Bayer CropScience

2mEPSPS PROTEIN

HEAT STABILITY STUDY

DATA REQUIREMENT
No applicable guidelines

REPORT OF STUDY SA 07185
Sponsor identification number: Lynx-PSI N°TX99L033

REPORT AMENDMENT N°1

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: SEPTEMBER 27, 2007
REPORT AMENDED N°1 ON: NOVEMBER 27, 2007

REPORT AMENDMENT

Report amendment N°1:

Reason: **Update in Summary and Conclusion Chapters.**

Due to a typo error, the sentence
"It is concluded that the 2mEPSPS protein is partially heat-stable up to 90°C for 30 minutes and markedly degraded at 90°C for 60 minutes."

replaces

"It is concluded that the 2mEPSPS protein is partially heat-stable up to 90°C for 30 minutes and markedly degraded at 90°C for 30 minutes."

Study Director:

Date: November 27, 2007

D. ROUQUIE

Bayer CropScience

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DATA REQUIREMENT
No applicable guidelines

REPORT OF STUDY SA 07185
Sponsor identification number: Lynx-PSI N°TX99L033

AUTHOR / STUDY DIRECTOR: D. ROUQUIE

TESTING FACILITY:

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355, rue Dostoïevski
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06903 Sophia Antipolis Cedex
France

SPONSOR:

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

STUDY COMPLETED ON: SEPTEMBER 27, 2007

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 reported was not performed in compliance with Good Laboratory Practice in that, it was not subjected to specific Quality Assurance inspections. It was performed according to standard operating procedures which were previously accepted and periodically inspected by the Quality Assurance Unit.

Author / Study Director:

Date: 27 September, 2007

D. ROUQUIE

Sponsor Representative:

Date: 27 SEPTEMBER 2007

p.o. A. CAPT Corinne HERQUET-GUICHENEY

Study Submitter:

Date:

FLAGGING STATEMENTS

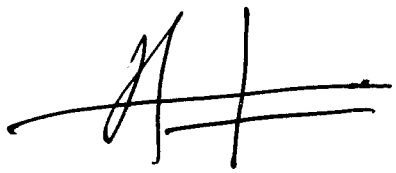
This page is reserved for flagging statements as may be required by US EPA.

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 27, 2007

D. ROUQUIE

STUDY PROFESSIONALS

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

STUDY DIRECTOR	:	D. ROUQUIE
RESPONSIBLE TECHNICIAN	:	A. ARNAUD
REPORT UNIT ASSISTANTS	:	P. ALMERAS

TABLE OF CONTENTS

SUMMARY

The 2mEPSPS protein encoded by the *2mepsps* gene was tested for stability at temperatures of 60, 75 or 90°C for periods of 10, 30 or 60 minutes. The protein was examined by SDS-PAGE and by Western blot analysis.

The 2mEPSPS protein encoded by the *2mepsps* gene showed no significant changes after heat treatment at up to 60°C for 10 minutes. Some 2mEPSPS protein degradation fragments are visible from the incubation at 60°C for 30 minutes. The intensity of these fragments increased with temperature and the duration of incubation. A marked but not complete degradation of the 2mEPSPS protein was observed after incubation at 90°C for 60 minutes.

It is concluded that the 2mEPSPS protein is partially heat-stable up to 90°C for 30 minutes and markedly degraded at 90°C for 30 minutes.

INTRODUCTION

This *in vitro* heat stability study (SDS-PAGE and Western blot) has been conducted to establish if heat treatment causes any structural changes to the 2mEPSPS protein.

The study protocol and amendment are presented in Attachment 1.

The study schedule was as follows:

Sponsor representative protocol approval date	August 27, 2007
Study initiation date*	August 27, 2007
Experimental starting date	August 28, 2007
Experimental completion date	August 31, 2007

* Date of protocol approval by Study Director

MATERIAL AND METHODS

1 - TEST PROTEIN

The test protein was supplied by the sponsor.

Identification	:	2mEPSPS protein (double mutant 5-enol pyruvylshikimate-3-phosphate synthase from maize)
Batch N°	:	LEJ 5837
Source	:	Bayer Bioscience N.V., Gent, Belgium
Purity	:	99.91 % 2mEPSPS protein
Concentration	:	0.971 mg/ml in the storage buffer (0.1 M Tris; 2.7 mM KCl; 137 mM NaCl; 1 mM DTT; pH 7.5).
Expiry date	:	October 24, 2008
Storage	:	Approximately -70°C

The certificates of analysis are presented in Attachment 2.

2 - PROTEIN SOLUTION

The test protein was diluted in storage buffer (0.1 M Tris; 2.7 mM KCl; 137 mM NaCl; 1 mM DTT; pH 7.5) to a concentration of 0.25 mg/ml.

3 - INCUBATION

The protein incubation was made in 1.5 ml microcentrifuge tubes in a temperature-controlled hot-block at 60, 75 or 90 °C for 10, 30 or 60 minutes.

As soon as samples were taken at the end of the incubation period, the reaction was terminated by placing the sample tubes on ice, and adding 14 µl distilled water. The sample tubes were aliquoted in 20 µl and stored frozen at approximately – 20°C until use for SDS-PAGE analysis.

Additional control samples were prepared:

- Two samples of a zero minute incubation of protein (kept at 4 °C);
- buffer solution without protein was heated at 60 °C for 60 minutes;
- buffer solution without protein was heated at 90 °C for 60 minutes.

4 - SDS-PAGE ANALYSIS

Gel electrophoresis was carried out following the method of Laemmli (1) using a BioRad Mini-Protean III cell. Prior to running SDS-PAGE 5 µl of 5X Laemmli buffer (40% glycerol, 5% β-mercaptoethanol, 10% SDS, 0.33 M Tris HCl; 0.05% bromophenol blue; pH 6.8) were added to 20 µl of test sample tubes before loading the gel. Samples of 15 µl were added to wells of an SDS-PAGE gel (15 well, 1mm 10-20% gradient polyacrylamide Tris/Tricine) (BioRad, France).

A Kaleidoscope pre-stained standard solution (Bio-Rad) in the first lane and a standard marker solution (2.5-200 kDa) (Mark 12, Invitrogen, France) in the last lane were used to provide reference points of known molecular weights on the gel.

A setting of 117 ± 7 volts was used at a constant voltage for approximately 90 minutes 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 were prepared, one was stained by a sensitive Coomassie blue method (Invitrogen, France) based on the work of Neuhoff *et al*. (2). After rinsing, the gel was scanned using a GS800 scanner (Biorad, France). The second gel was used to prepare the Western blot.

5 - WESTERN BLOT ANALYSIS

A PVDF membrane was placed on the gel in a Tris/Tricine buffer and an electrical current applied in order to transfer the protein bands onto the membrane. To reveal the 2mEPSPS protein bands and/or its fragments recognized by a specific anti-2mEPSPS protein antibody, the membrane was incubated in the presence of a specific polyclonal rabbit anti-2mEPSPS protein antibody (dilution 1/10000 in PBS /Tween 20 (0.02 %v/v)) for approximately 1 hour. Following a washing step, the hybridization of the primary antibody with the proteins immobilized on the membrane was revealed using a goat anti-rabbit polyclonal antibody coupled with alkaline phosphatase (dilution 1/10000 in PBS /Tween 20 (0.02 %v/v)). In order to detect the alkaline phosphatase enzymatic activity coupled to the secondary antibody, the ECL detection system (Amersham) was used.

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

1.1 SDS-PAGE analysis after Coomassie blue staining (Fig. 1)

As expected, there were no bands visible in the lanes with only buffer solution.

The expected molecular weight of the 2mEPSPS protein is approximately 47 kDa. SDS-PAGE analysis of the unheated 2mEPSPS protein sample showed one band after Coomassie blue staining of the gel, demonstrating that the stock protein was pure and non-degraded. As expected, this band was located in between the molecular weight markers 36.5 and 55.4 kDa.

For the heated 2mEPSPS protein samples, there were no visible changes of the band intensity at 60°C and 75°C from 10 up to 60 minutes. After 30 minutes of incubation at 90°C, the band was visible but with an intensity decreased compared to other heated samples at 60°C and 75°C. After 60 minutes at 90°C, the band was still visible with a marked decrease of intensity compared to all samples, including the unheated sample.

1.2 Western blot analysis (Fig. 2)

As expected, there were no bands visible in the lanes with only buffer solution.

Western blot analysis showed that the protein band in the unheated sample located in between the molecular weight markers 36.5 and 55.4 kDa was detected by an anti-2mEPSPS protein polyclonal antibody. This result confirmed that the main band observed in the SDS-PAGE analysis was the 2mEPSPS protein (approximately at 47 kDa). Some higher molecular weight smears were visible, certainly due to incomplete denaturation of the samples prior to loading on the gel.

For the heated samples, the intensity of the intact 2mEPSPS band was unchanged after incubation at 60°C or 75°C for 10 up to 60 minutes. At 90°C the intensity of the intact 2mEPSPS band was still unchanged after 10 and 30 minutes and was decreased after 60 minutes in accordance with the results obtained by SDS-PAGE analysis after Coomassie blue staining.

Interestingly, 2mEPSPS degradation fragments located in between the molecular weight markers 31.0 and 36.5 kDa were visible in all the heated samples except in the sample after incubation of 10 minutes at 60°C. The intensity of these fragments increased with the temperature and the duration of incubation. A marked degradation of the protein was observed after incubation at 90°C for 60 minutes.

CONCLUSION

The 2mEPSPS protein encoded by the *2mepsps* gene showed no significant changes after heat treatment at up to 60°C for 10 minutes. Some 2mEPSPS protein degradation fragments are visible from the incubation at 60°C for 30 minutes. The intensity of these fragments increased with the temperature and the duration of incubation. A marked but incomplete degradation of the 2mEPSPS protein was observed after incubation at 90°C for 60 minutes.

It is concluded that the 2mEPSPS protein is partially heat-stable up to 90°C for 30 minutes and markedly degraded at 90°C for 30 minutes.

PROTOCOL DEVIATION

There were no protocol deviations during the study.

Author / Study Director:

Date: September 27, 2007

D. ROUQUIE

REFERENCES

DART Numbers

1 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.

2 M-273056-01-1 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. 255-62.

ABBREVIATIONS

%	Percentage
µl	Microliter
°C	Degree Celcius
kDa	Kilodalton
ECL	Enhanced chemiluminescent
EDTA	Ethylendiamineteraacetic acid
2mEPSPS	Double mutant 5-enol pyruvylshikimate-3-phosphate synthase from maize
g	Gram
g/ml	Gram/milliliter
GLP	Good Laboratory Practice
kg	Kilogram
M	Molar
mM	Millimolar
mg	Milligram
mg/kg	Milligram/kilogram
ml	Milliliter
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
w/v	Weight/volume

FIGURES

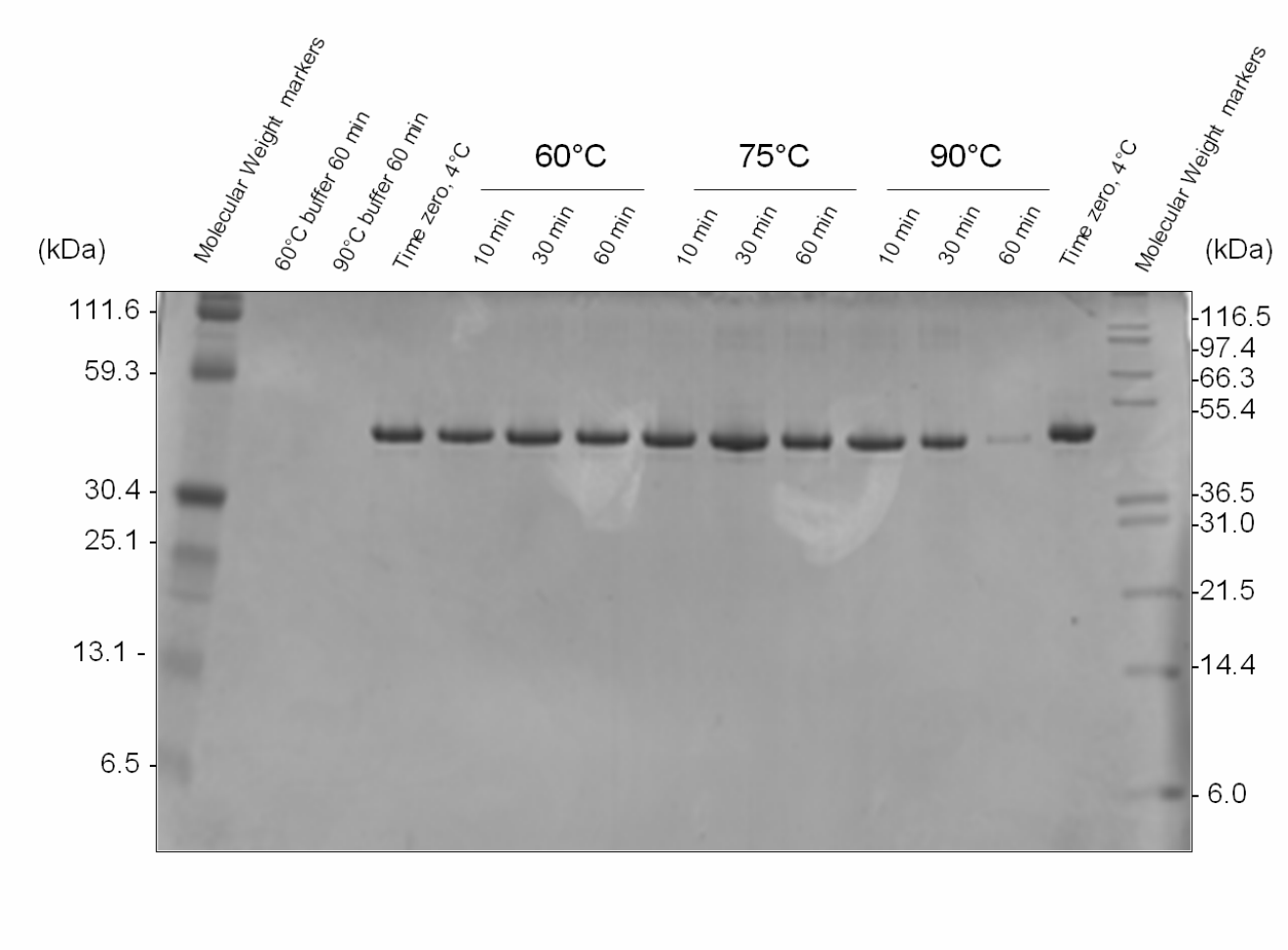
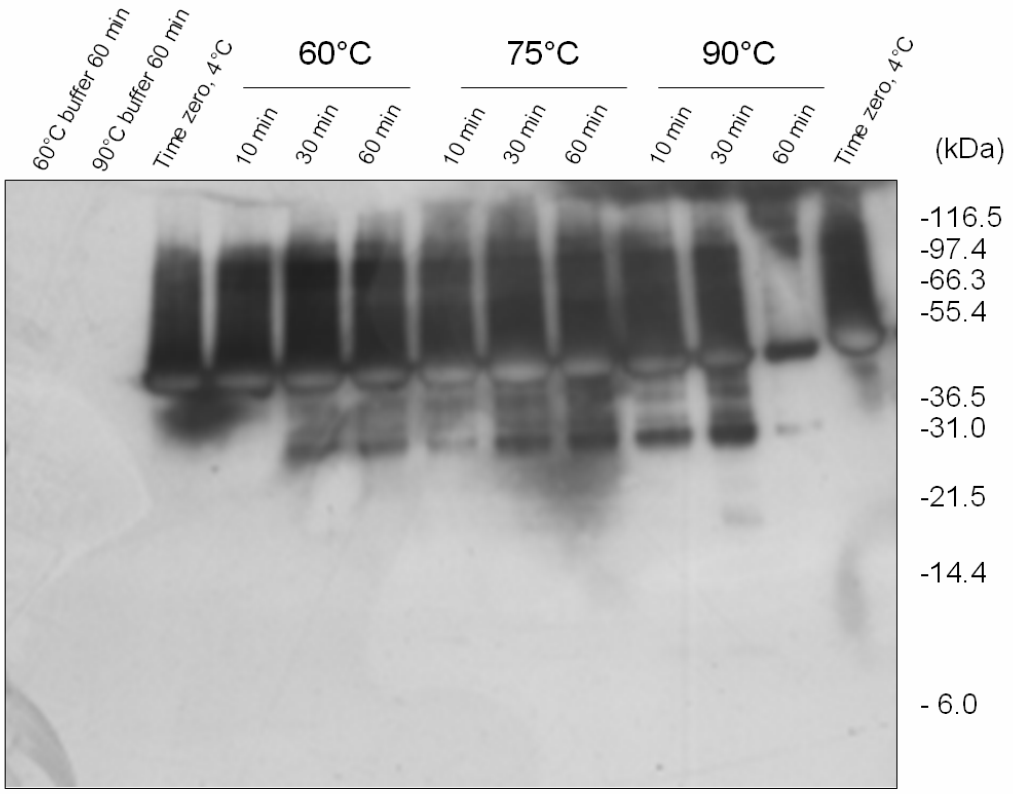

Molecular Weight markers
60°C buffer 60 min
90°C buffer 60 min
Time zero, 4°C
60°C
75°C
90°C
10 min
30 min
60 min
10 min
30 min
60 min
10 min
30 min
60 min
Time zero, 4°C
Molecular Weight markers
(kDa)
(kDa)
111.6
59.3
30.4
25.1
13.1
6.5
116.5
97.4
66.3
55.4
36.5
31.0
21.5
14.4
6.0

FIGURE 2 - WESTERN BLOT ANALYSIS OF 2MEPSPS PROTEIN AFTER INCUBATION AT 60, 75 OR 90°C FOR UP TO 60 MINUTES


60°C buffer 60 min
90°C buffer 60 min
Time zero, 4°C
60°C
10 min
30 min
60 min
75°C
10 min
30 min
60 min
90°C
10 min
30 min
60 min
Time zero, 4°C
(kDa)
-116.5
-97.4
-66.3
-55.4
-36.5
-31.0
-21.5
-14.4
- 6.0

ATTACHMENTS

ATTACHMENT 1 - **PROTOCOL**

2mEPSPS PROTEIN:
HEAT STABILITY STUDY

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 heat stability of the 2mEPSPS protein, encoded by the *2mepsps* gene. The rate of *in vitro* protein degradation after heat treatment will be observed by SDS-PAGE and Western-blot.

1.2 GOOD LABORATORY PRACTICE COMPLIANCE

This study will not be subjected to specific Quality Assurance inspections. However, standardized, routine operating methods similar to those which will be used in this study are periodically inspected.

1.3 REGULATORY GUIDELINES

No data requirement for this study.

2 STUDY PERSONNEL

2.1 STUDY DIRECTOR:

Date: August 27, 2007

D. ROUQUIE

2.2 SPONSOR REPRESENTATIVE:

Date: August 27, 2007

C. CAPT

2.3 OTHER STUDY PERSONNEL

Responsibility		Name
Replacement Study Director	:	J.B. RASCLE
In-life Supervisor	:	C. JAUSSELY
Responsible Technician	:	A. ARNAUD

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

3 PROPOSED DATES

Experimental starting date	:	August 28, 2007	
Experimental completion date	:	September 28, 2007	(estimated)

4 OVERVIEW OF STUDY DESIGN

A solution of the test protein will be heated to temperatures of 60, 75 or 90°C and samples taken for analysis at time-points of 0, 10, 30 and 60 minutes. The resultant protein solution will be analysed for presence of the parent protein and potential stable protein fragments by SDS-PAGE electrophoresis. In addition, a western blot analysis will be performed to evaluate the protein immunoreactivity by using a specific rabbit anti-2mEPSPS protein antibody.

5 MATERIALS AND METHODS

5.1 PROTEINS

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 number	:	LEJ 5837
Purity	:	99.91 % 2mEPSPS protein
Storage	:	Approximately –80°C
Certified through	:	October 24, 2008

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

5.2 PROTEIN SOLUTION

The test protein will be received as a stock solution of 0.971 mg/ml in the storage buffer (0.1 M Tris; 2.7 mM KCl; 137 mM NaCl; 1 mM DTT; pH 7.5).

The test protein will be diluted in the storage buffer to a final protein concentration of 0.25 mg/ml.

5.3 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.

5.4 HEATING

Nine 1.5 ml microcentrifuge tubes of 40 µl protein solution will be incubated to 60, 75 or 90°C in a hot-block for 10, 30 or 60 minutes.

Two additional samples will be kept at approximately 4°C to act as the time zero samples.

Two tubes of buffer only will be incubated as above for 60 minutes at 60 or 90°C.

5.5 SAMPLES

After the incubation period each sample must be placed immediately on ice and completed with 14 µl distilled water.

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

5.6 SDS-PAGE ANALYSIS

The method will be based on that of Laemmli (1970) using a BioRad Mini-Protean III cell (BioRad, France). Prior to running SDS-PAGE, 5 µl of 5X Leammli solution containing BME will be added to 20 µl of samples. Samples of 15 µl will be added to wells of an SDS-PAGE gel (15 wells, 1 mm 10-20% gradient polyacrylamide Tris-Tricine, BioRad). A few grains of sucrose may be added to the samples if a higher density is needed to allow correct deposition of samples into the wells.

The unheated samples and the control without protein will be treated the same way.

A Kaleidoscope pre-stained standard solution (Bio-Rad) in the first lane and a standard marker solution (2.5-200 kDa) (Mark 12, Invitrogen, France) in the last lane will be used to provide reference points of known molecular weights on the gel.

Loading order on gel:

Lane	Sample
1	Markers (Kaleidoscope)
2	60°C buffer 60 min
3	90°C buffer 60 min
4	Time zero 4°C
5	60°C 10 min
6	60°C 30 min
7	60°C 60 min
8	75°C 10 min
9	75°C 30 min
10	75°C 60 min
11	90°C 10 min
12	90°C 30 min
13	90°C 60 min
14	Time zero 4°C
15	Markers (Mark 12)

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 will be used to prepare the western Blot.

5.7 WESTERN BLOT ANALYSIS

A PVDF membrane will be placed on the PAGE-SDS gel in a Tris/Tricine buffer and an electrical current applied in order to transfer the protein bands onto the membrane. To reveal the 2mEPSPS protein bands and/or its fragments recognized by a specific anti-2mEPSPS protein antibody, the membrane will be incubated in the presence of a specific polyclonal rabbit anti-2mEPSPS protein antibody.

The hybridization of the antibody with the proteins immobilized on the membrane will be revealed using a goat anti-rabbit polyclonal antibody coupled with alkaline phosphatase. The hybridization bands will be visualized using the ECL detection system (Amersham).

5.8 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 protein blot is clearly visible at time zero (lanes 4 and 14 and is not visible in the lanes without protein (lanes 2 and 3).
- at least one marker lane is clearly visible to allow a molecular weight evaluation.

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.

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

9.1 GENERAL REFERENCES

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

ATTACHMENT 2 - **CERTIFICATES OF ANALYSIS**

Title

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

Batch n°: LEJ5837

Author

Nadine Bautsoens

Completed On
2nd March, 2007

Testing Facility

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

Study number

BBS06-012

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.

Study Director

Nadine Bautsoens
Expression and Protein Characterization
Molecular and Biochemical Analytical Services

02|03|2007
Date

STUDY IDENTIFICATION PAGE

Study start date:	October 24th, 2006
Experimental start date:	October 25th, 2006
Experimental Termination date:	October 27th, 2006
Study Completion date:	March 2nd, 2007
Test Facility Address:	Bayer BioScience N.V. Molecular & Biochemical Analytical Services GLP Test Facility Technologiepark 38 9052 Gent – Belgium Tel: +32 9-243 04 11 Fax: +32 9-224 06 94
Test Facility Manager:	Elizabeth Bates Address see Test Facility Tel: +32 9-243 04 25 Fax: +32 9-224 06 94 e-mail: elizabeth.bates@bayercropscience.com
Study Director:	Nadine Bautsoens Address see Test Facility Tel: +32 9-243 05 86 Fax: +32 9-224 06 94 e-mail: nadine.bautsoens@bayercropscience.com
Study Personnel:	Nadine Bautsoens Address see Test Facility Tel: +32 9-243 05 86 Fax: +32 9-224 06 94 e-mail: nadine.bautsoens@bayercropscience.com
Sponsor Representative:	Dominique Rouan Global Regulatory Affairs Manager Regulatory Affairs Address see Test Facility Tel: +32 9-243 04 21 Fax: +32 9-233 19 83 e-mail: dominique.rouan@bayercropscience.com

QUALITY ASSURANCE STATEMENT

Report **BBS06-012** Date: 01 MAR 2007
Page **5**

Quality Assurance (GLP)

Quality Assurance Statement

Title: **Certificate of analysis of 2mEPSPS protein produced from *E. coli* Batch LEJ 5837**

Study: BBS06-012

This study was periodically inspected and properly signed records of these inspections were submitted to test 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	24 OCT 2006	24 OCT 2006
Study conduct	26 OCT 2006	26 OCT 2006
Draft report	29 JAN 2007	30 JAN 2007
Study plan amendment	30 JAN 2007	30 JAN 2007
Draft report	19 FEB 2007	21 FEB 2007
Study plan amendment	22 FEB 2007	22 FEB 2007
Final report	28 FEB 2007	01 MAR 2007

S. Berghman / M. Lecleir
GLP Quality Assurance

01/03/07 01/03/2007

APPROVALS PAGE

Study Director / Author	Nadine Bautsoens	02/03/2007 Date
Test facility management	Elizabeth Bates	02/03/07 Date
Sponsor representative	Dominique Rouan	02/03/2007 Date

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUATION)

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 0.971 (±0.152) mg/ml and the purity was estimated to be 99.91%.

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 T13-01 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 AND STANDARDS

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 8-07) 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 PR006-1

Test Item ID:	T13-01
Test Item Identity:	2mEPSPS protein
Origin:	*Escherichia coli*
Batch n°:	LEJ5837
Expiry date:	October 24th, 2008
Buffer solution:	100 mM Tris (pH 7.5), 2.7 mM KCl, 137 mM NaCl, 1mM 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 T13-01

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 assay of the purified protein was also assayed and indicates that the protein has the correct enzymatic nature.

4.1 Quantification of the test item T13-01

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 concentrations and the standard deviation were calculated with the Microsoft Excel software (Table 1). The average protein concentration, estimated from the observed three data points, was 0.971 (± 0.152) mg/ml.

4.2 Molecular weight determination of the test item T13-01

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 electrophoresed using the SDS-PAGE technique according to SOP BBS 07/63/01 and the obtained gel (102606A, fig. 1) was stained according to SOP BBS 07/66/01.

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 $y = -0.7747x + 4.0839$. The obtained x-value is 4.69. The molecular weight of the test item corresponds to the inverse logarithm of the x-value and is estimated to be 49 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 T13-01

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 102606A with the FluorS™ MultiImager according SOP BBS04/40/00. 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 99.91%.

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 detection with antibodies.

The test item protein was separated from side products/protein trace amounts with the SDS-PAGE technique according to SOP BBS 07/63/01 and the obtained PAGE gel (102606B) was further processed according to SOP BBS07/64/01. Since the prestained molecular weight markers were visible on the membrane (102606C, 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 (102606C) makes them accessible for the reaction with a specific antibody. The membrane (102606C), 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 one major band to become visible (102606C, 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 colorimétric solution and stopped with the addition of 34% sodium citrate 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 0.0493 and 0.0596. The absorbance of the duplicates of the test item sample was 0.1595 and 0.1677.

5. RESULTS AND DISCUSSION

The protein concentration of the test item T13-01 was estimated to be 0.971 mg/ml and the purity is estimated to be 99.91%. 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 T13-01. Based on the analyses we identified the T13-01 test item to be 2mEPSPS. The concentration of the protein in the test item T13-01 was estimated at 0.971 mg/ml with an estimated purity of 99.91%.

7. ARCHIVING

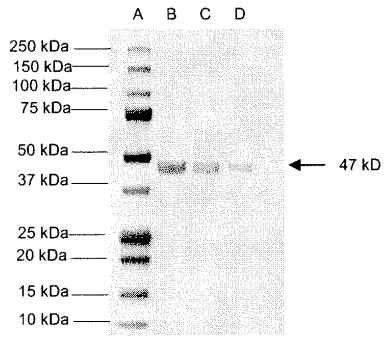
The study plan, the final report and the study data are archived under study number BBS06-012 in the GLP Test Facility Archives of Bayer BioScience N.V., Technologiepark 38, 9052 Gent, Belgium.

A sample of test item (T13-01) 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 T13-01

Subsample	Dilution factor	Measured concentration µg/ml	Concentration of test item mg/ml
PR006-1A	100	8.87	0.887
PR006-1B	125	7.04	0.879
PR006-1C	150	7.65	1.15

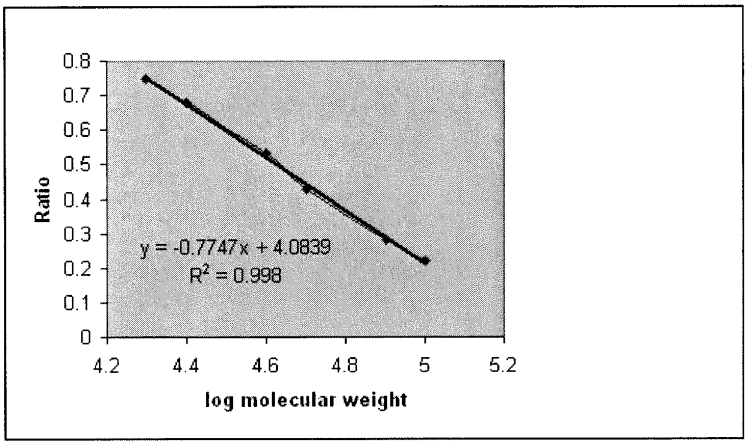
Figure 1: SDS-PAGE of test item T13-01(gel ID# 102606A)



Legend : A molecular weight marker
B 2 µg test item T13-01
C 1 µg test item T13-01
D 0.5 µg test item T13-01

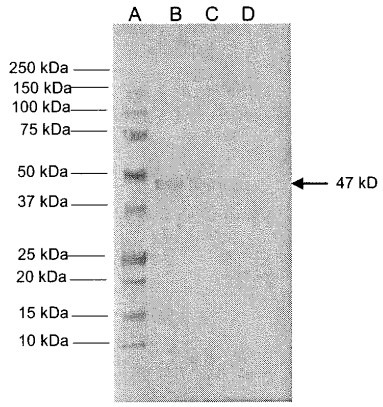
Figure 2: Plot standard curve molecular weight of test item T13-01

A	B	C	D	E
Molecular weight kDa	log Molecular weight	Migrated distance cm	Total distance cm	Ratio C/D
100	5	1.3	6	0.22
75	4.9	1.7	6	0.28
50	4.7	2.6	6	0.43
37	4.6	3.2	6	0.53
25	4.4	4.1	6	0.68
20	4.3	4.5	6	0.75



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

Figure 3: Western blot of test item T13-01 (blot ID# 102606C)



Legend : A molecular weight marker
B 0.6 µg test item T13-01
C 0.3 µg test item T13-01
D 0.1 µg test item T13-01

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No	Doc No	Author(s), year, title, source, edition, pages
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FINAL REPORT AMENDMENT

There is no final report amendment at this time.

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