the first fifteen cycles of Edman degradation was compared to that predicted from NcΔ15D gene. '|'indicates a match of the residues between two sequences. The single letter IUPAC-IUB amino acid code is: A, alanine; E, glutamate; H, histidine; P, proline; R, arginine; S, serine; T, threonine.

```
1   MAVTTRSHKA AAATEPEVVS TGVDAVSAAA PSSSSSSSSQ KSAEPIEYPD
51  IKTIRDAIPD HCFRPRVWIS MAYFIRDFAM AFGLGYLAWQ YIPLIASTPL
101 RYGAWALYGY LQGLVCTGIW ILAHECGHGA FSRHTWFNNV MGWIGHSFLL
151 VPYFSWKFSH HRHHRFTGHM EKDMAFVPAT EADRNQRKLA NLYMDKETAE
201 MFEDVPIVQL VKLIAHQLAG WQMYLLFNVS AGKGSKQWET GKGGMGWLRV
251 SHFEPSSAVF RNSEAIYIAL SDLGLMIMGY ILYQAAQVVG WQMVGLLYFQ
301 QYFWVHHWLV AITYLHHTHE EVHHFDADSW TFVKGALATV DRDFGFIGKH
351 LFHNIIDHHV VHHLFPRIPF YYAEEATNSI RPMLGPLYHR DDRSFMGQLW
401 YNFTHCKWVV PDPQVPGALI WAHTVQSTQ
```

Figure 3. **MALDI-TOF MS Coverage Map of the MON 87769-Produced NcΔ15D Protein**

The amino acid sequence of the MON 87769-produced NcΔ15D protein was deduced from the coding region of the full-length NcΔ15D gene present in MON 87769 (Girault *et al*., 2008). Boxed regions correspond to tryptic peptide masses that were identified from the ~46 kDa protein band using MALDI-TOF MS. In total, 15 fragments covering 45% (193 of 429 total amino acids) of the expected protein sequence were matched to expected masses.

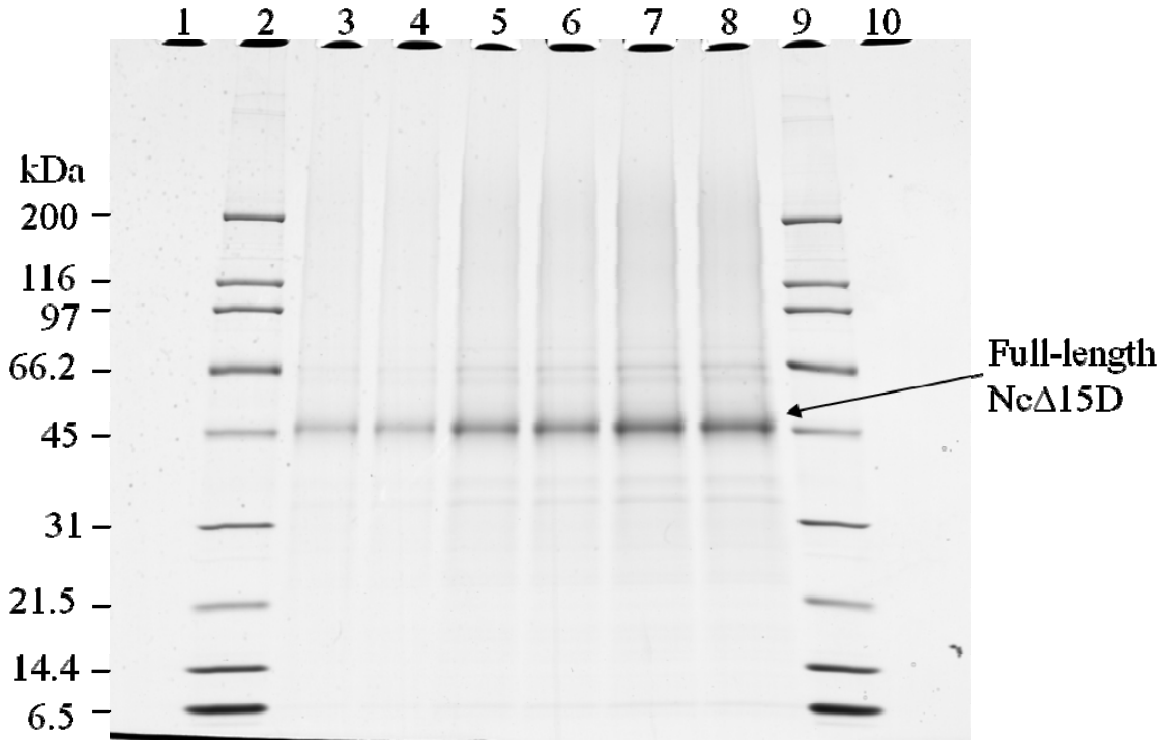

Figure 4. Molecular Weight and Purity Analysis of the MON 87769-Produced NcΔ15D Protein

Aliquots of the MON 87769-produced NcΔ15D protein were separated by SDS-PAGE, followed by Colloidal Brilliant Blue G. Approximate molecular weights (kDa) are shown on the left and correspond to the marker loaded in lanes 2 and 9. Arrow at right indicates NcΔ15D principal band.

Lane	Sample	Amount loaded (µg)
1	Empty	
2	BioRad Broad Range Marker	0.2
3	MON 87769-produced NcΔ15D protein	1
4	MON 87769-produced NcΔ15D protein	1
5	MON 87769-produced NcΔ15D protein	2
6	MON 87769-produced NcΔ15D protein	2
7	MON 87769-produced NcΔ15D protein	3
8	MON 87769-produced NcΔ15D protein	3
9	BioRad Broad Range Marker	0.2
10	Empty	

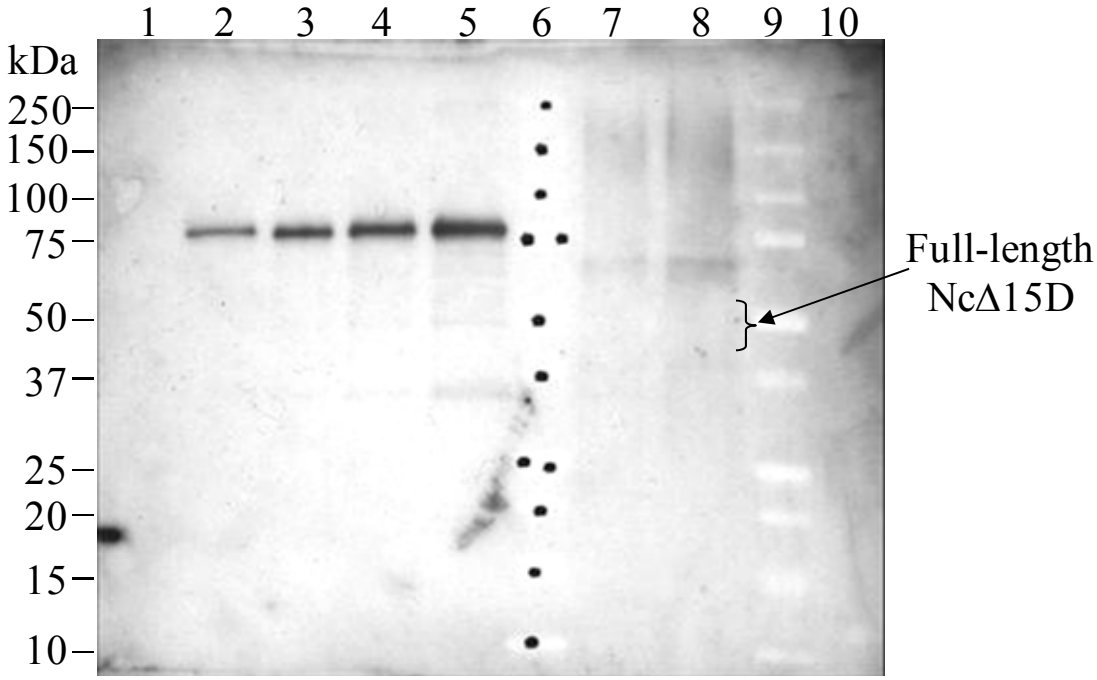

Figure 5. Glycosylation Analysis of the MON 87769-Produced NcΔ15D

Aliquots of transferrin (positive control) and MON 87769-produced NcΔ15D 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	0.05
4	Transferrin	0.10
5	Transferrin	0.20
6	BioRad Precision Plus MWT Marker	0.20
7	MON 87769-produced NcΔ15D protein	0.50
8	MON 87769-produced NcΔ15D protein	1.00
9	BioRad Precision Plus MWT Marker	0.20
10	Empty	

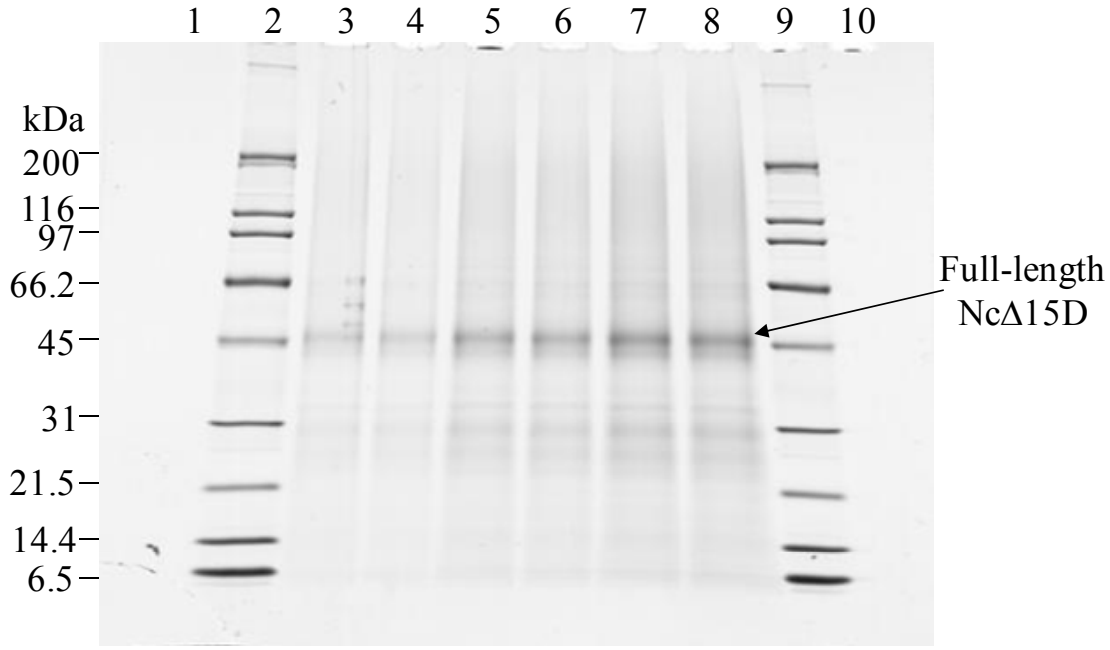

Figure 6. Storage Stability (Day 29) of the MON 87769-Produced NcΔ15D Protein Stored at 4 °C

Aliquots of the NcΔ15D sample stored at 4 °C were separated on 4-20% polyacrylamide gel and stained with Colloidal Brilliant Blue G. Approximate molecular weights (kDa) are shown on the left and correspond to the marker bands in Lanes 2 and 9. Arrow at right indicates the expected position of NcΔ15D. Figure 4 represents the Day 0 control.

Lane	Sample	Amount loaded (ug)
1	Empty	
2	BioRad Broad Range Marker	0.2
3	MON 87769-produced NcΔ15D protein	1.0
4	MON 87769-produced NcΔ15D protein	1.0
5	MON 87769-produced NcΔ15D protein	2.0
6	MON 87769-produced NcΔ15D protein	2.0
7	MON 87769-produced NcΔ15D protein	3.0
8	MON 87769-produced NcΔ15D protein	3.0
9	BioRad Broad Range Marker	0.2
10	Empty	

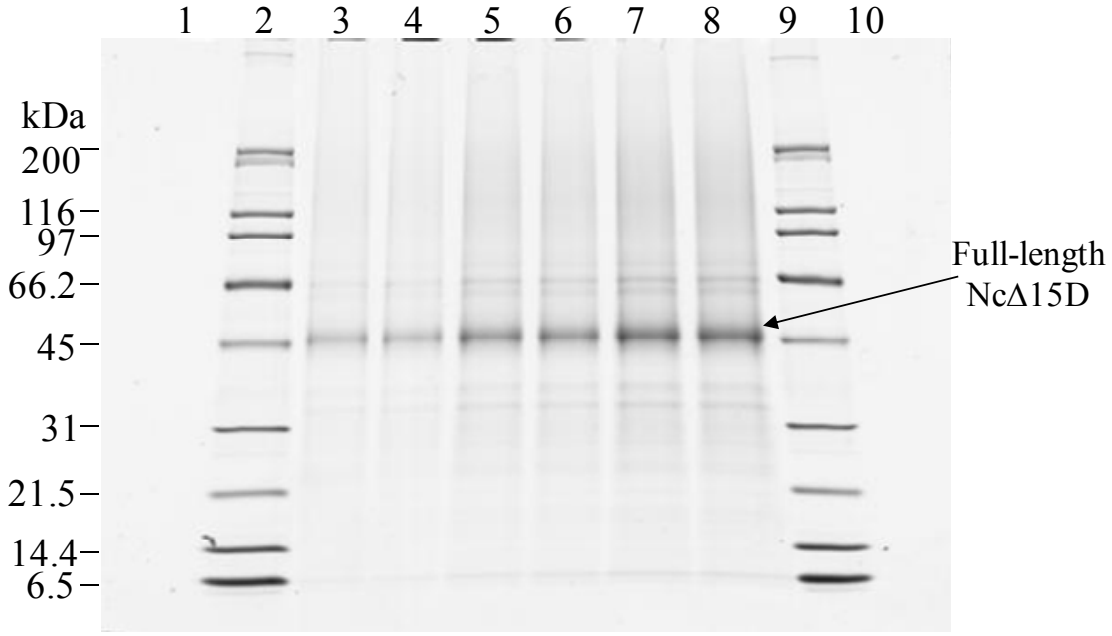

Figure 7. Storage Stability (Day 29) of the MON 87769-Produced NcΔ15D Protein Stored at -20 °C

Aliquots of the NcΔ15D sample stored at -20 °C were separated on 4-20% polyacrylamide gel and stained with Colloidal Brilliant Blue G. Approximate molecular weights (kDa) are shown on the left and correspond to the marker bands in Lanes 2 and 9. Arrow at right indicates the expected position of NcΔ15D. Figure 4 represents the Day 0 control.

Lane	Sample	Amount loaded (ug)
1	Empty	
2	BioRad Broad Range Marker	0.2
3	MON 87769-produced NcΔ15D protein	1
4	MON 87769-produced NcΔ15D protein	1
5	MON 87769-produced NcΔ15D protein	2
6	MON 87769-produced NcΔ15D protein	2
7	MON 87769-produced NcΔ15D protein	3
8	MON 87769-produced NcΔ15D protein	3
9	BioRad Broad Range Marker	0.2
10	Empty	

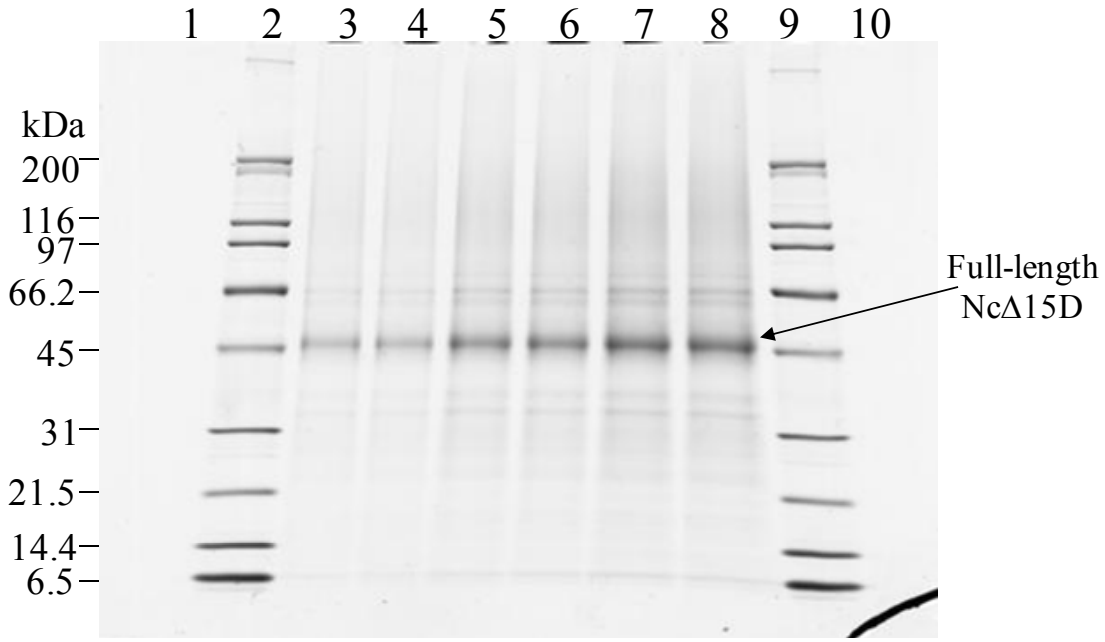

Figure 8. Storage Stability (Day 29) of the MON 87769-Produced NcΔ15D Protein Stored at -80 °C

Aliquots of the NcΔ15D sample stored at -80 °C were separated on 4-20% polyacrylamide gel and stained with Colloidal Brilliant Blue G. Approximate molecular weights (kDa) are shown on the left and correspond to the marker bands in Lanes 2 and 9. Arrow at right indicates the expected position of NcΔ15D. Figure 4 represents the Day 0 control.

Lane	Sample	Amount loaded (ug)
1	Empty	
2	BioRad Broad Range Marker	0.2
3	MON 87769-produced NcΔ15D protein	1
4	MON 87769-produced NcΔ15D protein	1
5	MON 87769-produced NcΔ15D protein	2
6	MON 87769-produced NcΔ15D protein	2
7	MON 87769-produced NcΔ15D protein	3
8	MON 87769-produced NcΔ15D protein	3
9	BioRad Broad Range Marker	0.2
10	Empty	

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 MW 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® Derivatiz ation

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 of protein purity and molecular weight (Purity Gels from 02/015/08 and the 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 x 36.3 µm from the list of resolutions and then click "Done". In the actual image acquisition step, the resolution setting of 42.3 x 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 x 36.3 as specified in BR-ME-0956-03 and the setting of 42.3 x 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 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, therefore there is no impact to the study by this deviation.
6) 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.

7) There were two documentation deviations (SOP AG-PO-1163-02 and BR-PO-0454-02) involving the X-ray film developer that occurred during this study. As the film developed correctly thus verifying the solutions' activity there was no impact to the data.

Appendix 3. Analytical Protein Standard Certificate of Analysis

Analytical Protein Standard Certificate of Analysis

MONSANTO *ANALYTICAL PROTEIN STANDARDS*

Sample Information:

Name of APS	**APS Lot Number**	**Expiration Date**
Soy-produced *Neurospora crassa* Delta-15 Desaturase [MON 87769]	10001516	July 31, 2008
Common or Alias Name(s) NcD15D	**Historical APS Lot Number** —	**Storage Requirements (until use)** –80 °C
Source Seeds of Soy MON 87769		**Comment(s)** None
Additional Background Information None		

Characteristic	Method	Assay Date	Result
Concentration	Amino Acid Composition	9 Feb 2008	0.62 mg/mL (total protein)
Purity	SDS-PAGE/Densitometry	14 Feb 2008	74%
Molecular weight	SDS-PAGE/Densitometry	14 Feb 2008	46.2 kDa
Identity	Immunoblot	05 Mar 2008	Confirmed – immuno-reactive band observed
Identity	N-terminal sequence	26 Feb 2008	Confirmed – AVTTRSHKAAAATEP
Identity	MALDI-TOF MS (Trypsinized)	27 Feb 2008	Confirmed sequence - 45.0 % 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 10001516, no significant degradation was observed for samples stored at –80°C or -20°C. However, the relative molecular weight of the NcD15D protein had changed when stored at 4°C.

Purity corrected concentration is 0.24 mg/mL (0.62 mg/mL × 0.74 ≈ 0.46mg/mL)

Quality Assurance Specialist — Date: 04/29/08

Analytical Protein Standards Officer — Date: 4/29/08

Page 1 of 1