

Document Cover Page

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Document Type	Report / File Number(s)	Date Issued (dd-MMM-yy)	Pages in Report
Research	DAI 1006	13-Jan-11	28
Document Title			
Production and Characterization of 2mEPSPS (DMMG) Protein for Supporting Regulatory Toxicology Study			
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Materials being tested:			
Active Ingredient(s) / Structural Gene(s) / Protein(s), etc.	Formulation Nos.	Product Name(s) (DAS only)	
2mEPSPS, DMMG			
Lab Notebook(s)	Information Release No.	GLP Substance Nos (TSN, AGR)	Other Batch/Lot Number(s)
E3493, E2911, E3256		TSN033171-0001	
Key Terms including compound numbers (ex: XDE-123)			
DMMG, 2mEPSPS, maize EPSPS, EPSPS TIPS mutation, Glyphosate, Pseudomonas fluorescens, protein expression and purification, Tox-lot protein production, SGF and SIF protein digestibility			

DMMG/2mEPSPS, or double mutant maize EPSPS was engineered from wild-type of maize EPSPS gene by site-directed mutagenesis. After confirming by DNA sequencing analysis and other biochemical method validation, a production strain was constructed in our *Pseudomonas fluorescens* expression system, with RCC number DPf21575 and pDAB105082. Large scale protein production was performed and subsequently chromatography purification produced approximately close to 47 gram DMMG with above 95% purity. The bulk protein sample was lyophilized in buffers containing stabilizers and additives, and the reconstituted sample was active in the enzymatic assay, and insensitive to Glyphosate. The protein had an expected total 444 amino acid length, with correct TIPS mutations in the correct positions by MALDI-TOF MS analysis. DMMG was readily digested by pepsin within 1 min in simulated gastric fluid; however, its half life by pancreatin treatment might be over 10-30 min span in simulated intestinal fluid.

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INTRODUCTION

The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) is a requisite and essential enzyme for the biosynthesis of aromatic amino acids and many aromatic metabolites such as lignin, flavonoids, and alkaloids utilized in the shikimate pathway (Herrmann 1995). The shikimate pathway occurs exclusively in plants, fungi, and bacteria but absent from animal, which makes it a great target for developing effective herbicide and antimicrobial agents (Coggins *et al.* 2003).

Specifically, EPSPS catalyzes the transfer of the carboxyvinyl moiety of phosphoenolpyruvate (PEP) to the 5'-hydroxyl group of shikimate-3-phosphate (S3P) to produce 5-enolpyruvyl-3-shikimate phosphate (EPSP) and inorganic phosphate (Pi) (see Fig. 1). EPSP is a precursor to the majority of aromatic compounds produced in the cells, including the aromatic amino acids.

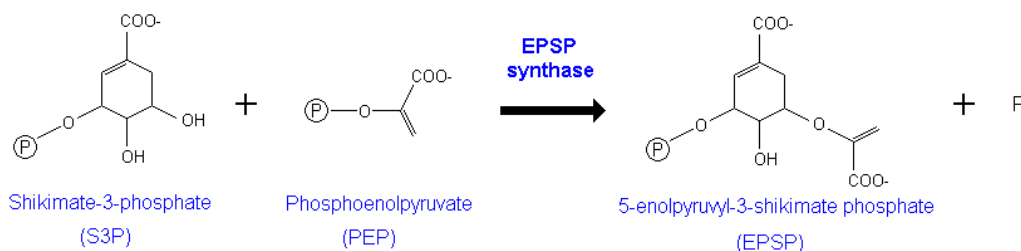


Figure 1. Reaction catalyzed by EPSPS

Glyphosate, a synthetic compound and active ingredient in the non-selective and broad spectrum herbicides RoundupTM and TouchdownTM, is a competitive inhibitor to EPSPS by mimicking the PEP oxonium ion and competitively occupying the same active binding site on the enzyme for PEP, thus effectively shutting down aromatic amino acid biosynthesis in plants (Steinrucken *et al.* 1980; Schonbrunn *et al.*, 2001). Glyphosate is simple to use, inexpensive, yet effective. Since its sale started from 1970s, it has become the most extensively used foliar applied herbicide for weed and vegetation control.

Many innate microbial EPSPS enzyme variants that are not inhibited by glyphosate through evolution selection are discovered, in particular those designated as Class II EPSPS which have higher K_i to glyphosate and low K_m for PEP. Those provided the mode of action for the development of glyphosate-tolerant crops. For instance, Roundup ReadyTM soybean and corn from Monsanto contain a CP4 EPSPS gene derived from *Agrobacterium* sp. strain CP4. CP4 EPSPS has a single amino acid residue difference in the active site from highly conserved glycine residue at position 100 in natural plant and bacterial enzymes to alanine (GA mutation), which renders the enzyme insensitive to glyphosate (Padgett *et al.* 1995). Similarly, a double mutant maize EPSPS, designated as DMMG or 2mEPSPS, contains two mutations at T102I and P106S, or TIPS mutation which tolerant to high dose of glyphosate was developed by Bayer CropScience (Lebrun *et al.*, 1996), and introduced into several crops including corn, cotton and others for commercialization (for example, GHB614 cotton, or GlytolTM).

Through commercial licensing, recently Dow AgroSciences is in collaborating with Stine/MSTech to develop soybean line with molecular stacking of our own AAD-12 trait and DMMG. Regulatory work for deregulating the product lines need to produce tox-lot DMMG for protein toxicity and eco-toxicity studies. Large scale production campaign was therefore performed to deliver the needed material. This report

described the development of a production strain in DOW proprietary *Pf* expression system for DMMG and documented the protein purification data, as well as protein characterization as part of the submission package.

MATERIALS AND METHODS

Bacterial Strains and Cloning Vectors: *E. coli* TOP 10 and BL21Star (DE3) competent cells were purchased from Invitrogen (Carlsbad, CA) and *E. coli* XL10-Gold competent cells were purchased from Stratagene (La Jolla, CA). *Pseudomonas fluorescens* (*Pf*) strain DC454, a modified DOW proprietary *Pf* host was obtained from DAS Research Cultural Collection (RCC). Plasmid pET28b(+) was purchased from Novagen (Madison, WI) and plasmid pDOW1169 was obtained from RCC. Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Xba I and Xho I were used for releasing DMMG fragment from pET28b(+) vector, and Spe I / Sal I double digested pDOW1169 was used in subcloning DMMG fragment.

Transformation and Plasmid Isolation: Following ligation reaction or other cloning/mutagenesis procedures, the recombinant plasmid DNA containing DMMG fragment in pET vector was transformed into chemically competent cells according to standard *E. coli* transformation protocol, LB/Kan plate was used for the selection, whereas the recombinant plasmid in pDOW vector was transformed into freshly prepared electrocompetent *Pf* using an established in-house procedure (Lin *et al.* 2005), and selected on M9 minimal salt plate with 2% Glucose. Plasmid DNA isolation was carried out by using Qiagen Miniprep kit (cat# 27106) starting from 1.5 ml overnight culture for *E. coli*, or 5 ml overnight culture for *Pf*. TOP10 *E. coli* cell was used for the recombinant pET plasmid for better stability maintenance and further manipulation.

Site-Directed Mutagenesis: The QuikChange Lightning kit (cat# 210518) from Stratagene was used to perform the mutagenesis reaction. Briefly, 10 or 100 ng of plasmid pDAB4743 was set up as template DNA in a 50 µl volume with appropriate reagents according to the suggested protocol, QC Primers 1 and 2 were used in one pair, and QC Primers 3 and 4 were used in another pair (see TABLE I). Thermal cycler (MJ Research, model PTC-225) program was consisting of 95 °C for 2 min, followed by 18 cycles of 95 °C 20 sec, 60 °C 10 sec, 68 °C for 2.5 min, then a final cycle of 68 °C for 5 min. The amplified samples were treated with Dpn I restriction enzyme at 37 °C for 5 min to degrade parental plasmid, immediately followed by transformation into XL10-Gold or BL21Star (DE3) competent cells.

Plasmid DNA Sequencing: Plasmid was purified using Qiagen Miniprep kit as stated before or Macherey-Nagel Nucleospin plasmid kit (Cat# 740-588.250), and restriction enzyme digestion was performed to validate the constructs. Total nine samples were selected and submitted to a contract service NWG/Operon (Huntsville, AL) for custom DNA sequencing. Four gene-specific sequencing primers (see TABLE I), plus two vector-derived primers (T7 promoter primer and T7 terminator primer) were used in the double-stranded sequencing reaction to cover the entire DMMG fragment and junction sequence at cloning sites.

Protein Expression Testing: For *E. coli* expression studies, only LB culture median was used in screening the positive clones. Briefly, 2.5 ml LB/Kan media were inoculated with a single colony of transformant at 37 °C placed in a 300 rpm shaker, after 3 h incubation, the temperature was reduced to 30 °C and 0.1 mM IPTG was added to the culture. After another 3 h post-induction, cells were harvested and a portion of the paste (corresponding to 0.5 ml culture) was used for the preparation of cell lystate. For *Pf* expression, 10 ml M9 salt median with 2% Glucose were used for preparing the starting seeds culture. Protein induction was

carried out in standard Dow *Pf* production media followed a typical 3 days monitoring procedure (Lin *et al.* 2006). Cells were resuspended in 10 mM Tris, pH 7.5/0.1 M NaCl, and sonicated on ice for approximately 30 sec using an ultrasound sonication device (Branson Sonifier 250, Danbury, CT). Soluble fraction, after spun at 14 krpm in microcentrifuge for 4 min was collected for SDS-PAGE gel analysis.

SDS-PAGE and Western Blot Analysis: SDS-PAGE was performed using a Novex Bis-Tris polyacrylamide gel (Invitrogen) and stained with Coomassie brilliant blue. For immunodetection, protein samples were transferred onto a nitrocellulose membrane using iBlot Dry Blotting System (Invitrogen, cat# IB1001). A mouse monoclonal antibody specific to DMMG (10B5.B4 with stock concentration 5.6 mg/ml) was obtained from our collaborator MST/Stine via DAS RSGA and used as primary detection antibody, followed by secondary goat anti-mouse IgG HRP conjugate and chemiluminescent detection. Standard operation procedures were followed for those routine analyses (For reference, see Lin *et al.* 2005).

Bulk Protein Production: Large scale expression was carried out using selected recombinant *Pf* host strain in shake flask. Briefly, 10 ml of overnight seeds culture grown in M9 salt media with 2% Glucose was transferred into 500 ml freshly prepared *Pf* production media in 2.8 L bottom-baffled Erlenmeyer flask. The culture was incubated at 28 °C and 300 rpm for 24 h, 1 mM final concentration of IPTG was added and continue to induce for 2 days. Cell paste was harvested by centrifugation at 10,000 x g for 20 min, and cells were stored at -80 °C prior to analysis. Several fermentation batches were conducted with total approximately 25 L culture had been collected to generate the cell mass for protein production.

Large Scale Protein Purification: DMMG protein was prepared from the soluble extract after cell lysis using Microfluidizer followed an established method (Lin *et al.* 2006), then purified using a three-step conventional column chromatography procedures. The soluble proteins in 50 mM Tris-HCl, pH 8.0 with 1 mM EDTA and 1 mM DTT were charged onto a Capto Q column (GEHC Index 70/500), and eluted by 0-1 M NaCl salt gradient. The main peak contains DMMG was pooled and 1.2 M ammonia sulfate was added, and then applied onto a Phenyl Sepharose HIC column (GEHC XK 50/60). The proteins were eluted by 1.2-0 M AmSO₄ gradient. Fractions contain DMMG were pooled and dialyzed against 10 mM Tris-HCl, pH 7.5, 1 mM DTT and finally polished on a Source 15Q column (GEHC XK50/30). Bulk protein sample was analyzed using various biochemical methods (details will be summarized in a separate report), and lyophilized using a freeze dryer (Virtis Genesis, model 25ES) (Lin *et al.*, 2006).

Enzymatic Activity Characterization: Enzyme kinetics of purified DMMG was assayed and calculated by measuring the amount of inorganic phosphate released in a reaction according to a modified procedure described by Lanzetta *et al.* (1979), and detailed condition of the colorimetric assay was supplied in Appendix I. Phosphoenolpyruvate (PEP) potassium salt was purchased from Sigma (St. Louis, MO), and shikimate-3-phosphate (S3P) triethylammonium salt was synthesized from shikimic acid (Sigma) and ATP using recombinant *E. coli* *AroL* according to a published method (Priestman *et al.* 2005). Optical density change at 660 nm over time was recorded in a Spectra-Max 190 plate reader (Molecular Device, Sunnyvale, CA). The data were fitted to the Michaelis-Menten equation for the determination of K_m and V_{max} . The IC₅₀ value for enzyme inhibition by glyphosate was also determined using constant 1 mM S3P in the reaction.

N-terminal Amino Acid Sequencing and MALDI-TOF MS: Purified DMMG was subjected for N-terminal AA sequencing analysis via Edman-degradation for 10 cycles using a Procise Protein Sequencer (Applied Biosystems). For peptide mass fingerprint, the protein sample was digested with trypsin, and peptides were purified with a C18 Zip Tip column. Mass spectral analyses were performed on a Voyager Biospectrometer (PerSeptive Biosystems, model DE STR). All the experiment details were followed in the standard operating procedures currently used in the Input Traits Discovery Department Protein Analytical Group. The data were uploaded into the software program PAWS for analysis.

Digestion in simulated gastric fluid (SGF) and simulated intestinal fluid (SIG): Protein digestibility assays were performed in accordance with standard UPS protocols with modification suggested by DAS RSGA Reg. Lab (see Xu, *et al*, 2005 for details on the preparation). Two reference proteins, bovine serum albumin (BSA) and β -Lactoglobulin were used as controls in both assays. The SGF assay was monitored for a period of 20 minutes, and SIF assay was monitored up to 1 hr period. The resulting reactions were analyzed by SDS-PAGE and followed by Western blot using anti-DMMG monoclonal antibody as described above and in the reference.

RESULTS AND DISCUSSION

Wild type of maize EPSPS sequence was initially synthesized in the early phase of the study, from an error using the external file (Lebrun WO 97/04103-A2 patent and EMBL accession A59404.1). The gene was designed based on bacterial codon-bias for optimal expression in *E. coli* and *Pf* hosts (detailed work will be reported in a separate document). All the bacterial expression constructs were made without the sequence of transit chloroplastid peptide, corresponding to mature and processed EPSPS protein. One of the construct, designated as pDAB4743 in our internal plasmid database was used as the parent template in the current study (notebook # E3492).

DMMG, or double mutant maize EPSPS gene was engineered by site-directed mutagenesis, converting from the wild type of maize EPSPS to a threonine to isoleucine and proline to serine (or TIPS) at two specific positions as a double mutant. Two sets of oligonucleotides, as listed in TABLE I, were designed in order to maximize the success of creating the desired mutations using Stratagene's mutagenesis kit. The only difference for the two pairs of primers is that one set has extra nt (39 nt vs. 27 nt for the short oligo set). The longer ones are considered more desirable especially with an increased melting temperature (T_m). Both sets of primers were used in the PCR reaction, followed the conditions suggested by the manufactures.

The resulting reactions were treated by restriction enzyme Dpn I to degrade the parent plasmid, then transformed either directly into an expression host *E. coli* strain BL21StarDE3 (Invitrogen), or a general cloning host *E. coli* strain XL10-Gold (Stratagene). A control reaction with pWhitescript plasmid and primers included in the kit on XL10-Gold cells yielded about close to 90% mutagenic rate, indicated by the blue/white appearance of the colony on selecting plate, which confirmed the kit was within its claimed specificity.

Based on the test results and prediction of mutagenesis efficiency, ten colonies each from the above experiments were randomly picked of the expression host strain BL21Star DE3, and screened for protein expression. Among twenty clones analyzed, all except one expressed a predicted close to 47 kDa inducible protein, as revealed by SDS-PAGE, and the exceptional one's 47 kDa band was not obvious (data not shown). Nine clones were further selected from this group, based on their inducible protein intensity (which relates to the protein expression level). Plasmid DNA was isolated as mini-prep, and double stranded DNA sequencing was performed, using the primers listed in TABLE I and standard vector-derived primers as described in the methods. All nine plasmids had identical sequence, with correct mutations at the specific sites. Those DNA sequencing files provided by NWG/Operon and maintained in our DAS local database are not included in this document. The resulting recombinant plasmid, with DMMG gene fragment (DMMG-A) in pET28b(+) vector via mutagenesis, is designated as pDAB4750.

The plasmid DNA of this construct was maintained in *E. coli* TOP10 cells, and bulked up for midi-scale isolation. The entire DMMG-A fragment was then released from the pET vector by restriction enzyme (Xba I and Xho I) digestion, and further ligated into pDOW1169 vector via Spe I and Sal I sites. The resulting plasmid pDAB105082, see Figure 2, was transformed into *Pf* host strain DC454, and validated by standard procedures and deposited to RCC as DPf21575.

The DMMG production strain in *Pf* host was first tested in small scale using 1 L shake flask and 200 ml

culture medium. A standard operation procedure for *Pf* cell growth and protein expression at constant 28 °C temperature was followed, which from previous experiments given rise the optimal expression for the wild type of maize EPSPS. SDS-PAGE analysis indicated that, similar to the wt maize EPSPS, DMMG was also expressed as soluble protein under this condition (data not shown). Large scale fermentation was subsequently scheduled and conducted using those conditions, in a set of 12 2.8 L shake flask each supplied with 500 ml culture medium. With the use of two temperature controlled shakers (New Brunswick Scientific, model Innova 44), 24 liters of culture were fermented in two consecutive weeks.

While large scale fermentation was in session, a small scale of pilot purification test was initiated using total 1 L of cell culture to scout the column chromatography selection. Similar to the wt maize EPSPS, DMMG was purified near to homogeneity by using sequential steps of IEX/HIC/high resolution IEX chromatography (data not shown). From the column separation standard point, the only observational difference is that DMMG has slightly weaker binding to the IEX resin, compared to wt maize EPSPS. This information was fitted into the process scale purification scheme, and arranged time to pack additional Source 15Q process columns.

The bulk fermentation cell pastes were divided into two aliquots, a Microfluidizer processor Model M110Y was used for the cell breakage and preparing of cell lysate. After clarification by high speed centrifugation and filtration through 0.45 um membrane, the soluble samples were analyzed by SDS-PAGE, shown in Figure 3. DMMG represents a dominant protein species in the total cell lysate. Large scale of chromatography was immediately followed.

Total soluble protein samples were allowed to bind to the capturing column CaptoQ, and after a brief (0.5 column volume) binding buffer wash, bound proteins were eluted using 0-1 M NaCl salt gradient. Figure 4 shows one of the two CaptoQ column chromatography profile. DMMG was eluted in the 0.1-0.3 M NaCl gradient as a main peak, as revealed by chromatography and gel analysis. The second step column separation is hydrophobic interaction chromatography (HIC) using Phenyl Sepharose High Performance resin. Three batches of separation were performed and a representative of chromatography and analysis was shown in Figure 5. And the last step is high resolution IEX with Source 15Q column purification. Due to unavailable of additional process scale column in the group, three lab scale (GEHC XK50/30) columns were packed and used in the process in order to complete the task in a short given time. Total 12 of chromatography were conducted to finish all the bulk DMMG from previous HIC separations, and one representative analysis is shown in Figure 6. After completion of those three steps of column separation, DMMG was purified near to homogeneity. The entire column purification processes were completed within one week, with the aid of using three AKTA units continually operated in the cold room.

Protein sample was buffer exchanged and lyophilized from final 1 L solution containing 10 mM Tris (1.21 g), 0.1 M NaCl (5.84 g), 1 mM DTT (0.15 g), and 1% Trahalose (10 g). Freeze-drying produced a final 70.5 g of dried fine powder. Subtracted by the mass from the sample buffer components and additives, approximately 50 g is expected as DMMG, which representing more than 70% dry weight of the final lyophilized powder.

Enzymatic activity of the purified DMMG was measured with S3P and PEP as substrates. The reaction is illustrated in Figure 1 and data shown in Figure 7. The released inorganic phosphate from the reaction was

quantified based on the malachite green dye. The calculated K_m and V_{max} for PEP from reconstituted DMMG are approximately 390 μ M and 13 μ mol/min/mg, respectively. The sensitivity of enzyme to glyphosate was also measured, as shown in Figure 7. DMMG is capable of tolerant to high concentration of glyphosate in the reaction, with a calculated LC_{50} about 53 mM. It should be noted that those numbers are for reference only, since the absolute or more accurate protein concentration was not available at the time of performing this test.

Size exclusion chromatography with an analytical Superdex 75 column XK 10/30, along with dynamic light scattering (DLS) analysis were deployed for characterization of the reconstituted bulk DMMG powder. As shown in Figure 8, a single peak was observed with protein eluted at half column volume (12 ml out of 24 ml column bed size), which corresponding to about 50 kDa size. DLS analysis also shown excellent sample polydispersity index, with majority (>99%) of the protein as monomeric form. Taking together, those data indicated that the protein was a monomer. The lyophilization process did not change any major aggregation and DMMG protein structure and conformation.

Further characterization of the protein sample using a specific mouse monoclonal antibody to DMMG is recorded in Figure 9. This antibody 10B5.B4 detected the full-length DMMG protein, no major or significant protein degradation of the sample was observed. However, under heavy amount of protein loading, dimeric or aggregated oligomers of DMMG were detectable (Figure 9). Also under overloading condition, a minor contaminant protein at about 25 kDa became visible. Since this protein is not immunoreactive to the anti-DMMG antibody, it is assumed as a host protein from *Pf* strain. Overall, purity of the DMMG sample by gel image analysis is determined to be above 95%. Sample was released to Reg. Lab and subjected for certificate of analysis, the report (Schafer and Embrey, 2010) is in agreement with the protein purity analysis.

The intact molecular weight of DMMG was measured by MALDI-TOF MS. The average molecular weight of DMMG (444 AA) is calculated as a theoretical 47286 Da, using PAWS program. The measured protein molecular weight is 47291 Da (see Figure 10). The detected MW matches to the predicted MW, suggesting that the protein is a full-length polypeptide. Also, peptide fingerprinting (PMF) analysis was performed on in-gel trypsin digested sample. 12 peptides were detected by MALDI-TOF MS, and matched the predicted DMMG sequence with overall 36% coverage for the entire 444 AA full-length polypeptide. Among those detected peptides, both N-terminal (1267.66 Da: A[2-13]K) and C-terminal (1678.67 Da: T[431-444]K) peptides were identified. In addition, the peptides covered the two mutation amino acid residues (1647.78 Da: E[92-106]R) and (2104.99 Da: S[107-128]R) were also identified (see Figure 10), which are completely different from the predicted wt maize EPSPS peptide mass (1634.8 Da and 2113.1 Da) for this location. (Note: those AA residue locations are based on translation sequence assignment, from M[1-445]N). Those data suggested that the protein was indeed the correct TIPS mutant of target protein.

The first 10 N-terminal amino acid sequences for the sample are AGAEEIVLQP, indicating that the first methionine residue for translation start was removed after protein synthesis. The actual AA sequencing chromatography is included in this report as Figure 11 for the purpose of any further document analysis. The data suggested that the final product of DMMG was a 444 AA polypeptide, based on N-terminal AA sequence and intact molecular weight analysis. However, this is not to exclude any small amount of 445 AA in the bulk sample. As we know the protein was synthesized starting from the initiation Met residue, as

a 445 AA precursor. Typically, this starting methionine residue will be removed by the methionine aminopeptidase and deformylase (Arie Ben-Bassat, 1991). However, if this post-protein synthesis process is not 100% complete inside of the cells, or other modification occurred on the initiating Met such as oxidation or formylation, it is possible in theory that this Methionine may be still attached to some extend on the final product. With very sensitive MS coupled with HPLC or LC/MS method deployed, even in very small amount (less than <0.01%) of not fully processed minor component in the total sample might be detectable. By the above analysis, we concluded that the majority protein of DMMG from this batch bulked material should be 444 AA long.

Protein digestibility in two *in vitro* simulated conditions, namely SGF and SIF, were performed on the reconstituted bulk material. SDS-PAGE and Western blot analysis on the testing DMMG sample were shown in Figure 12, whereas control samples (both positive and negative) in the reaction conducted at the same time were not shown in this report. For a 20 min reaction, DMMG was completely digested by SGF within 1 min, and no detectable protein fragment was seen on SDS-PAGE and by Western blot (upper panel in Figure 12). For SIF digestion, up to 60 min was tested (Figure 12, bottom panel). DMMG was completely disappeared within 10 min from the SDS-PAGE analysis. However, under an overloaded condition, DMMG and its degraded fragment were still detectable at 30 min time point by Western blot using chemiluminescent, a much more sensitive detection method.

Our result for DMMG on the SGF digestion is in agreement with literature data (Herouet-Guicheney *et al.*, 2009), but not on SIF digestion, in which they had shown that DMMG or 2mEPSPS was also completely degraded in SIF within 0.5 min. One possible explanation is that they had used much lower amount of sample for their analysis on the Western blot. On that particular report, they shown SGF digestion by SDS-PAGE analysis, but for SIF digestion, only Western blot was used, and the control sample at 0 time point was just at above the detection limit. Our Western blot signals for the untreated control DMMG were at least 100 x or 1,000 x stronger comparing to their untreated control. The digestibility of DMMG by SIF we observed here is therefore more or less like Monsanto CP4 EPSPS (Harrison *et al.* 1996). In their study, they measured the half-life of CP4 EPSPS in SIF was in 10 min or less range. In another study on CP4 EPSPS, a Japanese group reported that CP4 EPSPS in SIF had a half-life less than 30 min (Okunuki *et al.*, 2001). Furthermore, this group shown a pre-heating of CP4 EPSPS sample at 100 °C for 5 min prior to subject to SIF resulted in a dramatic decreased half-life, down within 5 sec. We could test those conditions to further evaluate the DMMG digestibility and heat stability.

CONCLUSION

DMMG/2mEPSPS was successfully engineered by site-directed mutagenesis from the wt maize EPSPS gene construct.

A production strain with high expression level of DMMG in *Pf* platform was constructed, validated, and delivered.

Large scale of protein production and purification campaign was initiated and completed within a short one month of period.

Close to 47 gram of pure protein (> 95% purity) in lyophilized form was delivered on time.

The total amino acid analysis for protein quantitation, and endotoxin test were not included at the time of sample releasing.

The final tox lot material was confirmed to be enzymatic active.

A summary report was issued and documented at the time of sample delivery.

ACKNOWLEDGMENTS

We would like to thank Terry Wright, Mark Krieger, Greg Bradfish, and Tom Meade for their support, encouragement, and helpful discussions. We also want to thank Rob Cicchillo and Samantha Griffin for performing enzyme assay.

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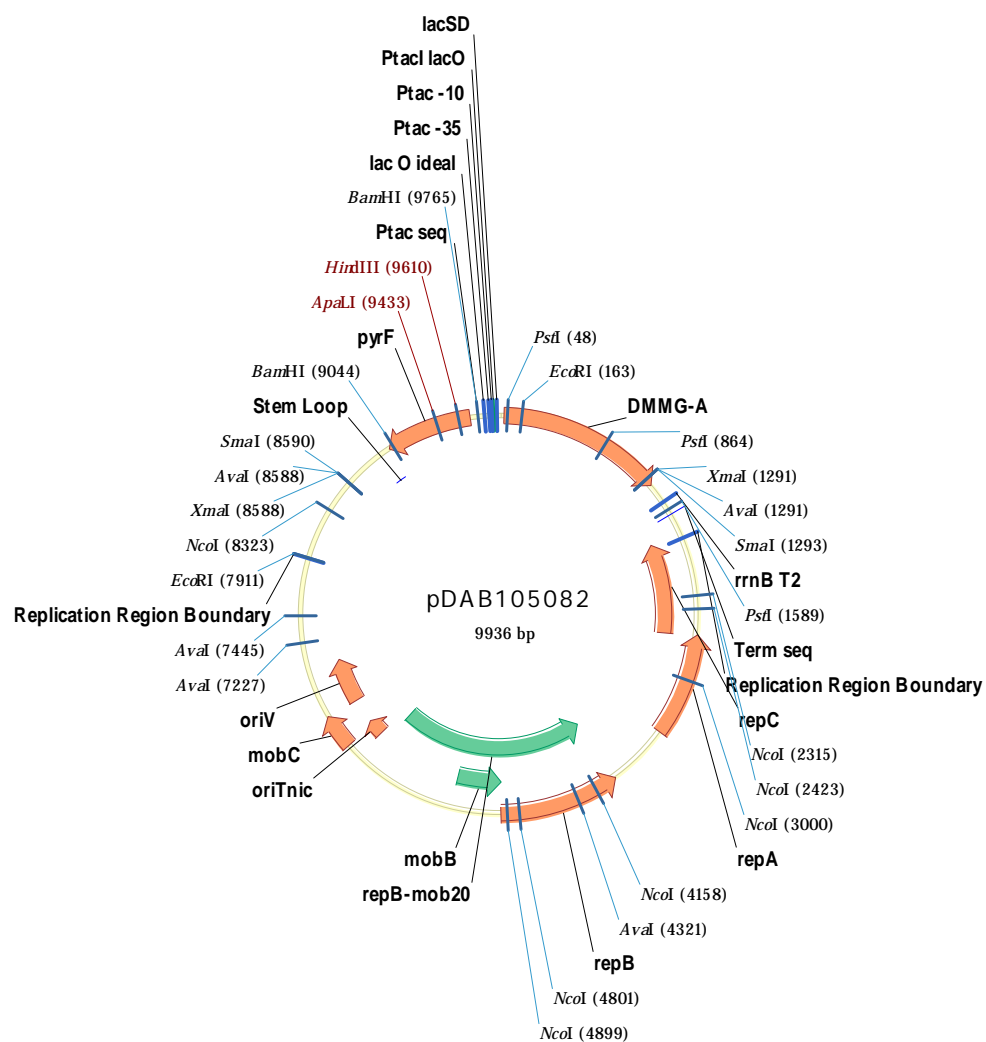
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TABLE I. Oligonucleotides used in this study

i.d. #	Nucleotide Sequence	Purpose
Seq Primer 1	GTGGATTGTTTCCTGGG	Sequencing
Seq Primer 2	ACCAGCGTGACCGTGAC	Sequencing
Seq Primer 3	ACCACGGCCAGGGTCATGGC	Sequencing
Seq Primer 4	TCCGTGCCCAGGAAACAATC	Sequencing
QC Primer 1	GCGGGTATTGCCATGCGTTCTTTGACG	Mutagenesis
QC Primer 2	CGTCAAAGAACGCATGGCAATACCCGC	Mutagenesis
QC Primer 3	GGCAATGCGGGTATTGCCATGCGTTCGTTGACGGCAGCG	Mutagenesis
QC Primer 4	CGCTGCCGTCAACGAACGCATGGCAATACCCGCATTGCC	Mutagenesis

Plasmid Map of pDAB105082



Translation of DMMG protein

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1  MAGAEEIVLQ PIKEISGTVK LPGSKSLSNR ILLLAALSEG TTVDNLLNS
51  EDVHYMLGAL RTLGLSVEAD KAAKRAVVVG CGGKFPVEDA KEEVQLFLGN
101 AGIAMRSLTA AVTAAGGNAT YVLDGVPRMR ERPIGDLVVG LKQLGADVDC
151 FLGTDCPPVR VNGIGGLPGG KVKLSGSISS QYLSALLMAA PLALGDVEIE
201 IIDKLISIPY VEMTLRLMER FGVKAEHSDS WDRFYIKGGQ KYKSPKNAYV
251 EGDASSASYF LAGAAITGGT VTVEGCGTTS LQGDVKFAEV LEMMGAKVTW
301 TETSVTVTGP PREPFGRKHL KAIDVNMNMK PDVAMTLAVV ALFADGPTAI
351 RDVASWRVKE TERMVAIRTE LTKLGASVEE GPDYCIITPP EKLNVTAIDT
401 YDDHRMAMAF SLAACAEVPV TIRDPGCTRK TFPDYFDVLS TFVKN

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Figure 2. Plasmid map for *Pf* production construct and deduced protein sequence for DMMG. DMMG gene fragment was cloned in-frame into pDOW1169 vector for *Pf* expression. Mutated residues from threonine to isoleucine and from proline to serine from the wild-type maize EPSPS are colored in Red.

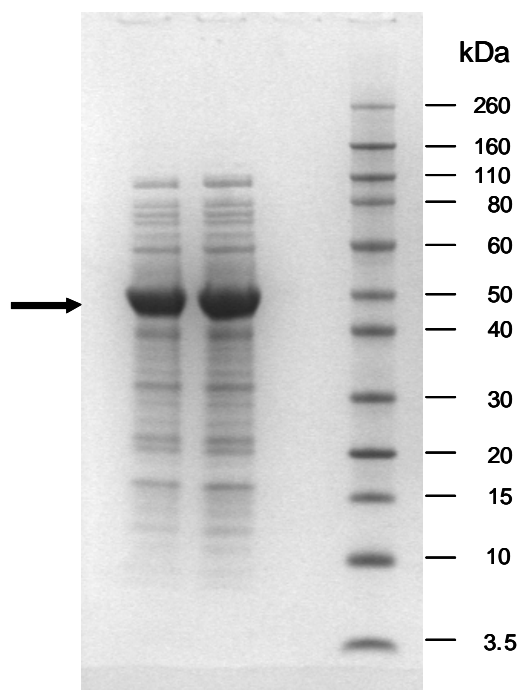
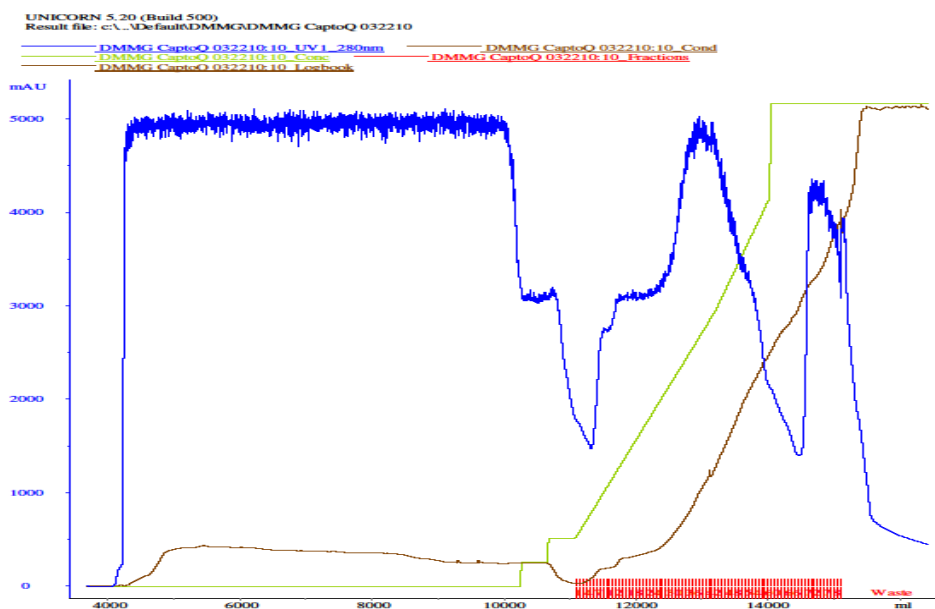


Figure 3. SDS-PAGE gel analysis of expressed DMMG from extracted soluble fraction. Samples were saved from two separate large scale protein extraction and analyzed on a 10% NuPAGE (Invitrogen) gel. Arrow indicates approximately 47 kDa DMMG protein band. Protein molecular weight (Sharp protein standard from Invitrogen) is labeled.

A. Chromatography



B. SDS-PAGE

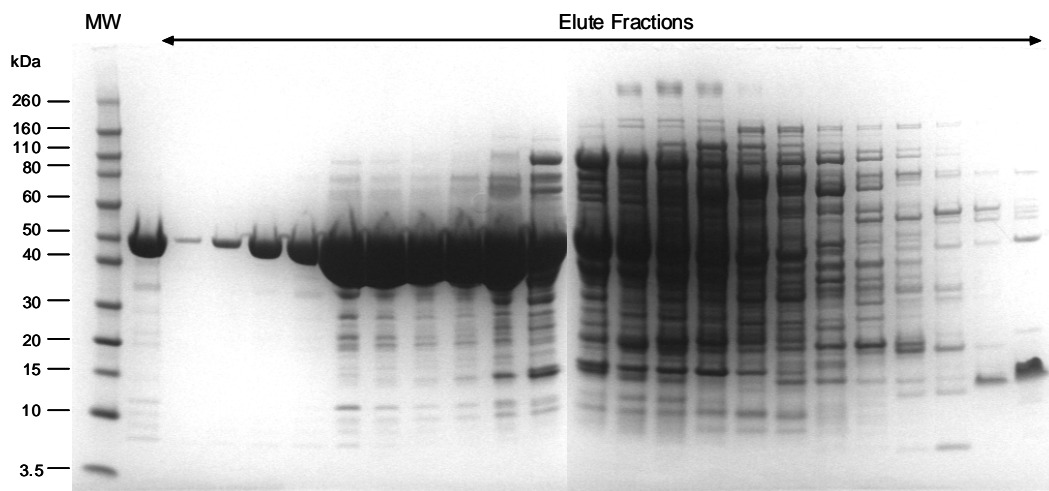


Figure 4. Ion-Exchange Chromatography on Capto Q column separation. A) Chromatography profile using CaptoQ Index 70/500 column. Proteins were eluted using 0-1 M NaCl gradient. B) SDS-PAGE analysis of major eluted protein peak on a 10% NuPAGE (Invitrogen) gel. DMMG was eluted in the early peak corresponding to 0.1-0.3 M NaCl salt gradient. Molecular weight standards (Sharp protein standard from Invitrogen) are labeled.

A. Chromatography



B. SDS-PAGE

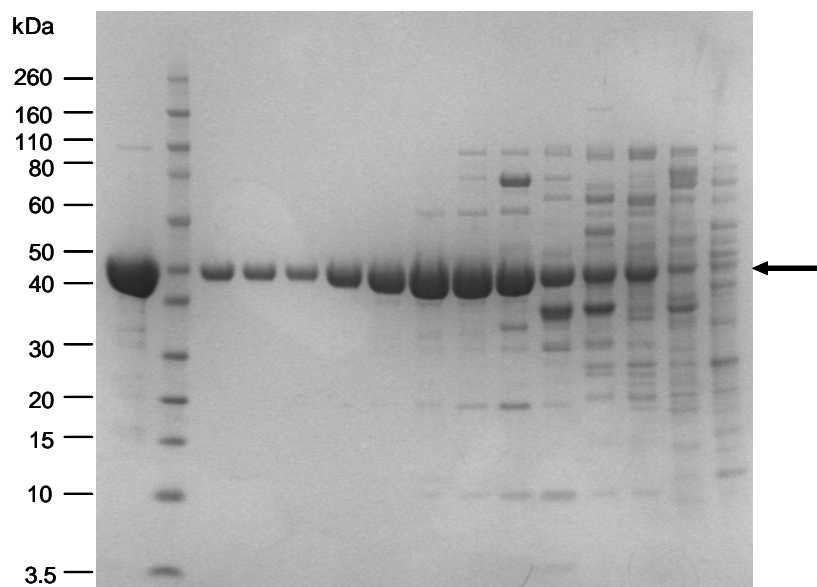
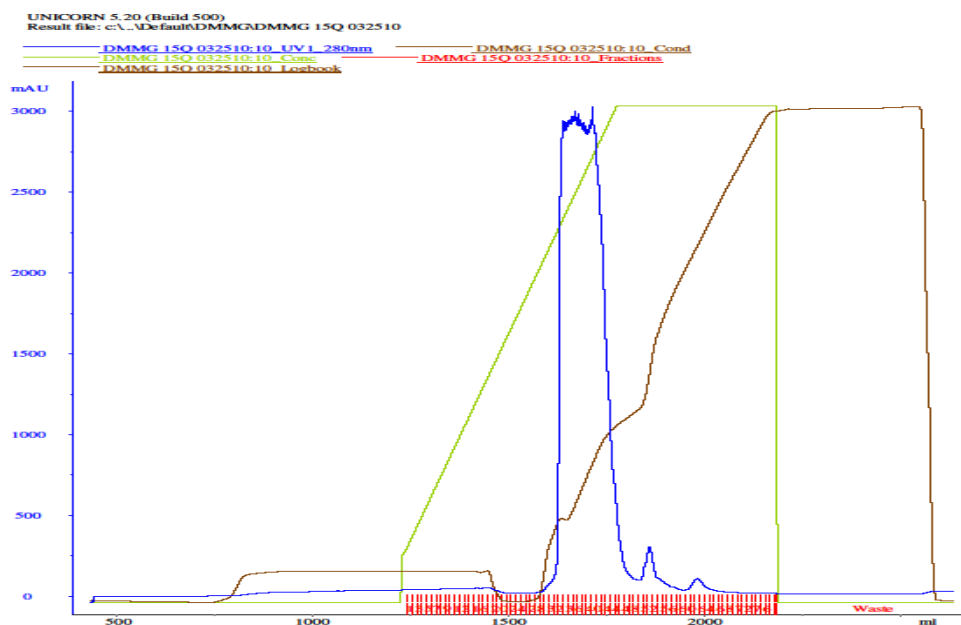


Figure 5. Hydrophobic Interaction Chromatography on Phenyl HP column separation. A) Chromatography profile using Phenyl HP XK50/60 column. Proteins were eluted from 1.2-0 M Ammonia sulfate gradient. B) SDS-PAGE analysis of major eluted protein peak on a 10% NuPAGE (Invitrogen) gel. Arrow indicates DMMG protein. Molecular weight standards (Sharp protein standard from Invitrogen) are labeled.

A. Chromatography



B.

SDS-PAGE

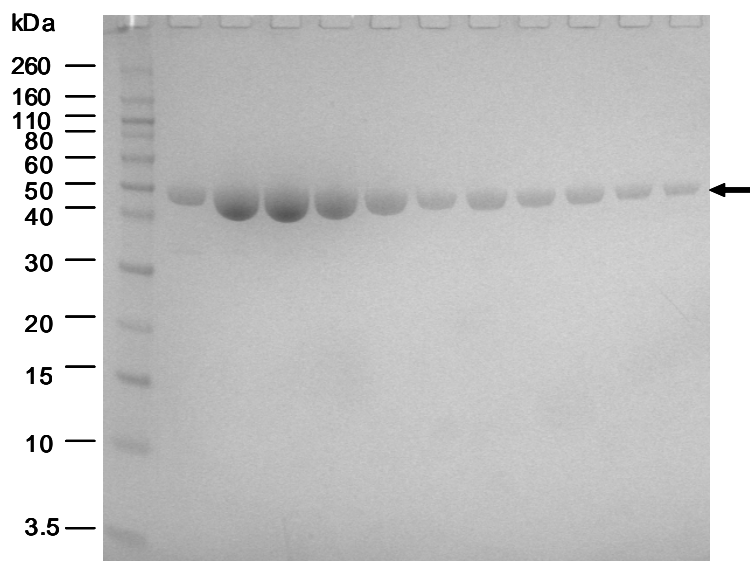


Figure 6. Source 15Q column separation. A) Chromatography profile using Source 15Q XK 50/30 column. Proteins were eluted using 0-1 M NaCl gradient. B) SDS-PAGE analysis of eluted protein peak on a 4-12% NuPAGE (Invitrogen) gradient gel. Molecular weight standards (Sharp protein standard from Invitrogen) are labeled. Arrow indicates the purified DMMG protein.

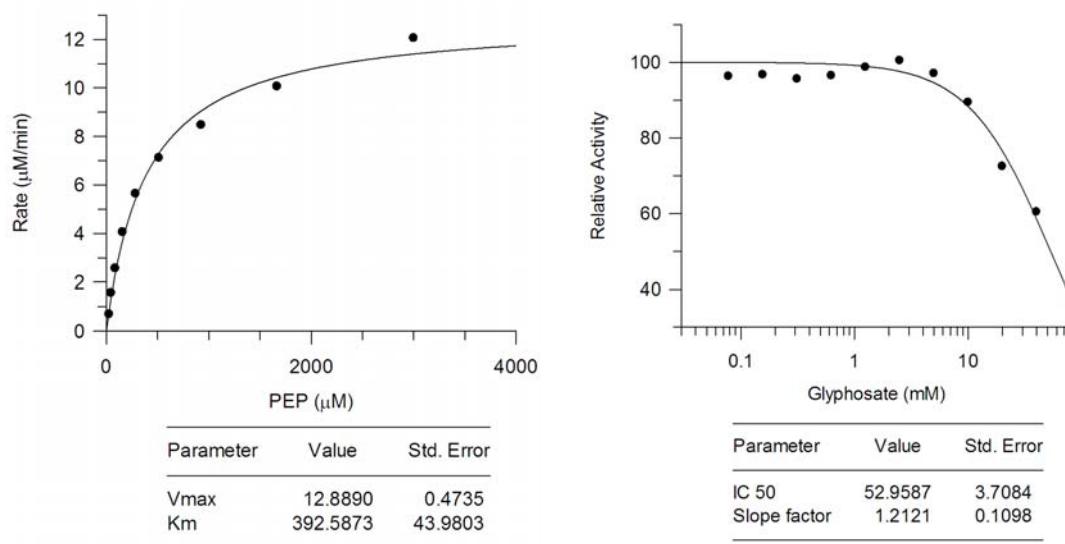
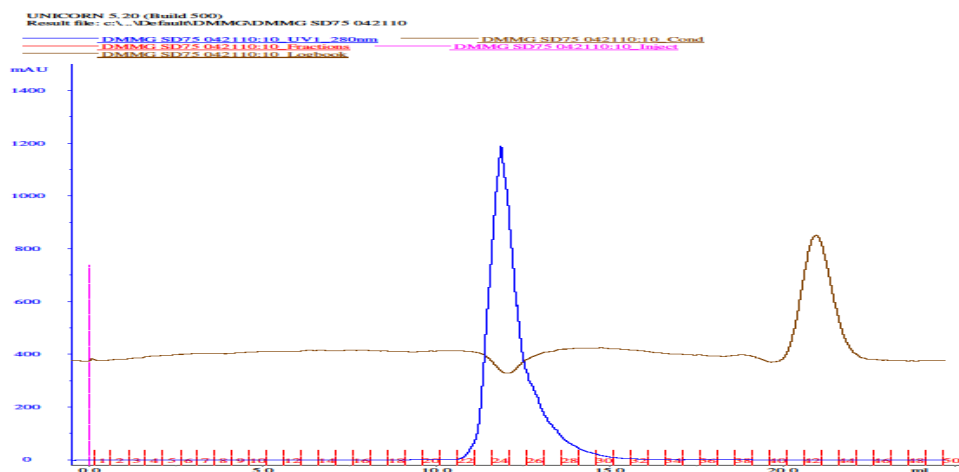


Figure 7. Enzyme assay. Left panel: activity of DMMG for substrate PEP using 1 mM S3P. Data were fit to the Michaelis-Menten equation for determination the K_m and V_{\max} values for PEP. Right panel: DMMG activity is not inhibited by the presence of high concentration of Glyphosate. Data were fit to the Michaelis-Menten equation for determination the LC_{50} of glyphosate.

A. Size exclusion chromatography (SEC) analysis



B.

Dynamic light scattering (DLS) analysis

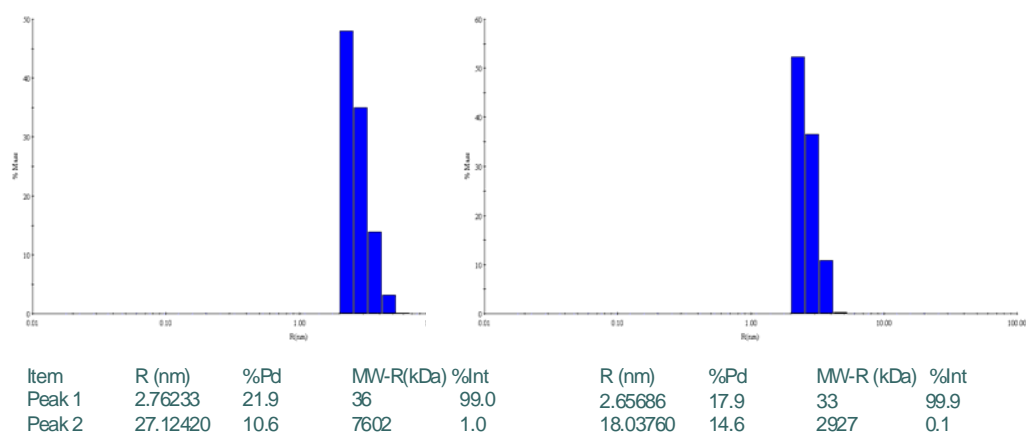


Figure 8. DMGM protein characterization by analytical gel filtration (A, top panel) and dynamic light scattering (DLS) (B, bottom panel) analysis. Approximately 0.3 mg of DMGM was injected onto a Superdex 75 10/300 GL analytical column for separation, intended for LC/MS analysis for the C-terminal AA determination. For determination of sample polydispersity index, the bulk sample prior to (left panel in B), and after (right panel in B) lyophilization was measured by DLS.

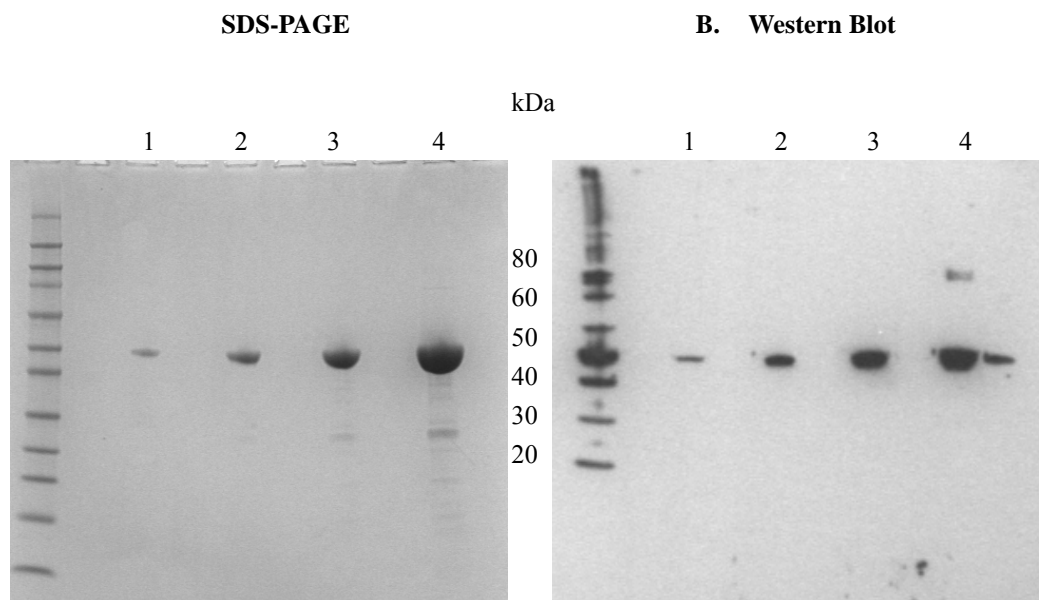
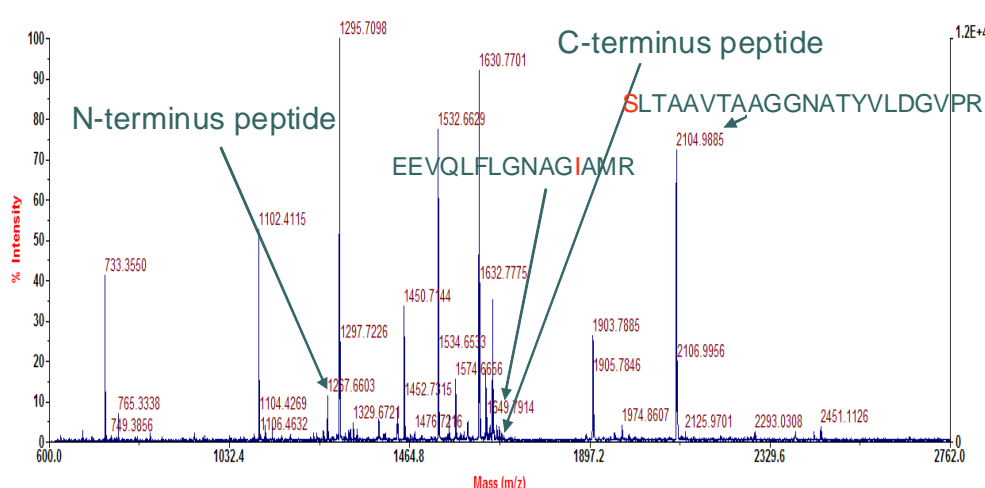


Figure 9. SDS-PAGE (A) and Western blot (B) analysis of final purified DMMG tox-lot sample. For SDS-PAGE, 0.5 μ g (lane 1), 1.5 μ g (lane 2), 5 μ g (lane 3) and 15 μ g (lane 4) of DMMG were analyzed on a 10% NuPAGE gel and stained by Coomassie Blue R-250. For Western blot, 1.5 ng (lane 1), 5 ng (lane 2), 15 ng (lane 3), and 50 ng (lane 4) of DMMG, were separated on a 10% NuPAGE gel and blotted for immuno-detection, as described in the Materials and Methods.

MALDI-TOF MS



PMF analysis detects 12 matched peptides, including N- and C-terminus and T102I and P106S mutation. Measured MW is 47291 Da, also matches theoretical MW of 47286 Da.

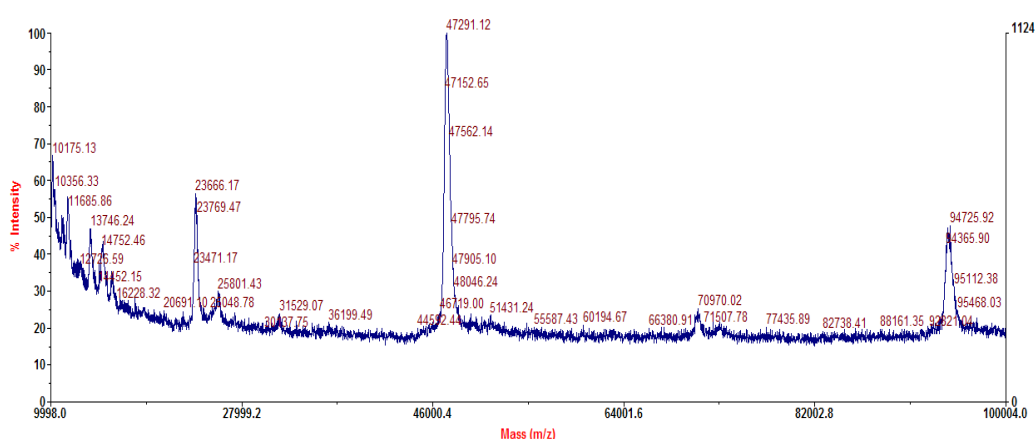


Figure 10. MALDI-TOF mass spectrometer analysis of DMMG. Top panel: Atryptic digestion for peptide mass fingerprinting. N-terminal peptide A₂-K₁₃ (mass 1267.66), C-terminal peptide T₄₃₁-K₄₄₄ (mass 1678.66), mutated residues in peptides E₉₂-R₁₀₆ (mass 1647.78) and S₁₀₇-R₁₂₈ (mass 2103.98) were detected and shown by the arrows. The assigned AA residue numbers are based on deduced DMMG sequence (see Fig.2). Bottom panel: Intact molecular weight (MW) determination. The MW was detected by MALDI-TOF MS using linear model with external BSA standard. The average MW of DMMG predicted by PAWS is 47286 Da, the measured one is 47291 Da.

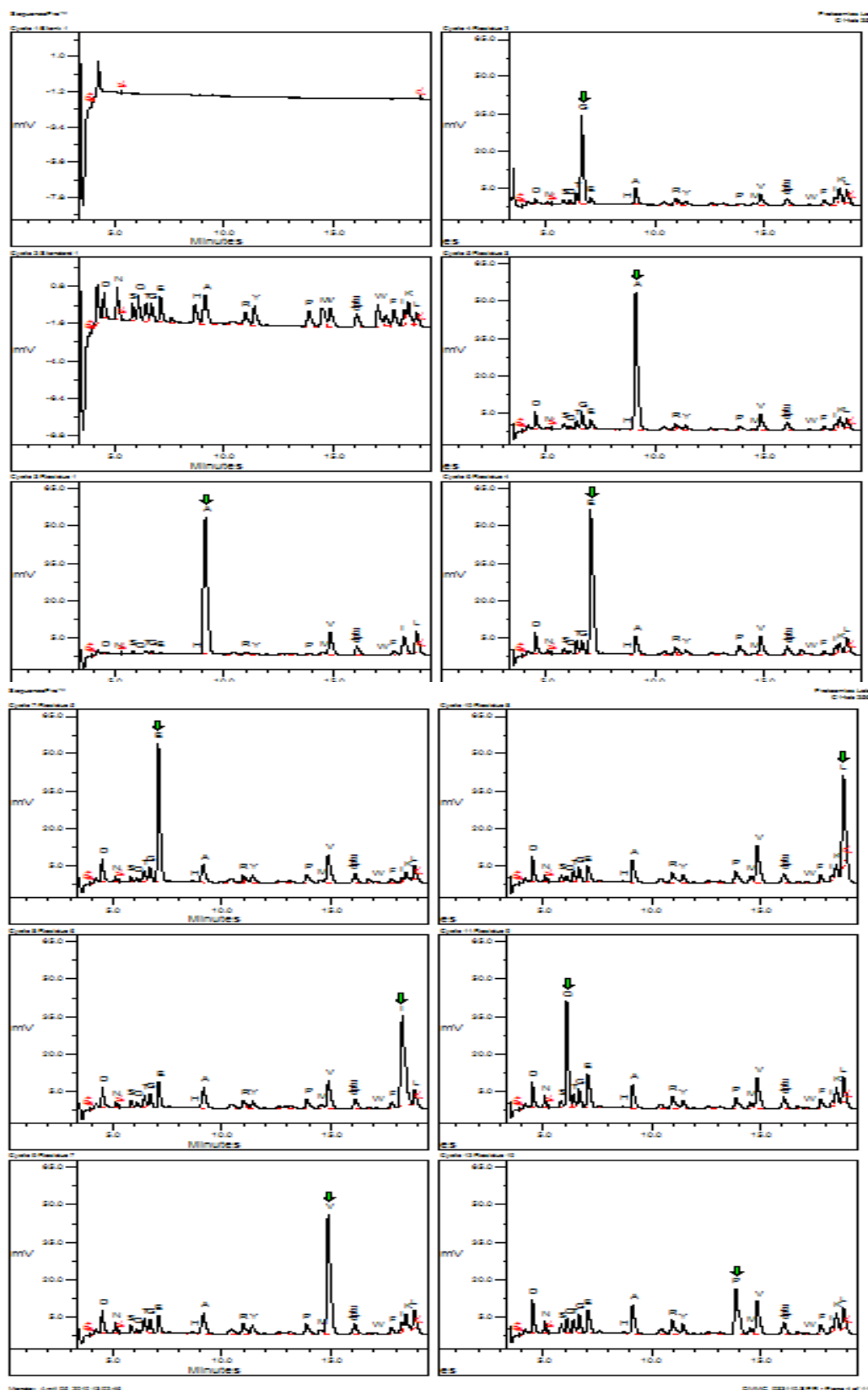
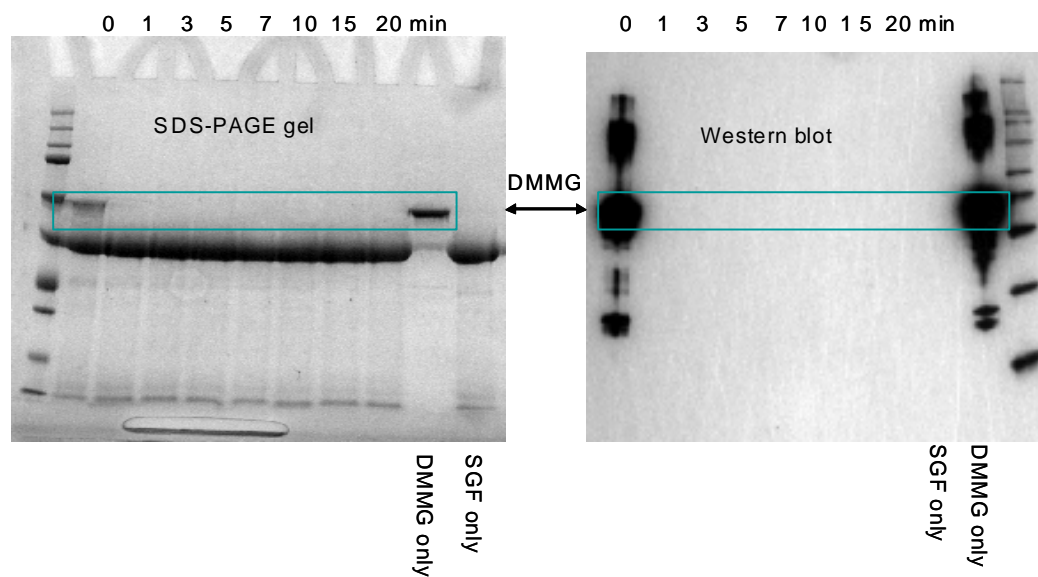


Figure 11. DMMG Amino Acid residue chromatography by Edman degradation reaction. The first 10 AA sequence is AGAEEIVLQP, which matches DMMG sequence from AA residue #2-#11.

Digestion in SGF



Digestion in SIF

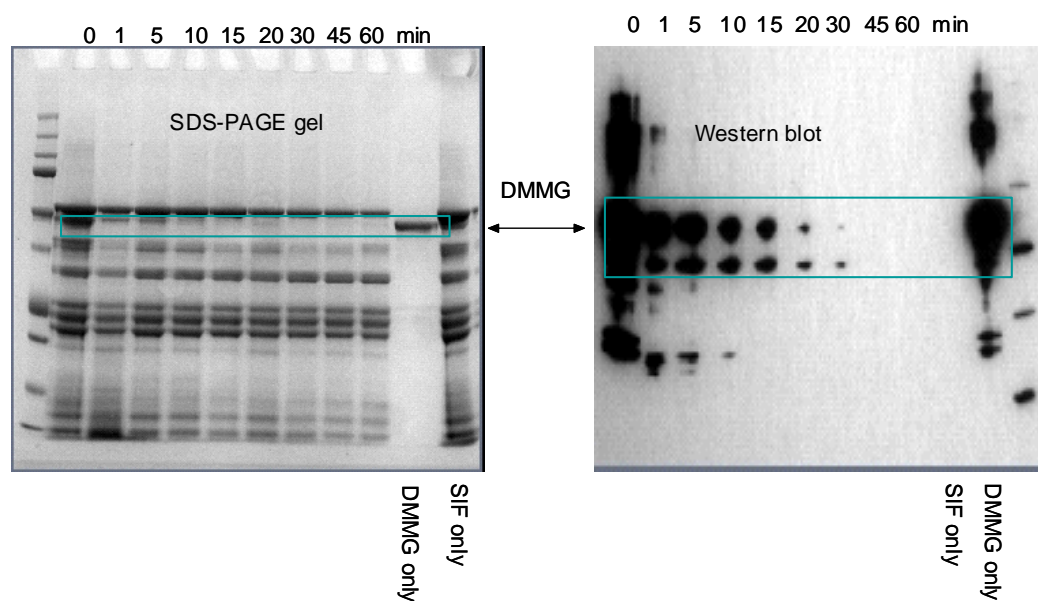


Figure 12. DMMG digestibility in SGF (top panel) and SIF (bottom panel). As described, digestion in SGF was carried on from 0 to 20 min, while digestion in SIF was conducted for 60 min period. Up to 1 μ g of DMMG from the reaction was applied on gel for separation, one set of gel was stained with Coomassie blue R-250, and another set of duplicate gel was blotted for immuno-detection. DMMG protein and its degraded products are shown in boxed area.

APPENDIX I. EPSPS Enzymatic Assays for Measuring Phosphate Releasing

All reactions are to be carried out at room temperature (25 °C) in 96-well ELISA plates. Prepare solutions and each substrate ahead of time, and store properly (4 °C for most of stock solutions and -20 °C for the chemical compounds).

PEP and S3P are made in water as 10 mM stock, and Glyphosate stock solution (500 mM) needs to be titrated with base (NaOH) to final pH 7.0 in order to maintain solubility in the reaction solution.

20 x Reaction buffer: 1 M HEPES, pH 7.5, 2 M KCl, and 40 mM DTT

Malachite Green/Ammonium Molybdate color solutions:

Malachite Green (MG) solution: 0.045% in di-water

Ammonium Molybdate (AM) solution: 4.2% in 4 N HCl

Mix three parts of MG with one part of AM for the working solution

A typical enzymatic reaction volume is set for 50 ul.

K_m Measurement:

1. Make PEP serial dilution in plate (two-fold dilution from working concentration)
2. Add water in the wells (to make up final volume of 50 ul)
3. Add 20 x buffer next (2.5 ul)
4. Add S3P (final 1 mM) (5 ul)
5. Add PEP (5 ul) to obtain the gradient concentration
6. Initiate reaction with enzyme (1-5 nM*) (final total added volume with enzyme is 50 ul)
7. Allow reaction to go for 3-5 min (vary time as needed, up to 15-20 min)
8. Quench reaction with 235 uL Malachite Green/Ammonium Molybdate solution
9. Allow color to develop for 1 min
10. Take endpoint reading at A₆₆₀ in the plate reader
11. Determine *K_m* based on those values

IC₅₀:

1. Make Glyphosate serial dilution (two-fold dilution from 80 mM, for example)
2. Add water as above described.
3. Add 20 x buffer next (2.5 ul)
4. Add PEP to follow (1 mM final)
5. Add Glyphosate gradient and mix well
6. Add S3P (1 mM final) after adding PEP and Glyphosate mixture
7. Initiate reaction with enzyme (5-25 nM**) (final total volume is 50 ul)
8. Allow reaction to go for 3-5 min (vary time as needed, up to 15-20 min)
9. Quench reaction with 235 uL Malachite Green/Ammonium Molybdate solution
10. Allow color to develop for 1 min
11. Take endpoint reading at A₆₆₀ in the plate reader
12. Determine IC₅₀ based on those values

Notes:

1. Watch for precipitations in wells. High amount phosphate produced and released will cause ppt in the reaction, if this happens, reduce amount of enzyme used in the reaction. * We have noticed the other DGT (all class EPSPS) was used in 50 nM in the reaction, but this DMMG is so active, in standard reaction it converts too much PO₄ and with the MG/AM color solution in HCl will form precipitate.
2. Make sure that PO₄ production is on your standard curve (meaning only 0-80 uM is in the right range). Increase or decrease enzyme concentration and/or adjusting reaction times (2-20 min range) could be used for this purpose.
3. When determine the enzyme LC₅₀ for Glyphosate, it maybe better to use slightly higher amount of enzyme** in the reaction, as we have noticed that LC₅₀ number increased with higher enzyme concentration, meaning higher amount of protein tolerant more Glyphosate in the assay. All other DGT EPSPS LC₅₀ for Glyphosate determination was used as 50 nM in the reaction.
4. Don't forget to run control line for background purposes. This line (all 12 wells) should have everything except enzyme. It was also noticed that Glyphosate has very high background in the assay.

References:

1. Forlani et al. (1994) 5-enol-Pyruvyl-Shikimate-3-Phosphate Synthase from Zea mays cultured cells. Plant Physiol. 104:1107-1114.
2. Lanzetta et al. (1979) An improved assay for nanomole amounts of inorganic phosphate. Anal. Biochem. 100:95-97.