Bayer CropScience

# HPPD W336 PROTEIN

# *IN VITRO* DIGESTIBILITY STUDY IN

# HUMAN SIMULATED INTESTINAL FLUID

**DATA REQUIREMENT**
**No applicable guidelines**

**REPORT OF STUDY SA 09052**
Sponsor identification number: Lynx-PSI N°TX99L051

**AUTHOR / STUDY DIRECTOR: J.B. RASCLE**

**TESTING FACILITY:**

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

**SPONSOR:**

Bayer AG
Bayer CropScience
Alfred Nobel Str. 50
40789 Monheim
Germany

**STUDY COMPLETED ON: SEPTEMBER 15, 2009**

# STATEMENT OF NO DATA CONFIDENTIALITY CLAIM

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA § 10 (d) (1) (A), (B) or (C).

Company Name:

Company Agent:

Title:

Signature:

Date: _____

These data are the property of Bayer CropScience, and as such, are considered to be confidential for all purposes other than compliance with FIFRA § 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality which may exist under any other statute or in any other country.

# GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The study here reported was performed in accordance with the principles of Good Laboratory Practice ("Bonnes Pratiques de Laboratoire") described in the following issue, with the exception of the test item solution, which was not analyzed for concentration, homogeneity and stability.

- Organization for Economic Cooperation and Development (O.E.C.D.) Principles of Good Laboratory Practice, 1997 (January 26, 1998).

- European Directive 2004/10/EC (February 11, 2004).

- French Decree N°2006-1523, regarding Good Laboratory Practice (December 04, 2006).

- U.S. Environmental Protection Agency (E.P.A.)
  40 CFR part 160
  Federal Insecticide, Fungicide and Rodenticide Act (FIFRA);
  Good Laboratory Practice Standards: Final Rule, August 17, 1989.

- Good Laboratory Practice Standards for Toxicology studies on Agricultural Chemicals, Ministry of Agriculture, Forestry and Fisheries (M.A.F.F.) in Japan, notification 11 Nousan N°6283, October 01, 1999, modified by: notification 12 Nousan n°8628, December 06, 2000.

Author / Study Director:

Date: September 15, 2009 J.B. RASCLE

Sponsor Representative:

Date: September 15, 2009 A. CAPT

Study Submitter:

Date:

# FLAGGING STATEMENTS

This page is reserved for flagging statements as may be required by US EPA.

# QUALITY ASSURANCE STATEMENT

The conduct of the study has been subjected to periodic inspections by the Bayer CropScience Sophia-Antipolis Quality Assurance Unit. The types and dates of inspections and dates of reporting to Study Director and management are given below:

| Type of Q.A. inspection | Study phases inspected | Date of Q.A. inspection | Date of reporting to Study Director | Date of reporting to Management |
|---|---|---|---|---|
| Study-based | Protocol | April 17, 2009 | April 17, 2009 | April 21, 2009 |
| Process-based | Western blot | April 30, 2009 | April 30, 2009 | May 05, 2009 |
| Study-based | Final report | September 02, 2009 | September 02, 2009 | September 10, 2009 |

This report has been audited by Quality Assurance personnel in accordance with the appropriate standardized operating methods. The reported results accurately reflect the original data of the study.

Quality Assurance Group Leader:

Date: September 15, 2009

G. ODAGLIA

# SIGNATURE

I, the undersigned, hereby declare that the work was performed under my supervision according to the procedures described and that this report provides a correct and faithful record of the results obtained.

There were no circumstances which affected the quality and integrity of the data.

Author / Study Director:

Date: September 15, 2009

J.B. RASCLE

# STUDY PROFESSIONALS

The following professionals were involved in the conduct of this study:

| | | |
|---|---|---|
| STUDY DIRECTOR | : | J.B. RASCLE |
| REPLACEMENT STUDY DIRECTOR | : | D. ROUQUIE |
| RESPONSIBLE TECHNICIAN | : | A. ARNAUD |
| REPORT UNIT ASSISTANT | : | M. VAGNER/P. ALMERAS |

# TABLE OF CONTENTS

# SUMMARY

The HPPD W336 protein (produced in *Escherichia coli*) was tested for stability in human simulated intestinal fluid (SIF) with pancreatin at pH 7.5 for incubation times from 0.5 to 60 minutes. The protocol was based on the International Life Science Institute (ILSI) protocol using simulated gastric fluid (1). This protocol was adapted to use SIF.

A solution of the test protein was incubated with simulated intestinal fluid (SIF) (a porcine pancreatin solution at pH 7.5) at approximately 37°C and samples were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resultant solutions were analyzed for the presence of the HPPD W336 protein or potential stable protein fragments by Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. The immunodetection was performed using a polyclonal antibody directed against HPPD W336 protein. Appropriate controls included HPPD W336 protein in buffer without pancreatin, the corresponding 10% loading condition (to verify the sensitivity of the detection procedure) and SIF without HPPD W336 protein.

The HPPD W336 protein (produced in *Escherichia coli*) was degraded very rapidly, in less than 30 seconds of incubation with SIF, in presence of pancreatin, at pH 7.5.

# INTRODUCTION

This *in vitro* human simulated intestinal fluid (SIF) digestibility study has been conducted to provide a GLP study carried out in line with a current internationally recognized protocol. This study is based on the protocol of the methods used in the 2004 ILSI ring trial (1). The method is based on the United States *Pharmacopea* (2). This protocol was adapted to use human Simulated Intestinal Fluids (SIF).

In this study the test material was the HPPD W336 protein (produced in *E. coli*).

The study protocol and its amendment are presented in Attachment 1.

The study schedule was as follows:

| | |
|---|---|
| Study initiation date* | April 17, 2009 |
| Sponsor representative protocol approval date | April 17, 2009 |
| Experimental starting date | April 20, 2009 |
| Experimental completion date | June 25, 2009 |

* Date of protocol approval by Study Director

# MATERIAL AND METHODS

## 1 - PROTEINS AND PANCREATIN

The test item HPPD W336 protein was supplied by BioAnalytics (Bayer CropScience NV, Ghent, Belgium).

| | |
|---|---|
| Identification ..... : | HPPD W336 protein (produced in *Escherichia coli*) |
| Batch N° ..... : | LB020309 |
| Description ..... : | Lyophilized powder |
| Purity ..... : | 96% ±2% |
| Storage ..... : | –74 + 10°C * |
| Certified through ..... : | June 2009 |

*: Storage condition at Sophia-Antipolis (SOP MTR00962)

The pancreatin protein was purchased from Sigma, France (Ref: P3292).

| | |
|---|---|
| Identification ..... : | Pancreatin (from porcine pancreas) |
| Appearance..... : | Tan powder |
| Concentration and purity.. : | Biological mixture of enzymes, concentration and purity not supplied by Sigma |
| Storage ..... : | Approximately -20°C |
| Certified through ..... : | Stable under storage conditions |

The reference protein item azoalbumin was purchased from Sigma (Ref: A2382).

| | |
|---|---|
| Identification ..... : | Azoalbumin |
| Appearance..... : | Orange powder with a red cast |
| Purity..... : | 78% |
| Storage ..... : | Approximately -20°C |
| Certified through ..... : | April 2011 |

The certificates of analysis are presented in Attachment 2.

## 2 - PROTEIN SOLUTIONS

The test protein was solubilized in 50 mM Tris pH 7.5 buffer at the final concentration of 2.5 mg/ml.

## 3 - TEST SYSTEM

The simulated intestinal fluid was prepared as follows:

- Preparation of 100 ml of I-con solution (6.8 g/l KH2PO4; titrate to pH 7.5 using NaOH)
- Preparation of the SIF solution by addition of pancreatin (1% w/v) to the I-con solution

In order to validate the activity of the pancreatin solution, a quality control procedure was carried out to demonstrate the degradation of the azoalbumin. The pancreatin solution was found to have a normal level of activity.

### 4 - DIGESTION INCUBATION

The protein incubation for the test and reference materials was made in 2 mL microcentrifuge tubes in a waterbath at approximately 37°C. For each test and reference protein solution, 80 µl was added to 1520 µl of SIF and mixed. Samples of 200 µl were taken at 0.5, 2, 5, 10, 20, 30 and 60 minutes. The tubes were agitated after each sampling and at approximately 45 minutes.
A dilution of the test protein solution at 1/10 in 50 mM Tris pH 7.5 buffer was prepared for the 10% loading control.

As soon as samples were taken, the reaction was terminated by adding the 200 µl sample to a tube containing 70 µl MiliQ water placed on ice. In addition, 70 µl of 5X Laemmli solution (supplemented with few grains of sucrose) was added to all samples, and all tubes were heated 10 min at >90°C.

Additional control samples were prepared:
- a zero minute incubation of protein (10 µl) with 'SIF without pancreatin' (190 µl);
- a zero minute incubation of the 1/10 diluted protein (10 µl) with 'SIF without pancreatin' (190 µl) (10% loading control);
- a 60 minute incubation of protein (10 µl) with 'SIF without any pancreatin' (190 µl);
- a 'time zero' sample was produced by adding the protein (10 µl) to SIF (190 µl) after the reaction was terminated as above;
- a sample of SIF alone before incubation and the reaction terminated as above;
- a sample of SIF alone after 60 minutes incubation and the reaction terminated as above.

An aliquot of 25 µl of the samples was used for SDS-PAGE and the remaining was frozen at -20°C.

### 5 - SDS-PAGE ANALYSIS

The method was based on the method of Laemmli (3) using a Mini-Protean III cell (Bio-Rad, France). Samples of 15 µl were added to wells of an SDS-PAGE gel (15 wells, 1 mm 10-20% gradient polyacrylamide Tris-Tricine, Bio-Rad).

Electrophoresis was carried out with a constant voltage set at 100 volts until the bromophenol line has reached the bottom of the gel.

Gels were stained by the Coomassie blue method (Invitrogen) based on the work of Neuhoff *et al.* (4). After rinsing, the gels were scanned using a GS800 scanner (Bio-rad). The gels are not stable for more than two weeks, so the raw data were the scanned images.

In addition, a second SDS-PAGE was used for the western blot analysis. This gel was loaded with approximately 1000 times less protein in quantity, to take into account the higher sensitivity of the western blot technique compared to that of Coomassie blue staining.

### 6- WESTERN BLOT ANALYSIS

A polyvinylidene fluoride (PVDF) membrane was placed on the SDS-PAGE gel in a Tris/Glycine buffer and an electrical current applied in order to transfer the protein bands onto the membrane. To detect the HPPD W336 protein bands and/or its potential fragments, the membrane was incubated in the presence of a specific rabbit polyclonal anti-HPPD W336 protein antibody.
The hybridization of the antibody with the proteins immobilized on the membrane was revealed using a goat anti-rabbit polyclonal antibody coupled with a peroxidase. The hybridization bands were visualized using enhanced chemiluminescent (ECL) detection system (Amersham chemiluminescent system, GE Healthcare Life Sciences, France).
The autoradiographs were scanned using a GS800 scanner (Bio-Rad) and resulting images were retained in the raw data and reported in the final report.

### 7 - DATA STORAGE

All raw data, supporting documents as well as protocol, protocol amendments, aliquot of the test substance solution and final report are maintained in the archive room. All of the above will be archived for at least 10 years in the designated areas at:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

# RESULTS AND DISCUSSION

## 1 - HPPD W336 PROTEIN (Fig. 1)

As expected, the SDS-PAGE analysis of the HPPD W336 protein solution revealed one band located above the 36.5 kDa molecular weight marker. The 10% HPPD W336 protein control was also visible with lower intensity than the undiluted HPPD W336 protein band.
The HPPD W336 protein band was visible at the 0 and 60 minute incubation times without pancreatin, with no decrease in stain intensity at 60 minutes compared to time 0.
At time 0 of incubation with SIF, the various pancreatin bands were clearly visible.

At time 0 of incubation, the HPPD W336 protein band was not clearly visible, suggesting that the HPPD W336 protein was immediately digested within a few seconds. At all subsequent incubation times, there were no HPPD W336 protein band and no smaller fragment bands observed.

This indicates a complete digestion of the HPPD W336 protein with less than 30 seconds in presence of pancreatin.

## 2 - WESTERN BLOT ANALYSIS (Fig. 2)

SDS-PAGE followed by western blot analysis of the HPPD W336 protein revealed one band located close to the molecular weight marker at 36.5 kDa at the 0 and 60 minute incubation times without pancreatin. The 10% HPPD W336 protein control was also visible, with lower intensity than the undiluted HPPD W336 protein band. The polyclonal anti-HPPD W336 antibody binding further confirmed the identity of the HPPD W336 protein.

At time 0 of incubation and at all subsequent incubation times, the intact HPPD W336 protein band was not visible anymore. There were no bands visible at a lower molecular weight.

The results of the western blot analysis indicate that more than 90% of the HPPD W336 protein are degraded in less than 30 seconds in presence of pancreatin.

# CONCLUSION

A complete digestion of the HPPD W336 protein (produced in *Escherichia coli*) was observed within few seconds of incubation with SIF, in presence of pancreatin, at pH 7.5.

# PROTOCOL DEVIATION

There were no protocol deviations during the study.

Author / Study Director:

Date: September 15, 2009

J.B. RASCLE

# REFERENCES

**DART Numbers**

1 M-229898-01-1 THOMAS et al. (2004): A multi-laboratory evaluation of a common *in vitro* pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology and pharmacology*, 39, pp. 87-98.

2 M-273056-01-1 UNITED STATES *PHARMACOPEA* (1990) Vol XXII, pp. 1788-1789, United States Pharmacopeia Convention, Inc, Rockville, MD.

3 M-223866-01-1 LAEMLLI U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970, 227, pp. 680-685.

4 M-273931-01-1 NEUHOFF V., AROLD N., TAUBE D., EHRHARDT W. (1988): Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis*, 9, pp. 255-262.

# ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| °C | Degree Celsius |
| µg | Microgram(s) |
| µl | Microliter(s) |
| ECL | Enhanced chemiluminescence |
| g | Gram(s) |
| g/l | Gram(s)/liter(s) |
| g/ml | Gram (s)/milliliter(s) |
| GLP | Good Laboratory Practice |
| $H_2O$ MilliQ | Ultrapure water |
| ILSI | International Life Science Institute |
| kDa | Kilodalton(s) |
| kg | Kilogram(s) |
| $KH_2PO4$ | Potassium phosphate monobasic |
| M | Molar |
| mg | Milligram(s) |
| mg/ml | Milligram(s)/milliliter(s) |
| min | Minutes(s) |
| ml | Milliliter(s) |
| mm | Millimeter(s) |
| mM | Millimolar |
| MW | Molecular Weight |
| NaOH | Sodium hydroxyde |
| pH | Potential of hydrogen |
| PVDF | Polyvinylidene difluoride |
| SDS-PAGE | Sodium dodecyl sulfate- polyacrylamide gel electrophoresis |
| SIF | Human simulated intestinal fluid |
| Tris | Tris hydroxymethyl aminomethane |
| USA | United States of America |
| w/v | Weight/volume |

# FIGURES



Note: For clarity reasons, only the molecular weight maker 12 is presented in the following figures. The kaleidoscope molecular weight maker lane can be seen on the raw data file pictures.

**Fig 1: Coomassie blue stained SDS-PAGE of HPPD W336 protein after incubation in human simulated intestinal fluid**

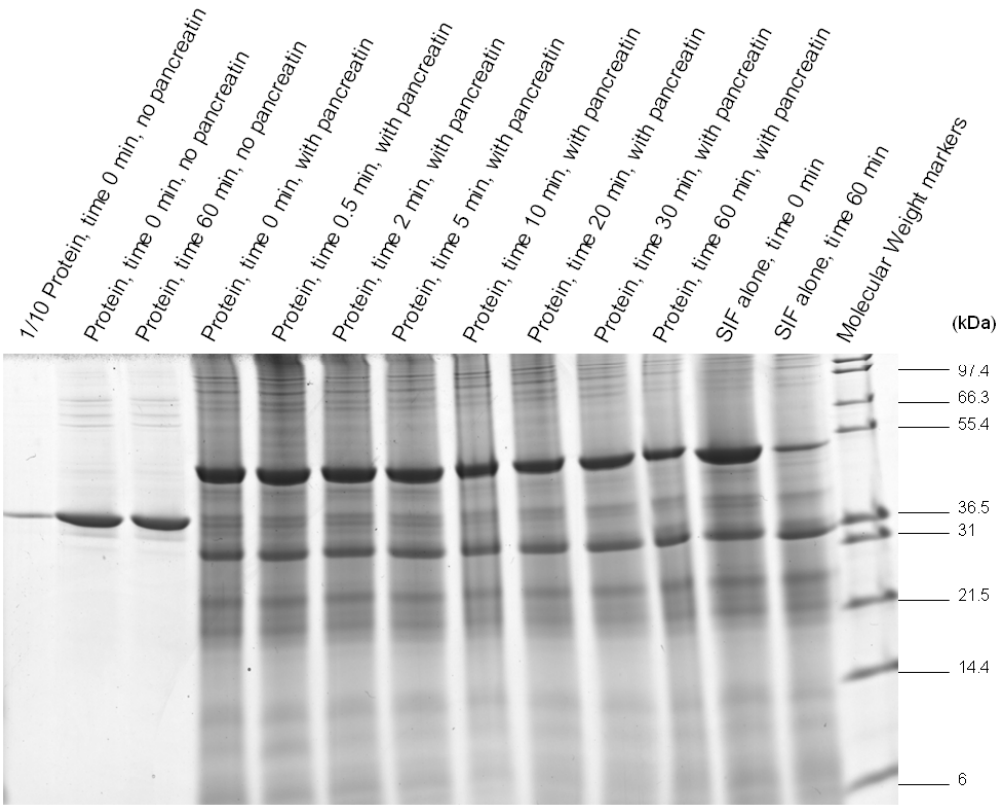
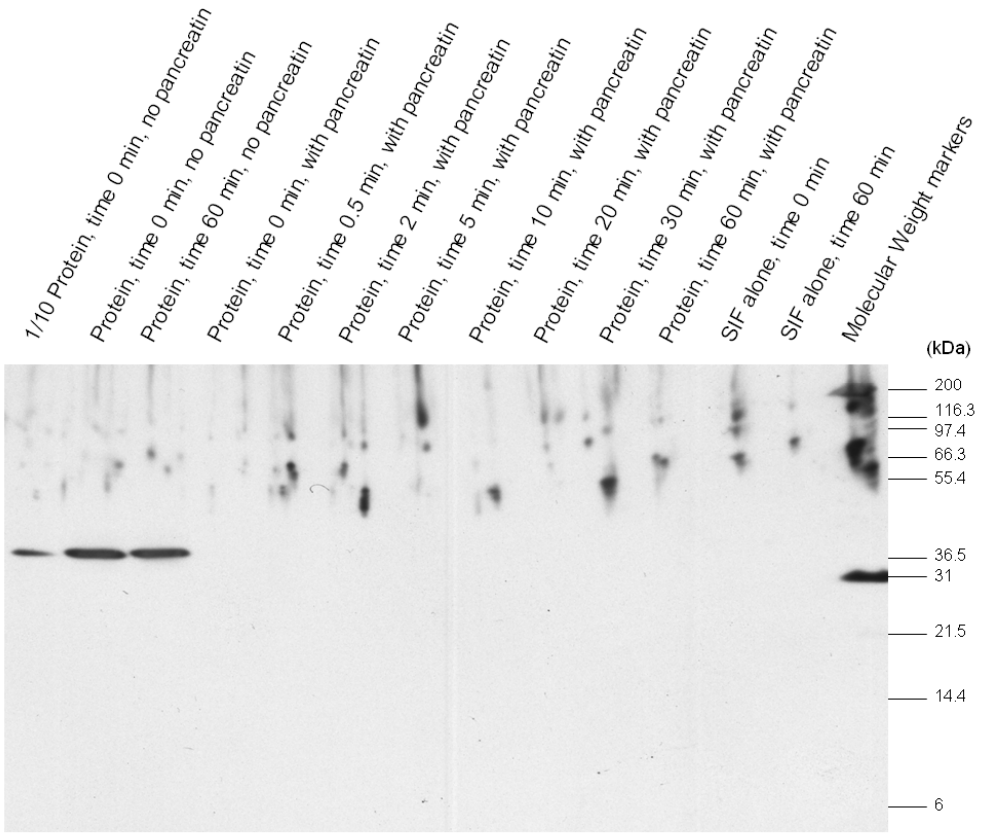


**Fig 2: Western blot of HPPD W336 protein after incubation in human simulated intestinal fluid**



# ATTACHMENTS

ATTACHMENT 1 – **PROTOCOL AND AMENDMENT**

**HPPD W336 PROTEIN**
***IN VITRO* DIGESTIBILITY STUDY IN HUMAN SIMULATED INTESTINAL FLUID**

**TESTING FACILITY:**
Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

**SPONSOR:**
Bayer AG
Bayer CropScience
Alfred Nobel Str. 50
40789 Monheim
Germany

## 1 GENERAL

### 1.1 PURPOSE OF STUDY

The present study is designated to determine the stability of the HPPD W336 protein, (produced in *E. coli*), by investigating the digestion of the protein using human simulated intestinal fluid (SIF). The *in vitro* protein degradation will be observed by Coomassie blue-stained SDS-PAGE and western-blot analyses and will give an indication of the potential stability of the protein in the digestive tract.

### 1.2 GOOD LABORATORY PRACTICE COMPLIANCE

This study will be performed in accordance with the principles of Good Laboratory Practice ("Bonnes Pratiques de Laboratoire") described in the following issues, with the exception of the test item solution, which will not be analyzed for concentration, homogeneity and stability.

- Organization for Economic Cooperation and Development (O.E.C.D.) Principles of Good Laboratory Practice, 1997 (January 26, 1998).
- European Directive 2004/10/EC (February 11, 2004).
- U.S. Environmental Protection Agency (E.P.A.)
  40 CFR Part 160
  Federal Insecticide, Fungicide and Rodenticide Act (FIFRA);
  Good Laboratory Practice Standards: Final Rule, August 17, 1989.
- Good Laboratory Practice Standards for Toxicology studies on Agricultural chemicals, Ministry of Agriculture, Forestry and Fisheries (M.A.F.F.) in Japan, notification 11 Nousan N°6283, October 01, 1999, modified by: 12 Nousan N°8628, December 06, 2000.
- French Decree N°2006-1523, regarding Good Laboratory Practice (December 04, 2006).

### 1.3 REGULATORY GUIDELINES

No data requirement for this study.

1.4 QUALITY ASSURANCE

The Quality Assurance Unit of Bayer CropScience, 355 rue Dostoïevski, BP 153, 06903 Sophia Antipolis Cedex, France, will undertake and document inspections while the study is in progress and will audit the study report.

## 2 STUDY PERSONNEL

2.1 STUDY DIRECTOR:

Date: April 17, 2009 J.B. RASCLE

2.2 SPONSOR REPRESENTATIVE:

Date: April 17, 2009 p.o. A. CAPT CORINNE HEROUET-GUICHENEY

2.3 OTHER STUDY PERSONNEL

| Responsibility | | Name |
|---|---|---|
| Replacement Study Director | : | D. ROUQUIE |
| Responsible Technician | : | A. ARNAUD |

Other study personnel will be identified as appropriate in the study file.

## 3 PROPOSED DATES

| | | | |
|---|---|---|---|
| Experimental starting date | : | April 20, 2009 | |
| Experimental completion date | : | May 07, 2009 | (estimated) |

## 4 OVERVIEW OF STUDY DESIGN

A solution of the test protein will be incubated with human simulated intestinal fluid (SIF) (a porcine pancreatin solution at pH 7.5) at approximately 37 °C and samples will be taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resultant solution will be analyzed for the presence of the HPPD W336 protein or potential stable protein fragments by western blot analysis. The immunodetection will be performed using a polyclonal antibody directed against HPPD W336 protein. Appropriate controls will include HPPD W336 protein in buffer without pancreatin, the corresponding 10% loading condition (to verify the sensitivity of the detection procedure) and SIF without HPPD W336 protein.

## 5 MATERIALS AND METHODS

5.1 PROTEINS AND PANCREATIN

The test item HPPD W336 protein will be supplied by BioAnalytics (Bayer BioScience NV, Gent, Belgium).

| | | |
|---|---|---|
| Test item identification | : | HPPD W336 protein (produced in *E. coli*) |
| Batch number | : | LB020309 |
| Purity | : | 96% ±2% |
| Storage | : | –74 + 10°C * |
| Certified through | : | Will be defined in the study report |

*: Storage condition at Sophia-Antipolis (SOP MTR00962)

The certificate of analysis will be attached to the study report.

The reference item (to show pancreatin activity) will be azoalbumin (A-2382, Sigma, France).

The pancreatin from porcine pancreas will be purchased from Sigma (Ref.: P3292).

5.2 PROTEIN SOLUTIONS

The test protein will be received lyophilized and will be solubilized in 50mM Tris pH 7.5 at the final concentration of 2.5mg HPPD W336 protein/ml.

Once the test protein is solubilised, it may be stored frozen at +5°C (±3°C) or colder.

Protein dissolution will be evaluated by visual inspection.

5.3 TEST SYSTEM

The simulated intestinal fluid will be prepared as follows:

- Preparation of 100 ml of I-con solution (6.8 g/l $KH_2PO_4$; titrate to pH 7.5 using NaOH)
- Preparation of the SIF solution by addition of pancreatin (1% w/v) to the I-con solution.

To ensure that the pancreatin is active, it will be tested against a pancreatin substrate, azoalbumin, using a spectrophotometric endpoint. The version of the spectrophotometric detection software is Cary 100 WinUV Version 3.00 (182). This test must show that the pancreatin is able to degrade azoalbumin rapidly.

5.4 5X LAEMMLI BUFFER

5X Laemmli buffer contains: 40% glycerol, 5% β-mercaptoethanol (BME), 10% SDS, 0.33 M Tris-HCl pH 6.8, 0.05% bromophenol blue.

5.5 DIGESTION

A dilution of the test protein solution at 1/10 in 50mM Tris pH 7.5 will be prepared for the 10% loading control.

The microcentrifuge tube of SIF for the serial digestion samples will be pre-warmed in a 37°C waterbath for approximately 2 minutes before time zero (prior to the addition of protein).

The 'serial digestion' tube prepared for the test protein digestion will contain 1520 µl of the SIF for sampling from 0.5 to 60 minutes.

Eighty µl of protein solution will be added (time zero) and briefly agitated then returned to the waterbath at 37°C.

At 0.5, 2, 5, 10, 20, 30 and 60 minutes, samples of 200 µl will be taken from the 'serial digestion' tube; prior to each sampling, tubes will be briefly agitated. An additional agitation at approximately 45 minutes without sampling will be performed.

Control tubes:

| | |
|---|---|
| I-con + protein 1/10 time 0 | 190 µl I-con (SIF without pancreatin) + 10 µl protein diluted to 1/10 at time zero |
| I-con + protein time 0 | 190 µl I-con (SIF without pancreatin) + 10 µl protein : sample at time zero |
| I-con + protein time 60 min | 190 µl I-con (SIF without pancreatin) + 10 µl protein : sample at 60 minutes (agitate and incubate in waterbath at 37°C) |
| SIF + protein time 0 | 190 µl SIF + 10 µl protein (added last after mixing and cooling on ice) |
| SIF alone, 0 min | 190 µl SIF + 10 µl $H_2O$ Milli Q: sample at time zero |
| SIF alone, 60 min | 190 µl SIF + 10 µl $H_2O$ Milli Q: sample at 60 minutes (agitate and incubate in waterbath at 37°C) |

5.6 SAMPLES

The reaction will be stopped as soon as samples are taken.

For the control tubes I-con + protein time 60 min and SIF alone time 60 min, each containing 200 µl of sample, the neutralisation solution (70 µl $H_2O$ MilliQ) will be added directly to the incubation tubes.

For the "serial digestion" samples and the other control tubes, the reaction will be terminated by adding the 200 µl of digestion sample to a tube containing 70 µl of $H_2O$ Milli Q and placed on ice.

In addition, 70µL of Laemmli solution (possibly supplemented with sucrose) will be added to all samples and heated for 10 minutes at >90°C.

Samples may be directly used for analysis on SDS-PAGE gels or frozen at approximately -20°C. When frozen, an aliquot of approximately 25µL may be prepared prior to freezing.

5.7 SDS-PAGE ANALYSIS

The method will be based on Laemmli's method (1970) using a Mini-Protean III cell (Bio-Rad, France). Samples of 15 µl will be added to wells of an SDS-PAGE gel (15 wells, 1 mm 10-20% gradient polyacrylamide Tris-Tricine, Bio-Rad).

Suitable molecular weight markers will be used to provide reference points of known molecular weights on the gel (Kaleidoscope, prestained standard, Bio-Rad and Mark 12, Invitrogen, France). Prior to running, 10 µl of 5X Laemmli will be added to 5 µl of Markers.

The test protein 'serial digestion' and control samples will be loaded on the same gel.

Loading order on each gel:

| Lane | Sample |
|---|---|
| 1 | Kaleidoscope |
| 2 | I-con + protein 1/10 time 0 |
| 3 | I-con + protein time 0 |
| 4 | I-con + protein time 60 min |
| 5 | SIF + protein time 0 |
| 6 | SIF + protein time 0.5 min |
| 7 | SIF + protein time 2 min |
| 8 | SIF + protein time 5 min |
| 9 | SIF + protein time 10 min |
| 10 | SIF + protein time 20 min |
| 11 | SIF + protein time 30 min |
| 12 | SIF + protein time 60 min |
| 13 | SIF alone, 0 min |
| 14 | SIF alone, 60 min |
| 15 | Marker 12 |

Electrophoresis will be carried out with a constant voltage set at 100 volts until the bromophenol line reaches the bottom of the gel.

Gels will be stained with Coomassie blue (Colloidal blue staining kit, Invitrogen, USA) and the gels will be scanned. The gels are not stable for more than a week, so the raw data will be the scanned image.

In addition, a second SDS-PAGE will be performed for the western blot analysis.

This gel will be loaded with approximately 100 times less protein in quantity to take into account the better level of detection of the western blot technique compared to the one of Coomassie blue staining. The dilution will be performed in 1X Laemmli solution (possibly supplemented with sucrose) prepared in $H_2O$ MilliQ.

5.8 WESTERN BLOT ANALYSIS

A polyvinylidene fluoride (PVDF) membrane will be placed on the gel in a Tris/Glycine buffer and an electrical current applied in order to transfer the protein bands onto the membrane. To detect the HPPD W336 protein bands and/or its potential fragments, the membrane will be incubated in the presence of a specific rabbit polyclonal anti-HPPD W336 protein antibody.

The hybridization of the antibody with the proteins immobilized on the membrane will be revealed by using a goat anti-rabbit polyclonal antibody coupled with a horseradish peroxydase. The hybridization bands will be visualized using chemoluminescent (ECL) detection system (Amersham, France).

The autoradiographs will be scanned (Bio-Rad) and resulting image will be retained in the raw data and reported in the final report.

5.9 DATA ANALYSIS

The results of a detailed visual inspection of the scans will be reported.

The gel will be considered to be valid if:

- the pancreatin bands are stable throughout the study on the gel.
- the protein or fragment bands are clearly visible at time zero (lanes 2 and 3) and not visible in the lanes without protein (lanes 13 and 14).
- at least one marker lane is clearly visible on the SDS gel to allow a molecular weight evaluation.

The study will be considered valid if the gels are valid.

The Coomassie blue-stained gel and the autoradiograph will be digitalized using high-resolution scanner (Biorad).

The time at which the test protein disappears will be reported. If stable fragments of the test protein are visible on the gels or on the blot, then their number, estimated molecular weight and time-course will be reported.

## 6 REPORTING

A copy of the draft report will be submitted to the Sponsor Representative and the Quality Assurance Unit for review. With the exception of the dated signature of scientists and other professional personnel, the draft report will contain all information and data to be included in the final report. The final report will include the information and data required by current internationally recognized regulations.

## 7 ARCHIVING

All raw data, supporting documents as well as protocol, protocol amendments and final report will be maintained in the archive room. An aliquot of the test substance solution will be kept in the area of the products storeroom defined for the archiving of test proteins.

All of the above will be archived for at least 10 years in the designated areas at:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

## 8 REFERENCES

LAEMMLI, U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970, 227, pp. 680-5.

PROTOCOL AMENDMENT

Protocol **SA 09052**

**HPPD W336 PROTEIN**
***IN VITRO* DIGESTIBILITY STUDY IN HUMAN SIMULATED INTESTINAL FLUID**

**Protocol amendment: N°1**

**Reason: Modification of the sample dilution factor for the western blot analysis**

Following initial analysis of the western blot data, it became clear that a repeat of this phase of the study would be required.

An additional dilution factor of the sample will be applied in order to take into account the better level of detection of the western-blot technique compared to one of Coomassie blue staining.

When compared to the Coomassie blue stained SDS-PAGE, the samples will now be loaded with approximately 1000 time less protein, and not 100 time less as originally tested, for the western blot.

Study Director:

Date: April 28, 2009

JB. RASCLE

ATTACHMENT 2 - **CERTIFICATES OF ANALYSIS**

Bayer CropScience



Title

**Certificate of analysis for the HPPD W336 protein produced in *E.coli* batch n°LB020309**

Author

**Veerle Habex**

Completed on

**April 24st, 2009**

Testing Facility

**BioAnalytics**
**Molecular Characterization**
**Bayer BioScience N.V.**
**Technologiepark 38**
**B-9052 Ghent**
**Belgium**

Study number

**BBS09-001**

Bayer CropScience



**STATEMENT OF DATA CONFIDENTIALITY CLAIMS**

This report is confidential. No part of the report or any information contained herein may be disclosed to any third party without the written prior authorisation of Bayer BioScience N.V.

Bayer CropScience



**GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT**

The undersigned hereby declares that the work to which this report refers was performed according to the procedures herein described and this report provides an accurate record of the results obtained. The study was conducted in accordance with the Good Laboratory Practice Standards as specified in the OECD/EU principles of Good Laboratory Practice.

Study Director

Veerle Habex
Molecular Characterization
BioAnalytics

24/4/09
Date

**STUDY IDENTIFICATION PAGE**

| | |
|---|---|
| Study Initiation date: | March 3rd, 2009 |
| Experimental start date: | March 9th, 2009 |
| Experimental Termination date: | April 6th, 2009 |
| Study Completion date: | April 24st, 2009 |
| Test Facility Address: | Bayer BioScience N.V.<br>BioAnalytics<br>GLP Test Facility<br>Technologiepark 38<br>9052 Ghent – Belgium<br>Tel: +32 9-243 04 11<br>Fax: +32 9-224 06 94 |
| Test Facility Manager: | Dirk Nennstiel<br>Address see Test Facility<br>Tel: +32 9-243 04 39<br>Fax: +32 9-224 06 94<br>e-mail: GLP_TFM@bayercropscience.com |
| Study Director: | Veerle Habex<br>Address see Test Facility<br>Tel: +32 9-243 05 84<br>Fax: +32 9-224 06 94<br>e-mail: veerle.habex@bayercropscience.com |
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Bayer CropScience



## QUALITY ASSURANCE STATEMENT

Report **BBS09-001** Date: 24 APR 2009

Quality Assurance (GLP)

**Quality Assurance Statement**

Title: **Certificate of analysis for the HPPD W336 protein produced in E. *coli* batch n° LB020309**

Study: BBS09-001

**This study was periodically inspected and properly signed records of these inspections were submitted to Test Facility management and the Study Director as listed below.**
**This report has been audited by the GLP Quality Assurance. The reported results accurately reflect the original raw data of the study.**

| Phase of Study | Inspection date | Reporting date |
|---|---|---|
| Study plan | 05 MAR 2009 | 06 MAR 2009 |
| Study conduct | 09 MAR 2009 | 09 MAR 2009 |
| Study conduct | 10 MAR 2009 - 13 MAR 2009 | 17 MAR 2009 |
| Study plan amendment | 30 MAR 2009 | 31 MAR 2009 |
| Study plan amendment | 07 APR 2009 | 07 APR 2009 |
| Draft report | 16 APR 2009 - 17 APR 2009 | 20 APR 2009 |
| Final report | 22 APR 2009 | 24 APR 2009 |

**L. Hottin**
GLP Quality Assurance

24/04/09

Bayer BioScience N.V. - BioAnalytics

Bayer CropScience



**APPROVALS PAGE**

| | | |
|---|---|---|
| Study Director / Author: | Veerle Habex | 24/4/09 Date |
| Test facility management/ Molecular characterization manager: | Dirk Nennstiel | 24/04/09 Date |
| Sponsor Representative: | Nicolas De Schrijver | 30/04/09 Date |

## TABLE OF CONTENTS



## LIST OF FIGURES



## LIST OF APPENDICES

**SUMMARY**

Bayer CropScience has introduced a *hppdPfW336* gene construct, conferring tolerance to isoxaflutole in *Glycine max* plants by means of particle bombardment. The explants were regenerated to whole plants and an elite event was selected on the basis of expression of the transgenic protein and agronomic performance.

Because the expression level of the HPPD W336 protein in transgenic plants is extremely low, safety studies are conducted with the HPPD W336 protein produced in *E. coli*.
In this study, the identity of the HPPD W336 protein produced in *E. coli*, batch LB020309 was confirmed by means of a molecular weight determination using SDS-PAGE, an immunoreactivity analysis using western blotting and an activity assay. The purity was determined by means of SDS-PAGE to be 96 ± 2%.

## 1. OBJECTIVE

In this study, the characterization of the HPPD W336 protein produced in *E. coli* batch n° LB020309 was performed by means of a concentration determination, a purity determination and a confirmation of the identity by means of a molecular weight determination, an immunoreactivity determination and an activity assay.

## 2. OVERVIEW OF EXPERIMENTAL DESIGN

In this study five analyses were performed on the resuspended test item T34-01. The concentration of the protein was determined by means of $OD_{280}$ measurement; the molecular weight and the purity of the protein were analyzed after sodiumdodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE); the identity of the protein was confirmed by western blotting and the activity of the protein was determined by means of the HPPD activity assay.

## 3. TEST ITEM, REFERENCE ITEM AND STANDARDS

### 3.1. Test item

The subject of this study, test item ID T34-01, was lyophilized HPPD W336 protein purified from *E. coli* batch LB020309. The test item was produced by Bayer BioScience N.V., Ghent, Belgium, as described in the study report 'Production of the HPPD W336 protein in *Escherichia coli* – Batches LB020309 and LB090309'.
Lyophilisation of the dialysed HPPD W336 protein was done in batches of ca. 1 mg (0.2 ml at 5 mg/ml).
Before the resuspension of the test item, the protein was stored in the ultrafreezer; after resuspension, the test item was stored in the refrigerator.

| | |
|---|---|
| Test Item ID: | T34-01 |
| Test Item Identity: | HPPD W336 protein |
| Origin: | *Escherichia coli* |
| Batch n°: | LB020309 |
| Expiry date: | The stability of the test item will be assessed in a quality management study, by means of a concentration determination, a molecular weight determination, a western blot analysis and an activity assay. The dates of the quality management studies for the HPPD W336 protein are June 2009, September 2009, December 2009, March 2010, September 2010, March 2011, September 2011, September 2012 and September 2013. The expiry date of the test item is guaranteed until the next quality management study. Based on the results obtained, the expiry date can be extended. The latest updates can be obtained upon request. |

### 3.2. Reference items

No reference items were used in this study.

### 3.3. Standards

As standard, the molecular weight marker 'Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)' was used to determine the molecular weight of the protein. The stock solution of this standard was stored in freezer 90FZ. The work solution was during the study conduct stored in refrigerator 91RF.

## 4. EXPERIMENTAL DESIGN

### 4.1. Resuspension of the test item

The content of 5 tubes each containing ca. 1 mg of the test-item T34-01, was resuspended individually in 1 ml of 50 mM Tris pH 7.5. The 5 mixtures were thoroughly vortexed, kept on ice for more than 15 minutes and vortexed again. The tubes were shortly spinned to collect all material at the bottom of the tube. The different suspensions were pooled and vortexed again. After resuspension, the sample was kept on ice or in the refrigerator. The redissolved protein was discarded after study completion.

### 4.2. Quantification of the test item

The concentration of test item T34-01 was determined by means of $OD_{280}$ measurement according to SOP BBS 07/80/00. Ten independent 1/2 dilutions of the test item in 50 mM Tris pH 7.5 were prepared. This dilution gave the appropriate $OD_{280}$ value between 0.2 and 0.8.
The calculation of the concentration was done using a validated excel sheet (Figure 1) as described in SOP BBS 07/80/00, knowing by the sequence of the protein that 1 $OD_{280}$ corresponds to a HPPD W336 concentration of 1.15 mg/ml (molar extinction coefficient = 35110; molecular weight = 40312 Da).

### 4.3. Molecular weight determination of the test item

To determine the molecular weight, the resuspended test item was analyzed by SDS-PAGE according to the SOP BBS 07/77/00. A NuPAGE® NOVEX Bis-Tris 10% gel was used in combination with a NuPAGE® MOPS SDS gel running buffer (Invitrogen). The concentration determined in section 4.2 was used to determine the volume of HPPD W336 protein to be loaded.

Loading order of gel G1-09-001:

Lane 1: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 2: 0.5 µg of resolved HPPD W336
Lane 3: 0.5 µg of resolved HPPD W336
Lane 4: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 5: 0.5 µg of resolved HPPD W336
Lane 6: 0.5 µg of resolved HPPD W336
Lane 7: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 8: 0.5 µg of resolved HPPD W336
Lane 9: 0.5 µg of resolved HPPD W336
Lane 10: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)

The gel was run at a constant voltage of 180 V during approximately 55 minutes. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue Staining according to SOP BBS 07/66/02. A photographic copy of the stained gel was made according to SOP BBS 07/42/01. This scan received the ID G1-09-001-F1 and is shown in Figure 2.

To dry the gel, the gel and 2 sheets of cellophane were soaked at least 2 min in Acrylamide gel drying solution. The gel was stretched between both cellophane sheets using the gel drying cassette of the DryEase Mini-Gel Drying system of Invitrogen. The gel was dried overnight and fixed to a white sheet of paper.

The molecular weight of the HPPD W336 protein was determined according to SOP BBS 07/42/02 using the scan with ID number G1-09-001-F1. In order to obtain a linear regression curve, the molecular weight proteins of 250 kDa, 150 kDa and 10 kDa were not taken into account.
The molecular weight and the value of the 95% confidence interval of the HPPD W336 protein were determined automatically in a validated Excel sheet (Figure 3).

### 4.4. Purity determination and immunoreactivity of the test item

To determine the purity and the immunoreactivity of the test item, the resuspended test item was loaded on a NuPAGE® NOVEX Bis-Tris 10% gel in combination with a NuPAGE® MOPS SDS gel running buffer (Invitrogen). The concentration determined in section 4.2 was used to determine the volume of HPPD W336 protein to be loaded.

Loading order of gel G2-09-001:

Lane 1: /
Lane 2: 2 µg of resolved HPPD W336
Lane 3: 2 µg of resolved HPPD W336
Lane 4: 2 µg of resolved HPPD W336
Lane 5: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 6: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 7: 0.03 µg of resolved HPPD W336
Lane 8: 0.01 µg of resolved HPPD W336
Lane 9: 0.003 µg of resolved HPPD W336
Lane 10: /

The gel was run at a constant voltage of 180 V during approximately 55 minutes. After electrophoresis, the gel was cut between positions 5 and 6. The proteins in lanes 1 to 5 were stained with Coomassie Brilliant Blue according to SOP BBS 07/66/02. A picture of the stained gel was made using the G-BOX (SOP BBS 04/77/01). This scan received the ID G2-09-001-F1 and is shown in Figure 4 – Panel A. The proteins in lanes 6 to 10 were transferred to a ProBlott membrane according to SOP BBS 07/64/03. This membrane received ID M2-09-001

#### 4.4.1.Purity analysis

Picture G2-09-001-F1 was analysed using the Genetools software to determine the protein purity of the test item (SOP BBS 07/42/01). The purity was calculated for the 3 lanes containing the HPPD W336 protein. The mean of these purity values and the standard deviation were calculated.

#### 4.4.2.Immunoreactivity of the test item by means of western blot

The membrane M2-09-001 was developed according SOP BBS 07/65/01. In this technique two antibodies were used:

- Mouse anti HPPD W336 (batch A36440-3), supplied by MS Technologies and tested at Bayer BioScience N.V. The antibody was stored in refrigerator 91RF and used in a 1:1500 dilution.
- Rabbit anti mouse-Alkaline Phosphatase (Sigma cat # A1902). The antibody was stored in the refrigerator 91RF and used at a 1:1000 dilution.

An electronical copy of the membrane was made according to SOP BBS 04/70/02. This scan (ID M2-09-001-F1) is presented in Figure 4 – Panel B. The original membrane was fixed to a white sheet of paper by means of a self-adhesive acetate plate sealer sheet.

### 4.5. HPPD W336 activity assay

HPPD W336 catalyzes the transformation of 4-hydroxyphenylpyruvate (HPP) into homogentisate. This activity is measured in a colorimetric method by determining the amount of HPP remaining in the assay mixture at the end of the incubation period after derivatisation with 2,4-Dinitrophenylhydrazine (DNP) (SOP BBS 07/62/00).

## 5. RESULTS

### 5.1. Quantification of the test item

The protein concentration of the test item was determined to be 1.01 ± 0.01 mg/ml (Figure 1).
Based on the volumes of the HPPD W336 protein subjected to lyophilisation and the amount described above, the amounts of HPPD W336 in the eppendorf tubes, the 15 ml and the 50 ml tubes were calculated to be either 1.01 ± 0.01 mg for the eppendorf tubes; 5.05 ± 0.05 mg or 25.25 ± 0.25 mg for the 15 ml tubes and 75.75 ± 0.75 mg for the 50 ml falcon tubes.

### 5.2. Molecular weight determination

A picture of gel G1-09-001 is shown in Figure 2. The calculation of the molecular weight is showed in Figure 3.

The molecular weight was calculated to be 40.7 ± 2.5 kDa. The accuracy of the technique is 4 kDa. Taken both together, the determined molecular weight fits with the deduced molecular weight of the HPPD W336 protein of 40.3 KDa.

### 5.3. Purity determination

The purity of the test item was assessed using gel G2-09-001 (Figure 4 – panel A). The purity of the test item was determined in all 3 lanes. The mean of the purity values and the standard deviation were calculated to be respectively 96 % and 2 %.

### 5.4. Immunoreactivity analysis

The western blot analysis (Figure 4 – panel B) revealed in each sample the expected HPPD W336 band, confirming the identity of the test item.

### 5.5. HPPD W336 activity determination

The activity analysis showed the activity of the HPPD W336 protein.

## 6. CONCLUSION

Biochemical analyses were performed to confirm the identity of test item T34-01. Based on the analyses the T34-01 test item was identified as HPPD W336 protein. The concentration of the protein in the test item T34-01 was determined at 1.01 ± 0.01 mg/ml with a purity of 96 ± 2 %. The activity of the test item was demonstrated.

## 7. ARCHIVING

The study plan, amendments and deviations, other study data, and the original of the final report will be archived in study file BBS09-001 at the BBS N.V. GLP test facility document archive at the test facility address.

One vial containing 1.01 mg of test item T34-01 was stored in the GLP Test Facility test and reference item archive at the test facility address.

## REFERENCES


| No | Doc No | Report No | Author(s), year, title, source, edition, pages |
|---|---|---|---|
| 1. | - - - - - - - | BIOX-013_ProtProd_243 | Beurms, L., Habex, V. – 2009 - Production of the HPPD W336 protein in *Escherichia coli* – Batch LB020309 and batch LB090309 |

**Figure 1: Determination of the concentration of the resuspended test item**

**Determination of Protein Concentration (OD280 method)**

**Average / Standard deviation OD values**

| | | Average | Standard deviation |
|---|---|---|---|
| OD260 | blanks | 0.0452 | 0.0013 |
| | samples | 0.2825 | 0.0037 |
| OD280 | blanks | 0.0408 | 0.0010 |
| | samples | 0.4119 | 0.0038 |
| Blank corrected OD280 | | 0.3712 | 0.0040 |

**Acceptance criteria**

| | |
|---|---|
| Average OD280 samples > average OD260 samples ? | Yes |
| Average OD280 blanks < 0,2 * average OD280 samples ? | Yes |
| Average OD280 samples ≥ 0,2 and ≤ 0,8 ? | Yes |
| Average OD260 samples ≥ 0,2 and ≤ 0,8 ? | Yes |
| **All acceptance criteria met?** | **Yes** |

**Calculation Protein concentration**

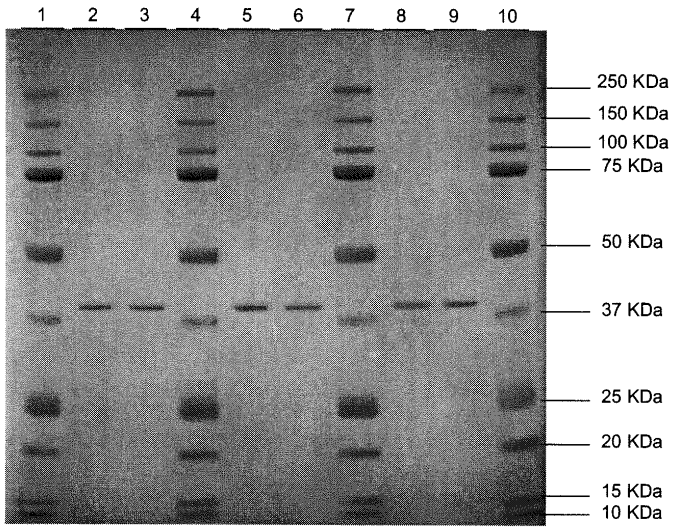
| | |
|---|---|
| Protein : | HPPD |
| Test item ID : | T34-01 |
| Dilution factor (1/x) : | 2 |
| Dilution buffer : | 50 mM Tris-HCl pH 7,5 |
| Batch N° dilution buffer : | 50 mM Tris pH 7,5 - 01 |
| Magellan workspace ID : | 09032009-001.wsp |

| | |
|---|---|
| Molecular Weight of HPPD : | 40312 Da |
| Molar extinction ratio of HPPD : | 35110 |
| Path length (300 µl): | 0.84 cm |

| | Calculated concentration | Standard deviation | Relative 95% confidence interval | **95% confidence interval** |
|---|---|---|---|---|
| Diluted sample | 0.51 mg/ml | 0.01 mg/ml | 0.66% | 0.51 ± 0 mg/ml |
| Undiluted sample | 1.01 mg/ml | 0.01 mg/ml | 0.66% | **1.01 ± 0.01 mg/ml** |

For calculations all available number of digits are taken into account.
The values displayed are rounded values, in order to improve readability of data.

**Figure 2: SDS-PAGE gel of test item T34-01 to determine the molecular weight (Gel ID G1-09-001)**




Loading order:
Lane 1: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 2: 0.5 µg of resolved HPPD W336
Lane 3: 0.5 µg of resolved HPPD W336
Lane 4: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 5: 0.5 µg of resolved HPPD W336
Lane 6: 0.5 µg of resolved HPPD W336
Lane 7: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 8: 0.5 µg of resolved HPPD W336
Lane 9: 0.5 µg of resolved HPPD W336
Lane 10: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)

**Figure 3: Determination of the molecular weight of the test item**

**Determination of the molecular weights of proteins**

**Migration distances**

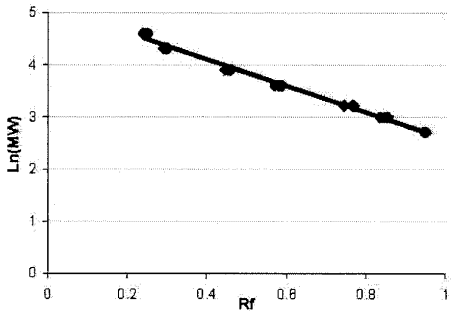
Marker: Precision Plus Protein Standards

| Molecular weight of bands (kDa) | Migration distance of bands (mm), measurement number | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| 250 kDa | | | | |
| 150 kDa | | | | |
| 100 kDa | 25.0 | 24.5 | 24.0 | 24.0 |
| 75 kDa | 30.0 | 29.5 | 29.0 | 29.0 |
| 50 kDa | 45.5 | 45.5 | 45.5 | 44.5 |
| 37 kDa | 58.0 | 58.5 | 58.0 | 57.0 |
| 25 kDa | 76.5 | 76.5 | 76.0 | 74.5 |
| 20 kDa | 84.5 | 85.0 | 84.5 | 83.5 |
| 15 kDa | 94.5 | 94.5 | 94.0 | 94.5 |
| 10 kDa | | | | |
| Total migration distance (mm) | 100.0 | 99.5 | 99.0 | 100.0 |

Sample: T34-01

| Measurement number | Migration distance (mm) | Total migration distance (mm) |
|---|---|---|
| 1 | 56.0 | 100.0 |
| 2 | 55.5 | 99.5 |
| 3 | 55.5 | 99.0 |
| 4 | 55.5 | 99.0 |
| 5 | 55.0 | 99.5 |
| 6 | 55.0 | 99.5 |

**Regression**




$y = -2.5446x + 5.1255$

$R^2 = 0.9925$

**Predicted Molecular Weights**

Marker : Precision Plus Protein Standards

| Known Molecular Weight of bands (kDa) | Predicted Molecular Weight (kDa), measurement number | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| 250 kDa | | | | |
| 150 kDa | | | | |
| 100 kDa | 89.1 | 89.9 | 90.8 | 91.4 |
| 75 kDa | 78.4 | 79.1 | 79.9 | 80.4 |
| 50 kDa | 52.9 | 52.6 | 52.3 | 54.2 |
| 37 kDa | 38.5 | 37.7 | 37.9 | 39.5 |
| 25 kDa | 24.0 | 23.8 | 23.9 | 25.3 |
| 20 kDa | 19.8 | 19.1 | 19.2 | 20.1 |
| 15 kDa | 15.2 | 15.0 | 15.0 | 15.2 |
| 10 kDa | | | | |

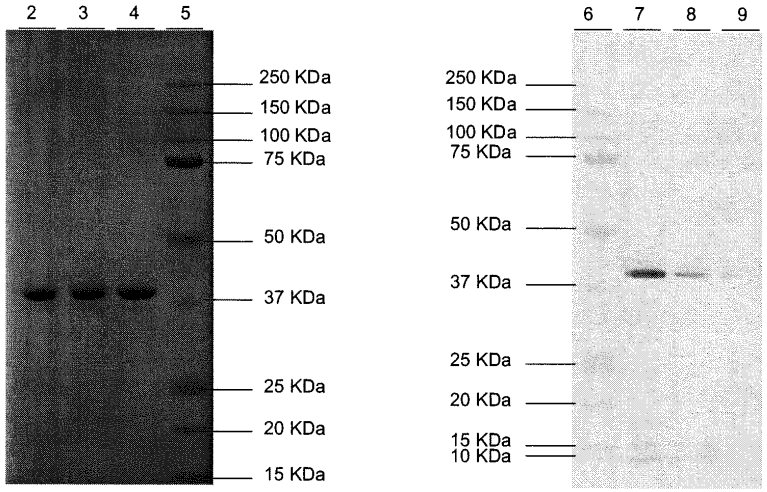
For calculations all available number of digits are taken into account
The values displayed are rounded values, in order to improve readability of data

Sample: T34-01

| Measurement number | Predicted Molecular Weight (kDa) |
|---|---|
| 1 | 40.5 |
| 2 | 40.7 |
| 3 | 40.4 |
| 4 | 40.4 |
| 5 | 41.2 |
| 6 | 41.2 |

| | |
|---|---|
| Predicted Molecular Weight : | 40.7 ± 2.5 kDa |
| Relative 95% confidence interval : | 6.1% |

**Figure 4: SDS-PAGE gel of test item T34-01 to determine the purity (Gel ID G2-09-001) and Western blot of test item T34-01 (Membrane ID M2-09-001)**




**Panel A: Coomassie stained part of gel G2-09-001** **Panel B: Western blot M2-09-002**

Gel G2-09-001 was after electrophoresis divided in 2 parts:

- The first 5 lines were coloured with Coomassie Blue (SOP BBS 07/66/02). This part of the gel was used to determine the purity of the test item.
- The last 5 lines were blotted (SOP BBS 07/64/03) and developed with as primary antibody mouse anti-HPPD and as secondary antibody Rabbit anti-mouse-Alkaline Phosphatase (SOP BBS 07/65/01). In this western blot, the immunoreactivity of the HPPD W336 protein was demonstrated.

Lane 1: /
Lane 2: 2 µg of the resolved HPPD W336
Lane 3: 2 µg of the resolved HPPD W336
Lane 4: 2 µg of the resolved HPPD W336
Lane 5: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 6: 5 µl of the Precision Plus Protein™ Standard Dual Color Marker (Bio-Rad)
Lane 7: 0.3 µg of the resolved HPPD W336
Lane 8: 0.1 µg of the resolved HPPD W336
Lane 9: 0.03 µg of the resolved HPPD W336
Lane 10: /

**Appendix 1: Overview of the analytical SOPs used in this study**

| SOP | Title | Reference |
|---|---|---|
| **BBS 07/42/01**<br>**BBS 07/42/02** | Fragment analysis using the gene tools software | Genetools user manual - Syngene |
| **BBS 07/62/00** | HPPD activity assay | |
| **BBS 07/64/03** | Electro transfer of proteins to membranes | Instruction manual Mini Trans-Blot® Electrophoretic Transfer Cell (Version M1703930 Rev.E) – BioRad<br>Instruction manual immobilization membranes ProBlott® - Applied Biosystems |
| **BBS 07/65/01** | Western blotting | |
| **BBS 07/66/02** | Coomassie Brilliant Blue Staining of gels | Instructions Gelcode® Blue Stain Reagent, version 0714.2 – Pierce Biotechnology |
| **BBS 07/77/00** | Sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) using NuPAGE® Novex Bis-Tris gels | NuPAGE technical guide – Invitrogen |
| **BBS 07/80/00** | Determination of protein concentration by OD280 measurement (Tecan method) | How to measure and predict the molar absorption coefficient of a protein - Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. and Gray, T. - Protein Sci. 1995 4:2411-2423. |

# Certificate of Analysis

SIGMA-ALDRICH

| | |
|---|---|
| **Product Name** | Pancreatin from porcine pancreas, powder, 4 × USP specifications, cell culture tested |
| **Product Number** | P3292 |
| **Product Brand** | SIGMA |
| **CAS Number** | 8049-47-6 |

| TEST | SPECIFICATION | LOT 117K1343 RESULTS |
|---|---|---|
| **APPEARANCE** | LIGHT YELLOW TO TAN POWDER | TAN POWDER |
| **DIGESTIVE POWER** | 4X USP SPECIFICATIONS | CONFORMS* |
| **CELL CULTURE TEST** | PASS | PASS |
| | | * SUPPLIER'S INFORMATION |
| **QC RELEASE DATE** | | DECEMBER 2007 |

Rodney Burbach, Manager
Analytical Services
St. Louis, Missouri USA

# Certificate of Analysis

SIGMA-ALDRICH

| | |
|---|---|
| **Product Name** | Azoalbumin |
| **Product Number** | A2382 |
| **Product Brand** | SIGMA |
| **CAS Number** | 102110-73-6 |

| TEST | SPECIFICATION | LOT 026K7565 RESULTS |
|---|---|---|
| **APPEARANCE** | ORANGE TO ORANGE-BROWN WITH A RED CAST POWDER | ORANGE POWDER WITH A RED CAST |
| **SOLUBILITY** | RED-ORANGE TO RED-BROWN SOLUTION AT 25MG/ML IN 0.01N HYDROCHLORIC ACID | RED-ORANGE SOLUTION |
| **PROTEIN BY LOWRY** | REPORT RESULT | 78% |
| **SUITABILITY** | SUITABLE FOR USE AS A TRYPSIN SUBSTRATE | CONFORMS |
| **UV-VIS SPECTRUM** | LAMBDA MAX AT 440NM IN 0.1N SODIUM HYDROXIDE | E1% = 28 AT LAMBDA MAX 440NM |
| **RECOMMENDED RETEST SOP QC-12-006** | 5 YEARS | APRIL 2011 |
| **QC RELEASE DATE** | | APRIL 2006 |

Rodney Burbach, Manager
Analytical Services
St. Louis, Missouri USA

# FINAL REPORT AMENDMENT

There is no final report amendment at this time.

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