SUMMARY

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STUDY TITLE

In Vitro Simulated Intestinal Fluid Digestibility Study of Microbially Derived Cry1F (tr)

DATA REQUIREMENTS

None

AUTHORS

V. A. Korjagin, A. D. Ernest

STUDY COMPLETED ON

December 5, 2000

PERFORMING LABORATORY

Global Environmental Chemistry Laboratory—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

GH-C 5146

In Vitro Simulated Intestinal Fluid Digestibility Study of Microbially Derived Cry1F (tr)

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The Cry1F protein remained undigested in SIF for the duration of the assay (120 min). The non-allergenic protein, acid phosphatase and the allergenic protein, ovalbumin also remained undigested for the duration of the assay. The allergenic protein, β-lactoglobulin, was digested between 1 and 5 minutes after addition to SIF.

The results from this study indicate the following:

1) The *in vitro* SIF model is apparently unable to discriminate between known non-allergenic and allergenic proteins using stability to proteolysis as an experimental endpoint.

2) The Cry1F protein remained undigested in SIF for the duration of the assay. Cry1F shares this and other characteristics with other Cry proteins that dissociate them from being regarded as potential food allergens.

STUDY TITLE

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DATA REQUIREMENTS

None

AUTHORS

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry1F (tr)

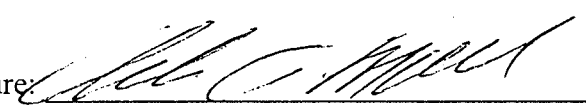
Title: *In Vitro* Simulated Intestinal Fluid Digestibility Study of Microbially Derived Cry1F (tr)

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: C. A. Mihaliak

Title: Regulatory Manager

Signature: 

Date: 11/9/00

*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 Intestinal Fluid Digestibility Study of Microbially Derived Cry1F (tr)

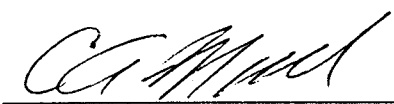
Study Initiation Date:	10/24/00	Study Completion Date:	December 5, 2000
Experimental Start Date:	10/24/00	Experiment Termination Date:	10/27/00

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

Organization for Economic Co-Operation and Development
ISBN 92-64-12367-9, Paris 1982

This study was not conducted under Good Laboratory Practices, and was not monitored by the Quality Assurance Unit.



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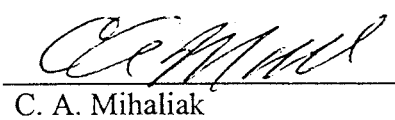

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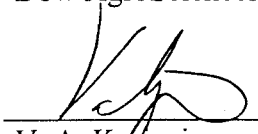

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V. A. Korjagin
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QUALITY ASSURANCE STATEMENT

Compound: Cry1F (tr)

Title: *In Vitro* Simulated Intestinal Fluid Digestibility Study of Microbially Derived Cry1F (tr)

Study Initiation Date: 10/24/00 Study Completion Date: December 5, 2000

NON-GLP STUDY

SIGNATURE PAGE

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TABLE OF CONTENTS

In Vitro Simulated Intestinal Fluid Digestibility Study of Microbially Derived Cry1F (tr)

ABSTRACT

Maize has been modified by the insertion of a gene from *Bacillus thuringiensis* (*B.t.*) subspecies *aizawai* strain PS81I. The *B.t.* gene encodes for an insecticidal delta-endotoxin designated as Cry1F. The purpose of this study was to evaluate the digestibility of the Cry1F protein in a simulated intestinal fluid (SIF) model. Reference proteins representing known non-allergenic (acid phosphatase; a common plant protein) and allergenic (β-lactoglobulin, a milk allergen and ovalbumin, an egg allergen) proteins were also included in the experimental design of this study for comparative purposes. The test and reference substances were subjected to SIF at specified time intervals and were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The Cry1F protein remained undigested in SIF for the duration of the assay (120 min). The non-allergenic protein, acid phosphatase and the allergenic protein, ovalbumin also remained undigested for the duration of the assay. The allergenic protein, β-lactoglobulin, was digested between 1 and 5 minutes after addition to SIF.

The results from this study indicate the following:

1) The *in vitro* SIF model is apparently unable to discriminate between known non-allergenic and allergenic proteins using stability to proteolysis as an experimental endpoint

2) The Cry1F protein remained undigested in SIF for the duration of the assay. Cry1F shares this and other characteristics with other Cry proteins that dissociate them from being regarded as potential food allergens.

INTRODUCTION

Maize has been modified by the insertion of a gene from *Bacillus thuringiensis* (*B.t.*) subspecies *aizawai* strain PS81I. The *B.t.* gene encodes for an insecticidal delta-endotoxin designated as Cry1F. The purpose of this study was to evaluate the digestibility of the Cry1F protein in a simulated intestinal fluid (SIF) model. Reference proteins representing known non-allergenic (acid phosphatase; a common plant protein) and allergenic (β-lactoglobulin, a milk allergen and ovalbumin, an egg allergen) proteins (1) were also included in the experimental design of this study for comparative purposes. The test and reference substances were subjected to SIF at specified time intervals and were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

EXPERIMENTAL

Test Substance

The test substance, microbially derived Cry1F protein (TSN101788) (Purity 11.4%) was obtained from the Test Substance Coordinator, Dow AgroSciences, 9330 Zionsville Road, Indianapolis, Indiana 46268-1054.

The recombinant Cry1F protein was prepared at Dow AgroSciences LLC, San Diego, California. A gene encoding the full-length Cry1F toxin (~130 kDa) was placed in *Pseudomonas fluorosescens* strain MR872. This strain was grown under large-scale fermentation conditions and the toxin extracted from concentrated cell pellets. The cell pellets were enzymatically lysed and full-length toxin was isolated as insoluble inclusions. These inclusions were washed extensively, suspended under alkaline conditions, and treated with trypsin to truncate the toxin. The soluble, truncated toxin (~65 kDa) was then diafiltered and finally concentrated by lyophilization (2). The resulting Cry1F powder was sent to the

Dow AgroSciences Indianapolis Test Substance Coordinator and entered into the Test Substance Database.

Reference Substances

The reference substances used in this study are listed in the following table:

Reference Substance	Product Name	ID Number	Reference
Non-Allergenic Protein	Acid Phosphatase Activity: 1.2 units/mg	Lot 30K7052	Sigma Catalog Number P-3752
Allergen Protein	β-lactoglobulin Purity: ≥90%	Lot 119H7007	Sigma Catalog Number L-7880
Allergen Protein	Ovalbumin Purity: ~ 99%	Lot 79H7003	Sigma Catalog Number A-2512
Molecular Weight Marker	Novex Mark 12 Wide Range Protein Standards	Lot MRK01005	Novex Catalog #LC5677, Molecular Markers of 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4, and 6 kiloDalton (kDa)

Test Methods

Equimolar (~0.074 mM) solutions of the test and reference substances were prepared as follows. TSN101788 (Cry1F protein) was reconstituted by weighing 4.8 mg of powder in a 4-mL test tube and adding 1 mL of H_2O. Acid phosphatase was reconstituted by weighing 5.1 mg of powder in a 4-mL test tube and adding 1 mL of H_2O. β-lactoglobulin was reconstituted by weighing 5.4 mg of powder in a 4-mL test tube and adding 2 mL H_2O. Ovalbumin was reconstituted by weighing 6.3 mg of powder in a 4-mL test tube and adding 2 mL of H_2O. Simulated intestinal fluid (SIF) containing approximately 1 % (w/v) pancreatin (Sigma Aldrich, St. Louis, MO) was prepared and adjusted to a pH of approximately 7.5 as described in the United States Pharmacopeia (3).

Ninety-five microliter aliquots of SIF were added to 1.5 mL microcentrifuge tubes and placed in a water bath set to 37 °C. After 2 minutes, 5 µL of each protein was then added to the individual tubes and a timer was set. Digestions were performed at time intervals of 1, 5, 15, 30, 60, 90, and 120 minutes for each protein. After each specified incubation interval, a 10-µL sample of each protein/SIF combination was removed and 18 µL of sample buffer (Invitrogen/Novex, Carlsbad, CA) containing fresh β-mercaptoethanol (Sigma Aldrich) was added to stop the reaction. The samples were immediately placed in a heat block set at 100 °C for 5 minutes then placed on wet ice until all of the time points were sampled. For each of the proteins above, an 'undigested control', incubated for 0 min and 120 min at 37 °C was prepared in the same manner except that water was substituted for SIF. These undigested controls were employed to determine the stability of the proteins under non-proteolytic conditions for the duration of the assay. An SIF reagent blank was prepared for each set of digestion reactions to determine enzyme stability during the assay. The digested proteins and their respective controls were stored in a freezer overnight.

For each individual protein sample, the entire sample volume (28 µL) was loaded on-to 4-20% polyacrylamide gels (Owl Separation Systems, Portsmouth, NH). Approximate protein concentrations are listed in Table 1.

The samples were then electrophoresed at 125V for 2 hours and 25 minutes. After separation the gels were stained for 3 hours with Coomassie Brilliant Blue dye (G250, Invitrogen/Novex) and then destained overnight.

RESULTS AND DISCUSION

Based on the presence of the SIF protein bands for both the 0 min (Figures 1-4, Lane 2) and 120 min (Figures 1-4, Lane 3) incubations at 37 °C, the SIF system appeared to be functional for the duration of the test. Similarly, the presence of the undigested protein control bands for both 0 min (Figures 1-4, Lane 4) and 120 min (Figures 1-4, Lane 5) incubations at 37 °C, indicates the test and reference proteins were stable for the length of the test.

The digestibility results for Cry1F, acid phophatase, β-lactoglobulin, and ovalbumin in SIF are summarized in Table 1. The test substance, Cry1F, remained undigested in the SIF for 120 min, the duration of the assay (Figure 1, lanes 6-12). The known non-allergenic protein, acid phosphatase, also remained undigested in SIF for the duration of the study (Figure 2, lanes 6-12). Of the two known protein allergens, one (β-lactoglobulin) was digested after 1 min and the other (ovalbumin) remained stable for the duration of the assay. The β-lactoglobulin protein was visible on the SDS-PAGE gel at the 1 min time point (Figure 3, lane 6), but not at later time points (Figure 3, lanes 7-12). Ovalbumin appeared to break down to a stable smaller molecular weight degradation product at the 1 min time point and remained visible on the SDS-PAGE gel for all subsequent time points of the assay (Figure 4, lanes 6-12).

Based on the results of this study the *in vitro* SIF model is apparently unable to discriminate between known non-allergenic and allergenic proteins using stability to proteolysis as an experimental endpoint; acid phophatase (non-allergenic protein) and ovalbumin (protein allergen) were both stable to digestion for the duration of the study. β-lactoglobulin (protein allergen) was readily digested (i.e., within 5 minutes after addition to SIF).

The Cry1F protein was stable to digestion in SIF for a period of 120 minutes. This property has been reported for a number of other Cry proteins (e.g., Cry1A(b) and Cry3A, [4]) and relates to the well known fact (5) that *Bt* protoxins (130-140 kDa in size) require processing via the alkaline pH and enzymatic conditions of the insect mid-gut to "protease-resistant cores" in order to exert their specific activity. Prior to use in this study, the Cry1F test substance was trypsinolysed to the truncated form ("Test Substance" section of this report), therefore, the observed behavior of the truncated protein in SIF was not unexpected.

The Cry1F protein has previously been shown to be rapidly digested (within 1-5 minutes, depending on pepsin concentration) in an *in vitro* simulated gastric fluid (SGF) model (2). The Cry1F protein shares these proteolytic characteristics in SGF and SIF systems as well as the absence of homology with known allergens with other Cry proteins (e.g., Cry1A(b), and Cry3A)

(4). These Cry proteins are not considered to share the characteristics of known food allergens (4).

CONCLUSION

In conclusion, the *in vitro* simulated intestinal fluid model is apparently unable to discriminate between known non-allergenic and allergenic proteins using stability to proteolysis as an experimental endpoint. Therefore, inferences regarding the potential allergenicity of proteins using this experimental system cannot be drawn. The Cry1F protein remained undigested in SIF for the duration of the assay (120 min). Cry1F shares this, and other characteristics with other Cry proteins that disassociate them from being regarded as potential food allergens.

ACKNOWLEDGMENTS

We would like to thank X. Xu for her technical contributions.

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4. U.S. EPA. 2000. Biopesticides Registration Action Document. *Bacilllus Thuringiensis* Plant Pesticides. U.S. Office of Pesticide Programs.

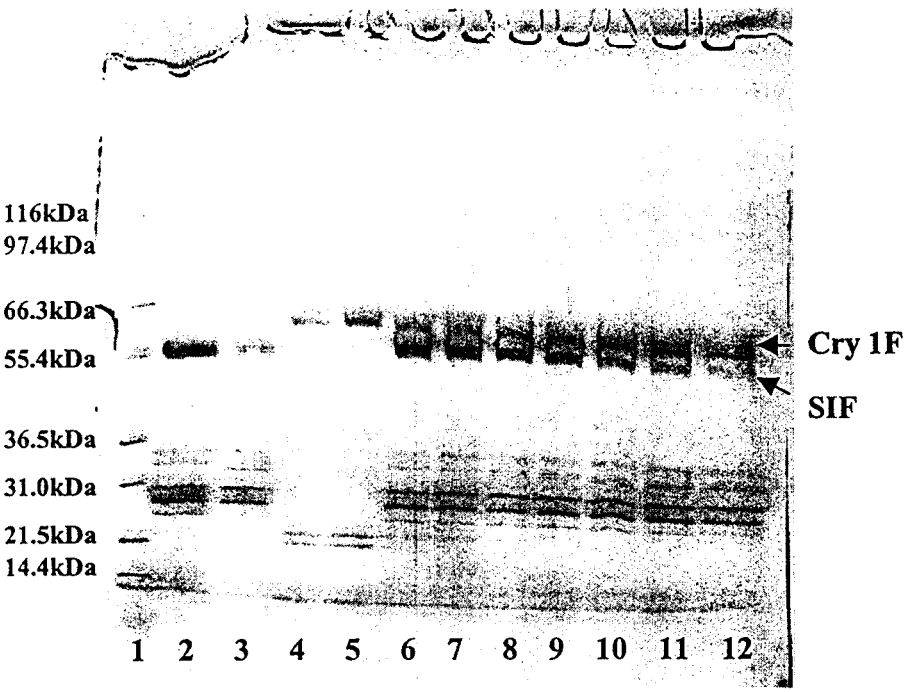
5. Gill, S. S., Cowles, E. A. and Pietrantonio, P. V. 1992. *The Mode of Action of Bacillus Thuringiensis Endotoxins*. Ann. Rev. Entomol., Volume 37: 615-636.

Table 1. Approximate Protein Concentration of Each Test Protein Subjected to SIF Digestion and Subsequently Analyzed by SDS-PAGE

Protein	Concentration of protein by weight (before digestion) added to the digestion reaction.	Concentration of protein by weight (before digestion) loaded in each lane for SDS-PAGE Analysis
Cry1F	~24 µg	~2.4 µg
Acid Phosphatase	~25.5 µg	~2.6 µg
β-lactoglobulin	~13.6 µg	~1.4 µg
Ovalbumin	~15.7 µg	~1.6 µg

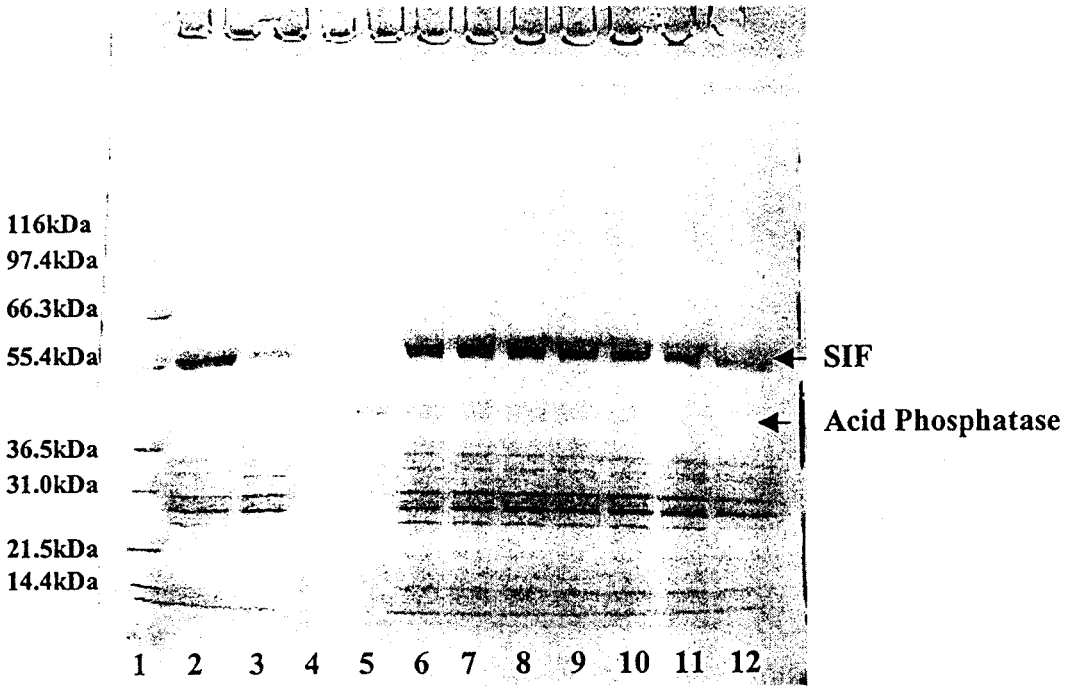
Table 2. *In Vitro* Digestibility Results in Simulated Intestinal Fluid

Known Allergenic Property	Protein	Stability in SIF (minutes)
Test Protein	Cry1F	> 120
Non-Allergen	Acid Phosphatase	> 120
Allergen	Ovalbumin	> 120
Allergen	β-Lactoglobulin	1-5



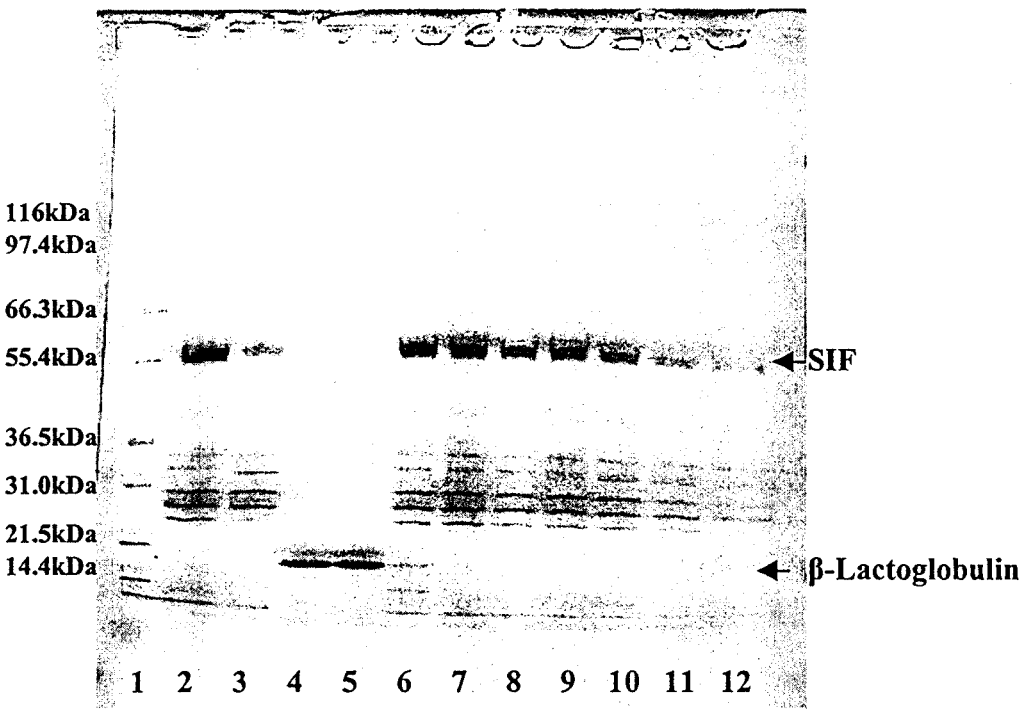
Lane assignments: (1) Mark 12 Wide Range Protein Standard, (2) SIF reagent blank, 0 minute incubation, (3) SIF reagent blank, 120 minute incubation, (4) undigested Cry1F (~2.4 µg protein), 0 minute incubation, (5) undigested Cry1F (~2.4 µg protein), 120 minute incubation, (6) 1 minute digestion, (7) 5 minute digestion, (8) 15 minute digestion, (9) 30 minute digestion, (10) 60 minute digestion, (11) 90 minute digestion, (12) 120 minute digestion.

Figure 1. SDS-PAGE Analysis of Cry1F Protein Subjected to Digestion in Simulated Intestinal Fluid



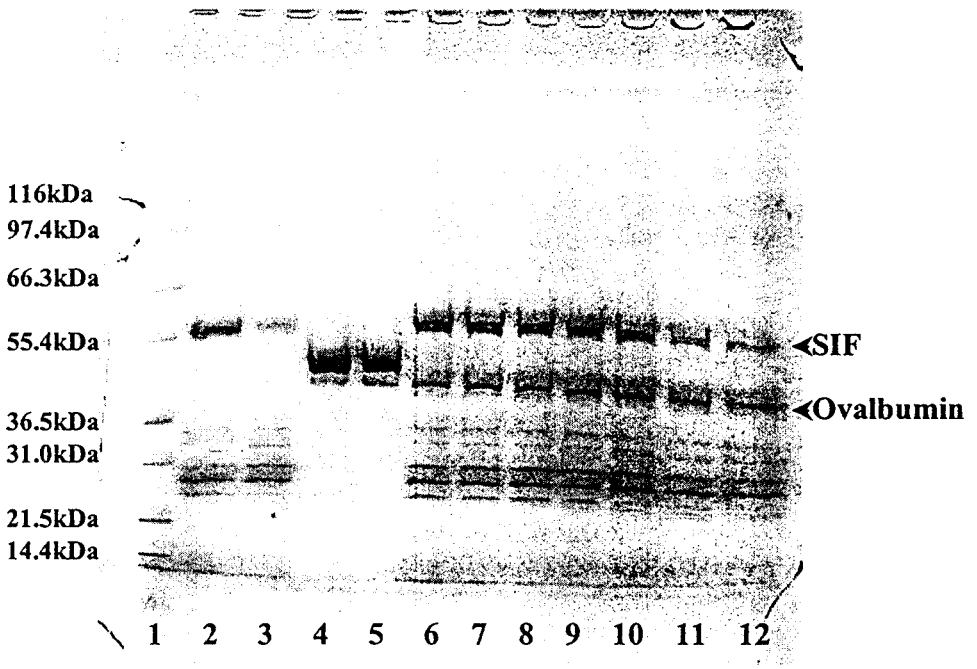
Lane assignments: (1) Mark 12 Wide Range Protein Standard, (2) SIF reagent blank, 0 minute incubation, (3) SIF reagent blank, 120 minute incubation, (4) undigested Acid Phosphatase (~2.6 µg protein), 0 minute incubation, (5) undigested Acid Phosphatase (~2.6 µg protein), 120 minute incubation, (6) 1 minute digestion, (7) 5 minute digestion, (8) 15 minute digestion, (9) 30 minute digestion, (10) 60 minute digestion, (11) 90 minute digestion, (12) 120 minute digestion.

Figure 2. SDS-PAGE Analysis of Acid Phosphatase Protein Subjected to Digestion in Simulated Intestinal Fluid



Lane assignments: (1) Mark 12 Wide Range Protein Standard, (2) SIF reagent blank, 0 minute incubation, (3) SIF reagent blank, 120 minute incubation, (4) undigested β-Lactoglobulin (~1.4 µg protein), 0 minute incubation, (5) undigested β-Lactoglobulin (~1.4 µg protein), 120 minute incubation, (6) 1 minute digestion, (7) 5 minute digestion, (8) 15 minute digestion, (9) 30 minute digestion, (10) 60 minute digestion, (11) 90 minute digestion, (12) 120 minute digestion.

Figure 3. SDS-PAGE Analysis of β-Lactoglobulin Protein Subjected to Digestion in Simulated Intestinal Fluid



Lane assignments: (1) Mark 12 Wide Range Protein Standard, (2) SIF reagent blank, 0 minute incubation, (3) SIF reagent blank, 120 minute incubation, (4) undigested Ovalbumin (~1.6 µg protein), 0 minute incubation, (5) undigested Ovalbumin (~1.6 µg protein), 120 minute incubation, (6) 1 minute digestion, (7) 5 minute digestion, (8) 15 minute digestion, (9) 30 minute digestion, (10) 60 minute digestion, (11) 90 minute digestion, (12) 120 minute digestion.

Figure 4. SDS-PAGE Analysis of Ovalbumin Protein Subjected to Digestion in Simulated Intestinal Fluid