Bayer CropScience

# 2mEPSPS PROTEIN

# *IN VITRO* DIGESTIBILITY STUDY IN

# SIMULATED INTESTINAL FLUID

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

**REPORT OF STUDY SA 06102**

**AUTHOR / STUDY DIRECTOR: D. ROUQUIE**

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: JULY 28, 2006**

# 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, Section 10(d)(1)(A)(B), or (C).

However, these data are the property of Bayer CropScience AG, and as such, are considered to be a trade secret and 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 country other than the USA.

Company: Bayer CropScience
Research and Development Department

Company Agent:

Title:

Signature:

Date: _____

The above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

# 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 software Cary 100 used to measure the optical densities for assessing the activity of pancreatin. In addition, the dosing suspensions were not analyzed for concentration, homogeneity or 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° 98-1312, on December 31, 1998 regarding Good Laboratory Practice.

- 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: 28 July 2006

D. ROUQUIE

Sponsor Representative:

Date: 28 July 2006

C. HERVOUET-GUICHENEY

Study Submitter:

Date:

# FLAGGING STATEMENTS

This page is reserved for flagging statements as may be required by E.P.A.

# 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 | Date of Q.A. inspection | Date of reporting to Study Director | Date of reporting to Management |
|---|---|---|---|
| Protocol | April 28,2006 | May 02, 2006 | May 11, 2006 |
| SIF test | May 03, 2006 | May 03, 2006 | May 05, 2006 |
| Report | July 24, 2006 | July 24, 2006 | July 27, 2006 |

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: July 28, 2006

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: July 28, 2006

D. ROUQUIE

# STUDY PROFESSIONALS

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

| | | |
|---|---|---|
| AUTHOR / STUDY DIRECTOR | : | D. ROUQUIE |
| RESPONSIBLE TECHNICIAN | : | B. LABORY-CARCENAC |
| REPORT UNIT ASSISTANTS | : | P. ALMERAS |

# TABLE OF CONTENTS

# SUMMARY

The 2mEPSPS protein 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 in accordance with the ILSI protocol using simulated gastric fluid (1). This protocol was adapted to use SIF.

The 2mEPSPS protein was degraded very rapidly in human simulated intestinal fluid, in less than 30 seconds of incubation, 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 full GLP study carried out in line with a current internationally recognized protocol. This study follows the protocol of the methods used in the 2004 ILSI ring trial (1). The method is based on the United States Pharmacopea (2).

In this study the test material was the 2mEPSPS protein. The sample used corresponds to the 2mEPSPS protein encoded by *2mepsps* gene. Furthermore, results from several laboratories world-wide validate the choice of these two reference proteins (1).

The study protocol and amendment are presented in Attachment 1.

The study schedule was as follows:

| | |
|---|---|
| Sponsor representative protocol approval date | April 27, 2006 |
| Study initiation date* | April 27, 2006 |
| Experimental starting date | May 03, 2006 |
| Experimental completion date | June 13, 2006 |

* Date of protocol approval by Study Director

# MATERIAL AND METHODS

## 1 – REAGENTS

### 1.1 Proteins

The test protein was supplied by the sponsor.

| | | |
|---|---|---|
| Identification | : | 2mEPSPS protein |
| Batch N° | : | LEJ5837 |
| Source | : | Bayer Bioscience N.V., Gent, Belgium |
| Description | : | Solution in Storage buffer (100 mM Tris/HCl; 2.7 mM KCl; 137 mM NaCl; 1 mM DTT; pH 7.5), concentration at 0.93 mg/ml |
| Purity | : | >95% |
| Expiry date | : | April 03, 2008 |
| Storage | : | Approximately -20°C |

The certificates of analysis are presented in Attachment 2.

### 1.2 Proteolytic enzyme

| | | |
|---|---|---|
| Identification | : | Pancreatin (from porcine pancreas) |
| Batch N° | : | 055K1193 |
| Source | : | Sigma, Reference P3292 |
| Description | : | Light yellow powder |
| Concentration and purity | : | Biological mixture of enzymes, concentration and purity not supplied by Sigma |
| Stability | : | Stable under storage conditions |
| Storage | : | Approximately -20°C |

The certificate of analysis is presented in Attachment 2.

## 2 – TEST SOLUTIONS

### 2.1 Protein solution

The 2mEPSPS protein was tested at a concentration of 0.93 mg/ml in storage buffer solution (100 mM Tris/HCl; 2.7 mM KCl; 137 mM NaCl; 1 mM DTT; pH 7.5).

### 2.2 Digestive solution

The human simulated intestinal fluid (SIF) consisted of the intestinal control solution (I-con):

- 0.68 g $KH_2PO_4$
- 19 ml 0.2 M NaOH
- Titrated to pH 7.5 using dilute NaOH then the volume adjusted to 100 ml with distilled water.

The pancreatin was added to the I-con solution to give a 1 % (w/v) pancreatin solution.

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

### 3 – DIGESTION INCUBATION

The protein incubation for the test and control materials was made in 2 ml microcentrifuge tubes in a waterbath at approximately 37°C. For each test and control 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 45 minutes.

As soon as samples were taken, the reaction was terminated by adding the 200 µl sample to a tube containing 70 µl distilled water placed on ice.

Additional control samples were prepared:
- a zero minute incubation of protein (10 µl) with 'SIF without any pancreatin' (190 µl);
- 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 20 µl of the samples was used for analysis on SDS-PAGE gels and the remaining was frozen at -20°C.

### 4 – SDS-PAGE ELECTROPHORESIS

Gel electrophoresis were carried out, following the method of Laemmli (3) using a BioRad Mini-Protean III cell. Prior to running SDS-PAGE, 10 µl of 5X Leammli solution was added to 20 µl of digestion samples and heated for 10 minutes at more than 90°C before loading the gel. Samples of 12 µl were added to wells of an SDS-PAGE gel (15 well, 1mm 12-20% gradient polyacrylamide Tris/Tricine)(BioRad, France).

A suitable marker solution (2.5-200 kDa) was used to provide reference points of known molecular weights on the gel (Mark 12, Invitrogen, France).

A setting of 117 ± 7 volts was used at a constant voltage for approximately 2 hours to give gels with a correct appearance (near straight lines for sample front and a front finishing near the top of the gel).

5- WESTERN BLOT ANALYSIS

A nitrocellulose membrane was placed on the second gel in a Tris/Tricine buffer and the protein transfer was made by passing an electrical current from the gel to the membrane leaving an imprint of the proteins on the nitrocellulose.

The membrane was rinsed in PBS/Tween 20 (0.2% v/v) for approximately 2.5 hours then after washing, incubated with the specific anti-2mEPSPS protein antibody (rabbit anti-2mEPSPS antibody, supplied by the sponsor) at 2 µl per 20 ml PBS/Tween 20 buffer for approximately 1 hour. Following a washing step, the membrane was incubated with an alkaline phosphatase linked goat anti-rabbit antibody in a similar buffer for approximately 1 hour. In order to detect the alkaline phosphatase enzymatic activity, the membrane was placed in a detection solution of bromo-chloro-indoyl-phosphate and nito-blue-tertrazolium in a dark room for approximately 20 minutes, then washed and dried. The membrane was scanned using a Scan Jet ADF (Hewlet Packard)

6 - DATA STORAGE

All raw data, supporting documents, as well as protocol, protocol amendments and final report are maintained in the document 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 - 2mEPSPS PROTEIN (Fig. 1)

As expected, the SDS-PAGE analysis of the 2mEPSPS protein solution revealed one band located between the molecular weight markers at 36.5 kDa and at 55.4 kDa. The 2mEPSPS protein band was visible in the zero and 60 minute incubation time without pancreatin, with no decrease in stain intensity at 60 minutes. At time zero of incubation with SIF, the pancreatin band at approximately 31 kDa were clearly visible. At time zero of incubation, the 2mEPSPS protein band was so faintly visible on the gel that it was not clearly visible after scanning the gel. Digestibility was dramatically increased by pre-heating and the 2mEPSPS was immediately digested within few seconds. At all subsequent incubation times, there were no 2mEPSPS band observed and no smaller fragment bands.

This indicates a complete digestion of the 2mEPSPS protein (encoded by the *2mepsps* gene) with less than 30 seconds.

## 2 - DISCUSSION

The 2mEPSPS protein was degraded very rapidly with no fragment protein visible in less than 30 seconds of incubation with SIF, in presence of pancreatin, at pH 7.5.

# CONCLUSION

The 2mEPSPS protein was degraded very rapidly in human simulated intestinal fluid, in less than 30 seconds of incubation, in presence of pancreatin, at pH 7.5.

# PROTOCOL DEVIATION

Figure 1: Prior to running SDS-PAGE, 10 µl of 5X Leammli solution was added to 20 µl of digestion samples and heated for 10 minutes at more than 90°C before loading the gel. Samples of 12 µl were added to wells of an SDS-PAGE gel (15 well, 1 mm 12-20% gradient polyacrylamide Tris/Tricine)(BioRad, France).
Loading order on the gel:

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

Markers N°2: Kaleioscope pre-stained standard (Biorad, reference number 1610324).

For the western blot analysis, a nitrocellulose membrane was used instead of the PVDF membrane.

It is the opinion of the Study Director that this deviation did not affect the results of the study.

Author / Study Director:

Date: July 28, 2006

D. ROUQUIE

# 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. 680-5. |

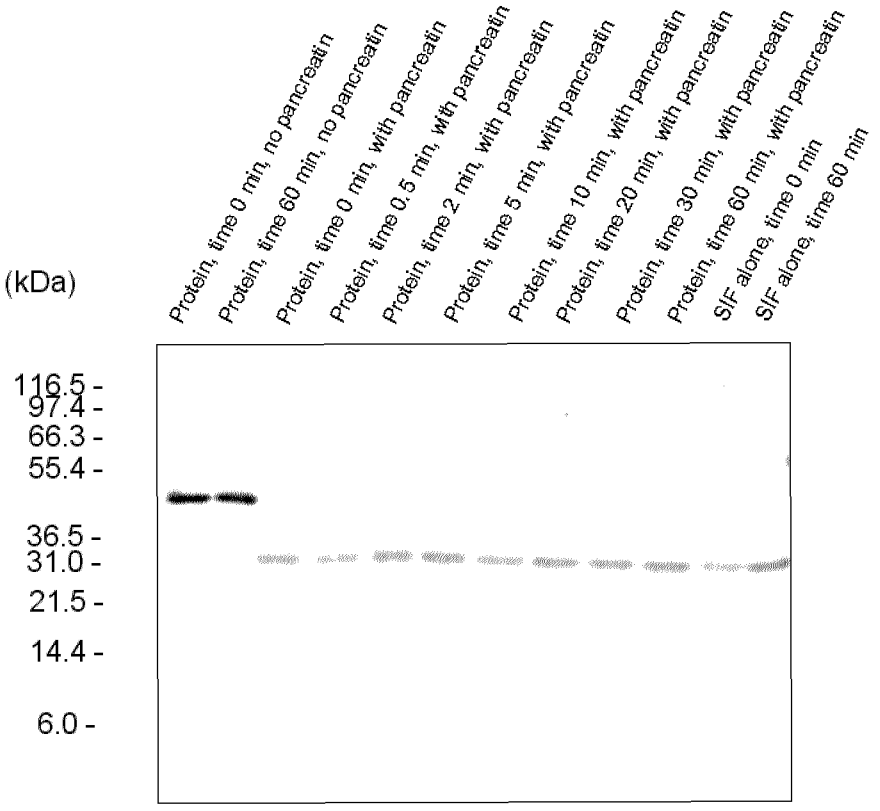
# ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| °C | Degree Celsius |
| µg | Microgram(s) |
| µl | Microliter(s) |
| AZA | Azoalbumin |
| EDTA | Ethylen-diamine tetra acetic acid |
| g | Gram(s) |
| g/ml | Gram (s)/milliliter(s) |
| GLP | Good Laboratory Practice |
| ILSI | International Life Science Institute |
| kDa | Kilodalton(s) |
| kg | Kilogram(s) |
| M | Molar |
| mg | Milligram(s) |
| mg/ml | Milligram(s)/milliliter(s) |
| min | Minutes(s) |
| ml | Milliliter(s) |
| mM | Millimolar |
| PAGE | Polyacrylamide gel electrophoresis |
| pH | Potential of hydrogen |
| SDS | Sodium dodecyl sulfate |
| SIF | Human simulated intestinal fluid |
| USA | United States of America |
| w/v | Weight/volume |

# FIGURE

FIGURE 1 - WESTERN BLOT OF 2mEPSPS PROTEIN AFTER INCUBATION IN HUMAN SIMULATED INTESTINAL FLUID

Fig. 1


(kDa)
Protein, time 0 min, no pancreatin
Protein, time 60 min, no pancreatin
Protein, time 0 min, with pancreatin
Protein, time 0.5 min, with pancreatin
Protein, time 2 min, with pancreatin
Protein, time 5 min, with pancreatin
Protein, time 10 min, with pancreatin
Protein, time 20 min, with pancreatin
Protein, time 30 min, with pancreatin
Protein, time 60 min, with pancreatin
SIF alone, time 0 min
SIF alone, time 60 min
116.5 -
97.4 -
66.3 -
55.4 -
36.5 -
31.0 -
21.5 -
14.4 -
6.0 -

# ATTACHMENTS

ATTACHMENT 1 - **PROTOCOL AND AMENDMENT**

**2mEPSPS PROTEIN**

***IN VITRO* DIGESTIBILITY STUDY IN SIMULATED INTESTINAL FLUID**

**TESTING FACILITIES**

Bayer CropScience
355, rue Dostoïevski
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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 2mEPSPS protein, encoded by the *2mepsps* gene by investigating the digestion of the protein using simulated intestinal fluid. The rate of the protein degradation *in vitro* will be observed and will give an indication of the stability of the protein and its speed of destruction in natural digestive process.

### 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 control and test material expiry dates and that the dosing suspensions were not analyzed for concentration, homogeneity and stability:

-O.E.C.D. (Organization for Economic Cooperation and Development) Principles of Good Laboratory Practice, 1997 (January 26, 1990).

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

-U.S. E.P.A. (Environmental Protection Agency)
. 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°98-1312 regarding Good Laboratory Practice, December 31, 1998.

### 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 27, 2006 D. ROUQUIE

2.2 SPONSOR REPRESENTATIVE:

Date: April 27, 2006 by C. HEROUET-GUICHENEY

2.3 OTHER STUDY PERSONNEL

| **Responsibility** | **Name** |
|---|---|
| In-life Supervisor | JAUSSELY Catherine |
| Responsible Technician | LABORY-CARCENAC Bénédicte |

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

## 3 - PROPOSED DATES

Experimental starting date : May 03, 2006
Experimental completion date : May 31, 2006 (estimated)

## 4 - OVERVIEW OF STUDY DESIGN

A solution of the test protein will be incubated with simulated intestinal fluid (SIF) (a porcine pancreatin solution at pH 7.5) at 37 ± 1° C and samples taken for analysis at timepoints of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resultant protein solution will be analyzed for potential presence of the 2mEPSPS protein and its stable protein fragments. A western blot method coupled with an SDS-PAGE electrophoresis may be used to detect the protein using a specific anti-2mEPSPS protein antibody. Appropriate controls will allow an evaluation of the rate and degree of digestion under these test conditions. Controls will include 2mEPSPS protein in buffer without pancreatin and SIF without 2mEPSPS protein.

## 5- MATERIALS AND METHODS

5.1 PROTEINS AND PANCREATIN

The pancreatin used will be from Sigma, batch N° 055K1193 (P-3292).

The test protein will be supplied by the sponsor (Bayer BioScience NV, Gent Belgium)

Test Protein Name: 2mEPSPS protein encoded by the *2mepsps* gene.

Batch N°: LEJ5837
Purity >90 % 2mEPSPS
Storage: approximately – 20 ° C.
Certified through at least 6 months.

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

The quality-control protein (to show pancreatin activity) will be azoalbumin (Sigma A-2382, batch N°129H7665).

5.2 PROTEIN SOLUTIONS

The test protein will be received as a stock solution of 0.931 mg/ml in the storage buffer (0.1M Tris/HCl; 2.7 mM KCl; 137 mM NaCl; 1 mM DTT; pH 7.5).

5.3 SIMULATED INTESTINAL FLUID

The intestinal control solution (I-con) will be prepared as follows:
19 ml 0.2M NaOH
0.68 g $KH_2PO_4$
Titrate to pH 7.5 using dilute NaOH
Add distilled water to a volume of 100 ml.

This solution may be stored for up to 4 months at 4°C.

The above I-con will be mixed with the pancreatin at a concentration to give a 1% w/v pancreatin solution. It will be used within 2.5 hours (being kept on ice when not in use).

To ensure that the pancreatin is active, it will be tested against a pancreatin substrate, azoalbumin, using a spectrophotometric endpoint. 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, 0.05% bromophenol blue (adjusted to pH 6.8). The mix without the BME is stable for one year, after addition of BME solution is stable for 1 month when stored at room temperature.

5.5 DIGESTION

A 2 ml microcentrifuge tube of SIF for the serial digestion samples, and a series of control tubes will be warmed to approximately 37° C in a waterbath for approximately 2 minutes before the time zero (addition of protein and/or sampling).

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

Control tubes:

| | |
|---|---|
| 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 | 190µl I-con (SIF without pancreatin) + 10 µl protein : sample at 60 minutes (agitate and incubate in waterbath) |
| SIF + protein time 0 | 190 µl SIF + 70 µl distilled water + 70 µl 5X Laemmli buffer + 10 µl protein (added last after mixing and cooling on ice) |
| SIF alone, 0 min | 190 µl SIF + 10 µl distilled water: sample at time zero |
| SIF alone, 60 min | 190 µl SIF + 10 µl distilled water: sample at 60 minutes (agitate and incubate in waterbath) |

5.6 SAMPLES

The reaction will be stopped as soon as samples are taken.
For the serial samples, the reaction will be terminated by adding the 200 µl of digestion sample to a tube containing 70 µl distilled water.

Samples may be directly used for analysis on SDS-PAGE gels or frozen at -20°C.

5.7 SDS-PAGE ELECTROPHORESIS

The method will be based on that of Laemmli (1970) using a BioRad Mini-Protean III cell. Prior to running SDS-PAGE, 5 µl of 5X Leammli solution will be added to 20 µl of digestion samples and heated for 10 minutes at >90°C before loading the gel. Samples of 15 µl will be added to wells of an SDS-PAGE gel (15 wells, 1mm 10-20% gradient polyacrylamide Tris-Tricine) (BioRad, France). A few grains of sucrose may be added to the samples at the last heating stage if a higher density is needed to allow correct deposition of samples into the wells.

A suitable marker solution (2.5-200 kDa) will be used to provide reference points of known molecular weights on the gel (Mark 12, Invitrogen, France).

Loading order on gel:

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

A setting of 117 ± 7 volts at a constant voltage for approximately 90 minutes will be selected to give gels with a correct appearance (near straight lines for sample front and a front finishing near the top of the gel).

Two gels will be prepared, one will be stained using the Coomassie blue method and the will be scanned. The gels are not stable for more than a week, so the raw data will be the scanned image. The second gel may be used to prepare the Western Blot.

5.8 WESTERN BLOT ANALYSIS

A PVDF membrane may be placed on the SDS gel in a Tris/Glycine buffer and an electrical current applied in order to transfer the protein bands onto the membrane. To potentially reveal the 2mEPSPS protein bands and/or its fragments recognized by a specific anti-2mEPSPS antibody, the membrane will be treated with a specific anti-2mEPSPS antibody.

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 blots are generally stable throughout the study.
- the protein or fragment blots are clearly visible at time zero (lanes 2 and 4) and not visible in the lanes without protein (lanes 12 and 13).
- 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 and the digestion of azoalbumin gives results that are in line with expected data (rapid digestion).

If stable fragments of the protein are visible on the gel, then their intensity and time-course will be reported.

The time at which the parent protein disappears will be reported as the time for protein digestion.

## 6 - STATISTICAL ANALYSES

No statistical analysis will be performed.

## 7 - REPORTING

### 7.1 INTERIM REPORTS

Any unexpected findings during the course of the study will be reported to the Sponsor Representative.

### 7.2 FINAL REPORT

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.

## 8 - ARCHIVING

All raw data, supporting documents as well as protocol, protocol amendments, an aliquot of the formulated test substance reference sample and final report will be 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

## 9 - 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 06102

**2mEPSPS PROTEIN**
***IN VITRO* DIGESTIBILITY STUDY IN SIMULATED INTESTINAL FLUID**

**Protocol amendment: N°1**

**Reason(s):**
Reason 1- Typing Error
In paragraph 1.2, the date of the O.E.C.D. (Organization for Economic Cooperation and Development) Principles of Good Laboratory Practice, 1997 should read as (January 26, 1998) and not (January 26, 1990) as stated in the Study Protocol.

Reason 2 – Modification of the SDS-PAGE analysis procedure
As the BioRad 15-well SDS-PAGE gels are not available anymore from the supplier, they will be replaced by Biorad SDS-PAGE gels (10 wells, 1mm 10-20% gradient polyacrylamide Tris-Tricine). As a consequence, all the samples will be loaded on two gels and the sample loading order on the gels will be as follows:
Gel 1

| Lane | Sample |
|---|---|
| 1 | Markers |
| 2 | I-con + protein time 0 |
| 3 | I-con + protein time 60 min |
| 4 | SIF + protein time 0 |
| 5 | SIF + protein time 0.5 min |
| 6 | SIF + protein time 2 min |
| 7 | SIF + protein time 5 min |
| 8 | SIF alone, 0 min |
| 9 | SIF alone, 60 min |
| 10 | Markers |

Gel 2

| Lane | Sample |
|---|---|
| 1 | Markers |
| 2 | I-con + protein time 0 |
| 3 | I-con + protein time 60 min |
| 4 | SIF + protein time 10 min |
| 5 | SIF + protein time 20 min |
| 6 | SIF + protein time 30 min |
| 7 | SIF + protein time 60 min |
| 8 | SIF alone, 0 min |
| 9 | SIF alone, 60 min |
| 10 | Markers |

In paragraph 5.7, the sentence "Two gels will be prepared, one will be stained using the Coomassie blue method and the will be scanned" should be replaced by "Two gels will be prepared, Coomassie blue stained and scanned"

Study Director:

Date: May 09, 2006

D. ROUQUIE

ATTACHMENT 2 - **CERTIFICATES OF ANALYSIS**

Title

**Certificate of analysis for the 2mEPSPS protein produced in *E. coli***

**Batch n°: LEJ5837**

Author

**Nadine Bautsoens**
**Koen Hendrickx, Ph.D.**

Completed On
June 16, 2006

Testing Facility

**Molecular & Biochemical Analytical Services**
**Expression and Protein Characterization**
**Bayer BioScience N.V.**
**Technologiepark 38**
**B-9052 Gent**
**Belgium**

Study number

**BBS06-003**

## 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.

## GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The undersigned hereby declare 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 except for the following:

1. No validation of computerized systems attached to: multifunctional monochromator Tecan Safire², and autoclave was performed.

Study Director

Koen Hendrickx
Expression and Protein Characterization
Molecular and Biochemical Analytical Services

12/06/06
Date

**STUDY IDENTIFICATION PAGE**

| | |
|---|---|
| Study start date: | April 3th, 2006 |
| Experimental start date: | April 10th, 2006 |
| Experimental Termination date: | May 5th, 2006 |
| Study Completion date: | June 16, 2006 |
| Test Facility Address: | Bayer BioScience N.V.<br>Molecular & Biochemical Analytical Services<br>GLP Test Facility<br>Technologiepark 38<br>9052 Gent – Belgium<br>Tel: +32 9-243 04 11<br>Fax: +32 9-224 06 94 |
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## QUALITY ASSURANCE STATEMENT

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Quality Assurance (GLP)

**Quality Assurance Statement**

Title: **Certificate of analysis for the 2mEPSPS protein produced in *E. coli*.**

Study: BBS06-003

**This study was periodically inspected and properly signed records of these inspections were submitted to testing facility management and the study director as shown below.**
**This report has been audited by the GLP Quality Assurance. The reported results accurately reflect the original data of the study.**

| Phase of Study | Inspection | Report |
|---|---|---|
| Study plan | 06 APR 2006 | 06 APR 2006 |
| Study plan amendment | 07 APR 2006 | 07 APR 2006 |
| Study conduct | 10 APR 2006 | 10 APR 2006 |
| | 24 APR 2006 | 25 APR 2006 |
| | 04 MAY 2006 | 05 MAY 2006 |
| Draft report | 01 JUN 2006 | 02 JUN 2006 |
| Final report | 12 JUN 2006 | 12 JUN 2006 |

**S. Berghman**
GLP Quality Assurance

12/06/06

**APPROVALS PAGE**

| | | |
|---|---|---|
| Study Director / Author | Koen Hendrickx | Date 16/06/06 |
| Test facility management | Elizabeth Bates | Date 13/06/06 |
| Sponsor representative | Dominique Rouan | Date 13 June 2006 |
| Head of MBAS | Henk Joos | Date 16 June 2006 HJ |

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## SUMMARY

This study was undertaken to confirm the identity of the bacterially produced 2mEPSPS to be used in subsequent studies and to determine the concentration and purity of the produced protein.

Five tests were performed in this study: (1) The concentration of the protein was determined by the Bradford technique. (2) The molecular weight of the protein was estimated by SDS-PAGE. (3) The same SDS gel was used to estimate the purity of the test item. (4) The immunological relationship with 2mEPSPS was tested by western blotting. (5) The activity of the protein was determined in an activity assay.

The identity of the 2mEPSPS protein was confirmed based on the observed molecular weight, cross-reactivity with the antibodies previously raised towards 2mEPSPS and the activity of the protein. The concentration of the protein was estimated to be 0.93 mg/ml and the purity was estimated to be 99.52%.

## 1.OBJECTIVE

In this study, the purified 2mEPSPS protein produced in *Escherichia coli* was analyzed for its concentration, identity and purity.

## 2. OVERVIEW OF EXPERIMENTAL DESIGN

Five analyses were performed on test item T09-01 produced in *E. coli*. (1) The concentration of the protein was determined with the Bradford method (1976). (2) The molecular weight of the protein was determined after sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). (3) The purity was estimated by qualitative comparison of the protein bands in the SDS gel. (4) The identity of the protein was confirmed by western blotting. (5) The activity of the protein was determined with the malachite green dye method (Lanzetta *et al.*, 1979) with minor modifications.

## 3. TEST ITEM AND REFERENCE ITEM

### 3.1 Test item

The test item was purified protein produced in *Escherichia coli*. The protein was produced at Bayer HealthCare AG (Wuppertal, Germany) according to the purification protocol described in Priestman *et al.* (2005). One aliquot of protein was received. Upon arrival at the Bayer BioScience N.V. test facility, the frozen aliquot was stored in the ultrafreezer (112UF – position 8-03) until the analysis took place.
Protein stored in the ultrafreezer is stable for at least 2 years.
To perform the analysis an aliquot was taken from the test item stock solution. This aliquot received the identification number PR003-01

| | |
|---|---|
| Test Item ID: | T09-01 |
| Test Item Identity: | 2mEPSPS protein |
| Origin: | *Escherichia coli* |
| Batch n°: | LEJ5837 |
| Expiry date: | April 3$^{th}$, 2008 |
| Buffer solution | 100 mM Tris (pH 7.5), 2.7 mM KCl, 137 mM NaCl, 1 mM DTT |

### 3.2 Reference items

No reference items were used in this study.

### 3.3 Standards

The Precision Plus Protein™ Standard Dual Color Marker was supplied by BioRad. The stock solution of the Precision Plus Protein™ Standard Dual Color Marker was stored in freezer 90FZ located in room L79. After opening the Precision Plus Protein™ Standard Dual Color Marker was stored in refrigerator 91RF in room L79.

## 4. ANALYSIS OF THE TEST ITEM T09-01

The concentration of the protein was estimated and the purity plus the identity of the protein was confirmed on the basis of five techniques. The concentration of the protein was determined according to the method of Bradford (1976). The molecular weight is a distinctive characteristic of a protein and was estimated to confirm the identity by performing a denaturing SDS-PAGE analysis. The same gel was used to estimate the purity of the produced protein. Western blotting is the fourth technique used to confirm the identity of the protein. This technique demonstrates the immunological relationship of the investigated protein to 2mEPSPS proteins by the reactivity of available antibodies directed towards 2mEPSPS. The activity of the purified protein was also assayed and indicates that the protein has the correct enzymatic nature.

### 4.1 Quantification of the test item T09-01

The Coomassie Plus™ kit (Pierce) is a ready-to-use Bradford assay for protein quantification. When the coomassie dye of the kit binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant color change from brown to blue. To perform the assay in either test tube or microplate format: a small amount of protein sample is combined with the assay reagent, mixed well, incubated briefly and the absorbance at 595 nm is measured. Protein concentrations are estimated by reference to absorbances obtained from a series of standard protein dilutions, which are assayed alongside the unknown samples.

A dilution series of the test item protein was made to determine the concentration of the protein according to SOP BBS 07/60/00. The dilution series samples were mixed with the assay reagents of the Coomassie Plus™ kit. With the Magellan software, the concentration of the protein samples was estimated by fitting the measured absorbances to a standard curve of known dilutions of bovine serum albumin. A correlation coefficient of 0.99 was obtained for the standard curve. The average of the concentration and the standard deviation was calculated with the Microsoft Excel software (Table 1). The average protein concentration was estimated from the observed three data points. A large standard deviation was introduced by the dilution factor of the sample. A small error in the highest dilution gets multiplied by 400 and becomes much bigger. Therefore we consider the 100 fold dilution to be the most accurate. The 50 fold dilution would give us a smaller deviation but is situated in a less accurate part of the standard curve. The most accurate estimation is 0.93 mg/ml.

### 4.2 Molecular weight determination of the test item T09-01

SDS-PAGE is an analytical technique used to separate proteins through an acrylamide gel based on their relative molecular weight. The migration distance of a sample is directly proportional to the molecular size of the protein.

The test item protein was separated from side products/protein trace amounts with the SDS-PAGE technique according to SOP BBS 07/63/00 and the obtained gel (041106A, fig. 1) was stained according to SOP BBS 07/66/00.

The logarithm of the molecular weight of the standards was plotted versus the distance of migration with the Microsoft Excel software. A standard curve based on 6 data points was made covering the molecular weight range of the test item. The correlation coefficient of this standard curve was 0.99 (fig. 2). The molecular weight of the test item was extrapolated from the graph by using the equation $y = -0.684x + 3.7023$. The obtained x-value is 4.68. The molecular weight of the test item corresponds to the inverse logarithm of the x-value and is estimated to be 48 kDa. The molecular weight deduced from the amino acid sequence was 47 kDa. Since the error margin of the technique in the molecular weight region of the protein is calculated to be 3 kDa, the deduced molecular weight is confirmed within the limitation of the analysis.

### 4.3 Purity analysis of test item T09-01

The purity of the test item was estimated by quantification of the bands visible on the SDS gel and calculating the percentage of each band compared to the total density observed.

The purity of the test item was analyzed on the stained protein gel 041106A with the FluorS™ MultiImager according SOP BBS 04/40/00. A representative digitized image was made and an analysis report was prepared. Based on the relative density of each individual band, the purity of the test item was estimated to be 99.52%.

**4.4 Electrotransfer of proteins to a membrane**

SDS-PAGE was used to separate the proteins through an acrylamide gel in an electrical field based on molecular weight. Afterwards the separated proteins were transferred to a PVDF membrane to make them accessible for the detection with antibodies.

The test item protein was separated from side products/protein trace amounts with the SDS-PAGE technique according to SOP BBS 07/63/00 and the obtained PAGE gel (041106E) was further processed according to SOP BBS 07/64/00. Since the prestained molecular weight markers were visible on the membrane (041106F, fig. 3) we concluded that the transfer to the PVDF membrane was successful.

**4.5 Western blotting**

Rabbit antiserum anti 2mEPSPS
The rabbit anti 2mEPSPS was produced by and tested at Bayer Bioscience N.V. An aliquot of the antibody was stored in freezer 90FZ located in room L79 until used.

Goat antiserum raised against rabbit IgG coupled with Alkaline Phosphatase
The goat anti-rabbit antiserum coupled with alkaline phosphate was supplied by Sigma. The antibody was stored in the refrigerator 91RF in room L79 until used

The immobilization of proteins to a membrane (041106C) makes them accessible for the reaction with a specific antibody. The membrane (041106C), obtained by electrotransfer of the protein was treated according to SOP BBS 07/65/00.

Protein-free areas on the membrane were blocked overnight by incubation with StartingBlock™ (PBS) Blocking buffer (Pierce). After washing the membrane was incubated with a 1:10000 dilution of rabbit anti 2mEPSPS (Bayer Bioscience NV). After washing the reaction was incubated with a goat anti-rabbit antibody coupled to alkaline phosphatase (1:7000). Addition of substrate caused two bands to become visible. This result is in conflict with the SDS-PAGE data where only one band became visible after staining. The experiment was repeated and a membrane 041106F was generated. After staining only one major band became visible (fig. 3).

**4.6 EPSPS activity assay**

In the shikimate pathway, phosphoenolpyruvate and erythro-4-phosphate are converted to chorismate through seven enzymatic steps. EPSPS catalyzes the transfer of the enolpyruvyl moiety of PEP to the 5-OH hydroxyl group of shikimate 3-phosphate and releases inorganic phosphate. The EPSPS activity assay was measured according to a colorimetric method described by Forlani et al. (1994) and described in SOP BBS 07/74/00. The inorganic phosphate release is measured using the malachite green dye method (Lanzetta et al., 1979) with minor modifications described in SOP BBS 07/74/00.

Reaction mixtures were made according to SOP BBS 07/74/00 and placed at 37°C for 20 minutes. Reactions were made visible by a colorimetric solution and stopped with the addition of 34% sodiumcitrate after 60 seconds. Samples were transferred to a microtiterplate and the absorbance was read at 660 nm. The absorbance of the duplicates of the buffer control sample was 0.0537 and 0.0536. The absorbance of the duplicates of the test item sample was 0.1562 and 0.1597.

## 5. RESULTS AND DISCUSSION

The protein concentration of the test item T09-01 was estimated to be 0.93 mg/ml and the purity is estimated to be 99.52%. Based on the migration of the protein in the SDS-PAGE we confirmed the experimental molecular weight of the protein to be comparable to the deduced molecular weight from the amino acid composition, taking into account the error margin of the technique. As expected the test item reacts with an antibody directed towards 2mEPSPS. This reaction demonstrates the immunological relationship of the test item with 2mEPSPS. An activity assay confirmed the activity of the test item.

## 6. CONCLUSION

Biochemical analyses were performed to confirm the identity of test item T09-01. Based on the analyses we identified the T09-01 test item to be 2mEPSPS. The concentration of the protein in the test item T09-01 was estimated at 0.93 mg/ml with an estimate purity of 99.52%.

## 7. ARCHIVING

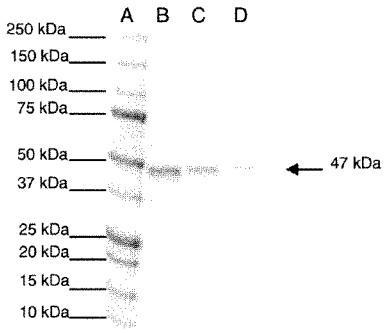
The study plan, the final report and the study data are archived under study number BBS06-003 in the GLP Test Facility Archives of Bayer BioScience N.V., Technologiepark 38, 9052 Gent, Belgium.

A sample of test item (T09-01) was archived in the archive ultrafreezer 113UF of the GLP Test Facility Archives of Bayer BioScience N.V., Technologiepark 38, 9052 Gent, Belgium (room number L57).

**Table 1: Protein concentration of test item T09-01**

| Subsample | Dilution factor | Measured concentration µg/ml | Concentration of test item mg/ml |
|---|---|---|---|
| PR003-1A | 400 | 4.54 | 1.82 |
| PR003-1B | 100 | 9.31 | 0.93 |
| PR003-1C | 50 | 14.74 | 0.74 |

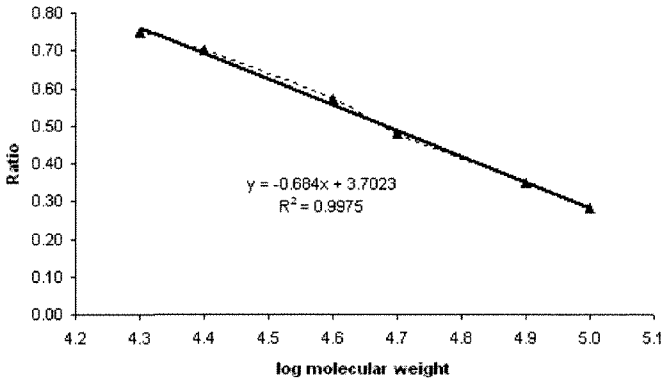
**Figure 1: SDS-PAGE gel of test item T09-01(gel ID# 041106A)**




Legend : A molecular weight marker
B 2 µg test item T09-01
C 1 µg test item T09-01
D 0.5 µg test item T09-01

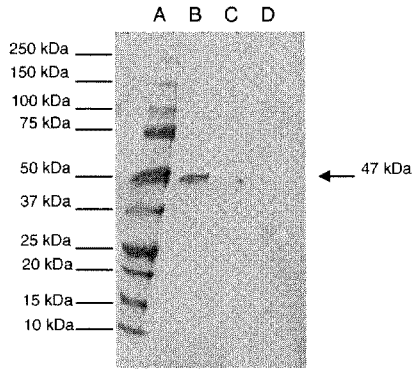
**Figure 2: Plot standard curve molecular weight of test item T09-01**

| A | B | C | D | E |
|---|---|---|---|---|
| Molecular weight kDa | log Molecular weight | Migrated distance cm | Total distance cm | Ratio C/D |
| 100 | 5.0 | 1.7 | 6.0 | 0.28 |
| 75 | 4.9 | 2.1 | 6.0 | 0.35 |
| 50 | 4.7 | 2.9 | 6.0 | 0.48 |
| 37 | 4.6 | 3.4 | 6.0 | 0.57 |
| 25 | 4.4 | 4.2 | 6.0 | 0.70 |
| 20 | 4.3 | 4.5 | 6.0 | 0.75 |




Legend : Plot of the logarithm of molecular weight of the standard proteins plotted against the ratio of the migrated distance and the total distance.

**Figure3: Western blot of test item T09-01 (blot ID# 041106F)**




Legend : A molecular weight marker
B 0.6 µg test item T09-01
C 0.3 µg test item T09-01
D 0.1 µg test item T09-01

**REFERENCES**


| No | Doc No | Author(s), year, title, source, edition, pages |
|---|---|---|
| 1. | M-268549-01-1 | Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Analytical Biochemistry 72, 248-254 |
| 2. | M-268612-01-1 | Lanzetta P.A., Alvarez L.J., Reinach P.S. and Candia O.A. (1979) An improved assay for nanomole amounts of inorganic phosphate. Analytical Biochemistry 100, 95-97 |
| 3. | M-269790-01-1 | Priestman M.A., Funke T., Singh I.M., Crupper S.S. and Schonbrunn E. (2005) 5-Enolpyruvylshikimate-3-phosphate synthase from Staphylococcus aureus is sensitive to glyphosate. FEBS Letters 579, 728-732 |
| 4. | M-268551-01-1 | Forlani G., Parisi B. and Nielsen E. (1994) 5-enol-Pyruvyl-Shikimate-3-Phosphate Synthase from Zea mays cultured cells. Plant Physiol. 105, 1107-1114 |

## Study data index

**Bayer BioScience N.V. Study number: BBS06-003**

**Study Title: Certificate of analysis for the 2mEPSPS protein produced in *E. coli***



Total number of study data pages: 71 pages

SIGMA-ALDRICH

# Certificate of Analysis

| | |
|---|---|
| **Product Name** | Pancreatin from porcine pancreas, powder, 4× USP specifications, cell culture tested |
| **Product Number** | P3292 |
| **Product Brand** | Sigma |
| **CAS Number** | 8049-47-6 |
| **Storage Temp** | −20°C |

| TEST | SPECIFICATION | LOT 055K1193 RESULTS |
|---|---|---|
| **APPEARANCE** | LIGHT YELLOW TO TAN POWDER | TAN POWDER |
| **DIGESTIVE POWER** | 4X USP SPECIFICATIONS | CONFORMS* |
| **CELL CULTURE TEST** | PASS | PASS |
| | | * SUPPLIER'S INFORMATION |
| **QC ACCEPTANCE DATE** | | MAY 2005 |

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

ATTACHMENT 3 - **GLP COMPLIANCE CERTIFICATES (AND ENGLISH TRANSLATION)**

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

**GROUPE INTERMINISTERIEL DES PRODUITS CHIMIQUES**

Paris, le 13 DEC. 2005

OBJET : Evaluation de la conformité aux B.P.L. selon la directive 2004/10/CE du 11 février 2004.

Consécutivement à votre engagement vis à vis du GIPC et du COFRAC et en application du décret n° 81-278 du 25 mars 1981 portant création d'un Groupe Interministériel des Produits Chimiques (GIPC), modifié notamment par le décret 90-206 du 7 mars 1990 et par le décret n° 98-1312 du 31 décembre 1998 concernant les bonnes pratiques de laboratoires, je vous confirme que le GIPC, au vu des résultats du contrôle exercé par le Comité français d'accréditation (COFRAC) - Section Laboratoires a décidé pour votre installation du statut suivant :

**Respect des principes de B.P.L.**
Domaines de reconnaissance :

- ☐ **2 - études de toxicité**
- ☐ **3 - études de mutagénicité**
- ☐ **4 - études écotoxicologiques sur les organismes aquatiques et terrestres**
- ☐ **8 - méthodes de chimie analytique et clinique**
- ☐ **9 - autres études (métabolisme animal)**

Date d'inspection : 26 & 27 octobre 2005 ☐ **inspection de renouvellement (i.r)**

Date de décision du G.I.P.C. : 9 décembre 2005
Date de prise d'effet : 27 octobre 2005
Année de première conformité : 1992
Durée de validité : 18 mois

P. O.
Le Président,

Pierre CREYSSEL
Conseiller d'Etat h.

BAYER CROPSCIENCE
355 rue Dostoievski
BP 153
06903 Sophia Antipolis Cedex

Secrétariat général du GIPC - DGE- Simap - 12, rue Villiot - 75572 Paris cedex 12
Téléphone : 01 53 44 96 10 – Télécopie : 01 53 44 91 72

**REPUBLIC OF FRANCE**

**INTERMINISTERIAL GROUP FOR CHEMICAL PRODUCTS**

**Paris, the 13 Dec. 2005**

SUBJECT: Evaluation of G.L.P conformity to directive 2004/10/CE of 11 February 2004

Following your commitment vis-a-vis GIPC and COFRAC and by applying for decree n°. 81-278 of 25 March 1981, supporting the creation of an Interministerial Group for Chemical Products (GIPC), modified, among others, by decree 90-206 of 7th March, 1990 and by decree n° 98-1312 of 31 December 1998 concerning good laboratory practice, I confirm that the GIPC, in view of the results of the inspection carried out by the French Accreditation Committee (COFRAC) - Tests Section has decided on the following status for your test installation:

**Observing the G.L.P principles**

Areas of recognition

☐ **2 - toxicity studies**
☐ **3 - mutagenicity studies**
☐ **4 - ecotoxicology studies on aquatic and terrestrial organisms**
☐ **8 - analytical and clinical chemistry methods**
☐ **9 - other studies (animal metabolism)**

Inspection date: 26 & 27 October 2005 ☐ renewal inspection (i.r)

Date of GIPC decision: 9 December 2005
Date of taking effect: 27 October 2005
Year of first conformity: 1992
Validity duration: 18 months

**The President**
**Pierre CREYSSEL**
State Advisor

Bayer CropScience
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**Telephone: 01 53 44 96 10 – FAX: 01 53 44 91 72**