In vitro Digestibility of *p*-Hydroxyphenylpyruvate Dioxygenase (AvHPPD-03) Protein under Simulated Mammalian Intestinal Conditions

Data Requirement: Not applicable

Author: [redacted]

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Company: *Syngenta Seeds, Inc.*

Company Representative: [redacted]

26 April 2010
Date

Senior Regulatory Affairs Manager

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STATEMENT CONCERNING GOOD LABORATORY PRACTICES STANDARDS

With the exceptions noted below, this study was conducted in compliance with the relevant provisions of Good Laboratory Practices Standards (US EPA 1989) pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act.

- The Invitrogen SeeBlue® Plus2 molecular weight protein standard characterization was not conducted under GLPS and was used as purchased.
- There were two instances in which raw data was not recorded promptly, although this information was accurately added in review.

Study Director:

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May 4, 2010
Date

Regulatory Scientist, Biochemical Assessment Team
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Submitted by:

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QUALITY ASSURANCE STATEMENT

Study Title: In Vitro Digestibility of *p*-Hydroxyphenylpyruvate Dioxygenase (AvHPPD-03) Protein under Simulated Mammalian Intestinal Conditions

Study Director: Stephanie Winslow

Study Number: TKRS0000158

Report Number: SSB-060-09

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 Dates
Audit Protocol	01-OCT-2009 - 01-OCT-2009	01-OCT-2009
Inspect Analytical	28-OCT-2009 - 28-OCT-2009	28-OCT-2009
Audit Final Report, 1st Audit	12-APR-2010 - 13-APR-2010	13-APR-2010
Audit Final Report, 2nd Audit	19-APR-2010 - 19-APR-2010	19-APR-2010

Prepared By: [redacted] Date: April 22, 2010

Senior Quality Assurance Auditor
Syngenta Biotechnology, Inc.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

AvHPPD-03	*p*-hydroxyphenylpyruvate dioxygenasefrom *Avena sativa*
Bis-Tris	bis (2-hydroxyethyl)-amino-tris (hydroxymethyl)methane
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
HPPD	*p*-hydroxyphenylpyruvate dioxygenase
LDS	lithium dodecylsulfate
LOD	limit of detection
MES	2-(*N*-morpholino) ethanesulfonic acid
mM	millimolar
N	normality
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
PVDF	polyvinylidene difluoride
SDS	sodium dodecylsulfate
SIF	simulated mammalian intestinal fluid
US EPA	United States Environmental Protection Agency
µg	microgram
®	registered trademark

SUMMARY

The susceptibility of *p*-hydroxyphenylpyruvate dioxygenase (AvHPPD-03) to proteolytic degradation in simulated mammalian intestinal fluid (SIF) containing pancreatin was evaluated *via* sodium dodecylsulfate polyacrylamide gel electrophoresis and Western blot analyses.

The AvHPPD-03 protein degraded rapidly upon exposure to SIF. No intact AvHPPD-03 (molecular weight approximately 47.0 kDa) was detected following its incubation in SIF for one minute. Smaller bands, most likely corresponding to degradation products of AvHPPD-03, were visible on the Western blot after incubation in SIF for one and two minutes; however, after five minutes of incubation in SIF, these bands were no longer visible.

The results of this study support the conclusion that AvHPPD-03 will be readily digested under typical mammalian intestinal conditions.

INTRODUCTION

The purpose of this study is to assess the *in vitro* digestibility of *p*-hydroxyphenylpyruvate dioxygenase (AvHPPD-03) in simulated mammalian intestinal fluid (SIF). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses were used to evaluate the *in vitro* digestibility of AvHPPD-03 in SIF over a 48 hour time course at 37ºC.

The AvHPPD-03 protein was prepared from a recombinant *Escherichia coli* strain expressing the novel gene, *avhppd-03* derived from oat (*Avena sativa*). The gene *avhppd-03* encodes a *p*-hydroxyphenylpyruvate dioxygenase (AvHPPD-03) enzyme that catalyzes the formation of homogentisic acid, the aromatic precursor of plastoquinone and vitamin E biosynthesis. In comparison with the native plant HPPD, the AvHPPD-03 isozyme from oat has reduced binding affinity for mesotrione, an herbicide that inhibits HPPD. Expression of *avhppd-03* in plants confers a mesotrione-tolerance phenotype.

MATERIALS AND METHODS

Microbially Produced AvHPPD-03

Microbially produced test substance containing AvHPPD-03 protein was prepared from an *E. coli* overexpression system by Syngenta Protein Science (Jealott's Hill International Research Centre, Bracknell, UK). The gene *avhppd-03* was synthesized, introduced into a pET24a vector, and transformed into *E. coli* strain BL21 (DE3) cells.

Prior to this study, AvHPPD-03 was prepared from *E. coli* cell paste. Briefly, *E. coli* cells were ruptured and the cell debris was removed by centrifugation. The supernatant was filtered and AvHPPD-03 was further purified using anion exchange chromatography, hydrophobic interaction, and gel filtration chromatography. The AvHPPD-03 containing fractions were pooled, concentrated, and lyophilized. The resulting dry formulation was designated AVHPPD-03-0209. The test substance was sent on dry ice to Syngenta Biotechnology, Inc. (Research Triangle Park, NC, USA), where it was stored at -20°C ± 8°C until further use.

This test substance was the source of AvHPPD-03 in this study. Prior to this study, AVHPPD-03-0209 was characterized in detail and was determined to contain 72.2% AvHPPD-03 by weight; the molecular weight of AvHPPD-03 was consistent with the predicted molecular weight of 47.0 kDa (Winslow 2009). For use in this study, AvHPPD-03-0209 was resolubilized in purified water.

In vitro Digestibility of AvHPPD-03 under Simulated Mammalian Intestinal Conditions

The SIF digestibility assay was performed at 37°C ± 2°C over a 48 hour time course with samples taken at zero, one, two, five, 10, 30, and 60 minutes, and two, three, six, 24, and 48 hours.

The SIF was prepared the day of use and consisted of 50 mM potassium phosphate monobasic, 38 mM sodium hydroxide (pH 7.5), and 10 mg pancreatin / ml (Sigma-Aldrich Cat. No. P7545) (USP 1990).

The digestion reaction was initiated by the addition of AvHPPD-03 to SIF at a ratio of 38 µg pancreatin to one µg AvHPPD-03; the reaction mixture was incubated at 37°C ± 2°C. At each time point, an aliquot of the reaction mixture was removed and mixed with stop solution to terminate the reaction. The samples were then incubated for 10 minutes at 95°C ± 5°C in preparation for SDS-PAGE analysis.

The time zero sample was prepared by adding an aliquot of AvHPPD-03 to a mixture of SIF and stop solution, after which it was then prepared for SDS-PAGE analysis as described. The mixture of the SIF with the stop solution ensures the inactivation of pancreatin before AvHPPD-03 is added for an accurate time zero. This time zero sample serves as an undigested control to which all samples are visually compared, allowing the digestion of AvHPPD-03 in SIF to be assessed over the time course.

The stop solution, containing lithium dodecylsulfate sample buffer, was preheated for two minutes at 95°C ± 5°C prior to use. Upon the addition of the stop solution, the pancreatin was inactivated by the elevation in temperature, thus, terminating the reaction. All samples were stored at -20°C ± 8°C until SDS-PAGE and Western blot analyses were conducted.

Table 1 details the composition of the SIF *in vitro* digestibility assay samples.

Assay Control Samples

Two controls were utilized in this study, an AvHPPD-03 control and an SIF control.

The AvHPPD-03 control contained AvHPPD-03 and SIF without pancreatin. This control examined the potential hydrolysis of AvHPPD-03 in SIF without pancreatin over the 48 hour time course.

The SIF control contained SIF and purified water, the solvent used to resolubilize AvHPPD-03. This control was examined to evaluate the potential for self hydrolysis of pancreatin over the 48 hour time course.

Both controls had the same concentration of AvHPPD-03 or pancreatin as initially contained in the *in vitro* digestibility assay samples. Both AvHPPD-03 and SIF controls were incubated at 37°C ± 2°C and sampled at zero, two, and 48 hours. Each control was combined with the stop solution and prepared for SDS-PAGE analysis in the same manner as described above for the *in vitro* digestibility assay samples. All assay control

samples were stored at -20°C ± 8°C until SDS-PAGE and Western blot analyses were conducted.

Table 1 details the composition of the assay control samples.

Table 1. SIF *in vitro* digestibility time course and control samples

Name	Composition	Time points examined	Purpose
SIF *in vitro* digestibility assay samples	AvHPPD-03, SIF	T0 (undigested control), 1, 2, 5, 10, 30, 60 minutes, and 2, 3, 6, 24, and 48 hours	Examined the *in vitro* digestibility of AvHPPD-03 in SIF
AvHPPD-03 control	AvHPPD-03, SIF without pancreatin	T0, 2 hours, 48 hours	Examined the potential hydrolysis of AvHPPD-03 in SIF without pancreatin
SIF control	purified water[1], SIF	T0, 2 hours, 48 hours	Examined the potential for self-hydrolysis of the pancreatin over the 48 hour time course

[1]Purified water was the solvent in which AvHPPD-03 was resolubilized
All samples and controls were analyzed *via* SDS-PAGE and Western blot analyses

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis Analysis

Equivalent volumes of each *in vitro* digestibility assay sample was subjected to SDS-PAGE under reducing conditions using a 4-12% Bis-Tris gel and 2-(*N*-morpholino) ethanesulfonic acid (MES) running buffer. Based on the concentration of AvHPPD-03 and pancreatin at the initiation of the digestion reaction, these volumes are equivalent to 1 µg AvHPPD-03 and 38 µg pancreatin. Volumes of the control samples, containing either 1 µg AvHPPD-03 or 38 µg pancreatin, were subjected to SDS-PAGE (described above). The molecular weight standard was the SeeBlue® Plus2 pre-stained standard. The gel was stained with Coomassie® blue and examined for the presence of bands consistent with the molecular weight of intact AvHPPD-03 (approximately 47.0 kDa).

The limit of detection (LOD) is defined as the lowest amount of analyte in a sample that can be detected. The LOD for SDS-PAGE was determined by subjecting serial dilutions of AvHPPD-03 to SDS-PAGE. (Amounts tested were 0.25, 0.125, 0.063, 0.031, and 0.016 µg of AvHPPD-03.) The gel was stained with a Coomassie® stain and the lowest amount of AvHPPD-03 visible on the gel was designated the LOD of AvHPPD-03 for SDS-PAGE.

Western Blot Analysis

Equivalent volumes of each *in vitro* digestibility assay sample was subjected to SDS-PAGE. Based on the concentration of AvHPPD-03 and pancreatin at the initiation of the digestion reaction, these volumes were equivalent to 5 ng AvHPPD-03 and 190 ng pancreatin (1:38). Volumes of the control samples, containing either 5 ng AvHPPD-03 or 190 ng pancreatin, were also subjected SDS-PAGE. The molecular weight standard was SeeBlue® Plus 2 pre-stained standard. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane *via* electroblotting. The membrane was probed with polyclonal rabbit antibodies capable of detecting AvHPPD-03. An alkaline phosphatase conjugated donkey anti-rabbit antibody was used to bind to the primary antibody. The protein was visualized by developing the blot with an alkaline phosphatase substrate solution. The Western blot was examined for the presence of bands consistent with the molecular weight of intact AvHPPD-03 (approximately 47.0 kDa) and AvHPPD-03 derived fragments.

The LOD for Western blot analysis was determined by subjecting serial dilutions of AvHPPD-03 to SDS-PAGE. (Amounts tested were 0.63, 0.31, 0.16, 0.078 and 0.039 ng AvHPPD-03.) The protein was transferred to a PVDF membrane and then probed with the same antibodies used to monitor the SIF assay. The lowest amount of AvHPPD-03 visible on the membrane was designated the LOD of AvHPPD-03 for the Western blot analysis.

Statistical Analysis

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

RESULTS AND DISCUSSION

Two methods (SDS-PAGE and Western blot) were used to analyze all samples and controls of the AvHPPD-03 *in vitro* digestibility experiment. The SDS-PAGE analysis provides information regarding the total protein present in each sample. The Western blot method allows for the specific analysis of the AvHPPD-03 protein within the *in vitro* degestibilty assay. Figures 1 and 2 show the results of the SDS-PAGE and Western blot analyses, respectively.

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis Analysis

The SDS-PAGE analysis (Figure 1) results suggest that AvHPPD-03 is readily digested after incubation in SIF for one minute. The band corresponding to AvHPPD-03 at time zero (Lane 9) is no longer visible after incubation in SIF for one minute (Lane 10). The additional bands in Lanes 10 through 20 are also seen in the SIF control (Lanes 2, 3, and 4) with the same intensity indicating these bands are from the pancreatin and are not due to breakdown fragments of AvHPPD-03.

The multiple protein bands present in the SIF control (Lanes 2, 3, and 4) are not observed in the AvHPPD-03 control (Lanes 5, 6, and 7). The AvHPPD-03 control does not contain pancreatin; therefore, the protein bands in Lanes 2, 3, and 4 are from the pancreatin alone. The protein bands due to pancreatin were also visible in the *in vitro* digestibility

assay samples (Lanes 9 through 20). There is significant degradation of the pancreatin observed in the SIF control most likely due to self hydrolysis, as shown by the decreasing intensity of the protein bands in the two and 48 hour samples (Lanes 3 and 4) compared to the time zero sample (Lane 2). This degradation is also apparent in the *in vitro* digestibility assay samples, Lanes 9 through 20.

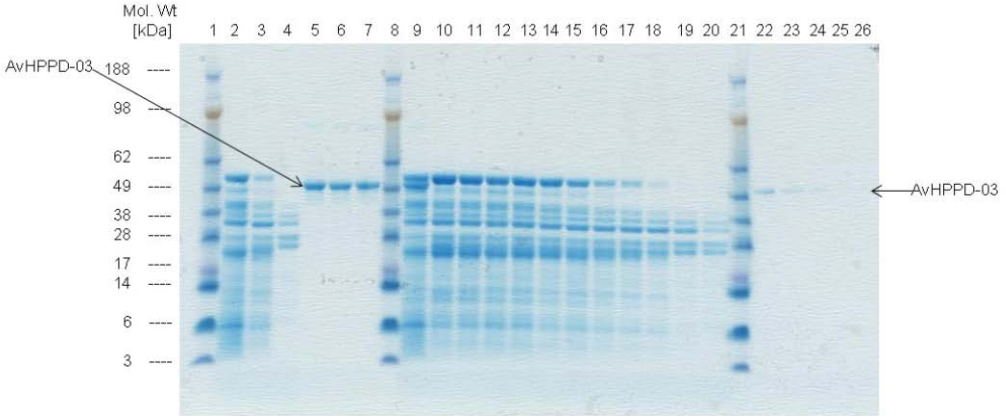
One of the protein bands, contributed by the pancreatin, has similar mobility to AvHPPD-03 at approximately 49 kDa. The decreasing intensity of this band in the SIF control at two and 48 hours (Lane 3 and 4, respectively) correlates well with the intensities observed in the SIF *in vitro* digestibility assay samples at two and 48 hours (Lane 16 and Lane 20, respectively). This observation strongly supports the conclusion that the 49 kDa band observed in the *in vitro* digestibility assay sample from one minute through two hours (Lane 10 through 16) is a pancreatin protein and not related to AvHPPD-03.

Similar band intensities were visualized between the time zero *in vitro* digestibility assay sample (Lane 9) and the AvHPPD-03 time zero control (Lane 5), confirming equal amounts were applied to SDS-PAGE.

The AvHPPD-03 protein incubated in SIF without pancreatin (AvHPPD-03 control) showed no significant degradation over the 48 hours (Lanes 5, 6, and 7), indicating that the hydrolysis of AvHPPD-03 seen for the SIF samples (Lanes 10 through 20) can be attributed to pancreatin.

Serial dilutions of AvHPPD-03 were analyzed by SDS-PAGE to determine the LOD, which was determined to be 0.031 µg AvHPPD-03 (Lane 25).

Figure 1. SDS-PAGE analysis of AvHPPD-03 following digestion in SIF



Lane 1: molecular weight standard
Lane 2: SIF control – time zero
Lane 3: SIF control – 2 hours
Lane 4: SIF control – 48 hours
Lane 5: AvHPPD-03 control (AvHPPD-03 in SIF without pancreatin) – time zero
Lane 6: AvHPPD-03 control (AvHPPD-03 in SIF without pancreatin) – 2 hours
Lane 7: AvHPPD-03 control (AvHPPD-03 in SIF without pancreatin) – 48 hours
Lane 8: molecular weight standard
Lane 9: *in vitro* digestibility assay - time zero
Lane 10: *in vitro* digestibility assay - 1 minute
Lane 11: *in vitro* digestibility assay - 2 minutes
Lane 12: *in vitro* digestibility assay - 5 minutes
Lane 13: *in vitro* digestibility assay - 10 minutes
Lane 14: *in vitro* digestibility assay - 30 minutes
Lane 15: *in vitro* digestibility assay - 60 minutes
Lane 16: *in vitro* digestibility assay - 2 hours
Lane 17: *in vitro* digestibility assay - 3 hours
Lane 18: *in vitro* digestibility assay - 6 hours
Lane 19: *in vitro* digestibility assay - 24 hours
Lane 20: *in vitro* digestibility assay - 48 hours
Lane 21: molecular weight standard
Lane 22: 0.25 µg AvHPPD-03 for LOD determination
Lane 23: 0.13 µg AvHPPD-03 for LOD determination
Lane 24: 0.063 µg AvHPPD-03 for LOD determination
Lane 25: 0.031 µg AvHPPD-03 for LOD determination
Lane 26: 0.016 µg AvHPPD-03 for LOD determination

The predicted molecular weight of AvHPPD-03 is 47.0 kDa.[1]

[1] The 47.0 kDa AvHPPD-03 protein band showed slightly lower mobility and, therefore an apparent higher molecular weight, when compared to the molecular weight standards on the gel and Western blot (Figure 1and Figure 2). The difference between the expected and observed molecular weights on the gel can be explained by the limitations of SDS-PAGE for accurate determination of molecular weight. Dube and Flynn (1988) have reviewed the reliability of SDS-PAGE for molecular weight determinations and concluded that the apparent molecular weight of a protein by this method is typically within 10% of its true molecular weight. This depends greatly on the similarity between the properties of the protein of interest and the proteins in the standard set (Sadeghi *et al*., 2003).

Western Blot Analysis

The Western blot analysis (Figure 2) results confirm that AvHPPD-03 is readily digested in SIF. The band corresponding to intact AvHPPD-03 at time zero (Lane 9) is no longer visible after incubation in SIF for one minute (Lane 10). This result strongly supports the conclusion that no intact AvHPPD-03 was present after one minute.

The digestion of AvHPPD-03 in SIF yielded three faint immunoreactive bands (approximately 28, 20, and 13 kDa) following incubation in SIF for one minute (Lane 10). These bands were also visible, although weaker in intensity, after two minutes of incubation (Lane 11). These bands were not present at time zero (Lane 9) or in the AvHPPD-03 controls (Lanes 5, 6, and 7), but they cross-reacted with the antibody that detected the intact AvHPPD-03 (Lane 9). This suggests the bands are degradation products of AvHPPD-03, resulting from pancreatin digestion. These degradation products were visible on the Western blot but not the SDS-PAGE analysis because Western blot analysis is much more sensitive than SDS-PAGE. Therefore, small quantities of protein can be detected by Western blot analysis that can not be detected by Coomassie® stained SDS-PAGE. After five minutes of incubation of AvHPPD-03 in SIF (Lane 12), none of these described protein bands were visible.

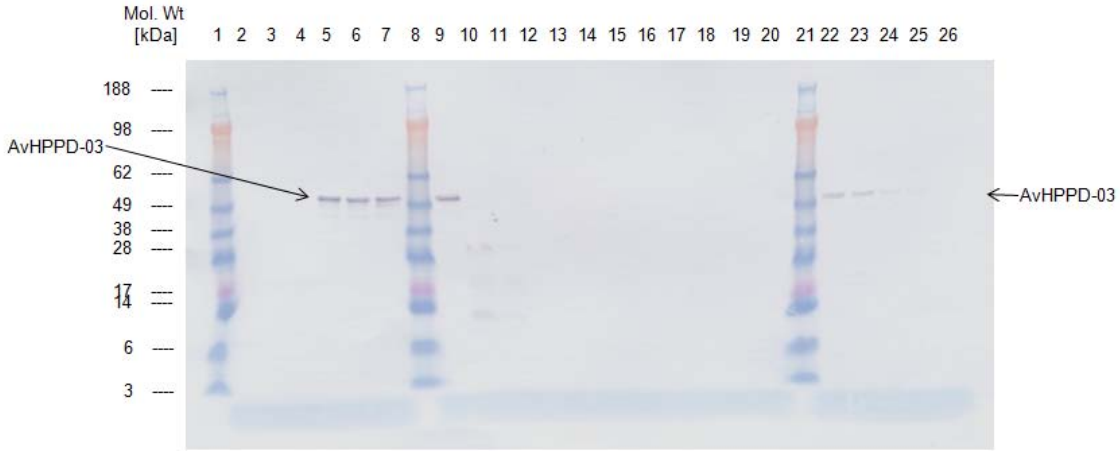
Similar band intensities were visualized between the time zero *in vitro* digestibility assay sample (Lane 9) and the AvHPPD-03 control at time zero (Lane 5), confirming equal amount were applied to the Western blot.

The 49 kDa band with similar mobility as AvHPPD-03 on the SDS-PAGE was not present on the Western blot (Lanes 10 through 17) confirming the band is a component of the pancreatin and not AvHPPD-03. This conclusion is supported by the fact that the antibody used in the Western blot method is specific for intact and breakdown fragments of AvHPPD-03 protein.

The AvHPPD-03 protein incubated in SIF without pancreatin (AvHPPD-03 control) showed no significant degradation over the 48 hours (Lanes 5, 6, and 7) indicating that the hydrolysis of AvHPPD-03 seen for the SIF samples (Lanes 10 through 20) can be attributed to pancreatin.

Serial dilutions of AvHPPD-03 were analyzed by Western blot to determine the LOD, which was determined to be 0.078 ng AvHPPD-03 (Lane 25).

Figure 2. Western blot analysis of AvHPPD-03 following digestion in SIF



Lane 1: molecular weight standard
Lane 2: SIF control – time zero
Lane 3: SIF control – 2 hours
Lane 4: SIF control – 48 hours
Lane 5: AvHPPD-03 control (AvHPPD-03 in SIF without pancreatin) – time zero
Lane 6: AvHPPD-03 control (AvHPPD-03 in SIF without pancreatin) – 2 hours
Lane 7: AvHPPD-03 control (AvHPPD-03 in SIF without pancreatin) – 48 hours
Lane 8: molecular weight standard
Lane 9: *in vitro* digestibility assay - time zero
Lane 10: *in vitro* digestibility assay - 1 minute
Lane 11: *in vitro* digestibility assay - 2 minutes
Lane 12: *in vitro* digestibility assay - 5 minutes
Lane 13: *in vitro* digestibility assay - 10 minutes
Lane 14: *in vitro* digestibility assay - 30 minutes
Lane 15: *in vitro* digestibility assay - 60 minutes
Lane 16: *in vitro* digestibility assay - 2 hours
Lane 17: *in vitro* digestibility assay - 3 hours
Lane 18: *in vitro* digestibility assay - 6 hours
Lane 19: *in vitro* digestibility assay - 24 hours
Lane 20: *in vitro* digestibility assay - 48 hours
Lane 21: molecular weight standard
Lane 22: 0.63 ng AvHPPD-03 for LOD determination
Lane 23: 0.31 ng AvHPPD-03 for LOD determination
Lane 24: 0.16 ng AvHPPD-03 for LOD determination
Lane 25: 0.078 ng AvHPPD-03 for LOD determination
Lane 26: 0.039 ng AvHPPD-03 for LOD determination

The predicted molecular weight of AvHPPD-03 is 47.0 kDa.[1]

Data Quality and Integrity

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

CONCLUSIONS

The AvHPPD-03 protein degraded rapidly upon exposure to SIF. No intact AvHPPD-03 was detected by SDS-PAGE or Western blot analyses after digestion in SIF for 1 minute. Furthermore; no AvHPPD-03 derived fragments were detected after incubation for five minutes. The results of this study support the conclusion that AvHPPD-03 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 Road, Research Triangle Park, NC 27709-2257, USA.

CONTRIBUTING SCIENTISTS

The analytical work reported herein was conducted by [redacted] at Syngenta Biotechnology, Inc., 3054 East Cornwallis Road, Research Triangle Park, NC 27709-2257, USA.

Reported by: [redacted] May 4, 2010
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Approved by: [redacted] April 23, 2010
Date

Team Leader, Biochemical Assessment Team
Regulatory Science and Trait Safety
Syngenta Biotechnology, Inc.

CRITICAL DATES

Study initiation date:	October 15, 2009
Experimental start date:	October 22, 2009
Experimental end date:	November 30, 2009

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