CONFIDENTIAL

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

Data requirement:	Not applicable
Author:	Andrea Nelson
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Report No.:	SSB-036-07
Performing Laboratory:	Syngenta Biotechnology, Inc. Regulatory Science and Trait Safety PO Box 12257 3054 East Cornwallis Rd. Research Triangle Park, NC 27709-2257, USA

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Company: *Syngenta Seeds, Inc.*

Company Representative:

Wendelyn Jones — 9/24/09
Regulatory Affairs Manager — Date

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STATEMENT CONCERNING GOOD LABORATORY PRACTICES

With the exceptions noted below, 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. The purity and solubility data for test substance PMI-0105 used in this study were obtained from a separate, ongoing GLP test substance characterization study for which the data had been audited prior to the initiation of the present study. The report for the PMI-0105 test substance characterization study was finalized after the initiation of the present study. The characterization of the Invitrogen SeeBlue Plus2 molecular weight standard was not conducted according to GLP.

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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 Intestinal Conditions

Study Director: Andrea Nelson

Study Number: PMI-07-04

Report Number: SSB-036-07

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 25, 2007	October 25, 2007
In-Progress Inspection	November 13, 2007	November 14, 2007
Final Report Audit (1st Audit)	May 22, 2008	May 23, 2008
Final Report Audit (2nd Audit)	October 29, 2008	October 29, 2008
Final Report Audit (3rd Audit)	September 10, 2009	September 10, 2009

Prepared by: Kimberly W. Hill Date: Sept. 10, 2009

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Bis-Tris	bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane
IgG	immunoglobulin G
LDS	lithium dodecyl sulfate
MES	2-(*N*-morpholino)ethanesulfonic acid
N	normal
PMI	phosphomannose isomerase
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SIF	simulated mammalian intestinal fluid
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
Tween 20	polyoxyethylene sorbitan monolaurate

SUMMARY

The susceptibility of phosphomannose isomerase (PMI) to proteolytic degradation in simulated mammalian intestinal fluid containing pancreatin was evaluated using sodium dodecylsulfate polyacrylamide gel electrophoresis and Western blot analysis. PMI from recombinant *Escherichia coli* was readily degraded in simulated mammalian intestinal fluid. The majority of intact PMI was digested after 5 minutes of incubation in simulated mammalian intestinal fluid and no intact PMI or degradation products were detected by Western blot analysis upon sampling of the reaction mixture after 15 minutes of incubation.

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

INTRODUCTION

The purpose of this study was to assess the *in vitro* digestibility of phosphomannose isomerase (PMI) in simulated mammalian intestinal fluid (SIF). 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 a 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 SIF over a 48-hour 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 and this vector was transformed into *E. coli* strain BL21(DE3)RP. The PMI in test substance PMI-0105 is predicted to be identical in amino acid sequence to the endogenous PMI protein produced in *E. coli* strain K12. 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 determined to be 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 SIF assessment.

Simulated Mammalian Intestinal Fluid

Simulated mammalian intestinal fluid (USP 1990) was freshly prepared and contained 50 mM potassium phosphate monobasic, 38 mM sodium hydroxide, 10 mg/ml pancreatin (Sigma-Aldrich Cat. No. P7545) and the pH was adjusted to 7.5 ± 0.05 using 0.2 N sodium hydroxide. Immediately before use, the proteolytic activity of pancreatin was confirmed using azoalbumin as the substrate. SIF without pancreatin was prepared for use as a negative control.

Time Course of Digestion in SIF

Except in the case of time-zero samples, the test protein solution was mixed with the SIF solution to give a final ratio of approximately 38 µg of pancreatin per 1 µg of PMI and incubated at 37°C ± 2°C. The SIF-test protein mixture was sampled after 1, 2, 5, 15, 30, and 60 minutes and 2, 3, 6, 24 and 48 hours of incubation. At each time point, an aliquot of SIF-test protein mixture was removed, and the reaction was quenched by adding it to a solution containing LDS Sample Buffer and Sample Reducing Agent that was preheated at 95 ± 5°C for 5 minutes. The sample was subsequently heated at 95 ± 5°C for 10 minutes. For the time-zero sample an aliquot of the SIF solution and an aliquot of the test protein solution (at the same ratio of pancreatin to PMI as described above) were added directly to the preheated LDS Sample Buffer and Sample Reducing Agent and incubated at 95 ± 5°C for 10 minutes.

As a negative control, an aliquot of the test protein solution added to the SIF without pancreatin was prepared in order to monitor the stability of the test protein over the 48-hour time course. Additionally, an aliquot of 50 mM Tris pH 7.0, which was the buffer used to produce the test protein solution, was added to the SIF in order to monitor the stability of pancreatin over the 48-hour time course. Both controls were sampled at 0, 2 and 48 hours and further treated as described above.

Samples produced from the above reactions were stored at –20°C ± 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 4%–12% Bis-Tris polyacrylamide gradient gel and MES running buffer. The molecular-weight standard was SeeBlue Plus2 pre-stained standard. The gel was stained with Coomassie blue and examined for the presence of bands corresponding to intact PMI (approximately 42.8 kDa) and PMI-derived fragments.

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 4%–12% Bis-Tris polyacrylamide gradient gel and MES running buffer. SeeBlue Plus2 pre-stained standard was used as the molecular weight standard. After electroblotting, the PVDF membrane was incubated with 1 µg/ml goat polyclonal antiserum specific for PMI. Alkaline phosphatase–conjugated donkey anti-goat IgG diluted to 1:3000 in Tris-buffered saline solution with Tween 20 was used to bind to the primary antibody and was visualized by development with alkaline phosphatase substrate solution. The Western blot was examined for the presence of intact immunoreactive PMI or other immunoreactive PMI-derived fragments.

Statistical Analysis

No statistical analysis was required for any parameter evaluated in this study.

RESULTS AND DISCUSSION

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

Following exposure to SIF for 15 minutes, no intact PMI (approximately 42.8 kDa molecular weight) or degradation products were evident, as assessed by Western blot analysis (Figure 2, Lane 12). The analysis of the SDS polyacrylamide gel showed that the SIF contained a protein that co-migrated with PMI which made it difficult to distinguish between the two proteins. This made it difficult to clearly follow the degradation of PMI on the gel. However, the intensity of the co-migrating bands appeared to diminish significantly after 15 minutes of incubation (Figure 1, Lanes 8-12). The diminished intensity was most likely the result of the digestion of PMI in the SIF. This result was confirmed by Western blot analysis which showed that an immunoreactive PMI band was visible at 1, 2, and 5 minutes (Figure 2, Lanes 9, 10, and 11) and completely disappeared after 15 minutes (Figure 2, Lane 12). Incubation of the SIF control solution for 48 hours resulted in significant degradation of the pancreatin itself, as shown by the decreasing intensity of the protein bands (Figure 1, Lanes 5, 6 and

7). The 48-hour SIF control showed faint bands of higher molecular weights when compared to the zero time point of the SIF control (Figure 1, Lanes 5 and 7). These unexpected higher molecular weight bands could be explained by dimerization of some proteins found in the pancreatin over the time course of the experiment (Figure 1, Lanes 7 and 19). SIF controls without pancreatin showed no significant degradation of PMI over the 48-hour time course (Figures 1 and 2, Lanes 2, 3 and 4).

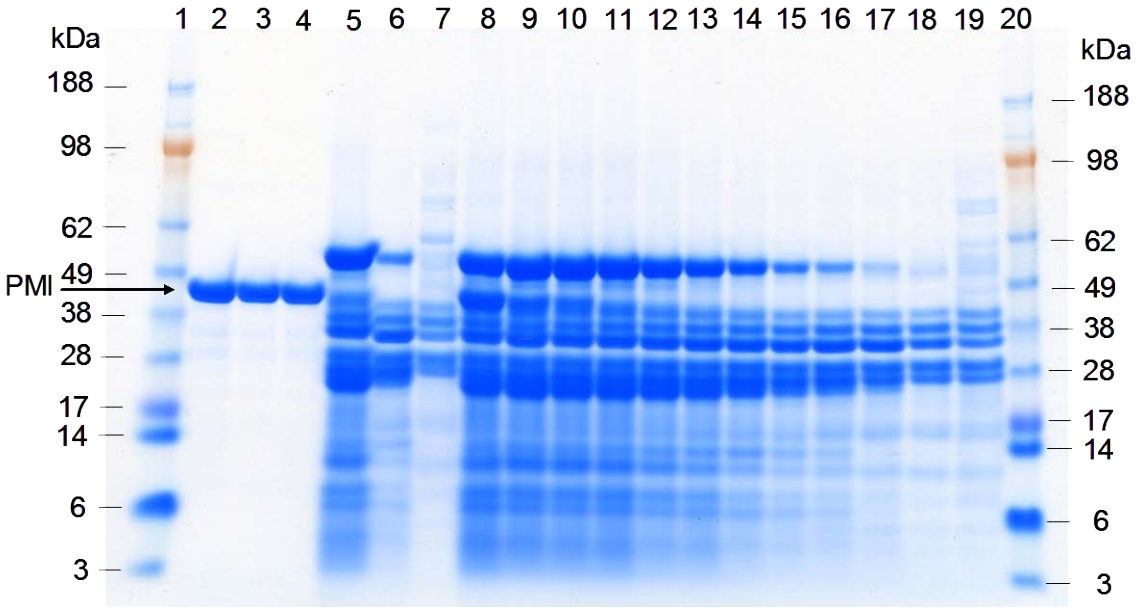

Figure 1. SDS-PAGE analysis of PMI (test substance PMI-0105) following digestion in SIF.

Lane 1: Molecular weight standard.
Lane 2: PMI incubated in SIF without pancreatin for 0 hours.
Lane 3: PMI incubated in SIF without pancreatin for 2 hours
Lane 4: PMI incubated in SIF without pancreatin for 48 hours
Lane 5: SIF incubated for 0 hours
Lane 6: SIF incubated for 2 hours
Lane 7: SIF incubated for 48 hours
Lane 8: PMI incubated in SIF for 0 minutes
Lane 9: PMI incubated in SIF for 1 minute
Lane 10: PMI incubated in SIF for 2 minutes
Lane 11: PMI incubated in SIF for 5 minutes
Lane 12: PMI incubated in SIF for 15 minutes
Lane 13: PMI incubated in SIF for 30 minutes
Lane 14: PMI incubated in SIF for 60 minutes
Lane 15: PMI incubated in SIF for 2 hours
Lane 16: PMI incubated in SIF for 3 hours
Lane 17: PMI incubated in SIF for 6 hours
Lane 18: PMI incubated in SIF for 24 hours
Lane 19: PMI incubated in SIF for 48 hours
Lane 20: Molecular weight standard.

The molecular weight of PMI corresponds to approximately 42.8 kDa.

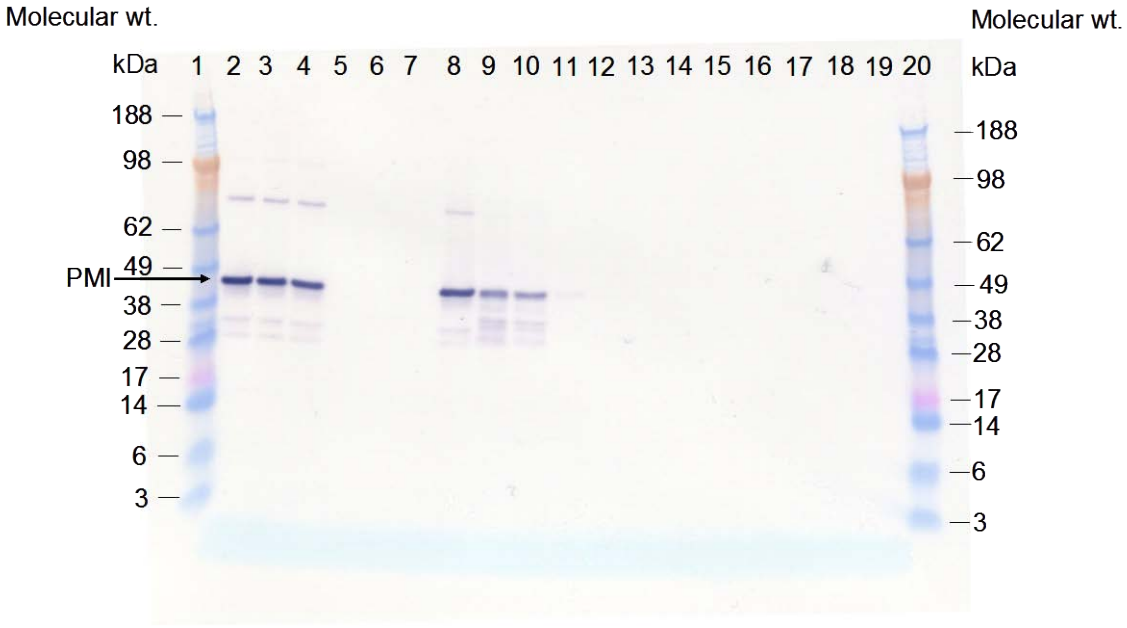

Figure 2. Western blot analysis of PMI (test substance PMI-0105) following digestion in SIF.

Lane 1: Molecular weight standard.
Lane 2: PMI incubated in SIF without pancreatin for 0 hours.
Lane 3: PMI incubated in SIF without pancreatin for 2 hours
Lane 4: PMI incubated in SIF without pancreatin for 48 hours
Lane 5: SIF incubated for 0 hours
Lane 6: SIF incubated for 2 hours
Lane 7: SIF incubated for 48 hours
Lane 8: PMI incubated in SIF for 0 minutes
Lane 9: PMI incubated in SIF for 1 minute
Lane 10: PMI incubated in SIF for 2 minutes
Lane 11: PMI incubated in SIF for 5 minutes
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Lane 16: PMI incubated in SIF for 3 hours
Lane 17: PMI incubated in SIF for 6 hours
Lane 18: PMI incubated in SIF for 24 hours
Lane 19: PMI incubated in SIF for 48 hours
Lane 20: Molecular weight standard.

The molecular weight of PMI corresponds to approximately 42.8 kDa.

CONCLUSIONS

The results of this study indicate that PMI is readily degraded under simulated mammalian intestinal conditions. The majority of intact PMI was digested after 5 minutes of incubation in simulated mammalian intestinal fluid and no intact PMI or

degradation products were detected by Western blot analysis upon sampling of the reaction mixture after 15 minutes of incubation. The results of this study support the conclusion that PMI will be readily digested under typical mammalian intestinal 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 27709-2257, USA.

CONTRIBUTING SCIENTISTS

The analytical work reported herein was conducted by Andrea Nelson, B.S., Ian Kietzman, B.S., and Stephanie Winslow, B.S. at Syngenta Biotechnology, Inc.

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CRITICAL DATES

Study initiation date:	November 6, 2007
Experimental start date:	November 7, 2007
Experimental end date:	April 6, 2009

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