CHAPTER THREE

Assaying plant formate-tetrahydrofolate ligase with monoglutamylated and polyglutamylated substrates using a fluorescence-HPLC based method

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Abstract

Formate-tetrahydrofolate ligase catalyzes reversible, ATP-dependent conversion of tetrahydrofolate and formate to 10-formyltetrahydrofolate, simultaneously releasing ADP and inorganic phosphate. This enzyme has traditionally been assayed in the direction of 10-CHO-tetrahydrofolate formation by lowering pH of the reaction post-incubation, thus converting the product of the reaction to 5,10-methenyltetrahydrofolate, which is then quantified spectrophotometrically. To increase sensitivity of the product detection, which is particularly useful when determining the kinetic parameters of the enzyme with polyglutamylated substrates, we have replaced the spectrophotometric detection with HPLC separation and fluorescence detection. In addition to the modified enzyme assay protocol, we are also providing protocols for producing recombinant formate-tetrahydrofolate ligase from Arabidopsis in *Escherichia coli* cells, producing crude Arabidopsis leaf and root extracts suitable for assaying this enzyme, and for synthesis of polyglutamylated tetrahydrofolate substrates.

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1. Introduction

Folates are a group of chemically related metabolites consisting of the cofactor tetrahydrofolate ($H_4PteGlu_n$), and its derivatives differing among each other in the oxidation state of the one-carbon group they carry. These derivatives are 10-formyl-($H_4PteGlu_n$), 5-formyl-($H_4PteGlu_n$), 5,10-methenyl-($H_4PteGlu_n$), 5,10-methylene-($H_4PteGlu_n$), and 5-methyl-($H_4PteGlu_n$). In both prokaryotic and eukaryotic organisms, these metabolites play key roles as one-carbon unit acceptors from and donors to multiple fundamental metabolic pathways including the biosynthesis of formyl-methionine-tRNA, pantothenate, purine, thymidylate, S-adenosylmethionine (AdoMet), methionine, serine, glycine, and the catabolism of histidine (Cossins, 1987; Hanson & Gregory, 2011; Hanson & Roje, 2001; Roje, 2006). In addition to the essential role in the biosynthesis of various metabolites**,** one-carbon metabolism has recently been shown to contribute a methyl group for DNA and histone methylation, thus playing an important role in the regulation of epigenetic modification (Groth et al., 2016).

The chemical structure of $H_4PteGlu_n$ (can be divided into three moieties: tetrahydropterin (H_4Pte), *para*-aminobenzoate (pABA), and the polyglutamate tail (Glu_n) (Fig. 1). In the cytosol of plant cells, the pterin moiety is synthesized from guanosine triphosphate (GTP) (Hanson & Gregory, 2011). Within plastids, chorismate from the shikimate pathway is employed as the precursor of pABA (Hanson & Gregory, 2011). Then, pABA and the pterin moiety are transported into mitochondria and

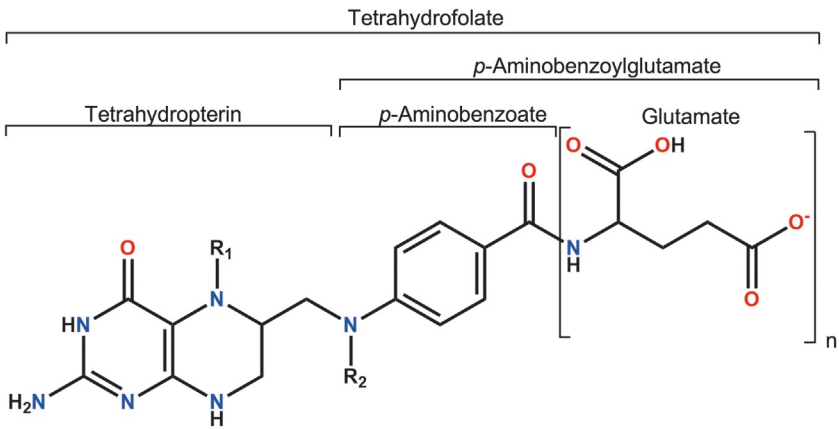

Fig. 1 Chemical structure of tetrahydrofolate ($H_4PteGlu_n$).

condensed to form dihydropteroate, which is subsequently glutamylated and reduced to produce $H_4PteGlu_1$ (Hanson & Gregory, 2011). The $H_4PteGlu_1$ molecules are conjugated with additional glutamate residues to produce $H_4PteGlu_n$ via gamma-carboxyl group peptide linkages (Hanson & Gregory, 2011). In plants, the number of gamma-linked glutamates in the tail can vary from one to eight (Zheng, Ying, Song, & Cossins, 1992).

Metabolism of $H_4PteGlu_n$-bound one-carbon units (C_1 metabolism) consists of reactions that either add or remove one-carbon units from $H_4PteGlu_n$ or change the oxidation state of the C_1-$H_4PteGlu_n$ derivatives. Within C_1 metabolism, the ATP-dependent formyltetrahydrofolate ligase (FTHFL) is responsible for enzymatically transferring a formyl group from formate to $H_4PteGlu_n$, generating 10-formyl-$H_4PteGlu_n$ and simultaneously releasing ADP and inorganic phosphate. Notably, this reaction is biochemically reversible (Curthoys & Rabinowitz, 1972).

FTHFL was first discovered and biochemically characterized by Greenberg and his colleagues in their work with homogenates from pigeon and pig liver (Greenberg, Jaenicke, & Silverman, 1955). A subsequent study identified ADP as a competitive inhibitor of the enzyme (Himes & Rabinowitz, 1962a). Furthermore, both monovalent cations (NH_4^+, K^+ or Rb^+) and the divalent metal ion (Mg^{2+}) were found to be required for maintaining the structural integrity of the FTHFL enzyme and maximizing its enzymatic activity (Himes & Cohn, 1967; Welch, Irwin, & Himes, 1968).

The reaction mechanism, which involves a formyl phosphate intermediate in the reaction, has been investigated with the enzymes from *Clostridium cylindrosporum, Peptococcus aerogenes, and Saccharomyces cerevisiae* (Buttlaire, Balfe, Wendland, & Himes, 1979; Buttlaire, Himes, & Reed, 1976; McGuire & Rabinowitz, 1978; Mejillano, Jahansouz, Matsunaga, Kenyon, & Himes, 1989; Song, Jahansouz, & Himes, 1993). The enzyme from *Moorella thermoacetica* has been crystallized and modeled structurally as a complex with the catalytic intermediate formylphosphate and the product ADP or with inhibitory substrate analogs (Celeste et al., 2012). These studies have collectively provided the mechanistic understanding of how the enzyme uses formate to initiate the nucleophilic attack on the gamma phosphate of ATP, which leads to the production of 10-formyl-$H_4PteGlu_n$ through the formyl phosphate intermediate.

The existence of FTHFL enzyme activity in higher plant species was first shown by purifying the enzyme from spinach leaves (Iwai, Suzuki, & Mizoguchi, 1967). A subsequent study of the enzyme purified from

7-day-old pea seedlings provided the first and only evidence that a plant FTHFL enzyme binds to $H_4PteGlu_n$ more efficiently than to $H_4PteGlu_1$ (Kirk, Imeson, Zheng, & Cossins, 1994). In terms of the subcellular localization, FTHFL enzyme activity was detected predominantly in the cytosol, with only a small amount found in microsomes, mitochondria, and the nuclei in pea seedlings in an early study (Suzuki & Iwai, 1974). A later study showed an abundance of FTHFL activity in the cytosol in pea, but barely detected it in mitochondria with an immunoblotting technique (Chen, Chan, & Cossins, 1997). Based on these studies, the FTHFL reaction was postulated to exist in various subcellular compartments including mitochondria, chloroplasts, and the cytosol (Groth et al., 2016; Hanson, Gage, & Shachar-Hill, 2000; Peterhansel et al., 2010). In contrast, recent bioinformatic evidence suggests that FTHFL exclusively localizes to the cytosol in the examined algae and land plant species (Gorelova et al., 2019). A single copy of FTHFL is encoded in most of the algae and land plant species; however, two copies of FTHFL have been found in *Glycine* max, *Linum usitatissimum,* and *Populus trichocarpa* (Gorelova et al., 2019) with the two homologs of FTHFL in *Glycine* sp. and *Populus* sp. predicted to be cytosolic. The available bioinformatic evidence therefore suggests that FTHFL functions in the cytosol in plants, but this has yet to be confirmed experimentally.

The FTHFL enzyme has traditionally been assayed using a spectrophotometric assay, the sensitivity of which can be too low when assaying for kinetic constant determination with polyglutamylated substrates. To increase sensitivity of the product detection, we have therefore replaced the spectrophotometric detection with HPLC separation and fluorescence detection.

2. Methods

This method describes a fluorescence-HPLC assay for FTHFL using purified recombinant protein or crude plant extracts, together with a procedure to synthesize polyglutamylated tetrahydrofolate species. The standard method for assaying FTHFL employs spectrophotometric detection of the product after conversion to 5,10-methenyl-$H_4PteGlu_n$ at low pH. By switching from spectrophotometric to fluorescence-HPLC 5,10-methenyl-$H_4PteGlu_n$ detection, a higher level of sensitivity is accomplished. Since polyglutamylated $H_4PteGlu_n$ substrates required for the enzyme assay are not commercially available, a procedure to synthesize these substrates from commercially available precursors is also provided.

2.1 Expression and purification of recombinant plant FTHFL from *E. coli* cells

2.1.1 Materials, equipment, and reagents

- cDNA clone for AtFTHFL (stock#U15996 from Arabidopsis Biological Resource Center)
- pET-30 EK/LIC vector kit (Novagen, catalog number 69077)
- Primers FTFHL_F (GGGGACAAGTTTGTACAAAAAAGCAGGC TTACCGAGAGCATAGACCTCGAATATCT) and FTHFL_R (GGGGACCACTTTGTACAAGAAAGCTGGGTTCATCGCACT CTCTGTCGAAGTCTGA)
- *E. coli* expression strain BL21 (DE3) competent cells (Novagen, catalog number 69450)
- Pfu DNA polymerase
- Luria-Bertani (LB) medium, liquid
- Luria-Bertani (LB) medium with agar
- Bacto dehydrated agar
- Agarose, Type I, Molecular Biology Grade
- Eppendorf tubes
- 250-mL centrifuge tubes
- DNA Gel Loading Dye
- DNA ladder
- Protein ladder
- Kanamycin, 100 mg/mL stock solution
- Isopropyl β-thio-galactopyranoside, 1 M stock solution
- Agarose gel electrophoresis apparatus
- 1 M Tris-HCl pH 7.5 stock solution
- 0.5 M Imidazole pH 7.5 stock solution
- 1 M $MgCl_2$ stock solution
- 1 M phenylmethylsulfonyl fluoride (PMSF) stock solution
- Extraction buffer (25 mM Tris-HCl pH 7.5, 10 mM imidazole pH 7.5, 1 mM $MgCl_2$, 400 mM KCl, 10% (*v*/v) glycerol, 10 mM β-mercaptoethanol, and 1 mM PMSF)
- Binding buffer (25 mM Tris-HCl pH 7.5, 10 mM imidazole pH 7.4, 1 mM $MgCl_2$, 400 mM KCl, 10% (*v*/v) glycerol and 10 mM β-mercaptoethanol)
- Elution buffer (25 mM Tris-HCl pH 7.5; 500 mM imidazole pH 7.5; 1 mM $MgCl_2$, 400 mM KCl; 10% (*v*/v) glycerol; and 10 mM β-mercaptoethanol)

- Desalting buffer (100 mM Triethanolamine HCl buffer pH 7.5; 2.5 mM $MgCl_2$, 200 mM KCl; 10% (*v*/v) glycerol; and 10 mM β-mercaptoethanol)
- Recombinant enterokinase (Novagen, catalog number 69066)
- Quick Start™ Bradford Protein Assay kit (Bio-Rad, CA)
- Bovine serum albumin
- PD-10 desalting column (GE Healthcare, NJ)
- 37° incubator
- 37° incubator shaker
- Petri plates
- Erlenmeyer flasks
- Mini-bead beater homogenizer
- 0.1 mm diameter Zirconia/silica beads, Cat. No. 11079101z, Biospec products (www.biospec.com)
- Centrifuge
- Microfuge
- FPLC system
- FPLC HiTrap IMAC column (GE healthcare)
- Protein gel NuPAGE 4–12% Bis-Tris Gel (Invitrogen)
- NuPAGE MES SDS or NuPAGE MOPS SDS Running Buffer (Invitrogen)
- Protein gel electrophoresis apparatus
- Power source for DNA and protein gel electrophoresis
- Spectrophotometer
- Cuvettes for spectrophotometer

2.1.2 Protocol

(1) The full-length cDNA clone for AtFTHFL (stock#U15996) is available from the Arabidopsis Biological Resource Center (ABRC). This clone is available as a full-length cDNA open reading frame in the pENTR/SD-dTopo vector, which has been fully validated by sequencing. The cDNA is a subclone from the RIKEN cDNA clone RAFL05-13-P07

(2) The primers FTFHL_F (GGGGACAAGTTTGTACAAAAA AGCAGGCTTACCGAGAGCATAGACCTCGAATATCT) and FTHFL_R (GGGGACCACTTTGTACAAGAAAGCTGGGTTC ATCGCACTCTCTGTCGAAGTCTGA) are employed to amplify the cDNA and subclone it into the pET-30 EK/LIC vector using the pET-30 EK/LIC vector kit (Novagen, catalog number 69077),

following the manufacturer's protocols available with the kit. Subcloning into the pET-30 EK/LIC vector yields a clone with a cleavable N-terminal hexahistidine tag, which facilitates the downstream purification of the recombinant protein. This expression construct is designated as pET30-AtFTHFL

(3) The expression construct should be sequenced to confirm that the cDNA is in frame and error free before transforming it into the *E. coli* expression strain BL21 (DE3).

(4) For expression of the recombinant AtFTHFL protein containing an N-terminal hexahistidine tag, 0.1 L of LB media containing the final concentration of 100 $\mu g\,mL^{-1}$ kanamycin is inoculated with 1 mL of an overnight culture of pET30-AtFTHFL at 37 °C with shaking until the OD_{600} reaches 0.6

(5) The resulting culture is then induced by adding isopropyl β-thio-galactopyranoside to obtain the final concentration of 1 mM

(6) The induced culture is grown continuously at 15 °C for 16 h

(7) Bacterial cells are harvested by centrifugation at $3000 \times g$ for 15 min at 4 °C

(8) The cell pellets can be stored in a −80 °C freezer until needed

(9) To purify the recombinant protein containing the N-terminal hexahistidine tag, the cell pellet is resuspended in 1 mL of extraction buffer (25 mM Tris-HCl pH 7.5; 10 mM imidazole pH 7.4; 1 mM $MgCl_2$, 400 mM KCl; 10% glycerol; 10 mM β-mercaptoethanol; and 1 mM PMSF) in a 1.5-mL screw-cap vial to which around 0.5-mL of 0.1 mm diameter Zirconia/silica beads were added

(10) The vial is topped off with the extraction buffer so that the liquid forms a convex meniscus, and then closed with the screw cap. This is done to minimize the amount of air present in the closed vial and should be done wearing gloves and on top of a stack of paper towels in case that a small amount of the bacterial suspension leaks from the vial while the cap is screwed on

(11) The bead beating technique is then employed to break the cells by alternating 3 times beating for 1 min at maximum intensity in a mini-bead beater homogenizer with a 1-min incubation on ice

(12) After the last 1-min incubation on ice, the crude bacterial extract is transferred into a fresh tube and centrifuged at $20{,}000 \times g$ for 20 min at 4 °C

(13) The supernatant containing the soluble recombinant protein is collected and desalted into the binding buffer (25 mM Tris-HCl pH

7.5, 10 mM imidazole pH 7.5, 1 mM $MgCl_2$, 400 mM KCl, 10% glycerol and 10 mM β-mercaptoethanol).

(14) The recombinant AtFTHFL protein is purified from the crude bacterial extract on a nickel-charged HiTrap IMAC column using an AKTA FPLC system (GE Healthcare, NJ). The IMAC column is equilibrated with the Binding buffer, and the bacterial extract containing the recombinant protein is loaded onto the IMAC column

(15) The recombinant AtFTHFL is eluted using elution buffer (25 mM Tris-HCl pH 7.5; 500 mM imidazole pH 7.5; 1 mM $MgCl_2$, 400 mM KCl; 10% glycerol; and 10 mM β-mercaptoethanol) over a 20-column volume gradient from 10 to 500 mM imidazole. The collected fraction size is 0.5 mL. The recombinant protein elutes around 150 mM imidazole

(16) The collected gradient fractions are run on the NuPAGE 4–12% Bis-Tris Gel (Invitrogen), along with a protein size standard ladder, to identify fractions containing the recombinant protein

(17) The fractions containing the recombinant AtFTHFL protein are pooled and desalted using a disposable PD-10 desalting column (GE Healthcare, NJ), which had been equilibrated with desalting buffer (25 mM Tris-HCl pH 7.5; 2.5 mM $MgCl_2$, 200 mM KCl; 10% glycerol; and 10 mM β-mercaptoethanol)

(18) The N-terminal hexahistidine tag is cleaved off by incubating the purified protein in the desalting buffer with 0.1 U mL^{-1} of recombinant enterokinase enzyme (Novagen) at 30 °C for 4 h. If incomplete recombinant enterokinase cleavage of the histidine-tagged AtFTHFL is encountered under these conditions, the incubation time should be extended, or more recombinant enterokinase should be added to the digest

(19) The untagged recombinant AtFTFHL is separated from the uncut enzyme and the cleaved histidine tag by a second round of FPLC separation on the nickel-charged HiTrap IMAC column equilibrated with the binding buffer

(20) The cleaved AtFTHFL protein without the tag elutes in the flow-through, which is during the FPLC separation collected in 1-mL fractions

(21) The flow-through fractions are collected and run on the NuPAGE 4–12% Bis-Tris Gel (Invitrogen) together with a protein ladder and an uncleaved AtFTHFL sample as a control

(22) Fractions containing the untagged recombinant AtFTHFL are pooled and desalted using a disposable PD-10 desalting column (GE Healthcare, NJ), which had been equilibrated with desalting buffer

(23) Protein concentrations are quantified using the Quick Start™ Bradford Protein Assay (Bio-Rad, CA) and bovine serum albumin as the standard

2.2 Preparation of crude protein extract from plant tissues

2.2.1 Materials, equipment, and reagents

- 1 M Triethanolamine HCl buffer pH 7.5 stock solution
- 1 M $MgCl_2$ stock solution
- 100 mM PMSF stock solution
- Extraction buffer (100 mM Triethanolamine HCl buffer pH 7.5; 2.5 mM $MgCl_2$, 200 mM KCl; 10% (*v*/v) glycerol; 1 mM PMSF; and 10 mM β-mercaptoethanol)
- 0.5 mL ZebaTM Spin Desalting Columns (Thermo Scientific, IL)
- Liquid nitrogen
- Mortar and pestle
- 2-mL screw cap vials
- 15-mL Falcon tubes
- Microfuge
- Vortexer

2.2.2 Protocol

(1) This procedure has been tested with Arabidopsis leaves collected from plants grown in soil or the Murashige-Skoog (MS) medium, and with roots grown on plates. The collected plant tissue is frozen in liquid nitrogen, and then pulverized using a mortar and pestle. Absolute care must be taken to prevent the plant tissue from thawing during the grinding and weighing procedure in order to avoid loss of the enzyme activity. To accomplish this, the ground tissue is first transferred from the mortar into a larger tube, such as a 15-mL Falcon tube, which is then placed in a rack inside a Styrofoam container filled with 1–2 in. of liquid nitrogen. A 2-mL screw-cap vial is then pre-weighed, dipped in liquid nitrogen, and placed on an analytical balance. Around 100 mg of the plant tissue is quickly added to the vial with a metal spatula that is kept chilled inside the container with liquid nitrogen. The weight is written down, and the vial with the sample is quickly capped and returned to the liquid nitrogen container

(2) Any sample showing signs of thawing, which is usually evident as the change of color from very pale green into darker green, or as a wet appearance, should be discarded immediately, and step 1 should be repeated

(3) Extraction buffer (100 mM Triethanolamine HCl buffer pH 7.5; 2.5 mM $MgCl_2$, 200 mM KCl; 10% glycerol; 1 mM PMSF; and 10 mM β-mercaptoethanol) is added to the ground tissue using 1 mL of the extraction buffer for 100 mg of tissue, and the vial is vortexed for 15–30 s to resuspend the plant tissue into the extraction buffer

(4) The tube is centrifuged in a microfuge at 20,000 × *g* for 5 min at 4 °C to pellet the cellular debris

(5) Only the clear supernatant is loaded into the 0.5 mL ZebaTM Spin Desalting Columns (Thermo Scientific, IL), which had been equilibrated with the extraction buffer, and collected

(6) The desalted samples are stored in a −80 °C freezer until needed

(7) Protein concentrations are quantified using the Quick Start™ Bradford Protein Assay (Bio-Rad, CA) and bovine serum albumin as the standard

2.3 Assay for FTHFL activity

2.3.1 Synthesis of polyglutamylated tetrahydrofolate species

2.3.1.1 Materials, equipment, and reagents

- Pteroyl(di-hepta)-γ-*L*-glutamic acid (Schircks laboratories, catalog numbers 16.252-16.255, 16.266 and 17.267; (http://www.schircks.ch)
- 50 mM Tris-HCl buffer, pH 7.8
- KBH_4
- β-Mercaptoethanol
- 1 N HCl
- 1 N NaOH
- 0.13 Sodium acetate, pH 6.9 containing 0.2 M β-mercaptoethanol
- 2 M Sodium acetate, pH 6.9 containing 0.2 M β-mercaptoethanol
- 3-mL Chromabond C_{18} Hydra Solid Phase Extraction column (Macherey-Nagel, Duren, Germany)
- 100 mM Tris(hydroxypropyl)phosphine
- Methanol
- 15-mL Falcon tubes
- FPLC
- Mono Q 5/50 GL column
- Spectrophotometer
- Spectrophotometer cuvette (quartz)

- Quartz cuvette for spectrophotometer
- Nitrogen gas tank with a regulator valve
- Glass pipette
- Flexible tubing

2.3.1.2 Protocol

(1) Polyglutamylated tetrahydrofolate species are not commercially available but can be synthesized from pteroylpolyglutamate precursors. These precursors are commercially available from Schircks laboratories (Jona, Switzerland), which is to the best of our knowledge the only commercial provider of these compounds. The pteroylpolyglutamate precursors are chemically reduced by potassium borohydride (KBH_4) to produce $H_4PteGlu_n$ using a modification of a published procedure (Scrimgeour & Vitols, 1966).

(2) The reaction mixture contains 10 mM pteroylpolyglutamate, which is dissolved right before use into the freshly degassed 50 mM Tris buffer, pH 7.8. Degassing of the Tris buffer is accomplished by slowly blowing nitrogen gas through the flask containing the buffer and is an important step that prevents premature folate degradation. A slow stream of nitrogen gas is accomplished by connecting flexible tubing to the regulator valve of the nitrogen gas tank on one end, and to a glass pipette on the other end. The glass pipette is then inserted into the flask with the buffer, and the stream of nitrogen is slowly turned on, making sure to avoid excessive gas flow, which would cause too much bubbling and cause the buffer to spill from the container

(3) After adding pteroylpolyglutamate to the degassed Tris buffer in a 15-mL Falcon tube, KBH_4 (5 mg/mL) powder is added next to initiate the pteroylpolyglutamate reduction to $H_4PteGlu_n$

(4) After a 10-min incubation at room temperature, 0.1 mL of 1 N HCl is added per mL of the reaction to destroy the unreacted borohydride. This reaction can produce some bubbling, so it is important to make sure that the reaction vessel is not filled all the way to the top

(5) The solution is neutralized rapidly with 0.1 mL of 1 N NaOH

(6) 5 mL of 0.13 M sodium acetate, pH 6.9, containing 200 mM β-mercaptoethanol is added, and the resulting solution is chromatographed immediately

(7) The synthesized $H_4PteGlu_n$ is purified on a Mono Q 5/50 GL column attached to an FPLC. Bound folates are eluted using a linear gradient of 0.13–2 M Sodium acetate, pH 6.9 containing 0.2 M β-mercaptoethanol

(8) The eluate is collected over 20 column volumes in 0.5 mL fractions following Besson, Rebeille, Neuburger, Douce, and Cossins (1993).

(9) The fractions containing $H_4PteGlu_n$ are verified spectrophotometrically at 298 nm

(10) The fractions containing $H_4PteGlu_n$ are pooled, and further purified with solid phase extraction on a 3-mL Chromabond C_{18} Hydra Solid Phase Extraction column (Macherey-Nagel, Duren, Germany) to remove sodium acetate and β-mercaptoethanol. The Chromabond column is activated following the manufacturer's instructions

(11) The $H_4PteGlu_n$ solution is loaded onto the column, and the flow-through is discarded

(12) The column is first washed with methanol, then with water containing 10 mM Tris(hydroxypropyl)phosphine in 1-mL increments. Elution of $H_4PteGlu_n$ is monitored spectrophotometrically at 298 nm

(13) After the $H_4PteGlu_n$ is eluted with 10 mM Tris(hydroxypropyl) phosphine, all the fractions containing $H_4PteGlu_n$ are combined, then aliquoted into 0.2 mL fractions, and stored at −80 °C until use

(14) The purified $H_4PteGlu_n$ concentration is determined spectrophotometrically at 298 nm using a molar absorption coefficient of 28,400 M^{-1} cm^{-1}. Repeated freezing and thawing of the prepared $H_4PteGlu_n$ solution should be avoided to prevent degradation. The solution should be taken out of the freezer and thawed right before the use

2.3.2 HPLC assay

2.3.2.1 Materials, equipment, and procedures

- $H_4PteGlu_n$ stock solution: $H_4PteGlu_1$ is available commercially, $H_4PteGlu_{n=2–8}$ can be synthesized described above
- 5,10-methenyl-$H_4PteGlu_n$: 5,10-methenyl-$H_4PteGlu_1$ is available commercially, 5,10-methenyl-$H_4PteGlu_{n=2–8}$ can be synthesized as described below
- 1 M Triethanolamine HCl buffer pH 7.5 stock solution
- 1 M $MgCl_2$ stock solution
- 1 M KCl stock solution
- 1 M ammonium formate stock solution
- 100 mM ATP stock solution
- 0.37 M HCl
- 27 mM phosphoric acid
- Xterra C18 Column (4.6 × 100 mm, 5 μm)
- HPLC with a fluorescence detector

2.3.2.2 Protocol

(1) FTHFL enzyme activity is assayed using a modification of the previously published procedure (Kirk et al., 1994), in which the accumulation of the 10-formyl-$H_4PteGlu_n$ product is measured spectrophotometrically after conversion to 5,10-methenyl-$H_4PteGlu_n$ with HCl. This spectrophotometric procedure was modified by replacing the product detection method with fluorescence-HPLC in order to increase the sensitivity of the method and enable more accurate assaying at low substrate concentrations, which is particularly important for determining the kinetic constants of FTHFL enzymes for the polyglutamylated $H_4PteGlu_n$ substrates

(2) The enzyme assay is performed by incubating protein extract in a reaction mix containing 100 mM Triethanolamine HCl buffer pH7.5, 2.5 mM $MgCl_2$, 200 mM KCl, 10% glycerol, 10 mM β-mercaptoethanol, 100 mM ammonium formate, a variable concentration of $H_4PteGlu_n$ (1 mM for the saturating conditions) and a variable concentration of ATP (2 mM for the saturating conditions) at 30 °C for 10 min

(3) The reactions should be started by adding enzyme (starting initial tests with 1 μg of the recombinant enzyme, or 100 μg of the crude plant protein extract is recommended) to the assay mixture

(4) Blanks are reactions to which the enzyme is added after the incubation

(5) The final assay volume of 50 μL is recommended when working with polyglutamylated substrates to keep the cost in check

(6) The enzymatic reaction is terminated by adding two assay volumes of 0.37 M HCl, which acidifies the reaction and converts the 10-formyl-$H_4PteGlu_n$ product of the reaction to 5,10-methenyl-$H_4PteGlu_n$

(7) The HPLC with fluorescence detection is used to measure the accumulated 5,10-methenyl-$H_4PteGlu_n$. 5,10-methenyl-$H_4PteGlu_n$ is separated isocratically on an Xterra C18 Column (4.6 × 100 mm, 5 μm) and is monitored with 360 nm excitation and 460 nm emission wavelengths. The mobile phase contains 20% (*v*/v) acetonitrile and 27 mM phosphoric acid, and the flow rate is 0.8 mL/min

(8) Peaks are quantified using a standard curve obtained by running a serial dilution of a solution containing an authentic 5,10-methenyl-$H_4PteGlu_1$ standard for the reactions with the monoglutamylated substrate

(9) For the reactions with the polyglutamylated substrate, 5,10-methenyl-$H_4PteGlu_{n=2–8}$ compounds needed as standards are not available commercially but can be synthesized from $H_4PteGlu_{n=2–8}$ using the

recombinant FTHFL enzyme. This is accomplished by running the reaction above to near-completion in the presence of a known amount of the $H_4PteGlu_n$ substrate, and then analyzing an aliquot for the remaining $H_4PteGlu_n$. The amount of the $H_4PteGlu_n$ remaining in the reaction is determined based on the comparison with an authentic standard, while the amount of the produced 5,10-methenyl-$H_4PteGlu_n$ is determined based on the reduction in the peak are for the $H_4PteGlu_n$ substrate

2.3.2.3 Precursor techniques

FTHFL enzyme activity is assayed using a modification of the previously published procedure (Kirk et al., 1994), in which the accumulation of the 10-formyl-$H_4PteGlu_n$ product is measured spectrophotometrically after conversion to 5,10-methenyl-$H_4PteGlu_n$ with HCl.

2.3.2.4 Safety considerations and standards

Standard safety procedures should be followed when working with bacteria and organic solvents.

2.3.2.5 Analysis and statistics

Product formation is quantified based on the standard curve obtained by running a serial dilution of a solution containing an authentic 5,10-methenyl-$H_4PteGlu_n$ standard under conditions described for the assays and blanks.

3. Summary

Described is a method for assaying FTHFL with purified recombinant protein or in plant extracts. In this method, the product formation is monitored after HPLC separation using a fluorescence detector. This method is a modification of the previously published procedure (Kirk et al., 1994) in which the product formation is quantified spectrophotometrically at 350 nm using the extinction coefficient of 24.9 μM^{-1} cm^{-1} after conversion to 5,10-methenyl-$H_4PteGlu_n$ with HCl. Protocols for preparing recombinant Arabidopsis FTHFL, and for synthesis of polyglutamylated $H_4PteGlu_n$ from commercially available precursors, are also provided. The main advantage of this assay is the lower product detection limit as compared to the standard spectrophotometric assay, while the major limitation is the need for HPLC instrument with a fluorescence detector.

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