Effect of Temperature on the Enzymatic Activity of *p*-Hydroxyphenylpyruvate Dioxygenase (AvHPPD-03) Protein

Data Requirement: Not applicable

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01 Dec 2010

Date

Senior Regulatory Affairs Manager

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STATEMENT CONCERNING GOOD LABORATORY PRACTICES STANDARDS

This study was conducted in compliance with the relevant provisions of Good Laboratory Practices Standards (40 CFR Part 160, US EPA 1989) pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act and subsequent revisions.

Study Director:

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QUALITY ASSURANCE STATEMENT

Study Title: Effect of Temperature on the Enzymatic Activity of p-Hydroxyphenylpyruvate Dioxygenase (AvHPPD-03) Protein

Study Director: [redacted]

Study Number: TK0022136

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	April 15, 2010	April 15, 2010
Inspect Analytical	July 15, 2010	July 19, 2010
Audit Final Report (1st Audit)	October 25 – 26, 2010	October 26, 2010
Audit Final Report (2nd Audit)	November 29, 2010	November 29, 2010

Prepared by: [redacted] Date: Nov 29, 2010

Staff Quality Assurance Auditor
Quality Assurance Unit
Syngenta Crop Protection, Inc.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

AvHPPD-03	*p*-hydroxyphenylpyruvate dioxygenase from *Avena sativa*
Bq	Becquerel
BTP	Bis-Tris propane
^{14}C	Carbon 14
dpm	disintegrations per minute
E. coli	*Escherichia coli*
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
HGA	homogentisic acid
HPLC/βRAM®	high pressure liquid chromatography / β-RAM®
HPP	*p*-hydroxyphenylpyruvate
HPPD	*p*-hydroxyphenylpyruvate dioxygenase
LSC	liquid scintillation counting
LOD	limit of detection
LOQ	limit of quantitation
µg	microgram
µM	micromolar
mg	milligram
ml	milliliter
mM	millimolar
N	Normal
pmol	picomole
sec	second
TCA	trichloroacetic acid
U	unit
US EPA	United States Environmental Protection Agency

SUMMARY

The purpose of this study was to investigate the effect of temperature on the enzymatic activity of *p*-hydroxyphenylpyruvate dioxygenase (AvHPPD-03) protein. The AvHPPD-03 protein was prepared from a recombinant *Escherichia coli* (*E. coli*) strain expressing the novel gene, *avhppd-03* derived from oat (*Avena sativa*). Aliquots of an aqueous solution of AvHPPD-03 were incubated for 30 minutes at 25°C, 37°C, 65°C, and 95°C. Following temperature treatment, enzymatic activity was assessed using a *p*-hydroxyphenylpyruvate dioxygenase (HPPD) enzymatic activity assay. An additional aliquot of the AvHPPD-03 solution, incubated at 4ºC, was used to establish the baseline enzymatic activity.

The percent enzymatic activity for AvHPPD-03 after temperature treatment at 25°C was 97.8%, when compared to the baseline enzymatic activity. Incubation at 37°C resulted in 74.1% loss of enzymatic activity. Following temperature treatment at 65°C and 95°C, the enzymatic activity of AvHPPD-03 was below the limit of detection for the HPPD enzymatic activity assay.

The data presented in this report supports the conclusion that the enzymatic activity of AvHPPD-03 is greatly decreased after incubation for 30 minutes at 37ºC. Enzymatic activity is no longer detected after incubation for 30 minutes at 65ºC and above, indicating the loss of its dioxygenase enzymatic function.

INTRODUCTION

The purpose of this study was to investigate the effect of temperature on the enzymatic activity of *p*-hydroxyphenylpyruvate dioxygenase (AvHPPD-03) protein. Aliquots of an aqueous solution of AvHPPD-03 were incubated for 30 minutes at 25°C, 37°C, 65°C, and 95°C. Following temperature treatment, enzymatic activity was assessed using an *p*-hydroxyphenylpyruvate dioxygenase (HPPD) enzymatic activity assay. Little or no loss of enzymatic activity is expected to occur while maintaining a protein solution at 4ºC; therefore, an additional aliquot of the AvHPPD-03 solution, incubated at 4ºC, was used to establish the baseline enzymatic activity.

The AvHPPD-03 protein was prepared from a recombinant *Escherichia coli* (*E. coli*) strain expressing the novel gene, *avhppd-03* derived from oat (*Avena sativa*). The gene *avhppd-03* encodes a HPPD enzyme, designated AvHPPD-03, that catalyzes the formation of homogentisic acid (HGA), the aromatic precursor of plastoquinone and vitamin E biosynthesis. Expression of *avhppd-03* in plants confers a mesotrione-tolerance phenotype.

MATERIALS AND METHODS

Microbially Produced AvHPPD-03

A microbially produced AvHPPD-03 was prepared from an *E. coli* expression system. The *avhppd-03* gene was introduced into a pET24a vector and transformed into *E. coli* strain BL21 (DE3) cells.

In August 2009, AvHPPD-03 was prepared from *E. coli* cell paste by Syngenta Protein Science (Jealott's Hill International Research Centre, Bracknell, UK). Briefly, *E. coli* cells were ruptured and the cell debris was removed by centrifugation. The AvHPPD-03 protein was further purified using anion exchange chromatography, hydrophobic interaction chromatography and gel filtration chromatography. The purified protein was pooled, concentrated, aliquoted, and lyophilized. The resulting lyophilized powder was designated test substance AVHPPD-03-0209. The test substance was shipped on dry ice to Syngenta Biotechnology, Inc. (Research Triangle Park, NC, USA), where it was stored at -20°C ± 8°C.

The test substance AVHPPD-03-0209 was the source of AvHPPD-03 in this study. AVHPPD-03-0209 was characterized in detail and was determined to contain 72.2% AvHPPD-03 by weight (Winslow 2009). For use in this study, AVHPPD-03-0209 was solubilized in purified water.

The AvHPPD-03 protein was sent on dry ice to Syngenta Crop Protection, Inc. (Greensboro, NC, USA), where it was stored at -20°C ± 8°C until further use. The temperature treatment and enzymatic activity assay was conducted at Syngenta Crop Protection, Inc. (Greensboro, NC).

Temperature Treatment

The temperature treatment was conducted in three individual sessions on separate days. For each session, the AvHPPD-03 was solubilized in purified water at a concentration of

5.31 µg/ml. Aliquots of the AvHPPD-03 solution were incubated at 25°C ± 2°C, 37°C ± 2°C, 65°C ± 2°C, and 95°C ± 2°C for 30 minutes. An enzymatic activity baseline control was prepared by incubating the AvHPPD-03 solution at 4°C ± 2°C for 30 minutes. Following temperature treatment, all samples were placed on ice until analyzed by an HPPD enzymatic activity assay.

Enzymatic Activity Assay

The enzymatic activity of AvHPPD-03 was determined using a radioactive $^{14}CO_2$ trapping HPPD enzymatic activity assay.

The HPPD enzyme catalyzes the formation of HGA and carbon dioxide from *p*-hydroxyphenylpyruvate (HPP) and molecular oxygen (Figure 1). The HPPD enzymatic activity assay determines the amount of radiolabeled $^{14}CO_2$ generated from a ^{14}C-labeled HPP substrate during the enzymatic reaction (Barta and Boger 1996).

Figure 1. Reaction catalyzed by HPPD

The enzymatic activity for AvHPPD-03 is reported as U/mg AvHPPD-03 where one unit of AvHPPD-03 activity is defined as the amount of enzyme required to catalyze the conversion of one µmol of HPP to produce one µmol of HGA and one µmol of CO_2 per minute under the described reaction conditions.

The enzymatic activity assays were performed at 25°C ± 0.2°C in duplicate at four time points (0, 1, 3, and 6 minutes).

A substrate mixture consisting of 50 µM unlabeled HPP in 50 mM Bis-Tris propane (BTP) buffer (pH 7) containing 25 mM sodium ascorbate, 4 µg/ml bovine catalase, and approximately 5 µM ^{14}C-HPP was prepared. Prior to use, the purity of ^{14}C-HPP was determined using a high pressure liquid chromatography (HPLC)/βRAM® method.

The substrate mixture was transferred into individual reaction chambers and capped with a tightly fitted rubber stopper. The specific radioactivity of the freshly prepared substrate mix was measured each individual day. Each enzymatic activity assay was initiated by adding AvHPPD-03. A suspended filter soaked with 1N sodium hydroxide was used to trap the CO_2 generated during the reaction. Reactions were then stopped after 0, 1, 3, and 6 minutes by addition of 0.6 N trichloroacetic acid (TCA).

Upon stopping each reaction, CO_2 trapping was allowed to continue for 90 minutes at 25°C ± 0.2°C. Radioactivity trapped within the filter was measured by liquid scintillation

counting (LSC). The total disintegrations per minute (dpm) for each time point was corrected for background by subtracting the measured radioactivity (dpm) for time zero.

Calculation of enzymatic activity

The total pmol of CO_2 produced was calculated:

$$\text{total } CO_2 \text{ (pmol)} = \frac{(\text{dpm measured at a given time} - \text{dpm measured at time zero})}{60\left(\frac{\text{dpm}}{\text{Bq}}\right) \times 0.67 \times \text{specific radioactivity of the substrate mix}\left(\frac{\text{Bq}}{\text{pmol}}\right)}$$

0.67 = the predetermined extraction efficiency of measuring the trapped $^{14}CO_2$ from the nozzle filter by LSC, which is 67%

60 (dpm/Bq) is the conversion factor of dpm to Bq

The CO_2 production rate (pmol/sec) was determined by plotting the amount of total CO_2 produced *vs*. time. The slope of the trendline represents the CO_2 production rate of AvHPPD-03.

The slope of the trendline was calculated:

$$y = mx + b$$

m = slope
b = y-intercept

A mean CO_2 production rate was calculated from duplicate activity assays. Enzymatic activity was then calculated as follows:

$$enzymatic\ activity = \text{pmol/sec} \times 60\,\text{amount of AvHPPD} - 03\,\text{in reaction (mg)}$$

60 = 60 seconds per minute

The relative enzymatic activity was calculated using the baseline enzymatic activity (4°C sample) set at 100% activity for each independent session. The mean relative enzymatic activity across the three sessions was then calculated and reported.

The limit of detection (LOD) of a method is defined as the lowest amount of analyte (in this case HPPD via detection of CO_2) in a sample which can be detected but not necessarily accurately quantitated. The limit of quantitation (LOQ) is the minimal concentration at which the production rate of CO_2 can accurately and precisely be measured. Prior to this study, the LOD and LOQ of the HPPD activity assay was determined to be 24.4 pmol CO_2 and 58.0 pmol CO_2, respectively (Emborsky 2010).

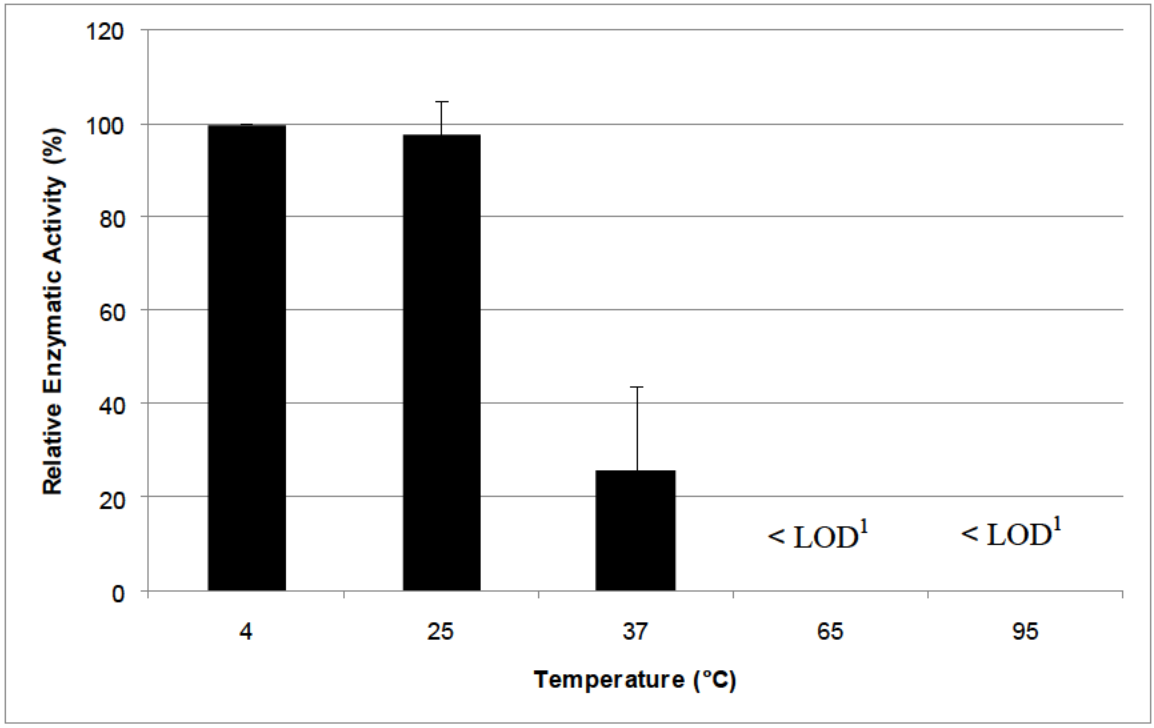
Statistical Analysis

Means and standard deviations were calculated using Microsoft Office Excel® 2007 software.

RESULTS AND DISCUSSION

The results demonstrated that AvHPPD-03 retains enzymatic activity following incubation for 30 minutes at 25°C (97.8%) when compared to the baseline enzymatic activity observed for the solution incubated at 4ºC (Figure 2 and Table 1). Incubation at 37°C resulted in 74.1% loss of enzymatic activity. Following temperature treatment at 65°C and above, the enzymatic activity of AvHPPD-03 was below the LOD of the enzymatic activity assay (Figure 2).

Figure 2. Relative enzymatic activity of AvHPPD-03 after temperature treatment (as measured by HPPD enzymatic activity assay)



[1]<LOD notes that the result was below the enzymatic activity assay LOD, 24.4 pmol CO_2

The error bars represent the standard deviation of triplicate sessions

Table 1. Relative enzymatic activity of AvHPPD-03 after temperature treatment (as measured by triplicate sessions of HPPD enzymatic activity assay)

	Session 1			Session 2			Session 3			Triplicate sessions	
Temperature (°C)	Mean[4] CO_2 production rate (pmol/sec)	Enzymatic Activity (U/mg)	Relative Enzymatic Activity[1] (%)	Mean CO_2 production rate (pmol/sec)	Enzymatic Activity (U/mg)	Relative Enzymatic Activity[1] (%)	Mean CO_2 production rate (pmol/sec)	Enzymatic Activity (U/mg)	Relative Enzymatic Activity[1] (%)	Mean[5] Relative Activity (%)	SD[6] (%)
4	2.86	0.66	100.0[3]	3.83	0.86	100.0[3]	3.79	0.86	100.0[3]	100.0	N/A
25	2.87	0.66	100.4	3.42	0.77	89.4	3.93	0.89	103.6	97.8	7.4
37	0.44	0.10	15.5	0.60	0.13	15.6	1.77	0.40	46.7	25.9	18.0
65	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]
95	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]	< LOD[2]

Due to rounding in Microsoft Office Excel® 2007, manual calculations may not result in the exact same number(s) shown here.

[1]Enzymatic activity retained relative to the baseline enzymatic activity observed for the solution incubated at 4ºC as measured by the HPPD enzymatic activity assay

[2] <LOD notes that the result was below the HPPD enzymatic activity assay LOD, 24.4 pmol CO_2 (Emborsky 2010)

[3]100% activity is equivalent to 0.66 U/mg for session one and 0.86 U/mg for sessions two and three

[4]The mean CO_2 production rate was calculated from the duplicate enzymatic activity assays for each independent session

[5]The Mean relative activity was calculated using the relative activity for the three independent sessions

[6]Standard deviation among the relative activity for the three independent sessions

Data Quality and Integrity

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

CONCLUSION

The data presented in this report supports the conclusion that the enzymatic activity of AvHPPD-03 is greatly decreased after incubation for 30 minutes at 37ºC. Enzymatic activity is no longer detected after incubation for 30 minutes at 65ºC and above, indicating the loss of its dioxygenase enzymatic function.

RECORDS RETENTION

Raw data, the original copy of this report, and other relevant records are archived at Syngenta Biotechnology, Inc., 3054 East Cornwallis Road, Research Triangle Park, NC 27709-2257, USA.

CONTRIBUTING SCIENTISTS

The analytical work reported herein was conducted by [redacted] at Syngenta Crop Protection, Inc. [redacted] at Syngenta Biotechnology, Inc.

CRITICAL DATES

Study initiation date:	April 29, 2010
Experimental start date:	July 13, 2010
Experimental end date:	August 10, 2010

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