

Assay for GTP Cyclohydrolase II Activity in Bacterial Extracts

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[Abstract] Riboflavin is the precursor of flavin nucleotides FMN and FAD, they play significant roles in all organisms. GTP is the initial precursor on riboflavin biosynthesis pathway and GTP cyclohydrolase II catalyzes the first step of this pathway. It converts GTP to 2,5-diamino-6-ribosylamino-4 (3H) -pyrimidinone 5'-phosphate. This protocol provides a reliable and fast method to assay GTP cyclohydrolase II activity from crude bacterial extracts. The product of the reaction catalyzed by GTP cyclohydrolase II, 2,5-diamino-6-ribosylamino-4 (3H) -pyrimidinone 5'-phosphate, is converted to its fluorescent derivative 6,7-dimethylpterin, which is then separated on a XTerra MS C18 column and detected using fluorescence HPLC system.

Materials and Reagents

1. *Sinorhizobium meliloti* (*S. meliloti* 1021, Galibert *et al.*, 2001)
Note: The protocol can be also applied to other bacteria.
2. Bio-Rad Protein Assay Kit I (Sigma-Aldrich, catalog number: 500-0001)
3. 50 mM Tris-HCl buffer (pH 7.5) (5 ml per sample)
4. 1x BugBuster reagent (Novagen, catalog number: 70921)
5. 0.5 M EDTA (pH 8.0) (2.5 µl per sample)
6. GTP (Guanosine 5'-triphosphate sodium salt hydrate) (Sigma-Aldrich, catalog number: G8877)
7. HPLC Standard: 6,7-Dimethylpterin (Schircks Laboratories, catalog number: 11.503)
8. Biotin
9. YMB medium (Somerville and Kahn, 1983) (see Recipes) (1 plate per sample)
10. MMNH₄ medium (Somerville and Kahn, 1983) (see Recipes) (13 ml per sample)
11. Lysis buffer (see Recipes) (1 ml per sample)
12. Desalting buffer (see Recipes) (3 ml per sample)
13. RibA assay buffer (see Recipes) (2.5 µl per sample)
14. Derivatization reagent (see Recipes) (50 µl per sample)
15. HPLC mobile phase (see Recipes)

Note: Except as otherwise noted, all other chemicals were obtained from Sigma-Aldrich.

Equipment

1. 14 ml Falcon tubes (BD Biosciences, catalog number: 352059)
2. Eppendorf 1.7 ml tubes (Thermo Fisher Scientific, catalog number: 14-222-168)
3. 0.22 μ m syringe filter (Microsolv Technology, catalog number: 58022-N04-C)
4. 2 ml Zeba Spin Desalting Columns (Thermo Fisher Scientific, catalog number: 87768)
5. Mini-centrifuge
6. Centrifuge
7. Shaker
8. HPLC: Waters Alliance 2695 HPLC system linked to a 2475 fluorescence detector
9. XTerra MS C18 column (4.6 x 100 mm, 5 μ m) (Waters, part number: 186000486)

Procedure

A. Cell extract preparation

1. Grow *S. meliloti* on YMB plate for 48 h at 30 °C. The optimal temperature for *S. meliloti* growth is between 28 °C and 30 °C.
2. Inoculate *S. meliloti* from the stock YMB plate to an OD₆₀₀ of ~0.1 in 3 ml MMNH₄ medium.
3. Grow cells for 48 h at 30 °C, 250 rpm in 14 ml Falcon or glass tubes.
4. Dilute the cells 20-fold into 10 ml fresh MMNH₄ medium and grow overnight at 30 °C, 250 rpm in 50 ml glass tubes or flasks.
5. Harvest cells from 10 ml culture by centrifugation at 3,600 x *g* for 15-20 min at 4 °C in 14 ml Falcon tubes.
6. Discard the supernatant and wash the cells with 5 ml 50 mM Tris buffer at 4 °C.
7. Resuspend the pellet in 1 ml of lysis buffer at 4 °C.
8. Incubate at room temperature for 15 min, without shaking.
9. Centrifuge the lysate at maximum speed for 15 min at 4 °C.
10. Measure protein concentration in the cell lysates using Bio-Rad Protein Assay Kit.

B. Enzymatic assay

This assay protocol was adapted from a method for assaying GTP cyclohydrolase II activity in the purified enzyme (Bacher *et al.*, 1997) and modified for using with bacterial extracts.

1. Desalt the cell lysate using 2 ml Zeba Spin Desalting Columns following the manufacturer's instructions. The column was equilibrated with 3 ml desalting buffer.

2. Add 25 μ l of the desalted whole cell lysate from the previous step in a total volume of 50 μ l reaction mixture containing 10 μ l 5 x RibA assay buffer, 5 μ l 10 mM GTP (prepare GTP in ultrapure water).
3. Incubate the assay at 37 °C for 30 min.
4. Terminate the assay by adding 2.5 μ l EDTA (0.5 M, pH 8.0).
5. Derivatize the product by adding 50 μ l of the derivatization reagent, followed by incubation at 70 °C for 20 min.
6. Clear the samples by centrifugation at 3,000 x g for 10 min at 4 °C.
7. Filter the supernatant using a 0.22 μ m syringe filter.
8. Analyze the derivatization products using HPLC with fluorescence detection.

C. HPLC purification and signal detection

1. Separate the fluorescent products on a XTerra MS C18 column (4.6 x 100 mm, 5 μ m) using a Waters Alliance 2695 HPLC system linked to a 2475 fluorescence detector. Excitation and emission wavelengths are 330 nm and 435 nm, respectively.

D. Data analysis

1. Quantify the products by comparison to standards. A standard curve was obtained by running sequential dilution of 6, 7-dimethylpterin (0.25 nM, 2.5 nM, 25 nM, 0.25 μ M, 2.5 μ M and 25 μ M) on HPLC.
2. Normalize the enzyme activity against protein concentration and assay incubation time in nmol/min/mg protein.
3. Perform three replicates on each assay. Data is the average $\pm$ S.E. of three replicates.

Note: The activities of GTP cyclohydrolase II vary in different organisms. The activity of purified recombinant enzymes have been reported from about 0.1- 182 nmol/min/mg protein, the activity in bacterial extracts can be lower (Herz et al., 2000; Kaiser et al., 2002; Yurgel et al., 2014).

Representative data

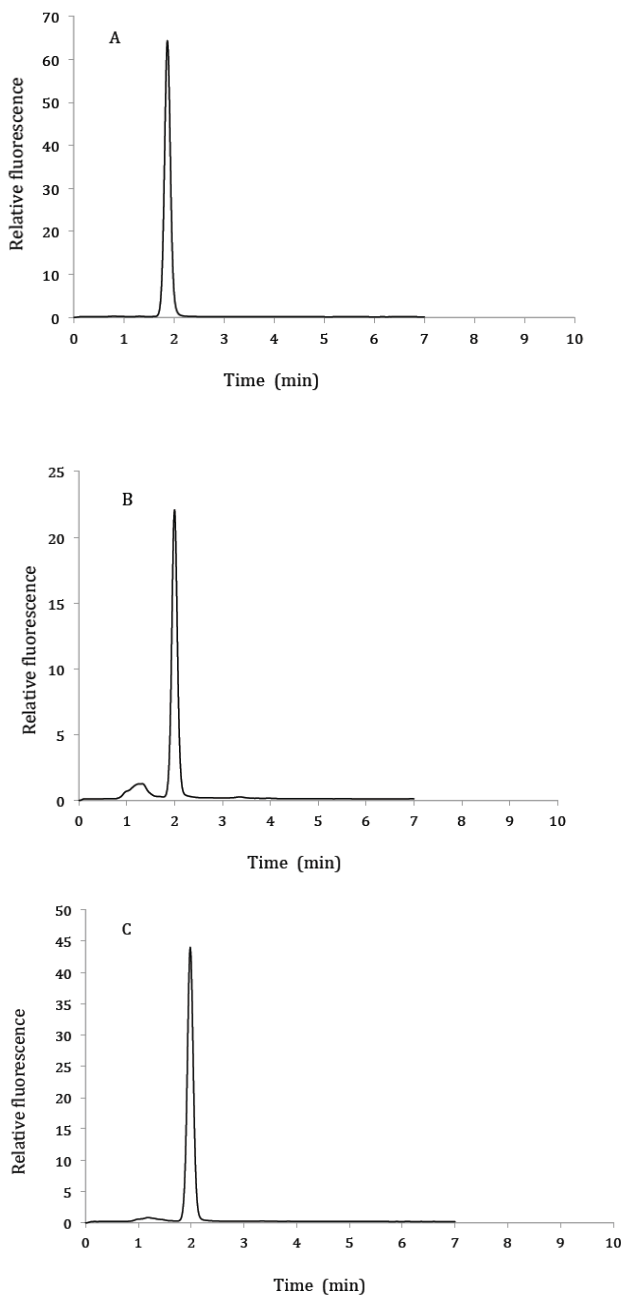


Figure 1. HPLC chromatograms of 2.5 μ M 6,7-dimethylpterin standard (A), product of GTP cyclohydrolase II assay from bacterial extracts (B) and co-elution of product from GTP cyclohydrolase II assay with 6,7-dimethylpterin standard (C). HPLC mobile phase: 10% methanol and 90% phosphoric acid (v/v). Excitation and emission wavelengths were 330 nm and 435 nm respectively.

Recipes

1. YMB media for *Rhizobium*

	1 L	Concentration
Yeast Extract	1 g	
Mannitol	10 g	54.9 mM
Agar	15 g	

Autoclave, cool to 55 °C, then add

YMB Salt I	10 ml
YMB Salt II	10 ml

YMB Salt I	1 L	Concentration
K ₂ HPO ₄	50 g	287.06 mM
NaCl	10 g	171.15 mM
d-H ₂ O	960 ml	

YMB Salt II	1 L	Concentration
MgSO ₄ ·7H ₂ O	20 g	81.11 mM
d-H ₂ O	1 L	

2. MMNH4 (minimal mannitol ammonia media for *Rhizobium*)

	per 1 L	Concentration
Mannitol	10.0 g	54.9 mM
NH ₄ Cl	0.5 g	9.34 mM
Agar (for plates preparation)	15.0 g	
d-H ₂ O	970 ml	

Autoclave, cool to 55 °C, then add:

Biotin (0.2 mg/ml in 50% EtOH)	1.0 ml
Thiamine (2 mg/ml), filter sterilized	1.0 ml
Min Man Salts I	10.0 ml
Min Man Salts II	10.0 ml

Min Man Salts I

	per 1 L	Concentration
K ₂ HPO ₄	100 g	574.12 mM
KH ₂ PO ₄	100 g	734.8 mM
Na ₂ SO ₄	25 g	174.8 mM
d-H ₂ O	1 L	

Min Man Salt II

	per 1 L	Concentration
FeCl ₃ .6H ₂ O	1.0 g	3.7 mM
Concentrated HCl	adjust pH to ~7.0 (~1drop)	
CaCl ₂ .2H ₂ O	10.0 g	68 mM
MgCl ₂ .6H ₂ O	25.0 g	123 mM
d-H ₂ O	1 L	
Autoclave		

3. Lysis buffer
 - 50 mM Tris-HCl (pH 7.5)
 - 10 mM MgCl₂
 - 1 mM Tris (hydroxypropyl) phosphine
 - 1x BugBuster reagent
4. Desalting buffer
 - 50 mM Tris-HCl (pH 7.5)
 - 1 mM Tris (hydroxypropyl) phosphine
 - 10% glycerol
5. RibA assay buffer
 - 100 mM Tris-HCl (pH 8.5) buffer
 - 5 mM MgCl₂
 - 5 mM dithiothreitol (DTT)
6. Derivatization reagent
 - 1% (v/v) diacetyl
 - 15% (w/v) trichloroacetic acid
7. HPLC mobile phase
 - 10% (v/v) methanol
 - 90% phosphoric acid (27 mM phosphoric acid)

Acknowledgments

This protocol was adapted from Yurgel *et al.*, 2014. This work was supported by the Agricultural Research Center (WNP-00773) at Washington State University and grant DE-FG0396ER20225 Vol. 27, No. 5, 2014 / 445 from the Energy Biosciences Program at the United States Department of Energy, and by grant NSF-MCB 1052492 to S. Rajamani. This activity was funded, in part, with an Emerging Research Issues Internal Competitive grant from the Agricultural Research Center at Washington State University, College of Agricultural, Human, and Natural Resource Sciences and Biologically-Intensive Agriculture and Organic Farming (BIOAg) Internal Competitive grant from The Center for Sustaining Agriculture and Natural Resources (CSANR) at Washington State University to S. Yurgel. We thank M. Kahn for discussions and the Washington State University Laboratory for Biotechnology and Bioanalysis for sequencing support.

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