

A Simple Continuous Assay for EPSP Synthase in Plant Tissue

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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 phosphorylate 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 than 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, Cajoacob C, Feng PCC, Heck GR, Qi Y, Flasiniski 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.) Padgett 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 A M R P L**

Resistant <i>Eleusine indica</i> :	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 L L
Yuan et al., 2005 Plant Prot. Bul. 47:251	G T A M R A L

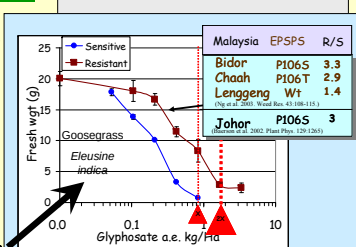
Resistant <i>Lolium rigidum</i> :	G T A M R A L
Yu et al., 2007 Planta 225:499	G T A M R T L
Wakelin & Preston, 2006 Weed Res. 46:432	G T A M R S L

Resistant <i>Lolium multiflorum</i> :	G T A M R S L
Perez-Jones et al., 2007 Planta	G T A M R L L

Resistant <i>Oryza sativa</i> :	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. <i>Z. mays</i> (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
<i>E. coli</i> (5)	1	0.16
Wt. <i>P. hybrida</i> (4)	10	0.12
Ph-R, P106S (4)	44	1
<i>E. indica</i> -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 (NH₄)₂SO₄ 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 (NH₄)₂SO₄ 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 Centrprep centrifugal filter devices (Amicon).

A. palmeri Ponder-S	Vol. mL	Assay (1.50)	Total O.D.	Units μmol/min	Total Units μmol/min	Units/Assay	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	Assay (1.50)	Total O.D.	Units μmol/min	Total Units μmol/min	Units/Assay	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

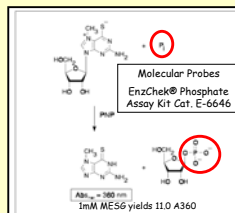


Figure 1. Enzymatic conversion of 2-amino-6-mercapto-7-methyl-purine riboside (MESG) to 2-amino-6-mercapto-7-methyl-purine riboside-5-phosphate (MESG-P) by the enzyme PNPase. The accompanying change in absorbance at 360 nm allows quantitation of inorganic phosphate (Pi) released in the reaction.

Webb WR. 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, Reinisch PS and Cardes OA, An Improved Assay for Nanomolar amounts of Phosphate. *Analytical Biochemistry* 100:95-97 (1979).

Phosphate Assay

- 1.) Add the following in order:
- 2.) 500μL 2X Assay buffer
- 3.) 250-500 μL Ultrapur phosphate-free water, HPLC-grade
- 4.) 200μL 1mM MESG
- 5.) 2μL 100U/mL PNPase
- 6.) 5μL x μL Phosphate standard

Follow kit protocol to make MESG, PNP, and Pi standard MS at 360nm. Use Ultrapur water to make all reagents.

EPSPS Assay in Order, at 25°C

- 1.) 500μL 2X Assay buffer
- 2.) 215-(y)-(x) μL Ultrapur 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

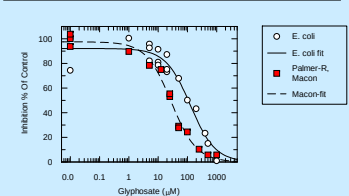
CONCLUSIONS

1. Crude EPSPS's from plant tissue can be stabilized and assayed using the Phosphate Assay kit.
2. The I₅₀ method is adequate to determine small differences in K_i 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).

Buffers

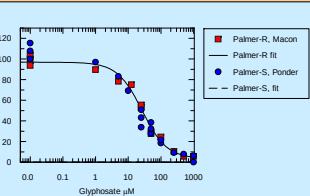
EXTRACTION BUFFER 1L, pH 7.0, @ 4°C			DIALYSIS BUFFER 1L, pH 7.0			EPSPS 2X Assay Buffer (store @ 4°C) pH 7.0		
100mM	MOPS	20.93g	10mM	MOPS	2.93g	500μL	2X Assay Buffer	
5mM	EDTA	1.86g	5mM	Glycerol	3mL	100mM	MOPS	
10% (v/v)	Glycerol	100mL	0.5mM	EDTA	0.186g	1mM	MgCl ₂	
50mM	KCl	3.73g	2.5mM	β-mercaptoethanol		10%	glycerol	
0.5mM	Benzamide	78mg				0.2mM	NaMoO ₄	
7.5mM	Potassium	5mg				0.2mM	NaF	
25mM	Trypsin Inhibitor	25mg						
4.2mM	Leupeptin	25mg						

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



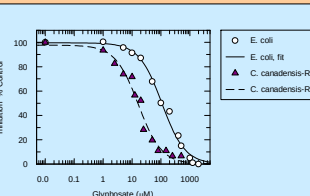
<i>E. coli</i>			Palmer amaranth-R, Macon		
Parameter	Value	Std. Error	Parameter	Value	Std. Error
Y Range	93.0953	3.7407	Y Range	97.7141	0.8082
IC 50	126.4367	25.4923	IC 50	126.4367	25.4923
Slope factor	1.0269	0.1747	Slope factor	1.0269	0.1747
Background	0.0000	0.0000	Background	0.0000	0.0000

R and S *Amaranthus palmeri* are the Same



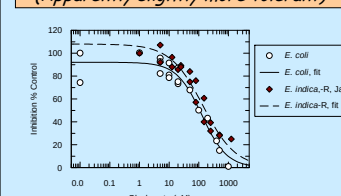
Palmer amaranth-R, Macon			Palmer amaranth-S, Ponder		
Parameter	Value	Std. Error	Parameter	Value	Std. Error
Y Range	93.0402	5.2670	Y Range	93.0381	0.0010
IC 50	26.4347	3.5827	IC 50	18.2148	2.0209
Slope factor	1.1467	0.1845	Slope factor	0.8670	0.0727
Background	3.4491	4.1253	Background	0.0000	0.0000

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



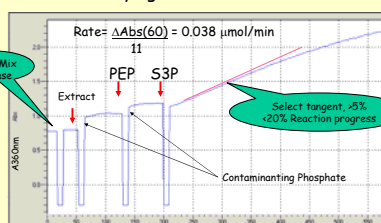
<i>E. coli</i>			<i>C. canadensis-R</i>		
Parameter	Value	Std. Error	Parameter	Value	Std. Error
Y Range	92.0953	3.7407	Y Range	97.7141	0.8082
IC 50	126.4367	25.4923	IC 50	126.4367	25.4923
Slope factor	1.0269	0.1747	Slope factor	1.0269	0.1747
Background	0.0000	0.0000	Background	0.0000	0.0000

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

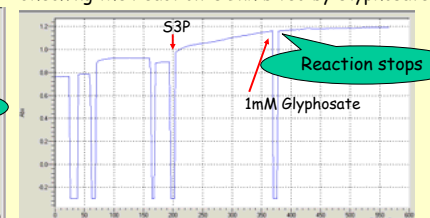


<i>E. coli</i>			<i>E. indica-R, Johor</i>		
Parameter	Value	Std. Error	Parameter	Value	Std. Error
Y Range	92.0953	3.7407	Y Range	108.0062	7.2543
IC 50	126.4367	25.4923	IC 50	138.7059	34.0175
Slope factor	1.0269	0.1747	Slope factor	0.7904	0.1093
Background	0.0000	0.0000	Background	0.0000	0.0000

Assaying "Ponder" crude



Checking the Reaction is Inhibited by Glyphosate



Examples of Raw Rate Data

