

Study Title

Phosphinothricin acetyltransferase (PAT) protein:
In Vitro Digestibility Study

Laboratory Project ID: DuPont-3365

Volume 4.

AUTHOR: [REDACTED]

STUDY COMPLETED ON: September 9, 1999

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
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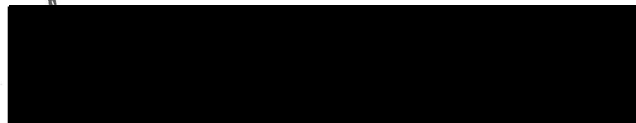
STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

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

These data are the property of Mycogen Seeds c/o Dow AgroSciences, and as such, are considered to be confidential for all purposes other than compliance with FIFRA §10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under any other statute or in any other country.

Company: Mycogen Seeds c/o Dow AgroSciences

Company Agent:



Registration Manager

Date:

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. FDA (21 CFR 58) Good Laboratory Practice for Nonclinical Laboratory Studies and EPA FIFRA (40 CFR 160), EPA TSCA (40 CFR 792) Good Laboratory Practice Standards, OECD Principles of Good Laboratory Practice (C(81)30(Final), Annex 2) and updated version (OCDE/GD(92)32), and MAFF Japan Good Laboratory Practice Standards (59 NohSan No. 3850), except for the item documented below. The item listed does not impact the validity of the study.

The test substance was characterized by the sponsor prior to the initiation of this study. Although the characterization was performed at a non-GLP-compliant laboratory, the accuracy of the data is considered sufficient for the purposes of this study.

Submitter: Mycogen Seeds c/o Dow AgroSciences
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San Diego, CA 92121-1718

Sponsor: Pioneer Hi-Bred International, Inc.
7250 NW 62nd Avenue
P. O. Box 552
Johnston, IA 50131

Study Director

[REDACTED]

Associate Scientist

9-Sep-1999
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QUALITY ASSURANCE STATEMENT

Haskell Sample Number(s):

24214

Dates of Inspections:

Conduct: August 18, 1999

Records, Reports: August 24, 1999

Dates Findings Reported to:

Study Director: August 25, 1999

Management: August 26, 1999

Reported by:

[REDACTED]

Quality Assurance Auditor

9-SEPT.-1999
Date

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CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Approved by Study
Submitter:

[REDACTED]

9/3/99
Date

Approved by Study
Sponsor:

[REDACTED]

9/8/99
Date

Issued by Study
Director:

[REDACTED]

9/9/99
Date

Associate Scientist

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STUDY INFORMATION

Substance Tested: PAT microbial protein (FL)

Synonyms/Codes:

- TSN101850
- PAT
- 1669:66-124 (Manufacturer's Lot No.)

Haskell Number: 24214

Composition: 84% PAT microbial protein
16% Proprietary ingredients

Known Impurities: Not supplied by the sponsor

Physical Characteristics: White solid

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Sponsor: Pioneer Hi-Bred International, Inc.
Johnston, IA 50131
U.S.A.

Study Initiated/Completed: 16-Aug-1999 / (see report cover page)

In-Life Initiated/Completed: 18-Aug-1999 / 24-Aug-1999

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STUDY PERSONNEL

Study Director: [REDACTED]

Management: [REDACTED]
[REDACTED]

Primary Technician: [REDACTED]

Toxicology Report Preparation: [REDACTED]

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SUMMARY

PAT microbial protein (H-24214) was mixed with simulated gastric fluid (SGF) containing proteolytic pepsin and digestion was evaluated out at a constant temperature. At various time points, aliquots of the digestion mixture were neutralized. Neutralized samples were denatured and subjected to electrophoresis. Proteins and digested fragments (if present) that were separated electrophoretically were visualized by staining with Coomassie. Bovine serum albumin (BSA) and β -lactoglobulin (β -lac) were chosen as positive and negative controls, respectively. Controls were treated under the same conditions as the test substance, however, selected time points were used.

Name of protein H-24214: PAT microbial protein
Amount of protein H-24214 in digestion: 8 μ g (assuming 100% solubility)
Name of positive control BSA
Amount of positive control in digestion 50 μ g
Name of negative control β -lac
Amount of negative control in digestion 50 μ g

Duration of Digestion

	Time 1	Time 2	Time 3	Time 4	Time 5	Time 6	Time 7
H-24214 (PAT)	5 sec	10 sec	20 sec	30 sec	1 min	5 min	10 min
PositiveControl (BSA)	5 sec	15 sec	1 min	ND	ND	ND	ND
Negative Control (β -lac)	1 min	5 min	10 min	ND	ND	ND	ND

sec: second
min: minute
ND: not determined

PAT was found to degrade below detectable levels within five seconds (Figure 1). All proteins and fragments in the gel were visualized by Coomassie staining. As expected, BSA, the positive control, was digested by the 1 minute time point (note the visibility of small fragments) and β -lactoglobulin, the negative control, was not digested within 10 minutes (Figure 2).

Under the condition of this study, PAT microbial protein was completely digested within 5 seconds in the simulated gastric condition thus indicating very low stability in the simulated gastric environment.

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INTRODUCTION

This purpose of this study was to evaluate the stability of the PAT microbial protein, in a simulated gastric model at various time points. The ability of food allergens to reach and cross the mucosal membrane of the intestinal tract is likely a prerequisite of food allergenicity.^(1,2) Clearly, a protein which is stable to the proteolytic and acidic conditions of the digestive tract has an increased probability of reaching the intestinal mucosa.^(1,2) Intact proteins are capable of crossing the mucosal membrane of the gut and entering the circulatory system.⁽³⁾ Thus, physiochemical properties that result in digestive stability can be used as an important indicator of allergenic potential.⁽²⁾ The converse is also true; properties that favor digestive instability can be used as indicators of a lesser allergenic potential. Allergenic proteins are typically 10-70 kD in molecular mass and often glycosylated. However, many proteins that are not allergenic share these two properties. Hence, they are not useful indicators of allergenicity.⁽²⁾ The *in vitro* digestibility study is designed to create a simulated gastric condition as a model of stomach digestion. Its purpose is to measure the presence of the intact protein at various time points during the digestion so that the stability of the protein in the gastric model can be assessed.

MATERIALS AND METHODS

A. Test Substance and Controls

The test substance, PAT microbial protein, was supplied by the sponsor as a white powder. It was determined by the sponsor that PAT is 85% soluble in water at a 1 mg/ml concentration. The test substance (2 mg) was added to water (0.25 ml) for digestibility testing. At this concentration the test substance was in suspension. A pilot study was conducted to determine a suitable concentration for detection with Coomassie stain and to determine the appropriate gel concentration to use for sufficient resolution. The concentration of PAT that was determined to be suitable for a working stock solution in the assay was 8 mg/ml resulting in a concentration of 0.4 mg/ml in each digestion time point. The selected gel was a 15% Tris/Glycine gel. The test substance was inverted and briefly vortexed to mix and maintain homogeneity before each aliquot for digestion was removed. Both the positive control (bovine serum albumin (BSA)) and negative control (β -lactoglobulin (β -lac)) were prepared at a stock concentration of 10 mg/ml in water to mimic the test substance conditions during the assay. The test substances and controls appeared to be stable under the conditions of the study. No evidence of instability, such as a change in color or physical state, was observed. Moreover, repeating the assay the next day resulted in similar staining intensities of the test substance control bands.

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B. Study Design

For each respective time point, 8 μ g (1 μ l) of PAT was mixed with simulated gastric fluid (SGF) at a pH of approximately 2.0 and containing approximately 0.3% pepsin (weight/volume) in 20 μ l total volume. The digestion was carried out at 37°C. After the appropriate incubation time, 8 μ l of cold neutralizing buffer (0.16 M sodium carbonate) was added and the digestion mixture was immediately placed on ice. For the controls, 50 μ g (5 μ l) of either BSA or β -lactoglobulin was mixed with SGF in a 100- μ l total volume. The digestion was carried out at 37°C. After the appropriate incubation times, 10- μ l aliquots were removed and mixed with 4 μ l of cold neutralizing buffer and placed on ice. All samples were neutralized with 1 volume of loading buffer containing sodium dodecyl sulfate (SDS), β -mercaptoethanol, Tris-HCl, glycerol, and bromophenol blue.

Duration of Digestion

	Time 1	Time 2	Time 3	Time 4	Time 5	Time 6	Time 7
H-24214 (PAT)	5 sec	10 sec	20 sec	30 sec	1 min	5 min	10 min
PositiveControl (BSA)	5 sec	15 sec	1 min	ND	ND	ND	ND
Negative Control (β -lac)	1 min	5 min	10 min	ND	ND	ND	ND

sec: second

min: minute

ND: not determined

For each protein (positive control, negative control, and test substance) an 'undigested control' was prepared by placing the same amount of protein as stated above into distilled and deionized water instead of SGF. Then, the appropriate amount of cold sodium carbonate was added to the 'undigested' mixture and kept on ice. All samples were then neutralized with 1 volume of loading buffer.

In addition, for the test substance protein, an 'inactivated SGF undigested control' was prepared by placing the same amount of protein as stated above into SGF that has been inactivated. This allows one to display a separation between the undigested test substance band and the pepsin band. To inactivate the SGF, 19 μ l was pretreated with 8 μ l of Na₂CO₃ and 28 μ l of loading buffer.

These two sample controls, 'undigested control' and 'inactivated SGF undigested control', also served as controls to verify that having a stock solution of 8 mg/ml, which was in suspension, was a viable approach to handling the test material in this assay.

All samples were then heat-denatured at 95°C \pm 2°C for 3 to 5 minutes. For the test substances, one-half of the sample volume (28 μ l) was subjected to electrophoresis on a 15% Tris-glycine-SDS gel. All proteins and digested fragments (if present) in the gel were stained with Coomassie blue protein stain (GelCode Blue - Pierce) for visualization.

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For the controls, the entire sample volume (28 μ l) was subjected to electrophoresis on a Tris-glycine-SDS gel. All proteins and digested fragments (if present) in the gel were visualized by Coomassie blue staining. A digital image of the gels was obtained in order to photographically capture the data. The gels were then dried (Promega Gel Drying Kit) and stored in the study records. The experiment was conducted twice for each protein sample to ensure reproducibility.

RECORDS AND SAMPLE STORAGE

Specimens (if applicable), raw data, and the final report will be retained at DuPont Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware, 19802.

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RESULTS AND DISCUSSION

PAT was found to degrade below detectable levels within five seconds (Figure 1). Intermittent trace fragments are visible at five seconds and are gone by ten seconds. All proteins and fragments in the gel were visualized by Coomassie staining. As expected, BSA, the positive control, was digested by the 1 minute time point (note the visibility of small fragments) and β -lactoglobulin, the negative control, was not digested within 10 minutes (Figure 2).

CONCLUSIONS

Under the condition of this study, PAT microbial protein was completely digested within 5 seconds in the simulated gastric condition thus indicating very low stability in the simulated gastric environment.

REFERENCES

1. Astwood, J.D., J.N. Leach, and R.L. Fuchs. Stability of food allergens to digestion *in vitro*. Nature Biotechnology (1996), 14: 1269-73.
2. Astwood, J.D., and R.L. Fuchs. Allergenicity assessment of foods derived from genetically modified plants. Food Technology (1996), 83-8.
3. Gardner, M.L.G. Gastrointestinal absorption of intact proteins. Annual Reviews of Nutrition (1988), 8: 329-50.

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FIGURES

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FIGURE 1

SDS-PAGE AND COOMASSIE ANALYSIS OF PAT MICROBIAL PROTEIN DIGESTED IN SIMULATED GASTRIC FLUID

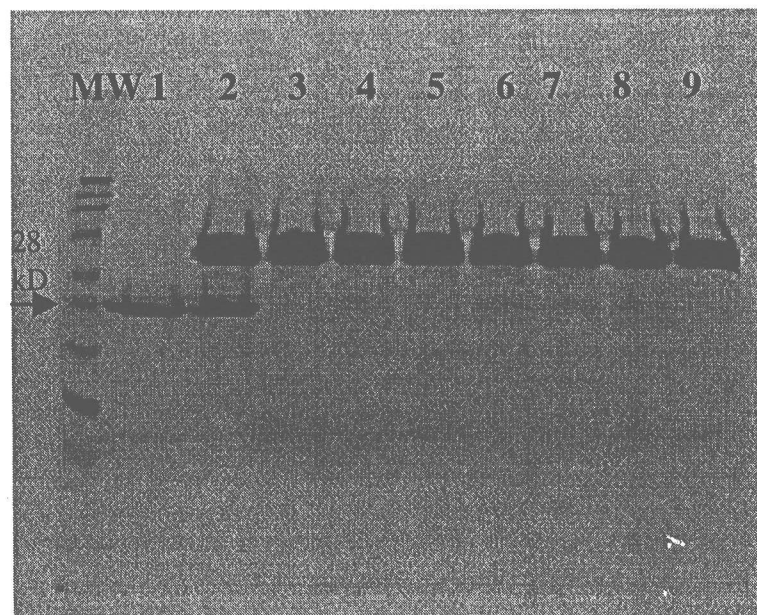
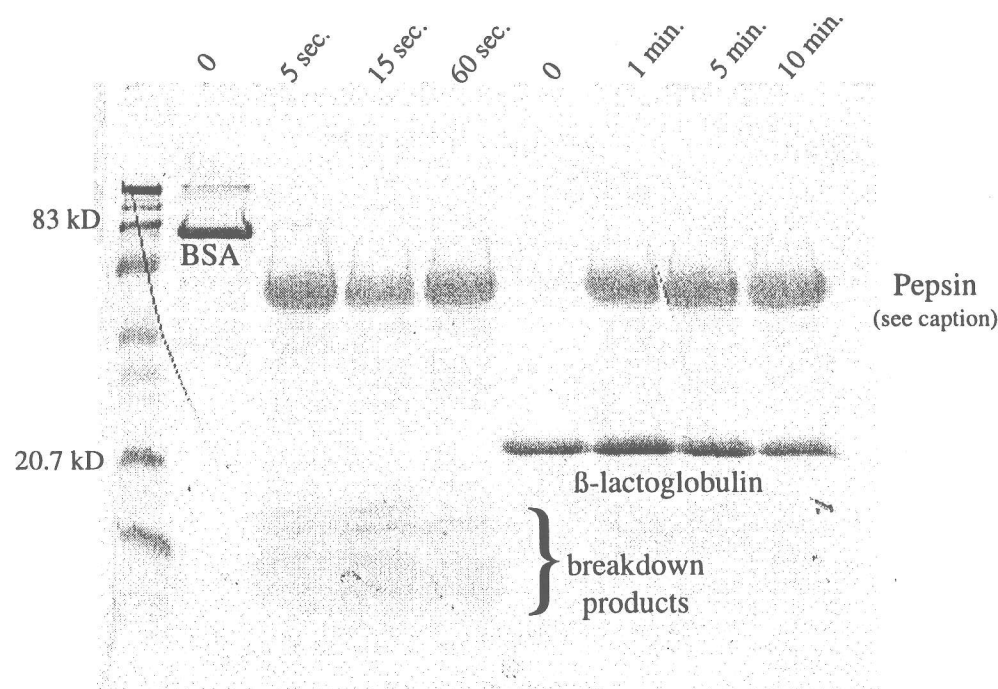


FIG. 1. SDS-PAGE and Coomassie stain analysis of PAT microbial protein samples digested in simulated gastric fluid. Lane 1: undigested control (~4 μ g); Lane 2: undigested control (~4 μ g) with inactivated SGF. Lanes 3 through 9 are individual assay time points: Lane 3: 5 seconds; Lane 4: 10 seconds; Lane 5: 20 seconds; Lane 6: 30 seconds; Lane 7: 1 minute; Lane 8: 5 minutes; Lane 9: 10 minutes. Lane MW: broad range molecular weight markers (Bio-Rad). The 28-kD arrow denotes the molecular weight marker that the test protein (starting material) eluted closest to.

FIGURE 2

SDS-PAGE AND COOMASSIE STAIN ANALYSIS OF BSA AND β -LAC DIGESTED IN SIMULATED GASTRIC FLUID



Positive (BSA "digestible") and negative (β -lactoglobulin "stable") controls. After incubating the proteins in a simulated gastric fluid containing pepsin, samples were run on a 15% Tris/HCl polyacrylamide gel for approximately 1 hour at 35 mA. As seen in the figure above, BSA was digested within 5 seconds. The β -lactoglobulin was still undigested at 10 minutes indicating a potential allergen. This figure is representative of what was actually observed in the current assay.