A Simple Continuous Assay for EPSP Synthase in Plant Tissue

R. Douglas Sammons, Julie Meyer, Erin Hall, Elizabeth Ostrander and Stephen Schrader

Monsanto Company, 800 North Lindbergh Blvd., St. Louis, Missouri 63167. rdsamm@monsanto.com

Abstract

The discovery of glyphosate-resistant weeds naturally leads to investigations of the mechanism of resistance. The most common mechanism is target site modification where a variant of the herbicide-targeted enzyme has been selected in the surviving weed.(1) Hence, determining the sensitivity of the enzyme to the herbicide can result in identifying the resistance mechanism. We present a method to assay EPSPS in a continuous phosphate release assay which allows an estimation of the of the inhibition constant for glyphosate by determining the I50. The assay is an adaptation of the commercial phosphate assay kit sold by Molecular Probes. The enzyme, purine nucleotide phosphorylase (PNPase), scavenges phosphate to phosphorylyze the nucleoside bond of 2-amino, 6-mercapto, 7-methyl-purine riboside (MESG) to create an increase in absorbance at 360nm with the release of the modified purine. Maintaining an excess of the coupling enzyme PNPase, allows the rate of phosphate produced in the EPSPS reaction to be determined.

The procedure for the extraction, concentration and stabilization of an EPSPS protein for enzyme assay is reported. We demonstrate that the EPSPS from glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*), is actually more sensitive to glyphosate then the very well studied *E. coli* EPSPS. Therefore, this glyphosate-resistant Palmer amaranth biotype does not appear to be utilizing a modified EPSPS for the resistance mechanism.

1.) Sammons RD, Heering DC, DiNicola N, Glick H and Elmore GA, Sustainability and Stewardship of Glyphosate and Glyphosate-Resistant Crops. *Weed Technology* **21**:347-354 (2007).

2.) Alibhai MF, Cajacob C, Feng PCC, Heck GR, Qi Y, Flasinski S and Stallings WC, Glyphosate Resistant Class I 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS). US2004/004636 (2004); WO 2004/07443.

3.) S. R. Baerson, D. J. Rodriguez, M. Tran, Y. M. Feng, N. A. Biest and G. M. Dill, *Plant Physiology*, **129**, 1265-1275 (2002).

4.) Padgette SR, Re DB, Gasser CS, Eichholtz DA, Frazier RB, Hironaka CM, Levine EB, Shah DM, Fraley RT, Kishore GM (1991) Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site. J Biol Chem 266: 22364-22369.

5.) Gruys KJ, Walker MC and Sikorski JA, Substrate Synergism and the Steady-State Kinetic Reaction Mechanism for EPSP Synthase from E.coli. *Biochemistry* **31**:5534-5544 (1992).

Background

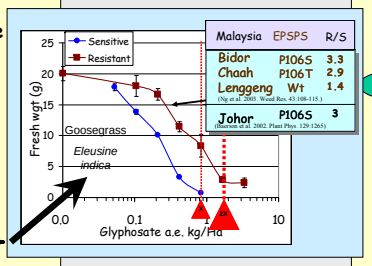
In Planta Point mutations in EPSPS

Consensus Plant Site:	G	T$_{102}$	A	M	R	P$_{106}$	L
Resistant *Eleusine indica:*	G	T	A	M	R	S	L
Baerson et al., 2002 Plant Phys. 129:1265	G	T	A	M	R	T	L
Ng et al., 2004 Aust. J. Ag. Res. 55:407	G	T	A	M	R	A	L
Yuan et al., 2005 Plant Prot. Bul. 47:251							
Resistant *Lolium rigidum:*	G	T	A	M	R	A	L
Yu et al., 2007 Planta 225:499.							
Wakelin & Preston, 2006 Weed Res. 46:432.	G	T	A	M	R	T	L
Resistant *Lolium multiflorum:*	G	T	A	M	R	S	L
Perez-Jones et al.,2007 Planta							
Resistant *Oryza sativa:*	G	T	A	M	R	L	L
Zhou et al. 2006 Plant Phys. 140:185	**Directed evolution**						

Overall 2-3X Resistance Recorded P106X

EPSPS Variants, Sensitivity to Glyphosate

EPSPS	K_m-PEP (µM)	K_i (µM)
Wt *Z. mays* (2)	27 ± 4	0.5 ± 0.1
Zm-**R**, P$_{106}$S (2)	17 ± 3	1 ± 0.1
Zm-**R**, P$_{106}$T (2)	25 ± 4	4 ± 0.6
E. coli (5)	1	0.16
Wt, P. hybrida (4)	10	0.12
Ph-**R**, P106S (4)	44	1
E. indica-S (3)	4	0.048
Ei-**R**, P106S (3)	7	0.76



Methods

Preparing Crude EPSPS from Leaf Tissue

1.) Weigh out frozen leaf tissue (young not fully expanded), chill mortar and pestles with dry ice, do not let tissue thaw, grind frozen leaf tissue to a fine powder.

2.) Transfer frozen ground tissue into a beaker on ice containing pre-chilled extraction buffer (1g:5mL) with 1% in polyvinylpyrrolidone(PVP) and fresh 5mM β-mercaptoethanol.

3.) Carry out all steps at 0-4°C, Homogenize for ~5 min. with stirring on ice, minimize foaming.

4.) Centrifuge 40 min. at 15,000 RPM @ 4°C, Decant clear supernatant thru cheesecloth into a beaker.

5.) SLOWLY add solid $(NH_4)_2SO_4$ with stirring to supernatant to make 45%, stir an additional 10 minutes. (Table, p. 291 of Deutscher's *Methods in Enzymology*, Vol. 182).

6.) Centrifuge 30 min. at 20,000 RPM @ 4°C, Decant supernatant and set pellet aside.

7.) SLOWLY add solid $(NH_4)_2SO_4$ with stirring to supernatant to 70%, stir an additional 10 minutes.

8.) Centrifuge 30 min. at 20,000 RPM @ 4°C, Decant supernatant and set aside; **collect pellet**.

9.) Dissolve pellet in extraction buffer with fresh 5 mM β-mercaptoethanol (~ 5-10mL)

10.) Dialyze overnight in 4L dialysis buffer, 30mm, 10kD MWCO Spectrum tubing @ 4°C with stirring.

11.) Concentrate protein using Y10K MW Centriprep centrifugal filter devices (Amicon).

A. palmeri Ponder-S	Vol. mL	A_{340}nm (1:50)	Total O.D.	Units µmole/min	Total Units µmoles/min	Units/ A_{340}nm	Fold Purification
Crude extract	250	0.553	6910	0.0655	16.4	0.0024	1
40% super.	250	0.279	3484	0.131	32.7	0.0094	4
70% super.	255	0.2695	3436	0	0	0	-
Crude isolate	3	1.0308	155	3.27	9.8	0.0635	26.8

A. palmeri Macon-R	Vol. mL	A_{340}nm (1:50)	Total O.D.	Units µmole/min	Total Units µmoles/min	Units/ A_{340}nm	Fold Purification
Crude extract	275	1.038	14273	0.0873	24	0.0017	1
40% super.	280	0.279	3902	0.0153	4.3	0.0011	0.7
70% super.	285	0.378	5381	0.0065	1.9	0.0003	0.2
Crude isolate	3	0.436	65.4	1.527	4.6	0.0701	41.7

Continuous Rate of Phosphate Appearance

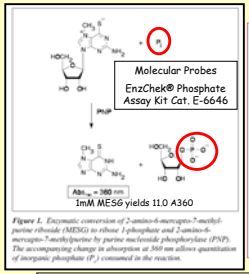


Figure 1. Enzymatic conversion of 2-amino-6-mercapto-7-methyl-purine riboside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP). The accompanying change in absorption at 360 nm allows quantitation of inorganic phosphate (P$_i$) consumed in the reaction.

1.) Coupled phosphate detection must be faster than EPSPS
- •PNPase, in excess
- •MESG, saturated

2.) Ab 360nm less than 3.0 O.D.units

3.) EPSPS initial rate that is linear &
- • Proportional to EPSPS
- • Independent of PEP & S3P

4.) No contaminants (especially Pi !!!)

5.) EPSPS substrates must have very low Pi levels

Webb MR, *A Continuous assay for inorganic phosphate and for measuring phosphate Release Kinetics in a Biological Systems. Proceedings of the National Academy of Sciences of the United States of America* **89**:4884-4887 (1992).
Originally phosphate assays were end point reactions according to, Lanzetta PA, Alvarez LJ, Reinach PS and Candia OA, An Improved Assay for Nanomolar amounts of Phosphate. *Analytical Biochemistry* **100**:95-97 (1979).

Phosphate Assay
Add the following in order:
1.) 500µL 2X Assay buffer
2.) 290-x µL Ultrapure phosphate-free water, HPLC-grade
3.) 200µL 1mM MESG
4.) 2µL 100U/mL PNPase
5.) x µL Phosphate standard
Follow kit protocol to make MESG, PNP, and Pi standard MS at 360nm.
Use Ultrapure water to make all reagents.

EPSPS Assay in Order, at 25°C
1.) 500µL 2X Assay buffer
2.) 215-(**y**)-(**x**) µL Ultrapure phosphate-free water, HPLC-grade
3.) 200µL 1mM MESG
4.) 10µL 100U/mL PNP
5.) 25µL 50mM PEP
6.) (**y**) µL glyphosate
7.) (**x**) µL dialyzed EPSPS
8.) 50µL 10mM S3P

Buffers

EXTRACTION BUFFER ,1L, pH 7.0, @ 4°C

100mM	MOPS	20.93g
5mM	EDTA	1.86g
10%	Glycerin	100mL
50mM	KCl	3.73g
0.5mM	Benzamidine	78mg
7.3uM	Pepstatin	5mg
25mg	Trypsin Inhibitor	25mg
4.2uM	Leupeptin	25mg

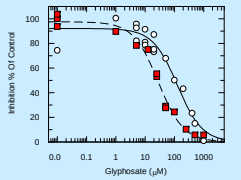
DIALYSIS BUFFER 1L, pH 7.0

10mM	MOPS	2.93g
5%	Glycerin	50mL
0.5mM	EDTA	0.186g
2.5mM	B-mercaptoethanol	

EPSPS 2X Assay Buffer (store @ 4 °C) pH 7.0

500µL	**2X Assay Buffer**
100mM	MOPS
1mM	$MgCl_2$
10%	glycerin
0.2mM	Na_2MoO_4
0.2mM	NaF

Resistant *Amaranthus palmeri* is more sensitive than *E. coli*



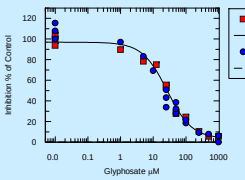
E. coli

Parameter	Value	Std. Error
Y Range	92.0953	3.7407
IC 50	126.4367	25.4923
Slope factor	1.0269	0.1747
Background	0.0000	0.0000

Palmer amaranth-R, Macon

Parameter	Value	Std. Error
Y Range	97.5326	2.6920
IC 50	27.9933	3.2338
Slope factor	1.0134	0.1174
Background	0.0000	0.0000

R and S *Amaranthus palmeri* are the Same



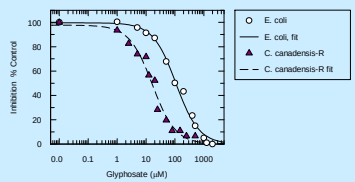
Palmer amaranth-R, Macon

Parameter	Value	Std. Error
Y Range	93.4042	5.2670
IC 50	26.4347	3.5827
Slope factor	1.1467	0.1845
Background	3.4491	4.1203

Palmer amaranth-S, Ponder

Parameter	Value	Std. Error
Y Range	0.0381	0.0010
IC 50	18.2148	2.0209
Slope factor	0.8670	0.0727
Background	0.0000	0.0000

Resistant *Conyza canadensis* is more sensitive than *E. coli*



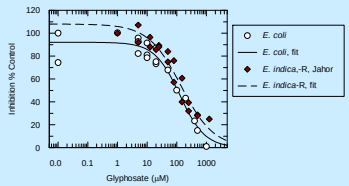
E. coli

Parameter	Value	Std. Error
Y Range	92.0953	3.7407
IC 50	126.4367	25.4923
Slope factor	1.0269	0.1747
Background	0.0000	0.0000

C. canadensis-R

Parameter	Value	Std. Error
Y Range	97.7141	0.8082
IC 50	16.5544	0.4880
Slope factor	1.0881	0.0347
Background	0.0000	0.0000

Resistant *E. indica* is Similar to *E. coli.* (Apparently slightly more tolerant)



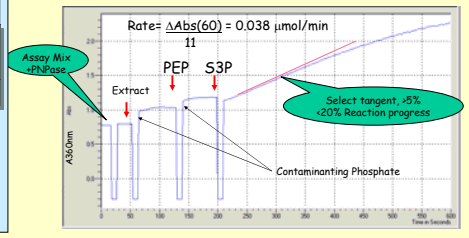
E coli

Parameter	Value	Std. Error
Y Range	92.0953	3.7407
IC 50	126.4367	25.4923
Slope factor	1.0269	0.1747
Background	0.0000	0.0000

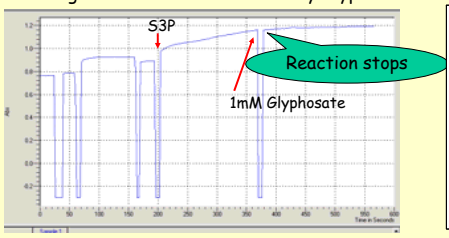
E. indica-R , Jahor

Parameter	Value	Std. Error
Y Range	108.0062	7.2543
IC 50	138.7059	34.0175
Slope factor	0.7904	0.1093
Background	0.0000	0.0000

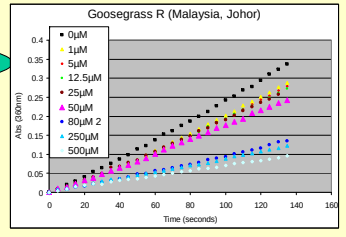
Assaying "Ponder" crude



Checking the Reaction is Inhibited by Glyphosate



Examples of Raw Rate Data



CONCLUSIONS

1. Crude EPSPS's from plant tissue can be stabilized and assayed using the Phosphate Assay kit.
2. The I_{50} method is adequate to determine small differences in Ki for glyphosate with crude EPSPS.
   - No difference in R and S horseweed, No EPSPS difference found in cloning.
   - Resistant goosegrass, *Eleusine indica*, Malaysia, Johor, with P106S-EPSPS is very slightly more tolerant than *E. coli* and more tolerant than other plant EPSPS's.
3. No support for a target-site modified EPSPS based on glyphosate sensitivity in *Amaranthus palmeri* from Georgia, Macon (Culpepper).