Effect of Temperature on Phosphomannose Isomerase as Contained in Test Substance PMI-0105 as Assessed by Enzymatic Activity

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Company: Syngenta Seeds, Inc. – Field Crops – NAFTA

Company Representative:

Ann Tuttle August 31, 2009

Ann Tuttle Date
Regulatory Affairs Manager

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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 (GLP) Standards, 40 CFR Part 160 (U.S. EPA 1989) pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act and subsequent revisions.

- The test substance PMI-0105 was characterized under GLP. While the test substance characterization and the enzyme activity reports were not finalized prior to the initiation of this study, they were finalized prior to the completion of this study (Nelson 2008) and (Nelson 2009) respectively.
- The plate imaging software and analysis software were not validated according to the GLPs.

Study Director

Glenn Mims — September 1, 2009

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QUALITY ASSURANCE STATEMENT

Study Title: Effect of Temperature on Phosphomannose Isomerase as Contained in Test Substance PMI-0105 as Assessed by Enzymatic Activity

Study Director: Glenn Mims

Study Number: PMI-07-05

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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 17, 2007	October 17, 2007
In-Progress Inspection	January 18, 2008	January 18, 2008
Final Report Audit (1st audit)	July 20-21, 2009	July 21, 2009
Final Report Audit (2nd audit)	July 30, 2009	July 30, 2009
Final Report Audit (3rd audit)	August 25, 2009	August 25, 2009
Final Report Audit (4th audit)	August 26, 2009	August 26, 2009

Prepared by: Connie L. Connor Date: August 26, 2009

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

loq	limit of quantitation
NADP	β-Nicotinamide adenine dinucleotide phosphate oxidized
NADPH	β-Nicotinamide adenine dinucleotide phosphate reduced
PMI	phosphomannose isomerase
STDEV	standard deviation
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
Tween 20	polyoxyethylene sorbitan monolaurate
U	units - One unit of PMI activity is defined as the amount of enzyme required to catalyze the conversion of 1 µmol of mannose 6-phosphate to fructose 6-phosphate per min (equivalent to 1 µmol NADP reduced to NADPH per min) under the described reaction conditions.

SUMMARY

The effect of elevated temperature on phosphomannose isomerase (PMI) protein was determined by incubating solutions of test substance PMI-0105 containing PMI for 30 minutes at temperatures ranging from 25°C to 95°C and subsequently analyzing them for specific phosphomannose isomerase activity. An additional sample of PMI solution was incubated at 4ºC to determine a baseline enzymatic activity.

The incubation of PMI at 25ºC and 37ºC resulted in no loss of enzymatic activity. Incubation at 65ºC resulted in loss of enzymatic activity below the enzymatic assay limit of quantitation and the incubation at 95ºC resulted in no detectable enzymatic activity.

The data presented in this study support the conclusion that PMI is labile upon heating at temperatures of 65ºC and above, as measured by loss of enzymatic activity.

INTRODUCTION

The purpose of this study was to investigate the effect of elevated temperature on the enzymatic activity of phosphomannose isomerase (PMI). The source of PMI in this study was test substance PMI-0105, which was prepared from a recombinant *Escherichia coli* over-expression system prior to the study (Attenborough 2005).

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 *E.coli manA* gene and express PMI protein are able to utilize mannose as a carbon source (Miles and Guest 1984).

In this study PMI, as contained in test substance PMI-0105, was exposed to a range of temperatures (25ºC, 37ºC, 65ºC and 95ºC) for 30 minutes. Additionally, the PMI test substance was incubated at 4ºC for 30 minutes as a control to determine the baseline PMI activity. Loss or decrease of specific PMI enzymatic activity was used to determine the effect of elevated temperature on the protein.

MATERIALS AND METHODS

Test Substance PMI-0105

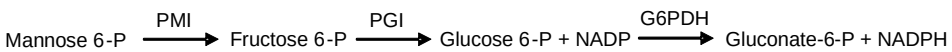
Prior to this study test substance PMI-0105 was prepared as described in Attenborough (2005) at Syngenta Protein Science (Jealott's Hill International Research Centre, Bracknell, UK). The *manA* gene was cloned into the inducible, over-expression vector pET-24a in *E. coli* strain BL21(DE3)RP to produce test substance PMI-0105 and has the same amino acid sequence as the endogenous PMI protein produced in *E. coli*. The PMI protein is used as a selectable marker in many of Syngenta's transgenic plant events including 3272 maize.

The lyophilized PMI sample preparation was designated test substance PMI-0105, shipped on dry ice to the Regulatory Science laboratory (Syngenta Biotechnology, Inc.) and stored at -20°C ± 8°C until further use. Test substance PMI-0105 was found to contain 89.5% PMI by weight (Nelson 2008).

Temperature Experiments and Enzymatic Activity Assays

Test substance PMI-0105 was dissolved in 50 mM Tris (pH 7.0) buffer to a final concentration of 5 µg PMI/ml. Three 1 ml aliquots of the test substance solution were then incubated at each temperature (25ºC ± 2ºC, 37ºC ± 2ºC, 65ºC ± 2ºC and 95ºC ± 2ºC) for 30 min. For a baseline control, three additional aliquots were incubated at 4ºC ± 2ºC for 30 min. After incubation each 1 ml (5 µg PMI/ml) solution was further diluted in 1 ml of 50 mM Tris (pH7.0) with 0.1% Tween 20 to a concentration of 0.1 ng PMI/µl.

The enzymatic activity of PMI was monitored for each treatment in triplicate (i.e. three 25ºC samples each were aliquoted three times into the assay for a total of nine reactions). The enzymatic reactions were conducted in Corning 96-well plates containing 10 mM mannose 6-phosphate , 1 mM β-nicotinamide adenine dinucleotide phosphate sodium salt hydrate, 2 U/ml phosphoglucose isomerase and 2 U/ml glucose 6-phosphate dehydrogenase in 50 mM Tris buffer (pH 7.0). The reaction was initiated by the addition of 5 ng PMI (50 µl of 0.1 ng/ul dilution) per well to the assay mixture (total reaction volume 200 µl per well). PMI activity was measured by the production of NADPH in a coupled reaction containing phosphoglucose isomerase (PGI) and glucose 6-phosphate dehydrogenase (G6PDH) based on the method described by Gracy & Noltmann (1968) and Gill et al. (1986) and validated for use in this study by Nelson (2009).



PMI = Phosphomannose isomerase, PGI = Phosphoglucose isomerase, G6PDH = Glucose 6 -phosphate dehydrogenase

The production of NADPH was monitored by measuring absorbance increase at 340 nm using a SpectraMax Plus 384 spectrophotometer (Molecular Devices) and SoftMax Pro software. One unit of PMI activity is defined as the amount of enzyme required to catalyze the conversion of 1 µmol of mannose 6-phosphate to fructose 6-phosphate per min (equivalent to 1 µmol NADP reduced to NADPH per min) under the described reaction conditions.

Statistical Methods

Mean values, standard deviations and relative activities were calculated using Microsoft Excel.

RESULTS AND DISCUSSION

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

Effect of temperature on PMI Enzymatic Activity

The results shown in Table 1 and Figure 1 indicate that incubation of test substance PMI-0105 at temperatures up to 37ºC for 30 minutes did not decrease the enzymatic activity of PMI. Incubation of test substance PMI-0105 at 65ºC for 30 minutes resulted in loss of enzymatic activity below the enzymatic assays limit of quantitation, and the incubation at 95ºC resulted in no detectable enzymatic activity.

Temperature (°C)	Mean Specific Activity (U^1/mg PMI)	STDEV[2] (U^1/mg PMI)	Relative Activity (%)
4 (control)	420	23	100
25	439	11	105
37	422	11	100
65	<loq[3]	<loq[3]	<loq[3]
95	not detectable[4]	not detectable[4]	not detectable[4]

Table 1. Effect of Temperature on Enzymatic Activity of PMI as contained in Test Substance PMI-0105

[1] One unit of PMI activity is defined as the amount of enzyme required to catalyze the conversion of 1 µmol of mannose 6-phosphate to fructose 6-phosphate per min (equivalent to 1 µmol NADP reduced to NADPH per min) under the described reaction conditions.
[2]For each temperature three aliquots were incubated and each aliquot was analyzed in triplicate for enzymatic activity as described in MATERIALS AND METHODS under Temperature Experiments and Enzymatic Activity Assays.
[3] Less than the limit of quantitation (<loq) for the enzyme assay (i.e. less than 52.2 milli-OD or 6.25 µg NADPH/ml as described in Nelson 2009).
[4] Not detectable (i.e. below 15.9 milli OD or 1.90 µg NADPH/ml as described in Nelson 2009).

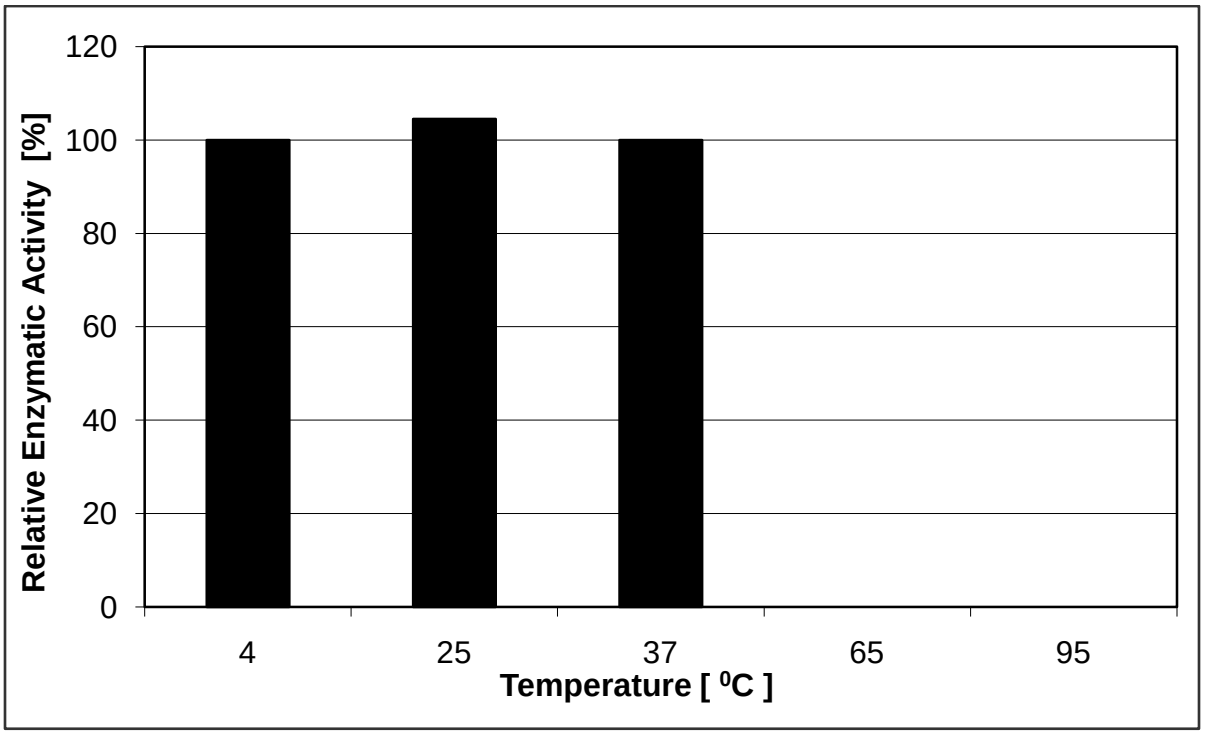


Figure 1. Effect of Temperature on Enzymatic Activity of PMI as contained in Test Substance PMI-0105.

CONCLUSIONS

The effect of temperature on the enzymatic activity of PMI as contained in test substance PMI-0105 was evaluated at various temperatures ranging from 25°C to 95°C in this study. The results indicate that PMI is labile upon incubation for 30 minutes at temperatures of 65°C and higher as shown by loss of enzymatic activity.

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 Glenn Mims B.S. and Andrea Nelson B.S. at Syngenta Biotechnology, Inc.

Reported by: Glenn Mims September 1, 2009

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Approved by: August 31, 2009

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CRITICAL DATES

Study initiation date:	October 18, 2007
Experimental start date:	January 18, 2008
Experimental termination date:	April 7, 2009

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