Study Title:

In Vitro Digestibility of Phosphomannose Isomerase (PMI) as Contained in Test Substance PMI-0105 Under Simulated Mammalian Gastric Conditions

Amended Report No. 1
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Author:

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Study No. PMI-07-03

Report No. SSB-034-07 A1

Performing Laboratory:

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STATEMENTS OF DATA CONFIDENTIALITY CLAIMS

1) The following statement applies to submissions to the Environmental Protection Agency in the United States of America.

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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: Syngenta Seeds, Inc. – Field Crops - NAFTA

Company Representative: Scott Huber

Title: Regulatory Affairs Manager

Signature: Scott Huber Date: 10/23/09

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STATEMENT CONCERNING GOOD LABORATORY PRACTICES

This study was conducted in compliance with the relevant provisions of Good Laboratory Practice Standards (U.S. EPA 1989) pursuant to the Federal Insecticide, Fungicide and Rodenticide Act, and subsequent revisions.

STUDY DIRECTOR:

Andrea Nelson — 10/23/09
Date

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QUALITY ASSURANCE STATEMENT

Study Title: *In Vitro* Digestibility of Phosphomannose Isomerase (PMI) as Contained in Test Substance PMI-0105 Under Simulated Mammalian Gastric Conditions

Study Director: Andrea Nelson

Study Number: PMI-07-03

Pursuant to Good Laboratory Practice Regulations (40 CFR Part 160), this statement verifies that the aforementioned study was inspected and/or audited and the findings reported to Management and to the Study Director by the Quality Assurance Unit on the dates listed below.

Inspection/Audit Type	Inspection/Audit Dates	Reporting Date
Audit Protocol	October 5, 2007	October 5, 2007
In-Progress Inspection	October 18, 2007	October 18, 2007
Final Report Audit (1st audit)	February 8 & 13, 2008	February 13, 2008
Final Report Audit (2nd audit)	March 31, 2008	March 31, 2008

Prepared by: Kimberly W. Hill Date: Oct. 23, 2009

Kimberly W. Hill
Senior Quality Assurance Auditor
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TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

Bis-Tris	bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane
EDTA	ethylenediaminetetraacetic acid
IgG	immunoglobulin G
LDS	lithium dodecyl sulfate
MES	2-(*N*-morpholino)ethanesulfonic acid
min	minute(s)
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SGF	simulated mammalian gastric fluid
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
Tween 20	polyoxyethylene sorbitan monolaurate

REPORT AMENDMENTS

AMENDMENT NO. 1: October 9, 2009

This amended report adds the description that no statistical analysis was performed for any parameter evaluated in the study and that no circumstances occurred during the conduct of the study that adversely affected the quality or integrity of the data generated. This change was made on page 9 of this report and affects the same page of the original report SSB-034-07. The corrected page in this amended report SSB-034-07 A1 is indicated as “REVISED”.

Syngenta Biotechnology, Inc.
Regulatory Science
Research Triangle Park, NC, USA

Report No. SSB-034-07 A1

In Vitro Digestibility of Phosphomannose Isomerase as Contained in Test Substance PMI-0105 Under Simulated Mammalian Gastric Conditions

SUMMARY

The susceptibility of phosphomannose isomerase (PMI) to proteolytic degradation in simulated mammalian gastric fluid containing pepsin was evaluated using sodium dodecylsulfate polyacrylamide gel electrophoresis and Western blot analysis. Phosphomannose isomerase from recombinant *Escherichia coli* was readily degraded in simulated mammalian gastric fluid. No intact phosphomannose isomerase (molecular weight approximately 42.8 kDa) or degradation products were detected by Western blot analysis following incubation in simulated mammalian gastric fluid for 1 minute.

The results of this study support the conclusion that phosphomannose isomerase protein will be readily digested under conditions representative of typical mammalian gastric conditions.

INTRODUCTION

The purpose of this study was to assess the *in vitro* digestibility of phosphomannose isomerase (PMI) in simulated mammalian gastric fluid (SGF). PMI catalyzes the reversible inter-conversion of mannose 6-phosphate and fructose 6-phosphate and has utility as a selectable marker for transformation of many plant species (Bojsen et al. 1994; Joersbo et al. 1998; Negrotto et al. 2000). Plant cells that have been transformed with the *Escherichia coli manA* gene (Miles and Guest 1984) and produce PMI protein are able to utilize mannose as a carbon source.

Test substance PMI-0105, a purified preparation of microbially produced PMI, was used as the source of test protein in this study. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were used to assess the digestibility of PMI in SGF over a 60 minute time course at 37°C.

MATERIALS AND METHODS

Test Substance PMI-0105

Test substance PMI-0105 was produced prior to this study in an *E. coli* over-expression system (Attenborough 2005). The *manA* gene was cloned into the inducible, over-expression pET-24a vector (Novagen; Madison, WI) and this vector was transformed into *E. coli* strain BL21(DE3)RP (Stratagene; La Jolla, CA, USA). The PMI in test substance PMI-0105 has the same amino acid sequence as the endogenous PMI protein produced in *E. coli*. The same PMI protein is used as a selectable marker in a number of Syngenta's transgenic plant events.

Test substance PMI-0105 was prepared from pooled batches of *E. coli* cell paste by Syngenta Protein Science (Jealott's Hill, Bracknell, Berkshire, UK) and shipped on dry ice to the Regulatory Science laboratory (Syngenta Biotechnology, Inc.) and stored at -20 ± 8°C until further use.

Test substance PMI-0105 was characterized in detail and was determined to contain 89.5% PMI by weight and the molecular weight of the PMI was 42.8 kDa (Nelson 2008).

To prepare the test protein solution for this study, the lyophilized test substance PMI-0105 was resuspended in 50 mM Tris buffer (pH 7.0) to produce a 5 mg PMI/ml solution which was subsequently used in the SGF assessment.

Simulated Mammalian Gastric Fluid

Simulated mammalian gastric fluid (USP 2000) containing NaCl at 2 mg/ml, 2,600 units pepsin/ml (Sigma-Aldrich Cat. No. P6887) and pH 1.2 ± 0.05 (adjusted with 6N HCl) was freshly prepared. Immediately before use, proteolytic activity was confirmed using azoalbumin as the substrate. SGF without pepsin was prepared for use as a negative control.

Time Course of Digestion in SGF

Except in the case of the time-zero sample, the test protein solution was mixed with the SGF solution to give a final ratio of approximately 10 pepsin activity units per µg of PMI (Thomas et al. 2004) and incubated at 37 ± 2°C. The SGF-test protein mixture was sampled after 1, 2, 5, 10, 30, and 60 min incubation. At each sampling point, an aliquot of SGF-test protein mixture was removed, and the reaction was quenched by addition of stop solution containing 200 mM sodium bicarbonate, 4X NuPage LDS Sample Buffer (Invitrogen Cat. No. NP0007) and 10X Sample Reducing Agent (Invitrogen Cat. No. NP0009) followed by heating at approximately 95 ± 5°C for 10 min. For the time-zero sample, an aliquot of the test protein solution was added to a mixture of SGF and stop solution (at the same ratio of PMI to SGF as described above), and the mixture was heated at 95 ± 5°C for 10 min.

For negative controls, an aliquot of the test protein solution added to the SGF without pepsin was prepared in order to monitor the stability of the test protein over the 60 min

time course. Additionally, an aliquot of 50 mM Tris buffer (pH 7.0) was added to SGF in order to monitor the stability of pepsin over the 60 min time course. Both controls were sampled at 0 and 60 min and further treated as described above.

Samples produced from the above reactions were stored at –20 ± 8°C until used for SDS-PAGE and Western blot analyses.

SDS-PAGE Analysis
Volumes equivalent to 2.5 µg of PMI based upon the initial concentration in the control and reaction samples were subjected to SDS-PAGE with a NuPAGE 4%–12% Bis-Tris polyacrylamide gradient gel (Invitrogen Cat. No. NP0322) and NuPAGE MES SDS running buffer (Invitrogen Cat. No. NP0002). SeeBlue Plus2 Pre-Stained Standard (Invitrogen Cat. No. LC5925) was used as the molecular weight standard. The gel was stained with Coomassie (Pierce Cat. No. 24592) and examined for the presence of bands corresponding to intact PMI (approximately 42.8 kDa) and PMI-derived polypeptides.

Western Blot Analysis
Volumes equivalent to 10 ng of PMI based upon the initial concentration in the control and reaction samples were subjected to SDS-PAGE with a NuPAGE 4%–12% Bis-Tris polyacrylamide gradient gel and NuPAGE MES SDS running buffer. After electroblotting, the polyvinylidene difluoride (PVDF) membrane was incubated with goat polyclonal antiserum specific for PMI (purified at Syngenta Biotechnology, Inc. (SBI), Research Triangle Park, NC, USA). Alkaline phosphatase–conjugated donkey anti-goat IgG (Jackson ImmunoResearch Cat. No. 705055147) diluted 1:3000 in Tris-buffered saline with Tween 20 (Sigma Cat. No. T9039) was used to bind to the primary antibody and visualized by development with alkaline phosphatase substrate solution (Sigma Cat. No. B1911). SeeBlue Plus2 Pre-Stained Standard was used as the molecular weight standard. The Western blot was examined for the presence of intact immunoreactive PMI or other immunoreactive PMI-derived polypeptides.

Statistical Methods
Statistical analyses were not required for any parameters evaluated in this study.

RESULTS

No circumstances occurred during conduct of this study that would have adversely affected the quality or integrity of the data generated.

Following exposure to SGF for 1 min, no intact PMI (approximately 42.8 kDa molecular weight) was evident, as assessed by SDS-PAGE and Western blot analysis (Figures 1 and 2, Lane 7). Faint, diffuse bands of lower molecular weight (approximately 4 kDa) were visible on SDS-PAGE starting at 1 min, diminished in intensity in the 2 min sample and were not detectable in the 5 min sample and later time points (Figure 1, Lanes 7 through 12). These bands did not cross-react with the anti-PMI antibody, as shown on the corresponding Western blot (Figure 2, Lanes 7 through 12).

SGF controls without pepsin showed no significant degradation of PMI over the 60-min time course (Figures 1 and 2, Lanes 3 and 4). Incubation of the SGF solution alone for 60 min showed no significant degradation of pepsin (Figure 1, Lanes 1 and 2).

CONCLUSIONS

The results of this study support the conclusion that PMI protein will be readily digested under conditions representative of typical mammalian gastric conditions.

RECORDS RETENTION

Raw data, the original copy of this report, and other relevant records are archived at Syngenta Biotechnology, Inc., 3054 East Cornwallis Rd., Research Triangle Park, NC, USA 27709.

STUDY PERSONNEL

Analytical work reported herein was conducted by Andrea Nelson, B.S. at Syngenta Biotechnology, Inc., Regulatory Science laboratory, 3054 East Cornwallis Rd., Research Triangle Park, NC, USA 27709.

CRITICAL DATES

Study initiation date: October 15, 2007
Experimental start date: October 18, 2007
Experimental end date: October 25, 2007

Reported by: Andrea Nelson — 10/23/09

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Regulatory Scientist, Regulatory Science

Date

Approved by: David Patton — October 23, 2009

David Patton,
Group Leader, Regulatory Science

Date

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Figure 1. SDS-PAGE analysis of PMI (test substance PMI-0105) following digestion in SGF

Lanes 1 and 2: SGF only incubated for 0 and 60 min, respectively.
Lanes 3 and 4: PMI incubated in SGF without pepsin for 0 and 60 min, respectively.
Lane 5: molecular weight standard.
Lane 6, 7, 8, 9, 10, 11 and 12: PMI incubated in SGF for time 0, 1, 2, 5, 10, 30 and 60 min, respectively.

The molecular weight of pepsin corresponds to approximately 35 kDa.
The molecular weight of PMI corresponds to approximately 42.8 kDa.

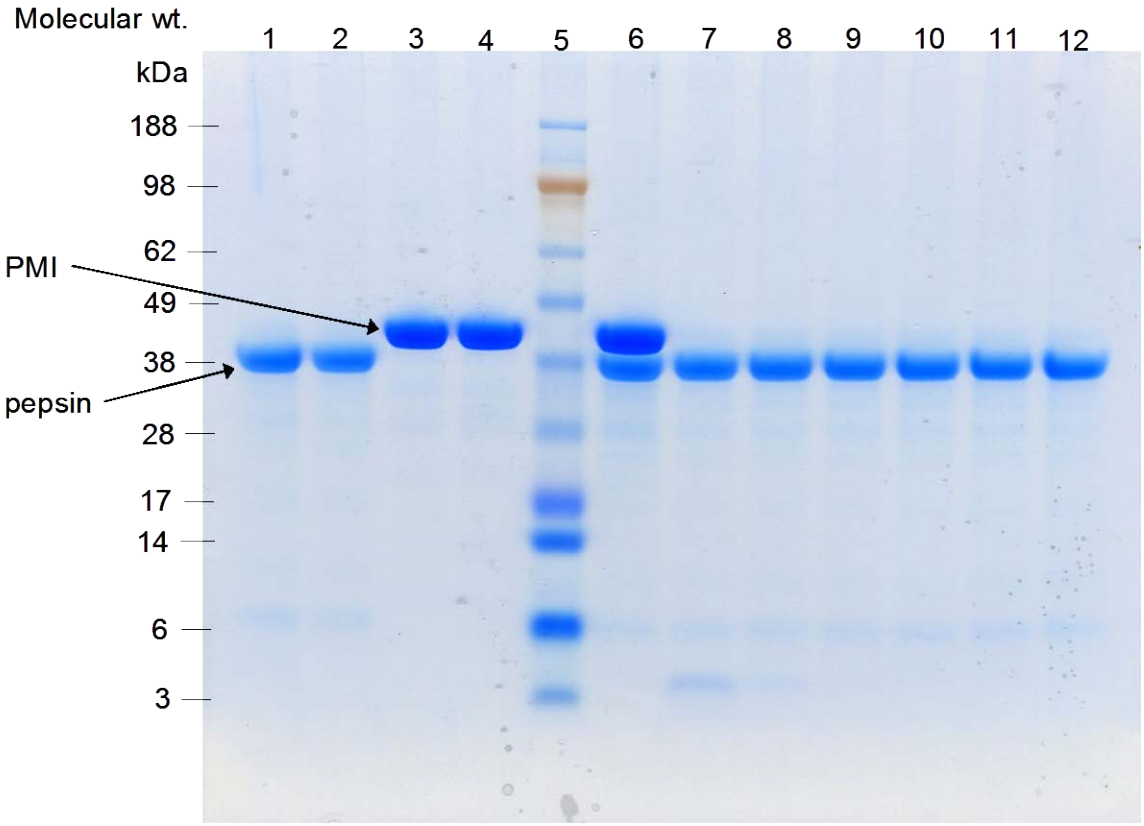

Figure 2. Western blot analysis of PMI (test substance PMI-0105) following digestion in SGF

Lanes 1 and 2: SGF incubated for 0 and 60 min, respectively.
Lanes 3 and 4: PMI incubated in SGF without pepsin for 0 and 60 min, respectively.
Lane 5: molecular weight standard.
Lane 6, 7, 8, 9, 10, 11 and 12: PMI incubated in SGF for time 0, 1, 2, 5, 10, 30 and 60 min, respectively.

The molecular weight of pepsin corresponds to approximately 35 kDa.
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