SUMMARY

(In accordance with 40 CFR part 152, this summary is available for public release after registration)

STUDY TITLE

In Vitro Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-1 (abbreviation AAD-1)

DATA REQUIREMENTS

N/A

AUTHOR(S)

S. K. Embrey, V. A. Korjagin

STUDY COMPLETED ON

9-Sept-2008

PERFORMING LABORATORY

Regulatory Sciences and Government Affairs—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

080062

In Vitro Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-1 (abbreviation AAD-1)

SUMMARY

Corn has been modified by the insertion of the *aad*-1 gene from *Sphingomonas herbicidivorans* which encodes the aryloxyalkanoate dioxygenase-1 (AAD-1) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and aryloxyphenoxypropionate (commonly referred to as "fop" herbicides such as quizalofop) herbicides, the latter of which may be used as a selectable marker during plant transformation and in breeding nurseries. The AAD-1 protein is approximately 33 kDa in size. The purpose of this study was to evaluate the stability of the AAD-1 protein in 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 digestions of the AAD-1 protein were 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 was used as a negative control since it is known to persist in SGF.

The AAD-1 protein is readily digested by pepsin (not detectable at 30 seconds) under simulated gastric conditions (pH 1.2, 37 °C) as demonstrated by both SDS-PAGE and western blot analyses.

STUDY TITLE

In Vitro Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-1 (abbreviation AAD-1)

DATA REQUIREMENTS

N/A

AUTHOR(S)

S. K. Embrey (317) 337-3674
[skembrey@dow.com]

STUDY COMPLETED ON

9-Sept-2008

PERFORMING LABORATORY

Regulatory Laboratories—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

080062

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: AAD-1

Title: *In Vitro* Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-1 (abbreviation AAD-1)

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

Company: Dow AgroSciences LLC

Company Agent: P. L. Hunst

Title: Regulatory Manager

Signature: Penny L. Hunst

Date: 9-Sept-2008

*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: *In Vitro* Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-1 (abbreviation AAD-1)

Study Initiation Date: 05/13/2008

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ENV/MC/CHEM(98)17, Paris January 26, 1998

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160, except the GLP status of commercial control and reference substances is not known and molecular weight markers were manually transferred from the nitrocellulose membrane after film development but membrane was not retained.

Penny K. Hunst — 9-Sept-2008

P. L. Hunst
Sponsor
Dow AgroSciences LLC
Date

Penny K. Hunst — 9-Sept-2008

P. L. Hunst
Submitter
Dow AgroSciences LLC
Date

9-Sept-2008

S.K. Embrey
Study Director/Author
Dow AgroSciences LLC
Study Completion Date

Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Compound: AAD-1 **Study ID:** 080062

Title: *In Vitro* Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-1 (abbreviation AAD-1)

Study Initiation Date: 13-May-2008 **Study Completion Date:** 9-Sep-2008

GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
13-May-2008	13-May-2008	Protocol Review
19, 20, 21-May-2008	28-May-2008	Preparation of Samples & Digestion with SGF and SDS-PAGE
13, 14, 18, 19-Aug-2008	19-Aug-2008	Report and Raw Data, Test Substance Container and Sample Verification

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.

Julie Schwake
Julie Schwake
Dow AgroSciences, Quality Assurance

9-Sep-08
Date

SIGNATURE PAGE

09 Sept 2008
Date

S. K. Embrey
Author
Dow AgroSciences LLC

09 SEPT 2008
Date

V. A. Kørjagin
Co- author
Dow AgroSciences LLC

09-Sept-2008
Date

R. A. Herman
Peer Reviewer
Dow AgroSciences LLC

09-SEP-2008
Date

B. W. Schafer
Peer Reviewer
Dow AgroSciences LLC

09-Sept 2008
Date

G. Shan
Science Leader
Dow AgroSciences LLC

09 Sept 2008
Date

K.A. Clayton
Global Leader Biotechnology
Dow AgroSciences LLC

STUDY PERSONNEL

Title: *In Vitro* Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-1 (abbreviation AAD-1)

Principal Analyst: (Principle Investigator) V. A. Korjagin

Analysts: S. K. Embrey, Dow AgroSciences LLC

V. A. Korjagin, Dow AgroSciences LLC

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In Vitro Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-1 (abbreviation AAD-1)

ABSTRACT

Corn has been modified by the insertion of the *aad*-1 gene from *Sphingomonas herbicidivorans* which encodes the aryloxyalkanoate dioxygenase-1 (AAD-1) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and aryloxyphenoxypropionate (commonly referred to as "fop" herbicides such as quizalofop) herbicides, the latter of which may be used as a selectable marker during plant transformation and in breeding nurseries. The AAD-1 protein is approximately 33 kDa in size. The purpose of this study was to evaluate the stability of the AAD-1 protein in 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 digestions of the AAD-1 protein were 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 was used as a negative control since it is known to persist in SGF.

AAD-1 protein is readily digested by pepsin (not detectable at 30 seconds) under simulated gastric conditions (pH 1.2, 37 °C) as demonstrated by both SDS-PAGE and western blot analyses.

ABBREVIATIONS

AAD-1	Aryloxyalkanoate Dioxygenase-1
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
µg	mirogram
µL	microliter
mL	milliliter
mM	millimolar
min	minute
MW	molecular weight
N/A	Not Applicable
ng	nanogram
OD	optical density
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
V	volt

INTRODUCTION

Corn has been modified by the insertion of the *aad*-1 gene from *Sphingomonas herbicidivorans* which encodes the aryloxyalkanoate dioxygenase-1 (AAD-1) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and aryloxyphenoxypropionate (commonly referred to as "fop" herbicides such as quizalofop) herbicides, the latter of which may be used as a selectable marker during plant transformation and in breeding nurseries. The AAD-1 protein is approximately 33 kDa in size. Along with many other tests that are conducted during the safety assessment of transgenic crops, the digestibility of the protein 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 rates of the pepsinolysis in SGF correlate with the digestibility of proteins in a human gastric system.

The purpose of this study was to evaluate the digestion of the AAD-1 protein in 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-1 protein 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 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 blotting of proteins to a nitrocellulose membrane, following SDS-PAGE and immunodetection with a protein specific antibody, is widely used to identify the authenticity of a molecule in a heterogeneous preparation.

EXPERIMENTAL

Test Substances

The recombinant AAD-1 protein (Lot Number: 480-15 (Batch 2)) was produced and purified from *P. fluorescens* by the DAS Supply R & D group in Indianapolis, IN. The protein preparation was sent to the Test Substance Coordinator at Dow AgroSciences also located in Indianapolis. The material was designated TSN105930. The purity was determined to be 36.1% (2).

Control Substances

The 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 075K7572	2-8 °C
β-lactoglobulin A	98%	Sigma catalog #L7880	Lot 037K7017	2-8 °C

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	Mark 12 Unstained Standard	399893	SDS-PAGE	Invitrogen Cat #: LC5677, Molecular Weight Markers of 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31.0, 21.5, 14.4, 6.0, 3.5* and 2.5* kDa
Prestained Molecular Weight Markers	Novex Sharp Protein Standard	419493	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.

*Molecular weight marker 3.5 and 2.5 kDa represent Insulin A and B chains which are unresolved when run on Tris-Glycine buffer systems.

Test Methods

Equimolar (~0.074 mM) solutions of the test and control substances were prepared as follows: 34.1 mg of AAD-1 (TSN 105930) was dissolved in a 15-mL tube by adding 5 mL phosphate buffered saline solution with Tween 20 (Sigma Aldrich, St. Louis, MO, catalog #P3563). BSA was solubilized by weighing 24.7 mg of powder in a 15-mL centrifuge tube and adding 5 mL of Milli-Q water. β-lactoglobulin A was solubilized by weighing 6.9 mg of powder in a 15-mL centrifuge tube and adding 5 mL of Milli-Q water. The varying amounts of the test and 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, 97% w/w pure, 3,370 units of activity/mg protein) was prepared as

recommended in the United States Pharmacopeia (1). Weighed out 0.1731 g of Pepsin and placed into 50mL of 34mM NaCl, pH 1.2.

The digestions for AAD-1 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 three proteins, AAD-1, BSA, and β-lactoglobulin A were digested as follows: Three 2.85-mL aliquots of SGF were placed in the 37 °C water bath. After 5 minutes, 150 µL of the 0.074 mM solutions of AAD-1, BSA, and β-lactoglobulin A were each added to a separate vial of the SGF and a timer was set. After each specified incubation interval, 100 µL of the reaction mixture was removed and added to tubes containing stop solution (40 µL 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 three proteins. An SGF control was prepared by substituting water for the sample protein and incubating for the duration of the experiment at 37 °C. The SGF control were prepared as follows: A 2.85-mL aliquot of SGF was heated in a 37 °C water bath for 5 minutes, 150 µL of milli-Q water was added and a timer was set. A 100 µL aliquot was immediately removed as the zero time point and placed into a tube containing the stop reaction (40 µL of 200 mM sodium carbonate, pH ~11.0), when all digestion reactions for all proteins were complete one final aliquot to was taken at the duration of the experiment. For each of the proteins above, a zero time point (neutralized control) was prepared as follows: First, a 2.85-mL aliquot of SGF was stopped with 1.2 mL 200 mM sodium carbonate, then 150 µL of the respective protein was added to the solution.

Aliquots of the neutralized and digested proteins were mixed with equal volumes of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, catalog #161-0737), containing 5% freshly added 2-mercaptoethanol (βME) (Bio-Rad Laboratories, catalog #161-0710) and heated for 5 minutes at ~95 °C. Additionally, aliquots of all samples and the AAD-1 neutralized control were initially diluted in PBST to a concentration appropriate for western blot analysis, then mixed with equal volumes of the Laemmli sample buffer preparation and heated.

Single 4-20% polyacrylamide gels (Bio-Rad Laboratories, catalog #345-0032) of BSA β-lactoglobulin A, and duplicate gels of AAD-1 were prepared. For each AAD-1 gel a 10-fold

dilution of AAD-1 protein 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 µL	~1.75 µg	N/A	N/A
β-lactoglobulin A	20 µL	~0.483 µg	N/A	N/A
AAD-1	20 µL	~0.879 µg	20 µL	~0.088 µg
10 % AAD-1	20 µL	~0.088 µg	20 µL	~0.0088 µg

The samples were then electrophoresed at a constant voltage of 180 volts per gel for ~45 minutes using Tris/Glycine/SDS buffer (Bio-Rad Laboratories, catalog #161-0772). After separation, three of the gels were stained with GelCode Blue stain (Pierce Chemical, Rockford, IL, catalog #24592). Proteins on the duplicate AAD-1 gel were electro-blotted to a nitrocellulose membrane (Bio-Rad Laboratories, catalog #162-0233) using a Bio-Rad Criterion Blotter at a constant voltage of 50 V for 1 hour. Tris/Glycine buffer (Bio-Rad Laboratories, catalog #161-0734) containing 20% methanol was used in the transfer. Following protein transfer, the membrane was blocked with phosphate buffered saline containing Tween 20 (Sigma, St. Louis, MO catalog# P-3563) and 5% powdered milk. For immunodetection of the western blotted membrane, a specific rabbit polyclonal antibody (PAb) against AAD-1 was used as the primary antibody (lot #DAS F1197-151). A conjugate of goat anti-rabbit IgG (H+L) horseradish peroxidase (Pierce Chemical, catalog #31460) was used as the secondary/detection antibody. Chemiluminescent detection solution (GE Healthcare Amersham Biosciences, Buckinghamshire, UK, catalog #RPN2132) was used for development and visualization of the immunoreactive

protein bands. The membrane was exposed to classic blue autoradiography film BX (MidSci, St. Louis, Missouri, catalog #EBA45) for various time points, and the film was developed.

STATISTICAL TREATMENT OF DATA

No statistical methods were used in this study.

RESULTS AND DISCUSSION

The positive and negative controls, BSA and β-lactoglobulin A, respectively, responded as expected (Table 1). BSA was not detected at the 30-second time point when subjected to the simulated gastric environment (Figure 1, lane 5). β-lactoglobulin A remained readily detectable for 16 minutes (the duration of the experiment) (Figure 2, lane 10). The test protein, AAD-1, was not detectable at the 30-second time point as demonstrated by both SDS-PAGE (Figure 3, lane 5) and western blot (Figure 4, lane 5) analyses. While faint SGF bands comigrated with the AAD-1 protein on the SDS-PAGE gel (Figure 3, lane 11), the western blot demonstrated that 10% of the initial AAD-1 protein was readily detectable and that no AAD-1 was detectable at or beyond the 30-second time point (Figure 4, lane 11).

CONCLUSION

AAD-1 protein is readily digested by pepsin (not detectable at 30 seconds) in simulated gastric fluid as demonstrated by both SDS-PAGE and western blot analyses.

ARCHIVING

The protocol, 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

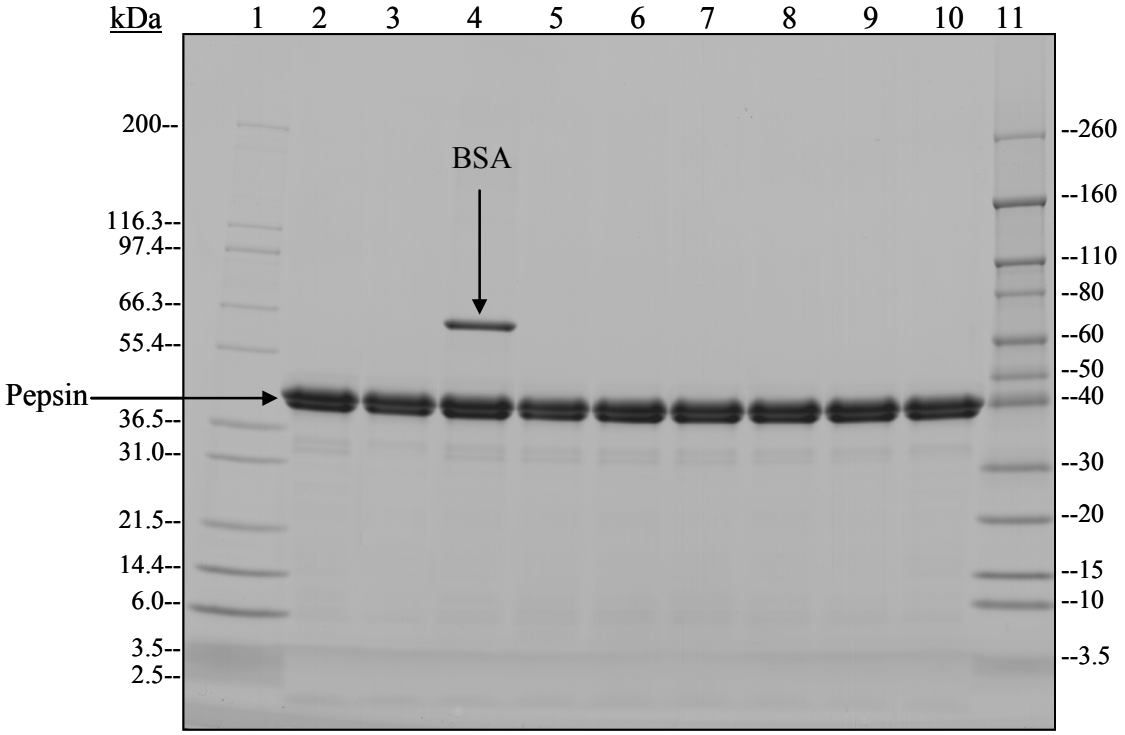
1. Board of Trustees (ed.) 1995. Simulated Gastric Fluid, TS, pp. 2053 in *The United States Pharmacopeia 23, The National Formulary 18.* United States Pharmacopeial Convention, Inc., Rockville, MD

2. Schafer, B. W. 2008. Certificate of Analysis for Test/Reference/Control/Substances AAD-1 Batch #1 (TSN106003) and Batch #2 (TSN105930). Dow AgroSciences BIOT08-162945

Table 1. Results of the In Vitro Digestibility Study of AAD-1 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
AAD-1	< 30 Seconds	< 30 Seconds

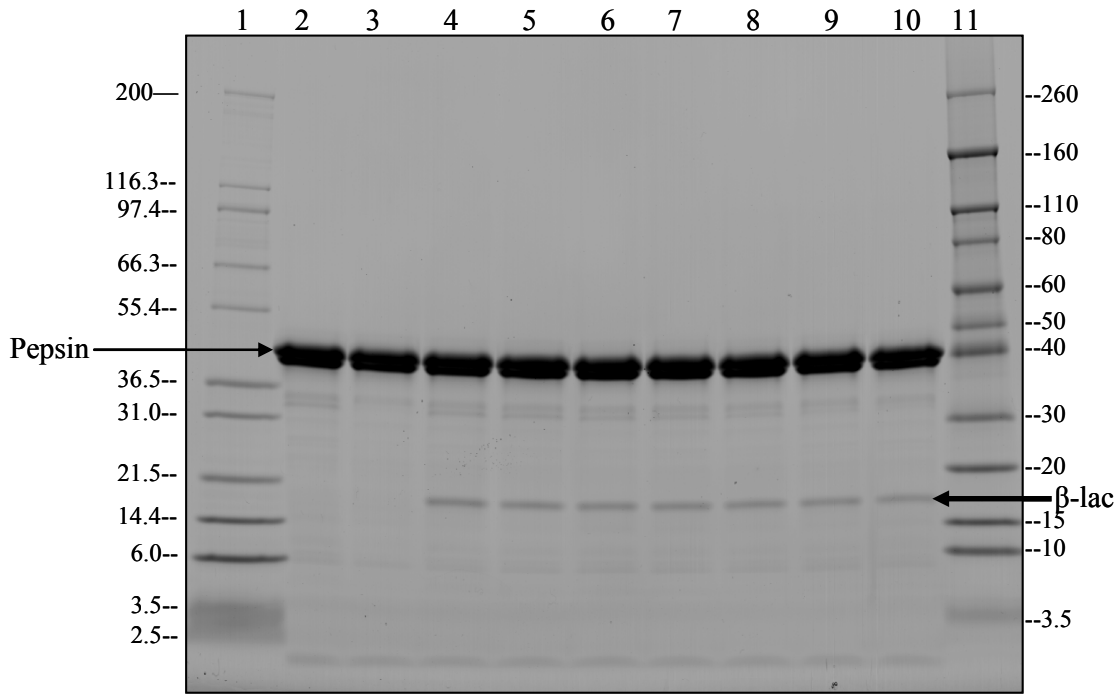
[a] Not applicable



The neutralized and digested BSA samples and SGF controls were held frozen for two days following the digestion. 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 into a Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V for ~45 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Pierce Chemical. Invitrogen Mark 12 molecular weight markers 3.5 and 2.5 kDa represent Insulin A and B chains which are unresolved when run on Tris-Glycine buffer systems.

Lane	Sample	Amount Loaded
1	Invitrogen Mark 12 MW markers	10µL
2	SGF Reagent Blank, 0 minute incubation	20 µL
3	SGF Reagent Blank, >16 minute incubation	20 µL
4	Neutralized BSA digestion	~1.75 µg
5	30-second BSA digestion	~1.75 µg
6	1-minute BSA digestion	~1.75 µg
7	2-minute BSA digestion	~1.75 µg
8	4-minute BSA digestion	~1.75 µg
9	8-minute BSA digestion	~1.75 µg
10	16-minute BSA digestion	~1.75 µg
11	Invitrogen Novex Sharp Prestained MW markers	10µL

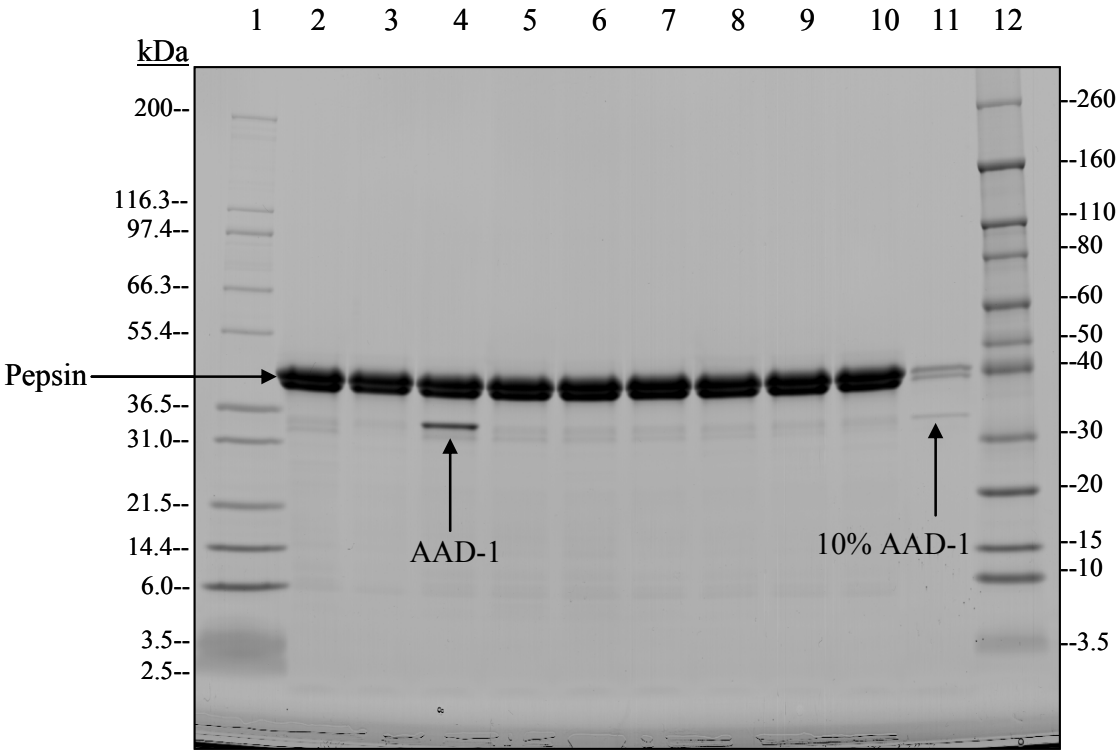
Figure 1. SDS-PAGE analysis of BSA (M.W. ~66 kDa) protein subjected to digestion in simulated gastric fluid.



The neutralized and digested β-lactoglobulin A (β-lac) samples and SGF controls were held frozen for two days following the digestion. 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 into a Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V for ~45 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Pierce Chemical. Invitrogen Mark 12 molecular weight markers 3.5 and 2.5 kDa represent Insulin A and B chains which are unresolved when run on Tris-Glycine buffer systems.

Lane	Sample	Amount Loaded
1	Invitrogen Mark 12 MW markers	10µL
2	SGF Reagent Blank, 0 minute incubation	20 µL
3	SGF Reagent Blank, >16 minute incubation	20 µL
4	Neutralized β-lac digestion	~0.483 µg
5	30-second β-lac digestion	~0.483 µg
6	1-minute β-lac digestion	~0.483 µg
7	2-minute β-lac digestion	~0.483 µg
8	4-minute β-lac digestion	~0.483 µg
9	8-minute β-lac digestion	~0.483 µg
10	16-minute β-lac digestion	~0.483 µg
11	Invitrogen Novex Sharp Prestained MW markers	10µL

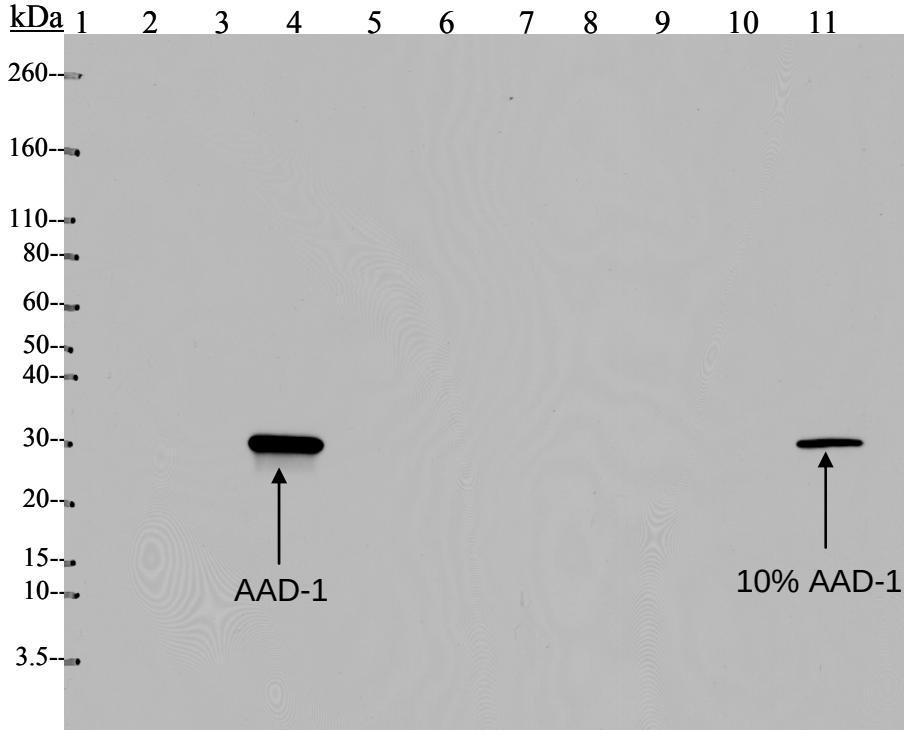
Figure 2. SDS-PAGE analysis of β-lactoglobulin A (M.W. ~18 kDa) protein subjected to digestion in simulated gastric fluid.



The neutralized and digested AAD-1 samples and SGF controls were held frozen for two days following the digestion. 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 into a Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V for ~45 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Pierce Chemical. Invitrogen Mark 12 molecular weight markers 3.5 and 2.5 kDa represent Insulin A and B chains which are unresolved when run on Tris-Glycine buffer systems.

Lane	Sample	Amount Loaded
1	Invitrogen Mark 12 MW markers	10µL
2	SGF Reagent Blank, 0 minute incubation	20 µL
3	SGF Reagent Blank, >16 minute incubation	20 µL
4	Neutralized AAD-1 digestion	~0.879 µg
5	30-second AAD-1 digestion	~0.879 µg
6	1-minute AAD-1 digestion	~0.879 µg
7	2-minute AAD-1 digestion	~0.879 µg
8	4-minute AAD-1 digestion	~0.879 µg
9	8-minute AAD-1 digestion	~0.879 µg
10	16-minute AAD-1 digestion	~0.879 µg
11	10% Neutalized AAD-1 digestion	~0.088 µg
12	Invitrogen Novex Sharp Prestained MW markers	10µL

Figure 3. SDS-PAGE analysis of AAD-1 (M.W. ~33 kDa) protein subjected to digestion in simulated gastric fluid.



The neutralized and digested AAD-1 samples and SGF controls were held frozen for two days following the digestion. 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 into two separate Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V per gel for ~45 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, one gel was stained with GelCode Blue stain from Pierce and the other gel was electro-blotted to a nitrocellulose membrane for 60 minutes under a constant charge of 50 volts. For immunodetection, the membrane was probed with an AAD-1 specific polyclonal rabbit antibody (Protein A purified: Lot #: DAS F1197-151, 1.6 mg/mL). 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 for various time points and subsequently developed with a film developer. The molecular weight markers were manually transferred to the film after development.

Lane	Sample	Amount Loaded
1	Invitrogen Novex Sharp Prestained MW markers	10µL
2	SGF Reagent Blank, 0 minute incubation	20 µL
3	SGF Reagent Blank, >16 minute incubation	20 µL
4	Neutralized AAD-1 digestion	~0.088µg
5	30-second AAD-1 digestion	~0.088µg
6	1-minute AAD-1 digestion	~0.088µg
7	2-minute AAD-1 digestion	~0.088µg
8	4-minute AAD-1 digestion	~0.088µg
9	8-minute AAD-1 digestion	~0.088µg
10	16-minute AAD-1 digestion	~0.088µg
11	10% Neutralized AAD-1 digestion	~0.0088µg

Figure 4. Western blot analysis of AAD-1 protein subjected to digestion in simulated gastric fluid.