

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-12  
(abbreviation AAD-12)

DATA REQUIREMENTS

N/A

AUTHOR(S)

S. K. Embrey, B. W. Schafer

STUDY COMPLETED ON

12-Sept-2008

PERFORMING LABORATORY

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

LABORATORY STUDY ID

080064

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(abbreviation AAD-12)

SUMMARY

Soybean has been modified by the insertion of the *aad-12* gene from *Delftia acidovorans* which encodes the aryloxyalkanoate dioxygenase-12 (AAD-12) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and pyridyloxyacetate herbicides and may be used as a selectable marker during plant transformation and in breeding nurseries. The AAD-12 protein is approximately 32 kDa in size. The purpose of this study was to evaluate the stability of the AAD-12 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 digestion samples of the AAD-12 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  $\beta$ -lactoglobulin A was used as a negative control since it is known to persist in SGF.

The AAD-12 protein was 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.

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*In Vitro* Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-12  
(abbreviation AAD-12)

DATA REQUIREMENTS

N/A

AUTHOR(S)

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STUDY COMPLETED ON

12-Sept-2008

PERFORMING LABORATORY

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: AAD-12

Title: *In Vitro* Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-12 (abbreviation AAD-12)

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: M. S. Krieger

Title: Regulatory Manager

Signature: 

Date: 11 September 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

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(abbreviation AAD-12)

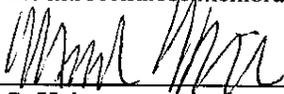
Study Initiation Date: 06/11/08

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

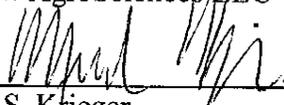
United States Environmental Protection Agency  
Title 40 Code of Federal Regulations Part 160  
FEDERAL REGISTER, August 17, 1989

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

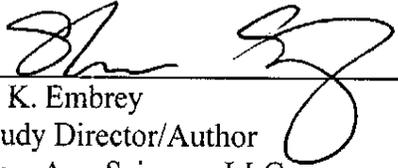
All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160 with the following exceptions: The GLP status of the commercial reference standards, such as bovine serum albumin,  $\beta$ -lactoglobulin A and protein molecular weight standards were unknown. The chain of custody of these standards was not monitored. Additionally, molecular weight markers were manually transferred from the nitrocellulose membrane after film development but membrane was not retained.

  
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12 Sep 2008  
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Study Completion Date

**Dow AgroSciences Quality Assurance Unit  
Good Laboratory Practice Statement Page**

**Compound:** AAD-12

**Study ID:** 080064

**Title:** *In Vitro* Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-12  
(abbreviation AAD-12)

**Study Initiation Date:** 11 Jun 2008

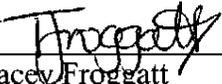
**Study Completion Date:** 12 Sept 2008

**GLP Quality Assurance Inspections**

<b>Date of GLP Inspection(s)</b>	<b>Date Reported to the Study Director and to Management</b>	<b>Phases of the Study which received a GLP Inspection by the Quality Assurance Unit</b>
5 June 2008	11 June 2008	Protocol Review
23, 25 June 2008	30 June 2008	Preparation of Samples & Digestion with SGF and SDS-PAGE
8, 9, 10 Sept 2008	10 June 2008	Report and Raw Data, Test Substance Container & Sample Verification

**QUALITY ASSURANCE STATEMENT:**

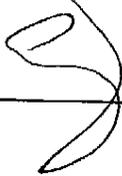
The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.

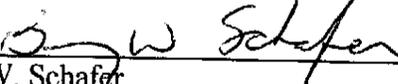
  
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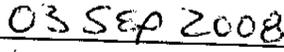
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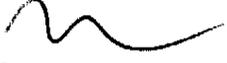
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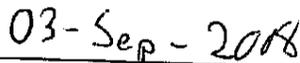
  
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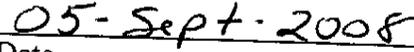
  
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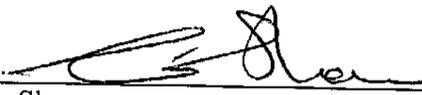
  
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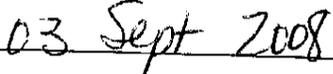
  
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## STUDY PERSONNEL

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

Principle Analyst: B. W. Schafer  
(Principle Investigator)

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*In Vitro* Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-12  
(abbreviation AAD-12)

ABSTRACT

Soybean has been modified by the insertion of the *aad-12* gene from *Delftia acidovorans* which encodes the aryloxyalkanoate dioxygenase-12 (AAD-12) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and pyridyloxyacetate herbicides and may be used as a selectable marker during plant transformation and in breeding nurseries. The AAD-12 protein is approximately 32 kDa in size. The purpose of this study was to evaluate the stability of the AAD-12 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 digestion samples of the AAD-12 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  $\beta$ -lactoglobulin A was used as a negative control since it is known to persist in SGF.

AAD-12 protein was 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-12	Aryloxyalkanoate Dioxygenase-12
AI	active ingredient
$\beta$ -lac	$\beta$ -lactoglobulin A
BSA	bovine serum albumin
DAS	Dow AgroSciences LLC
GLP	Good Laboratory Practice
HRP	horseradish peroxidase
kDa	kilodalton
M	Molar
$\mu$ g	microgram
$\mu$ L	microliter
mL	milliliter
mM	millimolar
min	minute
MW	molecular weight
N/A	Not Applicable
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
V	volt

## INTRODUCTION

Soybean has been modified by the insertion of the *aad-12* gene from *Delftia acidovorans* which encodes the aryloxyalkanoate dioxygenase-12 (AAD-12) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and pyridyloxyacetate herbicides and may be used as a selectable marker during plant transformation and in breeding nurseries. The AAD-12 protein is approximately 32 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 correlates with the digestibility of proteins in a human gastric system.

The purpose of this study was to evaluate the digestion of the AAD-12 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-12 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  $\beta$ -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-12 protein (Lot Number: 466-028A) 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 TSN030732-0002. The purity was determined to be 35.3% (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
$\beta$ -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 markers 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: 33.2 mg of AAD-12 (TSN030732-0002) 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 tube and adding 5 mL of Milli-Q water.  $\beta$ -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, 73% w/w pure, 3,410 units of activity/mg protein) was

prepared by weighing out 0.2307 g of Pepsin into 50mL of 34mM NaCl as recommended by the United States Pharmacopeia (1).

The digestions for AAD-12 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-12, BSA, and  $\beta$ -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  $\mu$ L of the 0.074 mM solutions of AAD-12, BSA, and  $\beta$ -lactoglobulin A were each added to a separate vial of the SGF and a timer was set. After each specified incubation interval, 100  $\mu$ L of the reaction mixture was removed and added to tubes containing stop solution (40  $\mu$ 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 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  $\mu$ L of milli-Q water was added and a timer was set. A 100  $\mu$ L aliquot was immediately removed as the zero time point and placed into a tube containing the stop reaction (40  $\mu$ L of 200 mM sodium carbonate, pH ~11.0), when all digestion reactions were complete one final aliquot 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  $\mu$ L 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 ( $\beta$ ME) (Bio-Rad Laboratories, catalog #161-0710) and heated for 5 minutes at ~95 °C. Additionally, aliquots of all samples and the AAD-12 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  $\beta$ -lactoglobulin A, and duplicate gels of AAD-12 were prepared. For each AAD-12 gel a 10-fold

dilution of AAD-12 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	40 $\mu$ L	~3.49 $\mu$ g	N/A	N/A
$\beta$ -lactoglobulin A	40 $\mu$ L	~0.97 $\mu$ g	N/A	N/A
AAD-12	40 $\mu$ L	~1.68 $\mu$ g	40 $\mu$ L	~0.17 $\mu$ g
10 % AAD-12	40 $\mu$ L	~0.17 $\mu$ g	40 $\mu$ L	~0.017 $\mu$ g

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-12 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 0.05% Tween 20 (PBST) and 5% powdered milk. For immunodetection, the membrane was probed with an AAD-12 specific polyclonal rabbit antibody (Protein A purified: Lot #: DAS F1197-167-2, 4.3 mg/mL). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase was used as the secondary antibody (Pierce, catalog #31460, lot# DA711252). 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 Amersham Hyperfilm MP autoradiography film (Amersham Biosciences GE Healthcare, Buckinghamshire, UK, catalog #28906845) and was subsequently developed with a film developer.

## STATISTICAL TREATMENT OF DATA

No statistical methods were used in this study.

## RESULTS AND DISCUSSION

The positive and negative controls, BSA and  $\beta$ -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).  $\beta$ -lactoglobulin A remained readily detectable for 16 minutes (the duration of the experiment) (Figure 2, lane 10). The test protein, AAD-12, 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. Additionally, both SDS-Page (Figure 3, lane 11) and western blot (Figure 4, lane 11) demonstrated that 10% of the initial AAD-12 protein was readily detectable and that no AAD-12 was detectable at or beyond the 30-second time point.

## CONCLUSION

AAD-12 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.

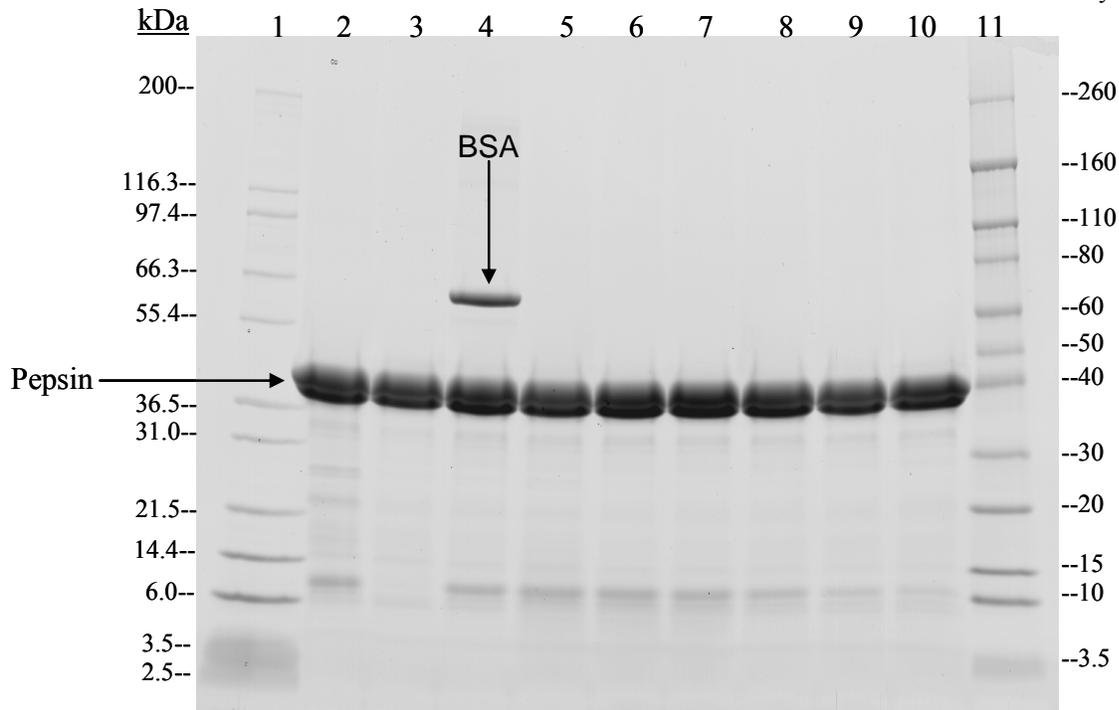
## REFERENCES

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 Aryloxyalkanoate dioxygenase (abbreviation: AAD-12) – TSN030732. Report Number: BIOT08-161536 (Unpublished).

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

<b>Protein</b>	<b>Detection by SDS-PAGE</b>	<b>Detection by Western Blot Analysis</b>
BSA	< 30 Seconds	<sup>a</sup> N/A
β- lactoglobulin A	> 16 Minutes	<sup>a</sup> N/A
AAD-12	< 30 Seconds	< 30 Seconds

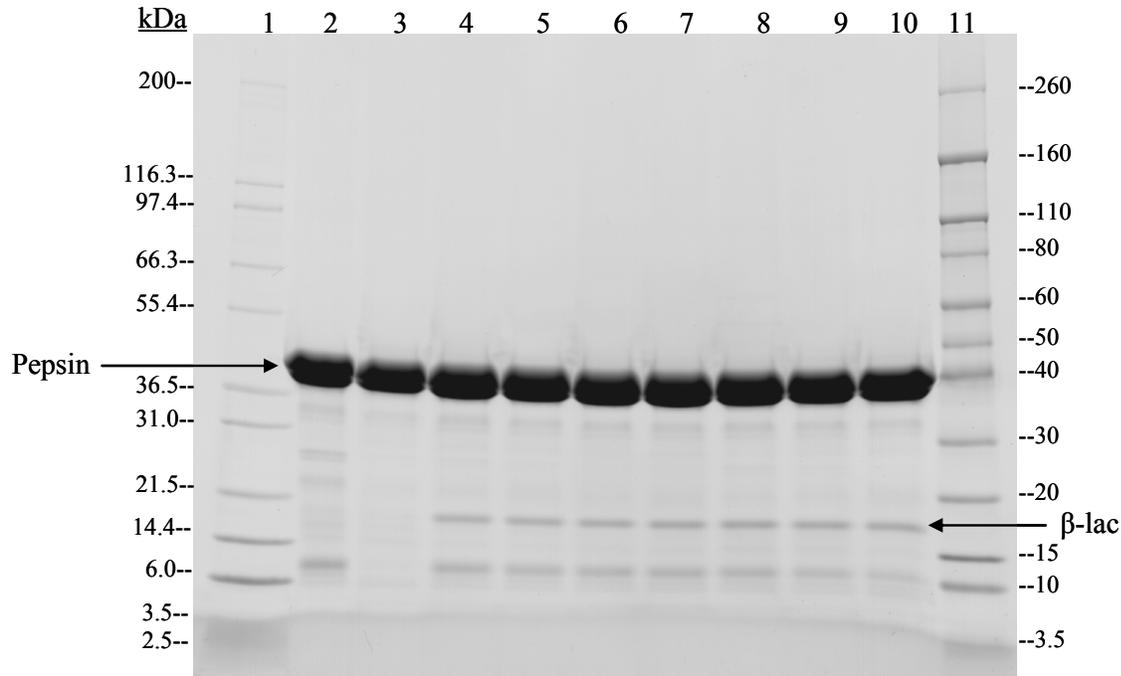
<sup>a</sup> Not applicable



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  $\sim 95^{\circ}\text{C}$ . The samples were loaded into a Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V per gel for  $\sim 45$  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	10 $\mu\text{L}$
2	SGF Reagent Blank, 0 minute incubation	40 $\mu\text{L}$
3	SGF Reagent Blank, >16 minute incubation	40 $\mu\text{L}$
4	Neutralized BSA digestion	$\sim 3.49$ $\mu\text{g}$
5	30-second BSA digestion	$\sim 3.49$ $\mu\text{g}$
6	1-minute BSA digestion	$\sim 3.49$ $\mu\text{g}$
7	2-minute BSA digestion	$\sim 3.49$ $\mu\text{g}$
8	4-minute BSA digestion	$\sim 3.49$ $\mu\text{g}$
9	8-minute BSA digestion	$\sim 3.49$ $\mu\text{g}$
10	16-minute BSA digestion	$\sim 3.49$ $\mu\text{g}$
11	Invitrogen Novex Sharp Prestained MW markers	10 $\mu\text{L}$

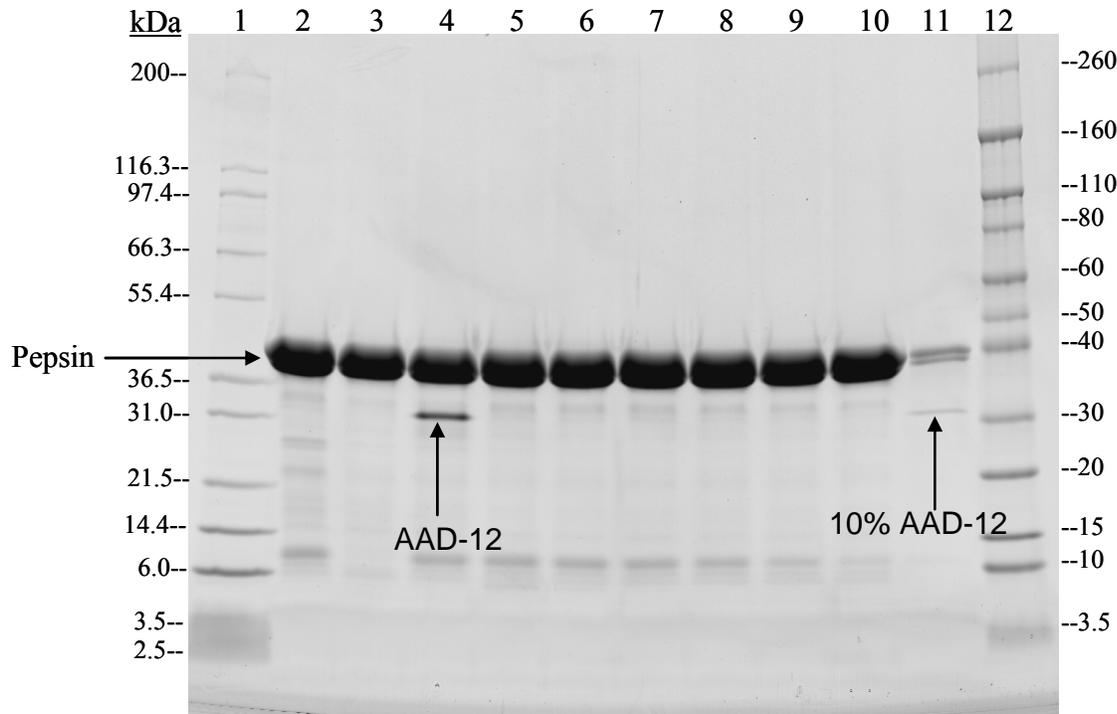
Figure 1. SDS-PAGE analysis of BSA (M.W.  $\sim 66$  kDa) protein subjected to digestion in simulated gastric fluid.



The neutralized and digested  $\beta$ -lactoglobulin A ( $\beta$ -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  $\sim 95$  °C. The samples were loaded into a Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V per gel for  $\sim 45$  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	10 $\mu$ L
2	SGF Reagent Blank, 0 minute incubation	40 $\mu$ L
3	SGF Reagent Blank, >16 minute incubation	40 $\mu$ L
4	Neutralized $\beta$ -lac digestion	$\sim 0.97$ $\mu$ g
5	30-second $\beta$ -lac digestion	$\sim 0.97$ $\mu$ g
6	1-minute $\beta$ -lac digestion	$\sim 0.97$ $\mu$ g
7	2-minute $\beta$ -lac digestion	$\sim 0.97$ $\mu$ g
8	4-minute $\beta$ -lac digestion	$\sim 0.97$ $\mu$ g
9	8-minute $\beta$ -lac digestion	$\sim 0.97$ $\mu$ g
10	16-minute $\beta$ -lac digestion	$\sim 0.97$ $\mu$ g
11	Invitrogen Novex Sharp Prestained MW markers	10 $\mu$ L

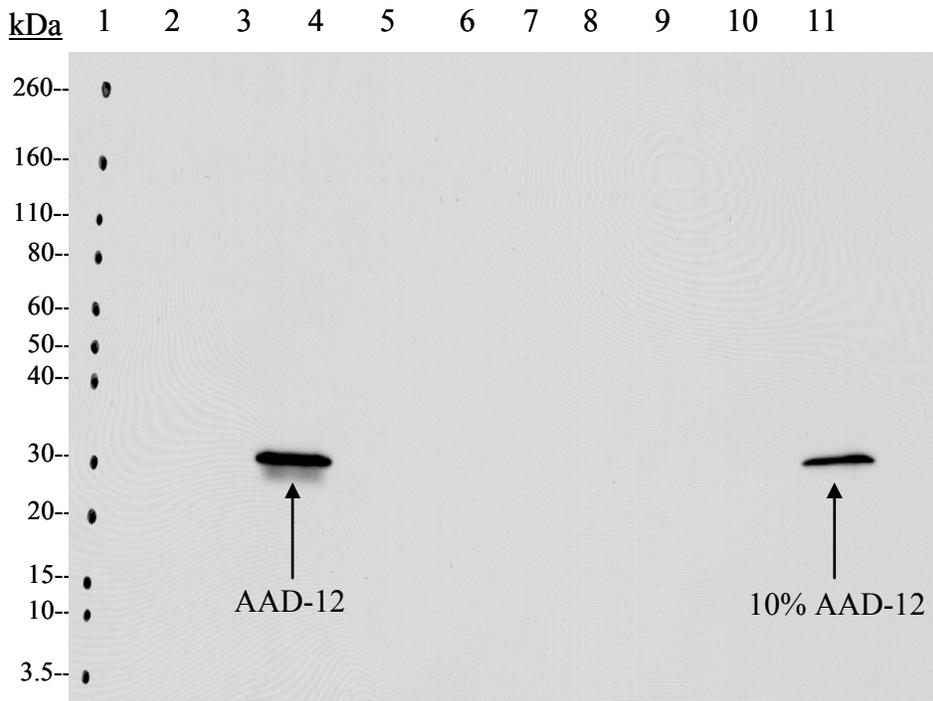
Figure 2. SDS-PAGE analysis of  $\beta$ -lactoglobulin A (M.W.  $\sim 18$  kDa) protein subjected to digestion in simulated gastric fluid.



The neutralized and digested AAD-12 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  $\sim 95^{\circ}\text{C}$ . The samples were loaded into a Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V for  $\sim 45$  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	10 $\mu\text{L}$
2	SGF Reagent Blank, 0 minute incubation	40 $\mu\text{L}$
3	SGF Reagent Blank, >16 minute incubation	40 $\mu\text{L}$
4	Neutralized AAD-12 digestion	$\sim 1.67$ $\mu\text{g}$
5	30-second AAD-12 digestion	$\sim 1.67$ $\mu\text{g}$
6	1-minute AAD-12 digestion	$\sim 1.67$ $\mu\text{g}$
7	2-minute AAD-12 digestion	$\sim 1.67$ $\mu\text{g}$
8	4-minute AAD-12 digestion	$\sim 1.67$ $\mu\text{g}$
9	8-minute AAD-12 digestion	$\sim 1.67$ $\mu\text{g}$
10	16-minute AAD-12 digestion	$\sim 1.67$ $\mu\text{g}$
11	10% Neutralized AAD-12 digestion	$\sim 0.17$ $\mu\text{g}$
12	Invitrogen Novex Sharp Prestained MW markers	10 $\mu\text{L}$

Figure 3. SDS-PAGE analysis of AAD-12 (M.W.  $\sim 32$  kDa) protein subjected to digestion in simulated gastric fluid.



The neutralized and digested AAD-12 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  $\sim 95^{\circ}\text{C}$ . The samples were loaded into a Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V for  $\sim 45$  minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the 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-12 specific polyclonal rabbit antibody (Protein A purified: Lot #: DAS F1197-167-2, 4.3 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 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	10 $\mu\text{L}$
2	SGF Reagent Blank, 0 minute incubation	40 $\mu\text{L}$
3	SGF Reagent Blank, >16 minute incubation	40 $\mu\text{L}$
4	Neutralized AAD-12 digestion	$\sim 0.17\mu\text{g}$
5	30-second AAD-12 digestion	$\sim 0.17\mu\text{g}$
6	1-minute AAD-12 digestion	$\sim 0.17\mu\text{g}$
7	2-minute AAD-12 digestion	$\sim 0.17\mu\text{g}$
8	4-minute AAD-12 digestion	$\sim 0.17\mu\text{g}$
9	8-minute AAD-12 digestion	$\sim 0.17\mu\text{g}$
10	16-minute AAD-12 digestion	$\sim 0.17\mu\text{g}$
11	10% Neutralized AAD-12 digestion	$\sim 0.017\mu\text{g}$

Figure 4. Western blot analysis of AAD-12 protein subjected to digestion in simulated gastric fluid.