**Study Title**

Assessment of the *In Vitro* Digestibility of the *Neurospora crassa* Δ15 Desaturase Protein (NcΔ15D) in Simulated Gastric and Simulated Intestinal Fluids.

**Authors**

Shefalee A. Kapadia, M.Sc., Thomas C. Lee, Ph.D., and Elena A. Rice, Ph.D.

**Report Completed On**

December 12, 2008

**Sponsor Representative**

Cherian George, Ph.D.

**Performing Laboratory**

Monsanto Company
Regulatory Product Characterization Team
Protein and Molecular Sciences Team
800 North Lindbergh Boulevard
St. Louis, Missouri 63167

**Laboratory Project ID**

Monsanto Study Number: REG-08-234
MSL: 0021427

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

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

**Statement of No Data Confidentiality Claims**

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

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

Company: Monsanto Company

Company Agent: _____

Title: _____

Signature: _____ Date: _____

**Statement of Compliance**

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

| | |
|---|---|
| Submitter | Date |
| Cherian George | 12-12-2008 |
| Cherian George, Ph.D.<br>Sponsor Representative | Date |
| S Kapadia | 12-12-2008 |
| Shefalee A. Kapadia, M.Sc.<br>Study Director | Date |

**Quality Assurance Unit Statement**

**Study Title:** Assessment of the *In Vitro* Digestibility of the *Neurospora crassa* Δ15 Desaturase Protein (NcΔ15D) in Simulated Gastric and Simulated Intestinal Fluids.

**Study Number:** REG-08-234

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

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

| Dates of Inspection / Audit | Phase | Date Reported To: | |
|---|---|---|---|
| | | Study Director | Management |
| 06/03/2008 | Digestive Fate | 06/06/2008 | 06/06/2008 |
| 07/23/2008; 07/29-31/2008 | Raw Data Audit | 07/31/2008 | 07/31/2008 |
| 11/06-07/2008 | Draft Report Audit | 11/10/2008 | 11/10/2008 |

**Todd Butzlaff**
Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

12/12/2008
Date

**Study Certification Page**

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

**Signatures of Final Report Approval:**

SAkapadia 12-12-2008

Shefalee A. Kapadia, M.Sc.
Study Director

Date

EARice December 12, 2008

Elena A. Rice, Ph.D.
Protein Team Lead

Date

## Study Information

| | |
|---|---|
| **Study Number:** | REG-08-234 |
| **MSL Number:** | 0021427 |
| **Title:** | Assessment of the *In Vitro* Digestibility of the *Neurospora crassa* Δ15 Desaturase Protein (NcΔ15D) in Simulated Gastric and Simulated Intestinal Fluids. |
| **Testing Facility:** | Monsanto Company<br>Regulatory Product Characterization Team<br>800 North Lindbergh Boulevard<br>St. Louis, Missouri 63167 |
| **Team Lead:** | Elena A. Rice, Ph.D. |
| **Study Director:** | Shefalee A. Kapadia, M.Sc. |
| **Contributors:** | Richard Thoma and Thomas C. Lee, Ph.D. |
| **Study Initiation Date:** | June 3, 2008 |
| **Study Completion Date:** | December 12, 2008 |
| **Specimen Retention:** | Specimens will be retained at Monsanto Company, St. Louis, as specified in Section 7.0 |
| **Records Retention:** | All study specific raw data, electronic files, study protocol, amendments, and final report will be retained at Monsanto Company, St. Louis. |

## Table of Contents

## List of Figures



## List of Appendicies

## Abbreviations and Definitions[1]

| | |
|---|---|
| BLG | β-lactoglobulin |
| CFR | Code of Federal Regulations |
| NcΔ15D | *Neurospora crassa* Δ15 Desaturase |
| DF | Dilution Factor |
| ECL | Enhanced chemiluminescence |
| *E. coli* | *Escherichia coli* |
| EPA | Environmental Protection Agency |
| FIFRA | Federal Insecticide, Fungicide and Rodenticide Act |
| GLP | Good Laboratory Practice |
| HRP | Horseradish peroxidase |
| IgG | Immunoblogulin G |
| ILSI | International Life Science Institute |
| LB | Laemmli buffer [62.5mM Tris-HCl, 5% (v/v) 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, 0.005% (w/v) bromophenol blue, 10% (v/v) glycerol, pH 6.8]. |
| 5× LB | Five times concentrated 1× LB |
| LOD | Limit of detection |
| MSL | Monsanto Scientific Literature |
| NFDM | Non-fat dry milk |
| PBST | Phosphate buffered saline - Tween® 20 |
| PTH | Phenylthiohydantoin |
| PVDF | Polyvinylidene difluoride |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SGF | Simulated Gastric Fluid |
| SIF | Simulated Intestinal Fluid |
| SOP | Standard operating procedure |
| T | Time |
| TCA | Trichloroacetic acid |
| U.S. | United States |

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

## 1.0 **Summary**

Monsanto Company has developed 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 integral membrane *Neurospora crassa* delta-15 desaturase (NcΔ15D) and integral membrane *Primula juliae* delta-6 desaturase (PjΔ6D) proteins. The expression of these two genes is driven by seed-specific promoters, resulting in the production of SDA only in soybean seeds.

The purpose of this study was to assess the *in vitro* digestibility of the NcΔ15D protein purified from immature seed of MON 87769 in simulated gastric fluid (SGF) containing a proteolytic enzyme, pepsin, and simulated intestinal fluid (SIF) containing a mixture of the enzymes called pancreatin. The digestibility of the NcΔ15D protein was also assessed in a sequential enzymatic digestion assay where the protein was digested in SGF followed by digestion in SIF. Digestibility of the NcΔ15D protein was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot methods. The extent of NcΔ15D protein digestion was evaluated by visual analysis of Colloidal Brilliant Blue G stained polyacrylamide gels or by visual analysis of developed western blot X-ray films.

The results of the study demonstrated that greater than 96% of the full-length NcΔ15D protein was digested within 30 s of incubation in SGF when analyzed using either stained gel or western blot with a NcΔ15D specific antibody. Several fragments (~17 kDa, ~12 kDa, ~5 kDa and ~4 kDa) were observed at various time points during digestion in SGF when specimens were analyzed using a stained gel. The fragments with molecular weight of approximately 17 kDa and 12 kDa were also observed on western blot X-ray films and, therefore, identified as products of proteolytic digestion of the NcΔ15D protein. The fragments with molecular weight of ~5 kDa and ~4 kDa were not observed on the western blot and, therefore, these fragments were N-terminally sequenced in an attempt to establish their identity. Sequence obtained for the fragment of ~4 kDa matched the expected sequence of the NcΔ15D protein starting at amino acid 376. The sequence obtained for ~5 kDa fragment did not match the expected sequence of the NcΔ15D protein and its identity was not established. Most likely this fragment originated from one of the soybean proteins that co-purified with the NcΔ15D protein. Digestion of the NcΔ15D fragments observed in SGF was further evaluated in SIF, where they were rapidly degraded upon exposure to SIF when analyzed using stained gel (<5 min) and western blot (<30 s).

The results of the study also demonstrated that greater than 96% of the full-length NcΔ15D protein was digested in SIF within 5 min when analyzed using western blot. No proteolytic fragments were observed at any time points.

The results of this study show that the integral membrane protein, NcΔ15D, is readily digestible in SGF and SIF. Rapid digestion of the full-length NcΔ15D protein and any

NcΔ15D protein fragments in SGF and SIF indicates that it is highly unlikely that the NcΔ15D protein will pose any safety concern to human health.

## 2.0 **Introduction**

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

A relationship between protein digestibility with pepsin at acidic pH in SGF and the likelihood of the protein being an allergen has been previously reported (Astwood et al., 1996), but this correlation is not absolute (Fu et al., 2000). The SGF assay protocol has been standardized based on results obtained from an international, multi-laboratory ring study (Thomas et al., 2004). The study showed that the results of *in vitro* pepsin digestion assays were reproducible when a standard protocol was followed. Using this protocol, the pepsin digestion assay was used to assess the susceptibility of the NcΔ15D protein to *in vitro* pepsin digestion.

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

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

## 3.0 **Purpose**

The purpose of this study was to assess the *in vitro* digestibility of the NcΔ15D protein in simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and in a sequential digestion where protein was first digested in simulated gastric fluid followed by the digestion in simulated intestinal fluid.

## 4.0 **Materials**

### 4.1 Test Substance

The NcΔ15D protein (Orion lot 10001516) was purified from immature MON 87769 soybean seed. The NcΔ15D protein is stored in a -80°C freezer in a buffer containing 50 mM sodium acetate, pH 5.6, 1 mM $MgCl_2$, 0.1 % Fos-choline 12, 0.5 M NaCl, and 10% glycerol.

### 4.2 Characterization of Test Substance

The characterization of the physicochemical properties of the test substance was performed under characterization plan 10001516 and is summarized in the Certificate of Analysis. The NcΔ15D has a concentration of 0.62 mg/ml, a purity of 74%, and an apparent molecular weight of 46.2 kDa as determined by SDS-PAGE. The N-terminal sequence of the NcΔ15D was also confirmed during characterization.

## 5.0 **Test Systems**

Two test systems, SGF and SIF, were utilized independently to test stability of the NcΔ15D protein and then in a sequential digestibility assay where NcΔ15D protein was first exposed to SGF followed by exposure to SIF.

### 5.1 SGF

SGF contained the proteolytic enzyme pepsin in a buffer adjusted to an acidic pH 1-2. The SGF was prepared using a highly purified form of pepsin (Catalog number P-6887, Sigma Company, St. Louis, MO). The SGF was formulated so that ten units of pepsin activity per μg of the NcΔ15D protein would be present in the digestion reactions. The amount of pepsin powder used to prepare SGF was calculated from the specific activity reported on the product label. Activity was assessed using a SGF activity assay, where one unit of activity is defined as a change in $A_{280\ nm}$ of 0.001 per min at 37 °C, measured as trichloroacetic acid (TCA) soluble products using hemoglobin as the substrate. The SGF activity assay was used to confirm the activity of the preparation before initiating the digestion of the NcΔ15D protein. The digestion of the NcΔ15D protein was assessed by SDS-PAGE stained gels and western blot analysis using a NcΔ15D specific antibody.

### 5.1.1 Justification for Selection of the SGF Test System

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

## 5.2 SIF

SIF contained a mixture of enzymes, known as pancreatin, in a buffer adjusted to neutral pH. SIF was prepared according to the method described in The United States Pharmacopoeia (USP 23, 1995). The pancreatin used for the preparation of SIF was obtained from Sigma Company (Catalog number P1500, St. Louis, MO). The SIF was formulated so that 55.3 µg of pancreatin powder would be present per µg of NcΔ15D protein in the digestion reactions. One unit of pancreatin activity in the SIF assay is defined as an increase in the absorbance at 574 nm of 0.001 per min at 37 ºC. The assay is based on the estimation of the amount of soluble peptides present in a trichloroacetic acid (TCA) solution after pancreatin digestion of resorufin-labeled casein. The SIF activity assay was used to confirm the activity of the preparation before initiating the digestion of the NcΔ15D protein. The digestion of the NcΔ15D protein was assessed by western blot analysis using a NcΔ15D specific antibody.

### 5.2.1 Justification for selection of the SIF Test System

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

# 6.0 Experimental Design

## 6.1 Digestibility of the NcΔ15D Protein in SGF

Digestibility of the NcΔ15D protein in SGF was evaluated over time by analyzing specimens from targeted incubation time points. A numerical code using the numbers 0 through 7 was used to distinguish incubation time points as follows:

| Targeted Incubation Time Point | Designation(s) |
|---|---|
| 0 min | SGF T0, SGF P0, SGF N0 |
| 0.5 min | SGF T1 |
| 2 min | SGF T2 |
| 5 min | SGF T3 |
| 10 min | SGF T4 |
| 20 min | SGF T5 |

| | |
|---|---|
| 30 min | SGF T6 |
| 60 min | SGF T7, SGF P7, SGF N7 |

SGF for the digestion was prepared to contain approximately 2532 U/ml of pepsin activity , by dilution of a stock SGF solution with SGF buffer lacking pepsin (10 mM HCl, 2 mg/ml NaCl, pH ~1.2). The digestion mixture was prepared by adding 522 µl of the NcΔ15D total protein to a tube containing 1278 µl of pre-heated (37.1 ºC, 5 min, 30 sec ) SGF which corresponds to 323.6 µg of NcΔ15D protein and 3236 U of pepsin, respectively. The tube contents were mixed by vortexing and immediately placed in a 37.3 ºC water bath. Specimens (200 µl) were removed at 0.5, 2, 5, 10, 20, 30 and 60 min (corresponding to specimen time points SGF T1 through SGF T7). Each 200 µl specimen was immediately placed in a tube containing the quenching mixture, consisting of 70 µl of 0.7 M sodium carbonate buffer (0.7 M $Na_2CO_3$, pH 11), and 70 µl of 5× Laemmli Buffer (LB)[5× LB, 312.5 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) Bromophenol Blue, and 50% (v/v) glycerol, pH 6.8].

The SGF T0 incubation specimen was prepared in a separate tube. One hundred and forty two µl of SGF (360 U of pepsin) was quenched by the addition of 70 µl of 0.7 M sodium carbonate buffer, and 70 µl of 5× LB prior to the addition of 58 µl (36 µg) of the NcΔ15D total protein.

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

### 6.1.1 SGF Experimental Controls

Experimental control specimens were prepared to determine the stability of the NcΔ15D protein in the test system buffer lacking pepsin (10 mM HCl, 2 mg/ml NaCl, pH ~1.2). These experimental control specimens were prepared in a similar manner as described in Section 6.1 for SGF T0, but the targeted incubation times were limited to 0 min (SGF P0) and 60 min (SGF P7).

Experimental control specimens were also prepared to determine the stability of the test system lacking the NcΔ15D protein. Protein storage buffer (50 mM Sodium acetate, pH 5.6, 1 mM $MgCl_2$, 0.1 % Fos-choline 12, 0.5 M NaCl, and 10% glycerol) was added to SGF in place of the NcΔ15D protein. These experimental control specimens were prepared in a similar manner as described above in Section 6.1 for SGF T0, but the targeted incubation times were limited to 0 min (SGF N0) and 60 min (SGF N7).

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

### 6.2 Digestibility of the NcΔ15D Protein in SGF followed by SIF

Digestibility of the NcΔ15D protein was assessed by digestion in SGF followed by digestion in SIF. The NcΔ15D protein was digested first in SGF as described in Section 6.1 for 2 min at which point the reaction was stopped by quenching with 0.7 M sodium carbonate buffer. It was then placed in SIF and digested as described in Section 6.3. The digestion of NcΔ15D in SGF followed by SIF was evaluated by SDS-PAGE stained gels and western blot analysis using a NcΔ15D specific antibody.

Digestion of the NcΔ15D protein in SGF followed by SIF was evaluated over time by analyzing specimens at targeted incubation time points. A numerical code using the numbers 0 through 7 were used to distinguish incubation time points according to the following:

| Targeted Incubation Time Point | Designations |
|---|---|
| **SGF system** | |
| 0 min | SEQ 0min |
| 2 min | SEQ 2min |
| **SIF system** | |
| 0 min | SEQ T0, SEQ P0, SEQ N0 |
| 0.5 min | SEQ T1 |
| 2 min | SEQ T2 |
| 5 min | SEQ T3 |
| 10 min | SEQ T4 |
| 30 min | SEQ T5 |
| 1 h | SEQ T6 |
| 2 h | SEQ T7, SEQ P7, SEQ N7 |

The SGF was prepared to contain approximately 2632 U/ml of pepsin activity. The digestion in SGF was prepared by adding 500 µl of the NcΔ15D total protein to a tube containing 1178 µl of pre-heated (37.3 ºC, 10 min) SGF, corresponding to 310 µg of NcΔ15D protein and 3100 U of pepsin, respectively. The tube contents were mixed by vortexing and immediately placed in a 37.3 ºC water bath. The tube was removed after 2 min, and the reaction was immediately quenched by adding 587 µl of 0.7 M sodium carbonate buffer. After quenching, an aliquot of 120 µl was removed for analysis, and mixed with 30 µl of 5× LB, and heated to 75-100 ºC for 5-10 min and designated as SEQ 2min.

For digestion in SIF, 1460 µl of the quenched SGF reaction mixture was added to 1110 µl of pre-heated (36.2 ºC, 10 min) SIF, corresponding to 200 µg SGF digested and quenched NcΔ15D protein (based on the pre-digested concentration) and 11.1 mg of pancreatin. The tube contents were mixed by vortexing and immediately placed in

a 37.4 ºC water bath. Digestion specimens (200 µl) were removed from the tube at 30 s, 2, 5, 10, 30 min, 1, and 2 h (corresponding to the specimen time points SEQ T1 through SEQ T7) and immediately placed in a tube containing 50 µl of 5× LB, heated to 75-100 ºC for 5-10 min, and frozen on dry ice for complete quenching.

The zero time incubation specimen for the SGF digestion phase (SEQ 0min) was prepared in a separate tube by first quenching 75 µl of SGF (198 U of pepsin) with 37 µl of sodium carbonate buffer, and 37 µl of 5× LB buffer and heating to 75-100 ºC for 5-10 min prior to the addition of 32 µl (19.8 µg) of the NcΔ15D total protein.

The zero time incubation specimen for the SIF digestion phase (SEQ T0) was prepared in a separate tube by first quenching 83 µl of SIF (0.83 mg) with 48 µl of 5× LB buffer and heating to 75-100 ºC for 5-10 min prior to the addition of 109 µl (15 µg, based on the pre digestion concentration) of the SGF digested and quenched NcΔ15D protein.

### 6.2.1 SGF followed by SIF Experimental Controls

Experimental control specimens for the SIF digestion phase were prepared to determine the stability of the NcΔ15D protein fragments in the SIF test system buffer lacking pancreatin (50 mM potassium phosphate monobasic, pH adjusted to 7.5 with sodium hydroxide). These experimental control specimens were prepared in a similar manner as described in Section 6.2 for SEQ T0, but the targeted incubation times were limited to 0 h (SEQ P0) and 2 h (SEQ P7).

Experimental control specimens were also prepared to characterize the test system (SIF) lacking the SGF digested and quenched NcΔ15D protein. Protein storage buffer (50 mM Sodium acetate, pH 5.6, 1 mM $MgCl_2$, 0.1 % Fos-choline 12, 0.5 M NaCl, and 10% glycerol) was added to SIF in place of the SGF digested NcΔ15D protein. These experimental control specimens were prepared in a similar manner as described above in Section 6.2 for SEQ T0, but the targeted incubation times were limited to 0 h (SEQ N0) and 2 h (SEQ N7).

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

## 6.3 Digestibility of the NcΔ15D Protein in SIF

Digestibility of the NcΔ15D protein in SIF was evaluated over time by analyzing specimens at targeted incubation time points. A numerical code using the numbers 0 through 8 was used to distinguish incubation time points according to the following:

| Targeted Incubation Time Point | Designations |
|---|---|
| 0 min | SIF T0, SIF P0, SIF N0 |
| 5 min | SIF T1 |
| 15 min | SIF T2 |
| 30 min | SIF T3 |

| | |
|---|---|
| 1 h | SIF T4 |
| 2 h | SIF T5 |
| 4 h | SIF T6 |
| 8 h | SIF T7 |
| 24 h | SIF T8, SIF P8, SIF N8 |

The digestion was prepared by adding 300 µl of the NcΔ15D to a tube containing 1.03 ml of pre-heated (36.7 ºC, 5 min) SIF, corresponding to 186 µg of the NcΔ15D total protein and 10.3 mg of pancreatin, respectively. The tube contents were mixed by vortexing and immediately placed in a 37.0 ºC water bath. Digestion specimens (100 µl) were removed at 5, 15, 30 min, 1, 2, 4, 8, and 24 h (corresponding to specimen time points SIF T1 through SIF T8) and immediately placed in a tube containing 25 µl of 5× LB, heated to 75-100 ºC for 5-10 min, and frozen on dry ice for complete quenching.

The zero time incubation specimen (SIF T0) was prepared in a separate tube by first quenching 103 µl of SIF (1.03 mg) with 33 µl of 5× LB buffer and heating to 75-100 ºC for 5-10 min prior to the addition of 30 µl (18.6 µg) of the NcΔ15D total protein.

### 6.3.1 SIF Experimental Controls

Experimental control specimens were prepared to determine the stability of the NcΔ15D protein in the test system buffer lacking pancreatin (50 mM potassium phosphate, pH 7.5). These experimental control specimens were prepared in a similar manner as described in Section 6.3 for SIF T0, but the targeted incubation times were limited to 0 h (SIF P0) and 24 h (SIF P8).

Experimental control specimens were also prepared to characterize the test system (SIF) lacking the NcΔ15D protein. Protein storage buffer (50 mM Sodium acetate, pH 5.6, 1 mM $MgCl_2$, 0.1 % Fos-choline 12, 0.5 M NaCl, and 10% glycerol) was added to SIF in place of the NcΔ15D protein. These experimental control specimens were prepared in a similar manner as described above in Section 6.3 for SIF T0, but the targeted incubation times were limited to 0 h (SIF N0) and 24 h (SIF N8).

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

## 7.0 Specimen Retention

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

## 8.0 **Analytical Methods**

Activity of the SGF and SIF were assessed using pepsin and pancreatin activity assays respectively. The digestibility of the NcΔ15D protein in SGF, and in SGF followed by SIF was assessed using stained SDS-PAGE gels and western blot analysis. The digestibility of the NcΔ15D protein in SIF was assessed using western blot analysis. The lower limit of detection (LOD) of the NcΔ15D protein was determined for stained SDS-PAGE gels and western blots. The identities of ~5 kDa and ~4 kDa fragments observed in SGF were assessed by N-terminal sequencing while the identities of the ~17 kDa and ~12 kDa fragments were determined by western blotting using a NcΔ15D specific antibody .

### 8.1 SGF Activity Assays

The SGF activity assay was used to confirm the suitability of the test system before its use with the NcΔ15D protein according to the current version of SOP BR-ME-0460. The assay is based on the ability of pepsin to digest denatured hemoglobin. Undigested hemoglobin was precipitated with TCA, and the amount of soluble peptides was estimated by measuring the absorbance at 280 nm. The amount of soluble peptides is directly proportional to the amount of protease activity. One unit of pepsin activity in this assay is defined as the amount of pepsin that will produce a change in absorbance at 280 nm of 0.001 per min at pH 1.2-2.0 at 37 ± 2 ºC. The SGF solution was formulated to contain 0.03 mg of powder per ml of SGF buffer. Acceptable specific activity (units/mg pepsin powder) for the SGF was equal to the specific activity determined by the manufacturer, ±1000 units/mg.

Because digestion of the NcΔ15D protein in SGF and in SGF followed by SIF were performed on the same day, only one SGF activity assay was performed. SGF solution was diluted to 0.03 mg of solid material (pepsin) per ml of SGF [the dilution factor (DF) was 26.7]. Acidified hemoglobin [2% (w/v), 5 ml] was added to each of three replicates of the test sample and blank sample and pre-warmed at 37 ± 2 °C for 5-10 min prior to starting the reactions. Diluted SGF (1 ml) was added to each replicate of test samples and both test and blank samples were incubated at 36.9 °C for an additional 10 min. The reactions were stopped by the addition of 10 ml of 5% (v/v) chilled TCA to the test and blank samples. Diluted SGF (1 ml) was then added to the blank samples. Samples were mixed and then incubated for another 5-10 min at 36.8 °C. Precipitated protein was removed by filtering the test and the blank samples using 0.8 µm syringe filters. Samples of the clarified test and blank samples were read at 280 nm in a Beckman DU-650 Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) The activity of pepsin was calculated using the following equation:

$$\frac{Mean\,Test_{A280\,nm} - Mean\,Blank_{A280\,nm}}{0.001 \times 10\,\text{min} \times 1\,ml} \times DF$$

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

### 8.2 SIF Activity Assay

The SIF activity assay was used to confirm the suitability of the test system before its use with the NcΔ15D protein according to the current version of SOP BR-ME-0461. One unit of pancreatin activity in this assay is defined as an increase in the absorbance at 574 nm of 0.001 per min at 37 ± 2 ºC. An acceptable specific activity for the SIF was defined as 11,000 ± 3,000 U/ml.

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

Because digestion of the NcΔ15D protein in SGF followed by SIF, and in SIF alone were performed on two separate days, two separate SIF activity assays were performed. For both assays, three activity replicates were incubated with 0.05× SIF (1× SIF was diluted to 0.05× SIF before the activity assay was initiated) for 15 min at 37.2 and 36.9 ºC for SIF activity-1 and SIF activity-2, respectively. Three blank replicates were incubated with 50 mM $KH_2PO_4$, pH 7.5 in place of SIF. The reactions was quenched by addition of chilled 5% (v/v) TCA to activity and blank replicates. The supernatants recovered after centrifugation were neutralized by the addition of assay buffer (500 mM Tris-HCl, pH 8.8), and the absorbance of the clarified activity and blank replicates was read at 574 nm using a Beckman DU-650 spectrophotometer. The activities of SIF solutions was calculated using the following equation:

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

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

### 8.3 SDS-PAGE and Colloidal Brilliant Blue G Staining

Specimens containing 1× LB from the SGF, and SGF followed by SIF *in vitro* digestions of the NcΔ15D protein were separated by SDS-PAGE using pre-cast tricine 10-20% polyacrylamide gradient mini-gels and tricine running buffer (Invitrogen, Carlsbad, CA) according to the current version of SOP BR-ME-0388.

The NcΔ15D protein was loaded at 0.8 µg per lane based on pre-digestion total protein concentration. All experimental controls were loaded at the same volumes as those containing NcΔ15D protein so that they would be comparable. Mark 12 molecular weight markers (Invitrogen, Carlsbad, CA) were loaded in parallel to estimate the relative molecular weight of proteins and peptides visualized by staining. Electrophoresis was performed at a constant voltage of 125 V for 81 and 90 min for SGF and SGF followed by SIF digestion specimens, respectively. After electrophoresis, proteins were visualized by staining the gels with colloidal Brilliant Blue G (Sigma, St. Louis, MO).

The colloidal Brilliant Blue G staining method was selected because it is an effective method for detecting nanogram quantities of a protein in a gel (Neuhoff et al., 1988). After separation of the proteins, the gels were fixed in a solution containing 7% (v/v) acetic acid and 40% (v/v) methanol for 30 min and 40 min, and stained for 17 h 53 min, and 18 h 41 min for SGF and SGF followed by SIF digestions of the NcΔ15D protein, respectively, in 1× Brilliant Blue G-colloidal stain solution containing 20% (v/v) methanol. The gels were destained for 25 s in 10% (v/v) acetic acid, 25% (v/v) methanol and then completely destained for ~6 h 50 min, and ~7 h 16 min for SGF and SGF followed by SIF digestions of the NcΔ15D protein, respectively, in a 25% (v/v) methanol solution. Images were captured using a Bio-Rad GS-800 densitometer (BioRad, Hercules, CA). The results of the *in vitro* digestibility of NcΔ15D in SGF and SGF followed by SIF were determined by visual examination of the stained gels.

The LOD of the NcΔ15D protein was determined using the colloidal Brilliant Blue G staining procedure. Various dilutions of the SGF zero time point (SGF T0) digestion specimen were loaded onto a separate gel that was run concurrently with the gel used to assess NcΔ15D protein digestibility in SGF. LOD samples were heated at 97.1 ºC for 3 min. Aliquots of the SGF T0 digestion specimen representing approximately 0.8, 0.5, 0.1, 0.05, 0.02, 0.01, 0.005, 0.0025, and 0.001 µg total protein per lane were used for the stained LOD gel.

### 8.4 Western Blot Analysis

Specimens from the SGF, SGF followed by SIF, and SIF *in vitro* digestions of the NcΔ15D protein were separated by SDS-PAGE using pre-cast tricine 10-20% polyacrylamide gradient mini-gels with tricine running buffer. The protein loaded in each lane was based on pre-digestion concentrations of the NcΔ15D protein. The digestion samples were diluted with 1× LB to a concentration of ~2 ng/µl, and ~15 ng of the NcΔ15D protein digestion specimens were loaded in each lane. The experimental controls were loaded in the same volumes as the digestion specimens. All samples were heated to 97.6, 95.8 and 95.7 °C for 3 min for the SGF, SIF and SGF followed by SIF digestions of the NcΔ15D protein, respectively, prior to loading on the gels. Electrophoresis was performed at 125 V for 81, 80, and 80 min for SGF, SIF and SGF followed by SIF digestions of the NcΔ15D protein, respectively. After

electrophoresis, the proteins were electrotransferred onto PVDF membranes with a pore size of 0.45 µm (Invitrogen, Carlsbad, CA) for 120 min at a constant voltage of 25 V. Prestained molecular weight markers (Precision Plus Dual color Protein Standards, Bio-Rad, Hercules, CA) were used to verify electrotransfer of the proteins to the membranes. Mark 12 unstained MWM were also loaded on the gel for SGF digestions of NcΔ15D to estimate the relative molecular weight of protein and peptides visualized by western blot analysis and compare them to the proteins and peptides visualized by staining. To visualize the Mark 12 unstained markers on the blot, the blot was washed with Milli Q water, 3 times (2-5 min for each wash), stained with Ponceau S stain for 30 s to 2 min. The visualized markers were designated on the blot, and then the blot was washed with Milli Q water for 30-60 s with several changes to remove the Ponceau S stain.

Proteins transferred to PVDF membranes were analyzed by western blot. The membranes were blocked overnight at ~4 ºC with 5% (w/v) non-fat dry milk (NFDM) in a phosphate buffered saline - Tween® 20 (PBST) buffer. All subsequent incubations were performed at room temperature. Goat anti–NcΔ15D affinity purified antibody (lot G-820096) was incubated with the membranes for 60 min at a dilution of 1:1500 in 1% (w/v) NFDM in PBST. Excess antibody was removed by three 10 min washes with PBST. The membranes were incubated with HRP-conjugated rabbit anti-goat IgG (lot G-836503C, Thermo Scientific, Rockford, IL) at a dilution of 1:10000 in 1% (w/v) NFDM in PBST for 50 min, and washed three times for 10 min with PBST. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ) and exposed to Hyperfilm ECL high performance chemiluminescence film (GE Healthcare, Piscataway, NJ). Films were developed using a Konica SRX101A automated film processor (Konica, Tokyo, Japan). The films were scanned using a Bio-Rad GS-800 densitometer to produce electronic images to be used as figures for reporting purposes.

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

The LOD for the western blot analysis procedure was determined for the NcΔ15D protein by loading various dilutions of the SGF and SIF zero time point (SGF T0 and SIF T0, respectively) digestion specimens on separate gels. These gels were run concurrently with the SGF and SIF digestion western blot gels, respectively, and subjected to the same western blot procedure as described above. The following approximate total protein loadings of the SGF T0 and SIF T0 were used for the western blot LOD analysis: 15, 10, 5, 2.5, 1, 0.5, 0.2, 0.1, 0.05, and 0.025 ng per lane.

## 8.5 N-Terminal Sequencing

N-terminal sequencing by Edman degradation was used to determine the N-terminal sequence of the transiently stable fragments with apparent molecular weights of ~5 kDa and ~4 kD observed in SGF.

### 8.5.1 Protein Blot for N-Terminal Sequence Analysis

The specimen SGF T3 was used to further characterize the transiently stable fragments with apparent molecular weights of ~5 kDa and ~4 kDa, respectively. This specimen corresponded to the 5 min digestion time point of the NcΔ15D protein which provided sufficient amount of the fragments for sequencing.

The SGF T3 specimen was loaded in 4 lanes at 2 µg per lane onto a tricine 10-20% polyacrylamide gradient 10-well gel. Precision Plus prestained molecular weight markers were loaded in parallel to verify electrotransfer of the protein to the membrane. Mark 12 unstained markers were also loaded in parallel to Precision Plus prestained markers to estimate the size of the stained bands observed. Electrophoresis was performed at a constant voltage of 125 V for 105 min. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 90 min at a constant voltage of 25 V. The blot was stained with Coomassie Blue R-250 stain (Bio-Rad, Hercules, CA) and then destained for ≥ 5 min with Coomassie Blue R-250 destain (Bio-Rad, Hercules, CA) to visualize the markers, and the transiently stable fragments generated from digestion of NcΔ15D protein in SGF. The blot was scanned using a Bio-Rad GS-800 densitometer to produce an electronic image.

### 8.5.2 N-Terminal Sequencing

The bands corresponding to the fragments with apparent molecular weights of ~5 kDa and ~4 kDa were each excised from the blot and sequenced. N-terminal sequence analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapillar 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 to chromatographically calibrate the instrument for the analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 picomole β-lactoglobulin, Applied Biosystems, Foster City, CA) was analyzed before and after the analysis of the two stable fragment bands to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

To establish identity of the ~ 5 kDa fragment, sequence data of the reported 9 amino acids was compared to the predicted NcΔ15D (pMON 77254) protein sequence (Girault et al., 2008). Similarly, for ~ 4 kDa fragment, sequence data of the reported 10 amino acids was compared to the expected NcΔ15D (pMON 77254) protein sequence (15 cycles were run, only 9 amino acids and 10 amino acids were reported for fragment of ~5 kDa and ~4 kDa, respectively, due to the loss of repetitive yield).

For the band with an apparent molecular weight of ~ 5 kDa, multiple amino acids were identified at several cycles indicating that multiple peptides were present in this band. Of the 9 sequencing cycles only 1 cycle produced an unambiguous amino acid call. The remaining cycles yielded multiple amino acids. The identified amino acids could not be matched to either the known NcΔ15D sequence nor the pepsin protein sequence. There was insufficient amino acid data (less than eight unambiguous amino acids as required by the study protocol) to perform a BLAST search therefore, the identity of the ~5 kDa fragment was not established.

Similarly, for the fragment of ~ 4 kDa, multiple amino acids were called at several cycles indicating that several peptides were present in this band. After comparing the experimental results to the known sequence of NcΔ15D, a match between experimentally obtained amino acids and expected NcΔ15D amino acid sequence was identified starting at position 376 of the protein sequence (Figure 5)

## 9.0 Control of Bias

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

## 10.0 Rejected Data

One set of N-terminal sequence analysis data was rejected because the N-terminal sequence analysis for β-lactoglobulin (BLG) failed to meet all system suitability requirements. An investigation found solvent leaks in both pumps from the HPLC portion of the N-terminal sequencing system. This resulted in delayed retention time for all phenylthiohydantoin (PTH) amino acids. Several late eluting amino acids (Tryptophan, Phenylalanine, Isoleucine, Lysine and Leucine) were not observed in the time allotted for each chromatogram.

## 11.0 Protocol Deviation

There was one study-specific SOP deviation. The protocol stated that SOP BR-ME-0461 will be followed for the preparation of 1× SIF and to assay the proteolytic activity of pancreatin in SIF. During the activity determination of pancreatin, it is required that after

each reaction is stopped upon addition of TCA, each tube should be incubated further in the water bath (37 ±2 ºC) for additional 10-15 min. However, a mistake was made and each tube was incubated for an additional 5 min instead of 10-15 min. There was no impact on the proteolytic activity of pancreatin in SIF because the calculated activity was within the acceptance criteria for the assay established in the SOP.

## 12.0 Results and Discussion

### 12.1 Pepsin Activity in SGF

To assess the suitability of the SGF test system used in this study, pepsin activity in SGF was evaluated prior to the use. The experimentally observed activity was 2643.7 units per mg of pepsin powder, which was within the acceptable interval of pepsin activity from 2280 to 4280 units per mg pepsin powder, and, therefore, the test system was shown to be suitable for use in this study.

### 12.2 Pancreatin Activity in SIF

To assess the suitability of the SIF test systems used in this study, pancreatin activity in SIF was evaluated prior to each assay The experimentally observed activities were 12312 and 12840 U/ml in the preparations used for the SGF followed by SIF, and SIF digestion assays, respectively. Both SIF preparations were within the acceptable interval of SIF activity (8,000 to 14,000 U/ml of SIF), and, therefore, the test systems were shown to be suitable for use in this study.

### 12.3 Digestibility of the NcΔ15D Protein in SGF

#### 12.3.1 Assessment of the NcΔ15D Protein Digestibility in SGF by Colloidal Brilliant Blue G Gel Staining of SDS-PAGE

The digestibility of the NcΔ15D protein in SGF was evaluated by visual analysis of colloidal Brilliant Blue G stained SDS-PAGE (Figure 1). The SDS-PAGE for the digestibility assessment (Figure 1, panel A) was run concurrently with a separate SDS-PAGE to determine the LOD of the NcΔ15D protein (Figure 1, panel B). The LOD of the full-length (~46 kDa) NcΔ15D protein was visually estimated to be 0.02 µg. The LOD estimated for the NcΔ15D protein was used to calculate the maximum amount of NcΔ15D protein that could remain visually undetected after digestion, which corresponded to approximately 2.5% of the total protein loaded:

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

$$\frac{0.02\,\mu g \times 100\%}{0.8\,\mu g} \cong 2.5\%$$

The gel used to assess the digestibility of the NcΔ15D protein in SGF (Figure 1, panel A) was loaded with ~0.8 µg of total protein (based on pre-digestion

concentrations) for each of the digestion specimens. Visual examination of the stained gel showed that the full-length NcΔ15D protein was digested within 30 s of incubation in SGF (Figure 1A, lane 5). Therefore, based on the LOD, more than 97% (100% - 2.5% = 97.5%) of the full-length NcΔ15D protein was digested within 30 s of incubation in SGF. Several fragments were observed in SGF for varying durations between 30 s and 60 min of the digestion. Fragments of ~17 kDa and ~12 kDa were observed up to 5 min and 10 min, respectively (Figure 1A, lanes 7 and 8). A minor band of ~7 kDa was observed only for 30 s (Figure 1A, lane 5) while two fragments of ~5 kDa and ~4 kDa were observed for 60 min and 20 min, respectively (Figure 1A, lanes 11 and 9).

No change in the full-length NcΔ15D protein band intensity was observed in the absence of pepsin in the experimental control specimens SGF P0 and SGF P7 (Figure 1A, lanes 3 and 12) indicating that the digestion of the NcΔ15D protein was due to the proteolytic activity of pepsin present in SGF and not due to instability of the protein while incubated at pH ~1.2 at ~37°C for 60 min.

In addition to the full-length NcΔ15D protein, three protein bands were observed at approximately 70 kDa, 60 kDa, and 7 kDa in the control specimens SGF P0, SGF T0 and SGF P7 (Figure 1A, lanes 3, 4, and 12). All three bands are thought to represent minor soybean proteins that were co-purified with the NcΔ15D protein because no distinct bands at these molecular weights were detected on immunoblots as described in the NcΔ15D characterization report (Dong, et al., 2008). Minor smearing in the high molecular weight area was also observed in the controls, which is attributed to higher order aggregation of NcΔ15D protein as described in the NcΔ15D characterization report (Dong et al., 2008).

The experimental control specimens SGF N0 and SGF N7 (Figure 1A, lanes 2 and 13), which evaluate the stability of the pepsin in the test system (SGF) lacking the NcΔ15D protein demonstrated that the pepsin was observed as a stained protein band at ~38 kDa throughout the experimental phase.

### 12.3.2 Assessment of the NcΔ15D Protein Digestibility in SGF by Western Blot Analysis

The digestibility of the NcΔ15D protein in SGF was also evaluated by western blot analysis (Figure 2). The western blot used to assess the stability of the NcΔ15D protein to pepsin digestion (Figure 2, panel A) was run concurrently with a western blot to determine the LOD of the NcΔ15D protein (Figure 2, panel B). The LOD of the full-length NcΔ15D protein was visually estimated to be 0.5 ng. The LOD estimated for the NcΔ15D protein was used to calculate the maximum amount of NcΔ15D protein that could remain visually undetected after digestion, which corresponded to 3.3% of the total protein loaded:

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

$$\frac{0.5ng \times 100\%}{15\,ng} = 3.3\%$$

The gel used to assess the NcΔ15D protein *in vitro* digestibility by western blot was loaded with 15 ng per lane of total protein (based on pre-digestion concentrations) for each of the digestion specimens. Western blot analysis demonstrated that the full-length NcΔ15D protein was digested below the LOD within 30 s of incubation in SGF (Figure 2A, lane 5). Based on the western blot LOD for the NcΔ15D protein it was concluded that more than 96% $(100\% - 3.3\% = 96.7\%)$ of the NcΔ15D protein was digested within 30 s. Two fragments with apparent molecular weights of ~17 kDa and ~12 kDa were observed up to 5 min and 10 min of the digestion, respectively (Figure 2A, lanes 7 and 9). These two fragment correspond to the ~17 kDa and ~12 kDa fragments observed on the stained gel and, hence, represent products of proteolytic degradation of the NcΔ15D protein. The fragments with molecular weight of ~7 kDa, ~5 kDa, and ~4 kDa were not observed on the western blot indicating that either the NcΔ15D specific antibodies do not detect these fragments or that these fragments are not derived from NcΔ15D protein.

No change in the full-length NcΔ15D protein band intensity was observed in the absence of pepsin in the experimental control specimens SGF P0 and P7 (Figure 2A, lanes 3 and 12), reaffirming that the NcΔ15D protein was stable in the test system without pepsin.

In addition to the full-length NcΔ15D protein, three minor bands at ~100 kDa, ~30 kDa, ~25 kDa and minor smearing in the high molecular weight were observed in the control specimens SGF P0, SGF T0 and SGF P7 (Figure 2A, lanes 3, 4,and 12). The ~ 100 kDa band may represent dimerization of the NcΔ15D protein. The smearing in the high molecular weight range has been are attributed to aggregation of NcΔ15D protein (Dong et al., 2008). Membrane proteins tend to aggregate after heating and can migrate as higher molecular weight aggregates on SDS-PAGE (McGregor et al., 2003; Von Jagow et al., 1994). The bands of ~30 kDa and ~25 kDa, most likely, represent degradation products of NcΔ15D protein as described in the NcΔ15D characterization report (Dong et al., 2008). No immunoreactive bands were observed in specimens SGF N0 and SGF N7 that represent test system experimental controls (Figure 2A, lanes 2 and 13). This indicates that non-specific interactions between the test system components and the antibodies were not observed under these experimental conditions.

### 12.4 Identification of Transiently Stable SGF Fragments by N-Terminal Sequencing

The fragments with molecular weights of ~5 kDa and ~4 kDa, observed on the stained gel for up to 60 min and 20 min, respectively, were not detected with NcΔ15D specific antibody and, therefore, were N-terminally sequenced.

The sequence obtained for ~5 kDa fragment did not match the expected sequence of the NcΔ15D protein and its identity was not established (see section 8.5.2.). Most likely this fragment originated from one of the soybean proteins co-purified with the NcΔ15D protein. Sequence obtained for the fragment of ~4 kDa matched the expected sequence of the NcΔ15D protein to a region of NcΔ15D sequence starting at amino acid 376 (Figure 5).

### 12.5 Digestibility of the NcΔ15D Protein in SGF Followed by SIF

#### 12.5.1 Assessment of the NcΔ15D Protein Digestibility in SGF Followed by SIF by Colloidal Brilliant Blue G Gel Staining of SDS-PAGE

To better understand stability of the NcΔ15D protein during gastrointestinal digestion, it was sequentially digested in SGF followed by digestion in SIF. The digestibility of NcΔ15D in SGF followed by SIF was evaluated by visual analysis of Colloidal Brilliant Blue G stained SDS-PAGE (Figure 3A). The gel used to assess the stability of the NcΔ15D protein to the digestion with pepsin in SGF followed by the digestion with pancreatin was loaded with ~0.8 µg total NcΔ15D protein (based on pre-digestion concentrations) for each of the digestion specimens. As expected, the full-length NcΔ15D protein was no longer observed at the 2 min digestion time point, while the ~17 kDa , ~12 kDa, ~5 kDa, and ~4 kDa fragments were observed as expected (Figure 3A, lane 3). After exposure to SIF, the ~17 kDa and ~12 kDa fragments were not visible at the 30 s digestion time point (Figure 3A, lane 5). The ~5 kDa fragment was not observed at the 5 min digestion time point (Figure 3A, lane 9). However, as discussed in section 12.4, the N-terminal sequencing results indicated that ~5 kDa fragment is not derived from NcΔ15D protein. The ~4 kDa fragment was not visible at the 2 min digestion specimen (Figure 3A, lane 8). These data clearly indicate that transiently stable fragments of NcΔ15D protein rapidly degrade upon short exposure to SIF.

No change in the intensities of the fragments were observed in the absence of pancreatin in the experimental controls SEQ P0 and SEQ P7 (Figure 3A, lanes 5 and 14). This indicates that digestion of the fragments was due to the proteolytic activity of pancreatin present in SIF and not due to the instability of the fragments at ~37 °C over the course of the experiment.

In addition to the full-length NcΔ15D protein, the banding and smearing pattern similar to that observed in the controls SGF P0, SGF T0 and SGF P7 as described in section 12.3.1 was also observed in the control SEQ 0min (Figure 3A, lane 2)

The experimental controls SEQ N0 and SEQ N7 (Figure 3A, lanes 4 and 15) demonstrated the integrity of the pancreatin in the test system over the course of the experiment.

### 12.5.2 Assessment of the NcΔ15D Protein Digestibility in SGF Followed by SIF by Western Blot Analysis

The digestibility of NcΔ15D protein in SGF followed by SIF was also evaluated using western blot (Figure 3B). The gel was loaded with ~15 ng total protein (based on pre-digestion concentrations) for each of the digestion specimens. Visual examination of the western blot showed that, as expected, the full-length NcΔ15D was observed only at the 0 min time point in the SGF phase (Figure 3B, lane 2). Two proteolytic fragments with molecular weight of ~17 kDa and ~12 kDa were observed at the 2 min time point in the SGF phase (Figure 3B, lane 3)

As expected, the ~17 kDa and ~12 kDa were observed in the experimental controls SEQ T0 (Figure 3B, lane 6), SEQ P0 and SEQ P7 (Figure 3B, lanes 5 and 14). No fragments recognizable by NcΔ15D specific antibody were observed at the 30 s digestion time point indicating their rapid degradation in SIF (Figure 3B, lane 7).

In addition to the full-length NcΔ15D protein, the banding and smearing pattern similar to that observed in the control specimens SGF P0, SGF T0 and SGF P7 as described in section 12.3.2 was also observed in the control SEQ 0min (Figure 3B, lane 2).

The experimental controls, SEQ N0 and SEQ N7 (Figure 3B, lanes 4 and 15), demonstrated absence of non-specific antibody interactions with the SIF test system during the SIF phase of the experiment.

## 12.6 Digestibility of the NcΔ15D protein in SIF

### 12.6.1 Assessment of NcΔ15 digestibility in SIF by Western Blot Analysis

The digestibility of the full-length NcΔ15D protein in SIF was evaluated by western blot (Figure 4). The western blot used to assess the *in vitro* digestibility of the NcΔ15D protein in SIF (Figure 4, panel A) was run concurrently with the western blot to determine the LOD (Figure 4, panel B) of the NcΔ15D protein. The LOD was visually estimated to be 0.5 ng. The LOD estimated for the NcΔ15D protein was used to calculate the maximum amount of NcΔ15D protein that could remain visually undetected after digestion, which corresponded to 3.3% of the total protein loaded:

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

$$\frac{0.5\ ng \times 100\%}{15\ ng} = 3.3\%$$

The gel used to assess digestibility of the NcΔ15D protein *in vitro* by western blot was loaded with 15 ng total protein (based on pre-digestion concentrations) for each of the digestion time points. Western blot analysis demonstrated that a band corresponding to the full-length NcΔ15D protein was digested below the LOD within 5 min of incubation in SIF (Figure 4A, lane 5). Therefore, based on the LOD, more than 96% (100% - 3.3% = 96.7%) of the full-length NcΔ15D protein was digested within 5 min. No proteolytic fragments of the NcΔ15D protein were detected at any time points. These data suggest that the full-length NcΔ15D protein degrades rapidly when exposed to pancreatin at neutral pH.

No change in the full-length NcΔ15D protein band intensity was observed in the absence of pancreatin in the experimental controls SIF P0 and SIF P8 (Figure 4A, lanes 3 and 13). This indicates that the NcΔ15D protein was stable in the test system without pancreatin at ~37 ºC over the course of the experiment. It is to be noted that the higher order aggregation of the NcΔ15D protein appears more intense in the SIF P8 control. This is because heating of the membrane proteins for a long duration (i.e. 24 h for SIF P8) leads to further aggregation of membrane proteins like NcΔ15D.

In addition to the full-length NcΔ15D protein, the banding and smearing pattern similar to that observed in the control specimens SGF P0, SGF T0 and SGF P7 as described in section 12.3.2 was also observed in the controls SIF P0, SIF T0, and SIF P8 (Figure 4A, lane 3, 4, and 13)

No immunoreactive bands were observed in specimens SIF N0 and SIF N8 that represent SIF test system experimental controls (Figure 4A, lanes 2 and 14).

The digestion of NcΔ15D protein in SIF was not evaluated using stained gels because the results of the NcΔ15D digestion in the sequential (SGF followed by SIF) enzymatic digestion assay clearly demonstrated that no stable fragments were observed after 2 min of incubation in SIF.

## 13.0 **Conclusions**

The results of the study demonstrated that greater than 96% of the full-length NcΔ15D protein was digested within 30 s of incubation in SGF when analyzed using either stained gel or western blot with a NcΔ15D specific antibody. Several fragments (~17 kDa, ~12 kDa, ~5 kDa and ~4 kDa) were observed at various time points during digestion in SGF when specimens were analyzed using a stained gel. The fragments with molecular

weight of approximately 17 kDa and 12 kDa were also observed on western blot X-ray films and, therefore, identified as products of proteolytic digestion of the NcΔ15D protein. The fragments with molecular weight of ~5 kDa and ~4 kDa were not observed on the western blot and, therefore, these fragments were N-terminally sequenced in an attempt to establish their identity. Sequence obtained for the fragment of ~4 kDa matched the expected sequence of the NcΔ15D protein starting at amino acid 376. The sequence obtained for ~5 kDa fragment did not match the expected sequence of the NcΔ15D protein and its identity was not established. Most likely this fragment originated from one of the soybean proteins that co-purified with the NcΔ15D protein. Digestion of the NcΔ15D fragments observed in SGF was further evaluated in SIF, where they were rapidly degraded upon exposure to SIF when analyzed using stained gel (<5 min) and western blot (<30 s).

The results of the study also demonstrated that greater than 96% of the full-length NcΔ15D protein was digested in SIF within 5 min when analyzed using western blot. No proteolytic fragments were observed at any time points.

The results of this study show that the integral membrane protein, NcΔ15D, is readily digestible in SGF and SIF. Rapid digestion of the full-length NcΔ15D protein and any NcΔ15D protein fragments in SGF and SIF indicates that it is highly unlikely that the NcΔ15D protein will pose any safety concern to human health.

## 14.0 References

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

Dong, J.G., Lee, T.C., Finnessy, J.J., and Rice, E.A. 2008. Characterization of *Neurospora crassa* Δ15 Desaturase Isolated from Immature Seeds of Soybean MON 87769. Monsanto Technical Report MSL-0021308. St. Louis, MO.

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

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

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

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

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

Neuhoff, V., Norbert, A., Taube, D., and Wolfgang, E. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 9: 255-262.

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

Girault, R., Song, Z., Pan, A., Feng, D., Rice, J.,. Tian, Q., Masucci, J. 2008. Molecular Analysis of Stearidonic Acid Producing Soybean MON 87769. Monsanto Technical Report MSL-19874 St. Louis, MO.

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

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

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

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

Von Jagow, G., Link, G.T., and Schagger, H. 1994. A practical guide to membrane protein purification. Academic Press.

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

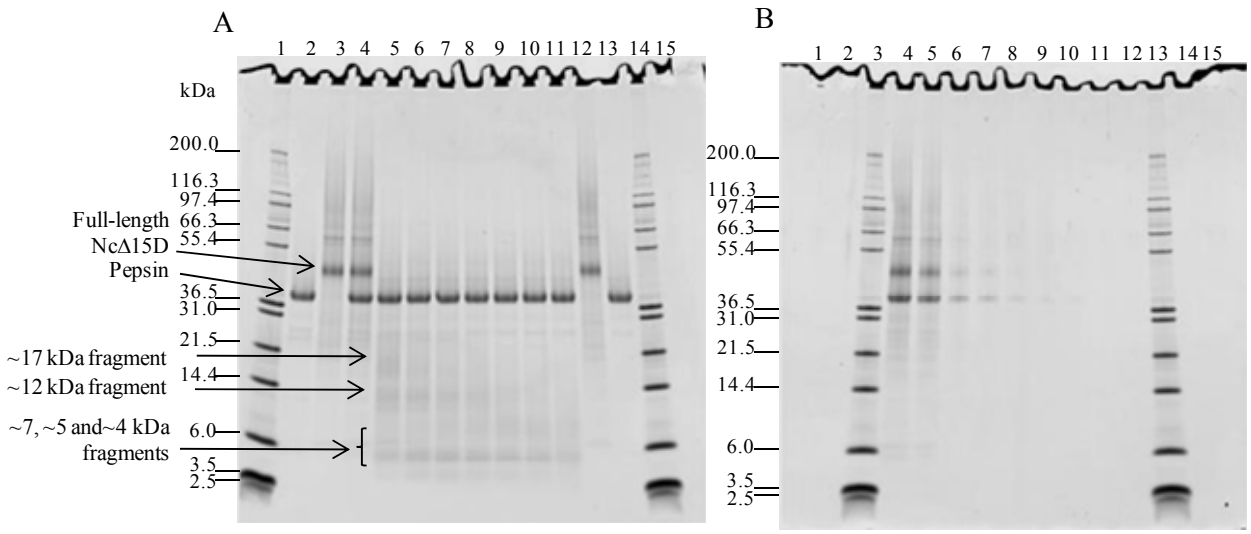


**Figure 1 SDS-PAGE Analysis of the Digestion of the NcΔ15D protein in SGF**

Colloidal Brilliant Blue G stained SDS-PAGE were used to analyze the digestibility of the NcΔ15D protein in SGF. **Panel A** corresponds to the NcΔ15D protein digestion in SGF. Based on pre-digestion protein concentrations, 0.8 µg of total protein was loaded in each lane containing the NcΔ15D protein. **Panel B** corresponds to the limit of detection of the NcΔ15D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel. In both gels, the NcΔ15D protein migrated to approximately 46 kDa and pepsin to approximately 38 kDa (indicated by arrows on the left).

| Lane | Sample | Incubation Time (min) | Lane | Sample | Amount (µg) |
|---|---|---|---|---|---|
| 1 | Mark 12 MWM | — | 1 | Blank | — |
| 2 | SGF N0 | 0 | 2 | Blank | — |
| 3 | SGF P0 | 0 | 3 | Mark 12 MWM | — |
| 4 | SGF T0 | 0 | 4 | T0, protein+SGF | 0.8 |
| 5 | SGF T1 | 0.5 | 5 | T0, protein+SGF | 0.4 |
| 6 | SGF T2 | 2 | 6 | T0, protein+SGF | 0.1 |
| 7 | SGF T3 | 5 | 7 | T0, protein+SGF | 0.05 |
| 8 | SGF T4 | 10 | 8 | T0, protein+SGF | 0.02 |
| 9 | SGF T5 | 20 | 9 | T0, protein+SGF | 0.01 |
| 10 | SGF T6 | 30 | 10 | T0, protein+SGF | 0.005 |
| 11 | SGF T7 | 60 | 11 | T0, protein+SGF | 0.0025 |
| 12 | SGF P7 | 60 | 12 | T0, protein+SGF | 0.001 |
| 13 | SGF N7 | 60 | 13 | Mark 12 MWM | — |
| 14 | Mark 12 MWM | — | 14 | Blank | — |
| 15 | Blank | — | 15 | Blank | — |

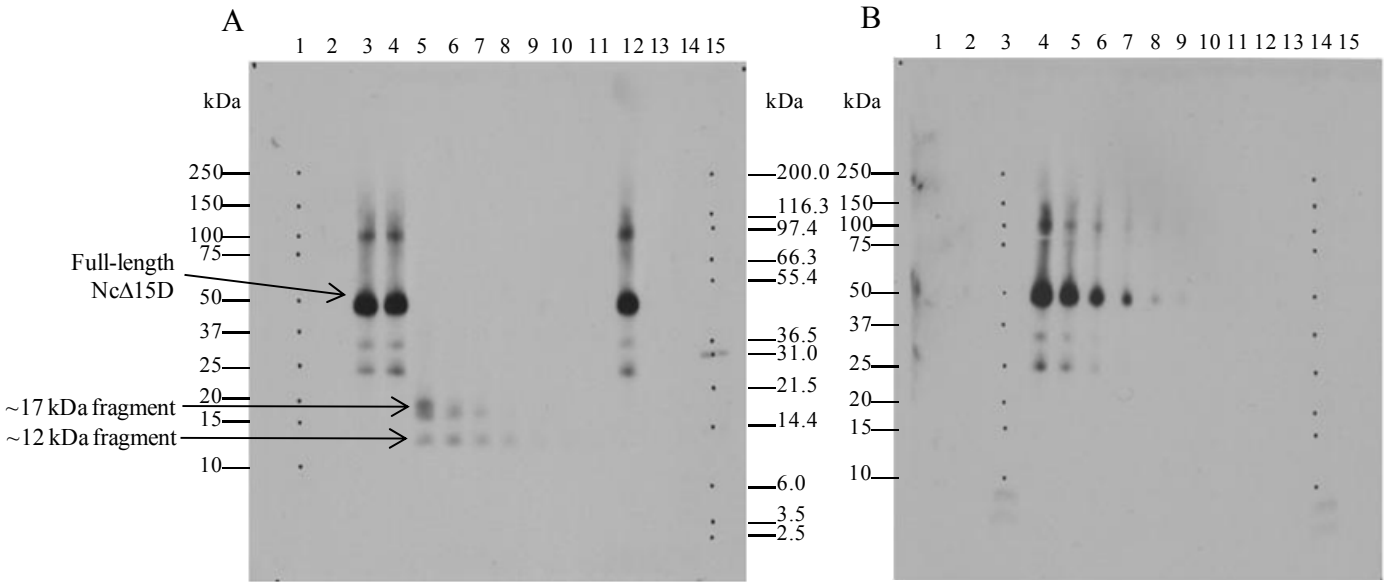


**Figure 2 Western Blot Analysis of the Digestion of the NcΔ15D protein in SGF**

**Panel A** corresponds to the NcΔ15D protein digestion in SGF. Based on pre-digestion protein concentrations, 20 ng (total protein) was loaded in the lanes containing the NcΔ15D protein. **Panel B** corresponds to the limit of detection of the NcΔ15D protein. Approximate molecular weights (kDa) are shown on the left and right for Panel A and on left for Panel B, correspond to the markers loaded on each gel. In both gels, the NcΔ15D protein migrated to approximately 46 kDa. A 3 min exposure is shown.

| Lane | Sample | Incubation Time (min) | Lane | Sample | Amount (ng) |
|---|---|---|---|---|---|
| 1 | Precision Plus MWM | — | 1 | Blank | — |
| 2 | SGF N0 | 0 | 2 | Blank | — |
| 3 | SGF P0 | 0 | 3 | Precision Plus MWM | — |
| 4 | SGF T0 | 0 | 4 | T0, protein+SGF | 15 |
| 5 | SGF T1 | 0.5 | 5 | T0, protein+SGF | 10 |
| 6 | SGF T2 | 2 | 6 | T0, protein+SGF | 5 |
| 7 | SGF T3 | 5 | 7 | T0, protein+SGF | 2.5 |
| 8 | SGF T4 | 10 | 8 | T0, protein+SGF | 1 |
| 9 | SGF T5 | 20 | 9 | T0, protein+SGF | 0.5 |
| 10 | SGF T6 | 30 | 10 | T0, protein+SGF | 0.2 |
| 11 | SGF T7 | 60 | 11 | T0, protein+SGF | 0.1 |
| 12 | SGF P7 | 60 | 12 | T0, protein+SGF | 0.05 |
| 13 | SGF N7 | 60 | 13 | T0, protein+SGF | 0.025 |
| 14 | Precision Plus MWM | — | 14 | Precision Plus MWM | — |
| 15 | Mark 12 MWM | — | 15 | Blank | — |

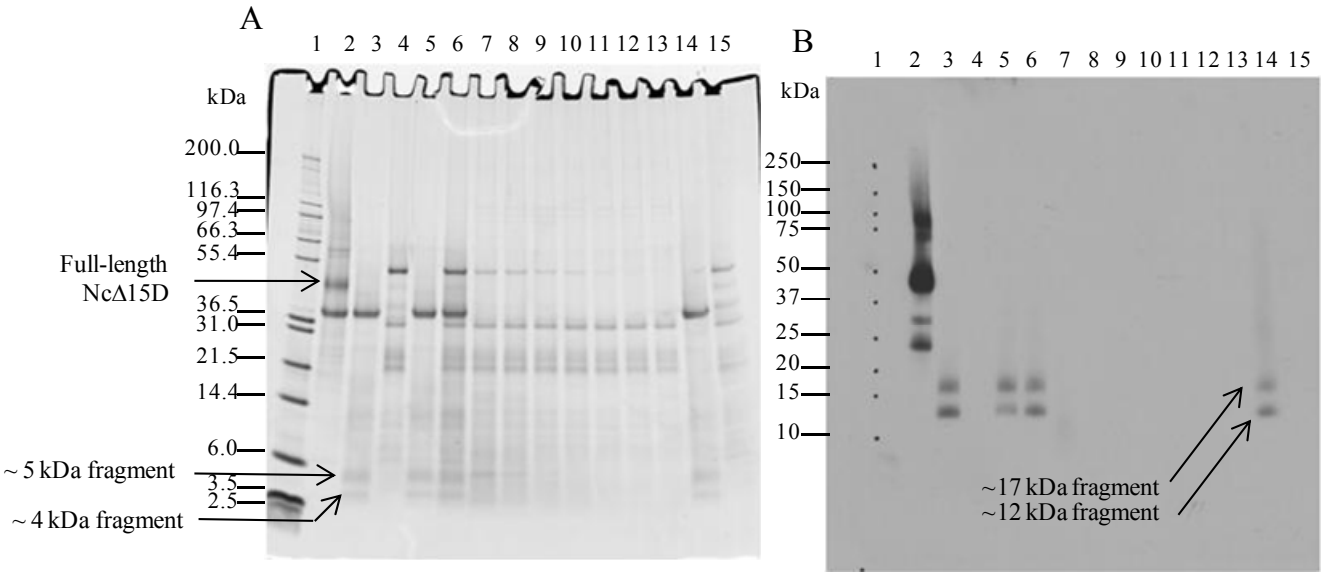


**Figure 3 SDS-PAGE and Western Blot Analysis of the Digestion of the NcΔ15D protein in SGF followed by SIF**

**Panel A** corresponds to the colloidal stained SDS-PAGE of the NcΔ15D protein digestion in SGF followed by SIF. Based on pre-digestion protein concentrations, 0.8 µg of total protein was loaded per lane containing NcΔ15D protein. **Panel B** corresponds to the western blot of the NcΔ15D protein digestion in SGF followed by SIF. Based on pre-digestion protein concentrations, 15 ng of total protein was loaded per lane containing the NcΔ15D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel. In both gels, the NcΔ15D protein migrated to approximately 46 kDa. A 3 min exposure is shown.

| Lane | Sample | Incubation Time | Lane | Sample | Incubation Time |
|---|---|---|---|---|---|
| 1 | Mark 12 MWM | — | 1 | Precision Plus MWM | — |
| 2 | SEQ 0min | 0 | 2 | SEQ 0min | 0 |
| 3 | SEQ 2min | 2 min | 3 | SEQ 2min | 2 min |
| 4 | SEQ N0 | 0 | 4 | SEQ N0 | 0 |
| 5 | SEQ P0 | 0 | 5 | SEQ P0 | 0 |
| 6 | SEQ T0 | 0 | 6 | SEQ T0 | 0 |
| 7 | SEQ T1 | 0.5 min | 7 | SEQ T1 | 0.5 min |
| 8 | SEQ T2 | 2 min | 8 | SEQ T2 | 2 min |
| 9 | SEQ T3 | 5 min | 9 | SEQ T3 | 5 min |
| 10 | SEQ T4 | 10 min | 10 | SEQ T4 | 10 min |
| 11 | SEQ T5 | 30 min | 11 | SEQ T5 | 30 min |
| 12 | SEQ T6 | 1 h | 12 | SEQ T6 | 1 h |
| 13 | SEQ T7 | 2 h | 13 | SEQ T7 | 2 h |
| 14 | SEQ P7 | 2 h | 14 | SEQ P7 | 2 h |
| 15 | SEQ N7 | 2 h | 15 | SEQ N7 | 2 h |

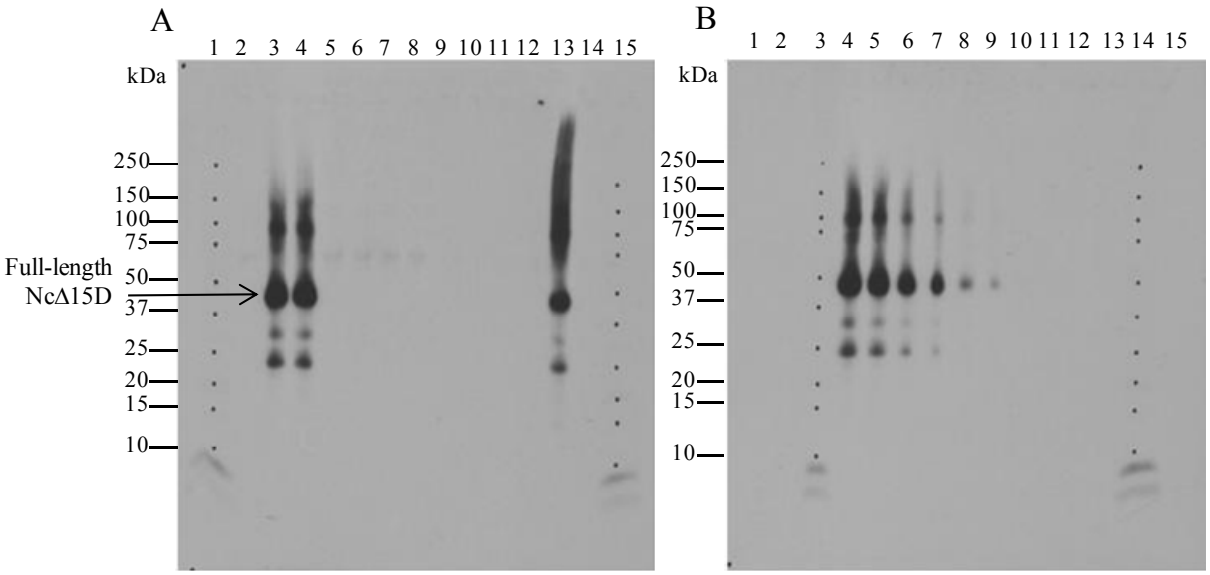


**Figure 4 Western Blot Analysis of the Digestion of the NcΔ15D protein in SIF**

**Panel A** corresponds to the NcΔ15D protein digestion in SIF. Based on pre-digestion protein concentrations, 15 ng (total protein) was loaded in the lanes containing the NcΔ15D protein. **Panel B** corresponds to the limit of detection of the NcΔ15D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel. In both gels, the NcΔ15D protein migrated to approximately 46 kDa. A 3 min exposure is shown.

| Lane | Sample | Incubation Time | Lane | Sample | Amount (ng) |
|---|---|---|---|---|---|
| 1 | Precision Plus MWM | — | 1 | Blank | — |
| 2 | SIF N0 | 0 | 2 | Blank | — |
| 3 | SIF P0 | 0 | 3 | Precision Plus MWM | — |
| 4 | SIF T0 | 0 | 4 | T0, protein+SIF | 15 |
| 5 | SIF T1 | 5 min | 5 | T0, protein+SIF | 10 |
| 6 | SIF T2 | 15 min | 6 | T0, protein+SIF | 5 |
| 7 | SIF T3 | 30 min | 7 | T0, protein+SIF | 2.5 |
| 8 | SIF T4 | 1 h | 8 | T0, protein+SIF | 1 |
| 9 | SIF T5 | 2 h | 9 | T0, protein+SIF | 0.5 |
| 10 | SIF T6 | 4 h | 10 | T0, protein+SIF | 0.2 |
| 11 | SIF T7 | 8 h | 11 | T0, protein+SIF | 0.1 |
| 12 | SIF T8 | 24 h | 12 | T0, protein+SIF | 0.05 |
| 13 | SIF P8 | 24 h | 13 | T0, protein+SIF | 0.025 |
| 14 | SIF N8 | 24 h | 14 | Precision Plus MWM | — |
| 15 | Precision Plus MWM | — | 15 | Blank | — |

| Amino acid residue # from the N-terminus → | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
|---|---|---|---|---|---|---|---|---|---|---|
| Predicted NcΔ15D Sequence[1] → | A | T | N | S | I | R | P | M | L | G |
| | \| | \| | \| | \| | \| | \| | \| | \| | \| | \| |
| Observed Sequence→ | A | T | N | S | I | X | (P) | (M) | (L) | (G) |

**Figure 5 N-Terminal Sequencing of ~4 kDa Fragment**

The amino acid sequence of the ~4 kDa stable fragment from the SGF T3 specimen. The sequence obtained was compared to the expected sequence of the NcΔ15D protein. The predicted amino acid sequence of the NcΔ15D protein was deduced from the coding region of the full-length *NcΔ15D* gene present in MON 87769 (Girault *et al*., 2007). The "X" refers to an undesignated call and ( ) denotes tenuous designation. [1]The single letter IUPAC-IUB amino acid code is A, alanine; T, threonine; N, asparagine; S, serine; I, isoleucine; R, arginine; P, proline; M, methionine; L, leucine; and G, glycine.

## Appendix 1 List of Applicable SOPs

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