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 Study of Double Mutant 5-Enol Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) Protein

AUTHOR(S)

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STUDY COMPLETED ON

05-Jul-2011

PERFORMING LABORATORY

Regulatory Sciences and Government Affairs—Indianapolis Lab
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LABORATORY STUDY ID

102106

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The purpose of this study was to evaluate the stability of the 2mEPSPS 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) and the 2mEPSPS protein was 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 2mEPSPS protein was readily digested by pepsin (not detectable at 1 minute) 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 Study of Double Mutant 5-Enol Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) Protein

DATA REQUIREMENTS

N/A

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: 2mEPSPS

Title: *In-Vitro* Simulated Gastric Fluid Digestibility of Double Mutant 5-Enol Pyruvylshikimate 3-Phosphate Synthase (2mEPSPS) Protein

- STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS:

  No claim of confidentiality, on any basis whatsoever, is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA sec. 10(d)(1)(A), (B), or (C) and which pertains to a registered or previously registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA sec. 10(g).

Company: Dow AgroSciences LLC

Company Agent: M. S. Krieger

Title: Regulatory Manager

Signature: 

Date: 15 June 2011

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 Double Mutant 5-Enol Pyruvylshikimate 3-Phosphate Synthase (2mEPSPS) Protein

Study Initiation Date: 30 - Dec - 2010

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 bovinge serum albumin, β-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.

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Study Completion Date

Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

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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
30-Dec-2010	30-Dec-2010	Protocol Review
17-Mar-2011	22-Mar-2011	Digestion
29, 30-Jun, 1-Jul-2011	1-Jul-2011	Report and Raw Data Review: Test Substance Container 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.

TFroggatt

Tracey Froggatt
Dow AgroSciences, Quality Assurance

5-Jul-2011

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In Vitro Simulated Gastric Fluid Digestibility Study of Double Mutant 5-Enol Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) Protein

ABSTRACT

The purpose of this study was to evaluate the stability of the 2mEPSPS 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) and the 2mEPSPS protein was also examined 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 2mEPSPS protein was readily digested by pepsin (not detectable at 1 minute) under simulated gastric conditions (pH 1.2, 37 °C) as demonstrated by both SDS-PAGE and western blot analyses.

ABBREVIATIONS

2mEPSPS	Double Mutant 5-Enol Pyruvylshikimate-3-Phosphate Synthase
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
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 with the 2mEPSPS, or double mutant maize EPSPS engineered from wild-type of maize EPSPS gene by site-directed mutagenesis. The 2mEPSPS trait confers tolerance to glyphosate. The 2mEPSPS protein is approximately 47 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 2mEPSPS 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 2mEPSPS 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 2mEPSPS protein (Lot Number: DMMG_033110) was produced and purified from *P. fluorescens*. The protein preparation was sent to the Test Substance Coordinator at Dow AgroSciences and the material was designated TSN033171-0001. The purity was determined to be 66.5% (2).

Control Substances

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

Control Substance	Purity	Supplier	Storage
Bovine serum albumin (BSA)	>99%	Sigma	2-6 °C
β-lactoglobulin A (β-lac)	100%	Sigma	2-6 °C

Reference Substances

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

Reference Substance	Product Name	Assay	Reference
Unstained Molecular Weight Markers	Novex Sharp Unstained Protein Standard	SDS-PAGE	Invitrogen Cat #: LC5801, Approximate 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 Pre-stained Protein Standard	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 test and control substances were prepared as follows: 26.2 mg of 2mEPSPS (TSN033171-0001) powder was dissolved in a container by adding 5 mL phosphate buffered saline solution with Tween 20 (PBST, Sigma). BSA was solubilized by weighing 24.7 mg of powder in a container and adding 5 mL of PBST. β-lactoglobulin A was solubilized by weighing 6.8 mg of powder in a container and adding 5 mL of PBST. 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, 92% w/w pure, 3802 units of activity/mg protein) was prepared by weighing out 0.1830 g of Pepsin into 50 mL of 34 mM NaCl as recommended by the United States Pharmacopeia (1).

The digestions for 2mEPSPS were performed for time intervals of approximately 1, 2, 4, 8 and 16 minutes in a water bath set to 37 °C. The three proteins, 2mEPSPS, 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 2mEPSPS, 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 PBST 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 PBST 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 were complete, one final aliquot was taken at the duration of the experiment. For each of the proteins above, a 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. The SGF was then stopped with 1.2 mL 200 mM sodium carbonate and 150 µL of the respective protein was added to the solution.

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

Single 4-12% polyacrylamide gels (Bio-Rad) of BSA and β-lactoglobulin A, and duplicate gels of 2mEPSPS were prepared. For each 2mEPSPS gel, a 10-fold dilution of 2mEPSPS 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	~0.486 µg	N/A	N/A
2mEPSPS	20 µL	~1.24 µg	40 µL	~0.124 µg
10 % 2mEPSPS	20 µL	~0.124 µg	40 µL	~0.0124 µg

The samples were then electrophoresed at a constant voltage of 150 volts for ~60 minutes using Tris/Glycine/SDS buffer (Bio-Rad). After separation, three of the gels were stained with GelCode Blue stain (Thermo-Pierce). Proteins on the duplicate 2mEPSPS gel were electro-blotted to a nitrocellulose membrane (Bio-Rad) using a Bio-Rad Criterion Blotter at a constant voltage of 100 V for 1 hour. Tris/Glycine buffer (Bio-Rad) 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% non-fat dried milk (Bio-Rad). For immunodetection, the membrane was probed with a 2mEPSPS specific polyclonal rabbit antibody (Lot: G2874, 3.3 mg/mL). A conjugate of goat anti-rabbit IgG and horseradish peroxidase was used as the secondary antibody (Thermo-Pierce). Chemiluminescent detection solution (GE Healthcare) was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to X-ray film (Thermo-Pierce) and was subsequently developed with an All-Pro 100 plus film developer.

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 1 minute 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 9). The test protein, 2mEPSPS, was not detectable at the 1 minute time point as demonstrated by both SDS-PAGE (Figure 3, lane 6) and western blot (Figure 4, lane 6) analyses. Additionally, both SDS-PAGE (Figure 3, lane 5) and western blot (Figure 4, lane 5) demonstrated that 10% of the initial 2mEPSPS protein was readily detectable and that no 2mEPSPS was detectable at or beyond the 1 minute time point.

CONCLUSION

The 2mEPSPS protein is readily digested by pepsin (not detectable at 1 minute) 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

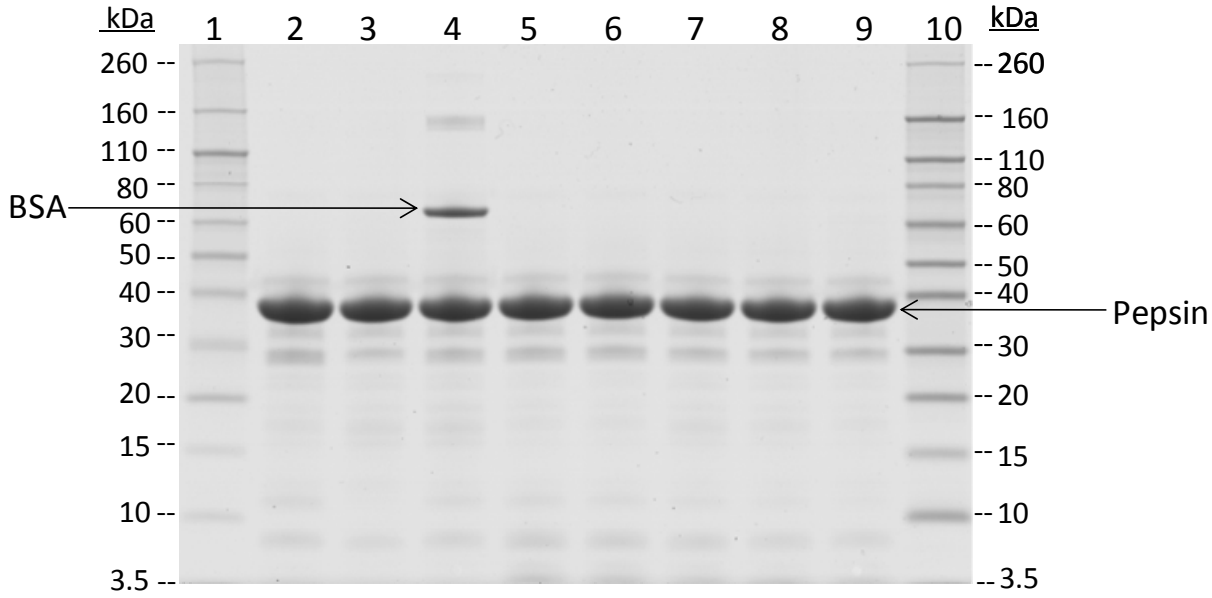
1. Board of Trustees (ed.) 1995. Simulated Gastric Fluid, TS, pp. 2053 in *The United States Pharmacopeia 23, The National Formulary 18.* United States Pharmacopeial Convention, Inc., Rockville, MD

2. Schafer, B. W. 2010. Certificate of Analysis for Test/Reference/Control/Substance: 2mEPSPS Protein (TSN033171-0001). Report Number: BIOT10-255698 (Unpublished)

Table 1. Results of the *In Vitro* Digestibility Study of 2mEPSPS in Simulated Gastric Fluid (SGF)

Protein	Detection by SDS-PAGE	Detection by Western Blot Analysis
BSA	< 1 minute	[a]N/A
β- lactoglobulin A	> 16 Minutes	[a]N/A
2mEPSPS	< 1 minute	< 1 minute

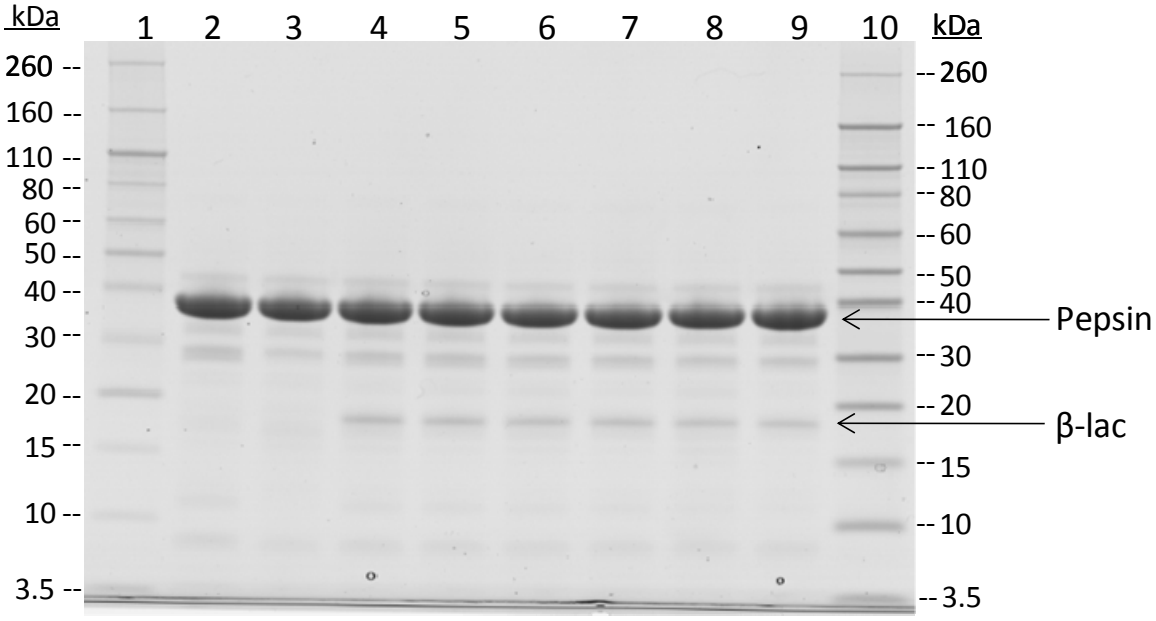
[a] Not applicable



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 into a Bio-Rad 4-20% Bis-Tris Criterion gel and electrophoresed at a constant voltage of 150 V per gel for ~60 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Thermo-Pierce.

Lane	Sample	Amount Loaded
1	Invitrogen Novex Sharp Unstained 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	1-minute BSA digestion	~1.75 µg
6	2-minute BSA digestion	~1.75 µg
7	4-minute BSA digestion	~1.75 µg
8	8-minute BSA digestion	~1.75 µg
9	16-minute BSA digestion	~1.75 µg
10	Invitrogen Novex Sharp Prestained MW markers	10µL

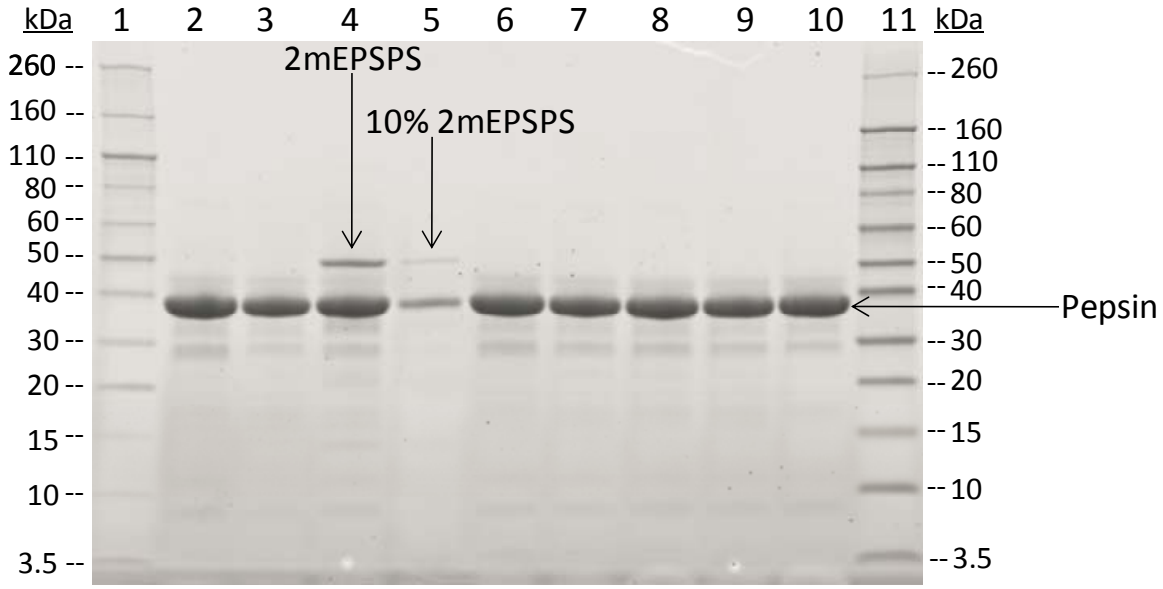
Figure 1. SDS-PAGE analysis of BSA (M.W. ~66 kDa) protein subjected to digestion in simulated gastric fluid.



The neutralized and digested β-lactoglobulin A (β-lac) samples 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% Bis-Tris Criterion gel and electrophoresed at a constant voltage of 150 V per gel for ~60 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Thermo-Pierce.

Lane	Sample	Amount Loaded
1	Invitrogen Novex Sharp Unstained 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	~0.486 µg
5	1-minute β-lac digestion	~0.486 µg
6	2-minute β-lac digestion	~0.486 µg
7	4-minute β-lac digestion	~0.486 µg
8	8-minute β-lac digestion	~0.486 µg
9	16-minute β-lac digestion	~0.486 µg
10	Invitrogen Novex Sharp Prestained MW markers	10µL

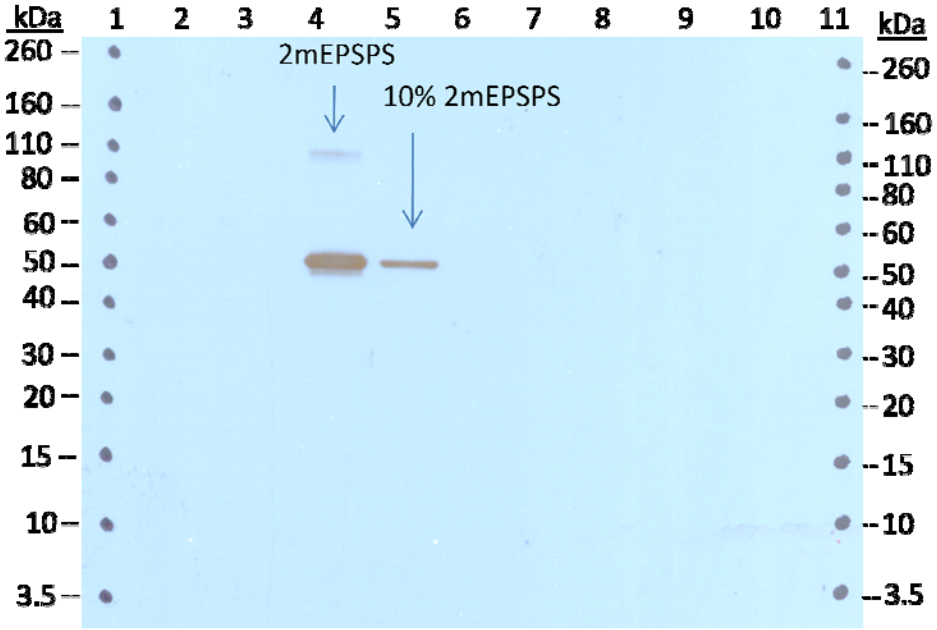
Figure 2. SDS-PAGE analysis of β-lactoglobulin A (M.W. ~18 kDa) protein subjected to digestion in simulated gastric fluid.



The neutralized and digested 2mEPSPS samples and SGF controls were held frozen 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% Bis-Tris Criterion gel and electrophoresed at a constant voltage of 150 V for ~60 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Thermo-Pierce.

Lane	Sample	Amount Loaded
1	Invitrogen Novex Sharp Unstained MW markers	10µL
2	SGF Reagent Blank, 0 minute incubation	20 µL
3	SGF Reagent Blank, >16 minute incubation	20 µL
4	Neutralized 2mEPSPS digestion	~1.24 µg
5	10% Neutralized 2mEPSPS digestion	~0.124 µg
6	1-minute 2mEPSPS digestion	~1.24 µg
7	2-minute 2mEPSPS digestion	~1.24 µg
8	4-minute 2mEPSPS digestion	~1.24 µg
9	8-minute 2mEPSPS digestion	~1.24 µg
10	16-minute 2mEPSPS digestion	~1.24 µg
11	Invitrogen Novex Sharp Prestained MW markers	10µL

Figure 3. SDS-PAGE analysis of 2mEPSPS (M.W. ~47 kDa) protein subjected to digestion in simulated gastric fluid.



The neutralized and digested 2mEPSPS samples and SGF controls were held frozen 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% Bis-Tris Criterion gel and electrophoresed at a constant voltage of 150 V for ~60 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 100 volts. For immunodetection, the membrane was probed with an 2mEPSPS specific polyclonal rabbit antibody (Lot: G2874, 3.3 mg/mL). A conjugate of goat anti-rabbit IgG 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	10µL
2	SGF Reagent Blank, 0 minute incubation	20 µL
3	SGF Reagent Blank, >16 minute incubation	20 µL
4	Neutralized 2mEPSPS digestion	~0.124 µg
5	10% Neutralized 2mEPSPS digestion	~0.0124 µg
6	1-minute 2mEPSPS digestion	~0.124 µg
7	2-minute 2mEPSPS digestion	~0.124 µg
8	4-minute 2mEPSPS digestion	~0.124 µg
9	8-minute 2mEPSPS digestion	~0.124 µg
10	16-minute 2mEPSPS digestion	~0.124 µg
11	Invitrogen Novex Sharp Prestained MW markers	10µL

Figure 4. Western blot analysis of 2mEPSPS protein subjected to digestion in simulated gastric fluid.