Study Title

Assessment of the *in vitro* Digestibility of the *Primula juliae* Δ6 Desaturase Protein (PjΔ6D) in Simulated Gastric and Simulated Intestinal Fluids

Authors

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

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Sponsor Representative
Cherian George, Ph.D.

Performing Laboratory

Monsanto Company
Regulatory Product Characterization and Detection Method Center
800 North Lindbergh Boulevard
St. Louis, Missouri 63167

Laboratory Project ID

Monsanto Study Number: REG-08-233
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Statement of Compliance

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

Submitter — Date

Cherian George — 1-22-2009

Cherian George, Ph.D.
Sponsor Representative — Date

Shefalee Kapadia — 1-22-2009

Shefalee A. Kapadia, M.Sc.
Study Director — Date

Quality Assurance Unit Statement

Study Title: Assessment of the *in vitro* Digestibility of the *Primula juliae* Δ6 Desaturase Protein (PjΔ6D) in Simulated Gastric and Simulated Intestinal Fluids.

Study Number: REG-08-233

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
08/04/2008; 01/14/2009	Raw Data Audit	08/07/2008; 01/16/2009	08/07/2008; 01/16/2009
10/31/2008; 11/03-05/2005; 01/14/2009	Draft Report Audit	11/05/2008; 01/16/2009	11/05/2008; 01/16/2009

Todd Butzlaff
Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

01/22/2009
Date

Study Certification Page

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

Signatures of Final Report Approval:

S Kapadia — 1-22-2009

Shefalee A. Kapadia, M.Sc.
Study Director — Date

Bhakta — 1.22.09

Tina Bhakta, Ph.D.
Principal Investigator — Date

E A Rice — January 22, 2009

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

Study Information

Study Number:	REG-08-233
MSL Number:	0021428
Title:	Assessment of the *In Vitro* Digestibility of the *Primula juliae* Δ6 Desaturase Protein (PjΔ6D) in Simulated Gastric and Simulated Intestinal Fluids.
Testing Facility:	Monsanto Company Regulatory Product Characterization and Detection Method Center 800 North Lindbergh Boulevard 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.
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Abbreviations and Definitions[1]

BLAST	Basic local alignment search tool
CFR	Code of Federal Regulations
CSPB	Cold Shock Protein B from *Bacillus subtilis*
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
PjΔ6D	*Primula juliae* Δ6 Desaturase Protein
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 the integral membrane *Primula juliae* delta-6 desaturase (PjΔ6D) and integral membrane *Neurospora crassa* delta-15 desaturase (NcΔ15D). The expression of these two genes are 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 PjΔ6D protein 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 PjΔ6D 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 PjΔ6D protein was assessed using sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and western blot methods. The extent of the PjΔ6D 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 99% of the full-length PjΔ6D protein was digested within 30 s of incubation in SGF when analyzed using stained gel, and greater than 96% was digested when analyzed using western blot with an antibody raised against full-length PjΔ6Dprotein. A fragment of ~10 kDa was observed by western blot in the 30 s digestion time point and was identified as a product of proteolytic digestion of the PjΔ6D protein. This fragment was digested within 2 min of incubation in SGF. On stained gel, fragments with molecular weights of ~4 kDa and ~ 5 kDa were observed in SGF for 10 min and 60 min, respectively. Fragment with molecular weight of ~ 3.5 kDa was observed from 10-60 min and, most probably, represents a product of further degradation of the ~4 kDa fragment. These fragments were not observed on western blot X-ray films and, therefore, they were N-terminally sequenced in an attempt to establish their identities. The sequences obtained for the ~ 4 kDa and ~ 3.5 kDa did not match the predicted sequence of the PjΔ6D protein and their identities could not be established. Most likely these fragments originate from one of the endogenous soybean proteins co-purified with the PjΔ6D protein. The N-terminal sequencing of the ~5 kDa fragment did not yield enough amino acid residues to allow a definitive sequence comparison to the PjΔ6D predicted sequence or to a protein database and, therefore, the origin of the fragment could not be established.

To better understand the fate of the PjΔ6D protein during gastrointestinal digestion, it was exposed to digestion with pancreatin in SIF following the digestion in SGF. After digestion of PjΔ6D protein in SGF for 2 min, the reaction was quenched and the mixture was exposed to further digestion in SIF. The digestibility of the PjΔ6D protein in SGF followed by the digestion in SIF was evaluated by visual analysis of Colloidal Brilliant

Blue G stained polyacrylamide gel or by visual analysis of western blot X-ray films. Visual examination of the stained gel demonstrated that transiently stable fragments with molecular weight of ~5 kDa and ~4 kDa observed after 2 min of digestion in SGF were rapidly digestible (<5 min) in SIF. Because the ~3.5 kDa fragment most probably represents a product of further degradation of ~4 kDa fragment and, appears later (10-60 min) during SGF digestion, this fragment was not observed in the SGF 2min time point during sequential digestion. Visual examination of the X-ray films showed that no immune-reactive fragments were observed at any time points in SIF. The data clearly indicate that the transiently stable fragments observed in SGF rapidly degrade upon short exposure to SIF.

Results of this study also demonstrated that greater than 84% of the full-length PjΔ6D protein was digested within 5 min of incubation in SIF with no proteolytic fragment(s) detected by western blot using PjΔ6D–specific antibody.

The results of this study show that the integral membrane protein, PjΔ6D, is readily digestible in SGF and SIF. Rapid digestion of the full-length PjΔ6D protein in SGF and the subsequent degradation of any fragments resulting from SGF digestion by SIF indicates that it is highly unlikely that the PjΔ6D 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). Allergenic proteins or their fragments when 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. The susceptibility of the PjΔ6D protein was assessed using this standardized *in vitro* pepsin digestion protocol.

To reach the intestinal mucosa, where antigen processing cells reside, protein or protein fragment(s) 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 digestion of a protein by pepsin in the gastric system

greatly reduces the possibility that any 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 fragment(s) is observed in SGF, further degradation of these fragments in SIF can be evaluated to better understand the fate of the protein during *in vivo* digestion. For example, following exposure to SGF, if 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, *in vitro* digestibility of protein in SIF is also used as a stand alone independent test system to assess the digestibility of food components (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 with *in vivo* digestion (FAO/WHO, 2001).

3.0 Purpose

The purpose of this study was to assess the *in vitro* digestibility of the PjΔ6D protein in SGF, SIF, and in a sequential enzymatic digestion where the protein was first digested in SGF followed by digestion in SIF.

4.0 Materials

4.1 Test Substance

The PjΔ6D protein (Orion lot 10001532) was produced and purified from immature MON 87769 soybean seed. The PjΔ6D 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 10001532 and is summarized in the Certificate of Analysis. The PjΔ6D protein had a total protein concentration of 0.52 mg/ml, a purity of 47%, and an apparent molecular weight of 45.9 kDa as determined by SDS- PAGE. The N-terminal sequence of the PjΔ6D was also confirmed during characterization.

5.0 Test Systems

Two test systems, SGF and SIF, were utilized independently and then sequentially to test stability of the PjΔ6D protein.

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 PjΔ6D protein would be present in the digestion reactions. The amount of pepsin powder used to prepare SGF was calculated from the specific activity of pepsin 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 activity of SGF preparation was confirmed prior to initiating the PjΔ6D digestion assay. The digestion of the PjΔ6D protein was monitored by SDS-PAGE stained gels and western blot analysis using a PjΔ6D 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 proteolytic enzymes known as pancreatin in a buffer adjusted to pH of ~7.5. SIF was prepared according to the current version of SOP BR-ME-0461 which is based on 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 P-1500, St. Louis, MO). The SIF was formulated so that 55.3 μg of pancreatin powder would be present per μg of PjΔ6D 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 activity of the SIF preparation was confirmed prior to initiating the PjΔ6D digestion assay. The digestion of the PjΔ6D protein in SIF was assessed by western blot analysis using a PjΔ6D 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 PjΔ6D Protein in SGF

Digestibility of the PjΔ6D 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	Specimen Code
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 2561 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 594 µl of the PjΔ6D total protein to a tube containing 1206 µl of pre-heated (36.5 ºC, 5 min) SGF which corresponds to 308.9 µg of PjΔ6D protein and 3089 U of pepsin, respectively. The tube contents were mixed by vortexing and immediately placed in a 36.5 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 thirty four µl of SGF (343 U of pepsin) was quenched by the addition of 70 µl of 0.7 M sodium carbonate buffer (0.7 M Na_2CO_3, pH 11), and 70 µl of 5× LB prior to the addition of 66 µl (34.3 µg) of the PjΔ6D 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 PjΔ6D 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 PjΔ6D protein. Protein storage buffer (50 mM sodium acetate, pH 5.6, 1 mM $MgCl_2$, 0.1 % fos-choline-12, 0.5 M NaCl, 10% glycerol) was added to SGF in place of the PjΔ6D 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 PjΔ6D Protein in SGF followed by SIF

Digestibility of the PjΔ6D protein was assessed by digestion in SGF followed by digestion in SIF. The PjΔ6D protein was digested first in SGF as described in Section 5.1 for 2 min at which point the reaction was stopped by quenching with 0.7 M sodium carbonate buffer (0.7 M Na_2CO_3, pH 11). It was then placed in the SIF assay and digested as described in Section 5.2.

Digestion of the PjΔ6D 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	Designation(s)
SGF system	
0 min	SEQ 0 min
2 min	SEQ 2 min
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 576 µl of the PjΔ6D total protein to a tube containing 1138 µl of pre-heated (36.9 ºC, 5 min) SGF, corresponding to 299.5 µg of PjΔ6D protein and 2995 U of pepsin, respectively. The tube contents were mixed by vortexing and immediately placed in a 36.9 ºC water bath. The tube was removed after 2 min, and the reaction was immediately quenched by adding 600 µl of 0.7 M sodium carbonate buffer (0.7 M Na_2CO_3, pH 11). 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, 1538 µl of the quenched SGF reaction mixture was added to 1100 µl of pre-heated (36.9 ºC, 5 min) SIF, corresponding to 200 µg SGF digested and quenched PjΔ6D 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 36.9 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 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 0 min) was prepared in a separate tube by first quenching 76 µl of SGF (200 U of pepsin) with 40 µl of sodium carbonate buffer, and 40 µl of 5× LB buffer and heating to 75-100 ºC for 5-10 min prior to the addition of 38 µl (20 µg) of the PjΔ6D totol 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 50 µl of 5× LB buffer and heating to 75-100 ºC for 5-10 min prior to the addition of 115 µl (15 µg, based on the pre digestion concentration) of the SGF digested and quenched PjΔ6D 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 PjΔ6D protein fragment 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 PjΔ6D protein. Protein storage buffer (50 mM sodium acetate, pH 5.6, 1 mM $MgCl_2$, 0.1 % fos-choline 12, 0.5 M NaCl, 10% glycerol) was added to SIF in place of the SGF digested PjΔ6D 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 PjΔ6D Protein in SIF

Digestibility of the PjΔ6D 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	Designation(s)
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 test substance to a tube containing 860 µl of pre-heated (37.3 ºC, 5 min) SIF, corresponding to 156 µg of the PjΔ6D total protein and 8.6 mg of pancreatin, respectively. The tube contents were mixed by vortexing and immediately placed in a 37.7 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 101 µl of SIF (1.01 mg) with 34 µl of 5× LB buffer and heating to 75-100 ºC for 5-10 min prior to the addition of 35 µl (18.2 µg) of the PjΔ6D total protein.

6.3.1 SIF Experimental Controls

Experimental control specimens were prepared to determine the stability of the PjΔ6D 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 PjΔ6D protein. Protein storage buffer (50 mM sodium acetate, pH 5.6, 1 mM $MgCl_2$, 0.1% Fos-choline 12, 0.5 M NaCl, 10% glycerol) was added to SIF in place of the PjΔ6D 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

Activities of the SGF and SIF were assessed using pepsin and pancreatin activity assays, respectively. The digestibility of the PjΔ6D protein in SGF, and in SGF followed by SIF was assessed using stained SDS-PAGE and western blot analysis. The digestibility of the PjΔ6D protein in SIF was assessed using western blot analysis. The lower limit of detection (LOD) of the PjΔ6D protein was determined for stained SDS-PAGE and western blots. Because fragments with molecular weights of ~5 kDa, ~4 kDa and ~3.5 kDa observed on stained gel were not recognized with PjΔ6D specific antibody, their identities were assessed by N-terminal sequencing.

8.1 SGF Activity Assays

The SGF activity assay was used to confirm the suitability of the test system before its use with the PjΔ6D 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 PjΔ6D 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 PjΔ6D 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 PjΔ6D protein in SGF followed by SIF, and in SIF were performed on two separate days, two separate SIF activity assays were performed. For both the activity 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:

$$\frac{Mean\ Activity_{\ A574\,nm} - Mean\ Blank_{\ A574\,nm}}{0.001 \times 15\,\text{min} \times 0.1\,ml \times 0.05}$$

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 $0.05\times$ 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 PjΔ6D 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 PjΔ6D 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 PjΔ6D 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 85 and 110 min for SGF and SGF followed by SIF specimens, respectively. After electrophoresis, proteins were visualized by staining the gel 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 stained for 18 h 30 min, and 16 h 18 min for SGF and SGF followed by SIF specimens, respectively, in 1× Brilliant Blue G-colloidal stain solution containing 20% (v/v) methanol. The gels were destained for 30 s in 10% (v/v) acetic acid, 25% (v/v) methanol and then completely destained for ~6 h 9 min 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 PjΔ6D in SGF and SGF followed by SIF were determined by visual examination of the stained gels.

The LOD of the PjΔ6D 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 PjΔ6D protein digestibility in SGF. LOD samples were heated at 98 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,SIF, and SGF followed by SIF *in vitro* digestions of the PjΔ6D 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 PjΔ6D protein. The digestion samples were diluted with 1× LB to a concentration of ~2 ng/µl, and ~15 ng of the PjΔ6D 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 95.2, 96.1, and 98 °C for 3 min each for the SGF, SIF, and SGF followed by SIF digestions of the PjΔ6D protein, respectively, prior to loading on the gels. Electrophoresis was performed at 125 V for 90, 80, and 110 min for SGF, SIF, and SGF followed by SIF digestions of the PjΔ6D 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 PjΔ6D 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 for SGF and SIF digestion blots and for 60 min at room temperature for SGF followed by SIF digestion blot, respectively 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– PjΔ6D sera (lot 10000821) was incubated with the membranes for 60 min at a dilution of 1:5000 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 G836503-C, Thermo Scientific, Rockford, IL) at a dilution of 1:10,000 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 blots were visually determined relative to the positions of the molecular weight markers.

The LOD for the western blot analysis procedure was determined for the PjΔ6D 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 assess the N-terminal sequence of the SGF transiently stable fragments with apparent molecular weights of ~5 kDa, ~4 kDa, and ~3.5 kDa.

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 and the specimen SGF T6 was used to characterize the transiently stable fragment with apparent molecular weight of ~3.5 kDa. These specimens correspond to the 5 min and 30 min digestion time points of the PjΔ6D protein and provided sufficient amount of the fragments for sequencing.

The SGF T3 specimen was loaded in 4 lanes at 4 µg per lane onto a tricine 10-20% polyacrylamide gradient 10-well gel. The SGF T6 specimen was loaded in 3 lanes at 3 µg per lane onto a different gel. Precision Plus prestained molecular weight markers were loaded in parallel to verify electrotransfer of the protein to the membranes. 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 75 min, and 85 min for SGF T3 and SGF T6 specimens, respectively. Electrotransfer to 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 90 min at a constant voltage of 25 V for both SGF T3 and SGF T6 blots. The blots were stained with Coomassie Blue R-250 stain (Bio-Rad, Hercules, CA) and then destained for $\geq$ 5 min with Coomassie Blue R-250 destain (Bio-Rad, Hercules, CA) to visualize the markers, and the stable fragments generated from digestion of PjΔ6D protein in SGF. The blots were 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 of ~5 kDa, ~4 kDa, and ~3.5 kDa were excised from the blot and each N-terminally sequenced. N-terminal sequence analysis for both blots 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 each analysis of the three transiently stable fragment bands to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

To establish identity of the transiently stable fragments, the acquired sequence data was to be compared to the predicted PjΔ6D protein sequence. Typically, 4 to 5 consecutive residues are required to compare sequence data to the predicted protein sequence, and 8 to 9 unambiguous consecutive residues are required for identification of protein using the BLAST (basic local alignment search tool) algorithm and protein database to avoid false positives. For the ~3.5 kDa fragment, only 4 consecutive amino acid residues were identified, which did not match the predicted PjΔ6D protein sequence. For the ~4 kDa fragment, multiple amino acids were identified at cycles 2-7, potentially indicating that multiple peptides were present in this band. The identified amino acids did not match the predicted PjΔ6D protein sequence. Because less then 8 unambiguous amino acids were identified for both ~3.5 kDa and ~4 kDa fragments, their identities could not be unequivocally established using BLAST search and protein database. The ~5 kDa fragment did not yield sufficient number of the consecutive amino acid residues to perform any comparisons to the predicted PjΔ6D protein sequence or a protein database. Thus, the fragment could have originated from the PjΔ6D protein or one of the co-purified soybean endogenous proteins. Therefore, the digestive fate of the PjΔ6D protein was further assessed in a sequential digestion of SGF followed by SIF.

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 stained gel for the LOD determination of PjΔ6D protein in SGF was rejected because the final scanned image of the gel was not of publishable standard. During the

image capture process the gel ripped at two locations resulting in a poor quality image. As a result of this, the stained gel for PjΔ6D protein digestion in SGF was also rejected since the protocol states that the "Digestion specimens and lower limit of detection samples will be analyzed concurrently to eliminate run-to-run variation" (Protocol Section 8.0, Control of Bias).

One stained blot prepared for N-terminal sequencing containing the PjΔ6D SGF T6 (30 min) time point was also rejected. This is because the resolution required for N-terminal sequencing after electro-transfer of the proteins on the blot between 6-2.5 kDa was not obtained.

11.0 Protocol Deviations

There was also 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 $\pm$ 2 ºC) for additional 10-15 min. However, a mistake was made and each tube was incubated for additional 5 min instead of 10-15 min. There was no impact on the proteolytic activity of pancreatin in SIF since the calculated activity was within the acceptance criteria for the assay established in the SOP.

12.0 Results and Discussion

12.1 Pepsin Activities is 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 of 2643.7 units per mg of pepsin powder was within the acceptable range of pepsin activity (i.e., 2280 to 4280 units per mg pepsin powder) and, therefore, suitable for the 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 range of SIF activity (i.e., 8,000 to 14,000 U/ml of SIF), and, therefore, suitable for the use in this study.

12.3 Digestibility of PjΔ6D Protein in SGF

12.3.1 Assessment of the PjΔ6D Protein Digestibility in SGF by Colloidal Brilliant Blue G Gel Staining of SDS-PAGE

The digestibility of the PjΔ6D 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 PjΔ6D protein (Figure 1, panel B). The LOD of the full-length (~46 kDa) PjΔ6D protein was visually estimated to be 0.005 µg. The LOD estimated for the PjΔ6D protein was used to calculate the maximum amount of PjΔ6D protein that could remain visually undetected after digestion, which corresponded to approximately 0.6% of the total protein loaded:

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

$$\frac{0.005\,\mu g \times 100\%}{0.8\,\mu g} \cong 0.63\%$$

The gel used to assess the stability of the PjΔ6D 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 PjΔ6D protein was digested within 30 s of incubation in SGF (Figure 1A, lane 5). Therefore, based on the limit of detection, more than 99% (100% - 0.63% = 99.37%) of the full-length PjΔ6D protein was digested within 30 s of incubation in SGF. Several fragments were observed in SGF for varying durations between 30 s to 60 min digestion specimens. Fragments of ~ 5 kDa and ~ 4 kDa was observed for up to 60 min and 10 min, respectively (Figure 1A, lanes 11 and 8). Additionally, a fragment of ~ 3.5 kDa was observed from the 10 min to 60 min, which most probably, represents a product of further degradation of the ~4 kDa fragment. (Figure 1A, lanes 8-11).

No change in the full-length PjΔ6D 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 PjΔ6D 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 PjΔ6D, minor smearing in the high molecular weight area was observed in the control specimens SGF P0, SGF T0 and SGF P7 (Figure 1A, lanes 3, 4, and 12) and on the LOD gel (Figure 1B, lanes 4-6). The smearing has been attributed to higher order aggregation of the PjΔ6D protein (Finnessy 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). Smearing in the low molecular weight region (between 31 and 14 kDa) is attributed to degradation products of the PjΔ6D protein. Additionally, a minor band at ~6 kDa was also observed in all the controls and most likely represents a soybean endogenous protein or its degradation product that co-purified with the PjΔ6D protein.

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 PjΔ6D 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 PjΔ6D Protein Digestibility in SGF by Western Blot Analysis

The digestibility of the PjΔ6D protein in SGF was also evaluated by western blotting (Figure 2). The western blot used to assess the stability of the PjΔ6D protein to pepsin digestion (Figure 2, panel A) was run concurrently with a western blot to determine the LOD of the PjΔ6D protein (Figure 2, panel B). The LOD of the full-length PjΔ6D protein was visually estimated to be 0.5 ng. The LOD estimated for the PjΔ6D protein was used to calculate the maximum amount of PjΔ6D 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 the PjΔ6D 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 PjΔ6D 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 PjΔ6D protein it was concluded that more than 96% $(100\% - 3.3\% = 96.7\%)$ of the PjΔ6D protein was digested within 30 s. A transiently stable fragment of ~10 kDa was observed at the 30 s digestion specimen (Figure 2A, lane 5). No immunoreactive bands were detected in the lanes corresponding to the 2 min to 60 min digestion specimens (Figure 2A, lanes 6-11) indicating that the ~ 10 kDa transiently stable fragment is rapidly digested in SGF within 2 min.

No change in the full-length PjΔ6D 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 PjΔ6D protein was stable in the test system without pepsin.

In addition to the full-length PjΔ6D protein, minor smearing in the high molecular weight range was observed in the control specimens SGF P0, SGF T0 and SGF

P7 (Figure 2A, lanes 3, 4, and 12) and on the LOD western blot (Figure 2B, lanes 2-4). Minor smearing in the high weight region on immunoblots has been attributed to aggregated PjΔ6D protein (Finnessy et al., 2008). Minor smearing below the PjΔ6D full-length band and minor bands at ~22 kDa, ~17 kDa, and ~12 kDa were also observed, which constitutes cleavage products of PjΔ6D protein.

No immunoreactive bands were observed in control 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, ~4 kDa, and ~3.5 kDa observed on the stained SGF gel for up to 60 min, 10 min, and 10-60 min, respectively, were not detected with PjΔ6D specific antibody and, therefore, were N-terminally sequenced.

As described in Section 8.5.2, for each of the ~4 kDa and ~3.5 kDa fragments, the obtained consecutive stretches of amino acid residues did not match the predicted PjΔ6D protein sequence, however, the number of determined amino acids was not sufficient to perform a BLAST search to the protein database. It is thus likely that these fragments originate from soybean proteins which co-purified with the PjΔ6D protein. Additionally, the ~5 kDa fragment did not yield sufficient number of consecutive amino acid residues to perform any comparisons to the predicted PjΔ6D protein sequence or a protein database and, therefore, the origin of the fragment could not be established.

12.5 Digestibility of PjΔ6D protein in SGF followed by SIF

12.5.1 Assessment of the PjΔ6D Protein Digestibility in SGF Followed by SIF by Colloidal Brilliant Blue G Gel Staining of SDS-PAGE

To better understand stability of the PjΔ6D protein during gastrointestinal digestion, it was sequentially digested in SGF followed by digestion in SIF. After digestion in SGF for 2 min, the reaction was quenched and the mixture was exposed to further digestion in SIF. The digestibility of PjΔ6D 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 PjΔ6D protein to the digestion with pepsin in SGF followed by the digestion with pancreatin was loaded with ~0.9 µg total PjΔ6D protein (based on pre-digestion concentrations) for each of the digestion specimens. As expected, the full-length PjΔ6D protein was no longer observed at the SEQ 2min digestion time point, while the ~5 kDa,

and ~4 kDa fragments were observed (Figure 3A, lane 3). Because the ~3.5 kDa fragment most probably represents a product of further degradation of ~4 kDa fragment and, appears later (10-60 min) during SGF digestion, this fragment was not observed in the SGF 2min time point during sequential digestion. After exposure to SIF, the ~5 kDa and ~4 kDa fragments were not visible at the 5 min digestion time point (Figure 3A, lane 9). Since the ~3.5 kDa fragment was observed in SGF digestion only between 10-60 min, we did not observe that fragment in 2 min digestion time point in SGF. These data clearly indicate that transiently stable fragments in SGF, rapidly degrade upon short exposure to SIF.

In addition to the full-length PjΔ6D, minor smearing in the high molecular weight area was observed in the control SEQ 0min, (Figure 3A, lane 2). The smearing is attributed to higher order aggregation of PjΔ6D protein. Smearing in the low molecular weight region (between 31 and 14 kDa) is attributed to degradation products of PjΔ6D protein.

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 PjΔ6D Protein Digestibility in SGF Followed by SIF by Western Blot Analysis

The digestibility of PjΔ6D 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 PjΔ6D was observed only at the 0 min time point in the SGF phase (Figure 3B, lane 2). No proteolytic fragments were observed in any other time point

In addition to the full-length PjΔ6D protein, minor smearing in the high molecular weight due to aggregated PjΔ6D was observed in the control SEQ 0min (Figure 3B, lane 2). Minor smearing below the PjΔ6D full-length band and minor bands at ~17 kDa were also observed, which constitutes cleavage products of PjΔ6D protein.

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 PjΔ6D Protein in SIF

12.6.1 Assessment of PjΔ6D digestibility in SIF by Western Blot Analysis

The digestibility of the full-length PjΔ6D protein in SIF was evaluated by western blot (Figure 3). The western blot used to assess the *in vitro* digestibility of the PjΔ6D protein in SIF (Figure 3, panel A) was run concurrently with the western

blot to determine the LOD (Figure 3, panel B) of the PjΔ6D protein. The LOD was visually estimated to be 2.5 ng. The LOD estimated for the PjΔ6D protein was used to calculate the maximum amount of PjΔ6D protein that could remain visually undetected after digestion, which corresponded to 16.6% of the total protein loaded:

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

$$\frac{2.5\ ng \times 100\%}{15\ ng} = 16.6\%$$

The gel used to assess *in vitro* digestibility of the PjΔ6D protein by western blot was loaded with 15 ng total protein (based on pre-digestion concentrations) for each of the digestion specimens. Western blot analysis demonstrated that a band corresponding to the full-length PjΔ6D protein was digested below the LOD within 5 min of incubation in SIF (Figure 3A, lane 5). Therefore, based on the LOD, more than 84% (100% - 16.6% = 84.3%) of the full-length PjΔ6D protein was digested within 5 min. No proteolytic fragments of the PjΔ6D protein were detected in any digestion specimens. These data suggest that the full-length PjΔ6D protein degrades rapidly when exposed to pancreatin at neutral pH.

No change in the full-length PjΔ6D protein band intensity was observed in the absence of pancreatin in the experimental control specimens SIF P0 and SIF P9 (Figure 3A, lanes 3 and 13). This indicates that the PjΔ6D protein was stable in the test system without pancreatin at ~37 ºC over the course of the experiment. It should be noted that the higher order aggregation of the PjΔ6D protein appears more intense in the SIF P8 control (Figure 3A, lane 13). This is because heating of the some membrane proteins for a long duration (i.e. 24 h for SIF P8) leads to further aggregation of membrane proteins like PjΔ6D.

In addition to the full-length PjΔ6D protein, minor smearing in the high molecular weight region was observed in the control specimens SIF P0, SIF T0 and SIF P7 (Figure 3A, lanes 3, 4, and 13). Minor smearing in the high weight region on immunoblots has been attributed to aggregated PjΔ6D protein (Finnessy et al., 2008). Minor smearing below the PjΔ6D full-length band and minor bands at ~22 kDa, ~17 kDa, and ~12 kDa were also observed, which constitutes cleavage products of PjΔ6D protein.

No immunoreactive bands were observed in specimens SIF N0 and SIF N9 that represent SIF test system experimental controls (Figure 4A, lanes 2 and 15).

13.0 Conclusions

The results of the study demonstrated that greater than 99% of the full-length PjΔ6D protein was digested within 30 s of incubation in SGF when analyzed using stained gel, and greater than 96% was digested when analyzed using western blot with an antibody raised against full-length PjΔ6Dprotein, respectively. A fragment of ~10 kDa was observed by western blot in the 30 s digestion time point and was identified as a product of proteolytic digestion of the PjΔ6D protein. This fragment was digested within 2 min of incubation in SGF. On stained gel, fragments with molecular weights of ~4 kDa and ~ 5 kDa were observed in SGF for 10 min and 60 min, respectively. Fragment with molecular weight of ~ 3.5 kDa was observed from 10-60 min and, most probably, represents a product of further degradation of the ~4 kDa fragment. These fragments were not observed on western blot X-ray films and, therefore, they were N-terminally sequenced in an attempt to establish their identities. The sequences obtained for the ~ 4 kDa and ~ 3.5 kDa did not match the predicted sequence of the PjΔ6D protein and their identities could not be established. Most likely these fragments originate from one of the endogenous soybean proteins co-purified with the PjΔ6D protein. The N-terminal sequencing of the ~5 kDa fragment did not yield enough amino acid residues to allow a definitive sequence comparison to the PjΔ6D predicted sequence or to a protein database and, therefore, the origin of the fragment could not be established.

To better understand the fate of the PjΔ6D protein during gastrointestinal digestion, it was exposed to digestion with pancreatin in SIF following the digestion in SGF. After digestion of PjΔ6D protein in SGF for 2 min, the reaction was quenched and the mixture was exposed to further digestion in SIF. The digestibility of the PjΔ6D protein in SGF followed by the digestion in SIF was evaluated by visual analysis of Colloidal Brilliant Blue G stained polyacrylamide gel or by visual analysis of western blot X-ray films. Visual examination of the stained gel demonstrated that transiently stable fragments with molecular weight of ~5 kDa and ~4 kDa observed after 2 min of digestion in SGF were rapidly digestible (<5 min) in SIF. Because the ~3.5 kDa fragment most probably represents a product of further degradation of ~4 kDa fragment and, appears later (10-60 min) during SGF digestion, this fragment was not observed in the SGF 2min time point during sequential digestion. Visual examination of the X-ray films showed that no immune-reactive fragments were observed at any time points in SIF. The data clearly indicate that the transiently stable fragments observed in SGF rapidly degrade upon short exposure to SIF.

Results of this study also demonstrated that greater than 84% of the full-length PjΔ6D protein was digested within 5 min of incubation in SIF with no proteolytic fragment(s) detected by western blot using PjΔ6D–specific antibody.

The results of this study show that the integral membrane protein, PjΔ6D, is readily digestible in SGF and SIF. Rapid digestion of the full-length PjΔ6D protein in SGF and the subsequent degradation of any fragments resulting from SGF digestion by SIF

indicates that it is highly unlikely that the PjΔ6D protein will pose any safety concern to human health.

14.0 References

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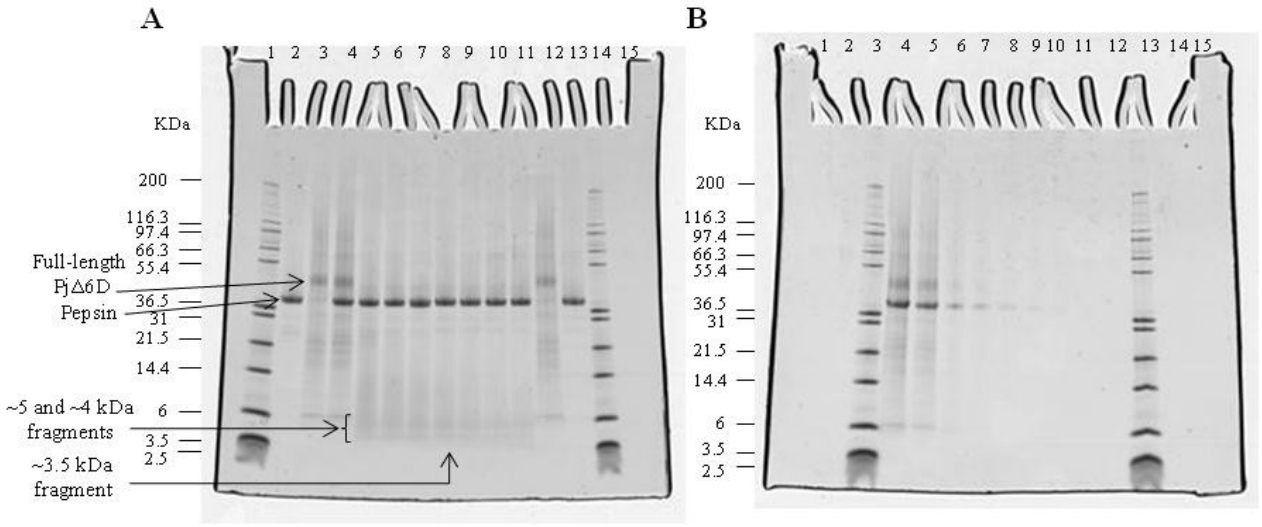

Figure 1 SDS-PAGE Analysis of the Digestion of the PjΔ6D protein in SGF

Colloidal Brilliant Blue G stained SDS-PAGE were used to analyze the digestibility of the PjΔ6D protein in SGF. **Panel A** corresponds to the PjΔ6D protein digestion in SGF. Based on pre-digestion protein concentrations, 0.8 µg of total protein was loaded in each lane containing the PjΔ6D protein. **Panel B** corresponds to the limit of detection of the PjΔ6D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. In both gels, the PjΔ6D 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	0.5
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	-

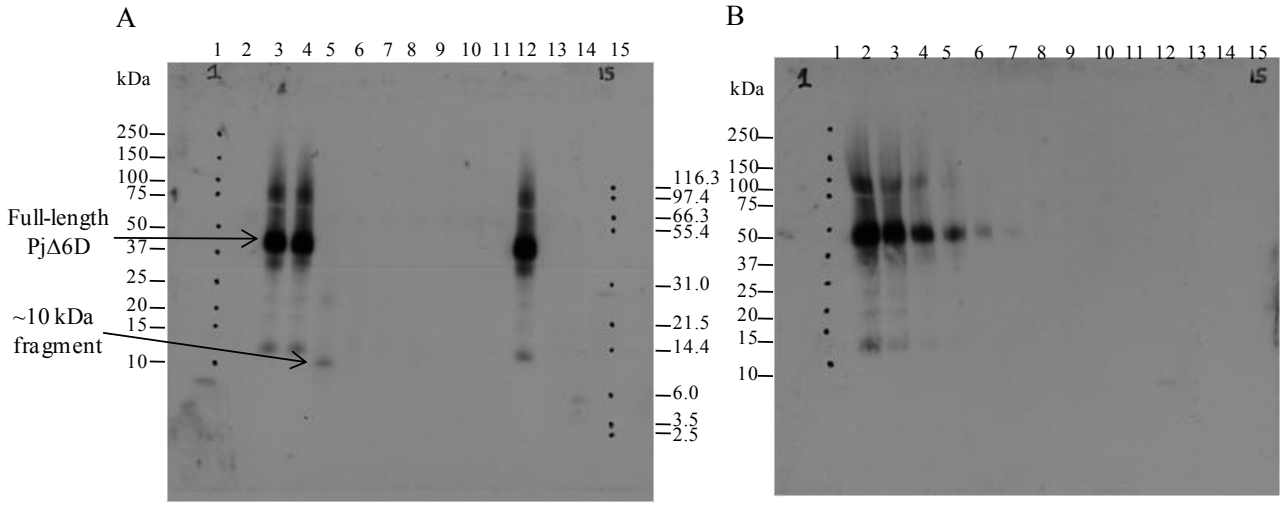

Figure 2 Western Blot Analysis of the Digestion of the PjΔ6D protein in SGF

Panel A corresponds to the PjΔ6D protein digestion in SGF. Based on pre-digestion protein concentrations, 15 ng (total protein) was loaded in the lanes containing the PjΔ6D protein. **Panel B** corresponds to the limit of detection of the PjΔ6D protein. Approximate molecular weights (kDa) are shown on the left and right of A and only on the left in B corresponding to the markers loaded in each gel. In both gels, the PjΔ6D 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	Precision Plus MWM	-
3	SGF P0	0	3	T0, protein+SGF	15
4	SGF T0	0	4	T0, protein+SGF	10
5	SGF T1	0.5	5	T0, protein+SGF	5
6	SGF T2	2	6	T0, protein+SGF	2.5
7	SGF T3	5	7	T0, protein+SGF	1
8	SGF T4	10	8	T0, protein+SGF	0.5
9	SGF T5	20	9	T0, protein+SGF	0.2
10	SGF T6	30	10	T0, protein+SGF	0.1
11	SGF T7	60	11	T0, protein+SGF	0.05
12	SGF P7	60	12	Mark 12 MWM	0.025
13	SGF N7	60	13	Blank	-
14	Precision Plus MWM	-	14	Blank	-
15	Mark 12 MWM	-	15		-

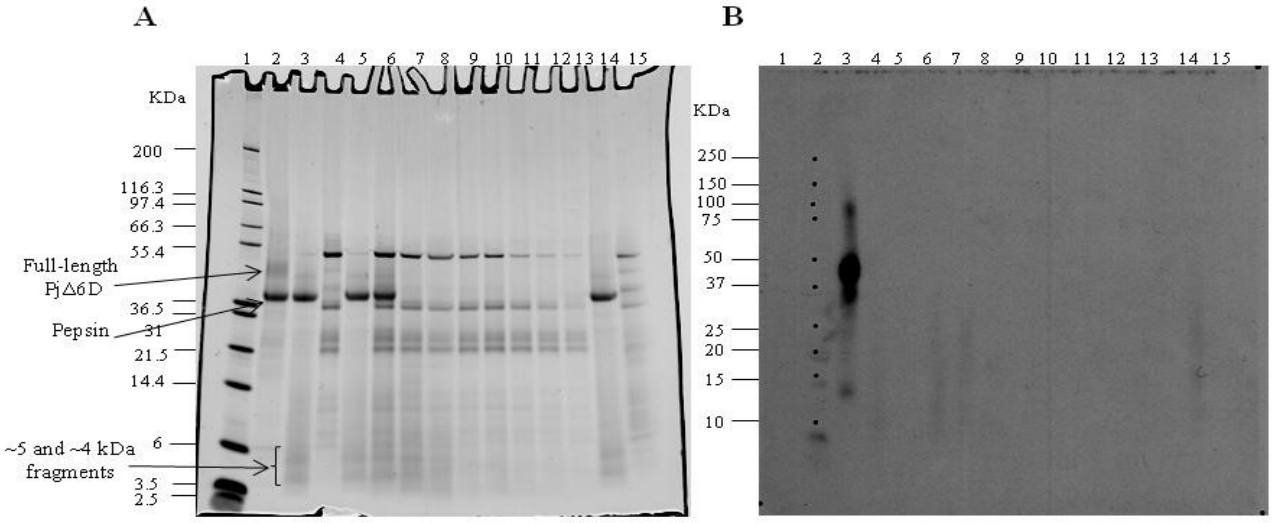

Figure 3 SDS-PAGE and Western Blot Analysis of the Digestion of the PjΔ6D protein in SGF followed by SIF.

Panel A corresponds to the colloidal stained SDS-PAGE of the PjΔ6D protein digestion in SGF followed by SIF. Based on pre-digestion protein concentrations, 0.9 µg of total protein was loaded per lane containing PjΔ6D protein. **Panel B** corresponds to the western blot of the PjΔ6D protein digestion in SGF followed by SIF. Based on pre-digestion protein concentrations, 15 ng of total protein was loaded per lane containing the PjΔ6D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel. In both gels, the PjΔ6D protein migrated to approximately 46 kDa. A 5 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

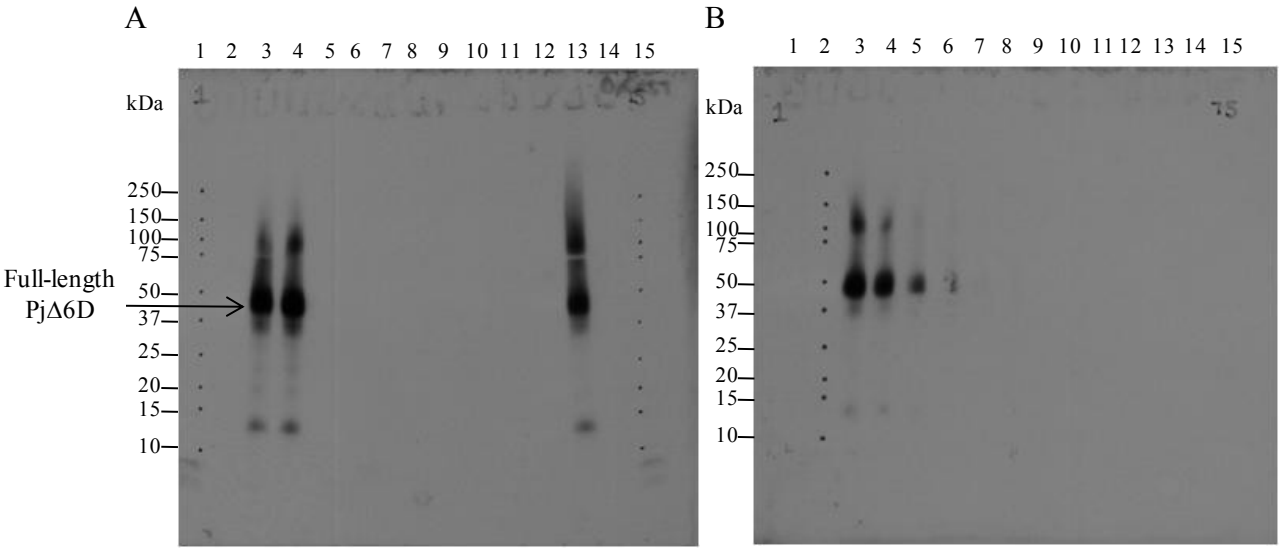

Figure 4 Western Blot Analysis of the Digestion of the PjΔ6D protein in SIF

Panel A corresponds to the PjΔ6D protein digestion in SIF. Based on pre-digestion protein concentrations, 15 ng (total protein) was loaded in the lanes containing the PjΔ6D protein. **Panel B** corresponds to the limit of detection of the PjΔ6D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. In both gels, the PjΔ6D 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	Precision Plus MWM	-
3	SIF P0	0	3	T0, protein+SIF	15
4	SIF T0	0	4	T0, protein+SIF	10
5	SIF T1	5 min	5	T0, protein+SIF	5
6	SIF T2	15 min	6	T0, protein+SIF	2.5
7	SIF T3	30 min	7	T0, protein+SIF	1
8	SIF T4	1 h	8	T0, protein+SIF	0.5
9	SIF T5	2 h	9	T0, protein+SIF	0.2
10	SIF T6	4 h	10	T0, protein+SIF	0.1
11	SIF T7	8 h	11	T0, protein+SIF	0.05
12	SIF T8	24 h	12	T0, protein+SIF	0.025
13	SIF P8	24 h	13	Precision Plus MWM	-
14	SIF N8	24 h	14	Blank	-
15	Precision Plus MWM	-	15	Blank	-

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	Electrotransfer 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