

SUMMARY

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STUDY TITLE

Heat Lability of Double Mutant 5-Enol Pyruvylshikamate-3-Phosphate Synthase (2mEPSPS)
Protein

DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

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STUDY COMPLETED ON

15-Jul-2011

PERFORMING LABORATORY

Regulatory Sciences and Government Affairs—Indianapolis Lab
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LABORATORY STUDY ID

110461

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Heat Lability of Double Mutant 5-Enol Pyruvylshikamate-3-Phosphate Synthase (2mEPSPS) Protein

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: 2mEPSPS

Title: Heat Lability of Double Mutant 5-Enol Pyruvylshikamate-3-Phosphate Synthase (2mEPSPS) Protein

- STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS:

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Company: Dow AgroSciences LLC

Company Agent: L. Han

Title: Regulatory Manager

Signature: 

Date: July 12, 2011

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STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Heat Lability of Double Mutant 5-Enol Pyruvylshikamate-3-Phosphate Synthase
(2mEPSPS) Protein


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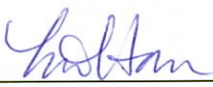
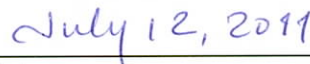
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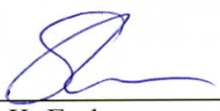

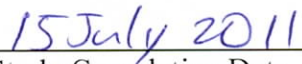
United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organization for Economic Co-Operation and Development
ENV/MC/CHEM(98)17, Paris January 26, 1998

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160 with the following exceptions. The GLP status of the protein molecular weight standards were unknown. The chain of custody of these standards was not monitored.

	
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**Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page**

Study ID: 110461

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GLP Quality Assurance Inspections

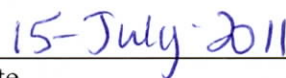
Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
11-May-2011	12-May-2011	Protocol Review
18-May-2011	18-May-2011	Heat Treatment and Activity Assay
24-June-2011	29-June-2011	Heat Treatment and Activity Assay (Repeat)
13-July-2011	14-July-2011	Raw Data and Report Review and Test Substance Container Verification

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.

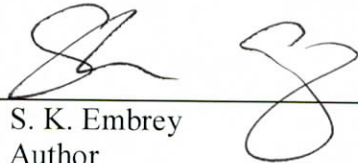


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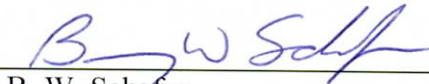
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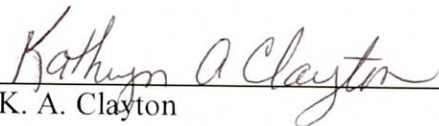
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Title: Heat Lability of Double Mutant 5-Enol Pyruvylshikamate-3-Phosphate Synthase
(2mEPSPS) Protein

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Heat Lability of Double Mutant 5-Enol Pyruvylshikamate-3-Phosphate Synthase (2mEPSPS) Protein

ABSTRACT

The thermal stability of the 2mEPSPS protein was evaluated by comparing heated 2mEPSPS protein solutions at 25, 37, 55, 75, and 95 °C for 30 minutes with a sample held on ice. At temperatures at or above 55 °C, the enzymatic activity of the 2mEPSPS protein was reduced by ≥ 73 %. At temperatures at or above 55 °C, the 2mEPSPS protein lost ≥ 90 % of its immunoreactivity as measured by a polyclonal antibody sandwich ELISA. SDS-PAGE analysis indicated that the molecular mass of the 2mEPSPS protein (approximately 47 kDa) was unchanged. These data indicate that industrial processing of the soybean grain would significantly degrade the tertiary structure of the 2mEPSPS protein, reduce its immunoreactivity, and significantly diminish its enzymatic activity.

ABBREVIATIONS

2mEPSPS	Double Mutant 5-enol Pyruvylshikimate-3-Phosphate Synthase
AI	active ingredient
DTT	Dithiothreitol
DAS	Dow AgroSciences LLC
ELISA	enzyme-linked immunosorbent assay
GLP	Good Laboratory Practice
kDa	kilodalton
M	Molar
µg	microgram
µL	microliter
mL	milliliter
mM	millimolar
min	minute
MW	molecular weight
ng	nanogram
OD	optical density
pAb	polyclonal antibodies
PBST	phosphate buffered saline with Tween 20, pH 7.4
KCl	potassium chloride
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TSN	test substance number
MW	molecular weight
N/A	Not Applicable
V	volt

INTRODUCTION

The objective of this study was to determine the stability of the 2mEPSPS protein after 30 minutes of exposure to heat at 25, 37, 55, 75, and 95 °C in buffer. Stability was measured based on the loss of immunoreactivity and enzymatic activity. Immunoreactivity was determined using a polyclonal antibody based sandwich ELISA specific for the 2mEPSPS protein. Enzymatic activity was determined using a colorimetric assay to detect the presence or absence of inorganic phosphate as measured by the malachite green dye assay when incubated with shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) as substrates (1,2). The stability of the primary protein structure was determined by Coomassie staining of the protein separated by SDS-PAGE.

MATERIALS AND METHODS

Test Substance

The 2mEPSPS protein (Lot Number: DMMG_033110) was produced and purified from *Pseudomonas fluorescens*. The protein preparation was sent to the Test Substance Coordinator at Dow AgroSciences and the material was designated TSN033171-0001. The concentration was determined to be 665 µg active ingredient (a.i.) per mg powder (3).

Reference Substances

The commercially available reference substance used is listed in the following table:

Reference Substance	Name	Assay	Reference
Unstained Molecular Weight Markers	Novex Sharp Unstained Protein Standard	SDS-PAGE	Invitrogen Cat #: LC5801, Approximate Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10 and 3.5 kDa
Prestained Molecular Weight Markers	Novex Sharp Protein Standard	SDS- PAGE	Invitrogen Cat #: LC5800, Approximate Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10 and 3.5 kDa
Protein Standard	2mEPSPS	ELISA	0.665 mg/mL

Heat Treatment of the 2mEPSPS Protein

The 2mEPSPS protein was dissolved in 50 mM HEPES, 100 mM KCl, 1 mM DTT, pH 7.4, at 1 mg of powder per mL of buffer by weighing 7.1 mg of lyophilized powder and adding 7.1 mL of buffer (665 µg of 2mEPSPS/mL). The solution was vortexed and aliquoted into 6 appropriately labeled tubes each containing 500 µL each. The original aliquot was held on ice and the others were heated at 25, 37, 55, 75, and 95°C for 30 min. After heat treatment, all samples were immediately placed on ice and assayed by colorimetric enzyme assay, ELISA, and SDS-PAGE.

Activity Assay of Heated and Non-Heated 2mEPSPS Protein

Enzyme activity of 2mEPSPS was determined based upon the presence or absence of inorganic phosphate as measured by the malachite green dye assay. When enzyme is incubated with shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) as substrates (1,2), the amount of color produced is a function of the concentration of phosphate. Assays were performed in a 96-well plate format with a total volume of 50 μ L. Typical assays contained 50 mM HEPES, 100 mM KCl, 1 mM dithiothreitol, pH 7.4, 1 mM S3P (shikimate-3-phosphate), 1mM PEP (phosphoenolpyruvate) and 0.07 μ M 2mEPSPS. Assays were initiated by the addition of 5 μ L (~0.07 μ M) of 2mEPSPS enzyme for each heat treatment. Reactions were terminated by the addition of 235 μ L of a 3:1 mixture of malachite green:ammonium molybdate solution. After 1 minute, the 96 well-plate was read on a Spectra-Max M2 plate reader (Molecular Devices) at 660 nm. Control reactions without the 2mEPSPS enzyme were used to correct for background absorbance.

ELISA Assay of Heated and Non-Heated 2mEPSPS Proteins

After treatment, all samples were kept on ice until analysis by a 2mEPSPS microtiter plate ELISA as described by the manufacturer (Acadia BioSciences, LLC). The samples were initially diluted 1:1000 in phosphate buffered saline 0.05% Tween (PBST) with 2x casein, and serially diluted (1:1) prior to being loaded into the wells of the ELISA plates. The assay used a sequential double antibody sandwich ELISA format. An aliquot of each sample was incubated with an immobilized anti-2mEPSPS polyclonal antibody in the wells of a coated plate, and then the unbound proteins were removed from the plate by washing with PBST. An excess amount of enzyme-conjugated polyclonal antibody was added to the wells and incubated at room temperature. The enzyme-conjugated antibodies bind with the target protein in the wells and form a “sandwich” with the immobilized antibodies. The presence of 2mEPSPS protein was detected by incubating the wells with enzyme substrate, generating a colored product. Since the target protein is bound in the antibody sandwich, the level of color development is related to the concentration of target protein in the sample (i.e., lower protein concentrations result in lower

color development). The absorbance at 450 nm minus absorbance at 650 nm was measured using a Spectra-Max M2 plate reader (Molecular Devices).

SDS-PAGE of Heated and Non-Heated 2mEPSPS Proteins

Diluted aliquots of the heat treated samples were mixed with equal volumes of 2x Laemmli sample buffer (Bio-Rad), containing 5% freshly added 2-mercaptoethanol (β ME, Bio-Rad) and heated for 5 minutes at $\sim 95^{\circ}\text{C}$. After a brief centrifugation, aliquots of the supernatants were loaded directly on the gel and the electrophoresis was conducted at a constant voltage of 150 V for ~ 60 minutes using XT MES buffer (Bio-Rad). After separation, the gel was stained with Gel Code Blue protein stain (Thermo-Pierce).

Data Analysis and Calculations

SOFTmax PRO software was used with the Molecular Devices plate reader. SOFTmax PRO allows the creation of computer generated data files containing all of the parameters required for acquiring and analyzing data from any MAXline instrument. The calibration curve for the 2mEPSPS ELISA kit was constructed using a quadratic curve regression of the known concentration of the standard calibration solutions and their subsequent absorbance (optical density).

The equation fits the best parabola to the standard curve based on Equation 1:

Equation 1

$$y = A + Bx + Cx^2$$

Where:

y = mean absorbance value (OD)

x = protein concentration

The SOFTmax PRO software was used to determine the amount of 2mEPSPS protein in each sample. The absorbance value and calculated concentration, as well as individual well results, mean sample result, standard deviation and the percent coefficient of variation are reported on the SOFTmax PRO data report.

STATISTICAL TREATMENT

Statistical treatment of the data in this study consisted of the calculation of means of the treatments.

RESULTS AND DISCUSSION

Treatment of Heated and Non-Heated 2mEPSPS Protein

To test heat lability of the 2mEPSPS protein, five treatment conditions were evaluated in this study. Proteins are usually sensitive to high temperatures and the degree of denaturing is dependent upon the temperature and duration of the heat treatment. Processing of soybeans at elevated temperatures is required because raw soybeans cannot be used in human foods as they contain many anti-nutritional factors (4). Fortunately, many of the factors are deactivated, modified, or reduced through proper heat-treatment. In soybean industrial processing, high heat treatments are usually involved in the extrusion or toasting processes. This extrusion process destroys the anti-nutrient factors and improves the nutritional quality of the soybean meal.

Activity Assay of the Heated and Non-Heated 2mEPSPS Protein

The 2mEPSPS protein activity was measured by a modified enzyme assay (1,2). The activities of the heated samples were compared with the sample held on ice (Table 1, Figure 1). As expected, the enzyme activity was significantly diminished after heating. At 55 °C, approximately 73% of the activity was lost and at temperatures above 75 °C, approximately 94%

of the activity was lost. Therefore, based on enzymatic activity it can be concluded that the 2mEPSPS protein is functionally unstable when heated.

Table 1. Summary of 2mEPSPS Enzyme Activity Results

Treatment Temperature	Time (min)	% Enzymatic Activity
Held on ice	NA	100
25 °C	30	102
37 °C	30	102
55 °C	30	27
75 °C	30	6
95 °C	30	6

Note: The relative activity of the treatment held on ice is designated as 100%. The results are averaged from enzymatic assay absorbance readings of serial dilutions of the 2mEPSPS. % enzymatic activity = (OD of heat treatment – blank) / (OD of sample held on ice – blank) x 100.

ELISA Analysis of the Heated and Non-Heated 2mEPSPS Protein

The immunoreactivity of each heated protein sample was compared with the 2mEPSPS protein held on ice (Table 2, Figure 1). Under the heat regimes (25 - 95 °C), the 2mEPSPS protein lost $\geq 90\%$ of its immunoreactivity based on a pAb sandwich ELISA at ≥ 55 °C. These results indicate that the epitopes (i.e., binding sites) for the polyclonal antibody used in the 2mEPSPS sandwich ELISA format were significantly altered once the protein was heated at or above 55°C. Therefore, based on pAb 2mEPSPS ELISA, it can be concluded that the 2mEPSPS protein is functionally unstable when heated.

Table 2. Summary of 2mEPSPS ELISA Results

Treatment Temperature	Time (min)	% Immunoreactivity
Held on ice	NA	100
25 °C	30	81
37 °C	30	103
55 °C	30	10
75 °C	30	5
95 °C	30	9

Note: The relative activity of the treatment held on ice is designated as 100%. The results are averaged from the predicted ELISA assay concentrations of serial dilutions of the 2mEPSPS. % Immunoreactivity = (ng/mL of each acceptable dilution of heat treatment /average ng/mL of sample held on ice) x 100.

SDS-PAGE Analysis of Heated and Non-heated 2mEPSPS Protein

In the toxicology-lot preparation of *Pseudomonas fluorescens* produced 2mEPSPS (TSN033171-0001), the major protein band, as visualized on Coomassie stained SDS-PAGE gels, is approximately 47 kDa (3). In the SDS-PAGE analysis, the 2mEPSPS protein held on ice or heated for 30 min at 25, 37, 55, 75, or 95 °C, showed that the protein molecular weight was as expected (Figure 2, Lanes 2-7). However, as the temperature increased, additional multimeric-aggregates became prevalent and some degradation of the protein's primary structure was evident.

CONCLUSIONS

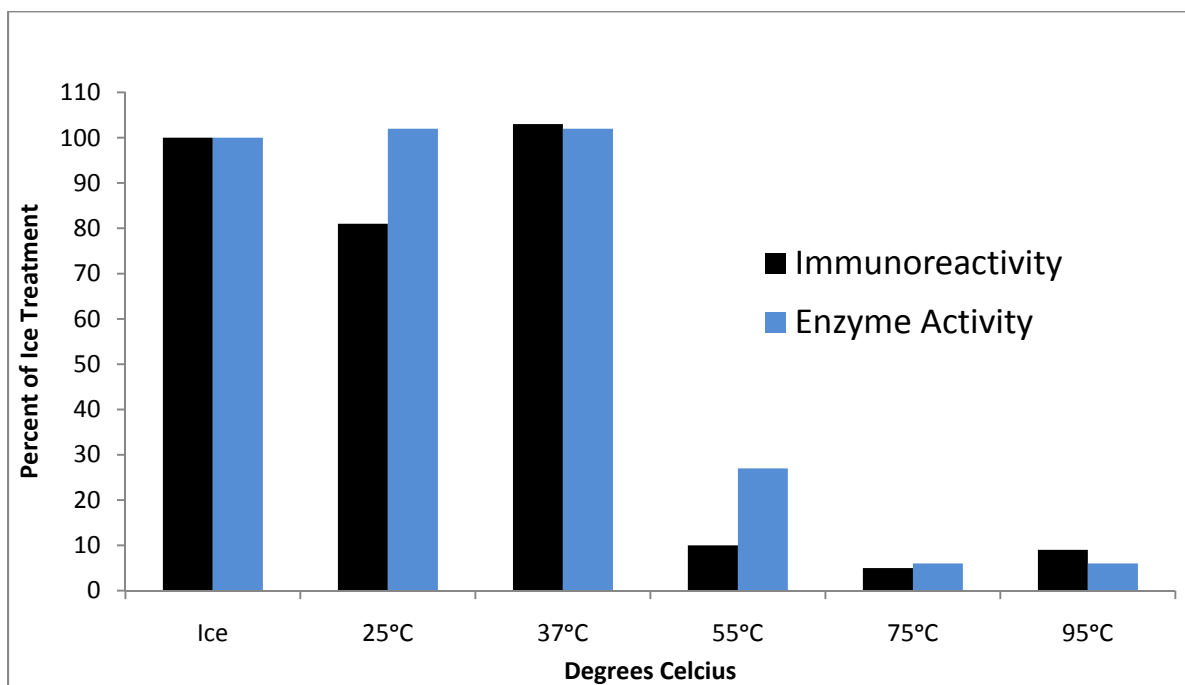
The thermal stability of 2mEPSPS protein was evaluated by heating protein solutions for 30 minutes at 25, 37, 55, 75 or 95 °C. Results indicated that the 2mEPSPS protein has significantly reduced immunoreactivity and enzymatic activity when heated.

ARCHIVING

The original version of the final report is stored in the Dow AgroSciences LLC archives at 9330 Zionsville Road in Indianapolis, Indiana 46268-1054.

REFERENCES

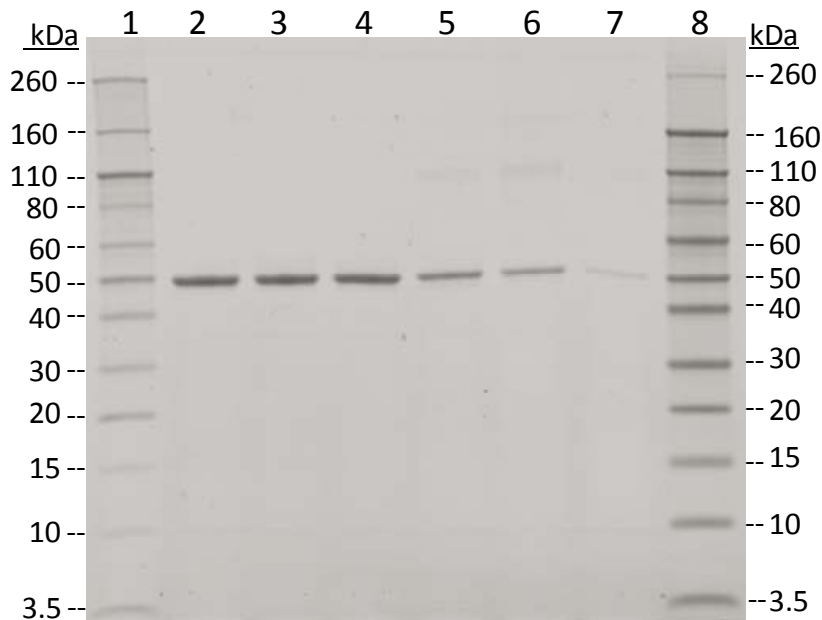
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For immunoreactivity, the sample held on ice was normalized to 100%. Serial dilutions were performed on each heat treatment to determine the appropriate dilutions within the linear range of the standard curve. Acceptable concentrations were compared to the sample held on ice and averaged for each heat treatment represented in the bar graph.

The relative enzymatic activity of the sample held on ice is designated as 100%. The results are from enzymatic assay absorbance readings (OD) of each heat treatment for the 2mEPSPS protein. The results are represented in the bar graph.

Figure 1. Enzymatic Activity and Immunoreactivity Analysis of Heated and Non-Heated 2mEPSPS Protein



The 2mEPSPS samples were mixed with equal volumes of Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at ~95 °C. The samples were loaded into a Bio-Rad 4-12% Bis-Tris Criterion gel and electrophoresed at a constant voltage of 150 V per gel for ~60 minutes using XT-MES buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Thermo-Pierce.

Lane	Sample	Amount Loaded
1	Invitrogen Novex Sharp Unstained MW markers	10 µL
2	2mEPSPS (TSN033171-0001) held on ice	~1.33 µg
3	2mEPSPS (TSN033171-0001) heated @ 25 °C, 30 min	~1.33 µg
4	2mEPSPS (TSN033171-0001) heated @ 37 °C, 30 min	~1.33 µg
5	2mEPSPS (TSN033171-0001) heated @ 55 °C, 30 min	~1.33 µg
6	2mEPSPS (TSN033171-0001) heated @ 75 °C, 30 min	~1.33 µg
7	2mEPSPS (TSN033171-0001) heated @ 95 °C, 30 min	~1.33 µg
8	Invitrogen Novex Sharp Prestained MW markers	10 µL

Figure 2. SDS-PAGE Analysis of Heated and Non-Heated 2mEPSPS Protein