

SUMMARY

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STUDY TITLE

In Vitro Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-12 (AAD-12) in
Soybean Leaf Extracts

DATA REQUIREMENTS

Not Applicable

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STUDY COMPLETED ON

03-Feb-2011

PERFORMING LABORATORY

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LABORATORY STUDY ID

110265

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The AAD-12 protein contained in crude soybean leaf extracts (in both transgenic tissue and non-transgenic tissue fortified with microbe-derived AAD-12) was readily digested by pepsin (not detectable at 30 seconds) under simulated gastric conditions (pH 1.2, 37 °C) as demonstrated by western blot analysis.

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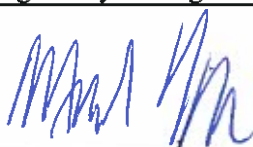
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(AAD-12) in Soybean Leaf Extracts


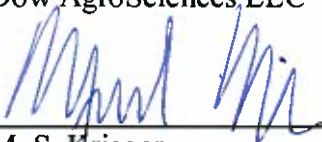
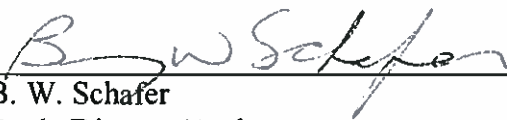
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This study does not meet requirements of 40 CFR Part 160.

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QUALITY ASSURANCE STATEMENT

Compound: AAD-12

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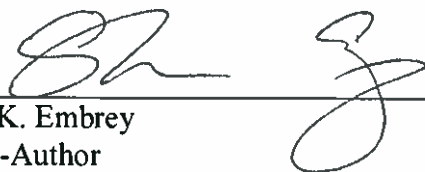
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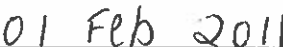
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In Vitro Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-12 (AAD-12) in Soybean Leaf Extracts

ABSTRACT

The purpose of this study was to evaluate the stability of the AAD-12 protein in transgenic soybean leaf extracts (event DAS-68416-4) upon exposure to simulated gastric fluid (SGF). Digestion samples of the transgenic soybean leaf extracts were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot.

The AAD-12 protein contained in crude soybean leaf extracts (in both transgenic tissue and non-transgenic tissue fortified with microbe-derived AAD-12) was readily digested by pepsin (not detectable at 30 seconds) under simulated gastric conditions (pH 1.2, 37 °C) as demonstrated by western blot analysis.

ABBREVIATIONS

AAD-12	Aryloxyalkanoate Dioxygenase-12
AI	active ingredient
β -lac	β -lactoglobulin A
BSA	bovine serum albumin
DAS	Dow AgroSciences LLC
GLP	Good Laboratory Practice
HRP	horseradish peroxidase
kDa	kilodalton
M	Molar
μ g	microgram
μ L	microliter
mL	milliliter
mM	millimolar
min	minute
MW	molecular weight
N/A	Not applicable
ND	Not detectable
ng	nanogram
PBST	phosphate buffered saline with Tween 20, pH 7.4
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SGF	Simulated Gastric Fluid
TSN	test substance number
w/	with
V	volt

INTRODUCTION

Along with many other tests that are conducted during the safety assessment of transgenic crops, the digestibility of proteins in simulated gastric fluid (SGF) is typically examined. Standard SGF contains 0.32% pepsin at pH 1.2 (1). Digestion of a protein in SGF is an enzyme-catalyzed hydrolysis of the protein under acidic conditions. It is generally believed that the rate of pepsinolysis in SGF correlates with digestibility in the human gastric system. The test substance used in this study was the AAD-12 (32 kDa) protein.

The purpose of this study was to evaluate the stability of the AAD-12 protein in transgenic soybean leaf extracts (event DAS-68416-4) upon exposure to simulated gastric fluid (SGF). The test and control substances were incubated with SGF for specific time intervals and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The AAD-12 protein contained or spiked into soybean extracts was also analyzed by western blot. Bovine serum albumin (BSA) was used as a positive control for the experiment since it is known to degrade readily in SGF, and β -lactoglobulin A (β -lac) was used as a negative control since it is known to persist in SGF.

The biochemical and immunological methods employed in this study are among those that are well established for protein analysis. SDS-PAGE separates proteins based on the apparent molecular weight (mass). Western blot and ELISA assays are widely used to identify or quantify specific proteins in a heterogeneous preparation. The SGF assay is routinely used in the safety assessment of novel recombinant proteins in regulatory studies (2).

EXPERIMENTAL

Test Substances

1) Lyophilized transgenic soybean leaf tissue of event DAS-68416-4. The leaf sample was collected at the V10 – V12 growth stage from field grown plants (DAS study number 101103). The tissues were harvested, frozen, lyophilized, and stored at -80 °C until use.

Test substance preparation: Immediately prior to exposure to SGF, 80.0 mg of the lyophilized transgenic tissue was resuspended in 1 mL of PBST and homogenized in a bead-mill (Geno-Grinder) for 3 minutes at 1500 strokes/min. The insoluble particulates were removed by centrifugation at ~20,000×g for 5 minutes and the supernatants were decanted and held on wet ice until exposure to SGF.

Control Substances

1) The commercially available positive and negative control substances used in this study are listed in the following table:

Control Substance	Purity	Reference	ID Number	Storage
Bovine serum albumin (BSA)	99%	Sigma catalog #A7638	Lot 029k7405	2 - 8 °C
β-lactoglobulin A (β-lac)	98%	Sigma catalog #L7880	Lot 097k7010	2 - 8 °C

2) Lyophilized non-transgenic leaf tissue (Maverick) fortified with microbe-derived AAD-12. The leaf sample was collected at the V10 – V12 growth stage from field grown plants (DAS study number 101103). The tissues were harvested, frozen, lyophilized, and stored at -80 °C until use. The tissue was fortified with recombinant AAD-12 protein (Lot Number: 466-028A) that was produced in *P. fluorescens* and purified to homogeneity by the DAS Supply R & D

group in Indianapolis, IN. The purified protein preparation was designated TSN030732-0002. The concentration of AAD-12 in the lyophilized preparation was determined to be 35.3% (3).

Control substance preparation: Immediately prior to exposure to SGF, 80.5 mg of the non-transgenic Maverick tissue was resuspended in 1 mL of PBST and 9.0 µg of microbe-derived AAD-12 was added to the vial. The tissue and recombinant protein were homogenized in a bead-mill (Geno-Grinder) for 3 minutes at 1500 strokes/min. The insoluble particulates were removed by centrifugation at ~20,000×g for 5 minutes and the supernatants were decanted and held on wet ice until exposure to SGF.

Reference Substances

1. The commercially available reference substances used are listed in the following table:

Reference Substance	Product Name	Lot Number	Assay	Reference
Molecular Weight Markers	Novex Sharp Unstained Standard	799764	SDS-PAGE	Invitrogen Cat #: LC5677, Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10, and 3.5 kDa
Prestained Molecular Weight Markers	Novex Sharp Protein Standard	764840	SDS PAGE/ Western Blot	Invitrogen Cat #: LC5800, Approximate Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10 and 3.5 kDa

Note: Reference substances were chosen as appropriate for the procedure used.

Test Methods

Equimolar (~0.074 mM) solutions of the control substances were prepared as follows: BSA was solubilized by weighing 24.7 mg of powder in a 15-mL tube and adding 5 mL of PBST. β-lac was solubilized by weighing 6.9 mg of powder in a 15-mL centrifuge tube and adding 5 mL of PBST. The varying amounts of the control substances reflect differences in purity and molecular weight. Simulated gastric fluid (SGF, pH ~1.2) containing a final concentration of

approximately 0.32% (w/v) pepsin (Sigma Aldrich, St. Louis catalog #P6887, 86% w/w pure, 4,220 units of activity/mg protein) was prepared by weighing out 0.195 g of Pepsin into 50 mL of 34 mM NaCl as recommended by the United States Pharmacopeia (1).

The digestions for the soybean extracts were performed for time intervals of approximately 30 seconds, 1, 2, 4, 8, and 16 minutes in a water bath set to 37 °C. The four samples; non-transgenic (Maverick) leaf extracts fortified with AAD-12, transgenic leaf extracts (event DAS-68416-4), BSA, and β -lac were digested as follows. Four 2.85-mL aliquots of SGF were placed in a 37 °C water bath. After 5 minutes, 150 μ L of the solutions of non-transgenic leaf extracts fortified with AAD-12, transgenic leaf extracts (event DAS-68416-4), BSA, and β -lac were each added to a separate vial of the SGF and a timer was set. After each specified incubation interval, 100 μ L of the reaction mixture was removed and added to tubes containing stop solution (40 μ L of 200 mM sodium carbonate, pH ~11.0). The stopped reactions were then placed on ice until all of the time points were sampled for the four test materials. An SGF control was prepared by substituting PBST for the sample protein/extract and incubating for the duration of the experiment at 37 °C. The SGF control was prepared as follows: A 2.85-mL aliquot of SGF was heated in a 37 °C water bath for 5 minutes, 150 μ L of PBST was added and a timer was set. A 100 μ L aliquot was immediately removed as the zero time point and placed into a tube containing the stop reaction (40 μ L of 200 mM sodium carbonate, pH ~11.0). When all digestion reactions were complete, one final aliquot was taken at the end of the experiment. For each of the proteins/extracts above, a zero time point (neutralized control) was prepared as follows: First, a 2.85-mL aliquot of SGF was heated in a 37 °C water bath for 5 minutes, stopped with 1.2 mL 200 mM sodium carbonate, then 150 μ L of the respective protein/extract was added to the solution.

Aliquots of the neutralized and digested samples were mixed with equal volumes of Laemmli sample buffer (Bio-Rad Laboratories, catalog #161-0737), containing 5% freshly added 2-mercaptoethanol (β ME) (Bio-Rad, catalog #161-0710) and heated for 5 minutes at ~95 °C. Aliquots of all samples and neutralized controls were mixed with equal volumes of the Laemmli sample buffer preparation and heated.

Single polyacrylamide gels (Bio-Rad, catalog #345-0123) of BSA, β -lac, and duplicate gels of the AAD-12 fortified soybean extract and the transgenic soybean extract were prepared. For each AAD-12 containing sample, a 10-fold dilution of the neutralized soybean extract was prepared from the neutralized SGF and all samples were loaded as described in the following table:

Protein	Volume of sample loaded per lane for SDS-PAGE analysis	Amount of protein loaded per lane for SDS-PAGE analysis	Volume of sample loaded per lane for Western blot analysis	Amount of protein loaded per lane for Western blot analysis
BSA	20 μ L	~1.7 μ g	N/A	N/A
β -lactoglobulin A (β -lac)	20 μ L	~0.48 μ g	N/A	N/A
Maverick soybean leaf extract fortified with AAD-12	20 μ L	4.3 ng	40 μ L	8.5 ng
10x dilution of Maverick soybean leaf extract fortified with AAD-12	20 μ L	0.43 ng	40 μ L	0.85 ng
Transgenic soybean leaf extract (DAS-68416-4)	20 μ L	N/A	40 μ L	N/A
10x dilution of transgenic soybean leaf extract (DAS-68416-4)	20 μ L	N/A	40 μ L	N/A

The samples were then electrophoresed at a constant voltage of 180 volts per gel for ~65 minutes using XT MES buffer (Bio-Rad, catalog #161-0789). After separation, four of the gels were stained with GelCode Blue stain (Pierce Chemical, catalog #24592). Proteins on the duplicate AAD-12 containing gels were electro-blotted to a nitrocellulose membrane (Bio-Rad, catalog #162-0233) using a Bio-Rad Criterion Blotter at a constant voltage of 100 V for 1 hour. Tris/Glycine buffer (Bio-Rad, catalog #161-0771) containing 20% methanol was used in the transfer. Following protein transfer, the membrane was blocked with phosphate buffered saline containing 0.05% Tween 20 (PBST) and 5% powdered milk. For immunodetection, the membrane was probed with an AAD-12 specific rabbit polyclonal antibody (Protein A purified: Lot #: DAS F1197-167-2, 1.0 mg/mL). A conjugate of goat anti-rabbit IgG (H+L) and

horseradish peroxidase was used as the secondary antibody (Pierce, catalog #31460). Chemiluminescent detection solution (GE Healthcare, catalog #RPN2132) was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to autoradiography film (Thermo Scientific, catalog #34091) and was subsequently developed with a film developer (Radiation Services, model #All Pro 100).

STATISTICAL TREATMENT OF DATA

No statistical methods were used in this study.

RESULTS AND DISCUSSION

The positive and negative controls, BSA and β -lac, respectively, responded as expected (Table 1). β -lac remained readily detectable for 16 minutes (the duration of the experiment) (Figure 1, lane 9). BSA was not detected at the 30-second time point when subjected to the simulated gastric environment (Figure 2, lane 4). The AAD-12 protein in the transgenic soybean extracts (event DAS-68416-4) and non-transgenic soybean extracts fortified with microbe-derived AAD-12, was not detectable at the 30-second time point as demonstrated by western blot analysis (Figures 5 and 6, lane 5). Additionally, the western blot (Figures 5 and 6, lane 4) demonstrated that a 10-fold dilution of the initial AAD-12 protein was readily detectable and that no intact AAD-12 or degradation fragments were detected at or beyond the 30-second time point.

CONCLUSION

The AAD-12 protein contained in crude soybean leaf extracts (in both transgenic tissue and non-transgenic tissue fortified with microbe-derived AAD-12) was readily digested by pepsin (not detectable at 30 seconds) under simulated gastric conditions (pH 1.2, 37 °C) as demonstrated by immunospecific western blot analysis.

ARCHIVING

The raw data and the original version of the final report will all be filed in the Dow AgroSciences LLC archives at 9330 Zionsville Road in Indianapolis, IN 46268-1054.

REFERENCES

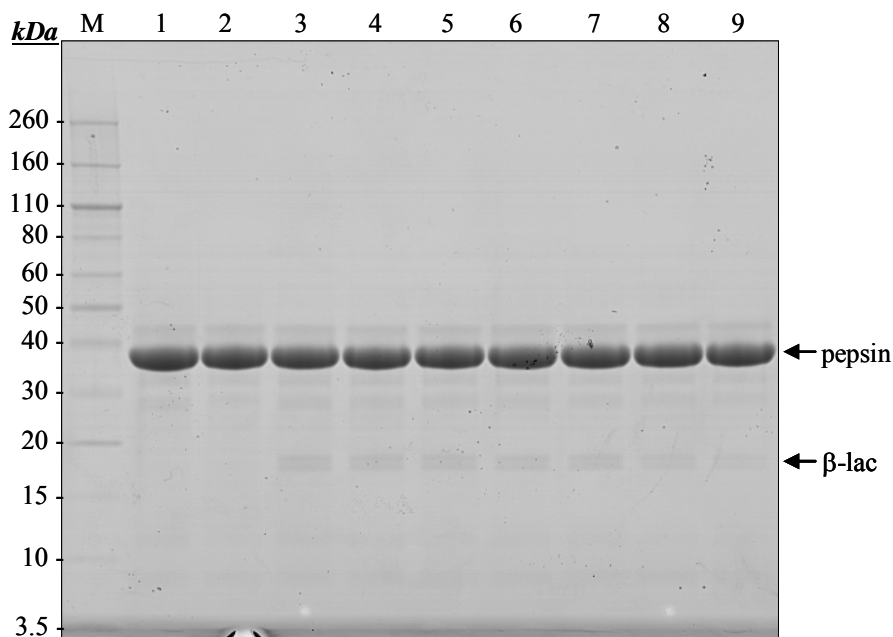
1. Board of Trustees (ed.) 1995. Simulated Gastric Fluid, TS, pp. 2053 in *The United States Pharmacopeia 23, The National Formulary 18*. United States Pharmacopeia Convention, Inc., Rockville, MD
2. Ladics, G. S., Current codex guidelines for assessment of potential protein allergenicity. *Food and Chemical Toxicology* 2008, 46, (10, Supplement 1), S20-S23.
3. Embrey, S. K. and Schafer, B. W. 2009. Certificate of Analysis for Test/Reference/Control/Substances Aryloxyalkanoate Dioxygenase (AAD-12) – TSN030732. Report Number: BIOT09-203009 (Unpublished report of Dow AgroSciences).

Table 1. Results of the *In Vitro* Digestibility Study of the AAD-12 protein in soybean leaf extracts incubated in Simulated Gastric Fluid (SGF)

Protein	Detection by SDS-PAGE	Detection by Western Blot Analysis
BSA	< 30 Seconds	^a N/A
β- lactoglobulin A	> 16 Minutes	^a N/A
Non-transgenic soy leaf extract (fortified w/ ADD-12)	^b ND	< 30 Seconds
Transgenic AAD-12 soy extract	^b ND	< 30 Seconds

^a Not applicable

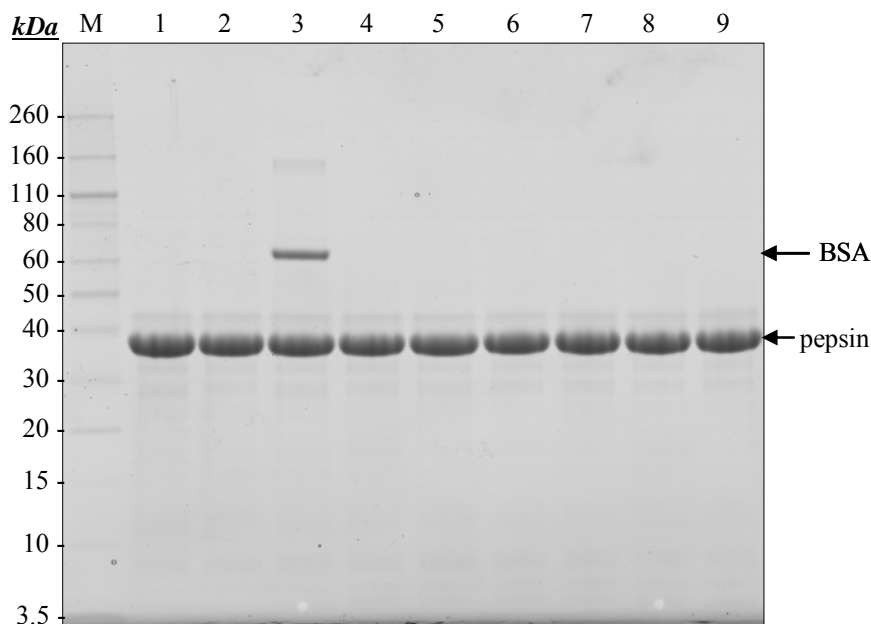
^b Not detectable



The neutralized and digested β -lac samples were mixed with equal volumes of Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at $\sim 95^{\circ}\text{C}$. The samples were loaded onto a Bio-Rad Criterion gel and electrophoresed at a constant voltage of 180 V per gel for ~ 65 minutes using MES XT buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Pierce Chemical.

Lane	Sample	Amount Loaded
M	Novex Sharp Unstained Standard	10 μL
1	SGF Reagent Blank, 0 minute incubation	40 μL
2	SGF Reagent Blank, >16 minute incubation	40 μL
3	Neutralized β -lac	~ 0.48 μg
4	30-second β -lac digestion	~ 0.48 μg
5	1-minute β -lac digestion	~ 0.48 μg
6	2-minute β -lac digestion	~ 0.48 μg
7	4-minute β -lac digestion	~ 0.48 μg
8	8-minute β -lac digestion	~ 0.48 μg
9	16-minute β -lac digestion	~ 0.48 μg

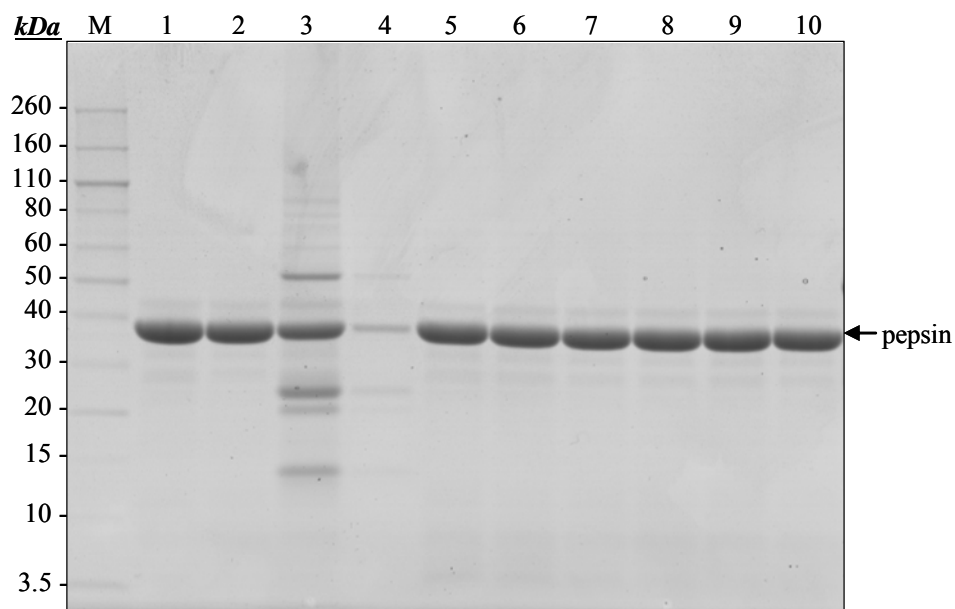
Figure 1. SDS-PAGE analysis of β -lactoglobulin A (M.W. ~ 18 kDa) protein subjected to digestion in simulated gastric fluid.



The neutralized and digested BSA samples were mixed with equal volumes of Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at ~95 °C. The samples were loaded onto a Bio-Rad Criterion gel and electrophoresed at a constant voltage of 180 V per gel for ~65 minutes using MES XT buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Pierce Chemical.

Lane	Sample	Amount Loaded
M	Novex Sharp Unstained Standard	10 μ L
1	SGF Reagent Blank, 0 minute incubation	40 μ L
2	SGF Reagent Blank, >16 minute incubation	40 μ L
3	Neutralized BSA	~1.7 μ g
4	30-second BSA digestion	~1.7 μ g
5	1-minute BSA digestion	~1.7 μ g
6	2-minute BSA digestion	~1.7 μ g
7	4-minute BSA digestion	~1.7 μ g
8	8-minute BSA digestion	~1.7 μ g
9	16-minute BSA digestion	~1.7 μ g

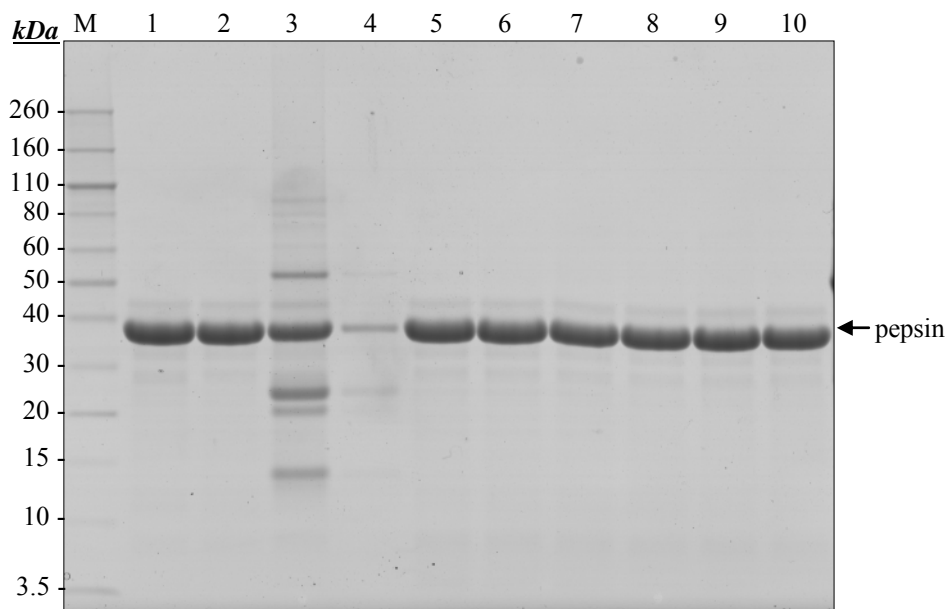
Figure 2. SDS-PAGE analysis of BSA (M.W. ~66 kDa) protein subjected to digestion in simulated gastric fluid.



The neutralized and digested transgenic soybean leaf extract (Event DAS-68416-4) samples were mixed with equal volumes of Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at ~95 °C. The samples were loaded onto a Bio-Rad Criterion gel and electrophoresed at a constant voltage of 180 V per gel for ~65 minutes using XT MES buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Pierce Chemical.

Lane	Sample	Amount Loaded
M	Novex Sharp Unstained Standard	10µL
1	SGF Reagent Blank, 0 minute incubation	20 µL
2	SGF Reagent Blank, >16 minute incubation	20 µL
3	Neutralized transgenic soybean leaf extract (Event DAS-68416-4)	20 µL
4	10x dilution of neutralized transgenic soybean leaf extract (Event DAS-68416-4)	20 µL
5	30-sec transgenic soybean leaf extract (Event DAS-68416-4) digestion	20 µL
6	1-min transgenic soybean leaf extract (Event DAS-68416-4) digestion	20 µL
7	2-min transgenic soybean leaf extract (Event DAS-68416-4) digestion	20 µL
8	4-min transgenic soybean leaf extract (Event DAS-68416-4) digestion	20 µL
9	8-min transgenic soybean leaf extract (Event DAS-68416-4) digestion	20 µL
10	16-min transgenic soybean leaf extract (Event DAS-68416-4) digestion	20 µL

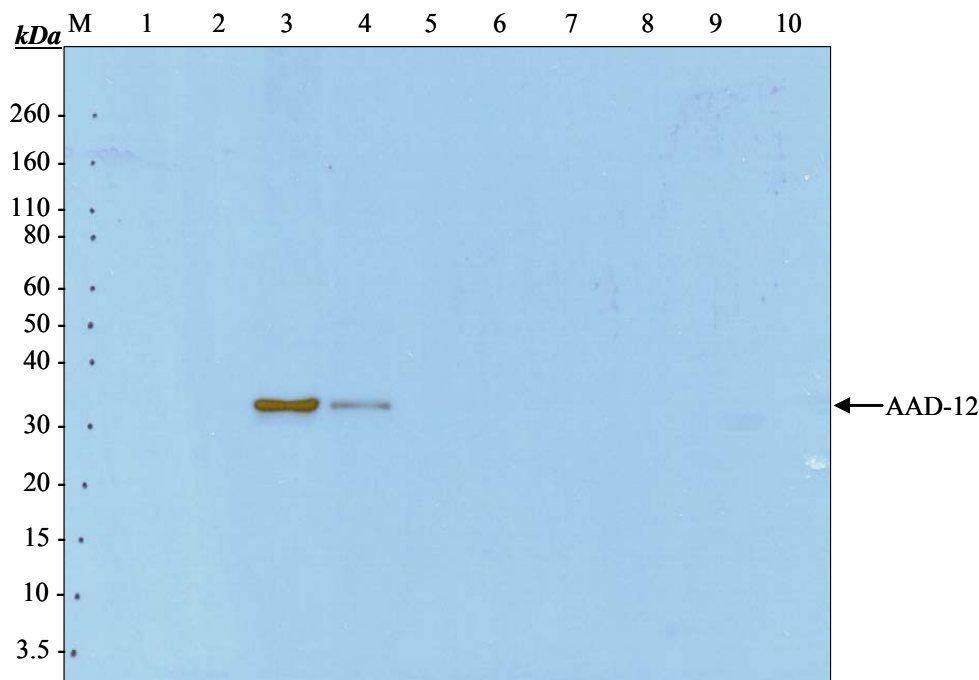
Figure 3. SDS-PAGE analysis of transgenic soybean leaf extract (Event DAS-68416-4) subjected to digestion in simulated gastric fluid.



The neutralized and digested Maverick soybean leaf extract (fortified with microbe-derived AAD-12) samples were mixed with equal volumes of Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at ~95 °C. The samples were loaded onto a Bio-Rad Criterion gel and electrophoresed at a constant voltage of 180 V per gel for ~65 minutes using XT MES buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Pierce Chemical. **Note:** Twenty-microliters of soybean extract were loaded per lane which contained 4.3 ng of microbe-derived AAD-12 protein, which is below the limit of detection of the GelCode Blue Coomassie stain

Lane	Sample	Amount Loaded
M	Novex Sharp Unstained Standard	10µL
1	SGF Reagent Blank, 0 minute incubation	20 µL
2	SGF Reagent Blank, >16 minute incubation	20 µL
3	Neutralized Maverick leaf extract fortified w/ AAD-12	4.3 ng
4	10x dilution of neutralized Maverick leaf extract fortified w/ AAD-12	0.43 ng
5	30-second Maverick leaf extract fortified w/ AAD-12 digestion	4.3 ng
6	1-minute Maverick leaf extract fortified w/ AAD-12 digestion	4.3 ng
7	2-minute Maverick leaf extract fortified w/ AAD-12 digestion	4.3 ng
8	4-minute Maverick leaf extract fortified w/ AAD-12 digestion	4.3 ng
9	8-minute Maverick leaf extract fortified w/ AAD-12 digestion	4.3 ng
10	16-minute Maverick leaf extract fortified w/ AAD-12 digestion	4.3 ng

Figure 4. SDS-PAGE analysis of Maverick soybean leaf extract (fortified with microbe-derived AAD-12) subjected to digestion in simulated gastric fluid.



The neutralized and digested transgenic soybean leaf extract (Event DAS-68416-4) were mixed with equal volumes of Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at ~95 °C. The samples were loaded onto a Bio-Rad Criterion gel and electrophoresed at a constant voltage of 180 V for ~65 minutes using XT MES buffer from Bio-Rad. After separation, the gel was electro-blotted to a nitrocellulose membrane for 60 minutes under a constant charge of 100 volts. For immunodetection, the membrane was probed with an AAD-12 specific polyclonal rabbit antibody (Protein A purified: Lot #: DAS F1197-167-2). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase was used as the secondary antibody. GE Healthcare chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to film and subsequently developed with a film developer. The molecular weight markers were manually transferred to the film after development.

Lane	Sample	Amount Loaded
M	Novex Sharp Unstained Standard	10µL
1	SGF Reagent Blank, 0 minute incubation	40 µL
2	SGF Reagent Blank, >16 minute incubation	40 µL
3	Neutralized transgenic soybean leaf extracts (Event DAS-68416-4)	40 µL
4	10x dilution of neutralized transgenic soybean leaf extract (Event DAS-68416-4)	40 µL
5	30-sec transgenic soybean leaf extract (Event DAS-68416-4) digestion	40 µL
6	1-min transgenic soybean leaf extract (Event DAS-68416-4) digestion	40 µL
7	2-min transgenic soybean leaf extract (Event DAS-68416-4) digestion	40 µL
8	4-min transgenic soybean leaf extract (Event DAS-68416-4) digestion	40 µL
9	8-min transgenic soybean leaf extract (Event DAS-68416-4) digestion	40 µL
10	16-min transgenic soybean leaf extract (Event DAS-68416-4) digestion	40 µL

Figure 5. Western blot analysis of transgenic soybean leaf extract (Event DAS-68416-4) subjected to digestion in simulated gastric fluid.



The neutralized and digested Maverick soybean leaf extract (fortified with microbe-derived AAD-12) was mixed with equal volumes of Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at ~95 °C. The samples were loaded onto a Bio-Rad Criterion gel and electrophoresed at a constant voltage of 180 V for ~65 minutes using XT MES buffer from Bio-Rad. After separation, the gel was electro-blotted to a nitrocellulose membrane for 60 minutes under a constant charge of 100 volts. For immunodetection, the membrane was probed with an AAD-12 specific polyclonal rabbit antibody (Protein A purified: Lot #: DAS F1197-167-2). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase was used as the secondary antibody. GE Healthcare chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to film and subsequently developed with a film developer. The molecular weight markers were manually transferred to the film after development. **Note:** Forty-microliters of soybean extract were loaded per lane which contained 8.5 ng of microbe-derived AAD-12 protein.

Lane	Sample	Amount Loaded
M	Novex Sharp Unstained Standard	10 μ L
1	SGF Reagent Blank, 0 minute incubation	20 μ L
2	SGF Reagent Blank, >16 minute incubation	20 μ L
3	Neutralized Maverick leaf extract fortified w/ AAD-12	8.5 ng
4	10x dilution of neutralized Maverick leaf extract fortified w/ AAD-12	0.85 ng
5	30-sec Maverick leaf extract fortified w/ AAD-12 digestion	8.5 ng
6	1-min Maverick leaf extract fortified w/ AAD-12 digestion	8.5 ng
7	2-min Maverick leaf extract fortified w/ AAD-12 digestion	8.5 ng
8	4-min Maverick leaf extract fortified w/ AAD-12 digestion	8.5 ng
9	8-min Maverick leaf extract fortified w/ AAD-12 digestion	8.5 ng
10	16-min Maverick leaf extract fortified w/ AAD-12 digestion	8.5 ng

Figure 6. Western blot analysis of Maverick soybean leaf extract (fortified with microbe-derived AAD-12) subjected to digestion in simulated gastric fluid.